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Low-temperature stress in plants: Molecular responses, tolerance mechanisms, plant biodesign and breeding applications

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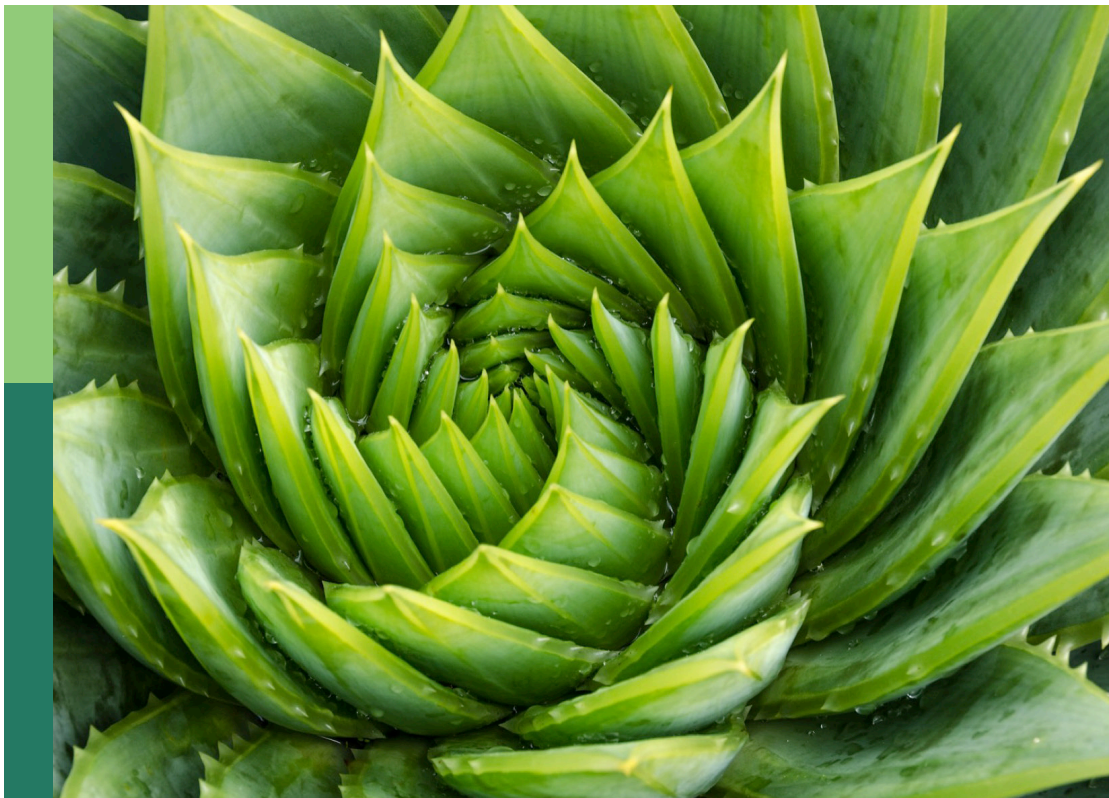

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Low-temperature stress in plants: Molecular responses, tolerance mechanisms, plant biodesign and breeding applications

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Editorial: Low-temperature stress in plants: molecular responses, tolerance mechanisms, plant biodesign and breeding applications

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Editorial on the Research Topic

Low-temperature stress in plants: molecular responses, tolerance mechanisms, plant biodesign and breeding applications

Low temperature stress is a major factor that affects the normal growth, development, and geographical distribution of plants, and has always been a hot topic in plant research. Against the backdrop of rapid development in biological breeding, deciphering the molecular mechanisms underlying plant responses to low temperature stress has become particularly urgent. In order to further refine the molecular mechanisms underlying plant responses to low temperature stress, this Research Topic of *Low-temperature stress in plants: molecular responses, tolerance mechanisms, plant biodesign and breeding applications* has collected seven original research articles and two review articles focusing on essential aspects of this field.

The degree of plant tolerance to low temperature stress varies significantly due to factors such as species, growth environment, and growth stage. Therefore, in the process of studying the molecular mechanisms underlying plant responses to low temperature stress, the selection of plant materials, design of low temperature environments, and evaluation of plant response phenotypic traits to low temperature stress are crucial considerations. The research papers collected on this topic involving seven plant species have significant differences in experimental design due to species variations. Regardless of the strategy used to study the molecular mechanisms underlying plant responses to low temperature stress, summarizing the research on this topic and previous results indicate that the main indicators for evaluating plant response to low temperature stress include ion permeability, soluble sugar content, CAT activity, SOD activity, POD activity, and

phenotypic traits associated with plant response to low temperature stress. Yang et al. find that low temperature stress significantly affects the yield and quality of rice grains during the grain-filling phase. In Song et al. study, Korean pine (*Pinus koraiensis* Siebold & Zucc) and Simon poplar (*Populus simonii* Carr.) trees were studied, and a detection method suitable for assessing freezing injury in woody plants was developed using electrical impedance spectroscopy (EIS) method. Additionally, the main environmental factors contributing to freezing injury in woody plants were identified.

High-throughput sequencing technology and molecular biology techniques are widely applied in the study of plant responses to low temperature stress. Integrating the research findings of Elakhdar et al., Wang et al. and Li et al. reveals the complex molecular mechanisms underlying plant responses to low temperature stress, which involve the coordinated regulation of multiple pathways. Among them, the widely studied pathways involved in these studies include the Ca^{2+} signalling, abscisic acid (ABA) signalling, mitogen-activated protein kinase (MAPK) cascade, and inducer of CBF expression 1 (ICE)-C-repeat binding factor (CBF) signalling pathways (Zhu, 2016; Kidokoro et al., 2022). Additionally, new plant low-temperature responsive genes have been discovered, such as the *CUC2* gene in *Brassica rapa* L and the *COMT*, *CCR*, *CAD*, *PER* and *F3'H* gene in *Prunus persica* L. Batsch in Tao et al. and Li et al. studies. The discovery of these low-temperature responsive genes in plants also provides genetic resources for molecular breeding of cold tolerance in plants. The role of non-coding RNAs in regulating plant responses to low temperature stress has been studied in *japonica* rice. Wang et al. unveils cold tolerance-related competing endogenous RNA (ceRNA) networks through whole-transcriptome profiling, identifying key components such as circular RNAs, microRNAs, and messenger RNAs. With WRKY transcription factors playing a pivotal role, the research highlights potential ceRNA networks crucial for enhancing cold tolerance. Meanwhile, previous studies have found that DNA methylation, histone acetylation, and chromatin accessibility play important roles in plant responses to low temperature stress (Ding et al., 2020). Therefore, it can be seen that molecular interactions and epigenetics are also important factors involved in plant responses to low temperature stress.

This Research Topic involves two review articles, Wang et al. providing an overview of the entire plant kingdom in response to low temperature stress, and Chen et al. focusing on the study of chrysanthemums response to low temperature stress. A comprehensive review of plant responses to low temperature stress from the perspective of the entire plant kingdom reflects the broad-spectrum nature of plant responses to low temperature stress. It provides a macro view, including topics such as plant hormone regulation, epigenetic modifications, and multiple molecular regulatory pathways. On the other hand, a specific species-focused review on chrysanthemums response to low temperature stress showcases more specific research methods and experimental results.

The future prospects of research on plant responses to low temperature stress are promising. With advancements in molecular biology and genetic engineering techniques, we can expect further elucidation of the underlying molecular mechanisms involved in cold tolerance and the development of novel strategies for enhancing low temperature stress resistance in plants. The emerging field of omics technologies, such as genomics, transcriptomics, proteomics, and metabolomics, offers powerful tools for comprehensively studying the molecular responses of plants to low temperature stress. By understanding the molecular basis of cold tolerance, researchers can potentially manipulate these genes to enhance the ability of plants to withstand freezing temperatures. Furthermore, the application of advanced breeding techniques, such as marker-assisted selection and genome editing, holds great promise for improving low temperature tolerance in crops. By precisely targeting specific genes or genetic regions associated with cold tolerance, breeders can expedite the development of new varieties with enhanced resilience to low temperatures. Considering the current research on plant responses to low temperature stress, the major challenge lies in the plant genetic transformation system, particularly for non-model plants. Addressing this issue is crucial for studying the molecular mechanisms of plant responses to low temperature stress and accelerating the development of new varieties with enhanced cold tolerance. Therefore, to expedite the progress of molecular breeding for low temperature tolerance, constructing a plant genetic transformation system is the next urgent scientific problem to be addressed, building upon the research foundation of this Research Topic.

Author contributions

PL: Data curation, Writing – original draft, Writing – review & editing. TZ: Writing – review & editing. DD: Writing – review & editing. YL: Writing – review & editing. SA: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Integrated analysis of transcriptomics and metabolomics of peach under cold stress

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Low temperature is one of the environmental factors that restrict the growth and geographical distribution of peach (*Prunus persica* L. Batsch). To explore the molecular mechanisms of peach brunches in response to cold, we analyzed the metabolomics and transcriptomics of 'Donghe No.1' (cold-tolerant, CT) and '21st Century' (cold-sensitive, CS) treated by different temperatures (-5 to -30°C) for 12 h. Some cold-responsive metabolites (e.g., saccharides, phenolic acids and flavones) were identified with upregulation only in CT. Further, we identified 1991 cold tolerance associated genes in these samples and they were significantly enriched in the pathways of 'galactose metabolism', 'phenylpropanoid biosynthesis' and 'flavonoids biosynthesis'. Weighted gene correlation network analysis showed that soluble sugar, flavone, and lignin biosynthetic associated genes might play a key role in the cold tolerance of peach. In addition, several key genes (e.g., COMT, CCR, CAD, PER and F3'H) were substantially expressed more in CT than CS under cold stress, indicating that they might be major factors during the adaptation of peach to low temperature. This study will not only improve our understanding towards the molecular mechanisms of peach trees under cold stress but also contribute to the screening and breeding program of peach in the future.

KEYWORDS

peach, metabolome, RNA-seq, saccharides, phenolic acids, flavones

Introduction

Peach (*Prunus persica* L. Batsch) trees are widely planted across the world due to the delicious fruit and nutrition supplies (Wu et al., 2017; Li et al., 2021) and cold stress (e.g, chilling: < 20°C, freezing: < 0°C) can restrict its growth, development, yield and geographical distribution. To combat the low temperature, various substances and protective proteins are synthesized in plants to systematically regulate the osmotic potential, ice crystal formation, and the homeostasis of cell membrane (Ji et al., 2020;

Feng et al., 2021; Hao et al., 2022). In the past few decades, many studies have been demonstrated to elucidate the molecular mechanisms involved in the plant cold acclimation. For example, the CBF/DREB (C-repeat binding factor/dehydration responsive element binding factor) dependent signalling pathway has been reported in many plants as a conserved regulatory mechanism to defend cold (Shi et al., 2018; Cai et al., 2019; Liu et al., 2019; Hwarari et al., 2022). The CBF genes, which belong to the AP2/ERF (apetala 2/ethylene response factor) family, are regulators of various abiotic stresses including cold in plants (Riechmann and Meyerowitz, 1998; Ghorbani et al., 2020). When plants suffer from cold, regulatory proteins, including the positive regulators ICE1 (inducers of CBF expression 1), CAMTA3 (calmodulin-binding transcription activator 3) and BZR1/BES1 (brassinazole-resistant 1) and suppressors MYB15, PIFs (phytochrome-interacting factors) and EIN3 (ethylene-insensitive 3) (Shi et al., 2018; Liu et al., 2019), are expressed to modulate the CBF gene expression and subsequently the CBF proteins specifically bind to the conserved C-repeat (CRT)/dehydration-responsive elements (CRT/DRE; G/ACCGAC) of downstream cold-responsive (COR) genes (Chinnusamy et al., 2007; Song et al., 2021). In addition, CBF-independent regulatory pathways have also been identified in plants under cold stress, such as the plant hormones auxin, ethylene, gibberellins, abscisic acid, and jasmonic acid (Shi et al., 2015).

Since the first peach genome was reported in 2010, omics approaches (e.g., genomics, transcriptomics, proteomics, and metabolomics) have been widely used to investigate the roles of cold tolerance associated genes in peach (Muthuramalingam et al., 2022). Digital expression analyses of EST datasets identified two promoters – Ppbec1 encoding endochitinase (C2131) and Ppxero2 encoding dehydrin (C254) as cold-inducible promoters for peach and reported the heterologous regulation of these promoters in peach at low temperatures (Tittarelli et al., 2009). The expression of peach CBF gene PpCBF1 in apple has an enhancement effect of tolerance to freezing (Wisniewski et al., 2015). By using transcriptomics analysis Yu et al. identified 1891 differentially expressed genes (DEGs) in the peach plant in response to cold and the DEGs were significantly enriched in the pathways of 'metabolic pathway' and 'biosynthesis of secondary metabolites' (Yu et al., 2020). Among the 23 selected heat-responsive genes in peach fruit, more than 90% were identified by Lauxmann to be modulated by a short cold exposure (Lauxmann et al., 2012). A bulked segregant gene expression analysis performed by Pons et al. identified some cold-responsive genes, such as ICE1, CBF1/3, SAD1, ERD15 and some transcription factors (e.g., HOS9, MADS-box, MYB, NAC, PHD, TUB, WRKY) in peach mesocarp (Pons et al., 2014). Sanhueza and colleagues analysed the transcriptome profiles of peach under cold stress and reported some cold responsive genes in peach, such as spatula/Alcatraz and MYB (agamous-like) TFs (Sanhueza et al., 2015). By transcriptomic and metabolic analyses, Wang found that low temperature could cause higher rate of ethylene production and more rapid flesh softening, reduced internal browning of fruit, lower transcript levels of polyphenol oxidase and peroxidase, and higher lipoxygenase in peach fruit (Wang et al., 2017). Based on the metabolomic analysis, Wang reported enhanced fatty acid content, increased desaturation, higher levels of phospholipids and a preferential biosynthesis of glucosylceramide in the peach fruit under cold stress. The above omics studies are about the peach fruit under cold stress and large is unknow about the development of peach trees at low temperature environment. Only Yu et al. reported the transcriptome profiles of peach tree shoots during the processes of cold acclimation and deacclimation (Yu et al., 2020).

Previously, our lab identified 329 and 399 differentially expressed proteins in the cold-sensitive (21st Century, CS) and cold-tolerant (Donghe No.1, CT) peach trees, respectively, treated by cold for 48 h (Li et al., 2021). We found that the CT cultivar displayed amount of energy from metabolic pathways (e.g, carbon, starch and sucrose) and phenylpropanoid biosynthesis to resist cold stress. Moreover, peroxidase, flavonoid, carbonic anhydrase and harpin proteins displayed more abundance in CT. To explore the metabolites and genes related to the cold tolerance of peach tree, in the present study, we performed transcriptomic and metabolomic sequencing for the branch samples of the CT and CS peach trees under different cold temperatures. This is the first time to investigate the transcriptome profiles and metabolites of peach branch samples under cold stress using omics approaches. Our findings will enhance the knowledge of molecular mechanisms in peach trees in response to cold and will benefit the peach breeding program.

Material and methods

Plant materials and cold treatment

We collected the one-year-old branches from the grafted peach trees of 'Donghe No.1' (CT) and '21st Century' (CS) in their ecodormancy stage in the field in January 2021 and no permissions were required to collect these plants. The branch samples were placed in a chamber and cold-stratified at 4°C for 7 days. Next, the branch samples were divided into six groups and each group was well-wrapped by plastic bags. The samples of each group were then placed into one of the six programmable incubators with temperatures set at -5°C (control), -10°C, -15°C, -20°C, -25°C and -30°C for 12 h, followed by the treatment of cooling or heating rates at 4°C/h. Then, the middle parts of the branches were cut, quickly frozen in liquid nitrogen immediately and stored at -70°C until use (relative electrolyte leakage was measured without freezing). The relative electrolyte leakage (REL) of each sample was measured as described (Sun et al., 2021). Each treatment was repeat three times and served as independent biological replicates.

Metabolite analysis by LC-MS/MS

Extraction and analysis of metabolites were carried out in Metware Biotechnology Co. Ltd. (Wuhan, China). Briefly, after the branch samples were freeze-dried by vacuum freeze-dryer (Scientz-100F), they were grounded into fine powder in a mixer mill (MM 400, Retsch) with a zirconia bead for 1.5 min at 30 Hz.

The lyophilized powder (100 mg) was dissolved in 1.2 mL of 70% methanol solution, vortexed 30 s every 30 min for 6 times, and placed in the refrigerator at 4°C overnight. After the samples were centrifuged at 10000 × g for 10 min, the supernatant was aspirated and filtered through a 0.22 mm pore size membrane and stored in the injection bottle. Next, the samples were analysed by an UPLC-MS/MS system (UPLC, SHIMADZU Nexera X2; MS, Applied Biosystems 4500Q TRAP). We connected the effluent with an ESI-triple quadrupole-linear ion trap (Q TRAP)-MS, acquired the linear ion trap (LIT) and triple quadrupole (QQQ) scans from a triple quadrupole-linear ion trap mass spectrometer equipped with an ESI Turbo Ion-Spray interface (operated in positive ion mode), and controlled the scan using Analyst (v1.6.3, AB Sciex). Then, a scheduled multiple reaction monitoring method was used to quantify the metabolites and the collision energy and declustering potential were optimized for each precursor-product ion (Q1-Q3) transition to obtain maximal signal (Chen et al., 2013). The melatonin content was calculated from the quantitative data of melatonin and the standard curves acquired from an authentic melatonin standard. To identify cold-responsive metabolites in one-year-old peach branches, differentially expressed metabolites (DEMs) were screened using log2 fold change (log2FC) ≥ 1. Three biological replicates were used for the metabolomics analysis.

RNA-Seq and bioinformatics analysis

Total RNA was isolated from the one-year-old peach branch samples using the RNAprep Pure Plant kit (DP441, Tiangen, China). The RAN quantity and quality were evaluated by NanoPhotometer spectrophotometer (IMPLEN, CA, USA), Qubit 2.0 Fluorometer (Life Technologies, CA, USA) and Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Then, the poly(A) mRNA was enriched by magnetic beads with oligo (dT) and used to construct the cDNA libraries. The libraries were then sequenced on the Illumina Novaseq 6000 system with paired-end 150-bp (PE150) strategies in Metware Biotechnology Co. Ltd (Wuhan, China), as described (Chen et al., 2016; Chen et al., 2021).

Raw data were cleaned by fastp and the quality of clean data was evaluated by FASTQC, as described (Chen et al., 2016; Chen et al., 2018). Then, the high-quality clean reads were mapped to the peach reference genome (Prunus persica-genome.v2.0.a1) using HISAT2 (version 2.1.0) with default parameters (Pertea et al., 2016). We used FeatureCount program to count the number of reads aligned to each gene and normalized the gene expression using the FPKM (Fragments Per Kilobase of transcript per Million fragments mapped) method (Liao et al., 2014). To identify cold-responsive genes in one-year-old peach branches, DEGs were identified using DESeq2 with the criteria of |log2FC| ≥1 and FDR (False Discovery Rate) < 0.05 (Love et al., 2014; Chen et al., 2016). Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DEGs were performed by the cluster Profiler R package (Yu et al., 2012).

Weighted gene co-expression network analysis

We performed the weighted gene co-expression network analysis (WGCNA) to identify core genes with similar expression patterns that may participate in the cold tolerance of peach trees, as described (Langfelder and Horvath, 2008). The gene networks and top 30 hub genes within a module were visualized by Cytoscape (Shannon et al., 2003).

Quantitative real-Time PCR validation

Total RNA was extracted from the samples using the Yisheng RNA extraction kit (Yisheng, Shanghai, China) according to the manufacturer's instructions. We randomly selected 11 genes for the qRT-PCR experiment and used actin as the internal control. Forward and reverse primers were designed using the Prime3 and synthesized in BGI-Shenzhen. Then, the qRT-PCR reactions were conducted with a Yisheng SYBR Green Master Mix and a CFX*Real-Time System (Bio-Rad). Relative gene levels were calculated using the $2^{-\triangle\triangle Ct}$ method and the CT/CS sample at 0° C were used as the reference. Three biological and technical reactions were performed for a gene in one sample, and we have 9 reactions for one gene in the peach branch sample at each condition.

Results

Physiological differences between CT and CS in response to cold stress

We first estimated the electrolyte leakage rates (ELR), a parameter used to evaluate the plant cold resistance, of 1-year-old branches of 'Donghe No. 1' (CT) and '21st Century' (CS) treated by cold for 12 h under different temperatures (from -5 to -30°C) (Figure 1A). It showed that the ELRs were similar from -5 to -15°C but had significant difference from -20 to -30°C in the two peach cultivars. This observation was consistent with the colour changes in the pith and xylem of the branches (Figure 1B). It is notable that the ELR of CS was ranged in 18~23% under freezing environment and increased significantly to 27.4% at chilling stress (Figure 1A). While the ELR of CT was 21.8% at -20°C and increased to 29.3% at -30°C. These results first confirmed the characteristics of cold tolerance of the two peach cultivars. We also observed that low temperature damage to the cell membrane occurred in the 1-year-old CS peach branch at -20°C/12 h, resulting in necrosis in the pith tissue (Figure 1B). However, no exhibition and browning vascular were observed in the CT peach branch at chilling stress (Figure 1B). Thus, we selected the peach branch samples treated at -20, -25 and -30°C for 12 h to study the changes in the transcriptome profiles and metabolites of peach trees in response to chilling stress, and peach branch samples treated by cold at -5°C for 12 h were used as the control.

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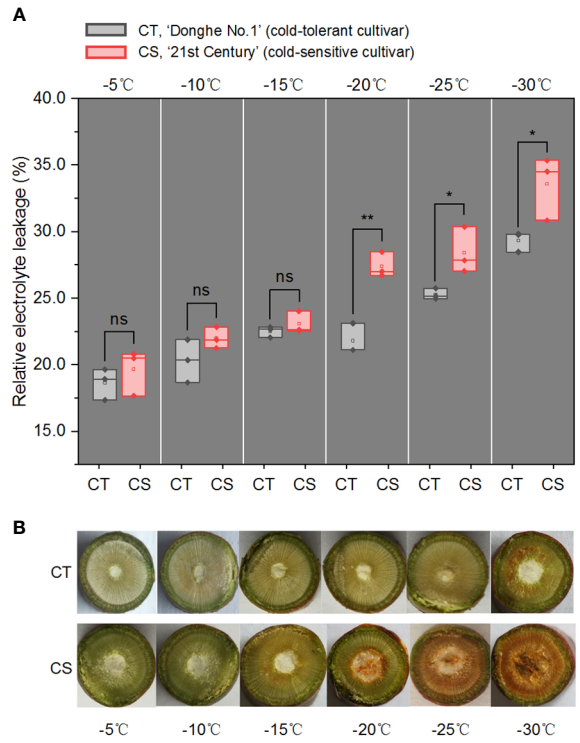

FIGURE 1
Relative electrolyte leakage and cross-section photos of peach branches under cold stress. **(A)** Relative electrolyte leakage (REL) of 1-year-old branches of CT and CS under cold stress for 12 h. CT, 'Donghe No.1' (cold-tolerant cultivar); CS, '21st Century' (cold-sensitive cultivar, CS). ns, not significant; *p < 0.05; **p < 0.01. The p values were calculated using the t-test for the difference in electrolyte leakage rates (ELR). **(B)** Cross-section photos of CT and CS under cold stress for 12 h.

Metabolome profiling of peach in response to cold stress

To analyze the metabolites between CT and CS peach genotypes at the four different treatment temperature points, we performed the UPLC-MS/MS analysis and identified a total of 1096 metabolites in all samples. They were classified mainly into the categories of flavonoids, phenolic acids, alkaloids, amino acids and derivatives, organic acids, terpenoids, lignans, coumarins and tannins (Table S1). Principle component analysis (PCA) of the metabolite profiles showed that the two cultivars were separated by PC1 (35.02%) and that samples collected at different temperature points were separated by PC2 (14.48%) (Figure 2A). It indicated that low temperature had profound impacts on the compound accumulation patterns in peach.

Next, we compared the metabolomic changes between CT and CS peach branches in response to cold. Initially, we identified 237 (53 upregulated and 184 downregulated), 235 (44 upregulated and 191 downregulated), 175 (82 upregulated and 93 downregulated) and 218 (41 upregulated and 177 downregulated) differentially expressed metabolites (DEMs) in A vs G, D vs K, E vs L and F vs M, respectively (Figure 2B; Table S2). Altogether, we identified 410 DEMs between CT and CS and they were mainly involved in flavonoids (128, 31.2%), phenolic acids (89, 21.7%), lipids (37, 9.0%), amino acids and derivatives (35, 8.5%), alkaloids (21, 5.1%), lignans and coumarins (11, 2.6%) and others (Figure 2C). Notably, among the changed flavonoids, two flavanone types (naringenin and homoeriodictyol), two flavone types (luteolin and apigenin) and one chalcone type (naringenin chalcone) were found with higher accumulation in CT than CS under cold stress (Figure 2D). In addition, other metabolites, such as saccharides (D-galactose, D-glucose, D-fructose, manninotriose, raffinose) and phenolic acids (p-coumaric acid, caffeic acid, ferulic acid and coniferin), also exhibited higher accumulation patterns in CT than CS (Figure 2D). Compared with CT, CS accumulated higher contents of flavonols (e.g., quercetin-3-O-(6''-O-acetyl) glucoside, kaempferol-3-O-(6''-malonyl) glucoside, quercetin-3-O-(6''-O-acetyl) galactoside) (Figure S1). Since some flavonoids, saccharides, and phenolic acids have been reported to be associated with plant cold tolerance, the accumulation patterns of these substances in CT probably provided strong cold tolerance capability.

Transcriptomic analysis of peach branches exposed to cold stress

We next performed transcriptome sequencing to study the gene expression changes in CT and CS peach branches under cold stress. In total, we obtained 1093 million clean reads for all samples and the quality control of each sample can be seen in Table S3. After the reads were aligned to the peach reference genome and gene sequences, the expression of all peach functional genes (25702 genes) for these samples can be found in Table S4. Like the metabolomic analysis, PCA of gene expression profiles showed that the samples from the different temperature points and their genotypes could be separated by PC1 (26.58%) and PC2 (14.62%), respectively (Figure 3A). Then, we identified 3998 DEGs between CT and CS peach branch samples treated by cold at the four temperature points for 12 h (Figure 3C), including 1893 (1005 upregulated and 888 downregulated), 1980 (1081 upregulated and 899 downregulated), 1623 (948 upregulated and 675 downregulated) and 2246 (1147 upregulated and 1099 downregulated) DEGs in the pairwise comparisons of A vs G (−5°C), D vs K (−20°C), E vs L (−25°C) and F vs M (−30°C), respectively (Figures 3B; Table S5). It is notable that 660 genes were deregulated in CT samples compared to CS samples at the four temperature points (Figure 3C).

Identification of cold tolerance associated genes in peach

Next, we compared the peach branch samples treated by different temperature points to the control sample (-5°C) and identified 5268 cold associated genes (CAGs) in CT. It showed in Figure 3D that 223 DEGs were commonly deregulated when the temperature dropped from -20 to -30°C (Table S6). In CS, we also identified 3998 DEGs in the peach branch samples under chilling stress and found 1991 DEGs shared by CT (Figure 3E; Table S7). Further, we analyzed the transcription factors (TFs) in the 1991

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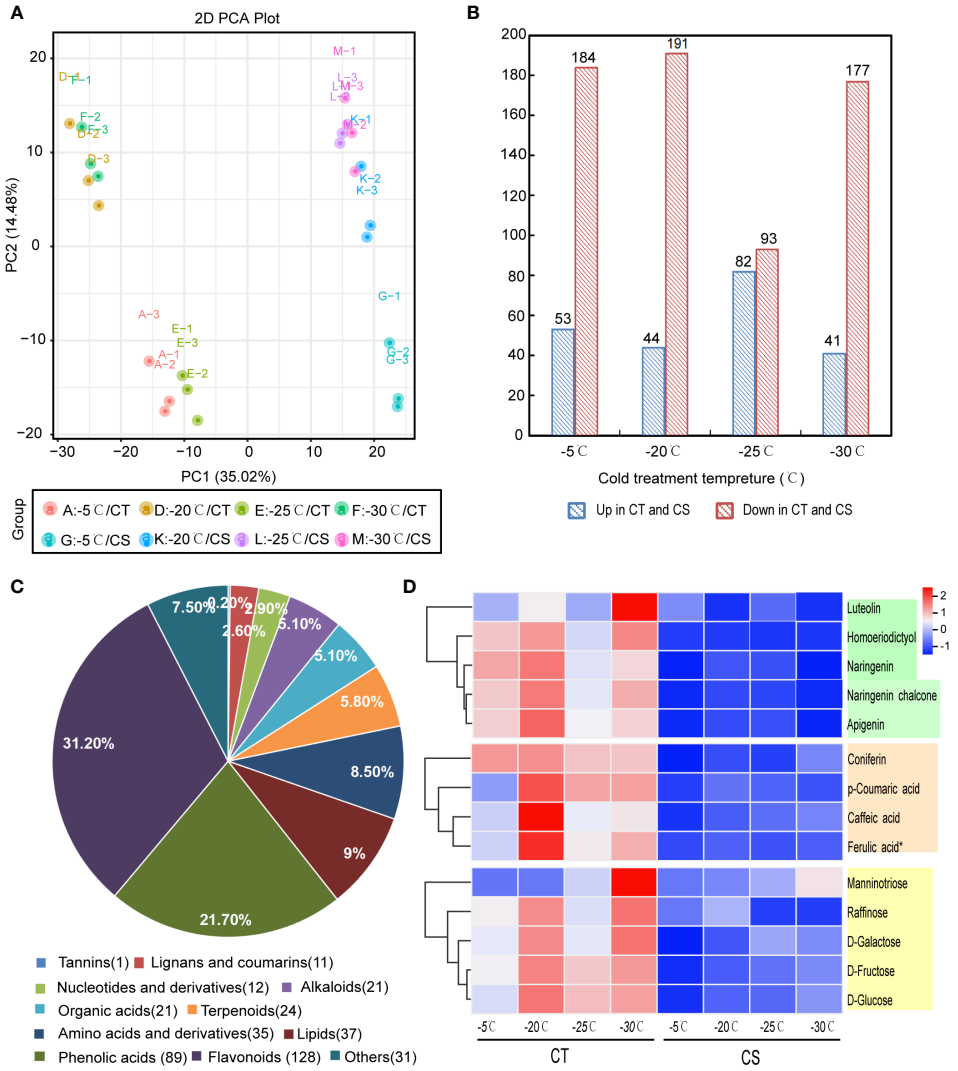

FIGURE 2
Metabolomics analysis of CT and CS under cold stress. **(A)** PCA of metabolites derived from CT and CS under cold. **(B)** Significantly differentially expressed metabolites (DEMs) between the CT and CS under cold stress. **(C)** Classification of DEMs in peach under cold stress. **(D)** The accumulation pattern of flavonoids (green), saccharides (orange), and phenolic acids (yellow) in CT and CS under cold stress. Scaled values of the relative contents of metabolites were used for z-scale normalization.

commonly deregulated genes of the two peach cultivars. It showed that 147 differentially expressed TF genes in peach branch samples in response to cold, including 23 AP2/ERF (15.65%), 18 NAC (12.24%), 16 MYB (10.88%), 12 bHLH (8.16%), 9 WRKY (6.1%) and 5 bZIP (3.40%) TFs (Table S7). Interestingly, we found seven AP2/ERF (Prupe.2G289500, Prupe.4G176200, Prupe.5G141200, Prupe.5G090100, Prupe.7G194400, Prupe.7G060700 and Prupe.8G224600), five MYB (Prupe.1G039200, Prupe.1G111700, Prupe.6G106200, Prupe.1G551400 and Prupe.8G223900), two NAC (Prupe.4G143600 and Prupe.4G186800) and one bHLH (Prupe.8G193900) with higher expression levels in CT than CS (Figure S2), indicating their potential roles in the regulation of cold tolerance associated genes in peach.

GO enrichment analysis of the 1991 common DEGs in CT and CS can be seen in Figure S3. It showed that 56, 49 and 35 DEGs were significantly enriched in the biological processes of 'regulation of defense response', 'response to nitrogen compound' and 'hormone biosynthetic process', respectively. In addition, 64 and 63 DEGs were enriched in the molecular functions of 'oxidoreductase activity' and 'transferase activity', respectively. The 42 genes enriched in the contents of sugars (hydrolase activity, hydrolyzing N-glycosyl compounds) indicated that they might be functional for peach branches in response to cold. Next, we analyzed the KEGG pathways enriched by the common DEGs in CT and CS (Figure 3F) and the top three significant enriched pathways were 'galactose metabolism', 'flavonoid biosynthesis' and 'phenylpropanoid biosynthesis'. As an important branch of the starch and sucrose pathway, genes involved in galactose biosynthetic were upregulated by cold stress. These results indicate that both sugars and flavonoids might play a key role in response to cold stress in peach.

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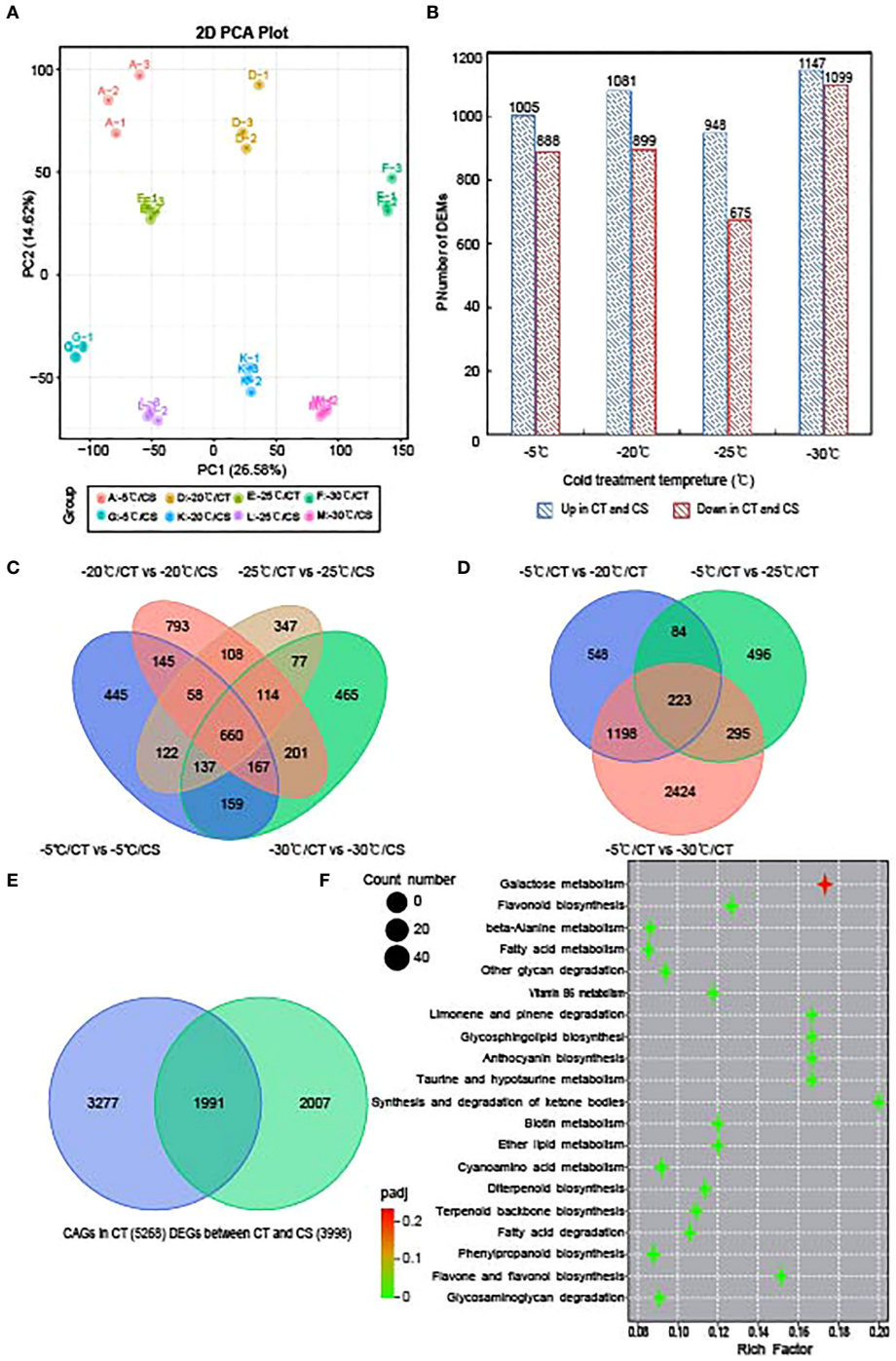

FIGURE 3
Transcriptome analysis of CT and CS under cold stress. **(A)** PCA of the gene expression profiles of CT and CS under cold stress. **(B)** Numbers of DEGs in in CT compared to CS under different temperatures. **(C)** Venn diagram of DEGs in CT compared to CS under different temperatures. **(D)** Venn diagram of DEGs in CT under chilling stress (-20°C, -25°C and -30°C) relative to control (-5°C). **(E)** Venn diagram of DEGs identified in CT and CS under chilling stress (-20°C, -25°C and -30°C) compared to control (-5°C). Blue: CT; green: CS. **(F)** KEGG pathway of the commonly deregulated genes in CT and CS under cold.

Identification of key genes and modules in response to cold stress by WGCNA

We next conducted WGCNA to identify co-expressed genes from the common 1991 DEGs in CT and CS peach branches under cold stress. A total of 9 modules of genes marked with different colors were identified (Figures 4A, B). The module-trait relationship analysis of the 24 samples revealed that saccharides (D-galactose, D-mannose, manninotriose) and phenolic acids (coniferin) were significantly associated with the genes in the turquoise module

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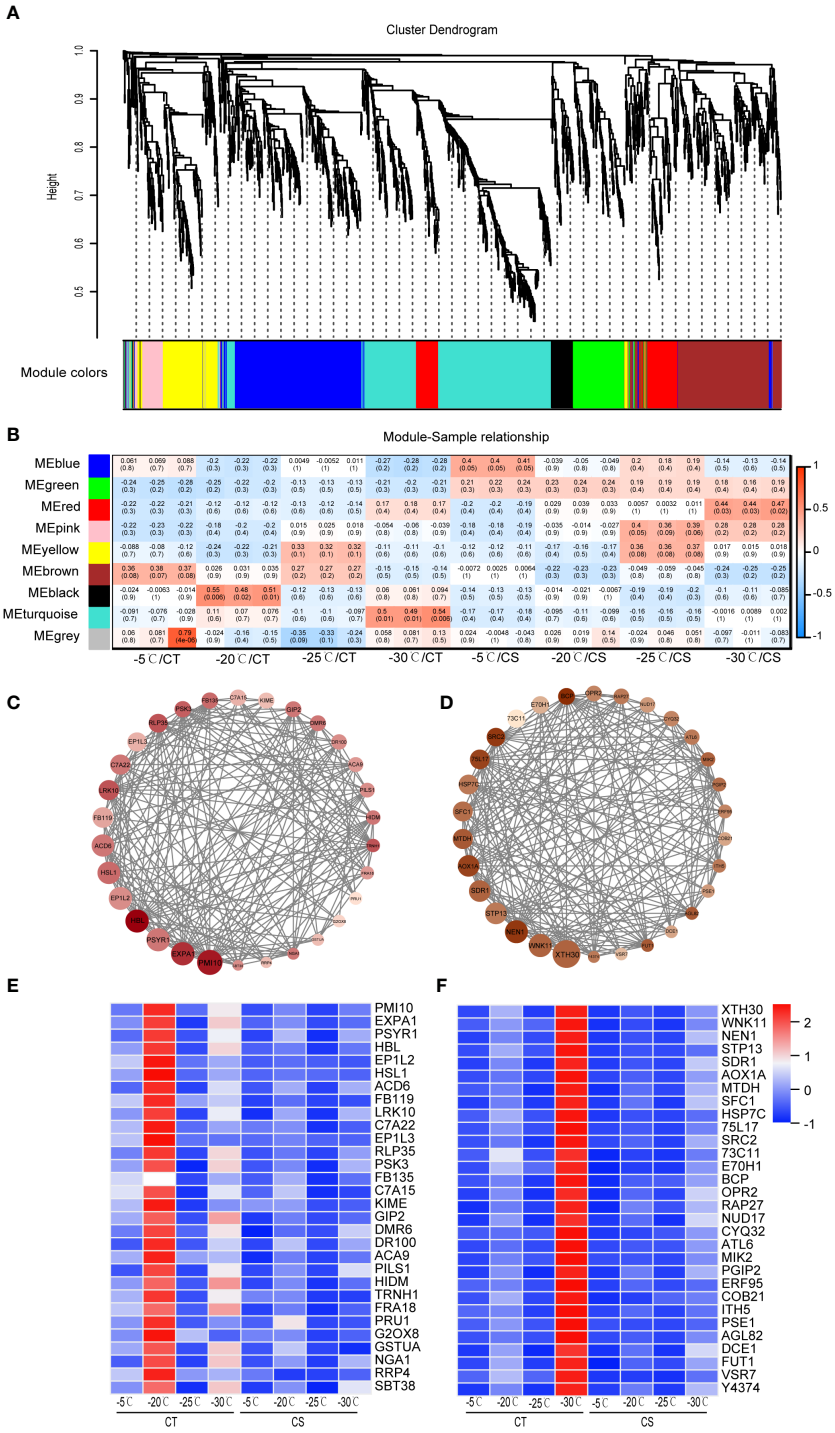

FIGURE 4
WGCNA identifies key modules of hub genes of peach in response to cold. **(A)** Hierarchical cluster tree showed 9 modules of co-expressed genes in peach under cold stress. **(B)** Heat map of correlations between metabolites and gene modules. Values in each cell represent the coefficient value of correlation (upper) and the p-values (lower in parentheses) of the module-trait association. **(C)** Protein-protein interaction network of co-expressed genes from the black module. **(D)** Protein-protein interaction network of co-expressed genes from the turquoise module. **(E)** Expression heat map of genes in the black module. **(F)** Expression heat map of genes in the turquoise module. Scaled values were used to present the FPKM of genes in the heat maps.

(R> 0.45, $p < 0.01$) and that flavonoids (prunetin, pratensein, genistein and 2'-hydroxygenistein) were related to the black module genes. According to the WGCNA edge weight values and node scores, we showed the expression levels of top 30 hub genes in the black module (Figure 4C). Notably, some genes involved in the abiotic stress processes were identified, including receptor-like protein kinase (HSL1, Prupe.1G444700), 2-hydroxyisoflavanone dehydratase (HIDM, Prupe.1G155100), cytochrome P450

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(C7A22, Prupe.1G291800), beta-amyrin 28-oxidase (C7A15, Prupe.4G103000), B3 domain-containing transcription factor (NGA1, Prupe.2G201000), and calcium-transporting ATPase 9 (ACA9, Prupe.3G018900). The turquoise module was found to be associated with the CT samples at -30°C (Figure 4B) and some TF genes (e.g., RAP27, ERF095 and AGL82) and several stress-related proteins/enzymes (e.g., HSP7C, WNK11, DCE1, MTDH, OPR2, CYQ32, 75L17 and 73C11) (Figure 4D). Both black and turquoise modules of genes were found to be upregulated in the CT samples at chilling stress but hardly expressed in CS samples, indicating their potential roles in cold tolerance of peach trees (Figures 4E, F).

Integrated analysis identifies important pathways for peach under cold stress

We next performed the integrated analysis of the transcriptome and metabolome results. Some common enriched pathways were identified, such as 'galactose metabolism', 'phenylpropanoid biosynthesis' and 'flavonoids biosynthesis'. It showed that the metabolites of galactose metabolism, such as galactose, raffinose, manninotriose, glucose and fructose, were markedly increased in CT than in CS under cold stress (Figures 2D, 5A). Notably, the metabolism of sugar compounds was higher in CT than CS peach branches, indicating their vital roles in the protection of peach trees against cold stress. In addition, we found that the transcriptome profiles of genes encoding structural enzymes in galactose biosynthetic pathways were correlated with the accumulation pattern in the two peach cultivars under cold stress (Figures 2D, 5B). For instance, three genes encoding galactinol synthases (EC=2.4.1.123, GOLS) were significantly upregulated in CT but not changed or only slightly upregulated in CS under cold stress (Figure 5B). We also found three raffinose synthases (RFS, also named as galactinol-sucrose galactosyltransferase, EC=2.4.1.82) induced by cold at -20 and -25°C only in CT peach branches (Figure 5B).

Likewise, we found that metabolite and gene expression profiles of lignin biosynthesis and flavonoids metabolism were much higher in CT than CS under cold stress (Figure 6). For example, coumaric acid, caffeic acid, ferulic acid and coniferin involved in the lignin biosynthesis, one of the main components of plant cell wall responding to biotic and abiotic stresses, were higher in CT than CS under cold stress (Figure 6A). Compared to CS, four key enzyme genes involved in the 'phenylpropanoid biosynthesis', including caffeic acid 3-O-methyltransferase (EC=2.1.1.68, COMT), cinnamoyl-CoA reductase (EC=1.2.1.44, CCR), cinnamyl-alcohol dehydrogenase (EC=1.1.1.195, CAD) and peroxidase 4 (EC=1.11.1.17, PER) were also found to be upregulated in CT in response to cold, especially at -30°C (Figures 6B; Table S5). In addition, we found one gene encoding flavonoid 3'-monooxygenase CYP75B137 (EC=1.14.14.82, F3'H) involved in the flavonoids biosynthesis was upregulated in CT but not changed in CS under cold stress (Figure 6B). These results indicate that the metabolites and genes involved in these pathways may play a vital role in protection of peach against cold stress.

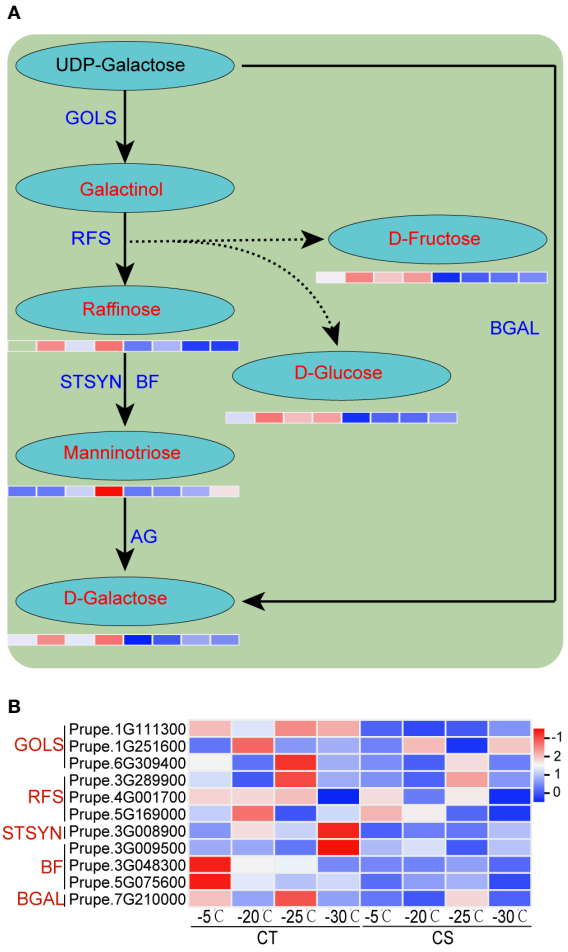

FIGURE 5
Integrated analysis of transcriptome profiles and metabolites of peach under cold stress. **(A)** The schematic diagrams of galactose metabolism pathway. Metabolites and structural enzymes are indicated in red and blue, respectively. The left and right four parts of the rectangles under the metabolites represented the expression levels of DEGs/DEMs in CT and CS, respectively. **(B)** Heat map of genes encoding the structural enzymes involved in the galactose biosynthetic pathways. Scaled values of FPKM were used.

RNA-seq data validation by qRT-PCR

We employed qRT-PCR to validate the expression patterns of 11 cold tolerance associated genes in the two peach cultivars under cold stress (Figure 7). First, we compared the gene expression levels in CT and CS. The expression of three genes (Prupe.1G111300, Prupe.1G251600, Prupe.6G309400) involved in the galactinol synthases, three genes (Prupe.3G289900, Prupe.4G001700, Prupe.5G169000) involved in raffinose synthase, and two genes (Prupe.5G075600, Prupe.3G0048300) involved in manninotriose synthases were found to be higher in CT than CS by qRT-PCR, similar to what was found in RNA-seq. Next, we compared the gene expression patterns in one peach cultivar under different cold temperatures and found that RNA-seq and qRT-PCR had excellent agreement (R^2 = 0.82663) (Figure 7). High agreement of

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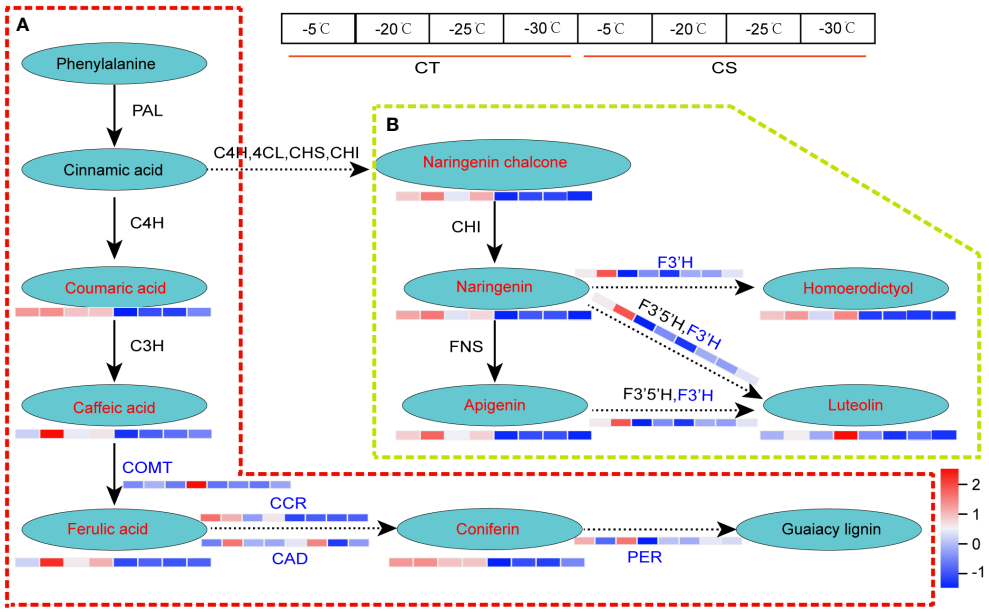

FIGURE 6
DEGs and DEMs involved in phenylpropanoid biosynthesis of peach under cold stress. **(A)** The DEGs and DEMs involved in the lignin biosynthesis. **(B)** The DEGs and DEMs involved in flavonoid metabolism. Letters in red and blue represent the metabolites and genes, respectively. The left and right four parts of the rectangles near the genes/metabolites represented the expression levels of DEGs/DEMs in CT and CS, respectively.

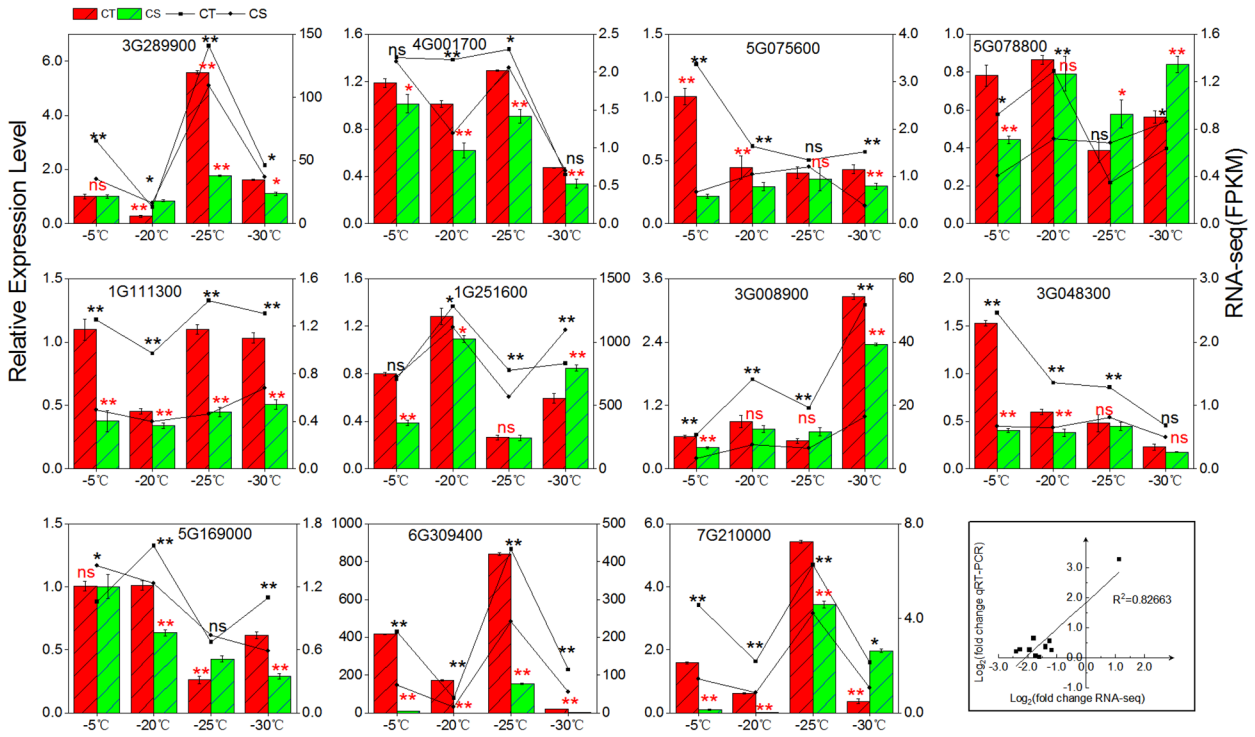

FIGURE 7
qRT-PCR validation. For the bars with standard errors and broken lines, log2 value of gene changes and log2 fold change were used for qRT-PCR and RNA-seq from three independent biological replicates (n = 3) using the $2^{-\Delta\Delta CT}$ method (left y-axis) and the FPKM value of RNA-Seq (right y-axis), respectively. ns, not significant; *, $p < 0.05$; **, $p < 0.01$.

RNA-seq and qRT-PCR in the gene expression patterns strongly supported the genes associated with cold tolerance of peach.

Discussion

Cold stress can seriously affect the growth and development of crops, resulting in a significant decline in crop yield, which is a universal and concerned problem in the world. In this study, we analyzed the transcriptomes and metabolomics of peach branches to explore the potential links between the expression of cold responsive genes and metabolites accumulation.

Galactose metabolism contributes greatly to cold stress in peach

Soluble sugars, as osmotic protective substances in plants, can improve the cell water potential, increase the cell water holding capacity and reduce the cytoplasmic freezing point (Brizzolara et al., 2018; Liang et al., 2022). Therefore, the change and range of soluble sugar content under low temperature stress are directly related to the cold tolerance of plants. In this study, the concentrations of soluble sugars (e.g., galactose, manninotriose, raffinose, glucose and fructose) were greatly increased in the two peach cultivars under cold stress. Further, their contents were higher in CT than CS (Figure 2D). It has been reported that chilling stress could cause a significant increase in glucose and fructose concentrations in wild strawberry (Shen et al., 2022). Similarly, we found that low temperature can cause a sudden increase in the electrolyte leakage rates (ELR) in CS rather than in CT under -20°C/12 h treatment (Figure 1A). Thus, it was proposed that the higher accumulation of soluble sugars may function in maintaining the stability of cell membrane structure in CT, which facilitated to its strong cold-tolerance ability. In addition, the accumulation patterns of galactose, manninotriose, raffinose, glucose and fructose between CT and CS correlated well with the expression profiles of their structural enzyme genes in the galactose metabolism pathway (Figure 5). Studies have shown that overexpression of cold-inducible galactinol and raffinose synthase increased the levels of galactinol and raffinose and further conferred higher tolerance to cold stress in transgenic rice (Shimosaka and Ozawa, 2015). Meanwhile, exogenous replenishment of raffinose could recover the cold tolerance in trifoliate oranges by the modulation of raffinose synthesis (Khan et al., 2021).These results indicated that genes and metabolites involved in galactinol metabolism may play a key role in cold tolerance of peach.

Transcription factors (TFs) have been reported to play a molecular switch role of many genes in plants under various environmental stresses (biotic and abiotic) (Rehman et al., 2021; Liu et al., 2022). As one of the most critical TFs functioning in the low-temperature signal transduction, C-repeat binding factors (CBFs) regulate the downstream gene expression, promote the synergy of multiple functional genes, and enhance the adaptability and resistance of plants (Gilmour et al., 2000; Liu et al., 2022). It has been reported that overexpression of CBFs can stimulate the synthesis of soluble sugars (e.g., sucrose, raffinose, glucose and fructose) and improve the cold tolerance of pomegranate (Wang et al., 2022). In this study, we identified three CBF genes (Prupe.4G242700, Prupe.5G090100 and Prupe.5G090000) were significantly upregulated in peach branches under cold stress (Table S5). In addition, CBF1, CBF2 and CBF3 are rapidly induced in response to low temperature, encode closely related AP2/ERF DNA-binding proteins that recognize the C-repeat (CRT)/dehydration-responsive element (DRE) DNA regulatory element present in the promoters of CBF-regulated genes in *Arabidopsis thaliana* (Park et al., 2015). We identified two genes encoding AP2/ERF (Prupe.2G220100 and Prupe.1G037700) domain-containing proteins in the turquoise module (Figure 4D, F). The AP2/ERF TFs have been reported to play a regulatory role in plants in response to abiotic stress (Xie et al., 2022; Yu et al., 2022). Overexpression of TERF2/LeERF2 TF in tomato and tobacco significantly increased their cold tolerance capacity (Zhang and Huang, 2010). Moreover, AP2/ERF TFs were highly expressed in peach with strong cold resistance under low temperature stress (Niu et al., 2020). These findings suggest AP2/ERF TFs closely related to the capacity of cold tolerance of peach, especially under chilling stress.

Several studies have shown that the abiotic stress tolerance of some plants is improved by the synthesis of protective metabolites (e.g., galactinol and raffinose) *via* the overexpression of heat shock TFs (HSFs) (Nishizawa et al., 2006; Lang et al., 2017). In the present study, galactinol and raffinose were specifically accumulated in CT under cold stress (Figures 2D, 5A). In addition, the gene expression of HSFs was significantly upregulated by cold and higher in CT than CS (Table S4, S5). Plant HSFs, as a kind of crucial regulators in network regulation, can respond to multiple biotic and abiotic stresses and confer various tolerances in plants. Therefore, it was proposed that the high expression of CBFs and HSFs in CT could be more beneficial to cope with cold stress.

Lignin biosynthesis plays key roles in cold stress in peach

Phenylpropanoid biosynthesis is one of the most important metabolisms in plants, generating an enormous array of secondary metabolites, such as lignin and flavonoid (Dong and Lin, 2021). Lignin in plants is synthesized *via* the lignin specific biosynthesis pathway which is a downstream pathway of the common phenylpropanoid pathway, and many enzymes are involved in this process (Figure 6). For instance, the higher expression of PALs (PUMP6L and V1SQAY) in cold-tolerant peanut could enhance the accumulation of coniferin accumulation and cold tolerance (Wang et al., 2021). In this study, we found that four key enzyme genes (Prupe.5G134400, COMT; Prupe.5G004900, CCR; Prupe.1G565200, CAD; Prupe.3G115300, PER) from the phenylpropanoid metabolism and downstream G-lignin biosynthesis were more abundant in CT than in CS (Figure 6; Table S7). COMT is a key enzyme of lignin synthetic pathway

which catalyzes the caffeic acid to generate ferulaic acid. In addition, CCR is the first key enzyme of lignin specific pathway and catalyzes the conversion of ferulaic acid to coniferin. Meanwhile, WGCNA analysis showed that CAD was relatively higher in CT than CS and identified as one of the hub genes in the turquoise module (Figures 4D, 5F). Overall, high expression of COMT, CCR, CAD and PER genes in CT could drive more carbon flux into the phenylpropanoid pathway and might be associated with the accumulation of coniferin and the enhancement of cold tolerance.

Flavonoids biosynthesis response to cold stress in peach

Flavonoids are the secondary major metabolites derived from the plant phenylpropanoid pathway that plays an important role during the plant development (Wang et al., 2020). They can affect the basic physiological metabolism, stress, and disease resistance response in plants. Studies have demonstrated that flavonoids play efficient roles in antioxidation and ROS scavenging. Under cold stress, 19 flavonoids were upregulated in the freezing-tolerant kiwifruit but not changed in freezing-sensitive kiwifruit (Sun et al., 2021). The over expression of flavonoid synthesis associated genes in plants can promote the accumulation of flavonoids and further significantly enhance the resistance of plants to environmental stresses. The C4H, a member of the cytochrome P450 monooxygenase superfamily, has been reported to controll the synthesis of p-coumaric acid from transcinnamic acid (Chen et al., 2007). The CHS catalyzes the condensation of malonyl-CoA and 4-coumaroyl-CoA to naringenin chalcone, which is the substrate for CHI and converted to naringenin (Rani et al., 2012). At the meantime, lack of F3'5'H in grapes can restrict the presence of quercetin, kaempferol, myricetin and syringetin derivatives (Bellés et al., 2008). In cucumber, C4H has been reported to be involved in the drought defense (Jeong et al., 2006). The CHS from Abelmoschus esculentus can regulate the flavonoid accumulation and abiotic stress tolerance in transgenic Arabidopsis (Wang et al., 2018). In this study, we found flavonoid 3'-monooxygenase (EC=1.14.14.82, F3'H) upregulated in CT under cold stress but not changed in CS peach branches at -20°C, indicating that F3'H may promote the accumulation of flavonoids and enhance the cold tolerance of peach (Figure 6). It has been reported that cold stress could lead to a significant increase in proteins (e.g., anthocyanidin reductase and flavonoid 3' hydroxylase) related to flavonoid biosynthesis in CT. Thus, it was proposed that the higher expression of flavonoid 3'-monooxygenase probably led to maintain a higher level of flavonoid 3' hydroxylase protein in CT, which suggested that they might play roles in mitigating the cold stress. Nagamatsu and colleagues reported that the overexpression of F3'H gene can increase the content of quercetin with antioxidant activity and improve the tolerance of soybean to environmental stress (Nagamatsu et al., 2007). The F3'H gene was also identified with higher expression in dormancy (cold-tolerant) variety than non-dormancy (cold-susceptible) variety of alfalfa under low temperature (Liu et al., 2022). Simultaneously, exogenous application of fulvic acid can improve the drought stress in tea by increasing F3'H activity, and enhance the yield of flavonoids, including kaempferol, quercetin and myricetin (Sun et al., 2020). Our results further demonstrated that the upregulation of some key biosynthetic genes and metabolites involved in flavonoids may play an essential role in response to cold stress in peach.

Conclusion

In the present study, we analyzed the metabolomic and transcriptomic profiles of CT and CS peach branches exposed to cold stress and identified a set of important cold-responsive genes and metabolites. Soluble sugars, flavonoids, and lignin, such as galactose, manninotriose, raffinose, glucose, homoeriodictyol, luteolin and coniferin, were found to play important roles in 1-year-old peach branches in response to cold. We found that the galactose metabolism, phenylpropanoid biosynthesis and flavonoids biosynthesis pathway were associated with the cold tolerance of peach. This is the first time to study the metabolomics and transcriptomics of peach branches under cold stress. The findings will improve our understanding towards the molecular regulation mechanisms of cold defense in peach and other plants.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA924510.

Author contributions

YL carried out the experiment, collected and organized data and wrote the manuscript. QT and ZW participated in designing the experiment and directed the study. RC, HC and SL, reviewed the manuscript and organized the data. GL, corresponding author, raised the hypothesis underlying this work, designed the experiment, and helped organize the manuscript structure. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1153902/full#supplementary-material

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Genome-wide association analysis provides insights into the genetic basis of photosynthetic responses to low-temperature stress in spring barley

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Low-temperature stress (LTS) is among the major abiotic stresses affecting the geographical distribution and productivity of the most important crops. Understanding the genetic basis of photosynthetic variation under cold stress is necessary for developing more climate-resilient barley cultivars. To that end, we investigated the ability of chlorophyll fluorescence parameters (F_VF_M, and F_VF_0) to respond to changes in the maximum quantum yield of Photosystem II photochemistry as an indicator of photosynthetic energy. A panel of 96 barley spring cultivars from different breeding zones of Canada was evaluated for chlorophyll fluorescence-related traits under cold acclimation and freeze shock stresses at different times. Genome-wide association studies (GWAS) were performed using a mixed linear model (MLM). We identified three major and putative genomic regions harboring 52 significant quantitative trait nucleotides (QTNs) on chromosomes 1H, 3H, and 6H for low-temperature tolerance. Functional annotation indicated several QTNs were either within the known or close to genes that play important roles in the photosynthetic metabolites such as abscisic acid (ABA) signaling, hydrolase activity, protein kinase, and transduction of environmental signal transduction at the posttranslational modification levels. These outcomes revealed that barley plants modified their gene expression profile in response to decreasing temperatures resulting in physiological and biochemical modifications. Cold

tolerance could influence a long-term adaption of barley in many parts of the world. Since the degree and frequency of LTS vary considerably among production sites. Hence, these results could shed light on potential approaches for improving barley productivity under low-temperature stress.

KEYWORDS

Hordeum vulgare L., chlorophyll fluorescence, photosystem II photochemistry, quantitative trait nucleotides (QTNs), Mixed linear model (MLM), Abscisic acid (ABA) signaling, protein kinase, post-transcription modification

1 Introduction

Environmental factors, particularly those affecting temperature and availability of water are the main factors of in plant growth and development. The low-temperature polar regions and Oceans cover about 80% of the Globe's land. One-third only of the global lands are free of ice, and 42% of this area is commonly under temperatures below -20°C (Junttila and Robberecht, 1999). In such regions, plants require specific processes to survive exposure to low temperatures. Most plants have developed a degree of cold tolerance, which is usually based on a combination of the length of exposure to cold stress and the minimum temperature experienced (Janska et al., 2010). Therefore, the growth and development of plants are affected by temperature variations in the majority of temperate regions on Earth (Nilsen et al., 1996). The problem is expected to expand due to climatic changes, particularly in Northern Europe and Canada. Cold stress impacts plant growth and crop productivity, causing substantial yield reductions (Junttila and Robberecht, 1999). Plants vary in their responses to freezing (<0°C) and chilling (0-15°C) temperatures (Browse and Xin, 2001). Damage appears when low temperatures overlap with sensitive stages of plant growth.

In barley (*Hordeum vulgare L.*), yield and grain quality are the key challenges in maintaining the constant growth of the agriculture industry. Though significant advances have been made in the genetic gains for yield and grain quality in barley, the crop rarely reaches its full yield potential because of seasonal variations including the low temperatures prevailing early and late in the growing season. Barley is mainly classified into three types of growth habits: spring, winter, and facultative. Winter barley is sown in the autumn because it requires a cold period (vernalization) to flower. Facultative types do not require vernalization, some lines possess levels of low-temperature tolerance comparable to winter barley. While, spring barleys are sown in the spring and do not require vernalization, and possess no perceivable level of low-temperature tolerance (Rizza et al., 2011; Zitzewitz et al., 2011). Spring barley is a main crop across the Canadian prairies and is used for food, malting, and general purposes (feed and forage). Spring barley yields about 20% less than winter barley, in areas where it is adapted (Kling et al., 2004). Though, spring barley genotypes are normally not as tolerant to cold as other winter cereals. Cold tolerance in barley has been a challenging phenomenon to develop with conventional breeding approaches. It is best to evaluate and screen breeding material under uniform cold stress in the laboratory as screening for winterhardiness in the field is rarely reliable. In 2021, barley-seeded areas consisted of 50.4% malting barley, 42.3% general purpose barley, and 2.3% food barley in the western Canada (Marta and Tricia, 2020). In poorer countries, barley is an essential food source (Grando and Macpherson, 2005), affording harvestable yields in locations that are harsh and marginal for crop production. Current research has categorized barley as a true functional food in more developed societies. The barley's grain is particularly high in soluble dietary fiber, which significantly declines the risk of serious diseases such as type II diabetes. The USA Food and Drug Administration (FDA) has approved cell-wall polysaccharides from barley grain as a human health claim (Collins et al., 2010).

Selection for cold tolerance/susceptibility in barley varieties can be associated with reliable screening techniques. Traditional methods, such as measuring survival rates and plant re-growth, are time-consuming and inaccurate in quantifying the level of cold tolerance (Novillo et al., 2004). Some techniques focus on injuries to the plasma membranes that result in the leakage of electrolytes from plant tissues (Steponkus et al., 1990). The electrolyte leakage is easily quantified by conductivity measurements (Rohde et al., 2004). For such investigations, plants are exposed to freeze-thaw cycles with minimum temperatures usually ranging from -1 to -50°C, and electrolyte leakage caused by cooling is then measured. The methods to quantify the cold tolerance of plants should be non-invasive and applicable at a high-throughput screening rate so that the underlying genetic determinations can be effectively quantified and established (Mishra et al., 2011). Evidence suggests that chlorophyll fluorescence emission can be used effectively in high-throughput screening of plants' low-temperature tolerance (Mishra et al., 2011).

Photosynthesis is one of the most essential and complex physiological mechanisms in all plants and is influenced in all periods by stresses (Baker, 1996). Because the photosynthesis mechanism regulates multiple cellular processes, including photosystems and photosynthetic pigments, electron transport system, and CO_2 reduction events, any stress-induced damage can

completely decrease the photosynthetic capacity of green plants (Lawlor and Cornic, 2002; Ashraf and Harris, 2013). Cold stress influences the photosynthetic apparatus through the suppression of photosystems and pigment modifications, thylakoid membranes, photosynthesis-related enzyme activity, chlorophyll fluorescence, gas exchange, and reduced CO_2 assimilation in addition to the electron transport rate. Photosynthesis converts light energy into redox equivalents (NADPH) and ATP, essential elements for plant growth and development. On the other hand, cold stress prevents thylakoid electron transport *via* aggregate membrane viscosity. It disorders light energy process trapping by Photosystem I and Photosystem II antenna. The enhancement of energy trapping beyond its regular ability results in a high-energy state, which eventually leads to the overproduction of reactive oxygen species (ROS) (Ashraf and Harris, 2013).

Chlorophyll fluorescence parameters have become a common and powerful technique in plant breeding to investigate the impact of stresses on the photosynthetic mechanism (Guidi et al., 2019). It can be used as a representative of plant stress as environmental stresses, such as extremes of temperature, water, and light availability can decrease the capability of a plant to metabolize normally. This can lead to an imbalance between the light energy absorption *via* chlorophyll and the utilization of energy in photosynthesis (Schreiber, 1986; Schreiber et al., 1986). Chlorophyll fluorescence is an indicator of photosynthetic energy that responds to alterations in Photosystem II (PSII) photochemistry and consequently represents a rapid and efficient tool to assess the capacity of the photosynthetic mechanism at low temperatures (Lichtenthaler and Rinderle, 1988). The function of the photosynthetic machinery can be evaluated by measuring the ratio of chlorophyll variable fluorescence (F_V) over the maximum fluorescence value (F_M), which reveals the efficiency of the excitation capture by open photosystem II reaction centers (Fracheboud et al., 1999; Rizza et al., 2001). An association between the decrease of F_V/F_M and frost tolerance during hardening and after freezing was found in winter wheat (Clement and Hasselt, 1996), spring and winter barleys, and rye (Smillie and Hetherington, 1983).

During cold acclimation, different physiological and biochemical modifications occur, such as the synthesizing of proline, soluble sugars, and cold-resistance proteins to maintain proteins (Hannah et al., 2010). These events play significant roles in the response to cold stress by controlling the ice crystal formation, osmotic potential, reactive oxygen species, and stability of cell walls and membranes (Ding et al., 2019). Some elements, including protein kinases, messenger molecules, phosphatases, and transcription factors (TFs), have been reported for cold-stress signaling pathways (Ding et al., 2019). Understanding the genetic regulation underlying the photosynthesis process under low-temperature stress in barley can facilitate the development of climate-resilient and high-yielding cultivars in a short period of time. Since the first draft reference of the barley genome was released (Schulte et al., 2009; IBGS et al., 2012), high-quality genome sequences have been published (Sato et al., 2016; Mascher et al., 2017; Dai et al., 2018). These tools facilitated barley research and became precious resources for the improvement and comparative genomics studies including genome-wide association studies (GWAS) and QTL mapping. In addition, advances in genome mapping and sequencing technologies have made possible the cost-effective assembly and sequencing of hundreds of genotypes with large-genome species, such as barley (5 Gb, haploid genome size) (Monat et al., 2019; Jayakodi et al., 2020). The 9K SNP array is a commonly powerful GWAS tool for identifying specific allele variants (Comadran et al., 2012).

Evidence of chlorophyll fluorescence and photosynthesis variability in barley has been reported (Kocheva et al., 2004; Guo et al., 2007; Bertholdsson et al., 2015), indicating opportunities for genetic improvement and selection. Several analyses have been reported for quantitative trait loci (QTL) for chlorophyll fluorescence under low oxygen concentration (Bertholdsson et al., 2015), post-flowering under drought (Guo et al., 2007), early short-time drought tolerance (Wojcik-Jagla et al., 2013). Understanding the mechanisms of low-temperature tolerance and barley productivity is one of the main challenges facing scientists and breeders today. So far, the effect of low temperatures on chlorophyll fluorescence as an indicator of photosynthetic energy conversion in barley plants is still well unknown. Therefore, the aims of the present study were (i) to investigate the response of barley genotypes at three- to four-leaf stages to low-temperature stress including cold acclimation and freezing shock; (ii) to identify genetic loci associated with the chlorophyll fluorescence trait, by using genome-wide association studies; (iii) to identify key genes related to chlorophyll fluorescence before and after the LTS conditions and, (iv) introgression of cold tolerance into spring barley.

2 Materials and methods

2.1 Plant materials

A panel of 96 spring barley genotypes from eight breeding programs of Western Canadian barley breeding programs was used in this study. All genotypes were evaluated at different times between 1994 and 2006 (Beattie et al., 2010) and selected based on their high seed yield and percentage of winter survival and cold hardiness (ranging from 50 to 100%). The association panel consists of advanced breeding lines, commercial varieties, two-rowed lines used to investigate beta-glucanase and limit dextrinase, and elite germplasm, which has been developed for the Western Two-Row Cooperative Registration. Several diversity array technology (DArT) markers were detected for grain quality using 91 genotypes of this panel and disease ratings for true loose smut and net blotch (Beattie et al., 2010). In our previous study, an important pattern of genetic diversity was detected in the population studied. Our previous study revealed that this panel is differentiated due to the ear-row type and breeding program origins into five subpopulations (Capo-Chichi et al., 2023).

2.2 Plant growth conditions

Two experiments namely cold acclimation and freezing shock evaluations were conducted to study the impact of the low-

temperature stress (LTS) on the 96 spring barley genotypes. In both experiments, ten seeds of each genotype were germinated in 8 cm×8 cm×7 cm pots containing pasteurized field soil (wet soil) in the growth chamber. The seeds in each pot were covered by an equal amount of soil to enhance uniform emergence. Pots were placed in a growth chamber at 20°C/15°C and a photoperiod of 12/12 h light/dark cycle. The experiments were designed in a completely randomized design (CRD) in three replicates. At the three-leaf seedling stage (on day 14), the germination rate was recorded for all genotypes then, seedlings were thinned to five plants per pot before the treatments.

2.3 Quantification of chlorophyll fluorescence

For cold acclimation treatment, seedlings at a three-leaf stage were placed in a programmable cold chamber. The initial temperature in the cold chamber was -1°C. The temperature was raised between 3°C and 5°C, then gradually decreased to -12°C over a duration of four hours (Supplementary Figure 1), to ensure that nucleation occurred evenly. For the freezing-shock treatment; pots were moved during the day from the growth chamber to a programmable cold chamber. The initial temperature was -18°C and the temperature was immediately raised to -6°C. Pots were exposed to temperatures that gradually declined from -6°C to -11°C for 75 min (0.06°C per min) (Supplementary Figure 1). In order to record chlorophyll fluorescence parameters for the same seedlings each time, the measured seedlings were numbered from 1 to 5 in both experiments. After the treatments, all seedlings were returned to the growth chambers (normal condition). A week later, a frost's survival rate in each pot was recorded and the genotypes were characterized according to their ability to tolerate the low temperatures. Frost survival was calculated visually twice; once after the acclimation using a 1-5 symptoms scale, where: 1 (dead plant), 2 (trace of life; low survival potential), 3 (intensive damage; less than half of the coleoptile leaf green), 4 (moderate to minimal damage; limited to leaf edges), 5 (no damage).

2.4 Fluorescence measurements

The fluorescence measurement values were collected from 96 genotypes after cold acclimation treatment, while after the freezing-shock treatment 22 genotypes died out completely, and the results were collected from the remaining 74 genotypes. The measurements were assessed in the greenhouse on the second leaf that completely expanded. A grid with a 33 mm hole diameter was clipped on the inner section of the leaf. For a dark-adapted period, the leaf clips were left for 20 min. The measurement probe was trimmed later and the reading values were taken by using a portable chlorometer OS-30P (Opti-Sciences). Plants were allowed to dark-adapt overnight to ensure that all PSII centers are open, and the lights were turned off in the greenhouse until measurements were concluded (between 1 and 3 a.m.). The chlorophyll fluorescence parameter was measured according to the formula; (Ghassemi-Golezani et al., 2008).

$$^{F_V}/_{F_M} = (F_M - F_0)/F_M$$

Where; F_0; F_M and F_V are primary fluorescence parameters while F_V/F_M and F_V/F_0 are fluorescence ratios as the following.

F_0: minimum fluorescence occurs while all antenna sites are supposed to be open (dark-adapted). F_M: maximum fluorescence intensity under exposure to the excitation source while all antenna sites are assumed to be closed. F_V: variable fluorescence. F_V/F_M: the maximum yield of primary photochemistry. F_V/F_0: maximum efficiency of PSII. The F_V/F_M is the ratio of variable fluorescence to maximal fluorescence, which is an indicator of maximum quantum efficiency and gives important information concerning the effect of environmental stress on the plant. The F_V/F_0 ratio is a very sensitive indicator of the maximum efficiency of photochemical processes in PSII and/or the potential photosynthetic activity of healthy and stressed plants (Lichtenthaler & Rinderle, 1988). Chlorophyll fluorescence measurements were recorded at three different times for both experiments: before the treatment (BF), two hours after the treatment (AF), and 24 hours after the treatment (DF). Heritability in the broad sense (H_b) was calculated before and after the treatments according to Elakhdar et al. (Elakhdar et al., 2016).

Heritability in the broad sense; $H_b^2 = V_G^2/V_P^2 \times 100$

, where, V_G; genetic variance and V_P; phenotypic variance

2.5 DNA isolation and genotyping

Genomic DNA was isolated from the young leaf of the 3-4 leaf stage using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNA quality was quantified at 230 nm and then qualified at 230/260 and 260/280 absorption ratios, respectively. Genotypes were genotyped on an Illumina 9K Barley Infinium iSelect SNP assay (Comadran et al., 2012) at the USDA-ARS genotyping laboratory (Fargo, ND). Physical positions of markers were taken from the barley pseudomolecule assembly by the James Hutton iSelect annotation (https://ics.hutton.ac.uk/50k/) and Barley DB: Barley Bioresources Database (http://earth.nig.ac.jp/~dclust/cgi-bin/index.cgi?lang=en). SNPs with unknown chromosomal position, monomorphism, and SNPs with missing values greater than 20% were eliminated. For association analysis, markers with genetic and physical positions and with minor allele frequency (MAF) of 0.05 or greater were used. After performing these filters, a total of 5063 high-quality SNP markers remained in the dataset and were used for subsequent GWAS.

2.6 Genome-wide association studies

Using the 5063 informative SNPs marker, a genome-wide association study (GWAS) was performed on 96 barley genotypes to identify genetic regions linked to photosynthesis after the cold.

GWAS was performed using a mixed linear model (MLM) (Zhang et al., 2005), using the *R* package GAPIT (Lipka et al., 2012). The MLM model was selected due to its strength and power for detecting marker/trait associations. This model is based on the genotype data (G), population structure (Q) as fixed effects, and the Kinship-matrix (K) as random effects (Pasam et al., 2012). In our previous study, Genome-wide LD decay was plotted as R^2 of an SNP marker against the corresponding genetic distance using the mixed-model method (Capo-Chichi et al., 2023). A strong LD with an approximate average value of 0.021 when the distance was 0.391 cM was observed among the 5063 marker pairs in the studied population. The kinship matrix was assessed using the whole set of markers. To identify significant quantitative trait nucleotides (QTNs), the Bonferroni corrected significance threshold was determined, based on the reduced marker set of 5063 SNP and a significant level of $p<0.001$ with a corresponding threshold of (-log10 p ≥ 3). Manhattan plots mapped the chromosome position on the x-axis against–$\log_{10}$ (*P*-value) on the y-axis of each marker. The quantile-quantile (QQ) was plotted between the observed and the expected -$\log_{10}$ P values.

2.7 Candidate gene prediction

Genes with significant markers associated with the chlorophyll fluorescence, their locations, and corresponding annotations were retrieved from the BARLEYMAP platform (http://floresta.eead.csic.es/barleymap/) (Cantalapiedra et al., 2015) version of the MorexV3 genome (Comadran et al., 2012). The physical positions of markers were revealed from the Barley Physical Map IBSC (IBGS et al., 2012), the POPSEQ map (Mascher et al., 2013), and the Morex Genome Map (Mascher et al., 2017). Gene Ontologies (GO) enrichments were obtained from the Amigo of Gene Ontology platform (http://geneontology.org) (Pomaznoy et al., 2018) and the Gene Ontology and GO annotation platform; QuickGO (https://www.ebi.ac.uk/QuickGO/). Gene co-expression for the identified genes was revealed from the Global gene co-expression networks (GCNs) database PlantNexus (Zhou et al., 2022).

2.8 Introgression of cold tolerance into spring barley from winter barley

Four two-rowed winter barleys '02Ab671', '02Ab431', '02Ab669' and '2Ab08X05W061-208' were obtained from the United States Department of Agriculture (USDA). The genotypes were selected based on excellent malt extract, high seed yield, and their high percentage of cold hardiness and winter survival (50 to 100% across different locations in the northern United States). Vernalization was performed in growth chambers at 5°C for 8 h of light for seedlings at the two-to-three-leaf stage. After ten weeks of vernalization, seedlings were moved to 20°C with 16/8 h light/dark, and humidity closely monitored. Upon flowering, eighteen crosses were made between spring and winter barleys (Supplemental Table 1).

3 Results

3.1 Germination and survival rates

Twenty-nine-day-old plants from 96 spring barley genotypes were used to study the impact of low-temperature stress: cold acclimation and freezing shock. The survival rates were assessed using a visual damage scale. In the cold acclimation and freeze shock experiments, the frequency of the genotypes, and distribution to germination were recorded for all genotypes (Figure 1). The results revealed that the time required for the emergence onset varied from 14 to 19 days for the genotypes studied (Figure 1). At 14 days, the emergence rates ranged from 0 to 38%, while on day 19, the emergence rates varied from 4 to 100%. The time required for 50% emergence ranged from 15 to 22 days, demonstrating that some genotypes have the ability to emerge faster than others. We observed several lines that exhibited adequate performance at 5°C.

Under cold acclimation, we observed that survival frequencies ranged from 30 to 100% with the majority grouped between 80 and 90% (Figure 2). Under freezing-shock stress, 22 genotypes were dead while survival rates ranged from 0 to 100% with the majority of the genotypes between 20 and 30% survival (Figure 2). The

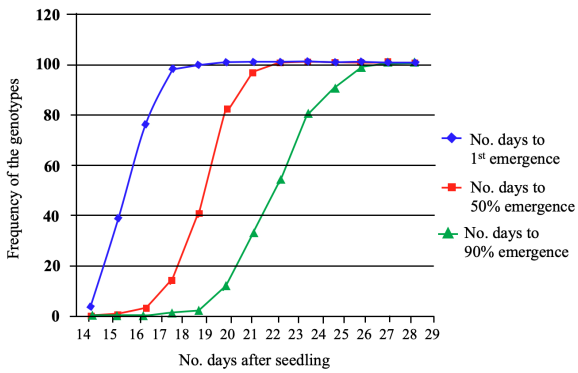

FIGURE 1
The emergence percentage (G%) of the first, 50% (T50) and greater than 90% (T>90) emergence of 96 spring barley genotypes. G% was calculated by dividing the number of emerged seedlings by the number of seeds planted for each seed plot and multiplying the product by 100.

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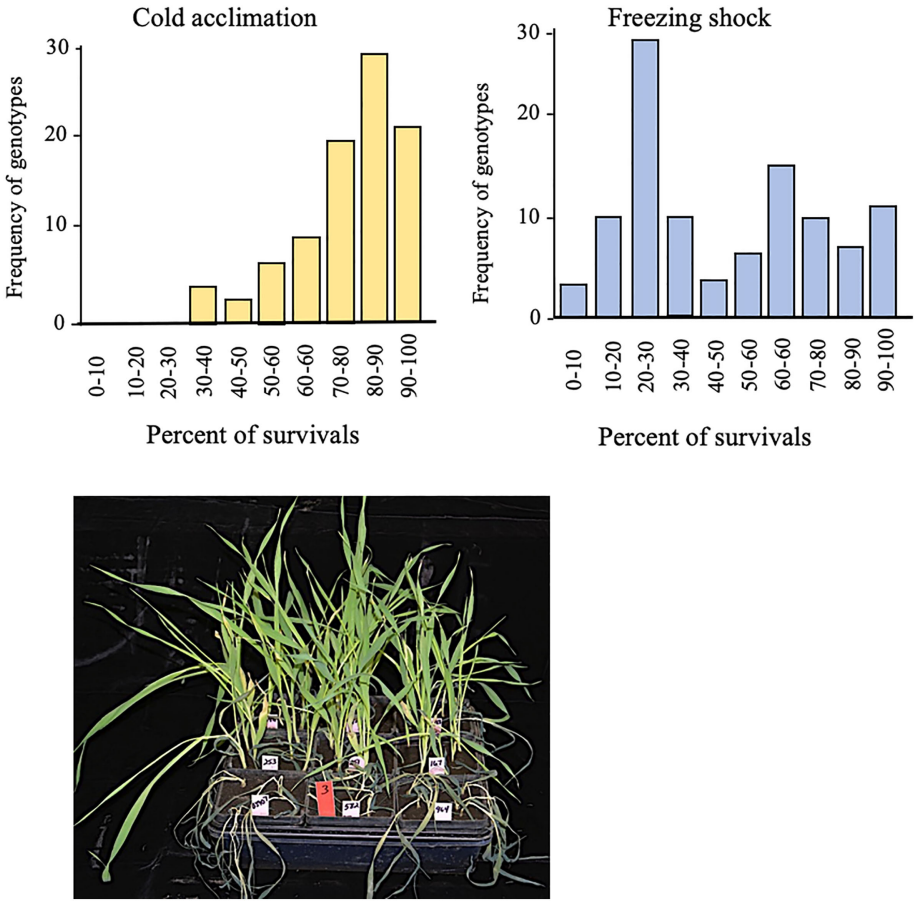

FIGURE 2
Survival rates distribution for 96 genotypes of spring barley one week old after low-temperature stress conditions. Cold acclimation (left) and freezing shock (right). View for the effect of freezing-shock treatment on the spring barley at the three-leaf stage (lower panel). Twenty-two genotypes died out completely. Low-temperature stress is induced at the three-leaf stage.

seedlings stressed under these conditions suffered leaf wilting as the duration of stress was prolonged. These findings suggest that the cold acclimation treatment improved freezing tolerance more than the freezing shock treatment.

3.2 Phenotypic evaluation of chlorophyll fluorescence under LTS

Five chlorophyll characteristics including parameters of transient fluorescence were collected for cold acclimation and freezing shock treatments. The parameters represent the photochemical efficiency of PSII behavior affected by LTS (Table 1). A high level of phenotypic variation was detected in all traits measured under both low-temperature stress conditions (Table 1). Before the treatments (BF), the highest H_b value was observed for F_V/F_0 (97.9% and 93.6% for cold acclimation and freezing shock treatments, respectively) (Table 1). Low to high values of heritability (H_b) were observed under LTS treatments. Two hours after treatments (AF), F_V/F_0 exhibited also the highest value of heritability H_b of 94.4% and 96.5%, cold acclimation and freezing shock, respectively, whereas, after 24 hours of stress (DF), F_V/F_0 and F_M had the highest values 92.2% and 73.5, respectively) (Table 1). These results suggest adequate variability and different responses to low-temperature stress that exists in the barley material studied. Normal distribution was detected for the chlorophyll fluorescence parameters, F_V/F_0 and F_V/F_M, before and after the low-temperature conditions (Figure 3). This indicates that barley's chlorophyll fluorescence traits are quantitatively inherited and controlled by multiple genes. In addition, the genotypes might vary in their photosynthetic mechanisms in response to low-temperature tolerance, which will impact the yield and ultimately the breeder. Thus, the difference in low-temperature tolerance between the genotypes provides a foundation for studying this phenomenon for GWAS and/or QTL studies.

Before the cold acclimation treatment (BF), the ratio of variable fluorescence to maximal fluorescence (F_V/F_M) values for lines ranged from 0.746 to 0.775, with a mean value of 0.76 (Table 1). The variable fluorescence to fluorescence occurring while antenna sites are supposed to be open (F_V/F_0) ranged from 2.943 to 3.453 with an average value of 3.22 (Table 1). After the exposure to low-temperature stress, the mean values of F_V/F_M and F_V/F_0 decreased significantly in both experiments compared with those before the treatment (Table 1). Two hours after cold acclimation stress (AF)

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TABLE 1 Chlorophyll characteristics-related traits under cold acclimation and freeze shock for 96 spring barley genotypes.

Treatment		Cold acclimatization					Freezing shock				
		Max	Min	Mean	SE	H^2_b (%)	Max	Min	Mean	SE	H^2_b (%)
	F_0	307	187	262.10	3.41	91.9	329.75	170.08	263.77	5.41	76.0
Before (BF)	F_V	1003	607	842.33	11.52	93.2	1016.67	558.42	844.94	15.57	57.4
	F_M	1308	797	1104.40	14.81	90.4	1345.33	730.17	1108.71	20.85	55.2
	F_V/F_M	0.775	0.746	0.76	0.00	95.1	0.78	0.75	0.76	0.00	53.9
	F_V/F_0	3.453	2.943	3.22	0.01	97.9	3.53	2.97	3.22	0.02	93.6
	F_0	357	183	260.03	5.21	88.1	313.17	179.33	261.44	3.88	86.6
2 hours (AF)	F_V	757	14	342.17	23.14	89.1	571.33	33.22	230.22	13.72	50.2
	F_M	1086	228	602.24	27.28	90.6	851.25	258.09	491.66	15.05	46.1
	F_V/F_M	0.667	0.062	0.39	0.02	90.5	0.55	0.10	0.30	0.01	81.4
	F_V/F_0	2.513	0.067	1.28	0.08	94.4	2.00	0.12	0.86	0.05	96.5
	F_0	295	111	209.42	5.45	97.0	270.58	127.08	194.26	4.99	72.8
	F_V	773	1	362.66	25.10	84.2	605.25	0.33	249.71	18.41	41.6
24 hours (DH)	F_M	1068	132	551.97	30.13	92.2	866.67	135.89	443.98	22.88	73.5
	F_V/F_M	0.714	0.001	0.38	0.02	88.4	0.656	0.014	0.298	0.02	72.2
	F_V/F_0	2.62	0.001	1.33	0.09	92.2	2.302	0.199	0.98	0.06	71.4

Max, Maximum; Min, Minimum; SE, the standard error. Broad-sense heritability (H_b). F_0; Minimum Fluorescence when all photosystem II (PSII) is open, F_V=F_M-F_0; Variable fluorescence, F_M; Maximum fluorescence when all PSII are closed, F_V/F_M; the ratio of photochemical efficiency of photosystem II (PSII), F_V/F_0; the ratio of maximum efficiency of photochemical processes in PSII of healthy and stressed plants.

treatment, the F_V/F_M values measured ranged from 0.062 to 0.667 with an average of 0.39 across the genotypes. The F_V/F_0 values ranged from 0.067 to 2.513, averaging 1.28. While the F_V/F_M values after 24 hours of cold acclimation treatment (DH) varied from 0.714 to 0.001, averaging 0.38 (Table 1). The F_V/F_0 values ranged from 0.001 to 2.62, averaging 1.33 (Table 1). After the freezing-shock treatment, 22 genotypes died out completely (Figure 2). F_VF_M values for genotypes ranged from 0.10 to 0.55 and 0.00 to 0.61 for AF and DH, respectively (Table 1). While the values measured for F_V/F_0 varied from 0.12 to 2.00, and from 0.00 to 2.15 for AF and DH, respectively (Table 1). The remaining 74 genotypes were used for the GWAS.

The result suggested that freezing-shock treatment caused a large decline in F_V/F_M and F_V/F_0 values in the non-hardy cultivars. During the recovery in the growth chambers, we found that this effect was irreversible and contingent on a threshold. In most cases, the irreversible effect was associated with F_V/F_M values below 0.220. For more hardy cultivars, the F_V/F_M values varied between 0.477 and 0.609 after freezing shock treatment, while the F_V/F_0 ranged from 1.832 to 2.413. Taken together, these results show that the decreases in the chlorophyll fluorescence indexes might reveal a reduction of PSII efficiency and plant death.

The comprehensive Pearson correlation analysis between the chlorophyll fluorescence-related traits; F_0, F_V, F_M, F_V/F_0, and F_V/F_M under low-temperature tolerance before (BF), two hours after (AF), and 24 hours after (DH) treatment are presented in histograms (Figure 4). For the AF readings, correlations between the parameters measured ranged from 0.628 between F_0 and F_V/F_0 to 0.992 between F_V and F_M at p = 0.0001. Meanwhile, a higher correlation (p = 0.0001) was observed under the DH stress condition between F_V and F_M, which ranged from 0.691 between F_O and F_V/F_M to 0.994 between F_V and F_M at p = 0.0001 (Figure 4A). A correlation analysis was also performed between the traits under freezing shock stress (Figure 4B). The correlations were moderate to high and ranged from 0.458 between F_M and F_0 to 0.968 between F_M and F_V under the AF stress condition (Figure 4 B). Under the DH condition, a positive correlation was detected between F_V/F_0 and F_V/F_M with all studied traits and ranged from 0.755 to 0.967 when p = 0.0001, respectively (Figure 4B).

3.3 Genomic regions associated with chlorophyll fluorescence traits

To investigate the genetic factors associated with low-temperature tolerance in barley, a GWAS analysis was conducted for the chlorophyll fluorescence related-traits from 96 spring barley genotypes with a set of 5063 high-quality SNP markers. The average r^2 value of the genome was 0.02, and the LD decay was found to start at an r^2 value of 0.38 and reached half-decay at 0.2, representing the genome's threshold distance for linkage analysis (Capo-Chichi et al., 2023). In total, 52 significant quantitative trait

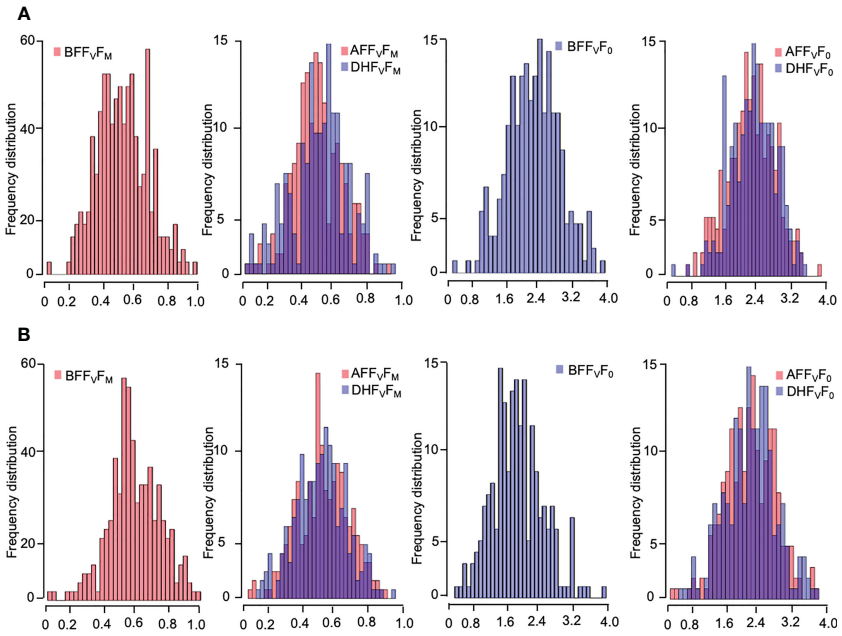

FIGURE 3
Frequency distribution of the chlorophyll fluorescence-related traits of four weeks-old barley plants under low-temperature stress; **(A)** cold acclamation and **(B)** freezing shock and overlap between them. Chlorophyll fluorescence parameters: $F_V/F_{M,}$ and F_V/F_0 values measured. Histograms with melt colors built with base R function (The R and Python graph galleries websites).

nucleotides (QTNs) were detected. Two significant SNPs ($P< 0.001$) were associated with chlorophyll fluorescence-related traits before low-temperature treatments, and 50 significant SNPs were observed under cold acclimation stress conditions (Table 2, Figure 5). All significant associations were found on chromosomes 1H, 3H, and 6H. While, under freezing-shock treatment, the remaining 74 cultivars used for the GWAS, the fluorescence data showed no significant associations between SNPs and chlorophyll fluorescence traits. Perhaps, this may be because of genetic drift, in which some versions of a gene have been lost because of random chance in this small population. Additionally, genetic diversity degrades more quickly in small populations than in large populations due to stochastic sampling error (genetic drift).

Before the low-temperature stress treatment, one significant MTA was identified for F_M and F_V, which is located on chromosome 1H (42.035-42.036 cM) (Table 2). The peak marker was SCRI.RS.152795 with likelihood ratio-based R^2 of 1.22 and explained 67.612 and 52.574% of the additive variances for F_M and F_V, respectively. The Manhattan and Q-Q plot for both F_M and F_V are shown in Figures 5A, B, respectively .

For the AF measurements under stress conditions, 39 significant QTNs were detected, three QTNs for F_M, ten QTNs for F_V, nine QTNs for $F_VF_{M,}$ and twenty for F_VF_0 (Table 2). Several QTNs were denoted in multiple chlorophyll fluorescence-related traits under both BF and/or AF conditions (Figure 6). There were reasonable correlations detected between chlorophyll fluorescence-

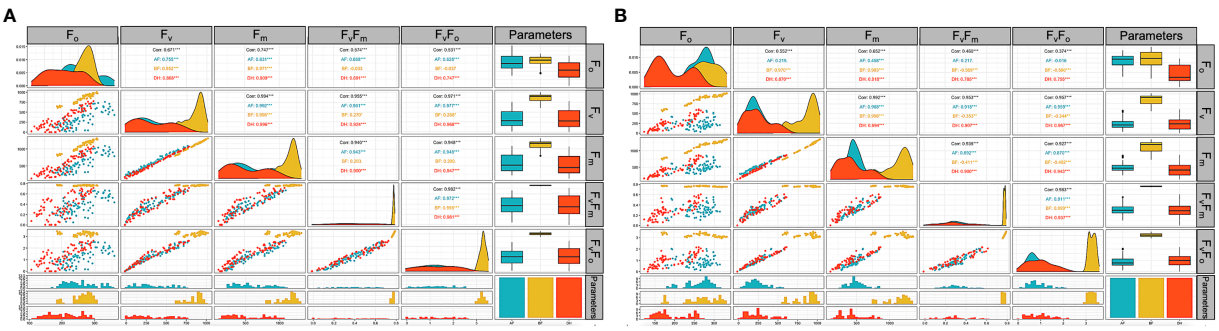

FIGURE 4
Correlation analysis of chlorophyll fluorescence parameters related traits of the three-leaf stage of 96 barley genotypes before treatment (yellow) low-temperature stress after two hours (blue) and 24 hours after the treatment (orange). Chlorophyll fluorescence parameters measured; F_0, F_V, F_M, $F_V/F_{M,}$ and F_V/F_0. **(A)**: cold acclimation treatment. **(B)**: freezing shock treatment. BF: Before treatment, AF: two hours after treatment. DH: 24 hours after the treatment. Correlation matrix built with base R function (ggally R package).

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TABLE 2 Significant quantitative trait nucleotides (QTNs) for chlorophyll fluorescence under cold acclimation condition.

Treatment	Trait	Marker	Chr	Position (bp)[a]	*P*-value	R^2	MAF	Allele	Effect
BF	F_M	SCRI.RS.152795	1H	420364483	7.75E-04	0.122	0.429	A/G	67.612
	F_V	SCRI.RS.152795	1H	420364483	7.90E-04	0.122	0.429	A/G	52.574
AF	F_M	SCRI.RS.219551	3H	431379904	6.15E-04	0.139	0.289	T/C	128.874
		SCRI.RS.155758	1H	539454103	4.33E-04	0.149	0.378	T/C	138.895
		SCRI.RS.165588	1H	539695184	3.54E-04	0.154	0.384	A/G	144.534
	F_V	SCRI.RS.219551	3H	431379904	3.59E-04	0.144	0.289	T/C	117.295
		BOPA1.7728.341	3H	434909031	5.94E-04	0.129	0.280	T/A	-114.505
		BOPA2.12.31368	3H	435526063	5.94E-04	0.129	0.280	A/G	-114.505
		SCRI.RS.181360	3H	440011663	5.94E-04	0.129	0.280	A/G	114.505
		SCRI.RS.138918	3H	446916578	5.94E-04	0.129	0.280	T/C	114.505
		BOPA1.ABC10084.1.2.363	3H	538660122	8.35E-04	0.120	0.179	C/G	-113.309
		SCRI.RS.144535	3H	538805589	8.35E-04	0.120	0.179	T/C	-113.309
		SCRI.RS.224360	3H	539228382	8.35E-04	0.120	0.179	T/C	113.309
		SCRI.RS.155758	1H	539454103	2.52E-04	0.154	0.378	T/C	123.775
		SCRI.RS.165588	1H	539695184	2.05E-04	0.160	0.384	A/G	128.682
	F_VF_M	SCRI.RS.202723	6H	13136770	8.74E-04	0.118	0.253	T/C	-0.084
		SCRI.RS.219551	3H	431379904	2.23E-04	0.157	0.289	T/C	0.094
		BOPA1.7728.341	3H	434909031	3.31E-04	0.145	0.280	T/A	-0.093
		BOPA2.12.31368	3H	435526063	3.31E-04	0.145	0.280	A/G	-0.093
		SCRI.RS.181360	3H	440011663	3.31E-04	0.145	0.280	A/G	0.093
		SCRI.RS.138918	3H	446916578	3.31E-04	0.145	0.280	T/C	0.093
		BOPA1.10126.999	3H	489991522	8.34E-04	0.119	0.295	A/G	-0.086
		SCRI.RS.155758	1H	539454103	6.70E-04	0.125	0.378	T/C	0.089
		SCRI.RS.165588	1H	539695184	5.43E-04	0.131	0.384	A/G	0.092
	F_VF_0	SCRI.RS.202723	6H	13136770	7.75E-04	0.118	0.253	T/C	-0.358
		SCRI.RS.219551	3H	431379904	1.53E-04	0.165	0.289	T/C	0.416
		BOPA1.7728.341	3H	434909031	2.58E-04	0.149	0.280	T/A	-0.407
		BOPA2.12.31368	3H	435526063	2.58E-04	0.149	0.280	A/G	-0.407
		SCRI.RS.181360	3H	440011663	2.58E-04	0.149	0.280	A/G	0.407
		SCRI.RS.138918	3H	446916578	2.58E-04	0.149	0.280	T/C	0.407
		BOPA1.2067.775	3H	449665313	9.70E-04	0.112	0.131	A/G	0.414
		BOPA2.12.31011	3H	462627529	9.70E-04	0.112	0.131	T/G	-0.414
		BOPA2.12.31393	3H	464907622	9.70E-04	0.112	0.131	T/C	-0.414
		BOPA1.1977.1385	3H	469771904	9.70E-04	0.112	0.131	A/G	-0.414
		BOPA1.2231.557	3H	473173134	9.70E-04	0.112	0.131	A/C	0.414
		SCRI.RS.114566	3H	482729752	9.70E-04	0.112	0.131	T/C	0.414
		BOPA1.4453.422	3H	482733343	9.70E-04	0.112	0.131	A/G	-0.414
		BOPA1.10126.999	3H	489991522	9.16E-04	0.113	0.295	A/G	-0.364
		SCRI.RS.137116	1H	536426484	8.61E-04	0.115	0.095	T/G	-0.477

(Continued)

TABLE 2 Continued

Treatment	Trait	Marker	Chr	Position (bp)[a]	*P*-value	R^2	MAF	Allele	Effect
		BOPA1.ABC10084.1.2.363	3H	538660122	5.66E-04	0.127	0.179	C/G	-0.386
		SCRI.RS.144535	3H	538805589	5.66E-04	0.127	0.179	T/C	-0.386
		SCRI.RS.224360	3H	539228382	5.66E-04	0.127	0.179	T/C	0.386
		SCRI.RS.155758	1H	539454103	9.83E-05	0.178	0.378	T/C	0.432
		SCRI.RS.165588	1H	539695184	7.95E-05	0.184	0.384	A/G	0.448
DH	F_M	SCRI.RS.155758	1H	539454103	3.10E-04	0.159	0.378	T/C	157.508
		SCRI.RS.165588	1H	539695184	2.55E-04	0.164	0.384	A/G	163.702
	F_V	SCRI.RS.155758	1H	539454103	2.19E-04	0.188	0.377	T/C	139.623
		SCRI.RS.165588	1H	539695184	1.73E-04	0.195	0.383	A/G	145.923
	F_VF_M	SCRI.RS.155758	1H	539454103	6.10E-04	0.144	0.378	T/C	0.114
		SCRI.RS.165588	1H	539695184	5.32E-04	0.147	0.384	A/G	0.118
	F_VF_0	SCRI.RS.155758	1H	539454103	3.47E-04	0.156	0.378	T/C	0.444
		SCRI.RS.165588	1H	539695184	2.95E-04	0.161	0.384	A/G	0.460

F_0; Minimum Fluorescence when all photosystem II (PSII) is open, F_V=F_M-F_0; Variable fluorescence, F_M; Maximum fluorescence when all PSII are closed, F_V/F_M; the ratio of photochemical efficiency of photosystem II (PSII), F_V/F_0; the ratio of maximum efficiency of photochemical processes in PSII of healthy and stressed plants.
BF; before the treatment AF; 2hr after the treatment. DH; 24hr after the treatment. Ch; chromosome number. [a] physical position of the markers based on (Mascher et al., 2017). MAF, minor allele frequency. Effect; additive effect. *Putative QTL that may be associated with multiple traits.

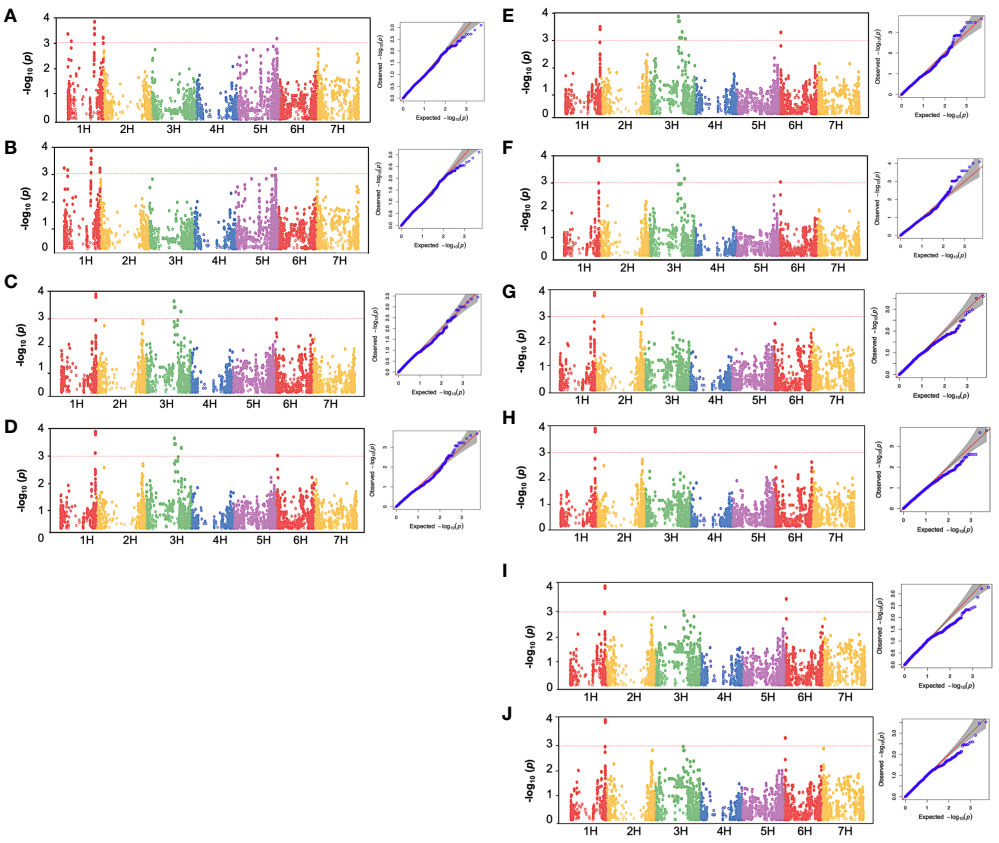

FIGURE 5
Manhattan plot and quantile-quantile (Q-Q) plot of GWAS for low-temperature stress in 96 spring barley genotypes showing significant association with chlorophyll fluorescence parameters. **(A)**: BFF_V, **(B)**: BFF_M, **(C)**: AFF_M, **(D)**: AFF_V, **(E)**: AFF_VF_M, **(F)**: AFF_VF_0, **(G)**: DHF_M, **(H)**: DHF_V, **(I)**: DHF_VF_M, **(J)**: DHF_VF_0. BF: Before cold treatment, AF: two hours after cold treatment. The x-axis indicates the relative density of identified reference genome-based SNPs mapped on seven chromosomes. The y-axis represents the -$\log_{10}$ *P*-values for the association power for each SNP with the trait expressed. The peaks above-dashed lines indicate significance thresholds (- log10 *P*< 3). Barley chromosomes1H to 7H are shown.

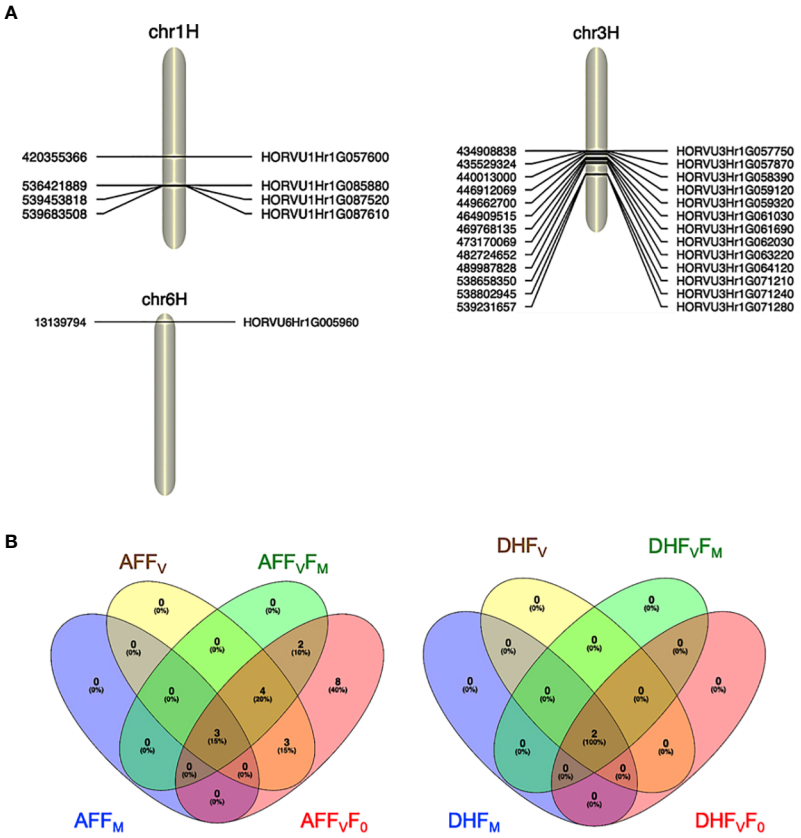

FIGURE 6
(A). Significant marker associated with cold tolerance on chromosomes 1H, 3H, and 6H. **(B)** Venn diagrams showing the significant SNPs identified for all the chlorophyll photosynthetic traits across experiments; before and after the cold acclimation stress. BF, Before cold treatment; AF, two hours after cold treatment. Chlorophyll fluorescence parameters: F_0, F_V, F_M, F_V/F_M, and F_V/F_0 values measured.

related traits (Figure 4A) and visual damage showing that some loci contributed to both phenotypes (i.e., cold acclimation damage/photosynthetic efficiency and tolerance). Among the significantly associated markers, four SNPs were detected for F_V, F_VF_0, and F_VF_M (Table 3 and (Figures 5D, E). For example, BOPA1.7728.341, BOPA2.12.31368, BOPA2.12.31368, SCRI.RS.181360, and SCRI.RS.13891 have founded in the interval region on chromosome 3H between 434909031 bp and 446916578 bp (Figure 5D). Further, five QTNs were associated with AFF_V, AFF_V/F_0. Of them, BOPA1.ABC10084.1.2.363, SCRI.RS.144535, and SCRI.RS.224360 were located between 538658350 bp and 539231657 bp in the interval region on chromosome 3H (Table 3). These results suggested that the QTNs identified in several traits could be considered to be more stable. For the DH readings, eight significant SNPs were detected for the chlorophyll fluorescence-related traits F_M, F_V, F_V/F_M, and F_V/F_0 (Table 3 and Figures 5G–J). All the above-mentioned related traits were associated with two markers SCRI.RS.155758 and SCRI.RS.165588, which were detected on chromosome 1H at the interval regions of 539454103 bp and 539695184 bp (Figure 6). The R^2 values ranged between 0.112 and 0.195 for the 52 SNP markers across different traits, suggesting the presence of major QTNs controlling different chlorophyll fluorescence parameters.

3.4 Prediction of candidate genes associated with significant SNPs

The genomic regions close to the associated SNPs detected in the GWAS were annotated using BARLEYMAP, and then the gene ontologies (GO) enrichments were performed by using Amigo (Tables 3 and 4). Of the 52 candidate genes identified by the GWAS dataset, we found that 15 genes were common among the different traits most of them with high confidence and predicted function (HC_G).

In the interval detected region between 420355366 bp and 420364697 bp on chromosome 1H, there is one gene (*HORVU1Hr1G057600*) associated with the F_M trait and encoding to PAX-interacting protein 1 (PAXIP1) (Tables 3). The PAXIP1 is involved in transcriptional regulation by histone methyltransferase (HMT) complexes and the DNA damage response. AF genes directly participate in the DNA repair pathway, post-translational modifications, and protein kinase which plays an important role in DNA replication and repair, transcription regulation, and chromosomal stability.

In the identified regions on chromosome 1H, between 539695184 bp to 536426484 bp, two common AF and DH genes (*HORVU1Hr1G087520*) belong to the E3 SUMO-protein ligase

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TABLE 3 Functional annotation of the candidate genes associated with chlorophyll fluorescence under cold acclimation conditions.

Marker	Trait	Chr.	Marker position (bp)	Gene ID[a]	Gene class[b]	start (bp)	end	Annotation
SCRI.RS.152795	BFF_M	1H	420364483	HORVU1Hr1G057600	HC_G	420355366	420364697	PAX-interacting protein 1
SCRI.RS.219551	AFF_M	3H	431379904					
SCRI.RS.155758	AFF_M, DHF_M, DHF_V, DHF_V/F_M, DHF_V/F_0	1H	539454103	HORVU1Hr1G087520	HC_G	539453818	539456544	E3 SUMO-protein ligase MMS21
SCRI.RS.165588	AFF_M, DHF_M, DHF_V, DHF_V/F_M, DHF_V/F_0	1H	539695184	HORVU1Hr1G087610	LC_U	539683508	539700690	unknown function
BOPA1.7728.341	AFF_V, AFF_V/F_M, AFF_V/F_0	3H	434909031	HORVU3Hr1G057750	HC_U	434908838	434910472	unknown protein
BOPA2.12.31368	AFF_V, AFF_V/F_M, AFF_V/F_0	3H	435526063	HORVU3Hr1G057870	HC_G	435529324	435531123	Mannan endo-1,4-beta-mannosidase 7
SCRI.RS.181360	AFF_V, AFF_V/F_M, AFF_V/F_0	3H	440011663	HORVU3Hr1G058390	HC_G	440013000	440017152	Bromodomain-containing factor 1
SCRI.RS.138918	AFF_V, AFF_V/F_M, AFF_V/F_0	3H	446916578	HORVU3Hr1G059120	HC_U	446912069	446922626	Chromosome 3B, genomic scaffold, cultivar Chinese Spring
BOPA1.ABC10084.1.2.363	AFF_V, AFF_V/F_0	3H	538660122	HORVU3Hr1G071210	HC_G	538658350	538660659	Mannan endo-1,4-beta-mannosidase 2
SCRI.RS.144535	AFF_V, AFF_V/F_0	3H	538805589	HORVU3Hr1G071240	HC_G	538802945	538807675	Protein kinase superfamily protein
SCRI.RS.224360	AFF_V, AFF_V/F_0	3H	539228382	HORVU3Hr1G071280	HC_u	539231657	539232192	undescribed protein
SCRI.RS.202723	AFF_V, AFF_V/F_0	6H	13136770	HORVU6Hr1G005960	HC_G	13139794	13140854	histone H2A 7
BOPA1.10126.999	AFF_V, AFF_V/F_0	3H	489991522	HORVU3Hr1G064120	HC_G	489987828	490006022	Alpha/beta hydrolase domain-containing protein 13
BOPA1.2067.775	AFF_VF_0	3H	449665313	HORVU3Hr1G059320	HC_G	449662700	449666771	V-type proton ATPase subunit E
BOPA2.12.31011	AFF_VF_0	3H	462627529					
BOPA2.12.31393	AFF_VF_0	3H	464907622	HORVU3Hr1G061030	LC_u	464909515	464910032	undescribed protein
BOPA1.1977.1385	AFF_VF_0	3H	469771904	HORVU3Hr1G061690	HC_G	469768135	469772099	Protein DEHYDRATION-INDUCED 19 homolog 3
BOPA1.2231.557	AFF_VF_0	3H	473173134	HORVU3Hr1G062030	HC_G	473170069	473175037	ROP guanine nucleotide exchange factor 5
SCRI.RS.114566	AFF_VF_0	3H	482729752	HORVU3Hr1G063220	HC_G	482724652	482734251	Splicing factor 3A subunit 3
BOPA1.4453.422	AFF_VF_0	3H	482733343					
SCRI.RS.137116	AFF_VF_0	1H	536426484	HORVU1Hr1G085880	HC_G	536421889	536430726	Ninja-family protein

F_0; Minimum Fluorescence when all photosystem II (PSII) is open, F_V=F_M-F_0; Variable fluorescence, F_M; Maximum fluorescence when all PSII are closed, F_V/F_M; ratio of photochemical efficiency of photosystem II (PSII), F_V/F_0; the ratio of maximum efficiency of photochemical processes in PSII of healthy and stressed plants. BF; before the treatment. AF; 2hr after the treatment. DH; 24hr after the treatment.

[a]The candidate genes and their corresponding annotations were obtained from BARLEYMAP (Cantalapiedra et al., 2015). http://floresta.eead.csic.es/barleymap/.

[b]HC_G; high-confidence gene with predicted function, HC_U, high-confidence gene without predicted function, LC_u, low-confidence gene without predicted function (MorexV3 2021 edition).

family which is involved in a DNA repair pathway. In addition, one gene has an unknown function. *HORVU1Hr1G085880* is another AF gene that encodes a ninja-family protein that plays a role in stress-related and growth-related signaling cascades (Table 3).

In the interval detected on chromosome 3H between 431379904 bp and 539228382 bp, there are six AF genes with undescribed protein annotations and ten annotated genes (Table 3). Of the ten genes, two (*HORVU3Hr1G057870* and *HORVU3Hr1G071210*) belong to the same enzyme family Mannan endo-1,4-beta-mannosidase 7 and Mannan endo-1,4-beta-mannosidase 2 protein, respectively. In addition to an enzyme (V-type proton ATPase subunit E), transcription factor (Bromodomain-containing factor 1), binding protein (Protein DEHYDRATION-INDUCED 19 homolog 3), transporter (ROP guanine nucleotide exchange factor 5) and splicing factor (splicing factor 3A subunit 3) (Table 3). The remaining genes are an Alpha/beta hydrolase domain-containing protein 13 and protein kinase superfamily protein.

In the interval region on chromosome 6H between 13139794 bp and 13140854 bp, one gene (*HORVU6Hr1G005960*) encodes histone H2A 7 protein, a core element of the nucleosome, which plays an important role in transcription regulation, DNA repair and replication and chromosomal stability (Table 3).

To provide more insight into the candidate gene products in the pathways related to low-temperature tolerance, three independent categories of gene ontology were categorized: cellular components (CC), biological processes (BP), and molecular functions (MF) were categorized. The discovered candidate genes were annotated, and several GO terms were observed to be mainly relevant to low-temperature tolerance and chlorophyll fluorescence (Table 4). We identified 16 GO terms using the discovered genes. We found that four unique GO terms, including the zinc ion binding term (GO:0008270), hydrolase activity (GO:0004553), carbohydrate metabolic process (GO:0005975), nuclear-transcribed mRNA catabolic process, no-go mRNA decay (GO:0005634) exhibited an overrepresentation of candidate genes linked with chlorophyll fluorescence-related traits under cold acclimation stress, AF and DH treatments. Interstitially, two GO terms GO:0005515 and GO:0005634 presented no-go mRNA decay as one of the mRNA surveillance pathways. We suggested that most of the identified genes might be directly and/or indirectly involved in the photosynthetic energy conversion in barley plants under low-temperature stress responses.

3.5 Cold tolerance introgression into spring barley

To develop new low temperature-tolerant varieties/germplasm of spring barley as a part of the current project, F_1 populations were produced from the cross-between winter and spring barleys. A single seed of each of the F_1 populations was planted per pot and growth habits were recorded. The results showed that winter barleys exhibit no flowers while all tillers of spring barleys exhibit fully flowered (Figure 7). We observed the number of tillers is high in F_1 populations in comparison with the spring type of the parental lines. F_2 populations were segregated for growth habits (Figure 7). The genotypes with spring growth habits were selected and evaluated for cold tolerance. We identified some spring F_2 plants earlier to flowering than the parental lines. These genotypes entered advanced generations (RIL_{2-5}) and were validated in field conditions to be used in the breeding programs towards the breeding of high and stable-yielding varieties in the Canadian prairies. We, therefore, suggest additional studies to refine and validate the cold hardiness of these lines such as QTL mapping to identify and characterize the candidate genes underlying this quantitative trait. Together, this study demonstrated that cold hardiness can be introgressed into spring barleys from winter barleys easily without the restriction of genes transferring related to vernalization requirement.

4 Discussion

Identifying and characterizing key genes underlying low-temperature tolerance has become the main priority for improving the hardiness of barley. A deeper comprehension of the regulation networking and pathways of these genes and their association with low-temperature stress (LTS) would assist in the illustration of how barley plants adapt to stress. Understanding these pathways might offer opportunities for increasing the levels of cold tolerance. Due to the complexity of injuries and symptoms, it is difficult to measure the cold tolerance of barley at the seedling stage. This study evaluated cold tolerance in barley using visual symptoms and chlorophyll fluorescence as indicators of photosynthetic energy conversion.

4.1 Natural variation in chlorophyll fluorescence traits

Under cold acclimation and freezing shock stresses, the F_VF_M and F_V/F_0 declined rapidly as an index of freezing tolerance and subsequent loss of viability (Table 1). We found that the time for full leaf wilting was shorter with the freezing shock treatment compared with the cold acclimation treatment. Evidence suggested that wilting was mainly initiated by reduced water- uptake, and the stomatal response to water stress was not influenced by lowered temperature (Benson, 2008). The reduction in osmotic potential accounted for an enhancement in sugars. Sugars are believed to function principally as cryoprotectants (Benson, 2008). The accumulation of these solutes at low temperatures is essential not only for freezing tolerance but also for the prevention of cell dehydration (Wang M. et al., 2021). Though other photosynthetic parameters might be used as indicators of viability, the F_VF_M parameter is suggested for some reasons. First, a minor change in the F_VF_M value is easily visible and indicates clearly that loss of viability is imminent. The consistency of the F_VF_M parameter also increases the ease with which a threshold level can be defined. More significantly, dissimilar light-dependent parameters, F_VF_M is obtained from specimens in the dark-adapted state, negating the need for an extended period of illumination before measurement. As a result, because the quantity of F_VF_M can be performed with a

TABLE 4 Gene Ontology annotation of the candidate genes on located on chromosomes 1H, 3H and 6H and their respective functional annotations.

Gene ID	InterPro	Gene Ontologies	Term	Definition	Synonyms		PFAM
HORVU1Hr1G057600	IPR001357 IPR016181						PF00533
HORVU1Hr1G087520	IPR004181 IPR013083	GO:0008270	zinc ion binding	Binding to a zinc ion (Zn).	Zn binding	MF	PF11789
HORVU1Hr1G087610							
HORVU3Hr1G057750							
HORVU3Hr1G057870	IPR013781 IPR017853 IPR018087	GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	Catalysis of the hydrolysis of any O-glycosyl bond.	O-glucosyl hydrolase activity	MF	
		GO:0005975	carbohydrate metabolic process	The chemical reactions and pathways involving carbohydrates, any of a group of organic compounds based of the general formula Cx (H_2O) y	carbohydrate metabolism	BP	
HORVU3Hr1G058390	IPR001487	GO:0005515	nuclear-transcribed mRNA catabolic process, no-go decay	The chemical reactions and pathways resulting in the breakdown of the transcript body of a nuclear-transcribed mRNA with stalls in translation elongation.	no-go decay/ no-go mRNA decay	BP	PF00439
HORVU3Hr1G059120							
HORVU3Hr1G071210	IPR018087 IPR001547 IPR013781 IPR017853	GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	Catalysis of the hydrolysis of any O-glycosyl bond.	O-glucosyl hydrolase activity	MF	PF00150
		GO:0005975	carbohydrate metabolic process	The chemical reactions and pathways involving carbohydrates, any of a group of organic compounds based of the general formula Cx(H_2O)y	carbohydrate metabolism	BP	
HORVU3Hr1G071240	IPR008271 IPR011009 IPR000719 IPR001245 IPR002290	GO:0004672	protein kinase activity	Catalysis of the phosphorylation of an amino acid residue in a protein, usually according to the reaction: a protein + ATP = a phosphoprotein + ADP.	protamine kinase activity	MF	PF07714
		GO:0005524	ATP binding	Binding to ATP, adenosine 5'-triphosphate, a universally important coenzyme and enzyme regulator.		MF	
		GO:0006468	protein phosphorylation	The process of introducing a phosphate group on to a protein.	protein amino acid phosphorylation	BP	
HORVU3Hr1G071280							
HORVU6Hr1G005960	IPR032454 IPR032458 IPR002119 IPR007125 IPR009072	GO:0046982	protein heterodimerization activity	Binding to a nonidentical protein to form a heterodimer.		MF	PF00125 PF16211
		GO:0000786	nucleosome	A complex comprised of DNA wound around a multisubunit core and associated proteins, which forms the primary packing unit of DNA into higher order structures	cytoplasmic nucleosome/ nuclear nucleosome	CC	
		GO:0003677	DNA binding	Any molecular function by which a gene product interacts selectively and non-covalently with DNA (deoxyribonucleic acid).	microtubule/ chromatin interaction structure specific DNA	MF	

(Continued)

TABLE 4 Continued

Gene ID	InterPro	Gene Ontologies	Term	Definition	Synonyms		PFAM
					binding plasmid binding		
		GO:0005634	nuclear-transcribed mRNA catabolic process, no-go decay	The chemical reactions and pathways resulting in the breakdown of the transcript body of a nuclear-transcribed mRNA with stalls in translation elongation	no-go decay no-go mRNA decay	BP	
HORVU3Hr1G064120	IPR029058 IPR029059						PF12695
HORVU3Hr1G059320	IPR002842	GO:0015991	proton transmembrane transport	The directed movement of a proton across a membrane.	hydrogen ion transmembrane transport	BP	PF01991
		GO:0033178	proton-transporting two-sector ATPase complex, catalytic domain	A protein complex that forms part of a proton-transporting two-sector ATPase complex and catalyzes ATP hydrolysis or synthesis. The catalytic domain (F1, V1, or A1) comprises a hexameric catalytic core and a central stalk, and is peripherally associated with the membrane when the two-sector ATPase is assembled.		CC	
		GO:0046961	proton-transporting ATPase activity, rotational mechanism	Enables the transfer of protons from one side of a membrane to the other according to the reaction: ATP + H2O + H+(in) = ADP + phosphate + H+(out), by a rotational mechanism.	ATP synthase activity	MF	
HORVU3Hr1G061030							
HORVU3Hr1G061690	IPR008598 IPR027935						PF05605 PF14571
HORVU3Hr1G062030	IPR005512	GO:0005089	guanyl-nucleotide exchange factor activity	Stimulates the exchange of GDP to GTP on a signaling GTPase, changing its conformation to its active form. Guanine nucleotide exchange factors (GEFs) act by stimulating the release of guanosine diphosphate (GDP) to allow binding of guanosine triphosphate (GTP), which is more abundant in the cell under normal cellular physiological conditions.	GDP-dissociation stimulator activity	MF	PF03759
HORVU3Hr1G063220	IPR031774 IPR000690 IPR021966 IPR024598	GO:0003676	nucleic acid binding	Binding to a nucleic acid.	base pairing	MF	PF16837 PF11931 PF12108 PF13297
		GO:0005634	nuclear-transcribed mRNA catabolic process, no-go decay	The chemical reactions and pathways resulting in the breakdown of the transcript body of a nuclear-transcribed mRNA with stalls in translation elongation.	no-go decay	BP	
		GO:0008270	zinc ion binding	Binding to a zinc ion (Zn).	Zn binding	MF	
HORVU1Hr1G085880	IPR012463 IPR032308						PF07897 PF16135

MF, Molecular Function; BP, Biological Process; CC, Cellular components.

single saturating pulse, a rapid evaluation of a large number of plants can be evaluated quickly in a short time.

An association between the decrease of F_V/F_M and frost tolerance during hardening and after freezing was observed in winter wheat (Clement and Hasselt, 1996). F_V/F_M values showed a significantly decreased in studied genotypes of oat (*Avena sativa* L.) during acclimation to low, nonfreezing temperatures. F_V/F_M measurement was also found to be highly associated with frost damage evaluation under field conditions (Rizza et al., 2001). The decreases in the chlorophyll fluorescence indexes in this study are revealing the reduction in PSII efficiency. This could lead to a decline in photosynthetic activity mainly because of a light-induced decrease in CO_2 assimilation and accumulation of reactive oxygen species (ROS), which, in turn, prevents protein synthesis (Baker,

FIGURE 7
Prospects for Growth Habit. Two F_1 populations were derived from a cross between 02Ab431 (♀- winter type) and Bentley (♂- spring type). F_2 population segregated for growth habit (spring versus winter type). The photo was taken 39 days after seeding. **(A)**: genotypes showing winter type. **(B)**: genotypes showing spring type with early flowering.

1996). Even though the decrease in photo-assimilation could be based on the damage to various elements of the photosynthetic apparatus, the term photoinhibition is commonly used to describe light-induced inhibition of the PSII activity (Murata et al., 2007). The produced ROS are accountable for the damage to PSII reaction centers by inhibiting the protein synthesis, which is required for the PSII repair, resulting in the stimulation of PSII photoinhibition (Murata et al., 2007). PSII is the most prone component to be destroyed in the thylakoid membranes. Hence, the main result of abiotic stress is to make PSII susceptible to photoinhibition. In this study, the association mapping materials were selected based on grain yield and a high percentage of winter survival. We found significant phenotypic variation among cultivars for chlorophyll fluorescence-related traits, including F_0, F_V, F_M, F_V/F_0, and F_V/F_M before (BF), two hours (AF), and 24 hours (DH) after low-temperature treatment. These outcomes suggest that this panel could be used as a genetic source of seedling low-temperature tolerance in Western Canadian barley breeding programs.

4.2 GWAS analysis provided potential SNP markers for chlorophyll fluorescence traits

The chlorophyll fluorescence traits characterized in this study are quantitative traits, thus, several genomic regions with small effects that contribute to the phenotype are expected. We first identified the genes having significant SNPs (Tables 2, 3) and discovered several candidate genes underlying chlorophyll fluorescence trait variation. Gene expression for some identified genes in different tissues of the barley plant was revealed from the Global gene co-expression networks (GCNs) database (Supplementary Figure 2).

Among these, 13 genes are associated with the chlorophyll fluorescence trait under cold acclimation stress. Interestingly, we found two SNP markers on chromosome 1H that have been reported to be correlated with environmental stress. On chromosome 1H, a peak SNP was discovered to be associated with AFF_M, DHF_M, DHF_V, DHF_V/F_M, and DHF_V/F_0 that encode E3 SUMO-protein ligase. SUMO is a small ubiquitin-related modifier conjugation that is essential for posttranslational modification controlled by environmental cues (Miura et al., 2007a; Miura and Hasegawa, 2010). SUMO conjugation/deconjugation was involved in responses to oxidative stress, heat shock, phosphate limitation, hypoxia, flowering, pathogen defense, and ABA signaling (Kurepa et al., 2003; Lois et al., 2003; Murtas et al., 2003; Miura et al., 2005; Yoo et al., 2006; Lee et al., 2007). In *Arabidopsis* AtSIZ1 SUMO E3 ligase functions are conserved in several environmental responses including cold tolerance responses, salicylic acid in plant defense, basal thermotolerance, flowering time regulation, and freezing tolerance (Miura et al., 2005; Yoo et al., 2006; Lee et al., 2007; Miura et al., 2007b). Overexpression of the rice *OsSIZ1* gene improved tolerance to drought, heat and salt tolerance in Arabidopsis (Mishra et al., 2018), and drought and heat tolerance in cotton (Mishra et al., 2017). Furthermore, they produced higher seed yields under different stress conditions and improved the photosynthesis rate of plants exposed to heat stress. The SUMO E3 Ligase SIZ1 is involved in anthocyanin accumulation under high light in *Arabidopsis* (Zheng et al., 2020), DNA demethylation (Kong et al., 2020), numerous abiotic stresses (Fang et al., 2022), phosphate homeostasis in rice (Pei et al., 2020), and protecting maize plants from paraquat toxicity (Wang H.et al., 2021). The *siz1* mutation exhibits freezing sensitivity as a decrease in expression of *CBF3/DREB1*, a transcription factor for acclimation to cold temperatures (Miura and Hasegawa, 2010). Recently, the *HORVU3Hr1G016010* gene encodes a SUMO found to be involved in the cold response in the VIR barley collection (2214 accession) (Sallam et al., 2021). In this investigation, we found that the *HORVU1Hr1G087520* on chromosome 1H encodes E3 SUMO-protein ligase associated with the chlorophyll fluorescence trait under low-temperature conditions and could regulate the photosynthesis rate. Photosynthetic proteins are responsible for providing the cells with the energy to assemble the defensive molecules in response to stress (Ashraf and Harris, 2013). Thus, E3 SUMO could be essential for cold acclimation tolerances in barley at the posttranslational modification level.

Further, we found a significant SNP associated with AFF_VF_0 under cold acclimation stress in a gene *HORVU1Hr1G085880* encoding Ninja-family protein. Ninja (NOVEL-INTERACTOR-OF-JAZ) protein regulates growth-related and stress-related signaling (Pauwels et al., 2010). ECAP NINJA is an adaptor protein linking JASMONATE-ZIM DOMAIN (JAZ) proteins with the co-repressor, TOPLESS (TPL), to regulate gene

suppression in Jasmonate (JA)-dependent the *Arabidopsis* root growth inhibition. ECAP interacts with JAZ6 and JAZ8, as an adaptor protein to inhibit the JA-responsive anthocyanin accumulation by TPR2 recruitment to the transcriptional complex (Li et al., 2020). Through protein Ninja, Jasmonate JAZ repressor proteins recruit the Groucho/Tup1-type co-repressor TPL and TPL-related proteins (TPRs) (Pauwels et al., 2010). In *Arabidopsis thaliana*, Ninja binding proteins AFP1 and AFP4 are key regulators of ABA signaling and stress responses in seedlings, as well as a negative regulator of jasmonate responses (Garcia et al., 2008). NINJA, the novel interaction of JAZ encodes a transcriptional repressor that participated in the jasmonic acid pathway in the leaf and root growth and development (Oblessuc et al., 2020). Phytohormones such as JA, ABA, and salicylic acid are essential elements of complex signaling networks of the stress response (Lee et al., 2007) and control gene expression.

Based on our results, we have proven that the post-translational modification of protein related to photosynthesis is critical for barley plants, which dynamically modulated their response to environmental changes under LTS conditions. We also reported that SUMO E3 ligase could accelerate the ubiquitin-proteasome system, by conjugation of small ubiquitin-like modifiers to target substrate proteins (cold tolerance-related genes) (Johnson, 2004). Ubiquitin could likewise alter some key TFs that facilitate plant adaptation to cold stress (Callis, 2014). Our results revealed that GWAS identified two SNPs on chromosomes 1H located between 539454103 bp (SCRI.RS.155758) and 536426484 bp (SCRI.RS.137116) linked to the E3 SUMO and Ninja proteins, respectively (Table 3).

Interestingly, after two hours of the cold acclimation stress, we discovered SNPs on chromosome 3H, detected at 440011663 bp within the *HORVU3Hr1G058390* gene encoding a Bromodomain-containing factor 1 protein was found to be associated with the F_V, $F_V/F_{M,}$ and F_V/F_0. Bromodomain-containing transcription factors control gene expression in three ways; (i) transcription activation, (ii) conserving transcription memory, and (ii) anti-silencing of the conserved chromosomal region (Loyola and Almouzni, 2004). The bromodomain proteins proceed with these functions *via* binding to acetylated histones and anchoring sequence-specific factors on the chromatin of target promoters. Further, bromodomain-containing proteins contain various families of 'epigenetic mark readers', which are basic to epigenetic gene regulation, and necessary for different environmental stress responses and cellular processes (Abiraami et al., 2022). In Arabidopsis, the bromodomain-containing transcription factors, GTE9, and GTE11 convert specific factors as BT2 to control gene expression. BT2 is a BTB domain-containing protein with an essential TAZ-zinc finger domain and calmodulin-binding domain C-terminal that responds to different metabolic and physiological responses (Mandadi et al., 2009). Loss-of-function mutants *bt2* exhibited a hypersensitive response to ABA- and sugar-mediated suppression of germination, suggesting the role of ABA in sugar signaling in germination and development. *BT2* expression was controlled by several abiotic and biotic stresses including cold, ABA, hydrogen peroxide (H_2O_2), and methyl jasmonate (Mandadi et al., 2009). Cold-stress signal perception is the first stage, which is completed by various pathways. Transcriptional regulations are the next factors that control ABA-dependent signaling pathways to prompt the expression of cold-regulated genes. Resulting in upregulating of hundreds of metabolite levels, some of which are identified to have protective results against the negative impacts of cold stress including soluble sugars, ROS, and photosynthetic metabolites (Heidarvand and Maali Amiri, 2010).

On the other hand, we found SNPs on chromosome 6H located within the *HORVU6Hr1G005960* gene that encodes histone H2A 7 protein, which has been associated with multiple traits, including F_V, F_V/F_0 under the cold acclimation condition. Histone H2A is one of the five key histone proteins engaged in the chromatin structure in eukaryotic cells that are responsible for maintaining the shape and structure of a nucleosome. Histone modification is an epigenetic mechanism involving changes in the chromatin structure of stressed genes *via* several chemical processes during the transcriptional and post-transcriptional modifications (Kim et al., 2015). These mechanisms play an essential role in plant survival under adverse environmental conditions (Elakhdar et al., 2022; Verma et al., 2022). In addition, epigenetic mechanisms might create stress memory which is convenient for the following generations (Verma et al., 2022), to regulate gene expression to cope with environmental stresses. Histone genes are suppressed by abiotic stresses such as drought (Kang et al., 2011), cold (Steward et al., 2000), and chilling tolerance (Zhang et al., 2012).

An SNP located at 538805589 bp within the *HORVU3Hr1G071240* gene on chromosome 3H associated with F_V, F_V/F_0 after two hours of the cold acclimation stress falls within protein kinase superfamily protein. Protein kinases belonging to a wide superfamily play an essential role in numerous biological processes of plant growth and stress tolerance. In parallel, several stress-inducible protein kinase families for instance calcium-dependent protein kinase (CDPK), mitogen-activated protein kinase (MAPK), and SNF1-related protein kinase (SnRK) are stimulated by ABA and different stress signals (Wrzaczek and Hirt, 2001; Ludwig et al., 2004). Gain- and loss-of-function findings have shown that signaling pathways resulting in cold, drought, salt, and tolerance are controlled through specific CDPK isoforms (Ludwig et al., 2004). Overexpression of Ca^{2+}-dependent protein kinase enhances the cold and salt/drought tolerance in rice plants (Saijo et al., 2000). *HORVU2Hr1G118320* gene encodes phosphatidylinositol kinase protein was previously found to affect cold response in the barley VIR collection (Sallam et al., 2021), maize (*Zea mays*) and *Arabidopsis thaliana* (Wang et al., 2018).

On chromosome 3H, we found an SNP located within the *HORVU3Hr1G064120* gene that encodes the Alpha/beta hydrolase domain-containing protein 13 associated with F_V, F_V/F_0. The α/β-hydrolase domain (ABHD) proteins are conserved in all organisms and belong to the α/β-hydrolase (ABH) superfamily. ABH family is involved in various processes including cell signaling, energy metabolism, growth, and development (Mindrebo et al., 2016), and in response to salinity stress (Liu et al., 2014). A few of α/β-hydrolase fold enzymes, for example, esterase, phospholipase D, and prolyl oligopeptidase (POP5) play a key role in responses to several abiotic stress including drought, salt, and chilling in addition to ABA signaling of plants (Wang, 2002; Hong et al., 2010; Tan et al., 2013; Liu et al., 2014).

On chromosome 3H, the detected SNP linked with F_VF_0 was found within the *HORVU3Hr1G059320* gene, which encodes V-type proton ATPase subunit E. V-ATPases are complexes of membrane-embedded proteins that function as ATP hydrolysis-driven proton pumps. V-ATPase maintains the pH and the regulates acidifying of intracellular compartments. In some cell types, it is aimed at the plasma membrane, where it regulates acidifying the extracellular environment (Vasanthakumar and Rubinstein, 2020). It was found that the survival of the cells was based strongly on adjusting or maintaining the V-ATPase activity under cold stress conditions (Dietz et al., 2001). Yoshida et al., (Yoshida et al., 1999), reported that plant sensitivity to low temperatures leads to an increase in frost and chilling hardiness including three vacuolar events related to V-ATPase activity. (a) Chilling stress leads to the suppression of V-ATPase activity. (b) Correspondingly, the formation of pH gradients is obstructed. (c) Then membranes adjusted their fluidity by improving the membrane content of unsaturated fatty acids. Evidence revealed that the association between stress injury and cytoplasm acidification was inveterate in studies relating V-ATPases from chilling-tolerant species for instance pea and chilling-sensitive species like mung bean (Hotsubo et al., 1998). The expression level of the wheat *E* subunit of the *V-type H^+-ATPase* gene was increased by cold, drought, salt, and exogenous ABA treatment (Zhang et al., 2014). An *in vitro* study found that chilling rice plants at 10°C increased the vacuolar-type ATPase activity (Orr et al., 1995). Cold-tolerant *Arabidopsis thaliana* and *Brassica napus* exhibited an upregulation in both proteins and subunit A mRNA in response to chilling at 2°C (Apse et al., 1999).

Another significant SNP was detected on chromosome 3H within the *HORVU3Hr1G061690* gene encoding the protein DEHYDRATION-INDUCED 19 associated F_VF_0. The Arabidopsis AtDi19 is a dehydration- induce protein that encodes a Cys2/His2 zinc-finger protein involved in high-salinity stress, ABA-independent dehydration, and light signaling events (Milla et al., 2006). *In vitro* assay showed that AtDi19-related proteins were phosphorylated through calcium-dependent protein kinases (CDPKs). These findings reveal that the post-translational modification could be significant in controlling the function of the AtDi19 (Milla et al., 2006). The Arabidopsis AtDi19-3 is also a transcriptional activator that participates in plant response to drought, salinity, ABA, and H_2O_2 events (Qin et al., 2014). In our study, we showed that the barley DEHYDRATION-INDUCED 19 is associated with chlorophyll fluorescence-related traits under low-temperature stress.

Another important genomic region F_VF_0 is on chromosome 3H, where their SNP is located within the *HORVU3Hr1G062030* gene that encodes ROP guanine nucleotide exchange factor 5. Guanine nucleotide exchange factors (RopGEFs) are activators of small GTPase proteins named ROPs in plants in turn regulate different cellular processes ranging from control growth to plant responses to environmental stimuli (Berken et al., 2005). The *Arabidopsis* RopGEF1 function as a negative regulator of signal transduction through the plant hormone ABA (Li et al., 2018). RopGEF1 was phosphorylated by calcium-dependent protein kinases CPK4. CPK4 stimulates RopGEF1 degradation. CPK4 also inhibits RopGEF1 activities in hairy root growth (Li et al., 2018). The *RopGEF1* (*HORVU3Hr1G085680.4*) gene expression is upregulated in salt-tolerant genotypes, which might be associated with salt stress (Chen et al., 2022). Taken together, our results could provide the basis for an advanced study into the function of barley RopGEF1 in cold acclimation tolerance.

Finally, an additional SNP was founded on chromosome 3H, within the *HORVU3Hr1G063220* gene that encodes subunit 3 of the splicing factor 3a protein complex (Splicing Factor 3A subunit 3) associated with F_VF_0. The *Arabidopsis* ROA1/RBM25 gene has been identified as a splicing factor required for the splicing of transcripts from several ABA signal transduction pathway genes (Zhan et al., 2015). The Arabidopsis *STA1* gene encodes a cold-induced pre-mRNA splicing factor, and *sta1-1* is cold-sensitive and defective in the splicing of the cold-induced *COR15A* gene. The splicing factor mutant *sta1-1* mutant displays that the STA1 protein regulates the splicing pattern and stability of some genes related to abiotic stress (Lee et al., 2006). The *sta1-1* and *rdm16-1* mutants exhibited hypersensitivity to ABA and salt stress in seed germination (Lee et al., 2006). In response to environmental stresses, mainly cold stress, plants modified their genome-wide alternative splicing profiles (Iida et al., 2004). Some plant hormones and environmental stresses, including cold and salt, dramatically adjust their expression and splicing profile of many important pre-mRNA splicing regulators mechanisms (Palusa et al., 2007).

The identified GO terms were related to molecular mechanisms of causal photosynthetic responses and low-temperature tolerance in barley. GO findings showed that hydrolase activity, carbohydrate metabolic process, nuclear-transcribed mRNA catabolic process, no-go mRNA decay, and protein kinase activity were enriched by identified genes linked with photosynthetic traits under cold acclimation conditions. Also, our results indicated that the zinc ion binding, protein phosphorylation, ATP binding, and guanyl-nucleotide exchange factor activity GO terms (Table 4) were enriched under cold acclimation conditions. These results indicate that barley plants have initiated growth and repaired damaged tissues under cold acclimation conditions. Recently a study of meta-analysis study determined the GO that plays a key role in the mechanism of barley responses to cold stress such as guanyl-nucleotide exchange factor activity, the mRNA surveillance pathway and starch and sucrose metabolism (Alamholo and Tarinejad, 2022).

Taken altogether, our functional annotation showed identified loci that were either within the known or close genes that play key roles in the photosynthetic signaling pathways, ABA signaling events, antioxidant biosynthesis, and posttranslational signals transduction. Overall, these outcomes suggest that various biological processes are involved in cold stress responses as well as post-transcriptional modification and epigenetics-mediated changes that may play essential roles in spring seedling responses to cold stress.

5 Conclusion

In this study, 96 spring barley genotypes were evaluated for chlorophyll fluorescence-related traits before and after the cold acclimation conditions. The genotypes were genotyped using the Barley 9K iSelect SNP Array. Our principal conclusions are the

following (1) Significant phenotypic variation among genotypes under low-temperature stress. (2) Several genomic regions are associated with chlorophyll fluorescence under cold stress. (3) GWAS analysis indicated that a total of two and fifty markers were significantly associated with chlorophyll fluorescence-related traits before and after the cold stress treatment, respectively. Thirty-nine significant QTNs and thirteen annotated candidate genes were identified. We first identified the genes having suggestive and significant GWAS SNPs and discovered several potential candidate genes underlying cold acclimation stress and/or chlorophyll fluorescence trait variation. Furthermore, the candidate genes were discovered around the significant SNPs mainly on chromosomes 1H, 3H, and 6H for cold-related-traits. Most of the candidate genes participate in plant response to abiotic stress at the post-transcriptional modification level including, for example, abscisic acid (ABA) signaling, hydrolase activity, protein kinase, and transduction of environmental signal transduction at the posttranslational modification levels. (4) Novel biparental populations ($RIL_{2\text{-}5}$) developed from a cross between spring x winter type which can be used to identify the new QTL for low-temperature tolerance in the spring barley in the future. Overall, our results provide fresh insight into potential low-temperature tolerance mechanisms in barley and the possibility of marker-assisted selection in the future.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://figshare.com/, https://figshare.com/ndownloader/files/38616278.

Author contributions

AE developed the article concept. LC-C prepared the material for genotyping. LC-C and JS physiological data collection and interpretation. AB provided the 91 varieties/breeding lines. AB made available the SNPs data. AE performed bioinformatics, data analyses, and interpretation of the results. AE wrote the manuscript. TK and AH reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

JS is employed by InnoTech Alberta Inc., Vegreville, Canada.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1159016/full#supplementary-material

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Cold tolerance identification of nine *Rosa* L. materials and expression patterns of genes related to cold tolerance in *Rosa hybrida*

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Members of the *Rosa* genus have a high ornamental value, but their cultivation area is limited by their sensitivity to cold temperatures. The aim of this study was to evaluate the cold tolerance of a range of *Rosa* materials, and then determine which genes were related to cold tolerance. Nine *Rosa* materials were subjected to a cold treatment. To identify genes related to cold tolerance, *R. hybrida* was treated at –15°C for 10 min, and leaves collected before and after this treatment were collected for RNA-Seq analyses. The transcript profiles of four DEGs (*POD17, NDUFA9, PMA1*, and *b-Amy1*) in *R. hybrida* were determined by qRT-PCR at 0 h, 1 h, 2 h, and 3 h at –15°C. Nine *Rosa* materials were subjected to a cold treatment, and the most cold-tolerant materials were identified as those that showed the lowest levels of electrolyte leakage and the best recovery after 30 d of growth. The most cold-tolerant materials were *Rosa hybrida, Rosa rugosa* 'Pingyin 12', and *Rosa rugosa*. In total, 204 significantly differentially expressed genes (DEGs) were identified, of which 88 were significantly up-regulated and 116 were significantly down-regulated under cold conditions. Gene Ontology classification and Kyoto Encyclopedia of Genes and Genomes pathway analyses showed that the DEGs were enriched in 57 pathways, especially starch and sucrose metabolism, phenylpropane biosynthesis, MAPK signaling, fructose and mannose metabolism, and oxidative phosphorylation. By transcriptional analysis, *PMA1*, which was related to H^+ ATPase activity, was continuously up-regulated, but the transcript levels of *POD17, NDUFA9*, and *β-Amy1* fluctuated during the freezing treatment. This research uncovered scarce cold-resistant materials and layed the foundation for further research on the cold tolerance mechanism of *Rosa* plants and the breeding of cold-tolerant varieties.

KEYWORDS

Rosa L., cold tolerance, RNA-Seq, qRT-PCR, resource evaluation

1 Introduction

There are about 200 species in the *Rosa* genus, of which 82 species are native to China. *Rosa* species are widely distributed in various climate zones around the world (Che et al., 2016). Among them, *Rosa chinensis* Jacq., *Rosa rugosa*, and *Rosa* sp. are the most representative, and are popular ornamental plants worldwide. There are many varieties of *Rosa* species, with rich colors and a strong scent. They not only have a high ornamental value, but are also important raw materials for the production of spices and foods. They also have a high nutritional and medicinal value (Guo et al., 2011). *R. chinensis* Jacq. is an evergreen and semi-evergreen low shrub. It is known as the 'Queen of Flowers' and is very popular with consumers. *R. chinensis* can be grown under a wide range of conditions and produces flowers in all four seasons. Its flowers show a diverse array of colors and types. It is an important plant for urban beautification and courtyard greening, but it is not sufficiently cold resistant to grow in northern areas of China. *R. rugosa* is an upright shrub that is not only attractive in its appearance, but also useful for making spices, cosmetics, and food. *R. rugosa* has strong tolerance to drought, saline-alkali soils, low-nutrient soils, and cold and other abiotic stresses, and it is an excellent ornamental plant breeding resource. However, different varieties of *R. rugosa* differ in their degree of cold tolerance. *Rosa* sp. are important wild resources with many excellent shapes and growth forms, beautiful flower colors, diverse flower types, high ornamental value, and strong cold resistance. With the increasing use of *Rosa* plants for planting, as cut flowers, and in products, there is an increasing demand for *Rosa* materials with particular characteristics. Through the joint efforts of breeders all over the world, many new varieties have been bred, but there are still resources that are underutilized and lack in-depth research.

As an important agronomic trait, cold resistance has attracted much attention. Cold temperatures can result in symptoms of wilting, yellowing, or necrosis, and these symptoms are related to changes in the physiological and biochemical functions of plant cells (Ruelland and Zachowski, 2010). The commonly used methods for identifying cold tolerance include electrolyte leakage, electrical impedance spectroscopy, chlorophyll fluorescence, differential thermal analysis, and whole plant freezing tests (Janská et al., 2010). Of those, the electrolyte leakage method is more accurate, requires less equipment and fewer samples, and is simple to operate. Thus, it is the most widely used.

Plants respond to low-temperature stress through two main processes: low temperature perception and signal transduction (Li, 2017). Low temperatures directly inhibit metabolism-related enzyme activity and alter gene expression in plants. Under low temperature, plants undergo extensive changes in their physiology and metabolism, including changes in cell membrane phase and permeability, and in the concentrations of hormones and cryoprotectant molecules (such as soluble sugars and proline). These changes result from changes in the expression of related genes. Recent research on the plant response to low temperatures has focused on the ICE1-CBF-COR transcriptional cascade signaling pathway (Tomashow, 1999; Chinnusamy et al., 2003). Under low-temperature stress, there are increased transcript levels of many genes encoding transcription factors, including ICE1 and CBF. ICE1 is an upstream transcription factor that regulates the transcription of *CBF* under low temperature. It is a constitutively expressed gene whose product is phosphorylated under cold stress. The CBF transcription factor activates the expression of many downstream COR (Li, 2017), thereby playing a very important role in cold adaptation. In addition to transcription factors, proteases are related to cold tolerance in plants. Teige et al. (2004) found that *MKK2* in the *Arabidopsis* MAPK signaling pathway is activated under cold and salt stress conditions, and regulates the expression of downstream genes involved in the low temperature response. Although previous studies have discovered many genes related to cold tolerance in plants, less is known about the mechanism of the perception of cold temperatures. It is important to explore this topic to breed new, cold-tolerant varieties of economically important plants.

RNA-Seq and analysis techniques have been applied in many fields, such as biology and medicine. RNA-Seq analyses can reveal new genes, gene transcripts with low abundance, and alternative splicing variants. Data generated in RNA-Seq analyses can provide important information about metabolic pathways and gene families involved in various responses, and are useful for evolutionary analyses (Song, 2016). Because RNA-Seq datasets are very large, they can enrich the genetic information available for a plant species. They can provide a large amount of related ESTs information, making it possible to detect changes in gene expression levels, and allowing for the discovery of new functional genes. The information generated in RNA-Seq analysis lays the foundation for subsequent gene cloning and the development of molecular markers such as SNP and simple sequence repeat markers, which are useful for mapping and breeding. RNA-Seq can reveal patterns of gene expression in specific tissues or cells, and reveal previously unknown small RNAs (Ke, 2016). In recent years, RNA-Seq technology has been widely used to study the mechanism of cold tolerance in many plants, such as *Arabidopsis thaliana* (Lee et al., 2005), wheat (Gulick et al., 2005), rice (Zhang et al., 2012), corn (Sobkowiak et al., 2014), and cassava (An et al., 2012). Analyses of cold-treated plant materials have generated a wealth of transcriptome data and revealed many transcription factors related to low-temperature signal transduction and the low-temperature stress response. This has established a baseline for research on other crops, and provided a way to unravel the complex mechanisms that underly tolerance to cold temperatures in plants.

Low-temperature stress is one of the main factors restricting the cultivation and production of *Rosa*. Low temperatures in winter negatively affect the quality of *Rosa* materials, resulting in shorter branches, smaller leaves, deformed petals, color degradation, and a prolonged growth period. This can cause substantial losses for producers (Zhu, 2017). In plants, cold resistance is the result of the combined effects of genetic factors and environmental factors (He, 1995), and comprehensive research using multiple methods from multiple perspectives is required to explore its mechanisms in detail. Low temperature stress damages the structure and function of plant biofilms, and affects the activity and abundance of various enzymes involved in physiological and biochemical processes. This leads to metabolic disorders that negatively affect plant growth, development, and yield, and limit plant cultivation and distribution.

Screening to identify cold-tolerant varieties has been conducted for a variety of plants, such as *Solanum lycopersicum*, *Rhododendron simsii*, *Camellia oleifera*, and *Zoysia japonica*. The identification of cold-tolerant *Rosa* materials is also advancing. At present, low temperatures in winter limit the geographical distribution of *Rosa*. Therefore, it is of great significance to identify cold-resistant *Rosa* materials to enrich the resources of high-quality cold-resistant varieties, and to guide people in the use of those varieties. The identification of cold-resistant *Rosa* materials will be useful to provide a scientific basis for the breeding and cultivation of cold-resistant resources, and to expand the cultivation and distribution range of *Rosa* plants.

To date, research on *Rosa* plants in China and elsewhere has focused on disease resistance and stress resistance mechanisms, breeding, flower aroma and essential oil composition, genetic relationships and classification of varieties, improvements in cultivation technology and cultivated varieties, cut flower preservation, wild resource distribution and ecology, essential oil extraction, factors contributing to endangerment, and protection strategies. However, few studies have focused on physiological and biochemical characteristics related to the cold tolerance of roses. In previous studies, the most and least cold-resistant genotypes were screened by evaluating the cold resistance of 17 different genotypes of courtyard roses. It was found that sucrose and oligosaccharides were related to the cold resistance of roses (Lin et al., 2019a). A study on the seasonal changes in the cold hardiness and carbohydrate metabolism of four garden *Rosa* cultivars revealed differences in the transcript levels of genes linked to dehydrin and carbohydrate metabolism during cold acclimation between the more cold-hardy 'Dagmar Hastrup' and the less cold-hardy 'Chandos Beauty' (Lin et al., 2019b).

In this study, we subjected *Rosa* materials to a low-temperature treatment and observed changes in certain biological characteristics and physiological indicators to evaluate their cold resistance. Then, by sequencing the leaf transcriptomes of a cold-resistant material before and after a low-temperature treatment, we identified DEGs related to cold resistance. We then analyzed the transcript profiles of four genes potentially involved in cold resistance during a 3-h cold treatment at −15°C using quantitative PCR (qPCR). The aims of this study were to select the varieties with higher cold tolerance from nine materials, to identify genes related to cold tolerance, and to explore the molecular mechanism of cold resistance of *Rosa*. The results of this study lay the foundation for the selection of cold-tolerant *Rosa* materials, the selection and manipulation of cold-tolerance related genes, and the breeding of new cold-tolerant varieties.

2 Materials and methods

2.1 Evaluation of cold tolerance of nine *Rosa* materials

2.1.1 Plant materials and low-temperature treatment

The nine *Rosa* materials were *R. hybrida*, *R. rugosa* 'Yilanxiao', *Rosa maximowicziana*, *R. rugosa* 'Pingyin 12', *R. rugosa*, *Rosa multiflora*, *Rosa multiflora hybrida*, *Rosa wichurana* 'Dian hong', and *Rosa wichuraiana*. All of these materials were provided by the farm of Beijing Academy of Agriculture and Forestry Sciences. The annual semi-lignified branches of biennial plants were cut to a length of about 10 cm, retaining two to three leaves, and the cuttings were made in a matrix seedbed of grassy soil and vermiculite (1:1) with sprinkler irrigation facilities. At about 40 d after cutting, the rooted cuttings were planted in pots and cultivated under open air conditions, with watering once every 2–3 d. After further 55 days of growth, the cuttings with good growth and high consistency in the material were selected for low temperature treatment.

For each variety, 16 rooted cuttings with strong, consistent growth were selected for the low-temperature treatment (4°C for 2 d, then 0°C for 4 d; light intensity of 350 lux; 12 h light/12 h dark photoperiod; and relative humidity of 70%–80%). After 0 d, 2 d, 4 d, and 6 d of the low-temperature treatment, six to eight mature leaves were collected from each material to measure REC using the method described by Campos et al. (2003). The initial conductivity (R1) at room temperature was measured using a thunder magnetic conductivity meter (DDSJ-308F, Shanghai, China). The pre-treated leaves were then placed in a boiling water bath for 30 min, and the conductivity (R2) was measured after cooling to room temperature, The leaves without low-temperature conditioning were used as control conductivity (R0). Relative conductivity was calculated as follows:

$$REC/\,\% = (R1 - R0)/(R2 - R0)*100$$

After 6 d of the low-temperature treatment, photographs were taken to record changes in morphology, and then four treated plants of each variety were placed in an ultra-low temperature freezer at −15°C for 3 h. At 0 h, 1 h, 2 h, and 3 h, three leaves were selected from the same positions on three plants of *R. hybrida*, wrapped in aluminum foil, placed in liquid nitrogen, brought back to the laboratory, and stored at −80°C until real-time fluorescence quantitative PCR analysis. Finally, all the materials treated at −15°C were allowed to recover in a greenhouse at a constant temperature (24°C) for 30 d with watering once every 4–6 d. These materials were photographed at intervals to record their growth and recovery.

2.1.2 Cold tolerance data analysis

Cynodon dactylon, *Vitis vinifera* and *Rosa* plants have evaluated the cold tolerance of plants by membership function method combined with various physiological and biochemical indexes (Zhang et al., 2007; Li et al., 2011; Deng et al., 2012). Therefore, we used the subordination function method to analyze the REC values of different materials. The materials were sorted according to the subordination function value to give a ranking of their cold tolerance. The larger the subordination function value, the stronger the cold resistance of the material.

The REC is positively correlated with cold tolerance; the formula used to calculate the subordination function value was as follows:

$$U_{ij} = (X_{ij}\text{-}X_{imin})/(X_{imax}X_{imin})$$

The REC is negatively correlated with cold tolerance; the formula used to calculate the subordination function value was as

follows:

$$U_{ij} = 1\text{-}(X_{ij} - X_{imin})/(X_{imax} - X_{imin})$$

where i represents a certain material, j is the index, U is the membership grade, X_{ij} is the measured value of the index in a certain material, X_{imin} is the minimum value of the index in material i, and X_{imax} is the maximum value of the index in material i.

2.2 Mining of DEGs related to cold tolerance in *R. hybrida*

2.2.1 Samples and cold treatment

We selected three plants of *R. hybrida* that produced new branches during the 30-d recovery period. The new branches were cut off and treated at −15°C until they wilted (about 10 min). Before and after this cold treatment, three leaves were selected from the same position on the plant, wrapped in aluminum foil, frozen in liquid nitrogen, and stored at −80°C until RNA extraction. The names of the leaf samples before and after treatment were as follows:

MGCK_1_15;
MGCK_2_18;
MGCK_3_19;
MGCL_1_12;
MGCL_2_13;
MGCL_3_14;

where MCCK denotes control leaves and MGCL denotes leaves subjected to the cold treatment.

2.2.2 Total RNA extraction and quality testing

Total RNA was extracted using the Takara MiniBEST Plant RNA Extraction Kit (Takara, Dalian, China). The concentration, purity, and integrity of the extracted RNA were tested using a NanoPhotometer® Spectrophotometer (Thermo Fisher, Beijing, China), a Qubit® 2.0 Fluorometer (Thermo Fisher, Beijing, China), and an Agilent 2100 RNA Nano 6000 Assay Kit (Agilent, Beijing, China).

2.2.3 Transcriptome library preparation

After checking the quality of the extracted RNA, mRNA was enriched by the magnetic bead enrichment method, and then fragmentation buffer was added to break the mRNA into fragments of about 200 bp. Then, random primer hexamers were added to synthesize cDNA. A nucleic acid purification kit (AMPure XP beads) was used for the first cDNA purification step. The purified cDNA fragments were subjected to end-repair, the addition sequencing adapters at both ends, and then secondary purification. After screening fragments of a suitable size, PCR amplification was conducted to obtain the final cDNA library.

After the library was constructed, the cDNA concentration was determined using a Qubit 2.0 Fluorometer and then adjusted 1 ng/μL. The insert size of the library was checked using an Agilent 2100 instrument to ensure the reliability of the results. An SYBR real-time fluorescent quantification kit (Bio-Rad, Hercules, CA, USA) was used to perform qPCR to determine the concentration of the constructed library, and to construct a standard curve. When the initial library concentration is >2 nM, sequencing can be performed on the HiSeq 2500 sequencing platform. For these analyses, we used a Bio-Rad CFX 96 fluorescent quantitative PCR instrument and separated the products by electrophoresis on 2% agarose gels. After generating clusters on cBot, the paired-end sequencing program (PE150) was run on the Illumina HiSeq 2500 sequencing platform, generating 100-bp or 125-bp paired-end sequencing reads.

2.2.4 Bioinformatics analyses

Sequencing quality assessment and transcript assembly: First, the original sequencing data were sorted and pre-processed. The data were filtered with TrimGalore software (Bolger et al., 2014) and the bases with a quality value greater than 20 were retained. Secondary screening was conducted to remove fragments shorter than 50 bp in length and those with only one end in paired-end sequencing. Because there was no reference genome, the clean reads were *de novo*-spliced to obtain the reference sequence for subsequent analysis. The clean reads were spliced using the Trinity method (Sammeth et al., 2008; Grabherr et al., 2013), and the longest transcript of each gene was selected as the unigene. Trinity combines the three independent software modules of Inchworm, Chrysalis, and Butterfly to process and splice a large amount of RNA-seq data.

Unigene functional annotation: The *R. hybrida* unigene sequences were searched against six major databases (including Swiss-prot database, GO database, and KEGG database) obtain gene function annotations. GO, or gene ontology is a structured standard biological annotation system whose information applies to each species and can classify gene function in international standards. In this experiment, the distribution of all the spliced Unigene in GO was analyzed. After the differential genes were screened, the distribution of differential genes in GO was also analyzed, to analyze the function of differential genes before and after low-temperature treatment. KEGG is the primary public database for the analysis of metabolic pathways and gene function in cells. The functions and pathways associated with DEGs were determined by KEGG pathway analysis.

Gene transcript level analysis: To analyze the gene expression levels in samples before and after the freezing treatment, the sequence reads were mapped against the reference sequence using Bowtie software, and htseq-count (v0.6.0) (Robinson et al., 2010) was used to estimate each transcript's readcount value. The gene expression level is expressed as the FPKM value, calculated as follows:

$$FPKM = \frac{\text{cDNA Fragments}}{\text{Mapped Fragments (millions)} \times \text{Transcript Length (kb)}}$$

where cDNA fragments represents the number of fragments obtained by paired-end sequencing aligned to a target transcript; mapped fragments (millions) is the total number of fragments aligned to all transcripts; and transcript length (kb) is the length of the transcript.

This experiment had three biological replicates, and the final expression value was the average of the three replicates.

Detection of DEGs from transcriptome data: The readcount data obtained in the gene expression level analysis were analyzed using the quantile standardization method with DESeq software. The threshold values were *p*-value<0.05 and FDR<0.05. Significant DEGs were defined as those with a -fold change of ≥2 (*p*-value<0.1, FDR<0.1) and common DEGs were defined as those with a -fold change of ≥1.5 between the two compared groups.

GO and KEGG analyses: We used BiNGO (v2.44) software (Maere et al., 2005) to perform GO enrichment analysis of the significant DEGs. This involved calculating the number of enriched genes per GO term in the overall background, and then applying a hypergeometric test to screen the GO terms that are significantly enriched with significant DEGs.

We used KOBAS 2.0 software (Sammeth, 2009) to detect pathways significantly enriched with DEGs in *R. hybrida* between before and after treatment at −15°C. According to the known pathways in KEGG, the hypergeometric test method to detect those significantly enriched with DEGs.

2.2.5 qPCR verification of DEGs detected from transcriptome sequencing data

To verify the accuracy of the transcriptome data, 10 unigenes related to the low-temperature response, as detected based on the results of transcriptome data and GO/KEGG analyses (Supplemental Table 9), were selected for qRT-PCR analysis. The data from the two analyses were compared in a correlation analysis. For the qRT-PCR method, see section 2.3 below.

2.3 Transcript profiles of cold tolerance-related genes in *R. hybrida*

Leaves of *R. hybrida* collected at different time points during the −15°C freezing treatment were used for the screening of internal reference genes and the analysis of DEG profile patterns.

2.3.1 cDNA synthesis

The cDNA was synthesized using a Promega Reverse Transcription System kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

2.3.2 Semi-quantitative RT-PCR

We selected three candidate reference genes for *R. hybrida*; *UBI1* and *ACT4*, which were used in a previous study on gene expression in *Rosa* (Meng et al., 2013), and *GAPDH*, which was used in a study on changes in gene expression of *Rosa viciifolia* under cold stress (Liu, 2017).

The internal reference genes were screened to determine which one was most suitable for our analyses using semi-quantitative RT-PCR. The 25-μL reaction mixture included 1 μL each of upstream and downstream primers (10 μM), and 2 × Novartis Taq Plus Master Mix (12.5 μL), 1 μL cDNA, and 9.5 μL ddH_2O. The analyses were conducted using a Bio-Rad T100 gradient PCR instrument, with the following thermal cycling conditions: 94°C for 4 min for pre-denaturation, followed by 30 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s, and then final extension at 72°C for 5 min. The sequences of the three internal reference primers tested are shown in Supplemental Table 1. The amplification reaction products were detected by 1% agarose gel electrophoresis.

2.3.3 Real-time quantitative PCR

The internal reference gene for qRT-PCR was *UBI1*, which was amplified using the primers described by Meng et al. (2013). Other gene primers were designed using Primer Premier 5 software, and the sequences are shown in Supplemental Table 1.

The qRT-PCR analyses were conducted using TB Green TM Fast qPCR Mix (#RR430A) on a CFX96 Real Time PCR Detection System. The thermal cycling conditions were as follows: pre-denaturation for 1 min at 95°C, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Melting curve analysis was conducted to confirm the specificity of the primers. The relative gene transcript levels were calculated as follows:

$$\text{Relative gene expression} = 2^{-}\Delta\Delta\text{Cq}$$

where△Cq refers to the difference between the Cq values of the target gene and the reference gene, and △△Cq refers to the difference between the△Cq values of the treatment and the control.

3 Results

3.1 Cold tolerance of nine *Rosa* materials

3.1.1 Changes in REC of *Rosa* materials under low temperature

The REC of the nine *Rosa* materials increased as the duration of the low-temperature treatment increased, but the extent of the increase differed among the materials (Figure 1). The overall REC increased by 16.1% after treatment at 4°C for 2 d, by 23.2% after treatment at 0°C for 2 d, and by 69.1% after treatment at 0°C for 4 d. For *R. multiflora*, the REC was almost unchanged after treatment at 4°C for 2 d and 0°C for 2, but reached the maximum value after treatment at 0°C for 4 d. During the low-temperature treatment, the REC of *R. hybrida* increased first and then decreased. The REC of the other materials did not increase significantly after treatment at 4°C for 2 d and 0°C for 2 d, but increased and reached the maximum value after treatment at 0°C for 4 d. Among the nine materials, *R. multiflora hybrida* showed the largest increase in REC after treatment at 0°C for 4 d (to 3.65 times that of the control group). These results showed that low-temperature treatment at 4°C for 2 d and 0°C for 2 d caused little damage to cell membranes. However, when the treatment time at 0°C increased to 4 d, there was substantially more damage to cell membranes. Thus, the longer the treatment time at low temperature, the greater the damage to the cell membrane system. This resulted in increased permeability and the release of electrolytes, which caused the REC value to increase.

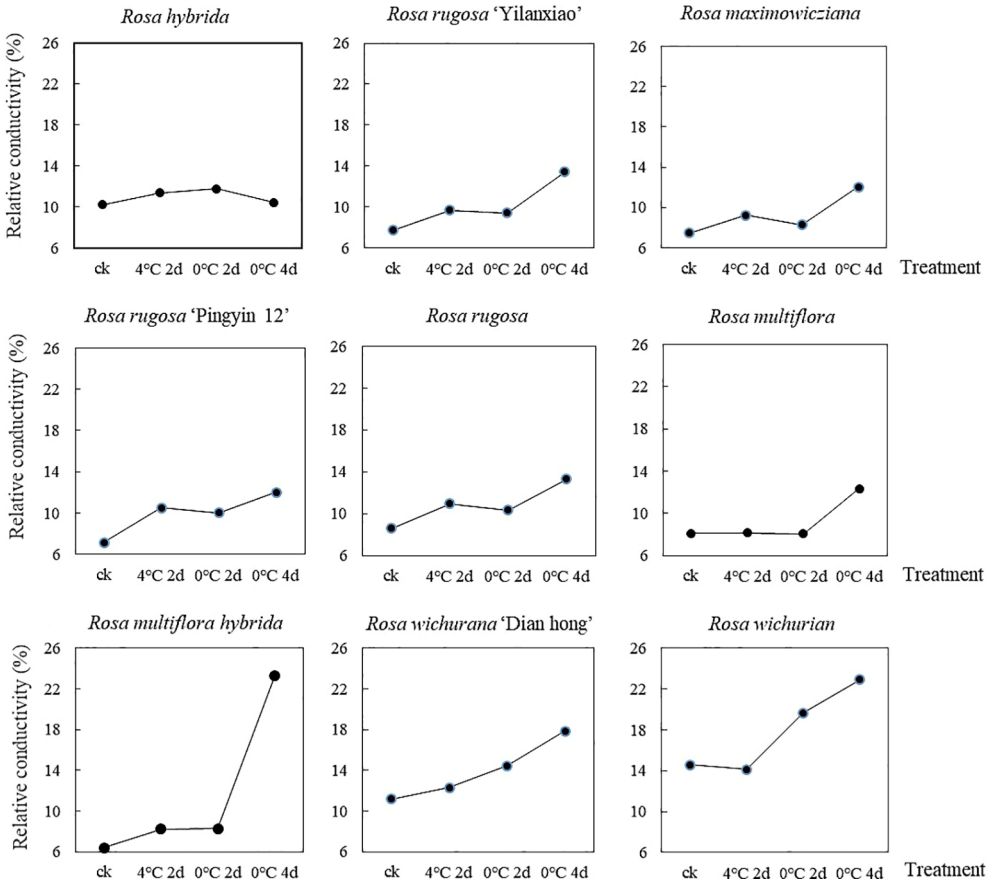

FIGURE 1
Changes in REC of nine *Rosa* materials under a low-temperature treatment.

To eliminate the influence of initial damage among the different materials, the membership function method was used to process the measured REC values of the nine materials. The larger the membership function value, the stronger the cold tolerance of the material. The nine materials were ranked, from highest membership function value to lowest, as follows: *R. rugosa* 'Pingyin 12' > *R. hybrida* > *R. rugosa* > *Rosa wichurian* > *Rosa wichurana* 'Dian hong' > *R. rugosa* 'Yilanxiao'> *Rosa maximowicziana* > *Rosa multiflora hybrida* > *Rosa multiflora.*

3.1.2 Changes in plant morphology under cold treatment

Leaves are the first organ to show symptoms of low-temperature stress. Figure 2 shows the morphology of the *Rosa* materials before the low-temperature treatment and during 30 d of recovery afterwards. During recovery, the leaves dried, shriveled, and lost their green color. Some became yellow. The branches changed in color and gradually became dehydrated.

The plant morphology changed by day 1 of recovery, with the leaves becoming wilted, drooped, and less glossy, and some branches showing color changes. White spots appeared on the leaves of *R. hybrida* and *R. rugosa* 'Pingyin 12', and the leaves of *R. rugosa* turned gray. Among all the materials, *R. rugosa* 'Yilanxiao' showed the smallest changes in leaf morphology. The branches of *R. rugosa* 'Yilanxiao' and *R. rugosa* remained purple, and branches of *R. rugosa* 'Pingyin 12' did not change color either. The stems of the other plants turned purple or black to varying degrees. The branches of *R. wichurian* were slender and widely spread. After the low-temperature treatment, the branches changed from purple to dark green, especially the lower branches, and their tips wilted.

At 15 d of recovery, the discoloration, yellowing, and dullness of the leaves had become more severe. The leaves of *R. rugosa* 'Yilanxiao', *R. rugosa* 'Pingyin 12', and *R. wichurana* 'Dian hong' were yellow, with obvious discoloration around the veins. The shoots of *R. multiflora* and *R. wichurian* were black and yellow. The branches on the upper third of *R. hybrida* and *R. wichurana* 'Dian hong' plants were yellow, but these two materials had produced new shoots at the base.

At 30 d of recovery, the branches of nine *Rosa* materials had changed color. There were multiple buds at the leaf positions on the shoots of *R. rugosa* 'Pingyin 12' and *R. rugosa*, but they were clustered together, indicating that the branches had not grown. Most of the branches on the other seven materials were still actively growing. As shown in Figure 2, new branches were present at the

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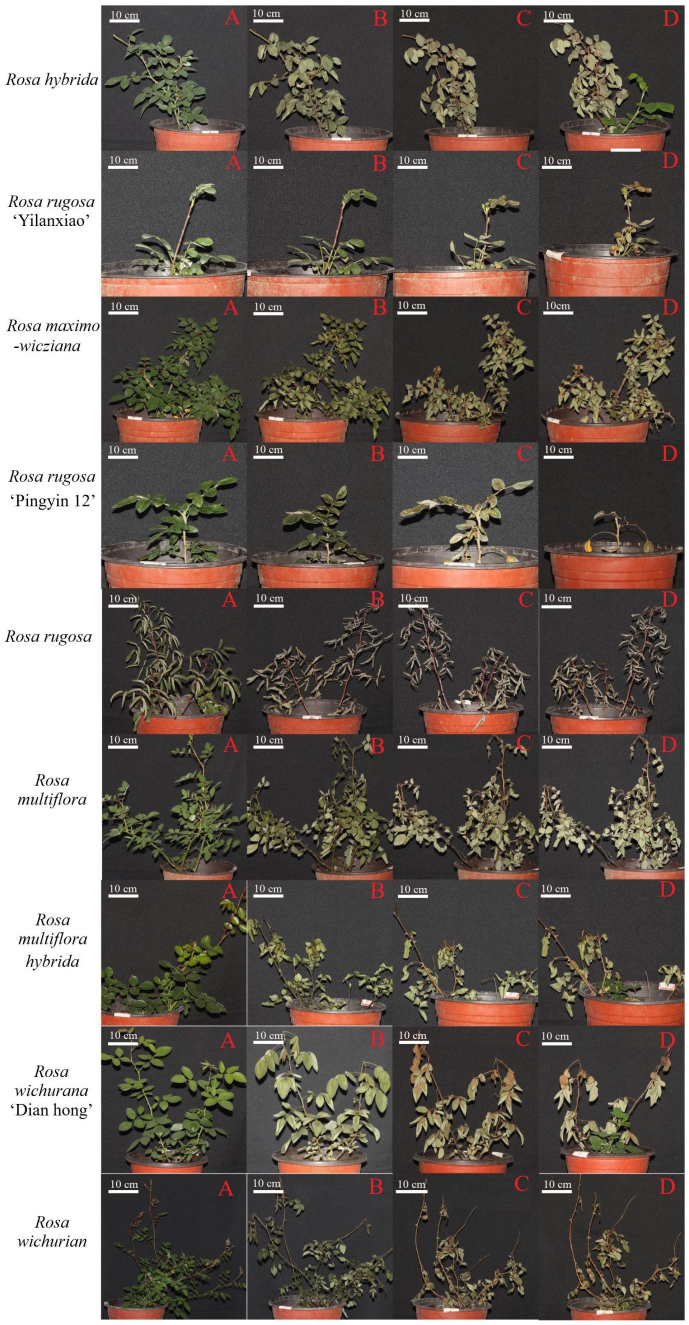

FIGURE 2
Morphology of *Rosa* materials before cold treatment **(A)** and at 1 d **(B)**, 15 d **(C)**, and 30 d **(D)** of recovery after the low-temperature treatment.

base of *R. hybrida*, *R. maximowicziana*, *R. multiflora hybrida*, *R. wichurana* 'Dian hong', and *R. wichurian* Both *R. hybrida* and *Rosa wichurana* 'Dian hong' had formed multiple compound leaves and showed the strongest recovery potential. *R. rugosa* 'Yilanxiao' and *R. multiflora* did not show form any new buds and did not survive.

On the basis of the results of the low-temperature treatments, *R. hybrida*, *R. rugosa* 'Pingyin 12', and *R. rugosa* were identified as the most cold-tolerant materials, while *R. rugosa* 'Yilanxiao' and *R. multiflora* were identified as the least cold tolerant. *R. hybrida* was chosen for transcriptome analysis because it showed a strong recovery potential after the low-temperature treatment.

3.2 Detection of DEGs based on transcriptome data

3.2.1 Phenotypic changes of *R. hybrida* plants before and after freezing treatment

To explore the molecular mechanism of cold resistance of *R. hybrida*, we selected *R. hybrida* plants after 30 d of recovery from the low-temperature treatment, and then subjected them to a freezing treatment (−15°C for 10 min). As shown in Figure 3, the leaflets of the three *R. hybrida* plants curled and drooped during the freezing treatment.

FIGURE 3
Morphological changes in *R. hybrida* plants after 10 min treatment at –15°C.

3.2.2 RNA sample quality

The six RNA samples produced clear bands, indicative of high integrity of the samples (Supplemental Figure 1). Analyses of the A260/A280 values (1.9≤A260/280 ≤ 2.1) (Supplemental Table 3) indicated that the samples were of sufficient quality for library construction.

As shown in Supplemental Figure 2, the cDNA library amplification curve had a single peak, with no contaminants or primer dimers. The initial concentration of the six samples was higher than 15.68 nM (Supplemental Table 4), so they met the requirements of the HiSeq 2500 sequencing platform. The size of the inserted fragments was suitable for further analysis. Thus, the quality of the constructed transcriptome library was sufficient for further processing steps.

3.2.3 Sequencing quality and transcript splicing

The six RNA samples from leaves of *R. hybrida* were subjected to RNA-Seq analysis. The total number of bases was 28.552 GB (at least 4.304 GB per sample). The unigene data from the six samples were combined to establish a *R. hybrida* unigene library. The Q20 base percentage was >95.63%, the Q30 base percentage was >89.97%, the GC content was >46.89%, and the average quality was higher than 37.71 (see Supplemental Table 5), indicating that the sequencing quality was sufficient for further analyses.

After splicing using Trinity software, the total number of bases was 261.64 MB, and 258,055 unigenes were obtained. The unigene length distribution curve was smooth (Figure 4). The average sequence length was 1013.9 bp, with the longest sequence being 27,201 bp. There were 76,022 unigenes longer than 2000 bp, and they accounted for 29.46% of the total unigene library. Further statistical information is provided in Table 1. The N50 (the length of the shortest contig for which longer and equal-length contigs cover at least 50% of the assembly) was 1,844. The larger the N50 value, the longer fragments are assembled and the better the assembly. This high N50 value confirmed the high integrity of the assembly.

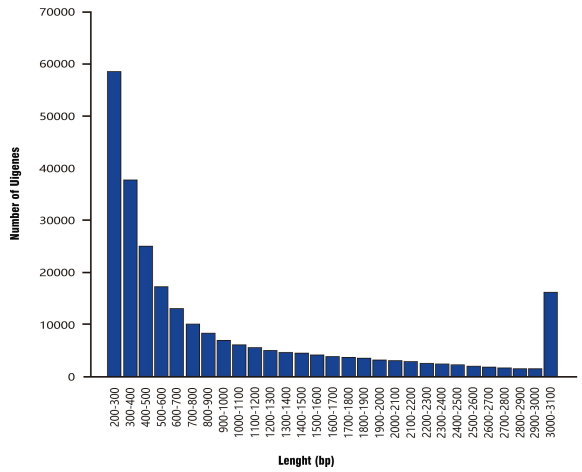

FIGURE 4
Distribution of unigene length in the library.

3.2.4 Unigene annotation

To explore the significance of differences in gene expression in *R. hybrida* under short-term freezing conditions, unigenes were annotated using GO, KEGG, and six other databases.

The 534 common DEGs were searched against the GO database. The unigenes with GO annotations were distributed in 29 subcategories of three major categories: Biological process (BP), Cellular component (CC), and Molecular function (MF). The DEGs were enriched in 12 subcategories of the BP category, eight subcategories of CC, and nine subcategories of MF. Among these subcategories, those enriched with large numbers of DEGs were protein binding (88 DEGs), catalytic activity (80 DEGs), metabolic process (58 DEGs), cellular process (57 DEGs), membrane (51 DEGs), cell (43 DEGs), and organelle (30 DEGs) (Figure 5).

Many genes contribute to normal biological functions in organisms. Pathway enrichment analysis can determine the most important biochemical metabolic pathways and signal transduction pathways involving the target gene, and this can shed light on the molecular mechanisms underlying physiological responses. In this experiment, 2192 genes were compared with the KEGG database. The common DEGs participated in 367 KEGG pathways. The pathways most enriched with the common DEGs were RNA transport, MAPK signaling, and starch and sucrose metabolism.

3.2.5 Transcriptome SNP analysis

An SNP is a change in a single nucleotide base in the genome. In simple terms, it is the same among different individuals. There is only one difference in the fact that most of the base sequences in the nucleotide sequence of a chromosome or the same site are the same. There are many SNPs in the genome, and multiple SNPs may be present on the same chromosome. SNP markers can be developed by screening for SNP sites closely linked to biological traits. Theoretically, each SNP site can have four different variants. Single nucleotide variants with a mutation frequency greater than 1% are called SNPs.

We used SAMtools/BCF tools to compare the sample genome with the reference genome, and then performed sorting, deduplication, and other processing steps. Finally, we used the mutation detection software SAMtools for SNP calling and filtering of the final SNP dataset. We

TABLE 1 Unigene length frequency distribution table.

Transcription length distribution	200–500 bp	500–1000 bp	1000–2000 bp	>2000 bp	Total
Unigene quantity	121081	55134	43801	76022	258055

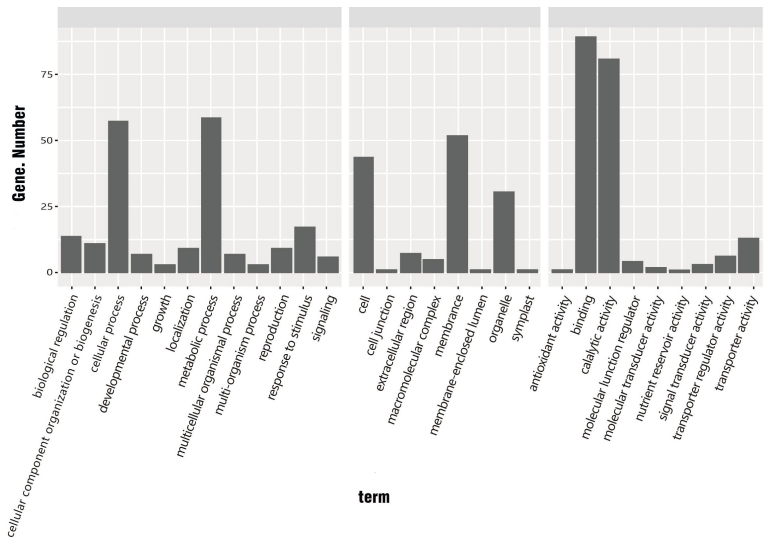

FIGURE 5
GO classification of common DEGs in *R. hybrida*, i.e., genes showing ≥1.5 log2-fold change between cold treatment and control. (BP, biological process; CC, cellular component; MF, molecular function).

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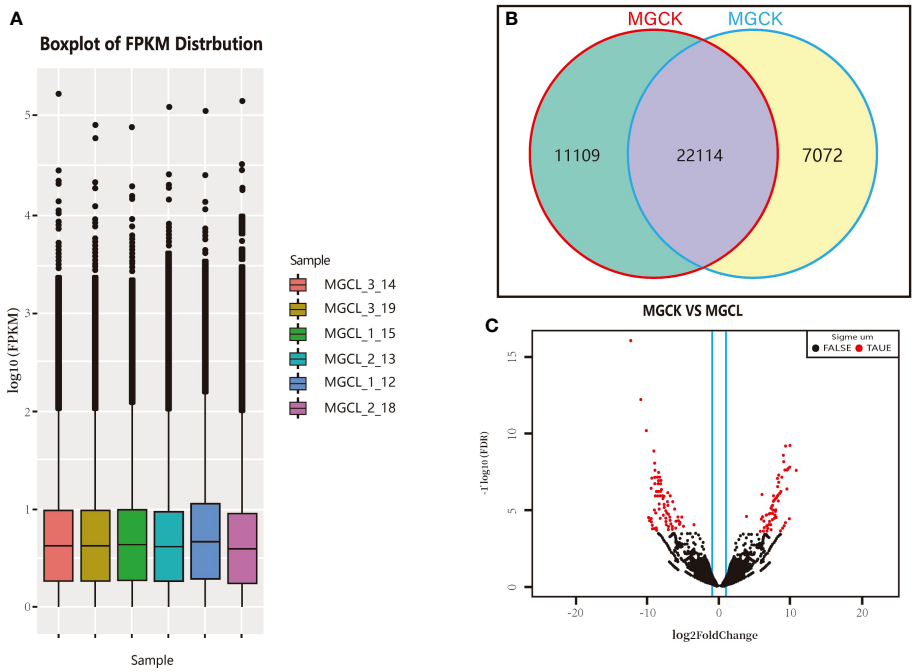

FIGURE 6
Gene transcript profiles in experimental materials and cluster volcanic plot of significant DEGs. **(A)** Log FPKM values. **(B)** Number of genes expressed in control (MGCK, no cold treatment) and under cold treatment (MGCL). **(C)** Cluster volcano plot of significant DEGs, red and black dots indicate genes showing significant and common DEGs, respectively, between control and cold treatment.

detected about 800,000 SNPs in each of the six samples (average value, 824,829.5; lowest value, 776,355) indicating that differences among the six samples were small, and the sequencing and splicing results were accurate (Supplemental Table 6).

3.2.6 Gene transcript level analysis

The FPKM value was obtained for each unigene as described above. The FPKM values spanned 10^0–10^5 orders of magnitude. The limits, median, and upper and lower quartiles of FPKM values, as well as deviations, were similar between the control (pre-freezing) and freezing treatment (−15°C for 10 min). The overall gene expression level after the freezing treatment was slightly higher than that before (Figure 6A). As shown in Figure 6B, 22,114 genes were expressed both before and after the low-temperature treatment, 11,109 genes were expressed only before the freezing treatment, and 7,072 genes were expressed only after the freezing treatment.

3.2.7 Screening to detect DEGs after freezing treatment

We used DESeq software to process the readcount values representing the gene expression levels, and used the quantile standardization method to screen significant DEGs between before and after the freezing treatment (genes with p-value<0.05, FDR<0.05, and |log2(fold change)|≥2T). The log2(fold change) indicates the size of the difference in gene transcript levels between two groups of samples, and the FDR is the correction value of the statistically significant p-value. As shown in Figure 6C, there were more significant DEGs than common DEGs. The distribution of the significant DEGs was more discrete than that of common DEGs.

We detected a total of 204 significant DEGs between before and after the cold treatment, accounting for 0.079% of all unigenes. Of them, 88 were significantly up-regulated and 116 were significantly down-regulated.

3.2.8 GO classification and enrichment analyses of significant DEGs

We conducted GO classification and enrichment analyses to explore the functions of the significant DEGs. In total, 137 significant DEGs were subjected to GO classification analysis. These DEGs were distributed in 27 subcategories within the main three categories of BP (12 subcategories), MF (9 subcategories), and CC (6 subcategories). Within the MF category, the subcategories most enriched with DEGs were ‘catalytic activity’ (67 DEGs), ‘protein binding’ (65 DEGs), ‘metabolic process’ (45 DEGs), ‘cell membrane’ (44 DEGs), ‘cellular process’ (39 DEGs), ‘cell’ (30 DEGs), and ‘organelle’ (20 DEGs). We note that there may be duplicate unigenes in each category (Figure 7A).

The results of GO enrichment for significant DEGs are shown in Figure 7B. In total, 47 significant DEGs were enriched in various categories and subcategories, with the BP category having the largest number of DEGs and the CC category having the fewest DEGs. Ten of the DEGs were enriched in the ‘RNA processing’ subcategory. Other subcategories enriched with significant DEGs under cold stress were ‘regulation of cellular carbohydrate metabolic process,’ ‘regulation of beta-glucan biosynthetic process,’ ‘regulation of beta-glucan metabolic process,’ and ‘malonyltransferase activity,’ which is related to the electron transfer chain. Therefore, these processes and pathways may be related to cold resistance in *Rosa*.

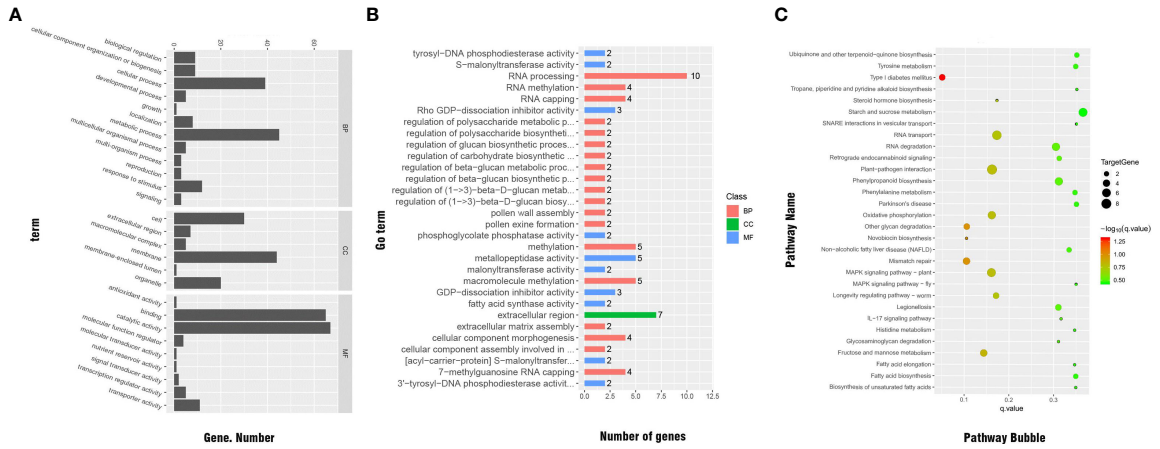

FIGURE 7
(A) GO classifications of significant DEGs. **(B)** Distribution of GO functional categories. **(C)** Functional annotations of significant DEGs by KEGG analysis.

3.2.9 KEGG pathway enrichment analysis of significant DEGs

The KEGG pathway enrichment analysis showed that, across the genome of the *R. hybrida*, 45 significant DEGs were enriched in 57 pathways in five categories and 16 subcategories. The KEGG classification results are shown in Supplemental Table 7. The pathways most enriched with DEGs were 'carbohydrate metabolism' (in the metabolism category) and 'signal transduction' (in the environmental information processing category).

The pathways most enriched with DEGs were 'RNA transport' (6 DEGs), 'starch and sucrose metabolism' (5 DEGs), 'phenylpropanoid biosynthesis'(5 DEGs), 'MAPK signaling pathway-plant' (5 DEGs), 'mismatch repair' (4 DEGs), oxidative phosphorylation (4 DEGs), and 'fructose and mannose metabolism' (4 DEGs) (Figure 7C).

Some of the pathways enriched with DEGs, including 'fatty acid elongation,' 'biosynthesis of unsaturated fatty acids,' and 'aminoacyl-tRNA biosynthesis' were related to the changes in unsaturated fatty acid content in biofilms and proline metabolism during the response to low-temperature stress. The 'oxidative phosphorylation,' 'glycolysis/gluconeogenesis,' and 'starch and sucrose metabolism' pathways were related to intracellular metabolism and the accumulation of protective substances against cold-induced damage. The 'plant hormone signal transduction' pathway was related to signal transduction and hormonal regulation under low-temperature stress. The pathways significantly enriched with DEGs are shown in Supplemental Table 8.

3.2.10 qPCR verification of DEGs identified from transcriptome data

Ten genes were selected for qRT-PCR analysis. Six of the 10 genes were up-regulated and four were down-regulated under freezing stress (Figure 8A). The trends in gene transcription detected in the qPCR analyses were the same as those detected from the transcriptome data (see Supplemental Table 9). A correlation analysis was conducted between the transcriptome sequencing log2 (Fold Change) values and the qRT-PCR relative expression log2 (Fold Change) values. The two sets of data were positively correlated, with a correlation coefficient (R^2) of 0.938 (Figure 8B). These results confirmed that the transcriptome data were accurate and reliable.

3.3 Trends in transcription of cold-tolerance-related genes in *R. hybrida*

3.3.1 Internal reference gene screening

We conducted semi-quantitative RT-PCR analyses to screen for an appropriate internal reference gene. The product amplified with *GAPDH* primers had two bands, indicative of poor primer specificity. Single bands were produced using the *UBI1* and *ACT4* primers, indicating that they had better specificity (Supplemental Figure 3). Thus, *UBI1* and *ACT4* were selected as potential internal reference genes for qRT-PCR.

The *UBI1* and *ACT4* genes were subjected to qRT-PCR, and the cq values obtained were processed and analyzed using NormFinder and BestKeeper software. The results showed that *UBI1* expression was more stable than *ACT4* expression (Table 2 and Supplemental Table 10). Therefore, *UBI1* was selected as the reference gene in this study.

3.3.2 Transcript profiles of cold-tolerance-related genes in *R. hybrida* at −15°C

After analyzing the results of transcriptome sequencing, we selected four genes related to cold tolerance for verification of their transcriptional profiles at different times points under −15°C treatment by qRT-PCR.

Note: * indicates significant difference ($P< 0.05$) compared with 0 h; ** indicates significant difference ($P< 0.01$) compared with 0 h.

As shown in Figure 9, we analyzed the transcript levels of four genes related to the cold response of *R. hybrida*. One of them encoded a POD, which participates in the enzymatic protection system in plants. During the 3-h freezing treatment, *POD17* was significantly down-regulated at 1 h, up-regulated at 2 h, and then down-regulated to the lowest level at 3 h. We speculated that the strongest response at 2 h would result in the production of POD to increase the capacity of the enzymatic protection system.

NDUFA9 encodes a subunit of NADH dehydrogenase, which catalyzes the transfer of electrons from NADH to coenzyme Q. This is a crucial enzyme for oxidative phosphorylation in mitochondria. During the 3-h freezing treatment, *NDUFA9* was down-regulated during the first two h, and then up-regulated by 3 h.

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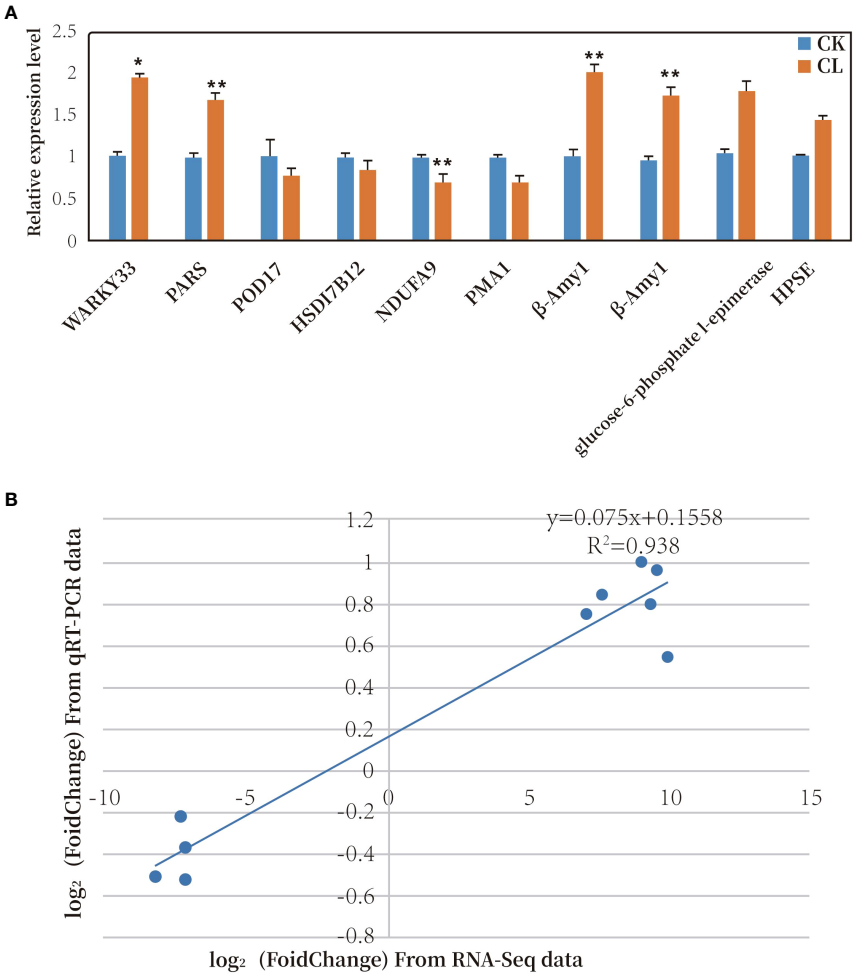

FIGURE 8
(A) Relative transcript levels of 10 DEGs as determined by qRT-PCR. * represents significant difference at $P< 0.05$ compared with control; ** represents significant difference at $P< 0.01$ compared with control. **(B)** Correlation analysis of results of RNA-Seq and qRT-PCR analyses of 10 DEGs. (CK, MGCK, no cold treatment; CL: MGCL, under cold treatment).

PMA1 encodes an ATPase. During the 3-h freezing treatment, *PMA1* was continuously and significantly up-regulated, with highly significant increased transcript levels at 2 h and 3 h (to 1.6 times that in the control).

The expression of *β-Amy1* increased first, then decreased, and then increased during the freezing treatment. It was significantly up-regulated at 1 h (to 1.2 times that in the control), decreased to its lowest level at 2 h, and then was up-regulated at 3 h. This fluctuation in the transcript level of *β-Amy1* in *R. hybrida* suggested that the abundance of amylase was regulated to control the soluble sugar (maltose) content in the cells.

TABLE 2 Expression stability of *UBI1* and *ACT4* analyzed by NormFinder.

Gene	stability	Sort
UBI1	0.018	1
ACT4	0.028	2

4 Discussion

4.1 Cold tolerance of *Rosa* materials

Plant cells sense low temperature through receptors on the membrane. The transmission of signals related to the low temperature response results in damage to the structure and function of the cell membrane system, changes in membrane permeability, and a large amount of electrolytes leaking from the membrane. This can be detected as an increase in REC. In cold-tolerant plants, the cell membrane permeability will change less and gradually recover. However, in cold-sensitive plants, the cells are more severely damaged and membrane permeability is greatly increased. If the cells cannot recover, this damage can result in plant death. In this study, we found that the REC values of two materials, *R. hybrida* and *R. rugosa* 'Pingyin 12', were lower than those of materials after cold treatment at 4°C for 2 d and 0°C for 4 d. The REC values of *R. hybrida* were particularly low, indicating that its membranes were not severely damaged by the cold treatment, and were able to recover afterwards. On the basis of this analysis, we

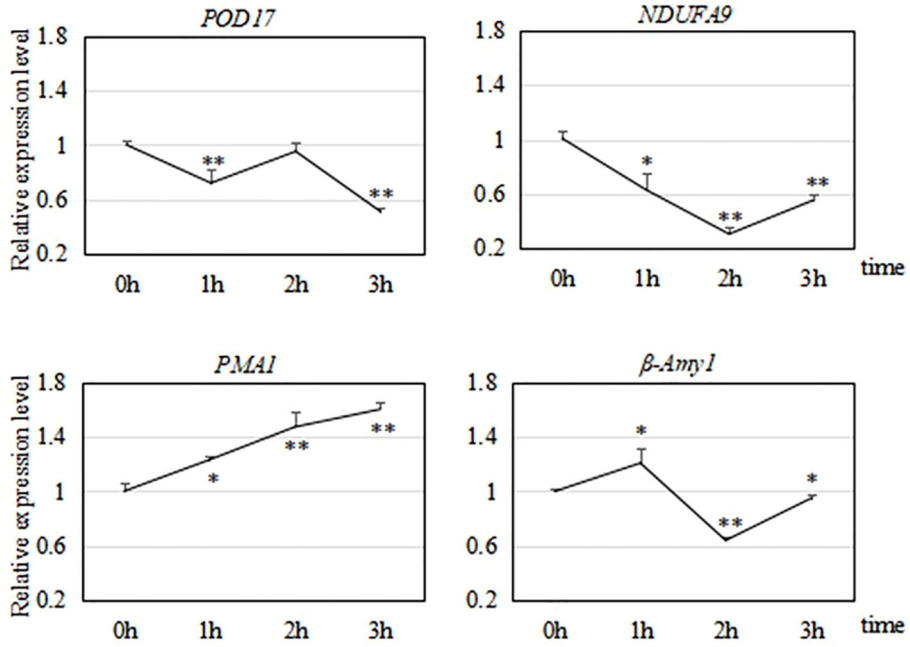

FIGURE 9
Relative transcript levels of four genes during a 3-h freezing (–15°C) treatment. * indicates significant difference ($P < 0.05$) compared with 0 h; ** indicates significant difference ($P < 0.01$) compared with 0 h.

concluded that *R. hybrida* and *R. rugosa* 'Pingyin 12' are cold-resistant *Rosa* materials.

In this study, whole plants in the *Rosa* genus were subjected to a low-temperature treatment to evaluate their cold tolerance. In other studies, such as those of Ren (2013) and Ma and Chen (1991), the cold tolerance of *Rosa* materials was assessed using isolated leaves and branches. One study assessed cold tolerance using dormant branches of *Rosa* plants, and measured changes in physiological indicators after a low-temperature treatment. Although that method is economical, convenient, and saves experimental space, the mechanical damage caused by cutting will affect the accuracy of the experimental results. In addition, because most of the branches and leaves are used, there is no way to observe the morphological changes to living plants after a low-temperature treatment. In this study, the cold tolerance of *Rosa* materials was evaluated by subjecting whole plants to a low-temperature treatment, and then assessing various physiological indicators and recovery. Compared with using cuttings, using whole plants provides a more accurate picture of how they respond to, and recover from, low-temperature stress.

Plant leaves produce carbohydrates through photosynthesis and then transport them to the branches and roots. The carbohydrates produced by photosynthesis are related to cold resistance (Liu, 2017). The sucrose produced in the leaves not only functions as a nutrient for transport to other organs *via* the phloem, but also as a response signal to various stresses. Price et al. (2004) found that a large number of stress-responsive genes, such as genes related to carbohydrate metabolism, signal transduction, and metabolite transport, are induced by glucose. Most of these genes are related to the cold resistance of plants, indicating that glucose in plant leaves plays an important role in signal transduction to establish cold resistance. Liu (2017) measured several physiological indicators in the branches and leaves of *Rosa sylvestris* and *C. odorifera* after a low-temperature treatment. Although the dynamic changes under low temperature were consistent for most indicators, they were inconsistent for soluble sugar content. In this study, we measured the REC of leaves after a low-temperature treatment and growth recovery after a −15°C treatment. We found that the results of growth recovery were consistent with the REC results. Therefore, determining the REC of leaves after a low-temperature treatment is a reliable method to assess the cold tolerance of plants.

According to our results, the most cold-resistant materials in this study were *R. hybrida* from Henan, *R. rugosa* 'Pingyin 12', and *R. rugosa* from Shandong, while *R. multiflora* and *R. wichurian* from Yunnan showed relatively weak cold resistance. This led us to speculate that their cold tolerance was related to their long-term growth environment. The most cold-tolerant materials are mainly grown in Northern China, while the most cold-sensitive materials are mainly grown in Southern China. It was speculated that differences in the cold tolerance of *Rosa* plants were related to the adaptability of provenances to their long-term growth environment.

4.2 Detection of DEGs from transcriptome data

The molecular mechanism of cold tolerance in plants is complex. The physiological and biochemical changes in plants caused by low temperature are not regulated by a single gene. A gene can participate in several different pathways, and can be regulated by multiple upstream genes. Therefore, it is important to study the whole functional network. By analyzing the transcriptome data, we detected a large number of DEGs in *R. hybrida* under low-temperature stress. In further research, it will be important to explore the roles of these DEGs,

and determine which ones encode key components in terms of cold resistance. In this study, we detected SNP markers for key genes related to cold resistance in *Rosa*. These may be useful for rapid estimates of the cold tolerance of various *Rosa* materials.

Transcription factors play an important role in the response to low-temperature stress. In this study, we detected significant up-regulation of the *WRKY33* transcription factor gene in the MAPK signaling pathway in *R. hybrida* under low-temperature stress. Many studies have demonstrated that this transcription factor is involved in resistance to stresses, including cold stress, in plants. (Jiang and Deyholos Michael, 2009). found that overexpression of *AtWRKY33* improved salt tolerance, and that ABA induced the expression of *OsWRKY08* and improved the osmotic stress resistance of transgenic *Arabidopsis* plants (Song et al., 2010). transferred soybean *WRKY21* into *Arabidopsis*, and found that it improved the cold tolerance of the transgenic plants. Another study demonstrated that *WRKY33* in alfalfa was up-regulated by low temperature (Feng et al., 2015). Further research is required to explore the roles of *WRKY33* in the cold response of *R. hybrida*, and to determine whether it can confer cold resistance.

The KEGG pathway enrichment analyses showed that POD is involved in three pathways simultaneously: 'phenylpropanoid biosynthesis,' 'metabolic pathways,' and 'biosynthesis of secondary metabolites.' Among all the pathways affected by the freezing treatment, 'synthesis of phenylpropanol substances,' was the most strongly affected. This pathway may be related to lignin synthesis. Lignin metabolism is related to plant cell differentiation, disease resistance, and fruit development (Yu et al., 2003). These results suggest that low temperature affects lignin synthesis in *Rosa*.

Liu (2017) conducted transcriptome analyses of *R. sylvestris*, and detected more DEGs in the branches than in the leaves. Branches connect leaves to roots. The phloem contains sugars, hormones, and other substances such as small RNAs (Lough and Lucas, 2006), which play a very important role in plant growth and signal transduction. Further experiments are required to explore how changes in gene expression affect the composition of substances in the branches and roots of *Rosa* plants under low-temperature stress.

4.3 Transcript profiles of cold tolerance-related genes in *R. hybrida*

Oxygen is involved in plant cell metabolism, but under low-temperature stress, the activity of enzymes related to metabolic activities decreases, and the oxygen utilization rate also decreases. This can lead to the accumulation of reactive oxygen species (ROS), which react with various cellular components because of their unpaired electrons and highly active state. This can result in abnormal cell metabolism and even death, thereby disrupting normal growth and development. The organs with more active metabolism produce more ROS. The antioxidant system can remove ROS and help to maintain the balance between their production and consumption (Dou, 2013). POD participates in the antioxidant system. Studies have shown that the activity and abundance of POD in grape branches increases after low-temperature acclimation, and these increases are greater in varieties with stronger cold resistance (Wang et al., 1996). In a study on winter wheat and spring wheat, Baek and Skinner (2003) found that at the early stage under a low-temperature treatment, there were increases in the transcript levels of genes encoding antioxidant enzymes such as mitochondrial manganese superoxide dismutase, cytosolic monodehydroascorbate reductase, chloroplast dehydroascorbate reductase, thylakoid-associated ascorbate peroxidase, and glutathione peroxidase. In this study, we found that *POD17* in *R. hybrida* was first down-regulated, then up-regulated, and then down-regulated under a 3-h freezing treatment. This may have increased the activity and/or abundance of POD around 2 h of freezing treatment, but further research is required to determine whether changes in gene transcript levels are consistent with changes in the levels of the corresponding protein.

NDUFA9 encodes a subunit of NADH dehydrogenase, which is the first enzyme in the oxidative phosphorylation pathway. This pathway oxidizes organic matter such as sugars, lipids, and amino acids to release energy and generate ATP to fuel all of the essential processes for plant life. Low-temperature stress in plants affects the expression of mitochondrial genes encoding NADH dehydrogenase subunits, resulting in defects in germination, growth, and development, and other changes in response to hormonal changes (Zeng et al., 2018). In this study, we found that *NDUFA9* in *R. hybrida* was down-regulated and then up-regulated during a 3-h freezing treatment. These results imply that this gene plays a role in the late stage of the cold-resistance response of *R. hybrida*.

PMA1 encodes an H^+-ATPase. In plants, H^+-ATPases participate in various processes, such as pH regulation within cells, cell elongation and growth, stomata opening and closing, and transmembrane transport. It is known as the "master enzyme" of essential plant processes (Yang et al., 2006). When cold-sensitive plants are subjected to low temperatures, the ATPase on the membrane is inactivated and its activity decreases, which weakens active transport, increases membrane permeability, and results in the passive export of potassium ions and sugars. The plasma membrane H^+-ATPase activity of eggplant leaves was found to double under mild freezing stress, but it decreased under more severe freezing stress. In the needles of very cold-hardly pine trees under severe freezing stress, the plasma membrane H^+-ATPase activity was found to be significantly increased (Mao et al., 2003). In this study, *PMA1* was gradually up-regulated in *R. hybrida* during the 3-h freezing treatment. This implies that the abundance of the proton transport ATPase increased to maintain active cell transportation, indicative of the low-temperature adaptability of this material.

Sugars function as osmotic regulation substances in plants, and their metabolism is greatly affected by low temperature. The accumulation of soluble sugars can alleviate the osmotic stress caused by low temperature and improve cold resistance (Chen, 2012). β-amylase catalyzes the formation of maltose from starch, and the accumulation of maltose helps to protect the electron transport chain and proteins in the chloroplast matrix under low-temperature stress. In another study, knock-out of the *Arabidopsis* β-amylase *BMY8* gene blocked maltose accumulation, resulting the accumulation of starch. The freezing resistance of the knock-out line was significantly lower than that of the wild type, and its photosystem II was more sensitive to cold stress (Kaplan and Guy, 2005). In the present study, we found that *β-Amy1* in *R. hybrida* was first up-regulated, then down-regulated, and then up-regulated during

a 3-h freezing treatment. We speculate that *β-Amy1* in *R. hybrida* was induced by low temperature at the initial stage of stress. This would increase starch degradation, leading to the accumulation of maltose, and consequently, less cell damage. As the duration of the freezing treatment extended, the tolerance limit was gradually reached and *β-Amy1* was down-regulated, so that the maltose content decreased. Thus, we speculate that this gene plays a very important role in the early stages of the response to freezing.

The expression of genes related to cold tolerance in plants is affected by multiple factors such as the duration (time) and intensity (temperature) of the cold treatment. Further studies should test the effects of cold treatments of different duration and intensity on gene expression in *Rosa*.

5 Conclusion

Nine *Rosa* materials were subjected to a low-temperature treatment to identify their degree of cold tolerance. *R. hybrida, R. rugosa 'Pingyin 12'*, and *R. rugosa* were selected as cold-resistant materials, that enriched the resources of high-quality cold resistant varieties, provided scientific basis for cold resistance breeding and cultivation management, and expanded the cultivation and distribution range of *Rosa* plants. We screened the transcriptome data to identify DEGs related to cold tolerance in *R. hybrida*. GO enrichment analysis revealed that genes showing significant changes in their transcript levels under short-term low-temperature stress were involved in sugar metabolism. The KEGG pathway enrichment results showed that 47 significant DEGs under low temperature were enriched in 57 pathways, Among them, Carbohydrate metabolism and Signal transduction were classified with the largest number of enrichment pathways The transcript profiles of four cold tolerance-related genes in *R. hybrida* were determined by qRT-PCR. The four genes, *POD17*, *NDUFA9*, *PMA1*, and *β-Amy1*, showed different expression patterns during a 3-h freezing treatment. All four of these genes were involved in the low temperature response in *R. hybrida*.

Plant cold resistance is the result of genetic factors and environmental factors, so it needs to be studied from various angles and by various methods. Although the selection of suitable planting land, increasing soil fertility and improving light and temperature conditions can improve the cold resistance of plants, the research on the molecular mechanism of cold resistance can lay the foundation for molecular breeding for cold resistance. Low temperature is a kind of abiotic environmental stimulus. In order to adapt to environmental changes, plants will produce various physiological responses and their own defense systems to resist cold and freezing. In this study, cold tolerance identification of *Rosa* materials and expression patterns of genes will lay a foundation for cold-resistant molecular breeding and mechanism analysis.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

XC, HW, QS, JX: Conceptualization, Methodology, Formal analysis, Resources, Writing-Reviewing and Editing, Funding acquisition. QS, HW: Investigation, Data Curation, Writing-Original draft preparation. DC: Writing-Reviewing, Validation. CL: Writing-Reviewing. HL: Methodology, Supervision. CH: Supervision, Funding acquisition, Project administration. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

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Research advance on cold tolerance in chrysanthemum

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Chrysanthemums are one of the top ten most well-known traditional famous flowers in China and one of the top four cut flowers worldwide, holding a significant position in landscape gardening. The cold temperatures of winter restrict the cultivation, introduction, and application of chrysanthemum, resulting in high costs for year-round production. This severely impacts the ornamental and economic value of chrysanthemum. Therefore, research on cold tolerance is of vital importance for guiding chrysanthemum production and application. With the development of genomics, transcriptomics, metabolomics, and other omics approaches, along with high-throughput molecular marker technologies, research on chrysanthemum cold tolerance has been continuously advancing. This article provides a comprehensive overview of the progress in cold tolerance research from various aspects, including chrysanthemum phenotype, physiological mechanisms, the forward genetics, molecular mechanisms, and breeding. The aim is to offer insights into the mechanisms of cold tolerance in chrysanthemum and provide reference for in-depth research and the development of new cold tolerance chrysanthemum varieties.

KEYWORDS

phenotype, physiological mechanism, the forward genetics, molecular mechanism, breeding

1 Introduction

Chrysanthemum (*Chrysanthemum* × *morifolium* Ramat.), a perennial herb of the *chrysanthemum* genus in the Asteraceae family, has a history of 4,000 years in Chinese written records (Zhang and Dai, 2013) and a cultivation history of more than 3,000 years (Zhang, 2017), with more than 3,000 varieties (Wang, 2020). Chrysanthemum is one of the top ten most well-known flowers in China. With a graceful posture, bright colours and pleasant flowers, it has high ornamental value. In addition, it also has edible, tea and medicinal functions. In ornamental plants, low-temperature stress not only affects their ornamental traits, such as flower shape and colour but also reduces their ornamental

quality (Li et al., 2008), and annual production is affected by the restriction of their growth and development (Ao et al., 2019). In addition, the winter production of chrysanthemums in most areas requires the use of facilities for heating cultivation, which greatly increases production costs, especially in the environment of northern facilities. There is a low-temperature "rosette" phenomenon in chrysanthemum production (Li et al., 2015), which seriously affects their ornamental value and economic benefits (Li et al., 2010). To date, studies on cold tolerance have been reported for a variety of plants, and plant cold tolerance is considered a complex quantitative trait (Guy, 2003; Wang et al., 2016; Zhang et al., 2018). Research on the cold tolerance in chrysanthemum has focused on evaluation systems (Anderson and Gesick, 2004; Xu and Chen, 2008); physiological, biochemical (Li et al., 2013; Wang et al., 2014), and genetic mechanisms (Ma, 2017; Chi et al., 2018); key gene mining (Chen et al., 2012; Ren et al., 2014); germplasm innovation (Zhang X. J. et al., 2011; Chen et al., 2012; Chi et al., 2018) and other relevant information.

This article provides a comprehensive review of the main cold tolerance evaluation indicators for chrysanthemum from both the phenotypic and physiological aspects. It also summarizes the existing research progress on the genetic and molecular mechanisms in chrysanthemum cold tolerance. Additionally, the breeding methods for cold resistant chrysanthemum are outlined (Figure 1). The aim is to offer a scientific basis for promoting excellent chrysanthemum varieties in cold regions, and to lay the foundation for further research into the mechanisms of chrysanthemum cold tolerance and the development of new cold resistant varieties.

2 Research advance on the phenotypes of cold tolerance in chrysanthemum

Low temperature is one of the main environmental factors affecting plant growth and development (Petrov et al., 2016; Xin et al., 2015), and low-temperature stress can result in phenotypic changes in plants. Many studies have analysed and evaluated their cold tolerance in chrysanthemum from the perspective of the foot bud (Anderson and Gesick, 2004), florescence (Li et al., 2013), relative conductivity (Li et al., 2009), semilethal temperature (Shi, 2013) and morphological structure (Xu et al., 2009).

2.1 Foot buds

Chrysanthemums are perennial herb flowers. After flowering, the ground part of the chrysanthemum withers, and foot buds sprout from the base to overwinter. The cold tolerance of the foot buds not only determines whether the chrysanthemum can safely

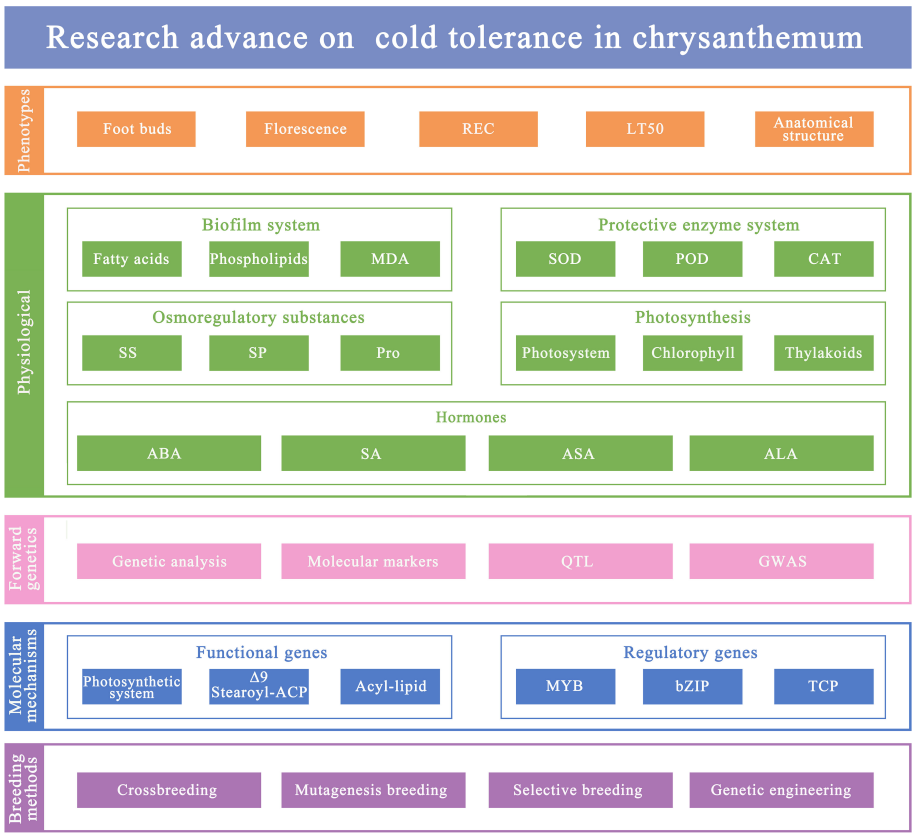

FIGURE 1
Review framework of cold cold tolerance in chrysanthemum.

overwinter but also affects the reproduction coefficient of the plant in the next year. Many studies have evaluated cold tolerance chrysanthemum mainly based on the number of foot buds and their growth. Anderson and Gesick (2004) found that the more foot buds that perennial overwintering roots had, the stronger the low-temperature tolerance of the variety was. Anderson and Gesick (2004) also judged the cold tolerance of different varieties by counting the number of foot buds in the 'Tianjian' autumn chrysanthemum. Zhang and Zhang (2014) used 15 groundcover chrysanthemums from the 'Jinling', 'Zhongshan' and 'Yuhua' series as experimental materials and evaluated their cold tolerance in terms of the frost damage grade of the petals and leaves of each variety, combined with the number of germinated foot buds in early spring. The petals of the 'Yuhua' series had the strongest cold tolerance, and the number of foot buds that germinated in early spring was also the highest. In addition, Kim and Anderson (2006) determined the cold tolerance of isolated foot buds and whole plants by two low-temperature treatment methods and established a method to evaluate cold tolerance in chrysanthemum. However, Li et al. (2014) found that the root rate, average number of roots and average root length of the foot buds of the groundcover chrysanthemum 'Wanfen' were the best when the cold storage temperature was 4°C and the cold storage time did not exceed 4 weeks, which allowed indirect evaluation of the cold tolerance of 'Wanfen'. In conclusion, the foot bud is an important phenotypic trait in the study of cold tolerance in chrysanthemum.

2.2 Florescence

The florescence is one of the most important phenotypic traits of chrysanthemum and can be divided according to natural florescence into spring chrysanthemum (late April to late May), summer chrysanthemum (late May to August), autumn chrysanthemum (late October to late November), and cold chrysanthemum (early December to February of the following year) (Wan, 2013). Related studies have found a correlation between chrysanthemum florescence and cold tolerance. In particular, Li et al. (2013) used early autumn chrysanthemums 'Taiping's Snare Drum' and 'Jin Fengling' and late autumn chrysanthemums 'Starlight Bright' and 'Mobao' as materials and found that the unsaturated fatty acid contents of 'Taiping's Snare Drum' and 'Starlight Bright' were significantly higher than those of the other two varieties at 16°C and 5°C. At -4°C and -8°C, the unsaturated fatty acid content of the early flowering variety 'Jinfengling' was significantly lower than that of the other three varieties, and the saturated fatty acid content was the opposite. It was speculated that the flowering period of autumn chrysanthemums had a certain relationship with the unsaturation of fatty acids and cold tolerance. Wang et al. (2014) performed cluster analysis of nine autumn chrysanthemum varieties and found that early flowering varieties had stronger cold tolerance, while late flowering varieties had weaker cold tolerance, and the flowering date was related to the cold tolerance of the chrysanthemums to some extent. Li (2010) analysed the cold tolerance of two different cut chrysanthemum varieties, 'Cold Purple' and 'Cold Yellow', and found that when the temperature was reduced to 9°C or 6°C, physiological obstacles began to appear in the flowering period. However, Xu and Chen (2008) ranked the cold tolerance of eight different chrysanthemums by comparing the semilethal temperatures (LT50) and found that there was no significant correlation between cold tolerance and florescence. In summary, there is a certain relationship between the florescence and cold tolerance in chrysanthemum, but the variety has a certain impact on this relationship, and the correlation between florescence and cold tolerance needs to be further studied.

2.3 Relative conductivity and semilethal temperature

When plants are under low-temperature stress, it is mainly manifested as reduced fluidity, enhanced permeability, and different degrees of changes in the structure and composition of the cell plasma membrane, resulting in different degrees of electrolyte exosmosis (Xu, 2012). Therefore, relative conductivity (REC) can be used as an evaluation indexes of plant cold tolerance. Recently, the REC index has been directly used to evaluate the cold tolerance of many plants (Zeng et al., 2016; Wei et al., 2017; Yang et al., 2017), but it has rarely been used to study cold tolerance in chrysanthemum. Li et al. (2009) subjected autumn white chrysanthemum to low-temperature exercise at 4°C and low-temperature stress at -5°C and found that the REC of chrysanthemum leaves after low-temperature exercise was significantly lower than that of the control, indicating that low-temperature exercise can enhance the cold tolerance of chrysanthemum and alleviate the degree of damage to the chrysanthemum leaf film.

In the study of chrysanthemum cold tolerance, the LT50, calculated by combining the REC with a logistic equation, is generally used as an important index to evaluate cold tolerance (Liu et al., 1985; Wang, 1987). Shi (2013) calculated LT50 by measuring the membrane lipid physiological indices of leaves and roots under low-temperature stress, and a comprehensive evaluation of the cold tolerance of different autumn chrysanthemum varieties was conducted. Xu and Chen (2008) measured the REC of leaves of different chrysanthemum varieties during the whole process of the temperature drop to rise in autumn and winter, recorded and calculated changes in LT50 values, and found that the LT50 could be used as a reliable index for evaluating cold tolerance in chrysanthemum. At the same time, it was found that the cold tolerance of each chrysanthemum variety was relatively stable after gradually decreasing natural low-temperature exercise, and the evaluation result of cold tolerance was relatively reliable. Liu et al. (2014) determined the physiological indices of 18 *Spiraeas* varieties under low-temperature stress, obtained the membership function values of the physiological indices of cold tolerance and LT50s through REC, evaluated the cold tolerance in combination with field investigation, and screened out the varieties of *Spiraea* with strong cold tolerance. Li (2010) studied 'Cold Purple' and 'Cold Yellow' chrysanthemum flowers and found that the REC and LT50 were related to the tongue and

tube flowers, and the REC significantly increased with decreasing temperature. The cold tolerance of the 'Cold Yellow' variety was stronger than that of 'Cold Purple', and the cold tolerance of the tube flowers of both varieties was stronger than that of the tongue flowers. In conclusion, REC can be used as an evaluation index of cold tolerance alone, or LT50 can be calculated by combining the logistic equation for evaluation. Both REC and LT50 are important indices for evaluating cold tolerance in chrysanthemum.

2.4 Anatomical structure

Plants growing in cold areas in nature have been in low-temperature environments for a long period of time, and their morphological and anatomical structures have undergone a series of adaptive changes to the environment, which gradually resulted in cold-resistant plants. The leaf epidermal structure includes the epidermis, cuticle, mesophyll, palisade tissue and spongy tissue. Many studies have found that the components of the leaf epidermal structure are closely related to cold tolerance (Zhao et al., 2005; Cui and Ma, 2007). In addition, Ai et al. (2006) found that the ultrastructure of cells was closely related to cold tolerance.

In terms of chrysanthemum cold tolerance, Xu et al. (2009) found that the LT50 of chrysanthemum leaves was significantly negatively correlated with the palisade tissue thickness, palisade tissue tightness, epidermal thickness, and palisade tissue/spongy tissue ratio and significantly positively correlated with palisade tissue porosity. Through path analysis, it was also found that the anatomical structure of leaves not only directly affected the cold tolerance of the chrysanthemum but also indirectly affected the change in the soluble sugar (SS) content. A mathematical model for comprehensive evaluation of the cold tolerance chrysanthemum was established by selecting the palisade tissue/spongy tissue ratio, palisade tissue tightness, palisade tissue porosity and SS content change rate as indices. Hu et al. (2000) found that after low-temperature treatment, the ultrastructure of the leaf organelles of a cut-flower chrysanthemum showed obvious changes, including the degradation of large starch grains, an increase in the number of mitochondria and lipoid particles, and slight disturbance of the lamellar structure. The organelles of varieties with strong cold tolerance were relatively stable. In conclusion, the structural composition of the leaf epidermis and ultrastructural changes in organelles can also be used as evaluation indices of cold tolerance in chrysanthemum.

There are relatively few studies on the phenotype of cold tolerance in chrysanthemum, mainly focusing on the aspects of the foot bud, florescence, REC, LT50 and leaf epidermal structure, among which the relationship between florescence and cold tolerance needs further investigation. In addition, research on evaluation indices that can directly evaluate chrysanthemum cold tolerance is insufficient, and it is necessary to strengthen research on the phenotype of cold tolerance in combination with new technologies and new theories to lay a foundation for future physiological and molecular studies in chrysanthemum.

3 Research advance on the physiological mechanisms of cold tolerance in chrysanthemum

Compared with the study of chrysanthemum phenotypic indices, the study of physiological mechanisms can more directly reflect the degree of low-temperature stress in plants. Many studies on the physiological mechanisms of cold tolerance in chrysanthemum have mainly focused on the biofilm system, protective enzyme system, osmotic regulatory substances, photosynthesis and hormones.

3.1 Biofilm system

Biofilms provide not only a relatively stable environment for cells but also a medium for transport, energy transfer and information exchange between cells and the outside world. Lyons (1970) believe that the cell membrane is the initial site damaged by low temperature, and its integrity and permeability are closely related to plant cold tolerance. To date, many studies of biofilms and cold tolerance have mainly focused on the changes in fatty acid, phospholipid and malondialdehyde (MDA) contents.

3.1.1 Fatty acids

Studies on plant cold tolerance have found that fatty acids play an important role in biofilm structure (Li and Li, 2009), and the proportion and content of unsaturated fatty acids in membrane lipids are closely related to plant cold tolerance (Murata and Los, 1997). Under normal conditions, membrane lipids are in the liquid crystal phase, and at low temperature, plants maintain the state of membrane lipids by reducing the unsaturation of membrane fatty acids (Lyons et al., 1973; Li et al., 2019). Murata et al. (1992) believed that the higher the content of unsaturated fatty acids in membrane lipids is, the stronger the cold tolerance of plants is. Related studies in chrysanthemum have mainly concentrated on the contents and proportions of saturated and unsaturated fatty acids in leaves and roots. Li et al. (2013) found that the main membrane lipid fatty acids in the leaves and roots of the autumn chrysanthemum early flowering varieties 'Taiping Xiaogu' and 'Jin Fengling' and the late flowering varieties 'Xingguang Canlan' and 'Mobao' of autumn chrysanthemums mainly included palmitic acid (C16:0), linoleic acid (C18:2) and linolenic acid (C18:3). The main unsaturated fatty acids were C18:3 in the leaves and C18:2 in the roots, and the saturated fatty acid was C16:0. In addition, it was found that the fatty acid contents in leaves and roots showed different low-temperature response mechanisms, and there were obvious differences between the C18:3/oleic acid (C16:1) + C18:2 ratios in the leaves and the C18:2/C16:1 + C18:2 + C18:3 in the roots. The cold tolerance of C18:3 in leaves was greater than that of C18:2, while that of C18:2 in roots was greater than that of C18:3. The C18:3/C16:1 + C18:2 and C18:2/C16:1 + C18:2 + C18:3 ratios can be used as effective indicators to identify differences in the cold tolerance of leaves and roots between varieties. Wang et al. (2014)

used nine autumn chrysanthemum varieties and found that the unsaturated fatty acid with the highest content in leaves was C18:3. The C18:3/C16:1 + C18:2 ratio significantly changed under low-temperature stress and could be used as an effective index to identify cold-tolerant autumn chrysanthemum varieties. In addition, through cluster analysis, the autumn chrysanthemum varieties were divided into three categories according to the strength of their cold tolerance, and the three varieties with strong cold tolerance were 'Starlight Bright', 'Mobao' and 'Sandalwood Lion', while according to their LT50 values, the varieties with strong cold tolerance were 'Starlight Bright', 'Mobao' and 'Tongque Chunshen'.Comprehensive analysis identified the late flower varieties 'Starlight Bright' and 'Mobao' as having the strongest cold tolerance.

In conclusion, cold tolerance in chrysanthemum is related to the content of unsaturated fatty acids, and the main components of unsaturated fatty acids in leaves and roots are different: (C18:3) in leaves and C18:2 in roots.C18:3/C16:1 + C18:2 and C18:2/C16:1 + C18:2 + C18:3 can be used as important indicators to identify the differences in leaf and root cold tolerance between varieties. In addition, Li et al. (2013) and Wang et al. (2014) came to consistent research conclusions on leaves, which may be due to a significant overlap in the experimental materials. However, the cold-resistant varieties screened by Wang et al. (2014) using two different evaluation methods had certain differences, possibly due to the characteristics of the varieties themselves, and further in-depth research is needed.

3.1.2 Phospholipids

Phospholipids are both functionaly and structurally active substances that have important physiological functions in biological activities, and certain amounts of phospholipids are present in all biological tissues (Yang, 2012). Plant biofilms are the first to be destroyed when plants are subjected to low temperature and freezing damage, and they play a key role in plant cold tolerance (Steponkus, 1984). Studies on cold tolerance in chrysanthemum have mainly focused on the contents of phospholipids in different parts and the response mechanism to low temperature. Yang (2012) found that the main component of phospholipids in leaves and roots was phosphatidylethanolamine (PE) in various chrysanthemum varieties, but its content was different and was generally higher in leaves than in roots. However, phosphatidylglycerol (PG), phosphatidylcholine (PC) and phosphatidylserine (PS) were not detected or were detected in trace amounts. In addition, the membrane component contents in leaves and roots of different chrysanthemum varieties had different response mechanisms to low temperature. The PE content in the leaves of most chrysanthemum varieties increased with decreasing temperature, while that in the roots increased, and the increase in the PE content contributed to the increase in the unsaturated fatty acid content in the phospholipid membrane. In conclusion, PE in phospholipid components has a certain relationship with cold tolerance. Under low-temperature stress, the PE content in leaves increases, while the PE content in roots varies, and the higher the PE content is, the higher unsaturated fatty acid content is, which indirectly indicates that the cold tolerance of leaves is stronger than that of roots.

3.1.3 Malondialdehyde

During stress, plants produce excess free radicals, which trigger or aggravate membrane lipid peroxidation. Both intermediate free radicals and MDA, the final product of membrane lipid peroxidation, can seriously damage biofilms (Chen, 1991; Yang, 2018). Low-temperature stress can cause changes in the permeability of the plant cell membrane, with the semipermeability of the cell membrane reduced or lost, and increase the content of MDA. The change in the MDA content can reflect the degree of cell membrane disturbance. Xu et al. (2010) found that with decreasing temperature, the MDA content in the leaves of five groundcover chrysanthemum strains increased to varying degrees, and lipid peroxidation in the cell membrane continuously increased, resulting in a sudden increase in electrolyte exosmosis due to cell membrane damage. Li (2010) studied cold tolerance in the 'Cold Purple' and 'Cold Yellow' varieties and found that the MDA content was closely related to the tongue and tube flowers of 'Cold Purple'. When the temperature was lower than 6°C, the MDA content in the tongue flowers of 'Cold Purple' continuously increased. In a study of 'Jinlong Tengyun' chrysanthemum, Cheng et al. (2018) also found that the overall MDA content in leaves increased with decreasing temperature. Li et al. (2011) found that in a low-temperature environment, the protective enzyme system was deactivated, and the MDA content first decreased and then increased. Li et al. (2009) found that the MDA content in the leaves of autumn white chrysanthemum after low-temperature exercise was lower than that in the control group, which could weaken the peroxidation of membrane lipids and promote cold tolerance.

As shown in Table 1, the MDA content of different chrysanthemum varieties increased overall with decreasing temperature. Once the MDA content increased, the degree of lipid peroxidation of the cell membrane intensified, the plant cell membrane system began to be persecuted, and the membrane system lost its original selective permeability, resulting in electrolyte leakage and a decrease in cellular water potential, and the cold tolerance gradually weakened. This change is irreversible. According to Li's research, the MDA content of chrysanthemums undergoing low-temperature exercise is lower than that of chrysanthemums without low-temperature exercise, which can weaken the peroxide effect and reduce the MDA content. However, the time of low-temperature exercise and low-temperature stress is relatively short, which can further explore the relationship between the duration of low-temperature exercise and MDA content.

3.2 Protective enzyme system

Reactive oxygen species (ROS) produced by plant metabolism mainly include oxygen-containing substances such as hydrogen peroxide (H_2O_2), superoxide anion (O^{2-}) and hydroxyl radical

TABLE 1 Changes in MDA content in different chrysanthemum varieties under low-temperature.

Chrysanthemum variety	Temperature range	Change trend	Reference
Ground-cover chrysanthemum	-5°C~14°C	Increase	Xu et al., 2010
'Cold yellow' 'Cold Purple'	3°C~9°C 3°C~9°C	Increase Increase	Li, 2010 Li, 2010
'Jinlong Tengyun'	-8°C~16°C	Increase	Cheng et al., 2018
Gazania rigens	-6°C~6°C	Initial decrease followed by an increase	Li et al., 2011
Autumn chrysanthemum	-5°C~4°C	Initial decrease followed by an increase and then a decrease	Li et al., 2009

(-OH) (Eva et al., 2002; Apel and Hirt, 2004). Plants produce more ROS under low-temperature stress, and a large amount of ROS accumulation can inactivate proteases and increase peroxidation of membrane lipids, causing serious damage to cells (Wang et al., 1986; Green and Fluhr, 1995; Dat et al., 2000; Hu et al., 2009; Xu et al., 2020). The protective enzyme system to remove ROS mainly consists of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and other components (Li et al., 2021). Among them, SOD, POD and CAT are more sensitive to low-temperature stress and are often used as important indicators to characterize plant cold tolerance of plants (Hao et al., 2014; Jiang et al., 2014).

3.2.1 Superoxide dismutase

The main function of SOD is to clear O^{2-} and catalyse the reaction of O^{2-} and H^{+} to produce H_2O. Under the stress of low temperature (Kayihan et al., 2012), the SOD enzyme activity of plants can increase. The increase in SOD activity can improve the cold tolerance of plants, but its effect is limited, and SOD cannot play a normal role under severe low-temperature stress. In the study of cold tolerance and SOD in chrysanthemum, the main focus has been on the change in SOD activity. Xu et al. (2010) found that the SOD activity in the leaves of five groundcover chrysanthemum strains increased with decreasing temperature, and the accumulation of SOD promoted cold tolerance in these strains. However, with the continuous decrease in temperature, the SOD activity decreased but was still higher than the initial SOD activity, indicating that the groundcover chrysanthemum strains had a strong ability to adapt to low-temperature stress. Li (2010) found that when the temperature was not lower than 6°C, the cold tolerance of the 'Cold Yellow' and 'Cold Purple' varieties was improved by adjusting SOD activity. When the temperature was further reduced, their own adjustment ability was weakened, resulting in low-temperature damage, and overall, the adjustment ability of 'Cold Yellow' was stronger than that of 'Cold Purple'. Cheng et al. (2018) found that under low-temperature stress, the SOD activity in the leaves of the chrysanthemum 'Jinlong Tengyun' showed an overall trend of first increased and then decreased with decreasing temperature, possibly due to low-temperature damage. Li et al. (2009) found that after low-temperature exercise, autumn white chrysanthemum still had higher SOD activity under low-temperature stress than that without low-temperature exercise. Bai et al. (2013) measured the SOD activity in overwintering leaves and foot buds of 'Purple Medal' in an open field and found that in the leaves, SOD activity first increased and then decreased with decreasing temperature, while the SOD activity in the foot buds first increased, then decreased and then increased again, indicating that the foot buds had strong adaptability to a low-temperature environment and stronger cold tolerance than the leaves.

In conclusion, SOD activity increased gradually overall with the decreasing temperature, but when the temperature exceeded the capacity of chrysanthemum plants, SOD activity decreased, and the plants began to suffer from low-temperature persecution. In addition, the cold tolerance of different chrysanthemum varieties is different, cold tolerance in different parts of the same plant is different, and the cold tolerance of the foot bud is stronger than that of the leaf.

3.2.2 Peroxidase

The main function of POD is to clear H_2O_2 in plants, generate H_2O and O_2, and reduce damage to plants (Chaoui et al.,1997). Under abiotic stress conditions such as low temperature (Zhang and Xu, 2009), POD activity increases, which helps improve plant tolerance to low temperature. However, when the low temperature exceeds the critical temperature and the ROS generation rate far exceeds the clearance capacity of POD, the POD activity decreases. Studies on chrysanthemum cold tolerance have mainly focused on the change in POD activity in response to temperature. Cheng et al. (2018) found that the POD activity in the leaves of the chrysanthemum 'Jinlong Tengyun' first increased and then decreased with decreasing temperature. Li et al. (2009) found that under -5°C stress, the POD activity in autumn chrysanthemum after low-temperature exercise was consistent with that in the control group, an initial increase followed by a decrease, and the POD activity after low-temperature exercise was stronger. Bai et al. (2013) determined the POD activity in the leaves and foot buds of 'Purple Medal' overwintering in an open field and found that the POD activity in both parts first decreased and then increased with decreasing temperature. However, starting with the flower organs, Li (2010) found that the changes in POD activity under low-temperature stress had a poor correlation with chrysanthemum varieties, and their cold tolerance at different flowering stages could not be judged, which was not suitable for the study of the tolerance indices of 'Cold Yellow' and 'Cold Purple' chrysanthemum. In conclusion, with decrease temperature, POD activity increased overall, but when the temperature exceeded the temperature tolerance range of the chrysanthemum plants, POD activity decreased, and ROS and H_2O_2 began to accumulate, causing stress. In addition, the cold tolerance of different varieties of

chrysanthemum is different, and the cold tolerance of flower organs of some chrysanthemum varieties is not suitable for studying changes in POD activity.

3.2.3 Catalase

CAT is a key enzyme in the removal of H_2O_2 in plants (Willekens et al., 1995), and its main function is to catalyze H_2O_2 to produce H_2O and O_2. CAT has a high enzyme activity rate and can efficiently remove H_2O_2, but it has a weak affinity for H_2O_2 and mainly removes high concentrations of H_2O_2 (Lu, 2007). Studies on cold tolerance chrysanthemum have mainly focused on changes in POD activity. Cheng et al. (2018) found that under low-temperature stress, CAT activity in the leaves of 'Jinlong Tengyun' chrysanthemum first increased and then decreased with decreasing temperature. Bai et al. (2013) measured the CAT activity in overwintering leaves and foot buds of 'Purple Medal' in an open field and found that the changes in CAT activity in the different parts were different. With the decrease in temperature, the CAT activity in the leaves first rised and then decreased, and that in the foot buds first increased, then decreased and then increased again. In conclusion, with decreasing temperature, CAT activity gradually increased overall, but when CAT activity reached an extreme value, the scavenging ability of CAT on H_2O_2 was the strongest, and the concentration of H_2O_2 began to decline, and the activity of CAT also declined. In addition, the cold tolerance of different varieties of chrysanthemum is different, the cold tolerance of different the same is different, and the cold tolerance of the foot bud is stronger than that of the leaf.

In addition, there are other enzymes that contribute to the response of chrysanthemum to low-temperature stress. Li et al. (2009) found that with increasing low-temperature stress time, the 5'-nucleotidase activity in chrysanthemum gradually decreased but overall, was significantly higher after low-temperature exercise than that in the control group, which could maintain the stability of the cell membrane and indirectly improve cold tolerance in chrysanthemum.

Table 2 shows that SOD, POD and CAT have a certain synergistic effect, and also have a certain temperature adjustment range. Under mild low-temperature stress, chrysanthemum plants produced a stress response, the activity of antioxidant enzymes increased, and the ROS scavenging ability improved, which can

TABLE 2 Changes in the protective enzyme activity of different chrysanthemum varieties under low-temperature.

Protective enzyme	Chrysanthemum variety	Temperature range	Change trend	Reference
SOD	Ground-cover chrysanthemum	-5°C~14°C	Initial increase followed by a decrease	Xu et al., 2010
	'Cold yellow'	3°C~6°C	Initial increase followed by a decrease	Li, 2010
	'Cold Purple'	3°C~6°C	Initial increase followed by a decrease	Li, 2010
	'Jinlong Tengyun'	-8°C~16°C	Initial increase followed by a decrease	Cheng et al., 2018
	Autumn chrysanthemum	-5°C~4°C	Initial increase followed by a decrease	Li et al., 2009
	'Purple Medal'	Natural wintering	Initial increase followed by a decrease (Leaf)	Bai et al., 2013
			Initial increase followed by a decrease and then an increase (Foot bud)	
POD	'Jinlong Tengyun'	-8°C~16°C	Initial decrease followed by an increase and then a decrease	Cheng et al., 2018
	Autumn chrysanthemum	-5°C~4°C	Initial increase followed by a decrease	Li et al., 2009
	'Purple Medal'	Natural wintering	Initial decrease followed by an increase	Bai et al., 2013
CAT	'Jinlong Tengyun'	-8°C~16°C	Initial increase followed by a decrease	Cheng et al., 2018
	'Purple Medal'	Natural wintering	Initial increase followed by a decrease (Leaf)	Bai et al., 2013
			Initial increase followed by a decrease and then an increase (Foot bud)	

alleviate low-temperature injury. With the decrease in temperature, the activity of antioxidant enzymes in plants began to decline, and the ability of plants to alleviate the damage from ROS became weak. As the temperature continued to decrease, it exceeded the ability of chrysanthemum to adjust. As its own adjustment ability was limited, the enzyme activity was significantly reduced, resulting in severely damaged from low temperature. In particular, the enzyme activity of CAT decreased within the temperature range of the chrysanthemum itself; this is related to the characteristics of CAT, which mainly removes high concentrations of H_2O_2. When H_2O_2 is jointly removed by highly active POD and CAT, the concentration of H_2O_2 decreases, and the activity of CAT may also decrease.

3.3 Osmoregulatory substances

Under low-temperature stress, plants can regulate cell osmotic pressure by producing and accumulating osmotic regulatory substances to avoid significant loss of cell water; this can alleviate the damage to plants caused by low temperature to a certain extent (Gao et al., 2016). Soluble sugar (SS), soluble protein (SP) and proline (Pro) are important osmoregulatory substances in plants, and SP content can reflect the response of plants to low temperature.

3.3.1 Soluble sugar

SS are not only important energy sources for plant respiration but also play a role in regulating cell osmotic pressure (Morsy et al., 2007), which can increase the concentration of cell fluid and increase cell water retention and nonfrozen water in tissues. The SS content is positively correlated with the cold tolerance of plants (Wang, 1987). Zheng et al. (1994) showed that some winter chrysanthemum varieties could withstand a low temperature of -20°C, and their cold tolerance was better than that of autumn chrysanthemum varieties because of their lower leaf water content and higher SS content. Mao et al. (2004) studied cold tolerance in the groundcover chrysanthemums 'Xiaruahuang' and '9871' and found that the water content of the plants decreased and the cold and freezing tolerance increased. There was a negative correlation between the water content and SS content in the chrysanthemum plants, and the relationship was more obvious when the temperature decreased. Xu et al. (2010) studied the cold tolerance of five groundcover chrysanthemum strains and found that with the decrease in temperature, the SS content began to increase the changes caused by low temperature. When the temperature dropped to a certain extent, the sugar contents of strains 074-17, 074-123, 074-237, and 074-428 stopped increasing and began to decrease, indicating that the groundcover chrysanthemums had a certain tolerance limit to low temperature, and the increase in the SS content also had a certain temperature range limit. Li (2010) found that with the decrease in temperature, 'Cold Yellow' had a stronger ability to enhance its cold tolerance than 'Cold Purple' by accumulating SS, and the cold tolerance of tubular flowers of the same variety was stronger than that of tongue flowers. Chen et al. (2018) studied cold tolerance in perennial chamomile and found that the SS content first increased and then decreased with decreasing temperature as a stress response, which could be used for the determination of cold tolerance, and the cold tolerance of perennial chamomile was good near 0°C. Cheng et al. (2018) found that the SS content in the leaves of the autumn chrysanthemum 'Jinlong Tengyun' first increased and then decreased with the extension of the low-temperature treatment time and the decrease in temperature, indicating that chrysanthemum cold tolerance could be improved through an increase in the SS content under mild low-temperature stress. However, with the continuous decrease in temperature, low-temperature stress prevented SS synthesis. They were even hydrolysed, further reducing the content. Bai et al. (2013) observed a 'Purple Medal' natural low-temperature strip and found that the SS content in the leaves significantly increased with decreasing temperature, while that in foot buds first increased, then decreased and then increased again.

In conclusion, the change in the SS content was positively correlated with cold tolerance. With the decrease in temperature, the contents of different SS increased overall, but when the temperature exceeded a certain range, SS synthesis is blocked, and the content began to decline. In addition, cold tolerance in different parts of the same plant was different, and tubular flowers had stronger tolerance than tongue flowers. The foot buds had stronger tolerance than leaves.

3.3.2 Soluble protein

SP, similar to SS, are nutrients in plant cells. Most of them are enzymes involved in various reactions; SP can also regulate cell osmotic pressure, and the increase in their accumulation can effectively prevent plant dehydration. The hydrophilic colloids of SP are strong, and an increase in their content can significantly enhance water retention by cells. Proteins also regulate gene expression during cold stress, resulting in improveed cold tolerance. Xu et al. (2010) studied cold tolerance in five strains of groundcover chrysanthemum and found that with decreasing temperature, the SP content increased, the cell fluid concentration was adjusted, and tolerance to low-temperature stress was observed. Li (2010) found that the SP contents of 'Cold Yellow' and 'Cold Purple' chrysanthemums first increased and then decreased with decreasing temperature. The ability of 'Cold Purple' to resist low-temperature stress through SP content adjustment was weaker than that of 'Cold Yellow', and 'Cold Purple' was more sensitive to low temperature; the cold tolerance of the tube flowers of the two varieties was stronger than that of the tongue flowers. Li et al. (2011) found that related protein synthesis genes were switched on at -3°C in *Gazania rigens*, thus increasing the cell protein concentration and decreasing the freezing point to resist low temperature. However, at -3°C to -6°C, the protein content dropped sharply, beyond the range of self-regulation. Chen et al. (2018) found that the SP content of *Gazania rigens* first increased and then decreased under low-temperature stress, which could be used for the determination of cold tolerance. Bai et al. (2013) determined the SP content in the leaves and foot buds of 'Purple Medal' overwintering in an open field and found that the SP content in both leaves and foot buds first decreased, then increased and then decreased again. In conclusion, with the decrease in temperature, the contents of different varieties increased overall, but when the temperature exceeded a certain range, the SP content began to decline. In addition, the cold

tolerance of different parts of the same plant was different, and tubular flowers had stronger tolerance than tongue flowers.

3.3.3 Proline

Pro is an important osmoregulatory substance in plant cells, which has strong hydration ability and can prevent cell dehydration under stress (Yao et al., 2000). Pro can also increase the hydrophilicity of proteins and maintain the conformation of enzymes at low temperatures (Jiang et al., 2002). The Pro content significantly increases under low-temperature stress, and its main function is to maintain cell water potential and enhance cell water retention under stress. Xu et al. (2010) studied the cold tolerance in five groundcover chrysanthemum strains and found that the free Pro content increased in the early stage of low-temperature stress, but as the temperature continued to drop, the free Pro content in the leaves of each strain decreased; as the temperature continued to decrease, the Pro content began to rapidly increase, indicating that it was sensitive to temperature stress. Thus, Pro content plays an important role in regulating cold tolerance in the groundcover chrysanthemums. Li et al. (2011) found that with the increasing intensity of low-temperature stress, the Pro content in the leaves of *Gazania rigens* first increased and then decreased. Li et al. (2009) found that after low-temperature exercise, the Pro content in autumn white chrysanthemum first increased and then decreased and was significantly higher than that in the control group. Bai et al. (2013) found that the Pro content in leaves and foot buds of 'Purple Medal' overwintering in an open field first decreased, then increased and then decreased again. In conclusion, with the decrease in temperature, the Pro content of different varieties increased overall, but when the temperature exceeded a certain range, the Pro contents began to decline. The Pro content of the ground cover first increased and then decreased with decreasing temperature, which is possibly related to the strong water absorption ability of the ground cover. In addition, the variation trends of SP and Pro in some chrysanthemum varieties were roughly the same, indicating that there was a certain relationship between them, and some proteins may regulate the expression of Pro-related genes.

Table 3 shows that with decreasing temperature, SS, SP and Pro contents showed a certain correlation. Among them, the contents of

TABLE 3 Changes in osmotic regulator content in different chrysanthemum varieties under low-temperature.

Osmotic regulator	Chrysanthemum variety	Temperature range	Change trend	Reference
SS	Ground-cover chrysanthemum	-5°C~14°C	Initial increase followed by a decrease	Xu et al., 2010
	'Cold yellow' 'Cold Purple'	3°C~12°C 3°C~12°C	Increase Increase	Li, 2010
	'Jinlong Tengyun'	-8°C~16°C	Initial increase followed by a decrease	Cheng et al., 2018
	Perennial chamomile	-10°C~5°C	Initial increase followed by a decrease	Chen et al., 2018
	'Purple Medal'	Natural wintering	Initial increase followed by a decrease	Bai et al., 2013
SP	Ground-cover chrysanthemum	-5°C~14°C	Increase	Xu et al., 2010
	'Cold yellow' 'Cold Purple'	3°C~12°C 3°C~12°C	Initial increase followed by a decrease Initial increase followed by a decrease	Li, 2010 Li, 2010
	Gazania rigens	-6°C~6°C	Initial increase followed by a decrease	Li et al., 2011
	Perennial chamomile	-10°C~5°C	Initial increase followed by a decrease	Chen et al., 2018
	'Purple Medal'	Natural wintering	Initial decrease followed by an increase and then a decrease	Bai et al., 2013
Pro	Ground-cover chrysanthemum	-5°C~14°C	Initial increase followed by a decrease and then an increase	Xu et al., 2010
	Gazania rigens	-6°C~6°C	Initial increase followed by a decrease	Li et al., 2011
	Autumn chrysanthemum	-5°C~4°C	Initial increase followed by a decrease	Li et al., 2009
	'Purple Medal'	Natural wintering	Initial decrease followed by an increase and then a decrease	Bai et al., 2013

SS and Pro showed more similar trends, which is possibly related to the relatively similar functions of SP and Pro. In addition, SP may indirectly regulate the expression of Pro biosynthesis-related genes and others, which needs to be further studied in combination with new technologies. As the intensity of external low-temperature stress increases and the stress time continues to exceed the temperature tolerance range of chrysanthemum, the related tissues and organs are damaged and unable to produce more SS, SP and Pro, thus reducing the content of osmoregulatory substances.

3.4 Photosynthesis

As the most important metabolic process in plants, photosynthesis refers to the process during which plants absorb light energy and convert it into stable chemical energy, assimilate CO_2 and H_2O, produce organic matter and release O_2 (Li et al., 2012), which is closely related to external environmental factors. Many studies have found that the photosynthetic rate of plants significantly decreases under low-temperature conditions (Rapacz et al., 2001; Guo et al., 2009; Yang et al., 2012). To date, many studies on photosynthesis and cold tolerance in chrysanthemum have mainly focused on the photosystem, chlorophyll content and thylakoid membrane lipid structure.

3.4.1 Photosystem

Low-temperature stress can damage plant photosystem II (PSII) and photosystem I (PSI), hinder photosynthetic electron transport, and generate excess light energy to destroy the photosynthetic reaction centre (Liu et al., 2001; Liu et al., 2009). Studies on the cold tolerance and photosynthetic system of chrysanthemum have mainly focused on the effects of changes in initial fluorescence (F_0), maximum photochemical efficiency (F_v/F_m), potential photochemical efficiency (F_v/F_0), photosynthetic electron transport quantum efficiency (Φ), photochemical quenching coefficient (Q_P) and nonphotochemical quenching coefficient ($N_{P,Q}$) on PSII and PSI under low-temperature stress.

To date, most studies on the chrysanthemum photosystem have only considered the photochemical reaction of PSII and rarely involved PSI. Liang et al. (2010) found that under low-temperature and low-light treatment, F_0 significantly increased, F_v/F_m significantly decreased, and Φ_{PSII}, Q_P and the apparent photosynthetic electron transfer rate decreased with increasing stress degree and stress time, which could be used as an evaluation index of chrysanthemum tolerance to low temperature and low light. Cheng et al. (2018) found that under 4°C treatment, F_v/F_m and Q_P of the autumn chrysanthemum 'Jinlong Tengyun' did not significantly change, while $N_{P,Q}$ increased, indicating that under short-term low-temperature stress, the PSII function of the chrysanthemum was affected and the chrysanthemum consumed excess excitation energy by increasing heat dissipation to protect the photosystem from damage. At -8°C, F_v/F_m and Q_P both significantly decreased, and $N_{P,Q}$ was almost 0, indicating that the photosynthetic system in 'Jinlong Tengyun' leaves was seriously damaged and that the photosynthetic capacity was essentially lost. Nie et al. (2020) also studied the autumn chrysanthemum 'Jinlong Tengyun' and found that at -4°C, F_v/F_m, F_v/F_0 and Q_p declined, while $N_{P,Q}$ first decreased and then increased, indicating that the chrysanthemum leaves responded to low-temperature stress by reducing PSII light energy utilization and photochemical efficiency and increasing heat loss. These results were consistent with the findings of Cheng et al. (2018), but the photosynthetic system was not seriously damaged in this study.

The photochemical reaction is a collaborative result of PSII and PSI, and the continuous excitation rapid chlorophyll fluorescence technique can accurately detect PSII and PSI related changes in plants (Li et al., 2005; Gao et al., 2013; Qi et al., 2015). Zhao et al. (2019) used this technology to study the cut-flower chrysanthemum 'White Ping-Pong' and found that under -4°C low-temperature stress, the openness of the photosystem reaction centre was reduced, and some reaction centres were inactivated, which caused serious damage to PSI and reduced the overall photosynthetic activity of the plant. Zhao (2019) conducted the same research on the chrysanthemum 'Tangyu Jinqiu' and found that the coordination between PSII and PSI was weakened at low temperature, the fluency of energy transfer was reduced, the energy utilization rate of PSI was decreased, PSI was seriously damaged, and the overall performance of the photosynthetic mechanism of the chrysanthemum leaves decreased at low temperature. The continuous excitation rapid chlorophyll fluorescence technique was not conducive to photochemical reactions and the accumulation of organic matter.

In summary, cold tolerance in chrysanthemum is mainly related to functional changes in the light system. Low-temperature stress causes changes in various photosynthetic indices and then affects the light system. The light system is protected from damage by increasing heat dissipation, but this protection has a certain temperature range beyond which serious damage occurs. In addition, continuous excitation rapid chlorophyll fluorescence technology allows quick and accurate study of changes in the chrysanthemum photosystem under low-temperature stress.

3.4.2 Chlorophyll

Under low-temperature stress, the decrease in plant photosynthesis is often related to changes in photosynthetic pigments. Chlorophyll is the main component of photosynthetic pigments in plants and is responsible for capturing and transferring light energy in the process of photosynthesis. In the study of cold tolerance in chrysanthemum, changes in the chlorophyll content may be different in response to different low-temperature stresses. Nie et al. (2020) found that under short-term low-temperature stress, the chlorophyll a/chlorophyll b ratio in chrysanthemum increased with decreasing temperature. Although the photosynthetic organs of PSII in plants were affected under low-temperature stress, the reaction centre was relatively stable, and therefore, it was inferred that chlorophyll a was more distributed in the PSII reaction centre and chlorophyll b was more distributed in the light-collecting pigment protein complex (Wang et al., 2003; Wang J. et al., 2006; Lichtenthaler and Buschaann, 2001; Ebrahimiyan et al., 2013). Cheng et al. (2018) found that the

contents of chlorophyll a, chlorophyll b and chlorophyll a + b in chrysanthemum leaves decreased trend with the extension of the low-temperature stress time and the decrease in temperature. The chlorophyll content of the chrysanthemum and the ability to capture and use light energy decreased, and the destruction of the photosynthetic structure was avoided, which was conducive to the adaptation of the chrysanthemum to low-temperature stress. In conclusion, chrysanthemums can cope with low-temperature stress through changes in chlorophyll content. Under short-term low-temperature stress, chlorophyll content increased to ensure the stability of the light reaction centre. Under long-term low temperature stress, chlorophyll content decreased to avoid the destruction of photosynthetic structure.

3.4.3 Thylakoids

The light reaction of photosynthesis is carried out on the thylakoid membrane, which has a high fluidity. Relevant studies have shown that low temperature can affect plant photosynthesis by affecting the structure of the thylakoid membrane (Dai et al., 2004). In addition, the photochemical reaction is the result of coordination between PSII and PSI, and improving the efficiency of PSII and PSI at the same time can enable efficient photosynthesis (Yao et al., 2009). In a study on the cold tolerance of chrysanthemum, Zhao (2019) found that the main fatty acids in the leaf thylakoid membrane were C16:0, followed by stearic acid (C18:0) and C16:1, and the contents of C16:0 and C18:0 gradually decreased with decreasing temperature under low-temperature stress, while that of C16:1 first decreased and then increased with decreasing temperature. The proportions of C18:2 and C18:3 increased with decreasing stress temperature. In addition, Zhao et al. (2019) found in their study on the cut-flower chrysanthemum variety 'White Ping-Pong' that the light-trapping pigment in leaves was degraded under low-temperature stress, resulting in reduced openness and partial deactivation of the PSII reaction centre, damage to the oxygen-releasing complex, and indirectly reduced activity of the PSI reaction centre. In addition, the PSI of cut chrysanthemum leaves was seriously damaged at -4°C, which blocked the energy transfer between thylakoids, reduced the protection ability of the photosynthetic system, and decreased the overall photosynthetic performance.

In summary, studies on the cold tolerance of thylakoids and chrysanthemums have mainly focused on the effect of changes in the unsaturated fatty acid content in membrane lipids on the structure of thylakoid membranes and the effect of degradation of light-capturing pigments in the light system on the light response. Cold tolerance in chrysanthemum can be judged by changes in the composition of the thylakoid membrane and the light response performance.

3.5 Hormones

To help plants cope with stress, exogenous hormones can be sprayed onto plants, and the content of endogenous hormones can be adjusted to cope with low-temperature stress. To resist low-temperature stress, plants maintain high endogenous abscisic acid (ABA) levels. Many studies on cold tolerance and hormones in chrysanthemum have been started by applying exogenous hormones such as salicylic acid (SA), acetylsalicylic acid (ASA) and 5-aminolevulinic acid (ALA).

3.5.1 Endogenous hormone ABA

ABA can inhibit cell division and elongation, thus hindering plant growth and promoting the formation of dormant buds to adapt to low-temperature stress (Thomashow, 1999; Jeon et al., 2010; Shi et al., 2012). Many studies have also confirmed that the endogenous ABA content of many plants significantly increased after low-temperature treatment and was higher in varieties with strong cold tolerance than in those with cold sensitivity (Chen et al., 2015). Zhang (2013) found that treatment of chrysanthemum with a low concentration of exogenous ABA could improve the antioxidant activity of 'Shenma' cut chrysanthemum cells under low-temperature conditions, reduce the content of ROS, reduce the damage caused by membrane lipid peroxidation, and enhance the osmotic regulation ability of cells, thus improving tolerance to low-temperature stress. ABA treatment also affected photosynthesis and dry matter accumulation in plants to a certain extent. In conclusion, the activities of antioxidant enzymes can be affected, the osmotic regulation ability can be enhanced, and cold tolerance in chrysanthemum can be indirectly improved through increases in the content of the endogenous hormone ABA.

3.5.2 Exogenous hormones SA, ASA and ALA

SA is a small-molecule phenolic substance that widely exists in plants and can significantly reduce the membrane permeability of plant cells under low-temperature stress and improve the cold tolerance of plants (Wang et al., 2002; Cai et al., 2007). At present, the application of exogenous hormones to improve the cold tolerance of plants is an important approach to extending the ornamental period and improving the ornamental value of chrysanthemums. Li Y. H. et al. (2010) used the autumn chrysanthemum early flower variety 'Tangyu Jinqiu' and late flower variety 'Xiangyun' as test materials and found that after application of 1.0 mmol/L SA, the Pro content, net photosynthetic rate, SOD activity and CAT activity in leaves were higher at all temperatures than those in the control group sprayed with distilled water, while the MDA content was lower than that in the control group. SA can significantly improve cold tolerance in chrysanthemum. Tian (2009) also studied 'Tangyu Jinqiu', an early flower variety of autumn chrysanthemum, and 'Xiangyun', a late flower variety, and found that external application of SA could alleviate the effects of low temperature by adjusting the REC and chlorophyll, MDA, SS and Pro contents, thus improving cold tolerance.

ASA is a derivative of SA. Yang and Zheng (2018) used 'Shenma' cut-flower chrysanthemum and found that exogenous ASA, $CaCl_2$ and ASA + $CaCl_2$ could significantly increase the activities of SOD, APX, and GR in chrysanthemum leaves. In addition, by improving the metabolic activity of the ASA-GSH circulatory system, the damage caused by ROS to chrysanthemum seedlings could be reduced, and the tolerance of chrysanthemum to

low temperature and low light could be improved. Moreover, the combination treatment with ASA and $CaCl_2$ was better than that of ASA or $CaCl_2$ alone.

ALA is a synthetic precursor of all porphyrins (chlorophyll, haem, photochromes, etc.) (Wang et al., 2005). Studies have found that low concentrations of ALA can regulate plant growth and development, promote crop yields and improve stress tolerance (Wang et al., 2003). In a study on the cold tolerance in chrysanthemum, Zhang et al. (2014) found that spraying 50 mg/L ALA on the leaf surface of 'Cold white' could significantly reduce the REC and MDA content, increase the chlorophyll, SS, and SP contents and SOD activity, and significantly increase the net photosynthetic rate, stomatal conductivity and transpiration rate in leaves. The photosynthetic capacity and cold tolerance of the cut-flower chrysanthemum were improved.

In conclusion, exogenous hormones sprayed on chrysanthemum can significantly improve physiological indices related to cold tolerance, such as the chlorophyll, MDA, SS, and Pro contents, REC, and SOD activity, and indirectly improve cold tolerance in chrysanthemum.

There are relatively few studies on the physiological mechanism of cold tolerance in chrysanthemums, mainly focusing on the biofilm system, protective enzyme system, osmotic regulatory substances, photosynthesis and hormones. However, there are relatively few studies on hormones and cold tolerance, and further analysis is needed on the effects of more endogenous hormones and exogenous hormones on cold tolerance. However, the correlation between physiological indicators and cold tolerance is not sufficiently clear, and it is necessary to combine genomics, transcriptomics, proteomics and other omics with new technologies to further study and analyse the physiological mechanisms of cold tolerance in chrysanthemum and, at the same time, lay the foundation for chrysanthemum molecular markers and breeding selection in the future.

4 Research advance on the forward genetics of cold tolerance in chrysanthemum

Compared with physiological indices, forward genetics studies can elucidate the genetic status of chrysanthemum cold tolerance. Many forward genetics studies on cold tolerance in chrysanthemums have mainly focused on genetic analysis, molecular markers, QTL mapping and genome-wide association analysis.

4.1 Genetic analysis

Chrysanthemums are crosspollinated plants; their genotypes are highly heterozygous, and their trait inheritance is extremely complex. In the early stage, genetic research on chrysanthemum characters was mainly conducted by investigating the separation ratios of characters in hybrid progeny populations and summarizing the genetic rules. Genetic analysis studies of chrysanthemum cold tolerance have mainly focused on the progeny separation ratio and mixed genetic model analysis. Zhang XJ et al. (2011) crossed a strain of the groundcover chrysanthemum 'Qiuyan', which has the strong cold tolerance *AtDREB1A* gene, as the maternal parent with the groundcover chrysanthemum 'Ya dong Zhiguang', which has excellent ornamental properties, and obtained 158 hybrid offspring. Among them, 55 hybrid offspring were examined by PCR and RT-PCR. There were 37 strains carrying and 18 strains not carrying the exogenous *DREB1A* gene, the ratio was approximately 2:1, indicating that the exogenous *AtDREB1A* gene could be stably inherited by offspring during sexual reproduction and that the offspring had strong cold tolerance.

The hybrid genetic model can be used to study the genetic mechanism of chrysanthemum through direct and indirect analyses. Chi et al. (2018) used the interspecific F_1 hybrid between the brain diploid-related species of intolerant *Chrysanthemum* and hardy *Chrysanthemum* as materials. They found that three traits were closely related to cold tolerance, namely the LT50, the number of foot buds and the height of foot buds, which had a large variation range, with coefficients of variation between 21.0% and 51.80%, and there was a certain degree of heterosis and superparent isolated individuals. In addition, genetic control mechanisms of the three cold tolerance traits were identified by the mixed genetic model as follows: the LT50 was controlled by two pairs of additive-dominant-epistatic major genes, the number of foot buds was controlled by one pair of additive-dominant major genes, and the foot buds was controlled by one pair of additive-dominant genes. Ma (2017) used 100 F_1 hybrid lines between strongly cold-tolerant 'Nannong Xuefeng' as the maternal parent and poorly cold-tolerant 'Mengbai' as the paternal parent and adopted the major gene plus multigene analysis method of the mixed genetic model of plant quantitative traits. The study found that chrysanthemum cold tolerance at the seedling and full flowering stages was mainly controlled by two pairs of major genes, which were manifested as additive, dominant and upper. However, no major gene controlling cold tolerance was found at the bud and foot bud stages, indicating that the genetic mechanisms of cold tolerance at different growth stages greatly differed. In addition, Ma found that the coefficient of variation in the cold tolerance of F_1 generation tongue flowers was 43.60%, and the variation range exceeded those of both parents, with positive and negative hyperparental separation, which provided a research basis for the selection of cold-tolerant hyperparental strains and the improvement of cold-tolerant varieties.

In conclusion, recent genetic analyses of cold tolerance are mainly based on the separation ratio of recrossed offspring and on the analysis of genetic mechanisms with the help of a mixed genetic model. Using the mixed genetic model, Chi et al. (2018) indirectly analysed the genetic mechanism of cold tolerance using three traits, the LT50, the number of foot buds and the height of foot buds, which are closely related to cold tolerance. Ma (2017) directly used the major gene plus multigene method to analyse the genetic mechanism of cold tolerance and provided some references for the genetic analysis of cold tolerance.

4.2 Molecular markers

Recent molecular marker research on the chrysanthemum cold tolerance is mainly based on traditional molecular markers to help elucidate the related quantitative genetic mechanism (Zhang et al., 2010; Ma et al., 2018; Ao et al., 2019; Xu et al., 2019), but the potential for molecular marker-assisted breeding is limited. To date, many studies on molecular markers of chrysanthemum cold tolerance have mainly focused on the localization of LT50 and phenotypic traits related to cold tolerance. Ma (2017) used character-marker variance analysis, combined with the characteristics of high sequence-related amplified polymorphism (SRAP) marker polymorphism, to study LT50 molecular markers of the cold tolerance of the F_1 chrysanthemum hybrid at different growth stages and obtained a total of 97 SRAP marker sites significantly related to the cold tolerance of chrysanthemum through one-way ANOVA. The contribution rates of a single marker site to coldtolerance variation ranged from 4.32% to 14.99%. In particular, the contribution rates of X-M18E7-133, X-M3E16-187, M-M13E19-135, XM-M21E15-143 and X-M3E16-187 were all above 10%, and the contribution rate of X-M3E16-187 was the highest (14.99%). These results demonstrate that cold tolerance chrysanthemum is a complex quantitative trait.

With the help of molecular marker technology, chrysanthemum varieties with strong cold tolerance can be screened using marker sites, and the phenotypic trait marker sites related to cold tolerance can also be unearthed, which will broaden the direction of research on cold tolerance in chrysanthemum. Yang et al. (2020) found three molecular markers (E11M24-3, E11M24-4 and E11M24-5) that were significantly correlated with the cold tolerance of tongue flowers by correlation analysis of the LT50 of 83 chrysanthemum tongue flowers, among which the E11M24-4 marker was also correlated with two phenotypic traits, the capitular diameter and the stem diameter. In addition, four cold-resistant chrysanthemum varieties (Qx034, Qx138, Qx145, and QD048) were identified. Xu et al. (2019) evaluated the LT50 values in the leaves of 83 cut chrysanthemum varieties at the seedling stage using electrical conductivity combined with a logistic equation, and 11 excellent allelic variation sites related to cold tolerance were detected through association analysis, among which 8 variation sites showed enhanced effects. The cold tolerance of cultivars with E7M12-13 was significantly higher than that of cultivars without E7M12-13, and six varieties with strong cold tolerance, namely Nannongjin lemon, Qx097, QD028, Qx049, Qx153 and Qx008, were found according to the synergistic sites. Xu et al. also found that three markers, E2M16-2, E2M16-1 and E11M23-14, related to cold tolerance were related to the flowering time, flower neck length and leaf edge serrated phenotypic traits, respectively, indicating that there was a certain correlation between cold tolerance and phenotypic traits in chrysanthemum.

In conclusion, the LT50 molecular marker is mainly used to study the tolerance in chrysanthemum, and chrysanthemum varieties with strong cold tolerance can be screened and identified using the marker sites. In addition, the phenotypic traits related to cold tolerance can be screened by molecular marker technology, laying a foundation for the breeding and application of cold-resistant chrysanthemums.

4.3 QTL mapping

With the development of molecular marker technology, QTL mapping, based on linkage mapping, provides an important means for genetic research on plant cold tolerance. In terms of cold tolerance, Ma (2017) further carried out QTL mapping of chrysanthemum cold tolerance based on the SRAP marker study and the genetic maps of 'Nannong Xuefeng' and 'Mengbai' and detected a total of 15 QTLs that were significantly correlated with chrysanthemum cold tolerance at four stages: the seedling stage, bud stage, full flower stage and foot bud stage. The contribution rate of a single QTL to explaining cold-tolerance variation ranged from 6.47% to 68.89%, and most of the QTLs were detected at more than two growth stages. In addition, the phenotype of a cold tolerance QTL obtained by this method was stable at different growth stages and was less affected by the environment, while other QTLs detected at a single growth stage were more affected by the environment, which further explained the correlation with cold tolerance at different growth stages. These findings also improved the possibility and predictability of selecting good genotypes for target traits in breeding. In conclusion, QTL mapping based on genetic maps is more convenient, intuitive and stable than traditional molecular markers, but QTL mapping requires genetic maps of hybrid parents. At present, there are few studies on the construction of genetic maps of hybrid parents of cold-resistant chrysanthemums, which can be further studied.

4.4 Genome-wide association studies

With the development of high-throughput sequencing technology, SNP markers based on genome-wide association studies (GWAS) have been applied to genetic studies of plant stress tolerance. At present, there are relatively few genome-wide association analyses of chrysanthemum cold tolerance. Fan et al. (2019) used 58 cut-flower chrysanthemums as materials for whole-genome association analysis to identify 24 SNP loci that were significantly correlated with cold tolerance at the foot bud stage, bud stage, leaves at the full flowering stage and tongue flowers at the full flowering stage and compared and analysed the specific locus amplified fragment (SLAF) tag sequences wherein significant SNP loci were located using the chrysanthemum transcriptome database. Five candidate genes were identified, among which CL2042.Contig4_All and Unigene40993_All may be associated with low-temperature stress. In conclusion, SNP markers based on GWAS are more convenient and accurate than traditional molecular markers and QTL mapping studies and effectively

broaden the genetic research basis of chrysanthemum cold tolerance. However, there are too few relevant studies, and more in-depth research is needed.

There are relatively few forward genetics studies on the cold tolerance in chrysanthemum, mainly focusing on genetic analysis, molecular markers, QTL mapping and genome-wide association analysis. QTL mapping and genome-wide association analyses of chrysanthemum cold tolerance are less common than those of other plants. However, Ma (2017) analysed hybrid progeny through a mixed genetic model using the major gene plus multigene method and QTL mapping and found differences in the results of the two methods of analysis for major gene mapping at the bud stage, indicating that different methods should be used for comparative genetic analysis. In addition, research on cold tolerance using forward genetics methods are insufficient, and it is necessary to combine genomics, transcriptomics, proteomics and other omics approaches to further study the genetic mechanisms of chrysanthemum cold tolerance from the perspective of reverse genetics, which also lays a foundation for the discovery of chrysanthemum cold tolerance genes and transcription factors and their application in chrysanthemum cold tolerance breeding.

5 Research advance on the molecular mechanisms of cold tolerance in chrysanthemum

In recent years, the molecular mechanisms of plant responses to low-temperature stress have been extensively studied. The expression of cold response genes can be induced under low-temperature conditions, which can reduce the damage to plants caused by low-temperature stress and thus enhance the tolerance of plants to low-temperature stress. Many studies on the molecular mechanisms of cold tolerance in chrysanthemum mainly focus on functional genes and regulatory genes, which play an important role in gene expression and signal transduction.

5.1 Functional genes

Functional genes are genes directly related to the improvement of plant cold tolerance, such as cold-induced genes, fatty acid desaturase genes and antioxidant oxidase genes, which have protective effects on the cell membrane (Huang and Chen, 2011; Yao, 2013). Studies on functional genes of cold tolerance in chrysanthemum have mainly focused on photosynthetic system genes and fatty acid desaturation genes.

5.1.1 Photosynthetic system genes

Light harvesting a/b-binding protein *CAB* gene in PSII of higher plants belongs to the gene of photosynthetic system (Raghvendra, 2001), and *CAB* can form light-trapping pigment protein complex with chlorophyll (Jansson, 1999). The light-trapping pigment complex can capture light energy and transfer it to the reaction center, maintain the thylakoid membrane structure, and regulate the energy distribution of PSI and PSII. Light-harvesting complex I chorophyll a-b binding protein (LHCI) is found on the thylakoid membrane (Green et al., 1991; Yu et al., 2001), including Lhca1, Lhca2, Lhca3 and Lhca4. In a study on the cold tolerance of the autumn chrysanthemum variety 'Tangyu Jinqiu', Zhao (2019) found that the *CmCAB* and *CmLhca2* showed a certain correlation with several chlorophyll fluorescence parameters. With the decrease in the stress temperature, the expression of the *CmCAB* gene was downregulated, and the number of pigment protein complexes was reduced, which weakened the absorption and transformation of light energy by PSII, reduced the degree of light inhibition, and alleviated the damage to PSII. The expression of the *CmLhca2* gene was continuously downregulated with decreasing stress temperature, and the LHCI content was reduced to alleviate the photoinhibition and damage to PSI caused by low temperature. However, abnormal changes occurred in the M_R/M_{R0} curve at -4°C, indicating that the low temperature caused relatively serious damage to PSI.

In summary, studies on the photosynthetic system genes *CmCAB* and *CmLhca2* of chrysanthemum found that the expression of these two genes was downregulated with decreasing temperature under low-temperature stress, thus reducing the degree of low-temperature damage to PSII and PSI. Various photosynthetic parameters in the reaction process of PSII and PSI are closely related to the chrysanthemum cold tolerance. *CmCAB* and *CmLhca2* can indirectly affect chrysanthemum cold tolerance.

5.1.2 Fatty acid desaturase gene

When plants are in a lower-temperature environment, the activity of desaturase increases, resulting in an increase in the content of unsaturated fatty acids, allowing the cell membrane to still maintain its original liquid state at a lower temperature. At low temperature, fatty acid desaturase has a certain protective effect on plant cells. According to the different substrates, vegetable fatty acid desaturases can be divided into acyl-ACP desaturases and acyl-ester desaturases.

5.1.2.1 Δ9 Stearoyl-ACP desaturase gene

Acyl-ACP desaturase in plants is mainly located on plastosomes and is water soluble (Li et al., 2009), and it is the only known soluble desaturase system. Among these enzymes, Δ9 stearoyl-ACP desaturase (SAD) is the most extensively tested and most common acyl-ACP desaturase. Nie et al. (2020) studied the chrysanthemum 'Jinlong Tengyun' and found that low-temperature stress changed the expression levels of the *CmSAD*, *CmFAD2* and *CmFAD7* genes, which further promoted an increase in the unsaturated fatty acid mass fraction and played a certain protective role in PSII. Miao et al. (2022) found that the *CmSAD* gene of the chrysanthemum 'Tangyu Jinqiu' was significantly upregulated at low temperature; however, the stearic acid content did not significantly change in the cell membrane, suggesting that stearic acid synthesis was not a single pathway. In addition, in the thylakoid membrane, *CmSAD* gene expression was significantly negatively correlated with the C18:0 content and positively correlated with the C18:1 and C18:2 contents. This gene has a

more direct regulatory effect on lipid unsaturation of the chrysanthemum thylakoid membrane; the expression of the *CmSAD* gene is upregulated, the concentration of the catalytic substrate is decreased, and the content of unsaturated fatty acids is increased.

5.1.2.2 Acyl-lipid desaturase gene

Acyl-lipid desaturases can be divided into two categories corresponding to different substrates: ω-6 and ω-3 fatty acid desaturases. The main ω-6 fatty acid desaturases are mainly encoded by *FAD2*, *FAD4* and *FAD6*, and the main ω-3 fatty acid desaturases are encoded by *FAD3*, *FAD7* and *FAD8*. Many studies have mainly focused on *FAD2* and *FAD7*. Wang et al. (2014) cloned the *CmFAD7* gene using the chrysanthemum 'Starlight Bright' as the material and found that under different treatment temperatures, the expression levels of the *CmFAD7* gene in leaves were higher than those in roots. With decreasing temperature, the expression level of *CmFAD7* in leaves first increased and then decreased, while that in roots peaked peak at 5°C and then significantly decreased. Li et al. (2015) also used the chrysanthemum 'Starlight Bright' as the material to study differences in the expression levels of cloned *CmFAD7* under low-temperature stress in leaves and roots by a real-time fluorescent quantitative PCR method. They found that under low-temperature stress, the expression of *CmFAD7* in the leaves of the chrysanthemum was upregulated and was higher than that in roots, and the content of linolenic acid was increased. When the temperature was reduced to -8°C, the expression levels in leaves and roots were lower. Using the chrysanthemum 'Tangyu Jinqiu', Zhao et al. (2019) found that the expression levels of the *CmFAD2* and *CmFAD7* genes were generally upregulated under low-temperature stress, which effectively improved the unsaturation of thylakoid membrane lipids and enhanced the stability of the thylakoid membrane.

In summary, through the mining of desaturase genes of different chrysanthemum varieties, it was found that different genes had different effects on different parts of chrysanthemum plants, but the mode of action was the same, and they all directly regulated the membrane desaturase content to cope with low-temperature stress.

5.2 Regulatory genes

Regulatory genes mainly regulate the expression of cold tolerance genes, cold signal transduction and other processes to improve plant cold tolerance. Transcription factors (TFs) are protein molecules located in the nucleus that can specifically interact with cis-acting elements of gene promoter regions, and their main function is to activate or inhibit gene transcription (Jain et al., 2008). In recent years, a series of transcription factors families related to plant cold tolerance, including the MYB, bZIP and TCP families, have been isolated and identified, and the regulatory mechanisms of related cold-tolerance genes have been studied (Bhattacharjee, 2005).

5.2.1 MYB transcription factors

MYB transcription factors contain highly conserved DNA-binding domains, typically consisting of 1 ~ 4 imperfectly replicated R junctions and 50 to 52 amino acid residues (Riechmann et al., 2000). MYB transcription factors play an important role in plant responses to cold stress, but the underlying mechanisms remain unclear. Yang et al. (2022) isolated the cold-induced R1-MYB transcription factor *DgMYB2* from *Chrysanthemum* and found that its overexpression enhanced cold tolerance, while antisense inhibition of *DgMYB2* resulted in decreased cold tolerance. An electrophoretic mobility shift assay (EMSA), chromatin immunoprecipitation (ChIP), luciferase complementary imaging (LCI) analysis and a dual-luciferase reporter gene assay (DLA) showed that *DgMYB2* directly targeted the MYB-binding site (CAACCA) in the *DgGPX1* promoter, increasing *GPX* enzyme activity and reducing ROS accumulation, thereby improving cold tolerance of daisy. He (2019) cloned *DgMYB1* from *Chrysanthemum* 'Shenma', transformed chrysanthemum by the *Agrobacterium*-mediated method to obtain two chrysanthemum lines, OE-18 and OE-25, with *DgMYB1* gene transfer and tested the cold tolerance of the two transgenic plants. The overexpression of *DgMYB1* in chrysanthemum decreased the REC and MDA content, increased the activities of SOD, POD and CAT, and increased the contents of SS, SP and Pro. These results indicate that *DgMYB1* can improve the tolerance of transgenic chrysanthemum to low-temperature stress, to a certain extent; it can be used as an excellent cold-tolerant plant breeding gene and can provide an effective gene reserve for new cold-tolerant chrysanthemum varieties.

5.2.2 bZIP transcription factors

In higher plants, the bZIP transcription factor family has a positively charged, highly conserved domain consisting of 60-80 amino acids, with an acid-activated base region at its N-terminus (Cao et al., 2012). bZIP transcription factors play a very important role in abiotic stress, such as low-temperature stress, but there are relatively few studies on the role of bZIP transcription factors in chrysanthemum cold tolerance. Bai et al. (2022) found that overexpression of *DgbZIP3* resulted in increased cold tolerance in chrysanthemum, while antisense inhibition of *DgbZIP3* resulted in decreased cold tolerance. EMSAChIP, LCI assay DLA showed that *DgbZIP3* could directly bind to the *DgPOD* promoter and activate its expression. *DgbZIP2* was identified as a *DgbZIP3*-interacting protein by yeast two-hybrid, coimmunoprecipitation, LCI and bimolecular complementary fluorescence assays. Overexpression of *DgbZIP2* led to increased cold tolerance, while antisense inhibition of *DgbZIP2* led to decreased cold tolerance. ChIP-qPCR showed that *DgbZIP2* was highly enriched in the *DgPOD* promoter. The DLA, EMSA and LCI assays further indicated that *DgbZIP2* could not directly regulate the expression of *DgPOD*. Wang (2019) cloned *DgbZIP2* from the chrysanthemum 'Shenma' and obtained transgenic plants. The study found that the expression of *DgbZIP2* was induced and upregulated under low-temperature stress, and the expression level was higher in leaves. In addition, the

phenotype and physiological index of cold tolerance of the transgenic plants were examined. The lodging, wilting and survival rates of the chrysanthemums overexpressing *DgbZIP2* were higher than those of wild-type chrysanthemums at low temperature; the activities of SOD, POD and CAT were increased, the content of MDA was decreased, and the SS, SP and Pro contents were increased. These results indicated that *DgbZIP*2 is a positive regulator of low-temperature stress, and the transgenic chrysanthemum plants had strong cold tolerance.

5.2.3 TCP transcription factors

During plant growth and development, long noncoding RNAs (lncRNAs) are at the core of gene regulatory networks, which are related to plant development, nutrient metabolism, and biological and abiotic stress processes. Although there are few studies on their functional mechanisms, many lncRNAs can act as scaffolds to mediate chromatin remodelling and histone modification to affect downstream gene expression (Lucia and Dean, 2011; Heo and Sung, 2011; Kim and Sung, 2017). Li et al. (2022) discovered a lncRNA, named *DglncTCP1*, that regulated transcribed *DgTCP1* from the TCP transcription factor. In response to low-temperature stress, the overexpression of *DgTCP1* and *DglncTCP1* enhanced chrysanthemum cold tolerance. In addition, LCI and DLA experiments showed that overexpression of *DglncTCP1* upregulated the expression of *DgTCP1* and that *DglncTCP1* may play a cis-regulatory role in the cold-tolerance regulation of *DgTCP1*. Using ChIP-qPCR, *DglncTCP1* was found to be a scaffold, and the histone-modified protein *DgATX* was recruited to *DgTCP1* to increase the level of H3K4me3, thus activating *DgTCP1* expression. *DgTCP1* can directly target *DgPOD*, promote its expression and reduce ROS accumulation, thus improving chrysanthemum cold tolerance.

Furthermore Huang et al. (2020) found that the acylation degree of *DgTIL1* increased at low temperature, and the interaction degree with *DgnsLTP* was enhanced, which was conducive to the increase of POD activity in chrysanthemum and the ability of chrysanthemum to resist low temperature. Bai et al. (2021) found that the expression of resistance related genes such as *DgCOR413*, *DgDREBa*, *DgCSD1* and *DgCSD2* in plants overexpressed with zinc finger protein *DgC3H1* increased, and the resistance to low temperature was significantly higher than that of wild type chrysanthemum.

In summary, through the molecular mechanisms by which the MYB, bZIP and TCP transcription factors regulate chrysanthemum cold tolerance, it was found that the transcription factors could activate the expression of target genes and increase catalase activity to reduce the accumulation of ROS, thereby indirectly improving chrysanthemum cold tolerance. Research on these three transcription factors continues. In particular, *DgMYB2* was only studied for its regulation of the downstream target gene *DgGPX1*. *DgbZIP3* was studied not only for its regulation of the downstream target gene *DgPOD* but also for the interaction between *DgbZIP3* and *DgbZIP2*. *DgbZIP2* could not directly activate the expression of *DgPOD* but coregulated *DgPOD* with *DgbZIP3*. However, the *DgTCP1* study introduced a new idea. Starting from the regulation of target genes by the lncRNA, it was found that *DglncTCP1* could act as a scaffold to mediate the recruitment of *DgATX* to *DgTCP1*, thereby increasing the level of H3K4me3 on *DgTCP1* and activating its expression. In addition, *DgTCP1*, as a transcription factor, can bind to the promoter of the target gene *DgPOD* to regulate its transcription, which provides a reference for the study of multiple transcription factors in one direction. Furthermore, *DgMYB1* and *DgbZIP2* were used in transgenic chrysanthemum experiments and were verified to have positive regulatory effects on low-temperature stress at the phenotypic and physiological levels.

There are relatively few forward genetics studies on on cold tolerance in chrysanthemum, mainly focusing on genetic analysis, molecular markers, QTL mapping and genome-wide association analysis. QTL mapping and genome-wide association analysis of chrysanthemum cold tolerance are less than those of other plants. However, Ma Jie analysed a hybrid progeny through the mixed genetic model using the major gene plus multigene method and QTL mapping and found differences in the results of the two methods of analysis for major gene mapping at the bud stage, indicating that different methods should be used for comparative genetic analysis. In addition, research on cold tolerance and forward genetics methods are insufficient, and it is necessary to combine genomics, transcriptomics, proteomics and other omics approaches to further study the genetic mechanisms of chrysanthemum cold tolerance from the perspective of reverse genetics, which also lays a foundation for the discovery of chrysanthemum cold tolerance genes and transcription factors and their application in chrysanthemum cold tolerance breeding.

There are relatively few studies on the molecular mechanism of cold tolerance in chrysanthemum, and they have mainly focused on functional genes and regulatory genes. Research on photosynthetic system genes in functional genes is still relatively limited, and further research is needed. Studies on desaturase genes are relatively sufficient, but the types and quantities of desaturase need to be further explored. In terms of regulatory genes, the functions of related transcription genes are mainly analysed from transcription factors, and related genes can also be cloned from chrysanthemum varieties to identify cold tolerance through transgenic plants. However, the number of transcription factors related to cold tolerance is still relatively small and needs to be further explored. These research findings lay a foundation for the breeding and application of new chrysanthemum varieties with cold tolerance and provide new breeding ideas.

6 Research progress on the breeding methods of cold tolerance in chrysanthemum

With the research and analysis of phenotypic evaluation indices, physiological mechanisms, forward genetics and molecular mechanisms, an increasing number of breeding methods are emerging for chrysanthemum. The demand for cold-resistant herbs in all four seasons is also increasing in urban landscaping, and the breeding of new cold-resistant chrysanthemum varieties has become an area of intense interest. Many studies on breeding methods for cold tolerance in chrysanthemum have mainly

focused on crossbreeding, radiation mutagenesis breeding, selective breeding and genetic engineering breeding.

6.1 Crossbreeding

Crossbreeding is a conventional method of flower breeding and one of the most widely used and effective breeding methods in China and around the world. The use of heterosis is one of the most important methods in the hybrid breeding of chrysanthemum, and the probability of hybridization in the hybrid progeny population is greater with a greater genetic locus difference between parents (Zhou and Duan, 1995; Chen et al., 2009). Studies on crossbreeding of cold tolerance in chrysanthemum have mainly focused on the selection of distant crosses, intergeneric crosses and heterosis.

For distant crossbreeding, Zhu et al. (2012) crossed 'Zhongshan Jingui' as the maternal parent and *Ajania nematoloba* as the male parent, and the cold tolerance of the F1 hybrid was significantly improved, but the ornamental value was worse than that of 'Zhongshan Jingui', and further improvement was needed. Afterwards, Zhu et al. used the F1 hybrid as the parent and 'Zhongshan Jingui' as the recurrent parent and obtained 17 backcross lines. The backcross offspring had stronger cold tolerance than 'Zhongshan Jingui' and not only inherited the cold tolerance characteristics of *Ajania nematoloba* but also improved the ornamental quality of the distant hybrid offspring. Deng et al. (2011) carried out intergeneric hybridization using embryo culture technology with 'Zhongshan Jingui' as the maternal parent and a wild chrysanthemum as the paternal parent and obtained five intergeneric hybrids. The determination of the LT50 and Pro and MDA contents showed that the hybrid offspring had the same cold tolerance characteristics as their parents, with high cold tolerance. In terms of heterosis, Ma (2017) used 'Nannong Xuefeng', which has strong cold tolerance, as the maternal parent and 'Mengbai', which has poor cold tolerance, as the paternal parent of the F_1 chrysanthemum hybrid in full bloom and found that the cold tolerance of the chrysanthemum tongue flowers had certain maternal inheritance. In addition, the cold tolerance of the tongue flowers was related to the number of days in full bloom. Therefore, varieties with strong cold tolerance and late flowering should be chosen as the mother. This finding provides a reference for the cultivation of strong cold tolerance lines.

In conclusion, crossbreeding chrysanthemums for cold tolerance has achieved certain research results in distant crosses, intergeneric crosses and heterosis, but it is still necessary to further use a variety of chrysanthemum materials to cultivate more chrysanthemum varieties with strong cold tolerance.

6.2 Mutagenesis breeding

Chrysanthemums are genetically highly heterozygous, and evolution proceeds from low to high allopolyploidy, which easily leads to complex changes in genetic factors and is more suitable for radiation mutagenesis. Radiation breeding has the characteristics of high mutagenesis efficiency and good repeatability (Techarang et al., 2018; Khitka et al., 2021) and has become an important means of chrysanthemum genetic improvement. To date, research on radiation breeding for cold-resistant chrysanthemum has mainly focused on different mutagenesis methods. Fu and Zheng (1994) treated materials selected for mutagenesis with ^{60}Co and selected eight new varieties of 'Hanju', whose natural flowering period was from late November to early January of the following year and which could flower normally at -2°C to -5°C with rich flowers of relatively high ornamental value. Ueno et al. (2003) used carbon ion-beam radiation produced by a TIARA accelerator to mutate leaves. After culturing and screening, 66 identifiable mutant materials, including those with early flowers, late flowers, a reduced number of axillary buds, and flowering at low temperature, were isolated from 13077 M1 plants. Ueno et al. (2013) also used this radiation source to radiate 'Shenma' plants and obtained new chrysanthemum varieties 'Xinshen' and 'Xinshen 2', which had a reduced number of axillary buds and could bloom normally under low-temperature conditions. In conclusion, there are still few studies on radiation breeding of cold-resistant chrysanthemums, which is possibly related to the uncontrollability of radiation mutagenesis, and further practical studies are needed.

6.3 Selective breeding

Selective breeding is the process of artificially selecting and propagating the characteristics of natural variation or artificial pollination variation in the planting process to cultivate new strains, which is one of the most important means of conventional plant breeding. Many studies in chrysanthemum have mainly focused on character selection, physiological indices and molecular marker-assisted breeding. In terms of character selection, Fu (1998) selected new cold chrysanthemum varieties with strong cold tolerance, 'Wanyu', 'Wanfenhe', 'Hongwanying' and 'Huangwanying', based on the morphological characteristics of the pollen. Miao et al. (2013) obtained a new chrysanthemum variety 'Yanzhilu' with strong cold tolerance after seed selection from the budding materials of the chrysanthemum variety 'Italian Red'. However, this method required certain experience, and there were certain accidental phenomena, which often resulted in the selected plants not carrying the target traits.

Regarding the selection and breeding of physiological indicators, a previous review of physiological indicators reported that SOD and POD activities and the MDA, SS, and Pro contents and other chrysanthemum physiological indicators can be determined (Xu et al., 2010; Li, 2010; Li et al., 2009; Cheng et al., 2018), and plants with strong cold tolerance can be evaluated after analysis. Compared with conventional selection and breeding methods based on traits, selection results based on physiological indicators are more accurate, but certain experimental conditions are needed. Marker-assisted selection (MAS) is a breeding technique that uses closely linked or coisolated markers to select individuals for target traits (genes), regardless of gene expression, growth stage and environmental factors (Wang L. S. et al., 2006), which can greatly shorten the breeding time. MAS has become a research focus in the field of plant genetics and breeding (Klie et al., 2016; Cobb et al., 2019). For MAS of chrysanthemum cold tolerance, Yang et al. (2017) obtained three

molecular markers through correlation analysis of chrysanthemum cold tolerance and LT50 and then selected four chrysanthemums with strong cold tolerance. Ma conducted a QTL mapping study on the cold tolerance data for the 'NannongXuefeng' × 'Mengbai' F_1 generation and found that the contribution rates of qBdsCTM33 and qFfsCTM33 on the M33 linkage group to cold tolerance at the bud and flowering stages were 65.78% and 68.89%, respectively. These two QTLs may be the main genes controlling cold tolerance, which can be verified in multiple environments in future studies and applied to the selective breeding of cold-tolerant chrysanthemums. Compared with conventional character selection breeding and physiological selection breeding, molecular marker-assisted breeding can select the target character more accurately. In conclusion, with the in-depth study of phenotypes and physiological and molecular mechanisms related to chrysanthemum cold tolerance, an increasing number of breeding methods have appeared, and they are faster and more accurate.

6.4 Genetic engineering breeding

Genetic engineering is based on the theory of molecular genetics and adopts various biotechnological methods to introduce and integrate foreign or original genes with different functions into plant cells and achieve effective expression of functional genes through transgenic means for the purpose of targeted improvement of plant quality and creation of new varieties (Zhang, 2013). Many studies in chrysanthemum have mainly focused on agrobacterium-mediated, asexual reproduction and gene recombination. Wu (2006) introduced the PEAMT gene into chrysanthemum using *Agrobacterium*, which increased the content of betaine in the plant, thus greatly enhancing its salt and cold tolerance. Hong et al. (2006) introduced the stress-induced transcription factor *DREB1A* into the 'Fall Color' groundcover chrysanthemum through the *Agrobacterium tumefaciens*-mediated method and somatic embryoblast pathway using the *AtDREB1A* transformation vector driven by 35S and rd29A as promoters, respectively, to make the transgenic plants more cold resistant. Chen et al. (2012) verified the tolerance of chrysanthemum plants with a transferred *CdICE1* gene to abiotic stresses, such as low temperature, drought and salt stress, and the results showed that the tolerance of the transgenic chrysanthemum plants to abiotic stresses was significantly improved. In terms of asexual reproduction, Li C. et al. (2010) observed an asexual progeny from the transfer of the *AtDREB1A* gene to 'Fall Color', studied by Hong et al. (2006), and found that the progeny plants obtained through tissue culture expansion had a certain cold tolerance, and their ability to overwinter in the open field was also significantly improved. In terms of gene recombination, Li et al. (2022) knocked out the *DgTCP1* gene in chrysanthemum using the CRISPR/Cas9 system and found that the cold tolerance of the *DgTCP1* mutant plants decreased, while that of chrysanthemum transgenic plants overexpressing *DgTCP1* increased, providing an important reference for improved low-temperature tolerance breeding. Yang (2021) transferred the *DgnsLTP* overexpression vector into wild type chrysanthemums by Agrobacterium mediated method, and obtained the *DgnsLTP* overexpression line *35S: DgnsLTP-1*, *35s: DgnsLTP-3*, and found that they had higher survival rate and stronger resistance to low temperature stress than wild type chrysanthemums. In summary, genetic engineering breeding of cold tolerance chrysanthemum is controllable, and certain research results have been achieved in the understanding of the physiological, genetic and molecular mechanisms in chrysanthemum. However, overall, genetic breeding research on cold tolerance chrysanthemum is still relatively limited, and further research is needed to meet the market demand for cold-resistant chrysanthemums.

There are relatively few studies on breeding methods for cold tolerance in chrysanthemum, mainly focusing on crossbreeding, mutagenesis breeding, selection breeding and genetic engineering breeding. Compared with the other three breeding methods, the research results for mutagenesis breeding are still insufficient, which is possibly related to the high genetic heterozygosity of chrysanthemum itself and the uncontrollability of mutagenesis breeding. More radiation materials and radiation technology should be used for mutagenesis breeding research. In addition, with the rapid development of various omics technologies, such as genomics, epigenomics, transcriptomics, proteomics, metabolomics and phenomics, and the significant reduction in breeding costs, multidimensional and multiomics research on chrysanthemum breeding can create better cold-resistant chrysanthemum varieties to meet the needs of scientific research and landscaping.

7 Conclusion and future prospects

In terms of chrysanthemum phenotype, characteristics such as foot buds, florescence, REC, LT50, and anatomical structure are important indicators for evaluating chrysanthemum cold tolerance. The quantity of foot buds, rooting rate, and root length can be visually observed in the field to assess the strength of chrysanthemum cold tolerance. REC generally increases gradually with decreasing temperature. Combining REC with the Logistic equation calculates the LT50, which directly reflects cold tolerance strength. Leaf anatomical structure can reflect chrysanthemum response to temperature changes, but observing the structure requires precise instruments and some anatomical experience, making it relatively challenging. Furthermore, there are discrepancies in the research results of cold tolerance and florescence in chrysanthemum, as shown by Li et al. (2013), Li (2010), and Xu and Chen (2008). The phenomenon of early-flowering varieties having stronger cold tolerance and late-flowering varieties having weaker cold tolerance needs further analysis and validation.

In terms of physiological mechanisms of chrysanthemum, there are various physiological indicators for cold tolerance identification, including the biological membrane system, protective enzyme system, osmotic regulatory substances, photosynthesis, and hormones. However, chrysanthemum research mostly focuses on the relationship between individual physiological indicators and cold tolerance. Therefore, it's crucial to select effective physiological indicators for rapid and accurate cold tolerance identification. Many scholars have started using multiple indicators to

comprehensively evaluate cold tolerance (Xu et al., 2009). Methods like membership functions and correlation analysis have been employed by Xu et al. (2010), Bai et al. (2013), and Qiao et al. (2023) to comprehensively assess the SOD, POD, and CAT activities activity and the MDA, SS, SP and Pro contents of different groundcover chrysanthemums, and ranked the strength of cold tolerance of different varieties. Therefore, establishing a reliable model for chrysanthemum cold tolerance evaluation through multiple physiological indicators is of significant importance for breeding new cold tolerance chrysanthemum varieties and evaluating cold tolerance across multiple varieties.

In the field of forward genetics, advancements have been made from traditional genetic analysis to molecular markers, QTL mapping, and GWAS. This includes processes like segregation ratio analysis, mixed inheritance model analysis, molecular markers, constructing hybrid parental genetic maps, and multi-variety association analysis. These processes progressively deepen and enhance the accuracy and efficiency of gene determination for traits. Regarding molecular mechanisms, chrysanthemum cold tolerance is controlled by polygenic effects. Cold tolerance genes mainly include functional genes directly related to cold tolerance and regulatory genes controlling cold tolerance gene expression and signaling. Functional genes include photosynthesis system genes and fatty acid desaturase genes, although they are less studied in chrysanthemum cold tolerance research. Regulatory genes, particularly transcription factors, are gaining attention. Many transcription factors associated with cold tolerance, such as AP2/EREBP, WRKY, MYB, bZIP, TCP, NAC, and Zinc-finger (Riechmann et al., 2000; Zhu et al., 2010; Iwase et al., 2011), have been identified. However, only MYB, bZIP, and TCP transcription factors have been identified in chrysanthemums so far, indicating the need for further research to discover other transcription factors.

In terms of chrysanthemum breeding, hybrid breeding remains the most commonly used method, involving interspecific hybridization, distant hybridization, and transgenic and non-transgenic hybridization to produce cold tolerance varieties. Mutational breeding is also an important method for genetic improvement of chrysanthemum, offering advantages such as rapid results. However, it comes with uncertainties and potential issues with inheritance due to chrysanthemum high genetic heterogeneity, as well as the mutagenic materials and techniques used. Selection breeding has evolved from traditional trait selection to molecular marker-assisted selection, enabling precise and rapid selection of target traits. Genetic engineering is a popular breeding approach, but its application in chrysanthemum cold tolerance research is relatively limited. This method involves introducing target genes into chrysanthemums through various techniques for validation, necessitating further in-depth research.

Based on the summarization and integration of research on chrysanthemum cold tolerance phenotype, physiological mechanisms, positive genetics, molecular mechanisms, and breeding methods, there remain numerous unresolved issues in the current study of chrysanthemum cold tolerance. The specific relationship between chrysanthemum cold tolerance and flowering stage requires further in-depth investigation. While LT50 may represent the results of most cold tolerance physiological indicators, within the intricate mechanism of low-temperature response, lipid metabolism also plays a crucial role. The relationship between fatty acid metabolism and LT50 needs further exploration. Epigenetics refers to the phenomenon where gene expression changes without altering the DNA sequence and is closely related to changes in external environmental conditions, making it suitable for studying chrysanthemum cold tolerance (Wang et al., 2022). Research on antifreeze protein genes (AFPs) has made significant progress in other plants but remains unexplored in chrysanthemum (Griffith et al., 1992; Urrutia et al., 1992; Duman and Olsen, 1993; Worrall et al., 1998; Yin et al., 2001). China possesses abundant chrysanthemum germplasm resources, and genetic breeding research has yielded fruitful results. However, research on breeding for cold tolerance is still insufficient. With the rapid development of high-throughput sequencing, proteomics, and metabolomics, the future holds the potential to better unveil chrysanthemum responses to varying levels of low-temperature stress at the RNA, protein, and metabolite levels. This includes discovering new genes, proteins, and differentially expressed metabolites related to cold tolerance, conducting multi-omics joint analyses, and systematically investigating the mechanisms underlying chrysanthemum cold tolerance. For example, chrysanthemum is rich in flavonoids, and the accumulation of flavonoids is positively correlated with the plant stress tolerance (Watanabe and Ayugase, 2015; Schulz et al., 2016; Zhou et al., 2021). While previous cold tolerance research in chrysanthemums mainly focused on single genes, cold tolerance is a quantitative trait controlled by multiple genes. In the future, employing the simultaneous transformation of multiple genes could enhance chrysanthemum cold tolerance.

Author contributions

QC: Writing – original draft. KG: Writing – review & editing. YX: Writing – review & editing. YS: Writing – review & editing. BP: Writing – review & editing. DC: Writing – review & editing. CL: Writing – review & editing. XC: Writing – review & editing. HL: Writing – review & editing. CH: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Cloning and functional analysis of the *BrCUC2* gene in *Brassica rapa* L

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The *CUP-SHAPED COTYLEDON2 (CUC2)* gene plays an important role in the formation of apical meristem and organ edges in plants. The apical meristematic tissue of *Brassica rapa* (*B. rapa*) is associated with cold resistance, however, the role of the *CUC2* gene in cold resistance of *B.rapa* is unclear. In this study, we used bioinformatics software to analyze the structure of *BrCUC2* gene, real-time fluorescence quantitative PCR to detect the expression level of *BrCUC2*, constructed transgenic *Arabidopsis thaliana* by the flower dipping method and subcellular localization for functional validation. The results showed that, we isolated a 1104 bp open reading frame of *BrCUC2* from the winter *B. rapa* cultivar 'Longyou 7'. The BrCUC2 contains a highly conserved domain belonging to the NAM superfamily. Its homologus *CUC* genes contain similar conserved motifs and are closely related to *Brassica oleracea* (*B.oleracea*), and the N-terminal of amino acid sequence contains NAC domain. BrCUC2 protein was localized in the nucleus and self-activation tests showed that pGBKT7-BrCUC2 had self-activation. Tissue-specific expression analysis and promoter β-Glucuronidase (GUS) activity showed that *BrCUC2* had high expression levels in *B. rapa* growth points and *A. thaliana* leaf edges, stems and growth points. After low-temperature stress, *BrCUC2* showed greater expression in 'Longyou 7,' which presents strong cold resistance and concave growth points, than in 'Longyou 99,' which presents weak cold resistance and protruding growth points. *BrCUC2* promoter contains multiple elements related to stress responses. *BrCUC2* overexpression revealed that the phenotype did not differ from that of the wild type during the seedling stage but showed weak growth and a dwarf phenotype during the flowering and mature stages. After low-temperature treatment, the physiological indexes and survival rate of *BrCUC2*-overexpression lines of *Arabidopsis thaliana* (*A. thaliana*) were better than those of the wild type within 12 h, although differences were not observed after 24 h. These results showed that *BrCUC2* improved the low-temperature tolerance of transgenic *A. thaliana* within a short time. It can provide a foundation for the study of cold resistance in winter *B. rapa*.

KEYWORDS

Brassica rapa, BrCUC2, functional analysis, low-temperature stress, transgenic Arabidopsis thaliana

Introduction

Rapeseed production is important for the sustainable supply of edible oil in China (Li et al., 2022). In northern China, the temperature can reach -32°C in winter, drought occurs in spring, and ecological conditions are harsh (Zhou et al., 2014). Wind erosion and farmland degradation are severe, and desertified areas are expanding. The successful northward migration of winter rapeseed, which represents the only winter oil seed crop, has provided economic benefits in the north (Ma et al., 2019b). Moreover, the plant has been utilized as a winter cover crop, which can effectively increase vegetation coverage in winter and spring, improve the multiple cropping index and land utilization rate, reduce soil wind erosion, increase organic matter content, protect farmlands, promote balanced development between local economic production, and achieve ecological and environmental protection(Guo et al., 2023).

As the main production region for winter rapeseed, the planting area of northern China accounts for approximately 90% of the total rapeseed area. *Brassica rapa* (*B. rapa*) is mainly distributed on the western Loess Plateau (Wu et al., 2022). Before overwintering, the aboveground parts of strong cold-resistant varieties grow slowly, and the leaves grow prostrate and enter the withered leaf stage earlier (Ma et al., 2019a). During the overwintering period, the growth point of winter rapeseed varieties with strong cold resistance sinks, and the growth occurs belowground. The central leaves cover the surface, such that the growth point can maintain a specific moisture content and remain in the soil layer at a relatively stable temperature. This can effectively prevent damage to rapeseed when the temperature changes dramatically and allow the plant to safely overwinter. Sun et al (Sun et al., 2011a) found that winter rapeseed varieties with different cold resistance have great differences in the growth points, and the strong cold resistance *B. rapa* varieties that can overwinter in northern regions such as Hexi Corridor in Gansu Province have the characteristics of concave growth points and creeping growth. (Niu et al., 2021). Therefore, an unknown regulatory mechanism may occur between the morphological characteristics of winter rapeseed with concave and raised growth points and their cold resistance.

The NAC family transcription factor *CUP-SHAPED COTYLEDON2* (*CUC2*) regulates the formation of various tissues and organs during plant growth and development (Hu et al., 2015), and plays a particularly important role in the formation of the apical meristem and maintenance of organ primordium edges (Aida et al., 1997; Olsen et al., 2005). In 1997, the *CUC2* gene was first isolated and identified in *Arabidopsis thaliana* (*A. thaliana*) (Niu et al., 2021), and its mutation caused developmental defects in the apical meristem (Aida et al., 1999; Maugarny et al., 2016). The *CUC2* gene can promote the aggregation of auxins in the organ primordium by regulating *PIN-FORMED 1* (*PIN1*), thus participating in the regulation of early ovule primordium formation (Kwon et al., 2006). In addition, *CUC2* can indirectly regulate the synthesis of *LATERAL SUPPRESSOR* (*LAS*) genes for lateral branch development (Reinhardt et al., 2003).

CUC2 is a negative regulator of plant growth that antagonizes cytokinins and auxins (Motte et al., 2011). It slows plant growth by inhibiting the division of plant cells and promoting the initiation of apical meristem development under the regulation of auxin. *CUC2* is gradually expressed and leads to changes in the plant growth stage, beginning in the spherical embryo stage and proceeding to the tip of the embryo during seed germination and the edge of the cotyledon after cotyledon formation (Nikovics et al., 2006). As the upstream gene of the apical meristem-forming factor Shoot meristemless (*STM*) (Balkunde et al., 2017), the *CUC2* gene is regulated by the activity of the *STM* gene (Spinelli et al., 2011) during the growth and development of the apical meristem of *A. thaliana* plants and restricted to the margins of the cotyledon and apical meristem. Apical meristem formation is regulated by regulating the expression of *STM* (Balkunde et al., 2017). Studies on *CUC2* promoters of *Betula platyphylla* (*B. platyphylla*) found that they contain many cis-acting elements associated with tissue-specific expression, hormone synthesis, environmental stress response, and transcription factors and are highly expressed in the apical meristem, leaf margins, and flower tissue organs (Aida et al., 1999).

The regulatory role of the *CUC2* gene in *A. thaliana* and *B. platyphylla* meristems has been studied. *CUC2* is a negative regulator of plant growth that plays a role in the apical meristem and organ edge formation, leaf edge morphogenesis, and growth and development (Aida et al., 1997). However, its role in the growth point of winter *B. rapa* remains unclear.

In this study, we cloned the *BrCUC2* gene from the growing point of winter *B. rapa* and analyzed its bioinformatics and tissue expression. The promoter sequence 2000 bp upstream of *BrCUC2* was cloned using Gateway technology. Online software was used to predict the cis-acting regulatory element of the promoter, and a promoter expression vector was constructed and transformed into *A. thaliana*. Transgenic plants were then identified by β-Glucuronidase (GUS) histochemical staining. This study reveals the role of *BrCUC2* at the growth point of winter *B. rapa* and lays a foundation for further studies on the transcriptional regulation of *BrCUC2*.

Materials and methods

Plant materials and growth conditions

The experimental *B. rapa* materials included the 'Longyou 7' (strong cold resistance) and 'Longyou 99' (weak cold resistance) varieties. Rapeseed seeds with excellent vitality were selected, treated with 10% H_2O_2 for 30 min, rinsed with sterile water 2–3 times, and germinated in Petri dishes containing wet filter paper under light conditions for 14 h at 30°C and dark conditions for 10 h at 28°C. After germination, the seeds were sown in pots and cultured in an artificial incubator (14 h light at 25°C, 10 h dark at 20°C) until the seven–leaves stage. Rapeseed seedlings were placed in incubators at 4, 0, and -4°C for the low-temperature treatment and 25°C (room temperature) as the control. After 72 h of treatment, the roots, stems, leaves, and growth cones of rapeseed were collected, immediately frozen in liquid nitrogen, and stored at

-80°C for real-time fluorescence quantitative expression analysis of extracted RNA. Three biological replicates were performed for each treatment.

Nicotiana benthamiana (*N.benthamiana*) seeds were vernalized at 4°C for three days and seeded in a sterilized soil matrix. A subcellular localization test was performed during the 4th week of growth. The growth chamber conditions were as follows: 16 h light, 8 h darkness, 25°C, and 65% relative humidity.

Cloning of the *BrCUC2* gene

The basic local allignment search tool (BLAST) alignment method of the national center for biotechnology information (NCBI) was applied to the *BrCUC2* gene coding region sequence reported in the GenBank database, and a coding sequence with 100% homology to *B. rapa* was obtained. Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) was used to design the gene cloning primers *CUC2*-A: 5′-ATGGACATTCCGTACTACCACTAT-3′ and *CUC2*-S: 5′-TAGTAATTCCATACGCAATCAAGT-3′. The cDNA at the 'Longyou 7' growing point at room temperature was used as a template for *in vitro* amplification by polymerase chain reaction (PCR). The amplification process was as follows: pre-denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58.4°C for 30 s, and extension at 72.0°C for 60 s; terminal extension at 72°C for 10 min; and preservation at 4°C. The size of the PCR products was determined by 1% agarose gel electrophoresis. Target bands were recovered using an agar DNA gel recovery kit (TaKaRa, Dalian, China). The purified PCR products were connected with the pMD-19T vector, incubated at 25°C overnight for 18 h, and transferred to *E. coli* DH5α receptor cells. Positive recombinants were screened and confirmed using sequencing.

Sequence alignment and evolutionary analyses of BrCUC2 proteins from different plant species

Bioinformatics was used to predict the molecular properties of the protein encoded by the *BrCUC2* gene. We used ProtParam's Expasy software (https://web.expasy.org/protparam/) to predict protein physicochemical properties, ProtScale (Expasy - ProtScale) (Wilkins et al., 1999) to analyze hydrophobicity, SignaIP (https://services.healthtech.dtu.dk/services/SignalP-5.0/) (Teufel et al., 2022) to predict signal peptides, TMHMM (https://services.healthtech.dtu.dk/services/TMHMM-2.0/) (Krogh et al., 2001) to predict transmembrane domains, SMART tool to predict conserved structural domains, SOPMA(https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa%20_sopma.html) (Geourjon and Deléage, 1995) and SWISS-MODEL (Waterhouse et al., 2018) (https://swissmodel.expasy.org/) to predict protein secondary and tertiary structures.

Download the homologous gene sequence of *BrCUC2* from NCBI database. Determine and visualize the distribution position of *BrCUC2* homologous gene on chromosome with TBtools software. Utilizing online software Gene Structure Display Server (GSDS) (Guo et al., 2007) (http://gsds.gao-lab.org/index.php) to analyse the gene structure of *BrCUC2* homologous gene in *B. rapa*. Based on the location information of the *BrCUC2* homologous gene on chromosomes, the chromosome location map of the CUC genes were drawn using by MapChart software (Voorrips, 2002). The conservative motifs of homologous gene were predicted by MEME (https://meme-suite.org/meme/tools/meme) (Bailey et al., 2009). and the protein sequences of different species of *Brassica napus*, *Arabidopsis lyrate*, *Asparagus officinalis*, *Arabidopsis thaliana*, *Brassica oleracea*, *Camelina sativa*, *Glycine max*, *Medicago truncatula*, *Nicotiana attenuata*, *Raphanus sativus*, *Ricinus communis*, *Sesamum indicum* and 'Longyou 7' were obtained by NCBI database BLAST comparison, The amino acid sequences were compared by DNAMAN software. Neighbor–joining method was used to construct the phylogenetic tree by MEGA7.0, and the value of Bootstrap method was 1000 (Hall, 2013; Kumar et al., 2016).

Real-time fluorescence quantitative PCR

RNA was extracted from the leaves, roots, stems, and growth cones of winter *B. rapa* 'Longyou 7' and 'Longyou 99' after low-temperature treatment and reverse-transcribed into cDNA using a real-time fluorescence quantification biotechnology kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The specifically designed primers are listed in Table 1. RT-qPCR was performed by adding a 20 μL system containing the target gene and reference gene to a 96–well plate using a fluorescence quantitative PCR apparatus. The reaction was repeated three times, and the reaction procedure was determined according to the manufacturer's instructions for the specific reagent. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Subcellular localization of BrCUC2

Using the coding sequence (CDS) amplification product of *BrCUC2* as a template, Gateway technology (Liang et al., 2013) was used to amplify the product using aatB joint-specific primers, and the amplified product was cloned into the pDNOR entry vector *via* the BP reaction. After the LR reaction, the gene was introduced into the pEarlygate101-GFP vector, and the constructed vector plasmid was transferred into *Agrobacterium* GV3101 using the electrical conversion method. Bacterial fluid was collected and

TABLE 1 Quantitative real-time PCR primer sequences.

Primer	Sequence 5′- 3′
CUC2-1	CCATCGCAGAGGTTGATCTT
CUC2-2	TCTCAGTCCCGTCGGATATT
Actin-F	TGTGCCAATCTACGAGGGTTT
Actin-R	TTTCCCGCTCTGCTGTTGT

resuspended in 1 mL of infiltration buffer, and set the optical density (OD) at 600 nm of the solution to 0.6. The lower epidermis of *N. Benthamiana* leaves was labeled with the bacterial solution using a sterile needle tube and then incubated in a 22°C culture room for 48 h. Labeled *N. Benthamiana* leaves were observed and photographed under a laser confocal microscope to determine the location of the genes.

Self-activation validation of BrCUC2

The constructed positive control plasmids pGBKT7-53 +pGADT7-T, negative control plasmids pGBKT7-Lam+pGADT7-T, and pGBKT7-CUC2+pGADT7 plasmids were transformed into yeast strain Y2HGold, respectively. The transformed strains were cultured on DDO/X(SD/-Leu/-Trp/-X-α-gal) medium and incubate in a 30°C incubator for 3–5 days. Select single colonies on QDO/X/A(SD/Leu/-Trp/-His/-Ade/-X-α-gal/AbA) medium. Observe the self-activation phenomenon of pGBKT7-CUC2 based on the growth status of yeast.

Promoter cloning and cis-acting regulatory element analysis

We searched the region approximately 2000 bp upstream of the *BrCUC2* sequence in the genome of *B. rapa* and designed specific primers using Primer Premier 5.0 software. The following primers were added to the Gateway vector connector sequence: *CUC2* F(5'-3'): AAAAAAGCAGGCTTCCAATATGACACTAATTATGC; *CUC2* R (5'-3'): AGAAAGCTGGGTCGAAGAACTGATGTTAAAAC. Amplification based on the Gateway technique was performed in the following PCR reaction system (total 25 μL): Prime STAR DNA polymerase, 0.25 μL; 5×PrimeSTAR buffer, 5 μL; 2.5 mM dNTP, 1 μL, forward and reverse primers, each 1 μL; template DNA, 2 μL; and ddH_2O, 14.75 μL. The PCR reaction procedure was as follows: pre-denaturation at 98°C for 10 s and a total of 30 cycles of denaturation at 98°C for 10 s and extension at 68°C for 1 min. The PCR reactions were performed in two rounds, and after two rounds of PCR, the target bands were cut and recovered, connected to the PMD19-T vector, and transformed into *E. coli* DH5α. The promoter sequences were then sequenced after PCR detection of the bacterial solution. *BrCUC2* promoter sequences were analyzed using the PlantCARE database (Lescot et al., 2002) to investigate cis-acting regulatory elements.

Genetic transformation and β-glucuronidase staining in *A. thaliana*

The constructed plant overexpression vector pEarlyGate101 was transformed into *Agrobacterium* GV3101 using the freeze–thaw method (Weigel and Glazebrook, 2006). When *A. thaliana* grew to the flowering stage, the *Agrobacterium* solution was activated and resuspended in a 5% sucrose solution. The absorbance at 600 nm was adjusted to 0.8, and 3/10000 surfactant (Silwet L-77) was added for later use. Culturing was performed in the dark at 25°C for 24 h and then in the light. The herbicide Glufosinate Ammonium (Basta) (1/10000) (Sangon Biotecch, shanghai, China) was sprayed for screening. After maturation, *A. thaliana* seeds were harvested from each plant.

Resistant *A. thaliana* plants were placed in a GUS staining solution, and wild-type *A. thaliana* was used as the control. The plants were kept at 37°C overnight, rinsed and soaked with 70% ethanol, and photographed under a microscope to observe GUS staining (Liu et al., 2017).

Screening, identification, and functional analysis of transgenic *A. thaliana*

After vernalization, the first generation T0 transgenic *A. thaliana* seeds were planted in sterilized nutrient soil, sprayed with Basta (10%) two weeks later for screening, and sprayed again three times a week. The surviving *A. thaliana* plants were harvested from the T1 generation seeds. The T1 generation seeds were seeded in 1/2 MS medium containing Basta and screened according to the 3:1 principle. T2 generation seeds were harvested after transplantation. The T2 generation seeds were planted in 1/2 MS medium containing Basta, and all surviving plants were transgenic homozygous plants. Subsequent experiments were conducted after transplantation. Genomic DNA of transgenic *A. thaliana* was amplified using the primers CUC2 F(5'-3'): CTATCCT TCGCAAGACCTTC and CUC2 R(5'-3'): TAATTCCAT ACGCAATCAAGT and identified *via* PCR and RT-qPCR analyses.

Phenotypic changes were observed in the third-generation transgenic lines and wild-type *A. thaliana* during the seedling, flowering, and mature stages. After four weeks of growth, they were exposed to -4°C for 3, 6, 12, and 24 h and then transferred to room temperature (25°C) to recover growth for seven days, and their survival rate was counted. *A. thaliana* leaves were collected at different treatment times, and their physiological indicators and gene expression levels were measured. Growth at room temperature (25°C) was used as a control. Superoxide dismutase (SOD) activity was determined using the nitroblue tetrazolium (NBT) photoreduction method (Durak et al., 1993). peroxidase (POD) activity was determined using the guaiacol method (Senthilkumar et al., 2021). Proline (PRO) content was measured using the acid ninhydrin method (Abrahám et al., 2010).

Results

Identification and analysis of CUC2 gene family in winter *B. rapa*

The NAC (PF02365) domain of CUC2 was determined using the Pfam database. Ten CUC genes of *B.rapa* were selected, and the homologous BrCUC2 protein sequences of different species were screened by BLAST-Protein (BlASTP) tool. DNAMAN software was used to compare the protein sequences of BrCUC2 and its homologous genes *CUC1*、*CUC3*, and *BrCUC2* (*B.rapa*, *B.napus*,

B.oleracea, *R.sativus, A. thaliana*) in different species (Figure 1). The results showed that all CUC protein sequences contained a highly conserved NAC domain at the N-terminus, including A, B, C, D and E subdomains. the subdomains A, C and D were highly conserved, while the subdomains B and E were structurally variable and might be involved in different biological functions. The integrity of the NAC domain is essential for CUC2 to promote the formation of adventitious buds in callus tissue (Taoka et al., 2004). The absence of any of the five motifs affects the formation of adventitious buds. The C-terminal domain of CUC2 protein contains three conserved motifs, namely the V motif (TEHVSCFS), L motif (SLPP), W motif (WNY), and two serine rich regions (S). Among them, the W motif is essential for the transactivation of the CUC2 protein, and the V motif contains miRNA binding sites, which is responsible for regulating *CUC2* gene activity. The intact CUC2 protein plays an important role in Shoot apical meristem (SAM) formation or plant organ separation. The BrCUC2 (Bra022685) protein sequence of *B.rapa* was 99.46% similar to the CUC2 protein of 'Longyou 7', and there were two amino acid mutation sites, alanine (A) of 'Longyou 7' was changed to valine (V), glycine (G) was changed to glutamic acid (E).The homology was 89.01% with *Raphanus sativus* (*R. sativus*), 97.28% with *Brassica oleracea* (*B. oleracea*) and 99.46% with *Brassica napus* (*B. napus*). Suggesting that the BrCUC2 (Bra022685) protein sequence is highly conserved during evolution process.

In order to further clarify the functional and evolutionary relationships of the *BrCUC2* gene in the growth cone of winter *B.rapa*, MEGA11 was used to construct a phylogenetic tree, and the evolutionary relationships were compared between CUCs and BrCUC2 proteins in 13 different species (including *B. napus*, *B. rapa*, *C. sativa*, *B. oleracea*, *R. sativus*, etc.) (Figure 2). The results showed that they were mainly divided into nine subfamilies, among which BrCUC2 (Bra002685.1) was more closely related to *B. oleracea*, *B. napus*, *R. sativus* and Bra003023. CUC1 (Bra001586) and CUC3 (Bra008259) were closely related to *Asparagus officinalis* (*A. officinalis*). It is speculated that the *BrCUC2* gene in winter *B. rapa* 'Longyou 7' is functionally similar to that in *B. napus*, *B. oleracea* and *R. sativus*.

Chromosome mapping and structure analysis of CUCs gene in winter *B. rapa*

Ten CUC genes in *B. rapa* were distributed on seven chromosomes (Figure 3A), two of which were distributed on chromosome A02, three on chromosome A03, and the rest on chromosomes A01, A04, A07, A09 and A10, respectively. The positions of introns and exons were determined by comparing the genomic DNA with the full-length cDNA of CUCs (Figure 3D). the results showed that Bra003023 contained three introns and the rest nine genes contained two introns. Ten CUC genes contained a total of 18 motifs, and all contained motif 1, motif 3 and motif 4, of which Bra022685 and Bra003023, Bra007855 and Bra032095, Bra021592 and Bra001586 contained the same motif, respectively. and the same subfamily of CUC proteins in the phylogenetic tree had the same motif, indicating that they were highly conserved (Figures 3B, C, S1; Table S1).

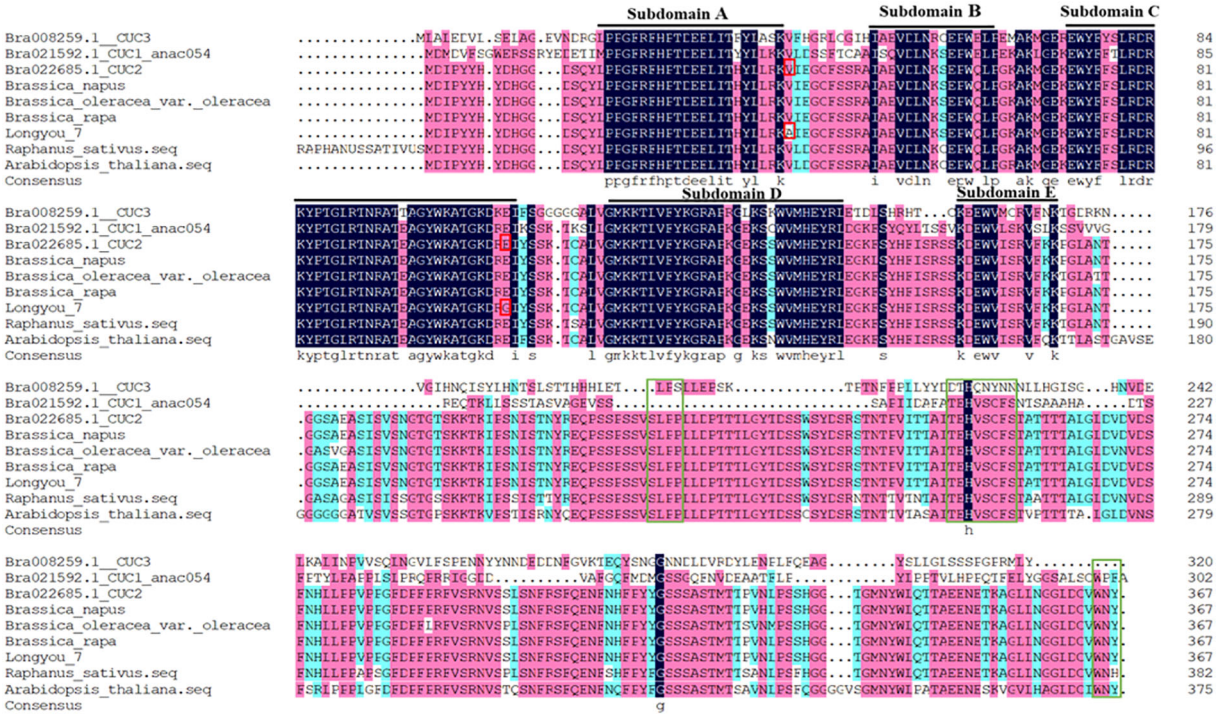

FIGURE 1
Amino acid sequence comparison of CUC2 protein in different species and homologous protein CUC. Highly conserved subdomains **(A–E)** are indicated by black lines. Amino acid mutation sites are indicated by red boxes. The L motif, V motif and W motif of C-terminal domain are represented by green boxes.

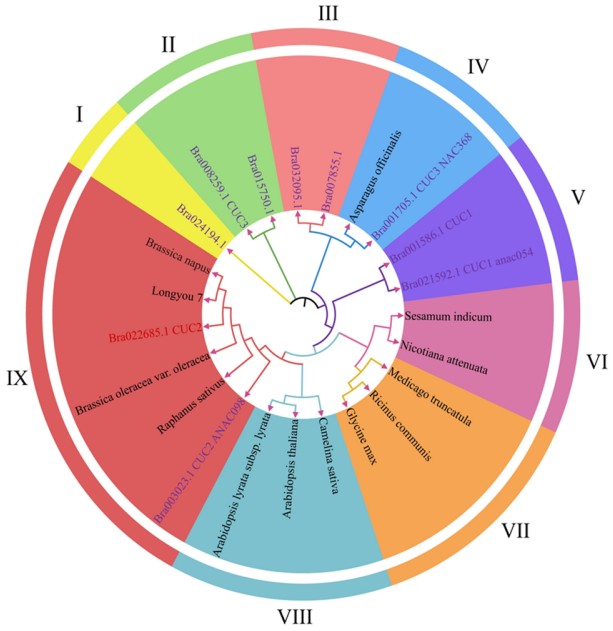

FIGURE 2
Evolutionary analysis of CUC2 in different species. The 9 subfamilies have different colors. Purple font represents members of the *BrCUC2* homologous genes, The others are *BrCUC2* in different species.

The BrCUC2 gene (1104 bp) was amplified using the cDNA template from the growing site of 'Longyou 7,' and the result was consistent with the predicted fragment size. The BrCUC2 gene sequence encodes 367 amino acids. The theoretical isoelectric point (PI) was 8.66, fat index was 58.71, instability index was 44.07, and total average hydrophilic value was -0.569. The BrCUC2 protein was predicted as an unstable hydrophilic protein (Figure S2A). No transmembrane regions and signaling peptides were detected (Figures S2B, C). The conserved domain was distributed among 18–144 amino acids and composed of 127 amino acids, and the conserved sequences belonged to the NAM superfamily (Figure S1). The secondary and tertiary structures of the BrCUC2 protein mainly consisted of irregular curling (65.12%), extended chain (16.25%), α helix (14.17%), and β angle (4.36%) (Figures S2D, E).

Prediction and functional analysis of cis-acting regulatory elements in the *BrCUC2* promoter region

Promoters are important gene elements that regulate gene expression. By analyzing the cis-acting elements, the pathway genes that may participate in abiotic stress-tolerance mechanisms can be better understood (Hernandez-Garcia and Finer, 2014). Using the genomic DNA of the 'Longyou 7' growth cone of winter *B. rapa* as a template, the *BrCUC2* promoter sequence with a length of approximately 2000 bp was amplified and sequenced after connecting to the vector.The sequencing was correctly used to prepare *Agrobacterium* liquid transformed *Arabidopsis*. PlantCARE and GSDS2.0 online software were used to analyze the *BrCUC2* promoter sequence and predict the cis-acting regulatory elements (Table 2; Figure 4). The results showed that the promoter region of this gene contains ABRE-acting elements involved in abscisic acid and stress responses, TGA elements involved in the auxin response, CAT-box elements involved in meristem expression. Tc-rich repeats are involved in stress response; ARE active elements are involved in anaerobic responses; Box4, GA-motif, GT1-motif, I-box, and TCT-motif active elements are involved in light response; and several CAAT-box and CAT-box elements are core promoters.

Subcellular localization and self-activation validation of BrCUC2

The fusion expression vector *PEarlyGate101 BrCUC2* GFP was constructed using Gateway technology, and tobacco leaves were transformed instantaneously with empty GFP as a control. After 48 h of growth in the dark, scanning and photography were performed using a confocal laser microscope. The results showed that the *BrCUC2* GFP fusion protein was distributed in the nucleus (Figure 5A).

To further characterize the function of BrCUC2, we investigated whether BrCUC2 has self-activating activity in yeast cells. The positive control pGBKT7-53+pGADT7-T, negative control pGBKT7-Lam+pGADT7-T, and pGBKT7-CUC2+pGADT7 were transformed into Y2HGold yeast cells and cultured on DDO/X (SD/-Leu/-Trp/-X-α-gal) and QDO/X/A (SD/-Leu/-Trp/-His/-Ade/-X-α-gal/AbA) medium, respectively. The results showed that the positive control combination grew and turned blue on DDO/X and QDO/X/A medium, while the negative control group grew but remained blue on DDO/X media, and did not grow on

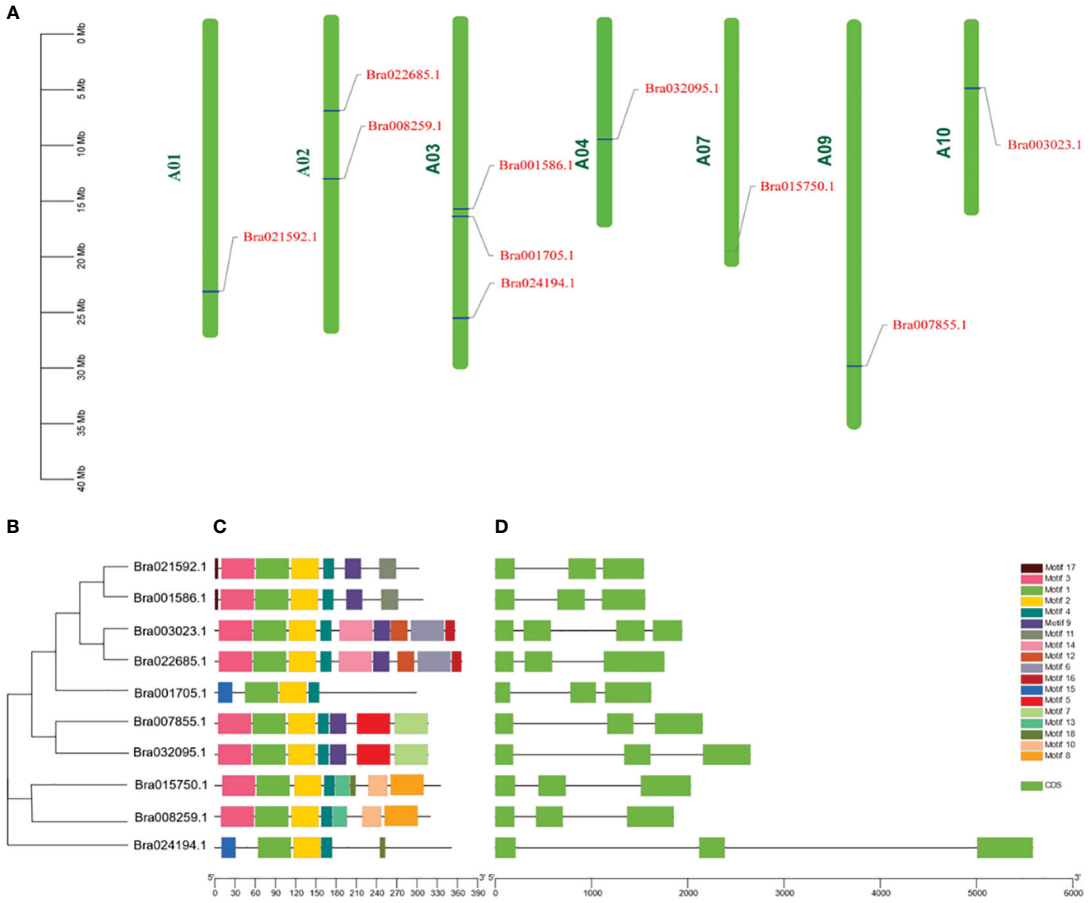

FIGURE 3
Chromosomal localization and structural analysis of the CUCs gene. **(A)** Chromosomal location distribution of CUCs genes. **(B)** Phylogenetic relationships of CUCs genes. **(C)** Schematic representation of conserved motifs in CUCs proteins. Different colored boxes represent different patterns. The black lines represent non-conserved sequences. **(D)** CUCs exon/intron structure. The green boxes represent exons; The space between boxes is the intron. The scale bars at the bottom of **(C, D)** represent the lengths of genes and motifs, respectively.Cloning and Sequence Analysis of the *BrCUC2* (Bra022685) Gene.

QDO/X/A media, indicating the success of the positive control and negative control experiments. The experimental group pGBKT7-CUC2+pGADT7 grew on DDO/X medium, grew and turned blue on both TDO/X (SD/-Leu/-Trp/-His/-X-α-gal) and QDO/X/A media (Figure 5B), indicating that the pGBKT7-CUC2 plasmid was successfully transferred into the yeast strain and showed self-activation in the yeast strain. Further inhibition experiments were conducted using (3-amino-1,2,4-triazole)3-AT, and the results showed that the growth turned blue on QDO/X/A/(1-5) mM 3-AT and weakened with the increase of 3-AT concentration. However, it did not grow on QDO/X/A/(7-15) mM 3-AT medium (Figure 5C), indicating that the QDO/X/A/7 mM 3-AT screening conditions can inhibit the self-activation phenomenon of pGBKT7-CUC2.

TABLE 2 Cis-acting elements and their functions in the *BrCUC2* promoter region.

Cis-acting element	Core sequence	Position	Function
ABRE	ACGTG	- 417, - 1522, - 1724	Involved in the abscisic acid responsiveness
ARE	AAACCA	1548	Essential for the anaerobic induction
AT-rich sequence	TAAAATACT	578	Element for maximal elicitor-mediated activation
Box 4	ATTAAT	766, - 1567, - 1373	Light responsiveness
CAAT-box	CAAT	5, -1831	Common cis-acting elements in promoter and enhancer regions
CAT-box	GCCACT	930	Related to meristem expression

(Continued)

TABLE 2 Continued

Cis-acting element	Core sequence	Position	Function
CGTCA-motif	CGTCA	419	Involved in the MeJA-responsiveness
GA-motif	ATAGATAA	-1239	Light-responsive element
GT1-motif	GGTTAAT	518	Light-responsive element
I-box	GATAAGGTG	-1237	Light-responsive element
O2-site	GATGATGTGG	1883	Involved in zein metabolism regulation
TC-rich repeats	GTTTTCTTAC	248	Involved in defense and stress responsiveness
TGA-element	AACGAC	- 735, - 1358	Auxin-responsive element
TCT-motif	TCTTAC	1032	Light-responsive element

Analysis of *BrCUC2* gene expression

To clarify the differences in the expression of the *BrCUC2* gene in various winter rapeseed tissues, RT-qPCR was performed to determine the relative expression levels of *BrCUC2* in the leaves, stems, growth cones, and roots (Figure 6A). The results showed that the expression of *BrCUC2* in different tissues of 'Longyou 7' and 'Longyou 99' was significantly different. The expression level in 'Longyou 99' was 10.9-times higher in the growth points than in the leaves, whereas that in 'Longyou 7' was 16.2-times higher in the growth cone than in the leaves, indicating that the *BrCUC2* gene mainly acts on the growth cone of winter *B. rapa*.

To verify the expression pattern of *BrCUC2*, GUS tissue staining was performed on wild-type and transgenic *A. thaliana* seedlings (Figure 6B). The results revealed that the GUS signal was absent in the wild-type *A. thaliana* but present on the leaf edges, stems, and growth points of transgenic *A. thaliana* seedlings, indicating that the BrCUC2 promoter cloned from winter *B. rapa* could drive downstream GUS reporter gene expression with tissue specificity.

To investigate the effect of cold stress on *BrCUC2* expression, 'Longyou 7' and 'Longyou 99' were treated at 0, 4, and -4°C, RT-qPCR was performed to measure the expression. The results showed differences in the relative expression of the *BrCUC2* gene among different tissues after low-temperature stress. As the temperature decreased, the expression of the *BrCUC2* gene was upregulated and reached the highest value at -4°C. The difference was most significant in stem and growth cone and higher in 'Longyou 7,' which presents strong cold resistance and a concave growth cone, than in 'Longyou 99,' which presents weak cold resistance and a convex growth cone. In the leaves, under normal temperature control, the expression of *BrCUC2* in 'Longyou 7' and 'Longyou 99' was downregulated at 4°C and 0°C, and after treatment at -4°C, the expression was 2.6- and 1.5-times higher than those in the control, respectively (Figure 6C). In the roots, the expression of *BrCUC2* in 'Longyou 7' and 'Longyou 99' was downregulated at 4°C and upregulated at -4°C and 0°C, respectively. After treatment at -4°C, the expression was 3.0- and 1.6- times higher than those in the control, respectively (Figure 6D). With a decrease in temperature, *BrCUC2* was upregulated in the stem and growth cone compared with that in the control (Figures 6E, F). After treatment at -4°C, the expression levels of *BrCUC2* in 'Longyou 7' and 'Longyou 99' were 18.3- and 8.5-times higher in the stem and 29.1 and 24.3-times higher in the growth cone than those in the control, respectively.

Screening and identification of the transgenic *BrCUC2* gene in *A.thaliana*

The first generation T0 *BrCUC2*-overexpressing plants were vernalized and planted in nutrient-rich soil. Two weeks later, they were sprayed with a 10% Basta solution. After exposure to the spray three times, seedlings that did not exhibit resistance to the herbicides turned yellow and died. Normal surviving seedlings were transplanted, and seeds were harvested from individual plants to obtain the T1 generation. The T1 generation of *A. thaliana* was selected on 1/2MS medium containing Basta according to the 3:1 principle. Seedlings with normal leaf and root growth were transplanted, and individual plants were harvested to obtain the T2 generation. The T2 generation of *A. thaliana* was screened for all surviving seedlings on 1/2MS medium containing Basta to obtain homozygous transgenic lines, which were then transplanted into pots for subsequent tests (Figure S3A).

After extracting *BrCUC2* transgenic *A. thaliana* DNA, PCR amplification results showed no amplification bands in the wild-

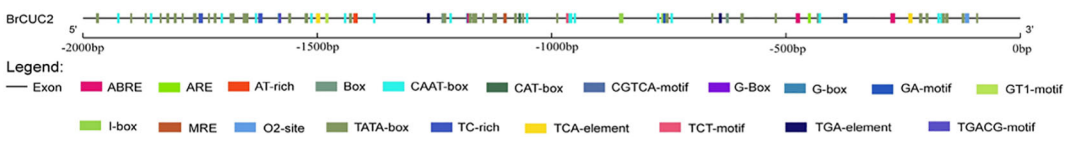

FIGURE 4
Promoter cis-acting regulatory element distribution. Different colored rectangular boxes represent individual cis-acting components.

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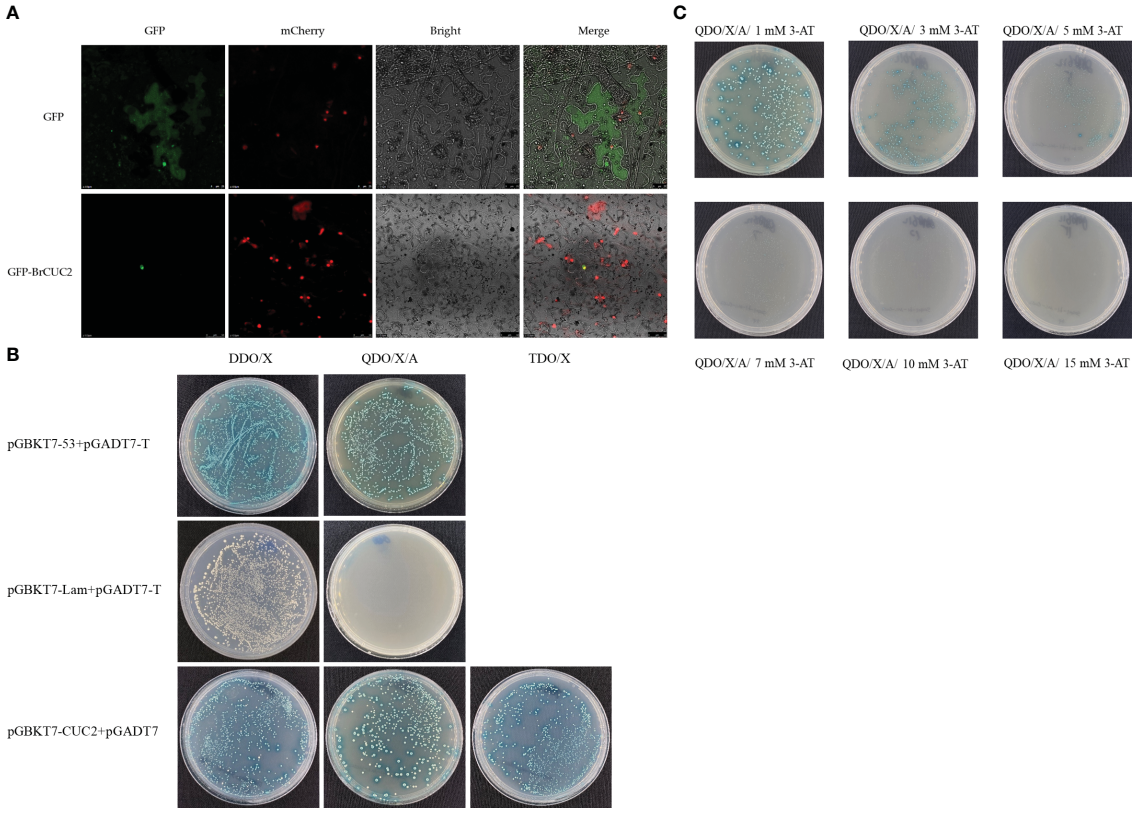

FIGURE 5
(A) Subcellular localization of PEarlyGate101-BrCUC2-GFP protein, empty GFP as a control, mCherry, a nuclear localization protein. **(B)** Self-activation verification of BrCUC2. The positive control pGBKT7-53+pGADT7-T, the negative control pGBKT7-Lam+pGADT7-T, and the experimental group pGBKT7-CUC2+pGADT7 were transferred to yeast strain Y2HGold, respectively. Cultured on DDO/X (SD/-Leu/-Trp/- X-α-Gal) and QDO/X/A (SD/Leu/-Trp/-His/-Ade/-X-α-Gal) medium, respectively. TDO/X (SD/-Leu/-Trp/-His/-X-α-Gal). **(C)** Screening of 3-AT inhibition at different concentrations in QDO/X/A medium. 3-amino-1,2,4-triazole(3-AT), 3-AT is a competitive inhibitor of histidine and can inhibit mild self-activation.

type *A. thaliana* single plants; however, 10 bands of approximately 1100 bp were amplified from 11 single transgenic plants. Among them, C2 had no bands, indicating that the transformation of the Br*CUC2* gene in C2 single plants failed, while the 10 remaining single plants were successfully transformed, indicating that they were transgenic plants, with C6 and C10 showing lighter bands (Figure S3B).

RT-qPCR identification was performed on 10 overexpression plants and wild-type transgenic plants, and the results showed that the *BrCUC2* gene was detected in all overexpression plants. The expression levels varied among different individual plants, with the highest expression levels of the *CUC2* gene in C3 and C9, which were 7.53 and 7.79 times higher than those in the WT (Figure S3C).

Phenotypic differences associated with the *BrCUC2* gene in *A. thaliana* treated at low temperature

Wild-type and *BrCUC2*-overexpressed *A. thaliana* phenotypes were observed at the seedling (4 weeks old), flowering, and mature stages under the same growth conditions (16h light/8 h dark cycle at 25°C). Comparisons between the wild-type and *BrCUC2*-overexpression *A. thaliana* plants showed that significant phenotype differences did not occur at the seedling stage but did occur at the flowering stage, with weaker growth and development and a dwarf phenotype (Figures 7A, B). The overexpression *A. thaliana* lines developed slowly and were set later than the wild-type at the mature stage. When wild-type *A. thaliana* plants were clamped and matured, the lateral branches of overexpression lines were still in the flowering stage, and the seed-setting rate was lower than that of the wild type (Figure 7C).

To verify the response of the *BrCUC2* gene to low-temperature stress in plants, 4-week-old wild-type and *BrCUC2*-overexpression plants were treated at -4°C for 3, 6, 12, and 24 h and then returned to room temperature (25°C) for 7 d to observe the phenotypes (Figures 7D, E). All plants grew normally after low-temperature treatment for 3 h; however, the survival rate of the wild-type plants was 72% after low-temperature treatment for 6 h and decreased to 8% after 12 h, with most of the leaves dying. After low-temperature treatment for 6 h, the survival rate of *BrCUC2*-overexpression plants was 99%; after 12 h, some leaves yellowed, and the survival rate was 62%, and after 24 h, the survival rate was 25%. These results indicate that *BrCUC2* overexpression enhances plant low-temperature tolerance over a short period.

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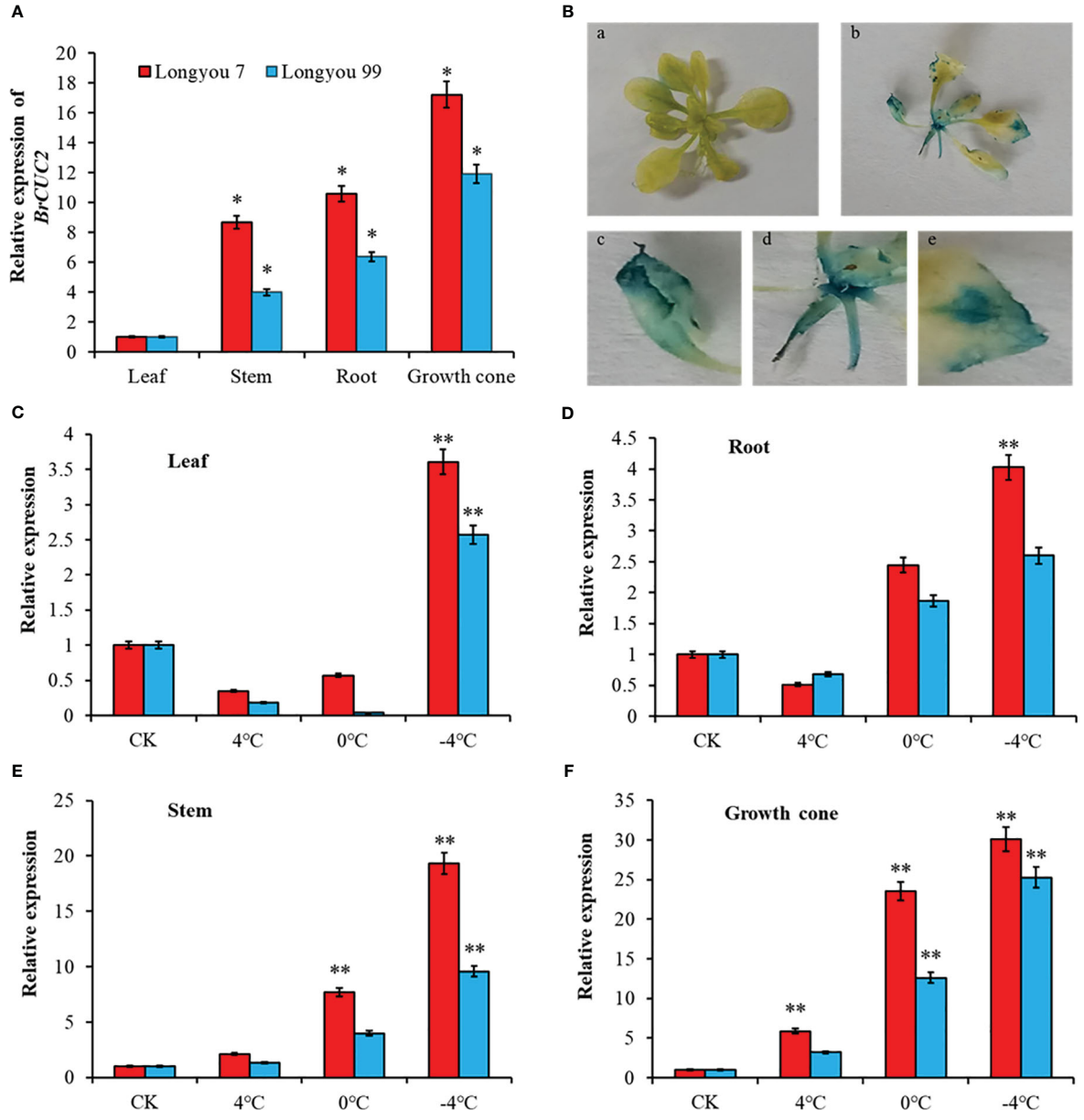

FIGURE 6
BrCUC2 tissue expression specificity. **(A)** Expression level of *BrCUC2* in different tissues. **(B)** GUS mapping of *BrCUC2* transgenic *Arabidopsis thaliana*. a, GUS staining of whole wild - type *A. thaliana*; b, GUS staining of whole transgenic *A. thaliana*; c-e, Partial magnification of b **(C)** Expression of *BrCUC2* gene in leaf under low temperature stress. **(D)** Expression of *BrCUC2* gene in Root under low temperature stress. **(E)** Expression of *BrCUC2* gene in Stem under low temperature stress. **(F)** Expression of *BrCUC2* gene in growth cone under low temperature stress. Symbols "*"and "**" indicate significant differences at *P values* of 0.05 and 0.01, respectively.

Physiological parameters of *BrCUC2* gene transfer in *A. thaliana*

The physiological indicators of wild-type and overexpressed *A. thaliana* after low-temperature treatment were analyzed after treatment at -4°C. The enzyme content in both plants gradually increased over time. The superoxide dismutase(SOD)activity、peroxidase (POD) activity and proline content in the *BrCUC2*-overexpressing plants were higher than those in the wild-type plants, reaching the highest values after 24 h (16.9%, 41.9%, and 22.9% higher than those in the wild-type, respectively) (Figure 8). This result indicates that the *BrCUC2* gene is involved in the response of winter *B. rapa* to low-temperature stress.

Discussion

The *A. thaliana* NAC family transcription factor *CUC2*, a negative regulator of plant growth (Takada et al., 2001), plays an important role in the formation of apical meristems and organ margins, leaf edge morphological formation, growth, and development. The formation of meristem edges in *A. thaliana* is

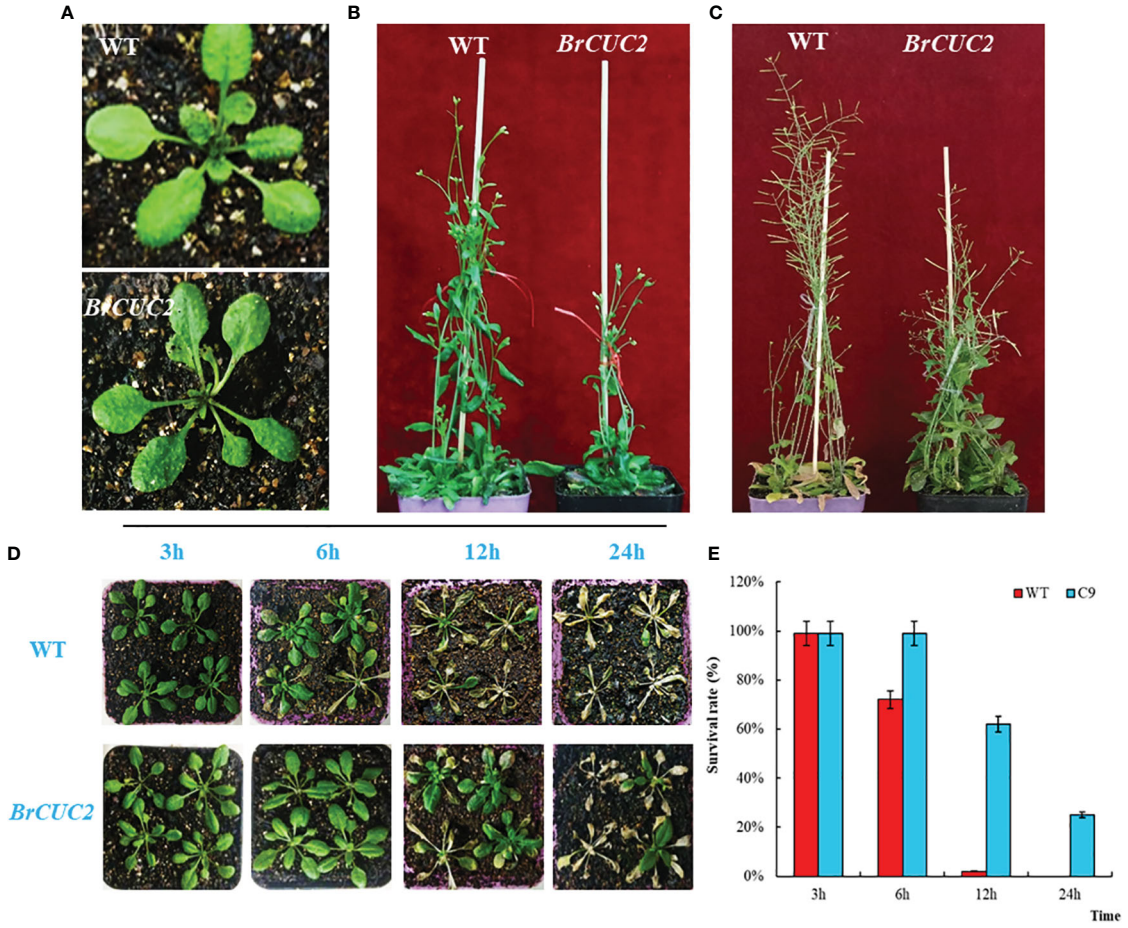

FIGURE 7
BrCUC2 transgenic *A. thaliana* phenotype. **(A)** Seedling stage, **(B)** Flowering stage, and **(C)** Maturity stage. **(D)** Phenotypes of transgenic and wild-type *A. thaliana* after low-temperature treatment at -4°C for 3, 6, 12, and 24 h. **(E)** Survival rate of plants after low-temperature treatment. C9: *BrCUC2* overexpressed plant.

mediated by the NAC family transcription factors *CUC1*, *CUC2*, and *CUC3* (Bertrand et al., 2003; Kamiuchi et al., 2014). The *CUC1* and *CUC2* are negatively regulated by *miR164* (Bhatt et al., 2004). Kwon et al (Kwon et al., 2006) found that the *CUC2* gene could fuse cotyledons and flower organogenesis in *A. thaliana*. Souer et al (Souer et al., 1996) showed that *CUC2* is highly homologous to petunia genes necessary for flower organ development, cotyledon separation, and apical meristem embryogenesis. In *A. thaliana*, the *CUC2* gene is necessary for the activation of the *STM* gene during embryonic development (Lenhard et al., 2002). Studies have shown

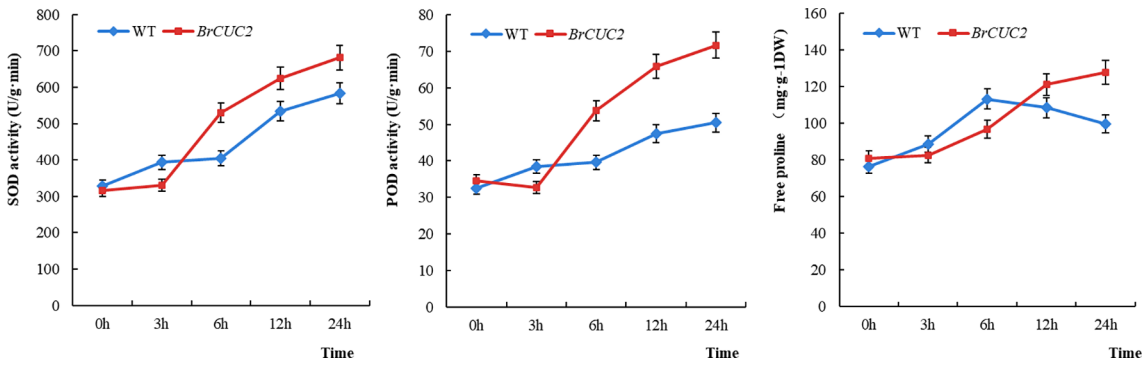

FIGURE 8
Physiological indices SOD、POD、Proline of *Arabidopsis thaliana* overexpressing *BrCUC2* gene at low temperature. WT: wild type, *BrCUC2*: overexpressed plant.

that *STM* can directly activate *CUC2* (Scofield and Murray, 2006), and mutations in the *CUC2* gene can change the cotyledon traits of *A. thaliana* and cause developmental defects in its apical meristem (Vroemen et al., 2003). *STM* or *CUC2* gene mutations affect the number of plant stem cells and lead to cotyledon fusion (Mallory et al., 2004). However, the regulatory mechanism of the *CUC2* gene in winter rapeseed growth sites has not yet been reported.

In the present study, the *BrCUC2* gene was cloned from the growth point of winter *B. rapa*, which controls the growth point differentiation of *A. thaliana*. The *BrCUC2* gene was expressed in the leaves, stems, roots, and growing points of winter *B. rapa*, with the highest expression level observed at the growing points. The relative expression level was as follows: the expression level of *BrCUC2* in the growing point of 'Longyou 99' was 10.9 times that in the leaves, and the expression level of *BrCUC2* at the growing point of 'Longyou 7' was 16.2 times that in the leaves. After 72 h of low-temperature treatment, the expression in the leaves was initially downregulated and then upregulated, while the expression in roots, stems, and growth points was upregulated and reached the highest value at -4 °C. The differences were most significant in the stems and growth points. The expression in 'Longyou 99,' which presents weak cold resistance and a raised growth point, was lower than that in 'Longyou 7,' which presents strong cold resistance and a depressed growth point. These results indicate that the *BrCUC2* gene might be induced by low-temperature stress and inhibit the growth of growing sites. Moreover, the high *BrCUC2* content caused the 'Longyou 7' growth point to sag and grow below the ground.

Bioinformatics analysis and functional verification showed that the *BrCUC2* gene contains a conserved NAM superfamily domain, *CUC2* is a transcription factor in the NAC family, and NAC domain proteins usually contain two relatively independent domains, one is a highly conserved N-terminal domain, divided into five (A-E) sub domains, responsible for DNA binding. The C-terminal region is highly divergent and serves as a transcriptional activation region, which endows NAC protein with functional variations (Bian et al., 2020). By comparing the protein sequences of BrCUC2 in different species and its homeotic genes, we found that the N-terminal and C-terminal of BrCUC2 contain NAC domains. Systematic evolution analysis shows that BrCUC2 is closely related to *B. oleracea*, and the CUCs gene contains similar conserved motifs, indicating that functional similarity was maintained during evolution.

Cloning the *BrCUC2* promoter sequence at the growth site of winter *B. rapa* showed that *BrCUC2* contained meristem-forming elements, indicating that the expression of *BrCUC2* would affect the formation of the meristem. The *BrCUC2* gene mainly present in the nucleus, and yeast self-activation verifies its self-activation activity, which may acts as a transcription factor. The *BrCUC2* promoter contains various photoresponsive elements, hormone- and stress-responsive elements. When plants are subjected to low temperature stress, transcription factors activate the expression of low-temperature -responsive genes by binding cis-acting elements on the gene promoter, thus regulating the signaling pathways in the plant to improve low temperature tolerance. The bZIP transcription factor binds to the ABRE element (ACGT) on the target gene promoter to regulate the expression of downstream low temperature response genes (Hwang et al., 2016). CsbZIP6 is a negative regulatory factor for low temperature stress response in tea plants, leading to a downregulation of downstream low temperature response gene expression and affecting the sensitivity of tea plants to ABA (Wang et al., 2017). The *Arabidopsis* gene AtbZIP1 binding ABRE action element and regulates plant response to low temperature stress through ABA-dependent signal transduction pathway (Sun et al., 2011b). *BrCUC2* promoter contains ABRE, ARE, CGTCA-motif, TC-rich repeats and TGA-element elements, which may be involved in the regulation of low temperature stress response. GUS is mainly observed in the leaf margins, stems, and meristems. Research on the expression pattern of the *BrCUC2* gene in winter rapeseed under low-temperature stress and GUS tissue staining showed that the light response elements, meristem formation, and stress-related elements contained in *BrCUC2* played an important role, which is consistent with previous research results on the *CUC2* promoter of *B. platyphylla*.

The natural environment is an important factor influencing plant growth and development. To ensure normal growth, plants must change their molecular and physiological mechanisms in response to external stress. SOD and POD, as scavengers of reactive oxygen species (ROS), can effectively protect plants from low temperatures and other abiotic stresses (Nadarajah, 2020).

Zhang et al (Zhang et al., 2020) found that overexpression of the *SikRbcs2* gene significantly increased SOD, POD, and CAT enzyme activity in tomato *SikRbcs2* transgenic *tobacco* under 4°C stress. The transgenic tomato *ZPR1* gene significantly enhanced *Arabidopsis* physiological activity and cold resistance (Li et al., 2012). The *cuc1-2* double mutant of *A. thaliana* has an abnormal stem end meristem tissue formation (Aida et al., 1997; Uauy et al., 2006). The plant transformed from *B. platyphylla* to 35S::BPCUC2 was shorter than that of the normal plant (Liu et al., 2018). This phenomenon may be due to *CUC2* overexpression, which affects plant growth by affecting the growth of plant meristems. In this study, we found no difference in the phenotypes of the wild-type and *CUC2*-overexpression strains at the seedling stage, whereas the growth characteristics of the overexpression plants were weaker and shorter during the flowering and maturation stages. This finding indicates that the *BrCUC2* gene, as a negative regulatory factor for plant growth, has an inhibitory effect on the growth cones of winter *B. rapa*. After low-temperature treatment, the physiological activity of the overexpression plants was significantly higher than that of wild-type *A. thaliana* only at 6 and 12 h, and there was no difference after 24 h, indicating that the *BrCUC2* gene can improve the low-temperature tolerance of transgenic *A. thaliana* over a short period.

Conclusion

The *BrCUC2* gene was cloned from the growth point of winter *B. rapa*. It encodes 367 amino acids, and the conserved sequence belongs to the NAM superfamily. Multiple sequence alignment revealed that the N-terminal amino acid sequence of BrCUC2 homologous CUC proteins contained a highly conserved NAC domain. Systematic evolution analysis showed that *BrCUC2* in winter *B. rapa* had the highest affinity for *B. oleracea*, *B. napus*,

and *R. sativus*. BrCUC2 is expressed in the nucleus and has self-activition. After low-temperature stress, the expression level of *BrCUC2* was the highest at the growth site. As the temperature decreased, *BrCUC2* was upregulated and presented higher expression in 'Longyou 7,' which presents strong cold resistance and a concave growth site, than in 'Longyou 99,' which presents weak cold resistance and a protruding growth site. The *BrCUC2* promoter sequence contains a variety of action elements related to light, hormone, and stress responses and meristem expression. GUS chemical tissue staining mainly revealed expression at the edges of the leaves, stems, and growth points in transgenic *A. thaliana*. The overexpression of the *BrCUC2* gene in *A. thaliana* is a characteristic of stunted plant development. After 12 h of low-temperature treatment, some leaves of the *BrCUC2*-overexpression *A. thaliana* withered and yellowed, and the survival rate was 62%; however, the survival rate of wild-type *A. thaliana* decreased to 8%, and most of the leaves died. After 24 h of low-temperature treatment, the physiological indicators SOD, POD, and PRO reached their highest values (16.9%, 41.9%, and 22.9% higher than that of the wild type, respectively). This result indicates that the *BrCUC2* gene can improve the low-temperature tolerance of transgenic *A. thaliana*. This study aimed to explore the regulatory mechanism of *BrCUC2* at the growth site of winter *B. rapa* to provide a foundation for studying cold resistance in winter *B. rapa*.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

XT: Data curation, Writing – original draft. YZ: Data curation, Writing – review & editing. JW: Data curation, Funding acquisition, Methodology, Writing – review & editing. RZ: Data curation, Writing – review & editing. JJ: Data curation, Writing – review & editing. RL: Data curation, Writing – review & editing. WM: Data curation, Writing – review & editing. YL: Data curation, Writing – review & editing. WW: Methodology, Writing – review & editing. YP: Methodology, Writing – review & editing. GY: Methodology, Writing – review & editing. LL: Methodology, Writing – review & editing. XL: Methodology, Writing – review & editing. LM: Funding acquisition, Methodology, Writing – review & editing. WS: Methodology, Writing – review & editing.

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Conflict of interest

Author YZ is employed by Gansu Yasheng Agricultural Research Institute Co. LTD Lanzhou, China.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1274567/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Conserved motifs logo in CUCs proteins.

SUPPLEMENTARY FIGURE 2
Structural characteristics of the *BrCUC2* gene.

SUPPLEMENTARY FIGURE 3
Screening of *BrCUC2* transgenic *Arabidopsis thaliana*.

SUPPLEMENTARY TABLE 1
Motif prediction of BrCUC2 protein by MEME server.

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The deterioration of starch physiochemical and minerals in high-quality indica rice under low-temperature stress during grain filling

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Low temperatures during the grain-filling phase have a detrimental effect on both the yield and quality of rice grains. However, the specific repercussions of low temperatures during this critical growth stage on grain quality and mineral nutrient composition in high-quality hybrid indica rice varieties have remained largely unexplored. The present study address this knowledge gap by subjecting eight high-quality indica rice varieties to two distinct temperature regimes: low temperature (19°C/15°C, day/night) and control temperature (28°C/22°C) during their grain-filling phase, and a comprehensive analysis of various quality traits, with a particular focus on mineral nutrients and their interrelationships were explored. Exposure of rice plants to low temperatures during early grain filling significantly impacts the physicochemical and nutritional properties. Specifically, low temperature increases the chalkiness rate and chalkiness degree, while decreases starch and amylopectin content, with varying effects on amylose, protein, and gelatinization temperature among rice varieties. Furthermore, crucial parameters like gelatinization enthalpy (ΔH), gelatinization temperature range (R), and peak height index (PHI) all significantly declined in response to low temperature. These detrimental effects extend to rice flour pasting properties, resulting in reduced breakdown, peak, trough, and final viscosities, along with increased setback. Notably, low temperature also had a significant impact on the mineral nutrient contents of brown rice, although the extent of this impact varied among different elements and rice varieties. A positive correlation is observed between brown rice mineral nutrient content and factors such as chalkiness, gelatinization temperature, peak viscosity, and breakdown, while a negative correlation is established with amylose content and setback. Moreover, positive correlations emerge among the mineral nutrient contents themselves, and these relationships are further accentuated in the context of low-temperature conditions. Therefore, enhancing mineral nutrient content and increasing rice plant resistance to chilling stress should be the focus of breeding efforts to improve rice quality.

KEYWORDS

rice, grain quality, low temperature, mineral nutrients, starch

1 Introduction

Rice (Oryza sativa L.) is a vital staple food that provides essential nutrients, including starch, protein, and various macro-micro nutrients, to more than half of the global population (Huang et al., 2016). With the world's population on the rise and increasing economic development, there has been an improvement in the yield potential and grain qualities of rice (Peng et al., 2009). Moreover, the nutritional quality of rice has garnered increasing attention in recent years due to its crucial role in human health, particularly in regions such as Asia, Latin America, and parts of Africa, where rice provides up to 76% of the caloric intake (Fitzgerald et al., 2009; Huang et al., 2016). The macro-micronutrients in rice are not only essential components of the human body, but also cofactors in many important biological processes (Huang et al., 2020). Inadequate consumption of macro-micro nutrients from food can have severe health consequences, especially among children and women in developing countries (Du et al., 2013). Zinc deficiency can lead to anorexia, stunted growth, and underdeveloped intelligence (Xi et al., 2016), while iron deficiency causes anemia in over two billion people globally (Zhang et al., 2020). Lack of calcium is the main cause of osteoporosis, and manganese deficiency can result in developmental and growth defects (Chen et al., 2005). Therefore, enhancing the nutrition of rice could be of significant benefit to human health, especially in developing countries.

Double-season rice cropping is a prevalent rice production system in central China and other parts of Asia, it involves sequential cultivation of early and late-season rice from March to November, leading to higher rice production per unit land area than single-season rice systems (Peng et al., 2009). Although late-season rice typically exhibits superior grain quality than early-season rice (Dong et al., 2017), its production is often impacted by lower temperatures from mid-September, leading to reduced grain yield and poor quality (Zeng et al., 2017; Zhang et al., 2017). Low temperatures have caused significant losses in rice production, with losses of up to 20% of total production reported in the last century (Xiao and Song, 2011). The southern regions of the mid-lower reaches of the Yangtze River have seen a significant increase in the area exposed to low temperatures over the 2010s (Wang et al., 2019). Moreover, addressing the gap in knowledge surrounding low-temperature stress and its impact on rice is also crucial, particularly in temperate and high-altitude areas in the tropics, where it is a significant factor affecting the growth and development of rice (Razafindrazaka et al., 2020).

Rice quality traits include milling recovery, physical appearance, cooking and eating qualities, and nutritional value. Starch, accounting for around 90% of milled rice weight, receives high attention for its physicochemical properties (Hu et al., 2020). The synthesis and accumulation of starch were blocked leading to the deterioration of cooking and eating quality for rice, especially under abiotic stress (Ahmed et al., 2015; Siddik et al., 2019; Wada et al., 2019). Previous studies have reported that low temperatures during the grain-filling stage increased amylose content (Zhu et al., 2017), improved the short chain of amylopectin (Chun et al., 2015), and decreased the amylopectin content and relative crystallinity in starch (Hu et al., 2020; Ai et al., 2023). Therefore, low temperatures could decrease starch physicochemical properties, and consequently deteriorate the cooking and eating quality of rice. Zhu et al. (2017) found that low temperatures increased protein content and gelatinization temperature while decreasing total starch content in Japonica rice. However, Hu et al. (2020) showed that the effect of protein and total starch content was not significant under low temperatures, but the gelatinization temperature decreased significantly. Additionally, some studies reported that the amylose and protein content decreased under low temperatures (Chun et al., 2015; Ai et al., 2023). Most previous studies have focused on the effect of low temperature on rice quality traits in japonica rice (Oryza sativa L. subsp. japonica) (Chun et al., 2015; Zhu et al., 2017; Hu et al., 2020) or inbred indica (Oryza sativa L. subsp. indica) (Lu et al., 2022). However, research on high-quality hybrid indica varieties has been limited, even though they have been bred to meet the demand for good eating and cooking quality in recent years (Zeng et al., 2022). Furthermore, the differences in quality characteristics, such as amylose content and physicochemical properties, between India and japonica rice are well-recognized (Feng et al., 2017). And comparative analyses of grain quality in response to high temperatures during the grain-filling stage between indica and japonica have been conducted (Fan et al., 2023). Therefore, it is crucial to investigate the effect of low temperature on high-quality indica varieties, which plays an essential role in maintaining food security and has been highly demanded by consumers in recent years.

While there has been extensive research on the effect of temperature on starch properties of rice, less attention has been paid to the impact of lower temperatures on mineral nutrients. The accumulation of mineral elements in rice grains is not only influenced by genetic factors but also constrained by external environmental conditions (Du et al., 2013; Huang et al., 2016). For instance, increasing atmospheric CO_2 or temperature can reduce the nutrient content, such as Zn and Fe, in wheat or rice (Myers et al., 2014; Chaturvedi et al., 2017). Moreover, the application of nitrogen fertilizer and water management can also affect the micro nutrient composition of grains. Gu et al. (2015) reported that increasing nitrogen fertilizer resulted in decreased levels of micro-nutrients such as Cu, Mg, and S, while Fe, Mn, Zn, Na, etc., increased. In contrast, Wang et al. (2018) found that a moderate level of nitrogen application was favorable for promoting the accumulation of micro-nutrients like Cu, Fe, Mn, and Zn in brown rice. Additionally, Xu et al. (2019) discovered that alternating wetting and moderate soil drying irrigation decreased the content of Cu, Fe, Mn, Mo, Se, and Zn in the brown rice. However, the effect of low temperature on the mineral nutrient composition of rice grains remains unclear. Furthermore, a complex correlation has been documented between within-grain minerals and minerals with different quality traits such as amylose content, protein content, gel consistency, and gelatinization temperature in rice (Jiang et al., 2007; Huang et al., 2016; Xi et al., 2016). However, the relationships between quality traits and mineral nutrients have not been explored under low-temperature conditions.

This study compares the grain quality traits and mineral nutrient contents of eight high-quality indica rice cultivars

subjected to either low-temperature stress or a control condition during the grain filling stage, which is the most critical for rice quality in response to extreme temperatures. Therefore, the primary objectives of the studies were to investigate (i) the impact of lower temperature on grain quality, and mineral nutrients in high-quality indica rice, and (ii) the association between grain quality traits and mineral nutrients exposed to lower temperature conditions.

2 Materials and methods

2.1 Plant materials

Eight high-quality indica varieties, Taoyouxiangzhan (TYXZ), Yliangyou911 (YLY911), Yuzhenxiang (YZX), Taiyou390 (TY390), Huanghuazhan (HHZ), Jinliangyouhuazhan (JLYHZ), Longjingyou 534 (LJY534), and Nongxiang 42 (NX42) were selected in this study. The experiment was conducted in the research farm at Hunan Agricultural University, Changsha city (14°C11′N, 121°C15′E, 21 m asl), Hunan Province, China. Twenty days of seedlings were manually transplanted into plastic pots (28cm and 32cm in internal diameter and height, respectively) to grow. Plants were given a basal dressing of 5g pot^{-1} [commercial fertilizer, 20-10-15 ($N\text{-}P_2O_5\text{-}K_2O$)] before transplanting. Water, weeds, pests, and diseases were completely controlled as requirements of local high-yield cultivation.

2.2 Temperature treatments and samplings

During the heading stage of the rice plants, two to three primary tillers which had headed on the same day from each plant were labeled. As previous research has shown that the second week of post-heading is the most critical period for rice quality in response to extreme temperatures (Siddik et al., 2019), a walk-in climate chamber-treated experiment was conducted to impose the temperature treatment at this period. Specifically, on the seventh day after marking, thirty plants were randomly selected and moved into two independent temperature-controlled growth rooms (2.6 m × 2.2m × 2.0 m in length, width, and height, respectively). The plants were subjected to two different temperature regimes for six consecutive days, a lower temperature of 19°C during the daytime (07:00 a.m. to 6:59 p.m.) and 15°C during nighttime (07:00 p.m. to 6:59 a.m.), and a control temperature of 28°C during the daytime and 22°C during nighttime. To monitor the temperature in the rice canopy, two stand-alone sensors (HOBO, MX2301A, USA) were placed in each growth room to measure the temperature at 10-minute intervals. Once the temperature treatments were completed, all plants were transferred outdoors. After physiological maturation, all the marked panicles on the individual plants for each variety and temperature treatment were collected. The panicles were manually threshed and then stored at room temperature for three months before determining the grain quality traits.

2.3 Determination of grain chalkiness

Three samples consisting of 200 grains each were randomly selected for each treatment, which was then dehulled. Grain chalkiness rate (%) and chalkiness degree were measured by a flatbed scanner (ScanMaker i800plus, MICROTEK, China) and analyzed with SC-E software (Hangzhou, Wanshen Detection Technology Co., Ltd., Hangzhou, China).

2.4 Total starch content, amylose, and amylopectin content

The total starch content was determined using the total starch kit (Suzhou Comin Biotechnology Co., Ltd, Suzhou, China) in accordance with the kit's protocol. The amylose content was measured using the amylose–iodine reaction with reference to the national standards of the People's Republic of China (GB/T 17891-2017). To measure the amylose content, rice flour (100mg) was mixed with 1 ml of 95% ethanol and 9 mL of 1 M NaOH, and then boiled for 10 min. After cooling, the volume was made up to 100 ml with distilled water. 5 ml of solution was added with 1 ml of 1M aqueous acetic acid and 2 ml of iodine solution (0.2 g iodine and 2.0 g potassium iodide in 100 ml aqueous solution). The volume was then made up to 100ml with distilled water and the absorbance of the solution was measured at 620 nm with a spectrophotometer. The amylopectin content was obtained by subtracting the amylose content from the total starch content, as described previously by Zhu et al. (2021).

2.5 Determination of protein content

Total protein content was measured using the method by Hu et al. (2020) with some modifications. In brief, it was determined indirectly using nitrogen concentration estimated by the semi-microKjeldahl method and a Kjeldahl conversion coefficient of 5.95 was used with reference to the national standards of the People's Republic of China (GB/T 5009.5-2016).

2.6 Determination of thermal properties

The thermal properties were determined using a differential scanning calorimetry analyzer (DSC 25, TA Instruments, USA). Five milligrams of starch were mixed with 10ul water, and then the mixture was hermetically sealed and left to stand at room temperature for 24 hours before being heated in the DSC. The DSC analyzer was calibrated using an empty aluminum pan as a reference. The sample pans were heated from 30°C to 95°C at a rate of 10°C min^{-1}. Onset temperature (To), peak temperature (Tp), conclusion temperature (Tc), and gelatinization enthalpy (ΔH) were calculated by the TA Universal Analysis 2000 software. The

gelatinization temperature range (R) and peak height index (PHI) were calculated as R = Tc−To and PHI =ΔH/(Tp− To) respectively (Ai et al., 2023).

2.7 Determination of pasting properties

The pasting properties of rice flour were evaluated using a rapid viscosity analyzer (RVA-3D, Newport Scientific, Australia). Specifically, 3 grams of rice flour were accurately weighed and placed into an RVA sample canister. Next, 25ml of ultrapure water was added to the canister, after which it was transferred into the RVA for testing. The temperature within the RVA tank was subjected to a heating-cooling program that commenced at 50°C for one minute, followed by a gradual increase to 95°C at a rate of 12°C per minute. The temperature was then maintained at 95°C for 2.5 minutes, after which it was decreased to 50°C at a rate of 12°C min^{-1} and maintained at 50°C for 2 minutes. The peak viscosity (PV), trough viscosity (TV), final viscosity (FV), breakdown value (PV - TV) and setback value (FV - PV) were analyzed by TCW (Thermal Cline for Windows) program (Jiang et al., 2022).

2.8 Macro and micro nutrients

The nutrients (B, Na, Mg, P, K, Ca, Mn, Fe, Cu, and Zn) were determined via an inductively coupled plasma mass spectrometer (ICP-MS; NexION300X; PerkinElmer, USA). Milled grains (0.50 g) were finely ground and wet-digested in a 50 ml conical flask using 10 ml mixed acid (4:1 HNO_3–$HClO_4$). After cooling, the digested

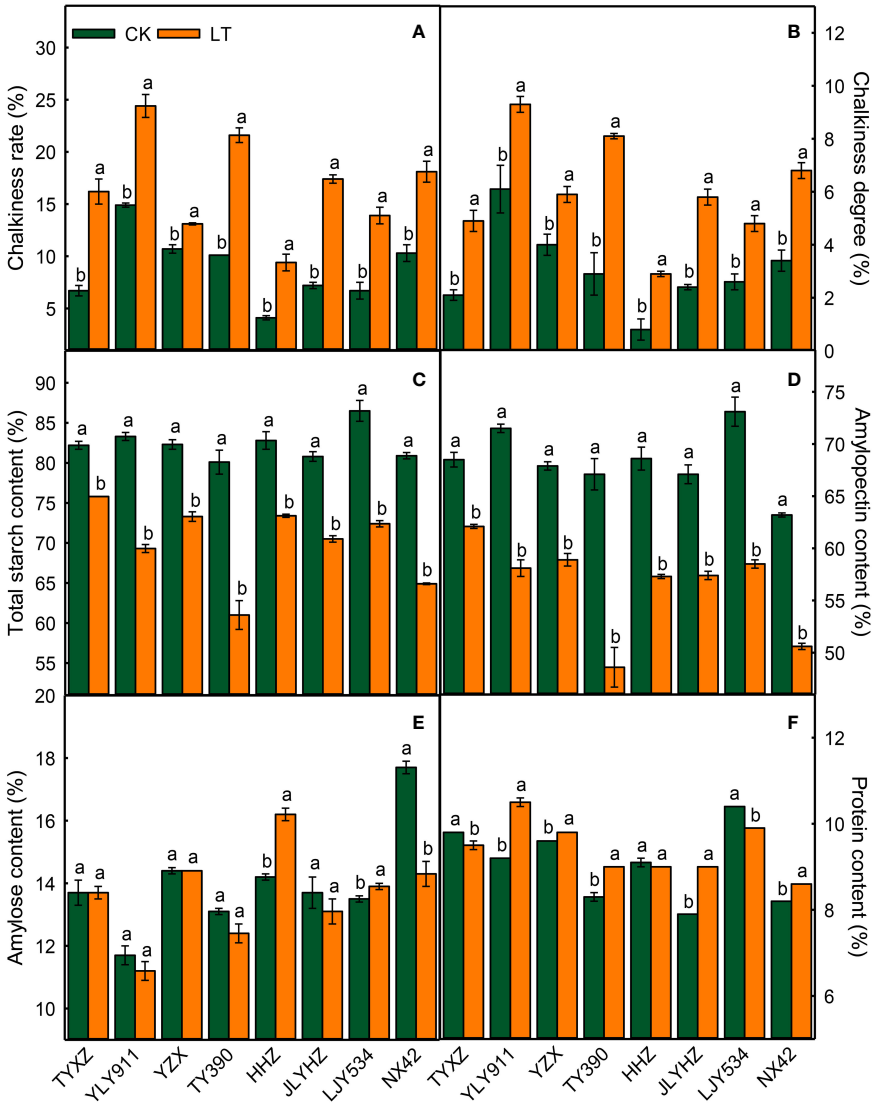

FIGURE 1
The impacts of low temperature on chalkiness rate **(A)**, chalkiness degree **(B)**, total starch content **(C)**, amylopectin content **(D)**, amylose content **(E)** and crude protein content **(F)** in high-quality indica rice. The values reported are mean ± standard error of three replicates. The different letters in each variety are statistically significant at $p < 0.05$. CK, control temperature treatment. LT, low-temperature treatment.

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solution was transferred to a 100 ml volumetric flask and brought to a final volume of 100 ml with double distilled water. The resulting solution was then filtered using a 0.45 um filter and collected in a fresh 10 ml plastic centrifuge tube for subsequent analysis by ICP-MS. Calibration curves and element contents were obtained using standard solutions of 32 elements (GNM-M323115-2013, 20DC821, China).

2.9 Statistical analysis

The statistical analysis employed in this study involved factorial analysis of variance (ANOVA), which was performed using the R language (version 4.1.0; http://www.R-project.org). Mean values ± standard error derived from three replications were reported in all tables and figures. To conduct multiple comparisons of variables with statistically significant differences ($p < 0.05$), Duncan's new multiple range method was utilized. The figures were plotted using the Sigma Plot software version 14.0 (Systat Software Inc., San Jose, CA, USA). The principal component analysis (PCA) and Pearson's correlation analysis were also carried out using the R language.

3 Results

3.1 Chalkiness, starch and protein content

Notably, exposure to low temperatures during the grain-filling stage resulted in a significant increase in both the chalkiness rate and degree across all varieties (Figures 1A, B). The largest increases in chalkiness rate (11.5%) and chalkiness degree (5.2%) induced by lower temperature were seen in variety TY390. Compared to the control treatment, lower temperature resulted in a significant decrease in both total starch and amylopectin content across all rice varieties, with the greatest reduction observed in TY390 (19.1% and 18.5% for total starch and amylopectin, respectively) and the least in TYXZ (6.4% for both total starch and amylopectin) (Figures 1C, D). Conversely, the content of amylose decreased significantly only in NX42, while increased significantly in HHZ and LJY534 (Figure 1E). Notably, the effect of lower temperatures on protein content varied among rice varieties (Figure 1F), with a significant reduction observed in TYXZ by 3.1% and in LJY534 by 4.8%, and a significant increase observed in YLY911, YZX, TY390, JLYHZ, and NX42 by 2.1%-14.1%.

TABLE 1 The impact of low temperature on the thermal properties of starch in high-quality indica rice.

Variety	Temperature treatment	To (°C)	Tp (°C)	Tc (°C)	ΔH (J g^{-1})	R (°C)	PHI
TYXZ	CK	67.2 ± 0.1 ^{b}	74.8 ± 0.0 ^{b}	78.0 ± 0.1 ^{b}	3.76 ± 0.02 ^{a}	10.75 ± 0.03 ^{a}	0.49 ± 0.00 ^{a}
	LT	69.9 ± 0.1 ^{a}	75.6 ± 0.1 ^{a}	78.6 ± 0.2 ^{a}	2.66 ± 0.04 ^{b}	8.66 ± 0.13 ^{b}	0.47 ± 0.00 ^{b}
YLY911	CK	75.6 ± 0.1 ^{b}	82.0 ± 0.1 ^{a}	85.5 ± 0.1 ^{a}	4.14 ± 0.03 ^{a}	9.93 ± 0.12 ^{a}	0.64 ± 0.00 ^{a}
	LT	76.7 ± 0.0 ^{a}	82.1 ± 0.0 ^{a}	84.9 ± 0.1 ^{b}	2.98 ± 0.01 ^{b}	8.21 ± 0.11 ^{b}	0.55 ± 0.00 ^{b}
YZX	CK	68.1 ± 0.2 ^{a}	73.3 ± 0.2 ^{a}	78.5 ± 0.5 ^{a}	5.95 ± 0.01 ^{a}	10.40 ± 0.28 ^{b}	1.15 ± 0.01 ^{a}
	LT	63.0 ± 0.1 ^{b}	72.5 ± 0.2 ^{a}	77.3 ± 0.1 ^{a}	5.56 ± 0.05 ^{b}	14.24 ± 0.17 ^{a}	0.58 ± 0.00 ^{b}
TY390	CK	77.0 ± 0.1 ^{a}	82.9 ± 0.1 ^{b}	86.3 ± 0.2 ^{a}	3.66 ± 0.02 ^{b}	9.28 ± 0.16 ^{b}	0.63 ± 0.01 ^{a}
	LT	76.5 ± 0.2 ^{b}	83.5 ± 0.1 ^{a}	86.8 ± 0.2 ^{a}	3.75 ± 0.02 ^{a}	10.28 ± 0.15 ^{a}	0.54 ± 0.01 ^{b}
HHZ	CK	67.5 ± 0.2 ^{a}	75.1 ± 0.1 ^{a}	79.8 ± 0.2 ^{a}	5.45 ± 0.01 ^{a}	12.27 ± 0.24 ^{a}	0.73 ± 0.01 ^{a}
	LT	67.5 ± 0.0 ^{a}	75.3 ± 0.1 ^{a}	78.5 ± 0.1 ^{b}	2.93 ± 0.02 ^{b}	11.01 ± 0.05 ^{b}	0.38 ± 0.01 ^{b}
JLYHZ	CK	75.8 ± 0.1 ^{a}	81.4 ± 0.2 ^{b}	85.7 ± 0.2 ^{a}	4.09 ± 0.04 ^{a}	9.81 ± 0.14 ^{a}	0.73 ± 0.02 ^{a}
	LT	76.7 ± 0.3 ^{a}	82.9 ± 0.2 ^{a}	86.1 ± 0.4 ^{a}	2.83 ± 0.03 ^{b}	9.37 ± 0.15 ^{a}	0.46 ± 0.00 ^{b}
LJY534	CK	67.3 ± 0.0 ^{a}	74.5 ± 0.1 ^{a}	77.9 ± 0.1 ^{a}	3.65 ± 0.02 ^{a}	10.61 ± 0.10 ^{a}	0.51 ± 0.01 ^{a}
	LT	67.9 ± 0.2 ^{a}	74.1 ± 0.1 ^{a}	77.3 ± 0.4 ^{a}	2.53 ± 0.01 ^{b}	9.39 ± 0.16 ^{b}	0.41 ± 0.00 ^{b}
NX42	CK	67.2 ± 0.1 ^{b}	72.4 ± 0.0 ^{b}	76.9 ± 0.1 ^{a}	6.06 ± 0.01 ^{a}	9.71 ± 0.05 ^{a}	1.18 ± 0.01 ^{a}
	LT	68.4 ± 0.1 ^{a}	73.6 ± 0.0 ^{a}	77.2 ± 0.1 ^{a}	3.42 ± 0.02 ^{b}	8.82 ± 0.07 ^{b}	0.67 ± 0.01 ^{b}
Analysis of variance							
Variety (V)		***	***	***	***	***	***
Temperature treatment (T)		ns	***	*	***	***	***
V*T		***	***	***	***	***	***

The values reported are mean ± standard error of three replicates. The different letters in the same column of each variety are statistically significant at $p < 0.05$. LSD (least significant difference) followed by *, ***, significance at 0.05 and 0.001, respectively, ns, non-significant. CK, control temperature treatment; LT, low-temperature treatment; To, onset temperature; Tp, peak temperature; Tc, conclusion temperature; ΔH, gelatinization enthalpy; R, gelatinization temperature range; PHI, peak height index.

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TABLE 2 The effects of low temperature on the pasting properties of high-quality indica rice.

Variety	Temperature treatment	Peak viscosity (cp)	Trough viscosity (cp)	Final viscosity (cp)	Breakdown (cp)	Setback (cp)
TYXZ	CK	3021.3± 6.3 ^{a}	2039.3 ± 10.3 ^{a}	2946.7 ± 8.2 ^{a}	982.0 ± 4.0 ^{a}	-74.7 ± 3.8 ^{b}
	LT	2782.7± 9.4 ^{b}	1906.7 ± 3.5 ^{b}	2811.0 ± 8.5 ^{b}	876.0 ± 7.0 ^{b}	28.3 ± 0.9 ^{a}
YLY911	CK	3627.0 ± 5.5 ^{a}	2225.7 ± 6.2 ^{a}	2976.0 ± 7.1 ^{a}	1401.3 ± 10.8 ^{a}	-651.0 ± 10.0 ^{b}
	LT	3004.3 ± 19.2 ^{b}	1962.0 ± 30.5 ^{b}	2705.3 ± 2.3 ^{b}	1042.3 ± 22.4 ^{b}	-299.0 ± 17.2 ^{a}
YZX	CK	3041.3 ± 3.8 ^{a}	1851.7 ± 39.9 ^{a}	2760.0 ± 15.0 ^{a}	1189.7 ± 36.1 ^{a}	-281.3 ± 11.4 ^{b}
	LT	2844.7 ± 7.8 ^{b}	1775.3 ± 7.9 ^{a}	2657.7 ± 14.8 ^{b}	1069.3 ± 14.9 ^{b}	-187.0 ± 17.3 ^{a}
TY390	CK	3426.0 ± 2.5 ^{a}	1908.7 ± 3.3 ^{a}	2742.3 ± 2.3 ^{a}	1517.3 ± 2.6 ^{a}	-683.7 ± 4.7 ^{b}
	LT	3017.0 ± 13.1 ^{b}	1780.7 ± 8.7 ^{b}	2649.0 ± 9.6 ^{b}	1236.3 ± 9.3 ^{b}	-368.0 ± 3.5 ^{a}
HHZ	CK	2965.7 ± 3.5 ^{a}	1918.7 ± 3.9 ^{a}	2850.7 ± 14.5 ^{a}	1047.0 ± 1.5 ^{a}	-115.0 ± 11.5 ^{b}
	LT	2698.3 ± 6.2 ^{b}	1891.7 ± 3.2 ^{b}	2885.0 ± 11.0 ^{a}	806.7 ± 7.0 ^{b}	186.7 ± 12.9 ^{a}
JLYHZ	CK	3312.0 ± 6.7 ^{a}	2087.7 ± 19.9 ^{a}	2972.7 ± 5.8 ^{a}	1224.3 ± 18.9 ^{a}	-339.3 ± 1.8 ^{b}
	LT	2829.0 ± 7.5 ^{b}	1837.0 ± 2.0 ^{b}	2696.0 ± 4.2 ^{b}	992.0 ± 9.3 ^{b}	-133.0 ± 3.6 ^{a}
LJY534	CK	2738.7 ± 17.9 ^{a}	1927.7 ± 12.5 ^{a}	2900.3 ± 8.7 ^{a}	811.0 ± 5.5 ^{a}	161.7 ± 9.3 ^{b}
	LT	2590.7 ± 4.7 ^{b}	1833.3 ± 5.0 ^{b}	2835.3 ± 9.1 ^{b}	757.3 ± 0.3 ^{b}	244.7 ± 5.2 ^{a}
NX42	CK	3475.7 ± 11.6 ^{a}	2420.3 ± 11.5 ^{a}	3345.3 ± 24.7 ^{a}	1055.3 ± 1.5 ^{a}	-130.3 ± 16.1 ^{b}
	LT	2984.3 ± 11.7 ^{b}	2109.3 ± 16.8 ^{b}	3078.3 ± 9.2 ^{b}	875.0 ± 5.3 ^{b}	94.0 ± 2.6 ^{a}
Analysis of variance						
Variety (V)		***	***	***	***	***
Temperature treatment (T)		***	***	***	***	***
V*T		***	***	***	***	***

The values reported are mean ± standard error of three replicates. The different letters in the same column of each variety are statistically significant at $p < 0.05$. LSD (least significant difference) followed by ***, significance at 0.001, CK, control temperature treatment. LT, low-temperature treatment.

3.2 Thermal properties

The results of the study showed that variety, temperature, and their interactions had a significant effect on To, Tp, Tc, ΔH, R, and PHI, except for varietal effects on To (Table 1). YLY911, TY390, and JLYHZ exhibited higher gelatinization temperatures (To, Tp, and Tc) compared to other varieties. The ΔH of YZX, NX42, and HHZ was also higher than that of other varieties, while HHZ had the highest R among all varieties. When exposed to lower temperatures compared to the control, the To significantly increased in TYXZ, YLY911, and NX42, by 1.2% to 4.0%, whereas it notably decreased in YZX by 7.5% and TY390 by 0.7%. Under low temperature conditions, Tp increased significantly in TY390, TYXZ, JLYHZ, and NX42 (0.7%-1.8%), while Tc increased greatly only in TYXZ (0.8%) and decreased significantly in YLY911 (0.7%) and HHZ (1.6%). Low temperature caused a significant decrease in ΔH, R, and PHI for all varieties, except for the ΔH of TY390 and R of YZX, TY390, and JLYHZ. The largest decrease in ΔH was observed in HHZ (46.2%), followed by NX42 with 43.6%. LJY534 had the highest decrease in R with 11.5%, and the greatest decrease in PHI was observed in YZX by 49.6%.

3.3 Pasting properties

As it was shown in Table 2, variety, temperature, and their interactions had a significant effect on peak viscosity, trough viscosity, final viscosity, breakdown and setback. Compared to the control, exposure to low temperature resulted in a significant decrease in the peak viscosity, trough viscosity, and final viscosity of all varieties except for the trough viscosity in YZX and the final viscosity in HHZ. YLY911 and NX42 exhibited the largest reduction in peak viscosity and trough viscosity, with a decrease of 17.23% and 12.9%, respectively, whereas LJY534 and HHZ showed the least decrease, with reductions of 5.4% and 1.4%, respectively. JLYHZ showed the greatest decrease in final viscosity, by 9.3%, whereas a slight increase of 1.2% was observed

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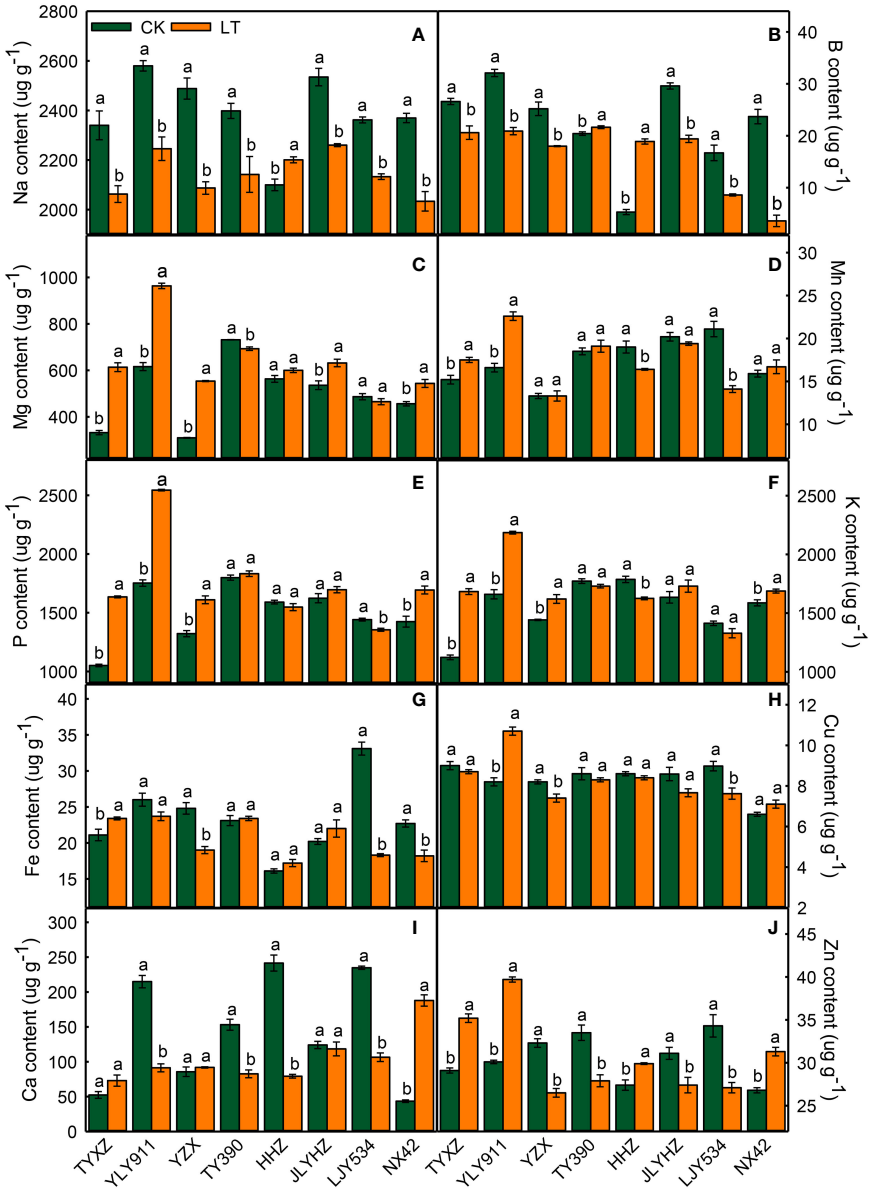

FIGURE 2
The impacts of low temperature on Na content **(A)**, B content **(B)**, Mg content **(C)**, Mn content **(D)**, P content **(E)**, K content **(F)**, Fe content **(G)**, Cu content **(H)**, Ca content **(I)** and Zn content **(J)** in high-quality indica rice. The values reported are mean ± standard error of three replicates. The different letters in each variety are statistically significant at $p < 0.05$. CK, control temperature treatment. LT, low-temperature treatment.

in HHZ under low-temperature conditions. The decrease in breakdown induced by low temperature was significant in all varieties, with the greatest decrease observed in YLY911 by 25.6% and the least in LJY534 by 6.6%. However, the setback of all varieties increased significantly under low temperatures, with the greatest increase observed in HHZ by 262.4% and the least in YZX by 33.5%.

3.4 Macro and micronutrients

Mineral nutrient accumulation differed significantly across varieties, temperature treatments and their interactions. Exposure to lower temperature resulted in significant reductions of Na and B in all varieties, except for Na in HHZ, B in TY390 and HHZ (Figures 2A, B). Meanwhile, Mg levels were significantly increased in most of varieties except for TY390, HHZ and LJY534 (Figure 2C). When plants were exposed to low temperatures, Mn levels increased significantly in TYXZ and YLY911 but decreased greatly in HHZ and LJY534 (Figure 2D). P and K levels significantly increased in four varieties (TYXZ, YLY911, YZX and NX42), with noticeably decreases in LJY534 and HHZ, respectively (Figures 2E, F). Fe increased significantly only in TYXZ and decreased in YZX, LJY534 and NX42 (Figure 2G). The Cu levels changed slightly due to lower temperatures, increasing significantly only in YLY911 and decreasing greatly in YZX and LJY534 (Figure 2H). Among all varieties, significant decrease of Ca under low temperature were recorded in YLY911, TY390, HHZ and

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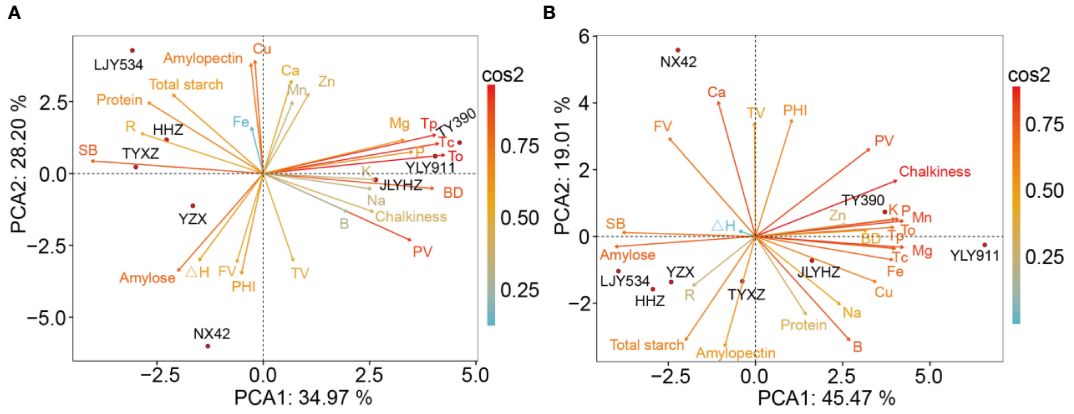

FIGURE 3
Principal component analysis of grain quality properties in high-quality indica rice under control **(A)** and low temperature **(B)** conditions. PV, peak viscosity; TV, trough viscosity; FV, final viscosity; BD, breakdown; SB, setback; To, onset temperature; Tp, peak temperature; Tc, tennination temperature; ΔH, gelatinization enthalpy; R, gelatinization temperature range; PHI, peak height index.

LJY534, but NX42 showed a noticeable increase in Ca (Figure 2I). Zn was affected by low temperatures in all varieties, with significant increases in TYXZ, YLY911, HHZ, and NX42, but decreased in remaining varieties (Figure 2J). YLY911 exhibited significant changes in nine elements, whereas TYXZ, YZX, NX42, and LJY534 had significant changes in eight elements.

3.5 Relationships among rice quality traits and varieties

Principal component analysis (PCA) was conducted on all quality traits under both control and low-temperature conditions (Figure 3). The analysis revealed the tradeoffs and synergies among the quality traits of different varieties, which were classified into three distinct clusters for each temperature condition. The first cluster consisted of LJY534, HHZ, TYXZ, and YZX, which exhibited high protein and starch content as well as a high setback value. The second cluster comprised YLY911, TY390, and JLYHZ, which displayed high grain chalkiness, gelatinization temperature (To, Tp, Tc), breakdown, peak viscosity, and mineral nutrient content. The third cluster exclusively included NX42, which had high trough viscosity, final viscosity, and PHI. Furthermore, it was observed that the mineral nutrient content decreased in the first cluster and increased in the second cluster under low-temperature conditions.

The results of Pearson's correlation analysis indicated significant relationships between various quality traits observed under two distinct temperature conditions (Figure 4). Notably, positive correlations were observed between grain chalkiness and To, peak viscosity, and breakdown, while negative correlations were observed with setback at the controlled temperature. Additionally, chalky was found to be negatively associated with starch and amylose content and positively associated with Tp, Tc and PHI under lower temperature conditions. The relationship between total starch or amylose content and To, Tp, Tc, peak viscosity, and breakdown was negative at the controlled temperature, with negative correlations becoming stronger at lower temperature. In contrast to total starch and amylose, protein content was found to be significantly correlated with thermal and pasting properties, including To, Tp, Tc, peak viscosity, trough viscosity, and breakdown, only at the controlled temperature, while the correlation was non-significant at lower temperature. Moreover, the present study identified noteworthy associations between quality traits and mineral nutrient contents in both controlled and low temperature conditions. Specifically, in the controlled condition, grain chalk exhibited a positive correlation with B and Na, while starch content showed a positive correlation with Ca and Fe. Conversely, amylose content was negatively related to Ca, Cu, and Zn. Notably, in contrast to control temperature, chalky grain, and amylose content demonstrated a greater number of positive and negative correlations with a range of mineral nutrient levels, including Mg, P, K, Mn, Fe, Cu, and Zn under low-temperature conditions. Minerals such as Na, Mg, P, and K were significantly correlated with To, Tp, Tc under both controlled and low temperature conditions, with the additional minerals B, Mn, Fe, and Cu showing positive associations with gelatinization temperature under low temperature conditions. Moreover, Mg, P, and K demonstrated positive correlations with peak viscosity and breakdown and negative correlations with setback under both controlled and low temperature conditions. In contrast, minerals Ca, Mn, and Fe were only significantly associated with pasting properties under low temperature conditions. Furthermore, significant positive correlations were observed among Mg, P, K, and Mn content under both temperature conditions. However, under low temperature conditions, Fe, Cu, and Zn exhibited a significant positive correlation with Mg, P, K, and Mn, which was not observed under controlled temperature conditions.

4 Discussion

4.1 Formation of grain chalkiness and changes in starch and protein in developing rice caryopses grown under low-temperature stress

Grain chalkiness is a notable visual trait identified as opaque white discoloration of the translucent endosperm, which is

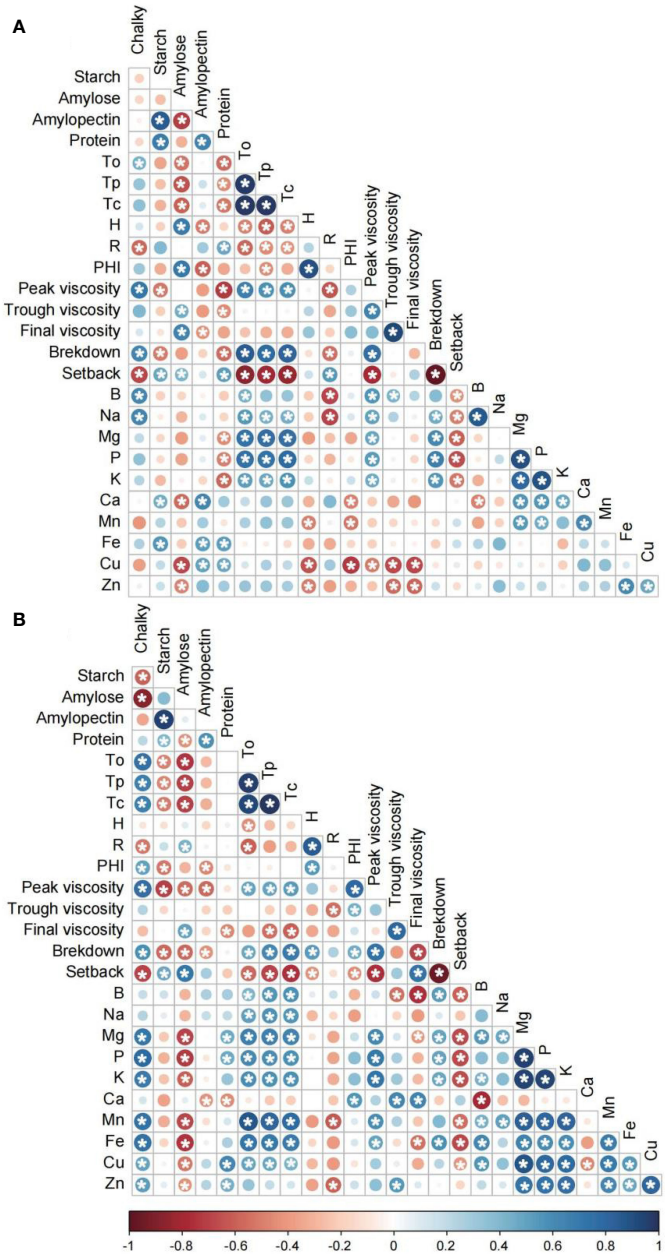

FIGURE 4
Correlation plot analysis of grain quality properties in high-quality indica rice under control **(A)** and low temperature **(B)** conditions. To, onset temperature; Tp, peak temperature; Tc, termination temperature;ΔH, gelatinization enthalpy; R, gelatinization temperature range; PHI, peak height index. P<0.05 followed by*.

significantly affected by extreme environmental temperatures (Wada et al., 2019). Our results were in accordance with previous studies indicating lower temperature occurring during grain development triggers an increase in grain chalkiness (Zhang et al., 2019). Low-temperature stress during grain ripening facilitates the formation of chalky grains through the loose packing of arranged starch granules and the presence of large air spaces between them (Gong et al., 2013). However, few studies have shown either a decrease in chalkiness rate or no significant changes caused by low temperatures (Siddik et al., 2019; Lu et al., 2022). This discrepancy could be attributed to variations in the treatment temperature levels and growth stages at which the temperature treatment is applied. In the present study, the low temperature was applied during the second week of post-heading, which is considered a critical period for rice quality in response to extreme temperatures (Siddik et al., 2019).

The endosperm of rice is primarily composed of starch, followed by protein, and is susceptible to environmental changes (Wada et al., 2019). Our study supports previous research suggesting that low temperature during grain filling period can lead to a decrease in total starch accumulation (Zhu et al., 2017; Chen et al., 2022). This reduction is caused by a reduced activity of

enzymes involved in starch synthesis, such as soluble starch synthase and starch branching enzyme (Chen et al., 2022), which results in lower levels of amylopectin and total starch contents. However, the effects on amylose content are inconsistent, with some studies reporting a decrease (Ai et al., 2023) and others an increase (Hu et al., 2020) in response to low temperature. In line with a previous study conducted on japonica rice (Zhu et al., 2017), two varieties demonstrated a significant increase in amylose under low temperatures while the other six varieties did not exhibit such an increase in this study. Lu et al. (2022) found that low temperature reduced amylose content in varieties with high amylose content but increased it in those with low amylose content, which discovered the impact of low temperature on amylose content varied depending on the initial amylose content of the rice varieties.

In this study, the protein content's response to low temperatures exhibited variations, with both increases and decreases observed. These fluctuations can be attributed to varietal disparities, which have also been corroborated in prior research. Notably, some studies report a decline (Chun et al., 2015), while others indicate an increase (Zhang et al., 2019), and still, another group observes no significant change (Hu et al., 2020). Typically, rice with lower protein content tends to exhibit better cooking quality. In this study, TYXZ exhibited a notable decrease in protein content while showing the least reduction in total starch under low-temperature treatment. This observation suggests that TYXZ, among the eight varieties studied, might possess superior cold tolerance. Varietal differences in amino acid content may underlie these discrepancies, as amino acids can serve as signaling molecules that regulate resistance to abiotic stress (Ashraf and Foolad, 2007). Additionally, changes in protein content under abiotic stress could be associated with translocation from vegetative organs and amino acid synthesis in the rice grain (Kang et al., 2022). The variation in protein content under low temperature among rice varieties suggests genotypic differences, offering an opportunity for breeding more climate-resilient crops that can help address the new challenges to global health (Myers et al., 2014).

4.2 Thermal and pasting properties in response to low temperature during grain filling

Starch gelatinization is an endothermic process whereby starch crystallinity is lost in starch granules under specific heat and moisture conditions. Gelatinization temperature refers to the temperature peak at which rice absorbs water and starch granules swell irreversibly. The higher the gelatinization temperature of the grain, the firmer the core of cooked rice. The gelatinization temperature range (R) and gelatinization enthalpy $\triangle$H are useful indicators of the energy required and cooking time necessary for starch gelatinization, and a higher $\triangle$H and R require more energy to dissociate the helix structure of starch (Jiang et al., 2022). Therefore, the cooking quality of rice is primarily determined by its starch gelatinization properties. In our study, significant decreases in both gelatinization enthalpy $\triangle$H, gelatinization temperature range R and PHI of starch under low temperatures were observed, which are consistent with previous research findings on japonica rice (Chun et al., 2015; Zhu et al., 2017). However, Ai et al. (2023) and Hu et al. (2020) found that the effect of low temperature on gelatinization enthalpy was not significant. These conflicting results may be attributed to the genetic diversity among rice, which was associated with factors such as amylose content, granular architecture, molecular weight distribution, and amylopectin fine structure (Kong et al., 2015). Previous studies also have indicated that the texture of cooked rice is associated with the fine structure of amylopectin, and that longer amylopectin chains forming double helices require higher temperatures to dissociate completely (Chun et al., 2015).

Rice with superior eating quality is characterized by higher breakdown and lower setback values, resulting in a soft texture after cooking and enhanced palatability. In this study, rice exposed to lower temperatures during grain filling exhibited a reduction in peak, trough, and final viscosities, a decline in breakdown, and an increase in setback, in comparison to rice exposed to the control temperature. These findings suggest that exposure to low-temperature stress during the grain filling period can negatively impact the eating quality of high-quality indica rice. Notably, these results align with previous research focused on japonica rice (Zhu et al., 2017; Hu et al., 2020). Moreover, the lower breakdown value and higher setback value observed under low-temperature conditions were associated with a decrease in crystallinity, a lower ratio of amylose to amylopectin, a decreased gelatinization enthalpy, and an increased size of starch granules (Hu et al., 2020; Ai et al., 2023).

4.3 Low temperatures during grain filling affect grain nutrient contents

Although rice is not known to be mineral-rich, it can still serve as an essential source of caloric energy and macro-micro nutrients for those who consume it as a staple food (Huang et al., 2020). Except for genotypic influences, environmental factors have been documented to affect minerals in rice grain (Du et al., 2013; Huang et al., 2016; Tiozon et al., 2023). Previous studies have shown that atmospheric CO_2 levels and temperature (Myers et al., 2014; Chaturvedi et al., 2017), application of nitrogen (Gu et al., 2015; Wang et al., 2018) and water management (Xu et al., 2019) could affect the mineral nutrients of rice grains. This research did the first effort to explore the effects of low temperatures on macro and micro nutrients. Low temperatures significantly affected the mineral elements of brown rice in all varieties, although the extent of the impact varied among the different elements and varieties. The results are in line with previous studies that reported inconsistent changes in mineral content in rice grains to environments or managements (Chaturvedi et al., 2017). Our study showed that low temperatures during the grain filling stage decreased the content of Na and B, while increasing the levels of K, Mg and P among most of the varieties. The impact of low temperatures on Ca, Mn, Fe, Cu, and Zn content varied among the different rice varieties. Mineral elements in rice grains have been supplied by

root uptake and translocation from vegetative plant tissues to developing rice grains during grain filling stage, and stomatal conductance and transpiration changing transpiration-driven mass flow of nutrients from root to apex organs (Houshmandfar et al., 2015). The present study provides a preliminary insight into the mineral accumulation in rice grains under low temperatures, while further investigation is needed to comprehensively understand the mineral uptake and distribution.

4.4 Relationships among rice quality traits were further enhanced under low temperatures

In this study, a negative correlation was observed between chalkiness and starch and amylose content when exposed to low temperatures. Additionally, Deng et al. (2021) reported a significant negative correlation between chalkiness and starch and amylose content under shading stress. Chalkiness is determined by the structure and arrangement of starch granules, where lower temperatures induce loosely packed, round starch granules in the chalk position due to defects in starch biosynthesis (Deng et al., 2018). The relationship between total starch or amylose content and gelatinization temperatures, peak viscosity, and breakdown was found to be negative at the controlled temperature, with the negative correlations becoming stronger at lower temperatures. Previous studies have also reported a negative correlation between amylose content and gelatinization temperature and breakdown, while a positive correlation with setback was observed (Zhong et al., 2022). However, the correlation between protein content and thermal and pasting properties was only significant at the controlled temperature, with no significant correlation at lower temperatures. Chun et al. (2015) reported a significant correlation between protein content, pasting viscosities, and setback value, with increasing treatment temperature. Furthermore, the palatability of cooked rice is influenced by the importance of starch components and protein content, indicating that amylopectin, amylose, and protein content are key chemical properties related to palatability (Chun et al., 2015). These findings suggest that protein content is associated with thermal and pasting properties, and its effect is amplified at higher temperatures. Moreover, the present study identified significant associations between quality traits and mineral nutrient contents under both controlled and low-temperature conditions. Xi et al. (2016) suggested that the mineral content of chalky grains was lower compared to translucent grains. However, our results align with Jiang et al. (2007) and Tiozon et al. (2023), who reported a significantly negative correlation between amylose content and K, Mg, and Mn levels. Additionally, significant positive correlations were observed among Mg, P, K, and Mn content under both temperature conditions, consistent with previous studies by Jiang et al. (2007) and Huang et al. (2016). Furthermore, the correlations between quality traits and mineral nutrients, as well as the relationships among mineral nutrient content, were more pronounced under low-temperature conditions than under controlled conditions. This finding is in line with Chaturvedi et al. (2017), who proposed that the associations between grain minerals and quality traits, such as protein and chalkiness, are further intensified under elevated CO_2 levels combined with high temperature.

5 Conclusions

The results showed that low temperature decreased starch, amylopectin content, pasting viscosity, and breakdown, and increased chalkiness and setback. Gelatinization temperature and pasting properties showed stronger correlations with chalk, starch, and amylose content than with protein content under low temperatures. Notably, low temperature exerted a notable influence on the mineral nutrient contents, although the extent of this impact varied among different elements and rice varieties. Additionally, the mineral nutrient exhibited positive relationships with chalkiness, gelatinization temperature, peak viscosity, and breakdown, while negative associations were found with amylose content and setback. Furthermore, correlations among mineral nutrients and other quality traits were further amplified in the presence of low temperatures. The findings offer a basis for identifying candidate quality traits and their associations with mineral accumulation and cold tolerance in high-quality rice. This knowledge can be applied to develop stress-resistant rice varieties with improved nutritional quality.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

JY: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. XZ: Data curation, Formal analysis, Writing – original draft. DW: Investigation, Validation, Writing – original draft. JW: Investigation, Validation, Writing – original draft. HX: Investigation, Validation, Writing – original draft. YX: Investigation, Validation, Writing – original draft. HJX: Project administration, Resources, Writing – review & editing. WS: Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

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Research progress on the physiological response and molecular mechanism of cold response in plants

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Low temperature is a critical environmental stress factor that restricts crop growth and geographical distribution, significantly impacting crop quality and yield. When plants are exposed to low temperatures, a series of changes occur in their external morphology and internal physiological and biochemical metabolism. This article comprehensively reviews the alterations and regulatory mechanisms of physiological and biochemical indices, such as membrane system stability, redox system, fatty acid content, photosynthesis, and osmoregulatory substances, in response to low-temperature stress in plants. Furthermore, we summarize recent research on signal transduction and regulatory pathways, phytohormones, epigenetic modifications, and other molecular mechanisms mediating the response to low temperatures in higher plants. In addition, we outline cultivation practices to improve plant cold resistance and highlight the cold-related genes used in molecular breeding. Last, we discuss future research directions, potential application prospects of plant cold resistance breeding, and recent significant breakthroughs in the research and application of cold resistance mechanisms.

KEYWORDS

low-temperature stress, physiological and biochemical metabolism, signal transduction, hormones, cold resistance mechanism

1 Introduction

The plant production process faces various adverse conditions, including salt-alkali, high temperature, low temperature, drought, pests, and other stresses. One of the common challenges is low temperature stress, which can be classified as cold damage (>0°C) and freezing damage (<0°C) (Ding et al., 2019). Both cold damage and freezing damage have detrimental effects on crop yield and quality (Aslam et al., 2022). They inhibit plant respiration and metabolism, reduce root water absorption rates, and lead to the yellowing of leaves and browning of fruits. Prolonged exposure to temperatures below 0°C damages the

cell membrane, resulting in cell death. Even when the temperature is above 0°C and no ice is present, water balance and physiological activities can still be disrupted, causing injury and death of plants.

Plants in the growth stage are particularly sensitive to temperature, and low temperatures significantly reduce flowering and fruit yield in tomatoes (Yang et al., 2021; Iovane and Aronne, 2022). Studies have shown that *Capsicum annuum* is susceptible to cold damage during postharvest storage and transportation, leading to symptoms such as concave skin surface, concave spots, brown calyx, water, and nutrient loss (Ding and Wang, 2018; Kong et al., 2018). In the case of *Solanum tuberosum*, different low-temperature treatments have varying effects. Leaves of *S. tuberosum* above 0°C can maintain normal growth, but temperatures below −2°C cause serious damage, such as wilting, leaf drop, waterlogging, and even death (Yan et al., 2021). Low-temperature stress inhibits seed germination and increases seedling mortality in *Brassica napus* (Zhu et al., 2021). *Cucumis sativus*, a cold-sensitive plant, exhibits reduced germination rates, shriveled and yellowish leaves, decreased seed set rates, and increased susceptibility to rot during storage and transport after cold stress (Zhang et al., 2021). *Solanum melongena* leaves undergo elongation, become soft and atrophied, and curl completely under low-temperature stress, with light green leaf spots appearing. *Zea mays* growth is adversely affected by cold stress, resulting in inhibited germination, stunted grain development, and a significant decrease in yield (Peleg and Blumwald, 2011).

However, most plants have evolved a strategy known as cold acclimation to adapt to low-temperature environments. This strategy enhances their cold tolerance by triggering physiological changes and cold response gene expression through a cascade of molecular events (Browse and Xin, 2001). Currently, the molecular mechanism underlying plant resistance to low temperatures remains insufficiently understood. Therefore, there is an urgent need to analyze the regulatory mechanism of plants under low-temperature stress and develop technology to improve the plants' resistance. This article comprehensively reviews the molecular mechanisms involved in plant responses to low-temperature stress, and the advances made in understanding the physiological, biochemical, and molecular signaling mechanisms underlying plant cold resistance. The roles of transcription factors (TFs), including C repeat binding factor (CBF), CBF expression inducer 1 (ICE1), APETALA2/ethylene-responsive factor (AP2/ERF), and dehydration-responsive element binding (DREB1), are extensively discussed in response to cold stress. It provides a theoretical basis for high-quality, high-yield, and stress-resistant plant cultivation and practical guidance for plant cold tolerance research.

Signaling pathway of plants' response to low-temperature stress

2.1 Low-temperature perception and transduction of calcium signals

Calcium (Ca^{2+}) serves as a universal second messenger in plant cells and plays a crucial role in plant growth, development, and responses to various biotic and abiotic stresses, such as cold, heat, and salt stress (Jiang et al., 2019). When exposed to low temperatures, changes in membrane fluidity and cytoskeletal rearrangement occur, leading to an influx of Ca^{2+} and an increase in intracellular Ca^{2+} levels (Dodd et al., 2010). The increased intracellular flow of Ca^{2+} is sensed by downstream calcium signal receptors and transmits Ca^{2+} signals further downstream, thus participating in signal transduction to induce the expression of cold response genes (Sanyal et al., 2016). Several calcium-responsive proteins, including calmodulin (CaM), CAM-like proteins (CML), calcium-dependent protein kinases (CDPK), and calcineurin B-like proteins (CBL), have been identified and play significant roles in responding to low-temperature stress (Reddy et al., 2011; Ma et al., 2015; Zhu, 2016). Studies conducted in *A. thaliana* have shown that the level of gene transcription induced by low temperature is positively correlated with the intensity of Ca^{2+} signals (Kleist and Luan, 2016). An increased concentration of intracellular Ca^{2+} promotes the production of inositol triphosphate (IP3), which amplifies the Ca^{2+} signal by regulating Ca^{2+} channels and further enhances the expression of cold response genes such as *CBF* and *COR* genes (Sulaiman et al., 2012). The specific components involved in the regulation of calcium signaling have been elucidated. For instance, the Ca^{2+} transporter *AtANN1* contributes to cold-induced calcium signaling by positively regulating cold tolerance through its effects on Ca^{2+} influx and the downstream CBF-COR-dependent cold signal transduction pathway in *A. thaliana* (Liu et al., 2021c). Similarly, the low-temperature receptor protein COLD1 interacts with G proteins to activate Ca^{2+} channels and enhance cold tolerance in *O. sativa* (Nawaz et al., 2014). Furthermore, calcineurin B-like protein-interacting protein kinase (CIPK) is another crucial component of Ca^{2+} signaling in the stress response. CIPK specifically interacts with CBLs, which control the localization and activation of CIPK. The CBL-CIPK signaling pathway plays a critical role in Ca^{2+} signaling in plants and is involved in the response to cold stress (Ormancey et al., 2017). Furthermore, a study (Yang et al., 2010) demonstrated that CRLK1 is a positive regulator of the plant response to low-temperature stress through gene knockout and complementation experiments. CRLK1 interacts with MEKK1 to activate the MAPK cascade reaction. The mitogen-activated protein kinase (MAPK) cascade has been extensively studied for its role in the response to low-temperature stress. This cascade typically consists of three protein kinases: MAP kinases (MAPKKK, MAP3K, or MEKK), MAP kinases (MAP2K, MKK, or MEK), and MAP kinases (MAPK). MKK2 and MAPKKK kinase MEKK1 are simultaneously activated by cold stress, and MKK2 induces the expression of *COR* genes, thereby improving plant cold tolerance (Teige et al., 2004). The MAP2K/MKK2 signaling pathway is activated by low temperatures and regulates the expression of downstream *COR* genes (Teige et al., 2004). While significant progress has been made in understanding the Ca^{2+} regulation mechanism in the plant response to cold stress, further research is needed to explore the interaction between Ca^{2+}-responsive transcription factors and the Ca^{2+} signaling pathway (Figure 1).

2.2 Signaling pathway and regulatory mechanism in response to low-temperature stress

2.2.1 CBF-dependent low-temperature response signaling pathway

The CBF-dependent low-temperature response signaling pathway is an essential mechanism for plants to cope with cold stress. CBF, which stands for C-repeat binding factor, is a group of plant-specific transcription factors that play a crucial role in regulating the expression of COR (cold-regulated) genes (Stockinger et al., 1997). The signaling pathway of *CBF* in response to low-temperature stress in higher plants has been extensively investigated. In the model plant *A. thaliana*, *CBF* genes belong to the dehydration-responsive element-binding protein (DREB) subfamily. They are regulated by the upstream transcription factor ICE (inducer of *CBF* expression) (Li et al., 2020). Consequently, *CBFs* are also known as *DREB1* genes, and their activation induces the downstream expression of *COR* genes to improve plant cold tolerance. This ICE-CBF-COR cascade is crucial in maintaining normal growth and development during cold stress. Several transcription factors, including the positive regulator CAMTA (calmodulin binding activator of transcription) and negative regulators such as MYB15 and HOS1, directly regulate *CBF* gene expression (Ding et al., 2019). When plants are exposed to cold stress, the signal transduction process is triggered, leading to the activation of ICE. ICE, in turn, promotes the transcription and expression of *CBF1/2/3* genes, which enhances the expression of *COR* genes (Hwarari et al., 2022). The ICE-CBF protein is regulated by ubiquitin, which improves plant cold tolerance (Sharma et al., 2021). Research has shown that the *ICE1* transcription factor is crucial for the expression of DREB1/CBF (Thomashow and Torii, 2020). ICE1, a structural protein of the basic helix–loop–helix (bHLH) domain of the MYC transcription factor, specifically recognizes cis-acting elements of MYC (CANNTG) in the promoter region of *CBF3/DREB1A* and binds to them, inducing the expression of *CBF/DREB1* and activating the cold tolerance regulatory pathway (Lee et al., 2005a; Wang et al., 2008). A study of *A. thaliana* discovered that the *ice1* mutation hampers *CBF1* expression, leading to decreased downstream expression of the *CBF* gene, while overexpression of ICE1 effectively enhances *CBF* expression and improves cold tolerance in *A. thaliana* (Chinnusamy et al., 2003). Furthermore, PUB25 and PUB26 interact with ICE1 to ubiquitinate ICE1 and stabilize its protein levels. The stabilized ICE1 protein then interacts with MYB15 to inhibit its DNA-binding activity, ultimately activating *CBF* gene expression (Wang et al., 2023c). Furthermore, MYB15, ICE1, and HOS1 have been identified as direct regulators of *CBF/DREB1* expression. ICE1 can interact with MYB15 and directly bind to the *MYB15* promoter region, leading to the downregulation of *MYB15* expression under cold stress (Agarwal et al., 2006b). The interaction between B1L (BYPASS1-LIKE) and TTL (TRANSTHYRETIN-LIKE) participates in the regulation of plant growth, development, and cold tolerance (Chen et al., 2020). The 14-3-3 proteins are a highly conserved family of acidic proteins widely expressed in eukaryotes and involved in various biological processes. B1L improves the stability of *CBF* by interacting with 14-3-3λ, thus inducing the expression of *COR* genes and improving cold tolerance in *A. thaliana* (Chen et al., 2019). ABI4 is a transcription factor of AP2/ERF (APETALA2/ethylene responsive

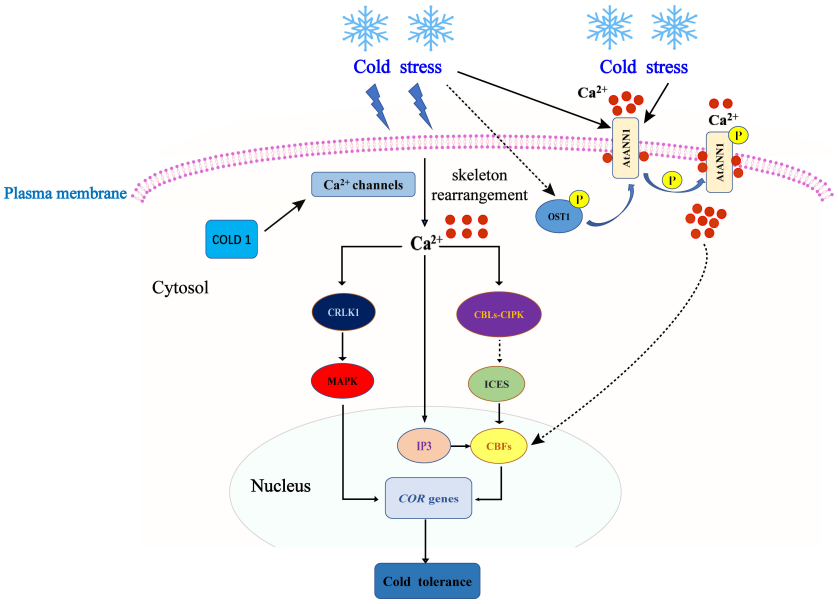

FIGURE 1
Ca^{2+} signal perception and transduction models. Cold stress induces extracellular Ca^{2+} influx, triggering the Ca^{2+} signal. Ca^{2+} promotes the production of IP3, further enhances the expression of the *COR* gene, and enhances plant cold tolerance. In *Arabidopsis thaliana*, OST1 kinase can regulate the expression of *CBF* and *COR* genes through phosphorylation of AtANN1 to enhance cold tolerance. Low temperature can activate the MAPK signaling pathway to regulate the expression of the downstream *COR* gene and enhance cold tolerance.

factor). ABI4 can regulate downstream gene expression by recognizing promoter motifs (Chandrasekaran et al., 2020). MdABI4 interacts with MdICE1 to enhance the transcriptional regulation of the downstream target gene *MdCBF1* by *MdICE1*. MdABI4 integrates jasmonic acid and abscisic acid signals to positively regulate cold tolerance in *M. domestica* through the JAZ-ABI4-ICE1-CBF cascade (An et al., 2022). PIF3 (phytochrome-interacting factors 3) is a bHLH transcription factor and a member of the PIF protein family (Ni et al., 1998). PIF3 acts as a negative regulator of plant cold tolerance by inhibiting the expression of the *CBF* gene. Two F-box proteins, EBF1 (EIN3-BINDING F-BOX 1) and EBF2 (EIN3-BINDING F-BOX 2), directly target PIF3 for degradation through the 26S proteasome pathway. This degradation leads to the activation of *CBF* gene expression (Jiang et al., 2017). Trx-h2 (Thioredoxin h2) interacts with CBFs at low temperatures to regulate CBF activity and protein structure. This interaction activates downstream *COR* gene expression and improves plant cold tolerance (Lee et al., 2021). Dehydrin, also known as LEA D-11 or LEA II (late embryogenesis abundant) protein induces cold acclimation in freezing-tolerant plants, enabling plants to survive under adverse conditions (Kosová et al., 2007). Dehydrin accumulates in plants when they are exposed to cold. Vernalization, the prolonged exposure of plants to low temperatures, is associated with a significant reduction in dehydrin accumulation, which delays the acquired frost tolerance of the plant (Kosová et al., 2011). There is a positive correlation between the relative accumulation of dehydrin protein and winter survival (WS) in winter wheat and barley (Vítámvás et al., 2019). Cold-induced dehydrin Lti30 binds to the membrane through its conserved K segment and reduces the membrane phase transition temperature (Eriksson et al., 2016). Lti30 binds to the lipid bilayer electrostatically, limiting the mobility of lipid and bound protein molecules and protecting the cell membrane by forming aggregates (Gupta et al., 2019). As a cold-induced CBF transcription factor in the *A. thaliana* genome, CBF1/2/3 can bind to the CRT/DRE/LTRE elements, thus increasing the tolerance to plant cold (Gilmour et al., 2004). Several dehydrin genes were identified in cold regulatory proteins whose promoters contained elements of CRT/DRE/LTRE (Zolotarov and Strömvik, 2015). Under cold stress, the ICE1-CBF cold response signaling pathway gene of the *DIICE1* overexpression strain was significantly upregulated compared with the wild type (Yang et al., 2019). In conclusion, the CBF-dependent low-temperature response signaling pathway involves multiple transcription factors and protein interactions that collectively regulate the expression of COR genes and enhance plant cold tolerance (Figure 2).

2.2.2 CBF-independent signaling pathways for low-temperature response

The regulation of *COR* genes is crucial for enhancing plant tolerance to cold during cold acclimatization. However, recent studies have revealed that only 10%–20% of *COR* genes in plant cells are regulated by CBF, while the majority of them participate in cold tolerance signaling pathways independently of CBF (Ding et al., 2019). For instance, the overexpression of *HsfC1* (heat shock transcription factor C1) in *A. thaliana* induces the expression of *COR* genes and improves cold tolerance (Jiao et al., 2022). A genome-wide association study on hypocotyl growth in *A. thaliana* demonstrated that *HsfA1d* (heat shock transcription factor A1d) promotes hypocotyl elongation under cold stress by binding to the promoters of two ribosomal protein genes, *RPL9* and *RPL18*, thus enhancing plant tolerance to low temperatures (Liu et al., 2021a). Moreover, the transcriptional regulatory factor BZR1 in the brassinosteroid (BR) signaling pathway regulates the expression of *CBF1* and *CBF2* and also independently controls *COR* genes. BZR1 regulates plant cold tolerance through both CBF-dependent and CBF-independent pathways (Li et al., 2017b). Initially identified as a *CBF-independent* gene in the cold tolerance signaling pathway, ZAT10 is downstream of *MAPK*, *APX*, and *CAT* (Gong et al., 2020). ZAT10 induces the expression of *COR* genes by regulating the expression of *MYB88/124*, thus enhancing the cold tolerance of *M. domestica* (Li et al., 2023). The overexpression of *CsPIF8* in *S. lycopersicum* and *V. vinifera* callus enhances citrus cold tolerance by increasing SOD activity and reducing O_2 levels, indicating that *CsPIF8* is involved in regulating cold tolerance independently of the CBF pathway (He et al., 2020). PeSTZ1 is induced under low temperature stress and directly binds to the PeAPX2 promoter, promoting its activation and reducing ROS accumulation under cold stress. This regulation of gene expression improves the cold resistance of *Populus euphratica* by regulating the expression of *COR* genes (He et al., 2019). Research has shown that BnHY5 regulates hypocotyl length in *B. napus* by regulating the downstream gene *PIF4* in the IAA pathway. It is speculated that *BnHY5* is a key gene involved in cold resistance and hypocotyl development in *B. napus* (Jin et al., 2022). Numerous CBF-independent signaling pathways for low-temperature response exist in plants, and further exploration is required to elucidate their regulatory mechanisms (Figure 2).

3 Low-temperature effect on the physiological and biochemical metabolism of plants

3.1 Effect of low temperature on membrane stability

The cell membrane, primarily composed of phospholipids, is highly sensitive to temperature and plays a crucial role in responding to low-temperature stimuli. It acts as the main sensor for abiotic stress signals, allowing plant cells to detect changes in cell membrane fluidity and protein conformation caused by low-temperature stress. Cell membrane fluidity, regulated by lipid composition and distribution, is a key factor in plant adaptation to temperature changes (Li et al., 2016). Prolonged exposure to cold temperatures can alter the lipid composition of plant cell membranes, causing a transition from a flexible, liquid crystal phase to a more rigid, solid gel phase. This decrease in membrane fluidity can impair its functionality and disrupt intracellular metabolism. Additionally, membrane and membrane-binding

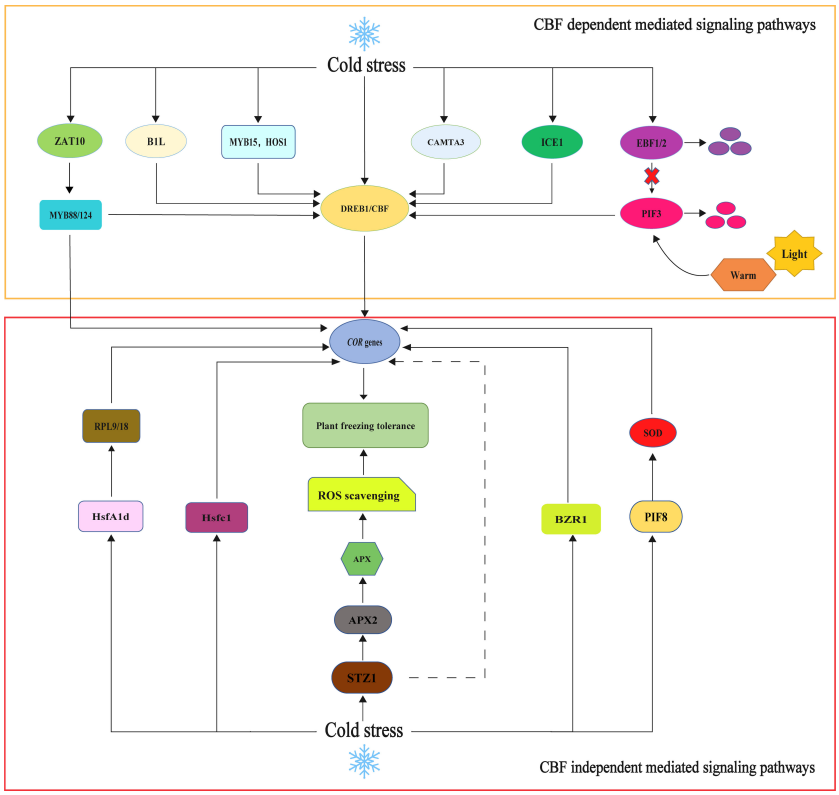

FIGURE 2
CBF-dependent and CBF-independent mediated signaling pathways models. Among the CBF-dependent pathways. The CBF-COR pathway is the primary cold stress signal regulation pathway. Cold stress can activate BIL, MYB15, HOS1, CAMTA3, ICE1, and ZAT10; regulate the expression of the *CBF* gene, thereby enhancing the expression of the *COR* gene; and regulate plant cold resistance. PIF3 can bind the promoter region of *CBFs*, inhibit the expression of *CBFs* and its downstream cold response genes, and negatively regulate the cold resistance of *Arabidopsis thaliana*. Under normal temperature and light conditions, EBF1/2 targets degrade PIF3 and induce *CBF* gene expression. Low-temperature and dark conditions promote the degradation of EBF protein, which makes PIF3 protein more stable. In the CBF-independent pathway, HsfA1d, Hsfc1, STZ1, BZR1, PIF8, and other transcription factors can directly regulate the expression of the *COR* gene independently of CBF and regulate plant cold tolerance.

enzyme activities may be affected (Sanchez et al., 2019). During freezing stress, when the temperature drops below 0°C, ice crystals can form in plant tissues, potentially leading to plant death. The obstruction of the cell membrane prevents intracellular fluid from freezing while extracellular water molecules form ice crystals. This rapid increase in osmotic pressure of the extracellular fluid can damage the cell membrane and denature its proteins, ultimately resulting in the loss of cellular integrity (Wang et al., 2023d). Low-temperature stress induced by freezing or cold temperatures also directly affects membrane integrity, molecular transport, cell signaling, and respiratory functions during seed germination (Hassan et al., 2021). Exposure to cold stress can increase membrane permeability, causing the leakage of essential electrolytes and cellular substances, such as amino acids, carbohydrates, unsaturated fatty acids, and metabolic compounds. This disturbance in ion balance inside and outside the cell can cause cell damage or death. Studies have shown that a higher unsaturated fatty acid content in the membrane of *A. hypogaea* corresponds to a lower membrane lipid phase transition temperature and stronger cold resistance (Sui et al., 2018). *Arabidopsis thaliana* exhibited a significant reduction in membrane lipid composition and severe membrane structure damage under cold stress (Kenchanmane Raju et al., 2018). Membrane stability is crucial for the survival of plant seedlings under low-temperature stress, and cold acclimation is closely associated with dynamic changes in lipid composition (Zheng et al., 2011; Degenkolbe et al., 2012). Exposing plants to low-temperature conditions can lead to bursts of ROS, which can induce membrane lipid peroxidation and increase levels of malondialdehyde (MDA). MDA can react with proteins and nucleic acids, disabling their function (Morales and Munné-Bosch, 2019). This process significantly increases membrane permeability, causing electrolyte leakage and irreparable cell damage. Therefore, MDA levels are used as an evaluation marker for lipid peroxidation, reflecting the extent of cell membrane damage and plant stress resistance (Morales and Munné-Bosch, 2019).

3.2 Influence of low temperature on fatty acid content

Lipids are essential components of biomembranes and play crucial roles in signal transduction and stress response (Suh et al., 2022). Moreover, they serve as fundamental building blocks for cell membranes, cuticle proteins, and wax constituents, forming a structural barrier against environmental stress (Upchurch, 2008).

The activity of alpha-linolenic acid lipase contributes to the restoration of membrane fluidity (Grechkin, 1998), potentially regulating plant defense gene expression against cold stress and enhancing plant resilience (Kachroo et al., 2001). Fatty acids and their derivatives play crucial roles in various biological processes, including growth, development, and cellular metabolism in plants and related tissues (Lee et al., 2003). During the reproductive development of male flowers, fatty acids play a vital role in forming the anther cuticle and pollen wall, ensuring fertility (Wan et al., 2020). The plant cell membrane, consisting of a phospholipid bilayer in a liquid crystal phase, undergoes a temperature-dependent transformation of glycerol ester, closely related to the degree of fatty acid unsaturation. Cold-resistant plants have higher levels of unsaturated membrane lipids, resulting in a lower transition temperature compared to cold-sensitive plants(Lyons, 1973). Low temperatures significantly reduce membrane lipid fluidity and contraction, immobilizing membrane proteins and altering the controlled entry/exit of substances, which can induce plant metabolic disorders (Sevillano et al., 2009). The fluidity and stability of the cell membrane are closely associated with plant cold resistance. Generally, plants with greater membrane fluidity and stability exhibit higher cold tolerance (Wu et al., 2022). The presence of unsaturated fatty acids helps maintain the fluidity of the cell membrane in plants (Miquel et al., 1993; Upchurch, 2008). Plants primarily regulate fatty acid desaturase gene activity to influence the composition of membrane fatty acids. Lipid desaturation is a common stress response aimed at maintaining membrane fluidity under low temperature stress (Kenchanmane Raju et al., 2018). Correlation studies have shown that cold-tolerant varieties of *Medicago sativa* have higher total and unsaturated fatty acid contents than sensitive varieties under low temperature stress (Tian et al., 2022). Furthermore, *S. tuberosum* acclimated to cold storage stores linoleic acid in leaf tissue membrane glycerides (18:2) during cold stress (Vega et al., 2004). Hexadecanetrienoic acid (16:3) and linolenic acid (18:3) are important components of membrane lipids. Increasing the TA content in chloroplast membranes enhances plant tolerance to cold during early growth stages (Iba, 2002). Moreover, low-temperature cold stress treatment significantly increases galactosyl diglyceride (DGDG) content in cotyledon lipids of *Phaseolus vulgaris* varieties, enhancing cold resistance (Wang et al., 2020b). Lipidomic studies of *Sorghum bicolor* after cold stress revealed increased conservative and cooling-induced lipid unsaturation, increased phosphatidylcholine unsaturation during cooling, and higher galactoid unsaturation (Marla et al., 2017). Investigations into the effect of low temperature on fatty acid unsaturation in *Lagenaria siceraria* and *cucumber* roots measured membrane fluidity using the double bond index (DBI) and showed that excessive accumulation of linolenic acid may trigger a hyperbolic reaction. Additionally, cold stress upregulates lipoxygenase (LOX) activity in *Ficus carica* leaves and gourd roots. The level of unsaturated lipids in the plasma membranes of the root is closely associated with a plant's cold tolerance capacity (Lee et al., 2005b). Linolenic acid serves as a precursor for synthesizing JA, which is formed after oxidation. Methyl jasmonate (MeJA) reduces oxidation and damage to plants during storage at low temperatures, thus promoting economic benefits (González-Aguilar et al., 2000; Ding et al., 2002; Gong et al., 2022). MeJA affects the expression of genes related to cold tolerance, protecting plants from low-temperature stress (Laura et al., 2018).

3.3 Influence of low temperature on organic osmolytes

Plants use two main mechanisms to accumulate organic osmolytes: uptake of inorganic ions from the external environment and synthesis of organic solutes within the cell. Osmotic regulation is crucial for plant protection against stress and relies on intracellular biosynthesis and absorption of specific substances. During abiotic stress responses, active solute accumulation and increased concentration of cell-soluble apoplastic fluid help maintain osmotic pressure, prevent excessive water loss, and protect against cell protoplast death, thereby enhancing plant stress resistance. The accumulation of soluble sugars (such as glucose, sucrose, fructose, and galactose) and soluble proteins is positively associated with plant cold resistance. These compounds help maintain osmotic potential, enhance water retention capacity, reduce the freezing point of cell fluids, alleviate damage to membrane structures under low-temperature stress, and prevent plant death. Free proline plays a role in maintaining the osmotic pressure balance between protoplasts and the surrounding environment. It promotes protein hydration after low-temperature stress, protects the spatial structure of enzymes, and improves plant resistance to low temperatures, thereby reducing damage (Fedotova and Dmitrieva, 2016). Proline promotes protein structural stability and membrane integrity through hydrogen bond interactions (Trovato et al., 2019). Studies have demonstrated that the contents of soluble sugars, proteins, and proline correlate with cold tolerance in plants (Li et al., 2012). *A. hypogaea* exhibits increased accumulation of organic osmolytes under cold stress, and cultivars with strong resistance to cold can accumulate higher levels of organic osmolytes to resist stress (Kavi Kishor and Sreenivasulu, 2014). Measuring proline and soluble sugar concentrations in *O. sativa* plants overexpressing $OsLTPL159^{IL112}$ after low temperature treatment showed that this overexpression improved the cold tolerance of early *O. sativa* seedlings by promoting cellulose deposition and enhanced the accumulation of organic osmolytes (Zhao et al., 2020). On the other hand, disruption of the proline-rich protein encoded by *OsPRP1* in *O. sativa* increased the plant's sensitivity to low temperatures at the seedling stage (Nawaz et al., 2019). Proline levels are positively associated with plant cold tolerance and can indicate cold tolerance and low-temperature stress (Ghosh et al., 2022). Long-term low-temperature stress increased the osmotic pressure of maize leaves and reduced cell damage (Guo et al., 2022). The content of betaine can also serve as an index to evaluate the plant's tolerance to cold. Betaine improves resistance to low temperature in plants under osmotic stress, maintains the stability of the structure and function of biological macromolecules (Huang et al., 2000), promotes seed germination (Park et al., 2004), enhances plant antioxidant capacity

(Wang et al., 2021a), and plays a vital role in maintaining normal respiration and photosynthesis.

3.4 Influence of low temperature on photosynthesis

Photosynthesis, the fundamental process for crop and energy plant production, is highly sensitive to changes in environmental temperature (Chen et al., 2015). Research has shown that 90%–95% of a plant's dry weight is derived from photosynthesis products, making them highly vulnerable to low-temperature stress (Chassot et al., 2001; Bilska and Sowinski, 2010). Many aspects of photosynthesis are affected by low temperature, including chloroplast structure, transpiration rate (Tr), photosynthetic pigment content, net photosynthetic efficiency (A), stomatal conductance (Gs), photochemical reactions, and the transport and distribution of photosynthetic products (Zhang et al., 2022a). For example, low-temperature treatment reduces the dry matter content of *A. hypogaea* seedlings, leads to a decrease in Gs and the transpiration rate of leaves, and causes a decrease in the photosynthetic rate (Pn) due to the interplay between stomatal and nonstomatal factors (Liu et al., 2013). Chloroplasts, which are one of the earliest organelles to sense low temperatures, play a vital role in plant growth and development (Liu et al., 2018). Low temperatures result in the destruction of organelle ultrastructure and affect the concentration of metabolites in chloroplasts (Ambavaram et al., 2014). Exposure to cold stress alters chloroplast membrane states and enzyme activities, leading to a decrease in photosynthetic effectiveness and the accumulation of excessive reactive oxygen species (ROS) and aberrant thylakoid morphology. Enzymatic reactions may slow down, and the high levels of ROS can damage membrane structures (Gan et al., 2019). These factors can lead to chloroplast dysfunction and degradation, ultimately affecting the activity of the light system (Liu et al., 2018). Under low-temperature stress, C_3 plants, such as chrysanthemum, exhibit reduced maximum PSII photochemical efficiency (*Fv*/*Fm*), PSII quantum yield, light energy capture efficiency, and apparent quantum efficiency, which inhibits photosynthesis (Ozturk et al., 2013). Wheat leaves subjected to low temperatures also experience a decrease in the photosynthetic rate, stomatal conductance, and transpiration rate, which impacts wheat's photosynthetic activity and leads to decreased yield (Zhang et al., 2022a). Low temperature diminishes the transfer of light energy and the conversion efficiency of PSII on the thylakoid membrane, reducing the capacity to absorb CO_2 and the electron transport ability, ultimately affecting photosynthesis and the formation and transport of photosynthetic products (Paredes and Quiles, 2015). Additionally, following low-temperature treatment, genes related to photosynthetic pathways are downregulated in *B. napus*, inhibiting the photosynthetic system, reducing the maximum quantum efficiency of *Fv*/*Fm*, and affecting light response and the Calvin cycle (Hussain et al., 2022). In conclusion, low temperatures can impact the efficiency of light energy use in plants, leading to a decrease in enzyme activity and ultimately affecting plant photosynthesis.

3.5 Influence of low temperature on oxidative stress

Reactive oxygen species (ROS) are chemically reactive substances that contain oxygen, such as superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), ozone (O_3), and singlet oxygen ($^1O_2$) (Apel and Hirt, 2004). $O_2^{\bullet-}$ can be produced by NADPH oxidase (NOXs) or by the reduction in O_2 by complexes I and III of the mitochondrial electron transport chain (ETC) (Murphy, 2009). Nicotinamide adenine dinucleotide phosphate (NADPH) produced through the pentose phosphate pathway is an important redox cofactor of the NOXs, which produce ROS (Sies and Jones, 2020). ROS produced by NADPH oxidase plays an important role in the plant's participation in environmental response (Kawarazaki et al., 2013). Mitochondrial complexes III (CIII) and CI are necessary for ROS production (Fernández-Agüera et al., 2015). Studies have shown that Na can act as a second messenger to regulate the production of ROS by regulating the fluidity of the mitochondrial inner membrane (Hernansanz-Agustín et al., 2020). Excessive accumulation of ROS can be toxic, causing damage to carbohydrates, proteins, membranes, and genetic materials within plant cells. It inhibits normal plant growth, and development, and can even lead to plant death (Hossain et al., 2015; Jain and Gould, 2015). ROS production primarily occurs in chloroplasts and mitochondria through electron transport. Higher plants also produce ROS during metabolic processes such as fatty acid β-oxidation. Cold stress reduces the photosynthetic rate and affects photosynthetic electron transport, which affects ROS production (Bai et al., 2022). Living organisms position two antioxidant systems: enzymatic antioxidant systems and non-enzymatic antioxidant systems. Enzymatic antioxidant systems include peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR). Non-enzymatic antioxidant systems include flavonoids, phenols, carotenoids, vitamin C, vitamin E, glutathione (GSH), and ascorbic acid (ASA). Under normal temperature conditions, ROS production is kept at a low level in cell membranes, mitochondria, chloroplasts, and peroxisomes, with chloroplasts and mitochondria being the main sites of ROS production in plants (Neill et al., 2002). Studies on multiple plant species such as *Oryza sativa* (Guo et al., 2022), *Z. mays* (Zhao et al., 2022), *Manihot esculenta* Crantz (Liao et al., 2022), and *Capsicum annuum* (Wang et al., 2022) have shown that the activity of antioxidant enzymes is significantly enhanced under low temperature stress. In peanut seedlings, the activity of SOD and CAT shows a marked increase in response to cold stress, followed by an increase in the activity of POD before eventually decreasing. These antioxidant enzymes work together to maintain an appropriate level of active oxygen, which protects the plants against potential oxidative damage (Xue Yunyun et al., 2018). At low temperatures, full-length OsCYP20-2 in chloroplasts can improve the activity of superoxide dismutase OsFSD2, enhance the ability of cells to remove reactive oxygen species, and improve the cold tolerance of *O. sativa* (Ge et al., 2020a). In recent years, proteomic analysis of *B. napus* has shown that the molecular

mechanism of the plant to enhance its tolerance to cold depends on the removal of ROS (Mi et al., 2021). It was reported that the activity of leaf antioxidant enzymes in plants under cold stress initially increased and then decreased over a certain period (Hongtao et al., 2017). Abscisic acid (ABA), an important plant hormone and growth regulator, has been found to improve cold tolerance by increasing the activity of antioxidant enzymes (Khorshidi and Nojavan, 2006). In the case of *Triticum aestivum*, treatment with ABA increases the activities of TaSOD, TaAPX, TaCAT, TaGR, TaDHAR, and TaMDHAR to enhance cold resistance (Yu et al., 2020). These findings highlight the importance of the redox system in plant response to low temperatures and the delicate balance between ROS production and scavenging for plant survival and adaptation to cold stress.

4 Molecular regulatory mechanism of plants' response to low-temperature stress

4.1 Relations between plant hormones and cold resistance

Plant hormones are trace organic compounds produced by plants that play a crucial role in the regulation of their growth, development, and response to stress. These hormones include indole acetic acid (IAA), gibberellic acid (GA), cytokinin (CK), ABA, ethylene (ET), brassinosteroids (BR), JA, salicylic acid (SA), and strigolactones (SL). SA has been found to play a significant regulatory role in combating cold stress. A medium concentration of SA was applied to watermelon plants to induce cold tolerance by influencing the expression of the *CBF* and *COR* genes, improving the activity of antioxidant enzymes, and improving cold tolerance (Cheng et al., 2016). Combining SA and trisodium phosphate (TSP) improved fatty acid desaturation efficiency, improved water retention in *C. annuum*, and reduced membrane damage at low temperatures (Ge et al., 2020b). In *Z. mays*, *O. sativa*, and *C. sativus* leaves, treatment with 0.5 mM SA reduced cold-stress-induced electrolyte leakage and increased the activities of glutathione reductase and guaiacol peroxidase (G-POD) (Kang and Saltveit, 2002). ABA, which typically accumulates under cold stress, has been found to correlate with cold tolerance positively. The ABA content in cultivars with strong cold tolerance was significantly higher than in cold-sensitive cultivars (Huang et al., 2017; Agurla et al., 2018). The combination of low temperature and ABA induced the expression of *CBF/DREB1* in *Vitis vinifera* seedlings and increased cold tolerance in dormant buds when ABA was applied to the leaves (Rubio et al., 2019). ABA enhances cold tolerance by increasing the activity of antioxidant enzymes and regulating stomatal opening and closing, thereby reducing CO_2 fixation and inhibiting the accumulation of reactive oxygen species (Lim et al., 2015). ABA and GA are the main endogenous regulatory factors, and ABA and GA have antagonistic effects on seed germination (Miransari and Smith, 2014). ABA induces dormancy, and GA regulates dormancy and germination. Cold stress during seed development enhances ABA accumulation and decreases GA content (Kendall et al., 2011; He et al., 2014). Low temperature regulates seed germination by mediating ABA and GA metabolism and signaling, and the two hormones antagonistically promote seed coat and endosperm rupture (MacGregor et al., 2015). As a plant growth regulator (PGR) that releases ethylene, ethephon can delay the flowering of many plants, greatly reducing the chance of freezing damage (Pahwa and Ghai, 2015). Ethephon can delay flowering and prolong the dormant period of flower buds by increasing cooling and improving the cold tolerance of flower buds (Durner and Gianfagna, 1991). The study showed that the appropriate application of ethylene prolongs the cooling accumulation period of peach buds, improves cold resistance, and delays flowering (Liu et al., 2021b). Low-temperature treatment induced the release of ethylene and the expression of *MdERF1B* (encoding an ethylene signal activator) in *M. domestica* seedlings. Overexpression of *MdERF1B* significantly enhances the cold tolerance of *M. domestica* and *A. thaliana* seedlings (Wang et al., 2021b). Ethylene can positively regulate the cold tolerance of *Poncirus trifoliata* (Zhang et al., 2022b). A-type response regulator *ZmRR1* in the CK signal transduction pathway is involved in the regulation of *Z. mays* cold tolerance, with natural variation in *ZmRR1* leading to differences in cold tolerance among *Z. mays* inbred lines (Zeng et al., 2021). JA and its derivatives, collectively known as jasmonate (Jas), are important molecules in regulating plant responses to biotic and abiotic stresses (Ruan et al., 2019). JA positively regulates leaves senescence, *CBF* expression, and downstream cold response genes, increasing cold tolerance in *O. sativa* (Du et al., 2013; Hu et al., 2017). A B-box protein (BBX) MdBBX37 was identified as a positive regulator of *M. domestica* cold stress resistance mediated by JA, shedding light on its function in jasmonate-mediated cold resistance (An et al., 2021). MeJA treatment increases cold tolerance, activates genes related to JA synthesis, increases endogenous JA content, and significantly increases antioxidant-related substance content in *C. annuum*, alleviating cold stress damage (Seo et al., 2020). BR regulates plant tolerance to low-temperature stress. It enhances cold tolerance by accumulating dephosphorylated forms of BZR1 and BES1, induces the expression of *CBF1* and *CBF2*, and positively regulates the cold tolerance of *A. thaliana*. *BZR1* acts upstream of *CBF1* and *CBF2*, directly regulating the expression of cold-tolerance-related genes (Li et al., 2017b). The phenylpropane group, including lignin, flavonoids, anthocyanins, and phenylpropane esters, is implicated in the response to cold stress. Upregulation of phenylpropanoid genes in *A. thaliana* seeds under cold stress is associated with a higher concentration of anthocyanins in the seed coat. Low temperatures can lead to cortical differences in tetrazolium, and low seed coat permeability or proanthocyanidin concentration can hinder seed dormancy after exposure to low temperatures (MacGregor et al., 2015). The early response genes of SL were identified in *A. thaliana*, and the molecular mechanism of SL anthocyanin accumulation and its regulation of plant growth

and development was elucidated (Wang et al., 2020a). SL promotes the expression of the *CBF* gene and improves the cold tolerance of *A. thaliana* by promoting anthocyanin synthesis (Wang et al., 2023b). In summary, plant hormones such as SA, ABA, ethylene, CK, JA, BR, and SL play significant roles in the regulation of plant cold tolerance. Understanding the interactions between these hormones and their signaling pathways is crucial for enhancing plant cold resistance and improving crop productivity (Table 1).

4.2 Epigenetic mechanisms

Low-temperature stress has been shown to induce epigenetic changes in plants, such as DNA methylation, chromatin modification, histone modification, and microRNA (miRNA) regulation. These epigenetic modifications play a crucial role in plant response to cold stress. For example, miRNA-mediated gene expression has been shown to play a crucial role in the response of plants to cold stress (Megha et al., 2018). MiRNA, a small noncoding RNA, regulates physiological processes and can help alleviate the harmful effects of abiotic stress on plants. The overexpression of *miR397a* induced the expression of cold-regulated *CBF* genes and downstream *COR* genes, thus improving the cold tolerance of *A. thaliana* (Dong and Pei, 2014). Upregulation of *miR393* under abiotic stress inhibits plant growth during stress, while cold stress leads to downregulation of *miR2004* in *A. thaliana* (Sunkar and Zhu, 2004). Cold-responsive miRNAs in *Z. mays* can regulate the size of *Z. mays* leaves under cold stress by modulating developmental transcription factors and maintaining redox homeostasis in plant cells (Aydinoglu, 2020). In *A. thaliana*, the overexpression of *miR402* can promote seed germination and enhance plant cold tolerance (Tiwari et al., 2020). OsmiR156 can enhance cold tolerance by targeting OsSPL3 to regulate the expression level of related transcription factor genes (Zhou and Tang, 2019). In *O. sativa*, OsmiR319 increases transcription levels of *OsDREB1A* by targeting OsPCF6 and OsTCP21, thereby clearing reactive oxygen species to improve plant cold tolerance (Wang et al., 2014). In general, only a few miRNAs may participate in plant cold tolerance through the CBF-COR pathway, laying a theoretical foundation for our study of the CBF-COR-independent pathway.

In *A. thaliana*, the highly expressed osmotic response protein HOS15 interacts with HD2C and binds to the promoter of *COR* genes, thus epigenetically regulating cold tolerance (Lim et al., 2020). Studies on *O. sativa* have shown that exposure to low temperatures can induce changes in the chromosome structure of the *OsDREB1b* promoter and upstream region. This can lead to histone H3 acetylation in the promoter regulatory region, altering the chromatin structure and impacting the cold tolerance of *O. sativa* (Roy et al., 2014). DNA methylation plays a crucial role in silencing endogenous gene transposons and regulating gene expression (Zhang et al., 2006). Cold stress can enhance the activity of transposons by altering the level of methylation of stress-responsive genes, leading to the induction of related cold resistance genes and enhanced cold tolerance. Significant changes in DNA methylation status have been observed in *Solanum*

TABLE 1 Examples of the role of plant hormones in cold stress responses.

Phytohormone		References
Salicylic acid (SA)	SA induces plant cold tolerance, affects the expression of *CBF* and *COR* genes, and improves antioxidant enzyme activity.	(Cheng et al., 2016)
	The desaturation efficiency of fatty acids can be improved by combining SA and TSP, enhancing water retention and alleviating membrane structure damage.	(Ge et al., 2020b)
	Cold tolerance is induced by SA, which enhances GR and G-POD activity.	(Kang and Saltveit, 2002)
Abscisic acid (ABA)	Enhanced cold tolerance is achieved through synergistic induction of *CBF/DREB1* expression by ABA and low temperature.	(Rubio et al., 2019)
	ABA enhances antioxidant enzyme activity, induces stomatal closure, and inhibits the accumulation of reactive oxygen species.	(Lim et al., 2015)
Gibberellic acid (GA)	Seed germination is regulated in an antagonistic manner by ABA and GA, promoting seed coat and endosperm rupture.	(MacGregor et al., 2015)
Ethylene (ET)	ET aids in cold resistance by enhancing cooling and prolonging the dormancy period of flower buds.	(Durner and Gianfagna, 1991)
	In *Malus domestica* seedlings, *MdERF1B* upregulates the expression of the low-temperature response gene *MdCBF1*. Additionally, *MdCIbHLH1* enhances the binding and transcriptional activation of *MdERF1B* to the target gene promoter in CBF-dependent pathways.	(Wang et al., 2021b)
	PtrERF9, induced by ethylene and low temperature, targets the ACC synthase gene *PtrACS1* in trifoliate citrus. This regulation affects the downstream target gene Glutathione S-transferase *PtrGSTU17* and ROS homeostasis in plants.	(Zhang et al., 2022b)
Cytokinin (CK)	*ZmRR1* enhances *Zea may's* cold tolerance by positively regulating the expression of cold response genes such as *ZmDREB1* and *CesA*.	(Zeng et al., 2021)
Jasmonic acid (JA)	JA positively regulates *CBF*, upregulates JA biosynthesis-related genes (including *AOC*, *AOS1*, *AOS2*, and *LOX2*), and promotes cell responses to cold stress through signaling genes (*COI1a* and *bHLH148*).	(Du et al., 2013; Hu et al., 2017)

(Continued)

TABLE 1 Continued

Phytohormone		References
	MdBBX37 directly binds to the *MdCBF1* and *MdCBF4* promoters to promote their transcription. It also interacts with *MdICE1* to enhance the transcriptional activity of *MdICE1* toward *MdCBF1*, thereby enhancing cold tolerance.	(An et al., 2021)
	MeJA induces the activation of JA synthesis genes, increasing the content of antioxidant metabolites.	(Seo et al., 2020)
Brassinosteroid (BR)	BR accumulation leads to BZR1 and BES1 dephosphorylation levels, inducing *CBF1* and *CBF2* upstream pathways to regulate cold tolerance gene expression.	(Li et al., 2017b)
Strigolactone (SL)	SL activates *BRC1* expression, upregulates *HB40* expression level, and increases ABA content in lateral buds. It also upregulates the expression of Anthocyanidin synthesis genes *DFR*, *ANS*, and *TT7* by activating *PAP1*, *PAP2*, *MYB113*, and *MYB114*, promoting the synthesis and accumulation of Anthocyanidin.	(Wang et al., 2020a)
	SL inhibits the expression of *WRKY41*, promotes the interaction between MAX2 and WRKY41, and degrades WRKY41 through the 26S proteasome pathway. This leads to increased expression of *CBFs* and downstream genes, enhancing cold tolerance in *A. thaliana*.	(Wang et al., 2023b)

lycopersicum after exposure to low temperatures. Cold stress downregulates the expression of the plant DNA demethylase *DML2*, resulting in increased methylation levels in the promoter region. This leads to gene silencing, decreased flavor quality, and reduced volatile production (Zhang et al., 2016). Histone H3 trimethylation at lysine 27 (H3K27me3), a common chromatin modification, is pivotal in plant growth and stress resistance. *V. vinifera* leaves treated at 4°C exhibited changes in H3K27me3 modification. Genes targeted by H3K27me3 showed significantly reduced expression compared to non-targeted genes, indicating the suppressive effect of this modification on gene transcription across the entire genome. The methylation of relevant genomic proteins was detected through chip-seq binding transcriptome analysis, providing insights into the regulatory mechanism of H3K27me3 in response to cold stress (Zhu et al., 2023). The vernalization pathway plays an important role in the regulation of *FLC*. The histone demethylases JMJ30 and JMJ32 regulate the vernalization rate of *A. thaliana* by activating *FLC* (Maruoka et al., 2022). Therefore, understanding the molecular mechanisms of cold tolerance in plants, including epigenetic modifications and gene function, is a significant challenge that requires further research.

TABLE 2 Cold stress-related genes.

Gene name	Species	Gene function	References
MYB15	*Arabidopsis thaliana*	MYB15 protein can interact with ICE1 and bind to the Myb recognition sequence of the *CBF* gene promoter. Overexpression of *MYB15* leads to reduced expression of the *CBF* gene and regulates plant cold tolerance.	(Agarwal et al., 2006a)
bHLH002	*Oryza sativa*	OsMAPK3 interacts with OsbHLH002 protein and phosphorylates it to activate *OsTPP1* and enhance the cold tolerance of rice.	(Zhang et al., 2017)
PUB25/26	*Arabidopsis thaliana*	Cold-activated OST1 phosphorylates PUB25 and PUB26, promoting the degradation of MYB15 and enhancing plant cold resistance.	(Wang et al., 2019)
OST1	*Arabidopsis thaliana*	Cold-activated OST1 phosphorylates BTF3L, enhances the interaction with CBF protein, and promotes the expression of *COR* genes.	(Ding et al., 2018)
ICE1	*Arabidopsis thaliana*	Cold-activated OST1 phosphorylation of ICE1 enhances its stability and transcriptional activity modulates plant cold tolerance.	(Ding et al., 2015)
MPK3/6	*Arabidopsis thaliana*	MPK3 and MPK6 interact with and phosphorylate ICE1, negatively regulating the cold tolerance of *Arabidopsis*.	(Li et al., 2017a)
B1L	*Arabidopsis thaliana*	B1L enhances cold tolerance by enhancing *CBF* stability, thereby inducing *COR* gene expression.	(Chen et al., 2019)
BZR1	*Arabidopsis thaliana*	*BZR1* triggers plant responses to cold stress by directly regulating the expression of *CBF1/2* or *COR* genes.	(Li et al., 2017b)
Trx-h2	*Arabidopsis thaliana*	Low-temperature-mediated translocation of h2-type thioredoxin can induce CBF structural transformation and functional activation through REDOX changes, thus improving plant cold tolerance.	(Lee et al., 2021)
WRKY41	*Arabidopsis thaliana*	WRKY41 can bind to the W-box motif of the *CBF*	(Wang et al., 2023b)

(Continued)

TABLE 2 Continued

Gene name	Species	Gene function	References
		promoter region and negatively regulate *CBF* gene expression and *Arabidopsis* cold tolerance.	
HY5	*Brassica napus*	Hypocotyl length was closely related to cold tolerance. *HY5* showed positive regulation on hypocotyl elongation.	(Jin et al., 2022)
BBX29	*Arabidopsis thaliana*	BBX29 can inhibit the expression of some cold response genes independent of the CBF pathway through specific binding to cis-acting elements such as G-box, thereby negatively regulating the cold tolerance of plants.	(Wang et al., 2023a)
NAC104	*Malus domestica*	MdNAC104 enhanced plant cold tolerance by promoting anthocyanin synthesis and expression of genes encoding antioxidant enzymes.	(Mei et al., 2023)
WRKY2	*Cynodon dactylon*	*CdWRKY2* promotes cold tolerance by mediating sucrose biosynthesis and the CBF signaling pathway.	(Huang et al., 2022)
PIF3	*Arabidopsis thaliana*	*PIF3* regulates plant cold tolerance by negatively regulating the expression of CBF pathway genes.	(Jiang et al., 2017)
CBF2	*Arabidopsis thaliana*	*MbCBF2* positively regulates cold tolerance by enhancing the activity of antioxidant enzymes and the expression of downstream-related genes.	(Li et al., 2022a)
MPK1/2	*Solanum lycopersicum*	SIMPK1/2-mediated phosphorylation of SIBBX17 enhances CBF-dependent cold tolerance.	(Song et al., 2023)
CBF1	*Citrus sinensis*	*CsCBF1* directly regulates the expression of arginine decarboxylase (ADC) and the synthesis of putrescine (Put) to coordinate cold resistance.	(Song et al., 2021)
CBF2/4	*Tectona grandis*	Genetic transformation experiments showed that *TgCBF2* and *TgCBF4* improved the cold resistance of *Arabidopsis* plants.	(Liu et al., 2023)
BIN2	*Hevea brasiliensis*	*HbBIN2* regulates plant cold tolerance by regulating *HbICE1* transcriptional activity and ROS homeostasis.	(Qiu et al., 2023)
CIPK18	*Vitis amurensis*	*VaCIPK18* overexpression positively regulates cold tolerance by reducing the production of reactive oxygen species.	(Yu et al., 2022)
CIPK13	*Capsicum annuum*	Overexpression of *CaCIPK13* improves cold tolerance by increasing anthocyanin content and active oxygen-scavenging enzyme activity.	(Ma et al., 2021)
NAM3	*Solanum lycopersicum*	*SlNAM3* regulates plant cold tolerance by increasing ethylene synthesis.	(Dong et al., 2022)
TCP1	*Chrysanthemum morifolium Ramat*	*DgTCP1* enhances cold tolerance by promoting peroxidase gene expression and reducing ROS accumulation.	(Li et al., 2022b)
WRKY40	***Kandelia obovata***	*KoWRKY40* endows transgenic *Arabidopsis* with cold tolerance by regulating the ICE-CBF-COR signaling pathway.	(Fei et al., 2022)

(*Continued*)

5 Summary and outlook of low-temperature resistance in plants

Temperature is a crucial environmental factor that significantly affects the growth of plants. Low temperatures can harm plants during winter, cause delays in sowing, and limit crop distribution. Therefore, it is imperative to enhance the cold resistance of plants and develop cold-resistant varieties for sustainable agricultural production in winter. Grafting is an alternative method to traditional breeding that can improve plants' tolerance to environmental stress (Venema et al., 2008). Studying genes related to plant frost resistance is particularly significant because it allows us to use genetic engineering to enhance the frost resistance of non-hardy plants, with evident potential applications. Furthermore, using tolerant rootstocks during plant cultivation has been found to enhance resistance to low temperatures (Lee et al., 2010). However, there is still a lack of a comprehensive and efficient evaluation system for assessing cold tolerance in plants. Additionally, the molecular mechanism underlying the regulation of crop low-temperature and cold resistance regulatory proteins remains unclear.

Enhancing plant cold tolerance depends on various factors, including genes related to cold resistance, external environmental factors, and physiological mechanisms of cold resistance. Enriching germplasm resources for cold tolerance can be achieved by selecting varieties with excellent agronomic traits and quality and crossing

them with varieties with strong cold resistance (Labroo et al., 2021). The determination of WCS120 protein content can also serve as a standard for selecting cold-resistant varieties (Vítámvás et al., 2010). The DHN5 protein, which is structurally similar to the WCS120 protein in wheat, can also be used as a marker for assessing frost tolerance (FT) in barley (Kosová et al., 2008; Kosová et al., 2013). The positive regulatory effect of CTB2 on cold tolerance in *O. sativa* has been confirmed through transgenic technology, contributing to the cultivation of cold-tolerant *O. sativa* germplasm resources (Li et al., 2021b). By closely examining QTLs associated with cold tolerance in *O. sativa* using genome-wide association studies (GWAS), researchers have conducted genetic analyses of cold tolerance-related genes, developed polymorphic molecular markers, and bred new cold-resistant varieties under cold stress conditions (Li et al., 2021a). Moreover, modern biotechnological advancements, including CRISPR/Cas9 technology, metabolomics, genomics, and proteomics, have facilitated genome sequencing in different cold-resistant plant varieties, enabling deeper investigations into the mechanisms of plant cold resistance. TALENs and CRISPR/Cas systems have revolutionized biological research, and their application to crop plants allows for targeting specific traits to improve crop productivity and ensure food security (Sedeek et al., 2019). Additionally, studies have shown that stimulating seeds with ROS nanoparticles (NPs) can enhance seed germination speed under stress conditions and improve seedling resistance to stress. Strategies based on this nanobiostimulant could promote sustainable agriculture by reducing the use of pesticides (Yan et al., 2023). This knowledge will facilitate the development of robust and resilient plant varieties that can withstand cold temperatures, ultimately addressing the issue of plant cold damage (Table 2).

Author contributions

YW: Writing – original draft, Writing – review & editing. JW: Writing – original draft, Writing – review & editing. RS: Writing – review & editing. WZ: Writing – review & editing. RG: Writing – review & editing. K-MZ: Writing – review & editing. X-LT: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Glossary

ABI	ABA insensitive
ACC	1-aminocyclopropane-1-carboxylic acid
ACS	1-aminocyclopropane-1-carboxylic acid synthase
ANS	anthocyanidin synthase
AOC	allene oxide cyclase
AOS	allene oxide synthase
BBX	B-box
BES	BRI1-EMS-suppressor
BRC	branched
bHLH	basic helix–loop–helix
BZR	Brassinazole-resistant
CBF	C-repeat binding factor
COI	coronatine insensitive
COR	cold regulated
DFR	dihydro-flavonol 4-reductase
DREB	dehydration-responsive element binding protein
ERF	ethylene response factor
G-POD	guaicol peroxidase
GR	glutathione reductase
GSTU	glutathione S-transferase U
HB	homeobox
HOS	high expression of osmotically responsive gene
HY	hypocotyl
ICE	inducer of CBF expression
LOX	lipoxygenase
MAX	more axillary growth
MYB	V-MYB avian myeloblastosis viral oncogene homolog
PAP	production of anthocyanin pigment
PIF	phytochrome-interacting factors
PUB	plant U-box
ROS	reactive oxygen species
RPL	ribosomal protein L
RR	response regulator
TSP	trisodium phosphate
TT	transparent testa
ZAT	zinc finger of *Arabidopsis thaliana*
BIN	BR-insensitive
CIPK	calcineurin B-like protein-interacting protein kinase
NAC	nascent polypeptide-associated complex
TCP	TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING.

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Experimental investigation of freeze injury temperatures in trees and their contributing factors based on electrical impedance spectroscopy

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In trees, injuries resulting from subfreezing temperatures can cause damage to the cellular biofilm system, metabolic functions, and fibrous reticulum, and even cell death. Investigating the occurrence of freezing damage and its contributing factors could help understand the mechanisms underlying freezing injury and prevent the subsequent damage in trees. To achieve this, a laboratory experiment was conducted using cut wood samples from Korean pine (*Pinus koraiensis* Siebold & Zucc) and Simon poplar (*Populus simonii* Carr.), and the effects of environmental freezing factors, including freezing temperatures, freezing duration, and cooling rate, on the temperature at which freezing injuries occur were examined using the electrical impedance spectroscopy (EIS) method. The semi-lethal temperature (LT50), as an indicator of freezing injury in wood tissue, was theoretically deduced based on the measured extracellular resistance (r_e) using EIS. The contributory factors to changes in LT50 were determined and their relationship was established. The results revealed that all freezing factors exhibited significant effects on electrical impedance characteristics (r_e, r_i, and τ), significantly influencing the LT50 of the wood. Random forest (RF) and support vector machine (SVM) models were used to assess the contribution of the freezing factors and moisture content (MC). Among the factors examined, freezing duration had the greatest impact on LT50, followed by the MC, whereas the contribution of the cooling rate was minimal. The model accuracies were 0.89 and 0.86 for Korean pine and Simon poplar, respectively. The findings of our study illustrate that the occurrence of freezing injury in trees is primarily influenced by the duration of freezing at specific subzero temperatures. Slow cooling combined with prolonged freezing at low subzero temperatures leads to earlier and more severe freezing damage.

KEYWORDS

freeze injury, semi-lethal temperature, electrical impedance spectroscopy, cooling rate, freezing duration

1 Introduction

Low temperature is one of the most detrimental environmental stressors for trees in temperate or boreal zones. Freezing damage to wood tissue, resulting from chilling and freezing, has garnered significant attention owing to its significant financial impact on global wood utilization annually (Snyder and de Melo-Abreu 2005; Gale and Moyer, 2017; León-Chan et al., 2017; Ouyang et al., 2019a; Guan et al., 2023). Further, low temperature is a major abiotic stress factor with profound effects on plant growth and development (Ritonga and Chen, 2020; Rahimi-Ajdadi, 2022). Although temperate or boreal trees can tolerate cold stress during winter and early spring, extremely low subzero temperatures (−50°) or sudden and rapid temperature drops can cause freezing-induced injuries, hindering the growth of trees and wood productivity (Zhang et al., 2022). Freezing of wood tissues can lead to frost cracking and freezing damage, and even threaten the survival of trees (Pearce, 2001; Guy, 2003). The damage to wood cell membranes is attributed to ice formation and cellular dehydration resulting from chilling and freezing (Shammi et al., 2022; Tian et al., 2022). Deep supercooling or extracellular freezing is the mechanism whereby wood tissues and organs adapt to subfreezing temperatures to resist freezing damage (Gusta and Wisniewski, 2013; Wisniewski et al., 2014a). However, if the temperature continues to decrease, the deeply supercooled water will ultimately freeze, leading to lethal injury to the tissue (Ashworth and Davis, 1984).

When trees are exposed to subfreezing temperatures for extended periods of time, ice can form in the apoplast of wood tissues, causing water to move from the protoplasm to the ectoplasmic space (Verhoeven et al., 2018; Ramirez and Poppenberger, 2020). Prolonged exposure can result in a continuous increase in ice crystal volume, puncturing the cell membrane and potentially leading to cell death (Romero Fogué et al., 2022; Mihailova et al., 2020; Prerostova et al., 2021). In addition to the duration of exposure to freezing temperatures, the rate at which the environmental temperature decreases is another factor that affects ice formation in wood tissues (Weiser, 1970). When the cooling rate is rapid, the water in the protoplasm does not have sufficient time to exfiltrate, which can potentially lead to the formation of intracellular ice crystals. This results in freezing injury reactions, such as cell membrane disintegration as well as plasma outflow, causing further damage and potentially leading to cell death (Hofmann and Bruelheide, 2015; Shin et al., 2015).

Freezing injury in plants refers to the damage caused to the cellular biofilm system, metabolic functions, and fibrous reticulum due to mechanical stress and secondary drought, ultimately resulting in cell death. The semi-lethal temperature at which 50% of plant tissue is damaged or killed (LT50) is considered a critical indicator of freezing injury to woody tissue (Repo et al., 1997; Repo et al., 2021). The degree of freezing injury is influenced by factors such as the duration of exposure to freezing temperatures, intensity of chilling stress, rate of cooling, and location of ice formation (Lim et al., 1998; Beck et al., 2004; Fujikawa et al., 2018; Kovaleski and Grossman, 2021). Therefore, understanding structural changes that occur in wood tissues at subfreezing temperatures is of practical importance for investigating the mechanism underlying freezing injury in trees.

Various methods have been used to assess freeze-injury in plant branches, shoots, and seedlings during controlled laboratory experiments (Endoh et al., 2014; Palacio et al., 2015). Freezing of water is an exothermic process. Differential thermal scanning (DSC) analysis has been used to determine the freezing temperature in wood tissues; previous studies have determined freezing time by observing the region of exothermic reactions in DSC curves (Neuner et al., 2010; Arias et al., 2015; Arias et al., 2017). Freezing processes in wood tissues typically involve two exothermic reactions: the first occurs at relatively higher temperatures and involves freezing of water in the extracellular spaces (Repo et al., 2022), whereas the second occurs at considerably lower temperatures and involves the freezing of supercooled water in intracellular spaces. In the case of some boreal and cold-temperate tree species, intracellular water is susceptible to supercooling and deep supercooling. In these species, tissue cells demonstrate resistance to ice nucleation activity and freezing of supercooled water within these cells is associated with the occurrence of exothermic processes at considerably lower temperatures. Some tree species are likely to undergo multiple exothermic reactions at lower temperatures (Räisänen et al., 2006; Repo et al., 2022).

In recent years, electrical impedance spectroscopy (EIS) has emerged as a nondestructive detection method widely used to assess physiological changes in various wood cells, tissues, and whole plants in response to freezing stress, cold acclimation, and decay (Luoranen et al., 2004; Wu et al., 2008; Zhang et al., 2010; Yue et al., 2018). The electrical impedance of the cellular structure of tissues comprises resistance and capacitance; the electrical impedance exhibits variation across different frequency ranges, and the EIS profile and its parameters directly reflect the physiological changes occurring in tissue cells (Ando et al., 2014; Repo et al., 2016; Jócsák et al., 2019; Wu et al., 2019). EIS parameters have been used to evaluate plant cold resistance by converting them into temperature response inflection points (LT50) (Repo et al., 2000; Wu et al., 2008; Zhang et al., 2010; Repo et al., 2021).

The northeast forest area (118°–135°E, 48°–55°N) constitutes the largest natural forest area in China, covering approximately 37% of the nation's total forested land. Trees in this region endure prolonged periods of subfreezing temperatures during the winter season. The historical lowest temperature recorded in this area reached a staggering −55.1°C. Freezing injury can occur in woody plants exposed to extreme low temperature.

Trees exposed to subfreezing temperatures for a long time can cause brittle breakage, frost cracking, large stuttering, and even death. Investigation of the temperature and conditions of freezing damage occurrence is the basis for understanding the mechanism

Abbreviations: R_e, Extracellular resistance (Ω); R_i, Intracellular resistance (Ω); r_e, Specific extracellular resistance (Ωm); r_i, Specific intracellular resistance (Ωm); I, Imaginary unit; ω, Angular velocity (rad·s^{-1}); Z, Complex impedance (Ω); ZRe, Real part of the impedance (Ω); ZIm, Imaginary part of the impedance (Ω); R_∞, Resistances in high frequency (Ω); R, Resistance difference between the direct current resistance and R_∞ (Ω); τ, Relaxation time (s); ψ, Distribution coefficient.

underlying freezing damage in trees, which is of great value for the resistance and acclimation of precious tree species to cold conditions. In the present study, we used EIS to evaluate the LT50 and cytoarchitectural states during freezing in the sapwood tissues of trees. We examined the effects of freezing factors on EIS characteristics and the LT50 of wood.

The objectives of this study were: (1) to investigate the critical temperature at which the freeze injury occurs in the trees subjected to subfreezing temperature environments by modelling the relationship between EIS characteristics and LT50, and (2) to examine the effects of exposure to subzero temperatures, freezing duration, cooling rate, and moisture on the LT50 of sapwood tissue.

2 Materials and methods

2.1 Materials and sample preparation

The experiments were conducted at the laboratory and logs of Korean pine (*Pinus koraiensis* Siebold & Zucc) and Simon poplar (*Populus simonii* Carr.) species were used for the experiments. The logs were obtained from the Hancong Ridge Forests in the Changbai Mountains, China. The Hancong Ridge Forests are located in the northeast region of China (Figure 1), and the area experiences subfreezing temperatures for over four months of the year (Figure 2). The logs used in this study were cut from standing trees on-site with a diameter of approximately 25 cm. They were then processed into small, flawless cubes of wood measuring 10 × 10 × 10 mm^3, separated into heartwood and sapwood. The cubes were numbered and wrapped in plastic film for preservation until further analysis.

2.2 Moisture content conditions

To determine the effect of the MC, we conditioned the wood samples to six MC levels including air-dry (15%), fiber saturation point (30%), and fully saturated wood (80%, 100%, 150% and 200%). Each group corresponding to a specific MC level consisted of 60 wood samples. All samples were air-dried naturally in a ventilated, cool place of the laboratory. A sample was randomly selected from each group and oven-dried at 105°C for 24 hours. During the drying process, six samples were weighed every 2 h until the weight difference between two consecutive measurements did not exceed 0.0002 g. The weights of the six samples were considered as completely dried weights of the corresponding group and recorded as M_0. The dried samples were stored in weighing bottles with a desiccant.

The target weight of each sample ($M_{i,\ i=1,2,3\ldots6}$) at each MC level was calculated using the Equation 1:

$$MC = \frac{M_i - M_0}{M_0} \tag{1}$$

Following this, all dried samples were soaked in beakers containing deionized water and sealed with Parafilm (Parafilm "M" Laboratory Film PM996, Bemis, WI, USA). After soaking for 12 h, the samples were air-dried with natural air. Five samples from each group were randomly weighed every 0.5 h and the weight was

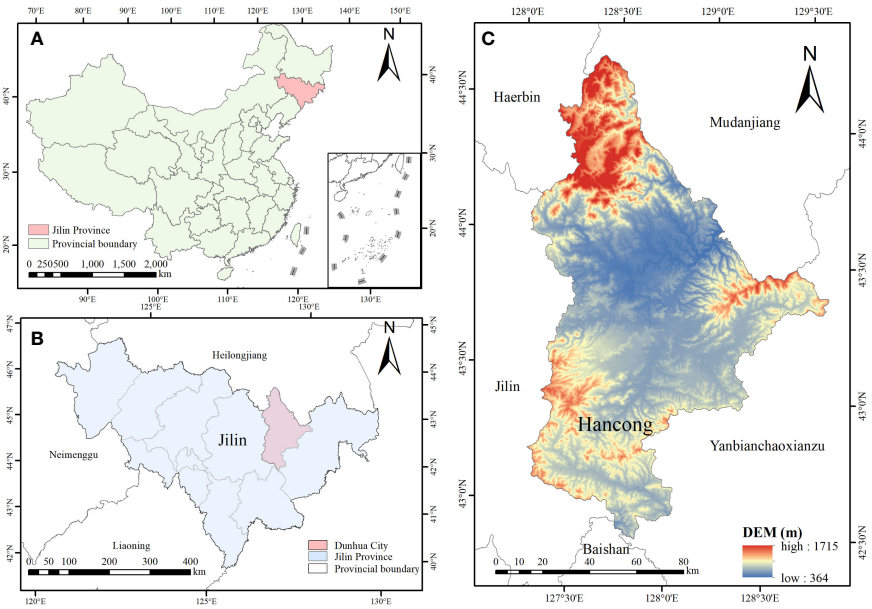

FIGURE 1
Geographical location maps of the Hancong forest region in Changbai Mountains. **(A)** The location of Changbai Moutains in China. **(B)** The location of Hancong Ridge in Jilin Province. **(C)** Digital elevation model (DEM) of Hancong Ridge.

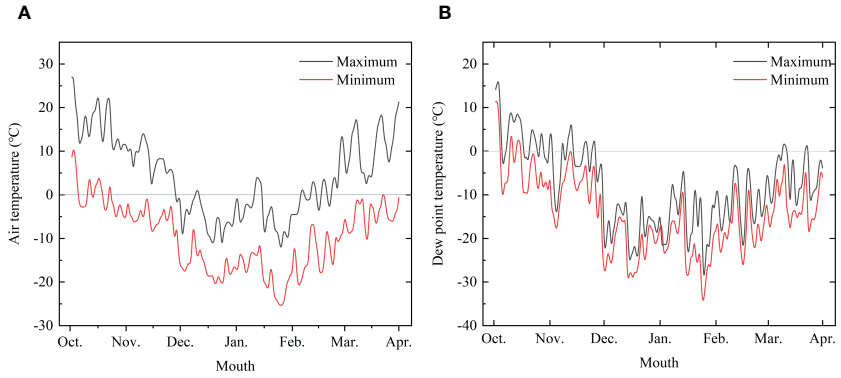

FIGURE 2
Temperature change trend monitored from October 2022 to March 2023. **(A)** Air temperature and **(B)** Dew point temperature.

recorded until it approached the target weight M_i over time. Finally, the conditioned samples were individually wrapped with plastic film to prevent moisture loss.

2.3 Differential scanning calorimetry measurements

The selected samples from the six MC groups were cut with a sharp blade into smaller cubic shapes measuring 2 × 2 × 2 mm^3. Two cooling rates, namely a slow rate of 2°C/min and a fast rate of 10°C/min, were set for the DSC tests. The samples were grouped based on the species and cooling rate into four groups: *Pinus*–slow-freezing (PiS), *Pinus*–fast-freezing (PiF), *Populus*–slow-freezing (PoS), and *Populus*–fast-freezing (PoF). To prepare the samples for the DSC, the samples were weighed and placed in a Φ5.4 mm × 2.6 mm crucible (TA-JYL0010, TA instruments, DE, USA). The high temperature exotherms and low temperature exotherms (HTEs and LTEs) during freezing were measured using the DSC device (TA Instruments Q20, TA instruments, DE, USA), starting from an initial temperature of 10°C. Each group of samples was cooled down to −80°C at both cooling rates, with eight repetitions at each rate. The obtained DSC curves were analyzed to determine the onset and termination temperatures of HTEs and LTEs, as well as the corresponding exothermic peaks.

2.4 Freezing condition control

A gas chamber was built as a sealed freezing room for wood sample temperature control. Liquid N_2 was injected into the gas chamber using a pump, and the flow rate of liquid N_2 was controlled by adjusting the pump pressure (Figure 3A). To align with the conditions set for the DSC tests, the cooling rates of 2°C/min and 10°C/min were maintained. Each species–cooling rate group (PiF, PiS, PoF, and PoS) comprised 48 samples. The samples were conditioned to the target MC level and then cooled down in an orderly manner to −10, −20, −30, −40, −50, −60, −70, and −80°C at both slow and fast cooling rates (Figure 3B). Thermocouples were connected to the samples to monitor the temperature changes during freezing, and liquid N2 was automatically refilled to maintain the desired temperature and cooling rates. The freezing duration for each sample at every temperature point was 0.5 and 1 h.

2.5 Electrical impedance spectroscopy

Conductive paint was evenly applied to both radial facets of the sample section. One end of a conductive wire was fixed on the surface of the wood sample using insulating rubber and the other end was attached to insulating jigs (TH26011BS, Tonghui Electronic Co. Ltd., Changzhou, China) using tinned copper. The impedance spectrum, including the real part (*ZRe*) and the imaginary part (*ZIm*), was measured using a high-frequency LCR digital bridge (Tong-Hui TH2826/A, Tonghui Electronic Co. Ltd., Changzhou, China) in the frequency range of 80 Hz to 1 MHz (Figure 3). The input voltage of the sine signal was set to 100 mV. The EIS parameters were modeled using the single-DCE model in the distributed circuit model based on *ZRe* and *ZIm*. The model is described by Equation 2 as follows:

$$Z = R_\infty + \frac{R}{1 + (i\tau\omega)^\psi}, \tag{2}$$

where R_∞ (Ω) represents the resistance at high frequencies, R (Ω) is the difference between the direct current resistance and R_∞ (Ω), i is the imaginary unit, ω ($2\pi f$) is the angular velocity, τ is the relaxation time, and ψ is the distribution coefficient.

The model parameters were fitted using ZSimpWin 3.60 software (ZSimpWin, AMETEK, Inc., USA). At low frequencies, the electrical double layer prevents the current from passing through the cell membrane, and it flows only in the apoplastic space (Ehosioke et al., 2020). The extracellular resistance (R_e) was calculated using Equation 3:

$$R_e = R_\infty + R \tag{3}$$

At high frequencies, the currents can pass through the cell membrane, flowing in both the apoplastic and symplastic spaces (Ehosioke et al., 2020). The intracellular resistance (R_i) is given by

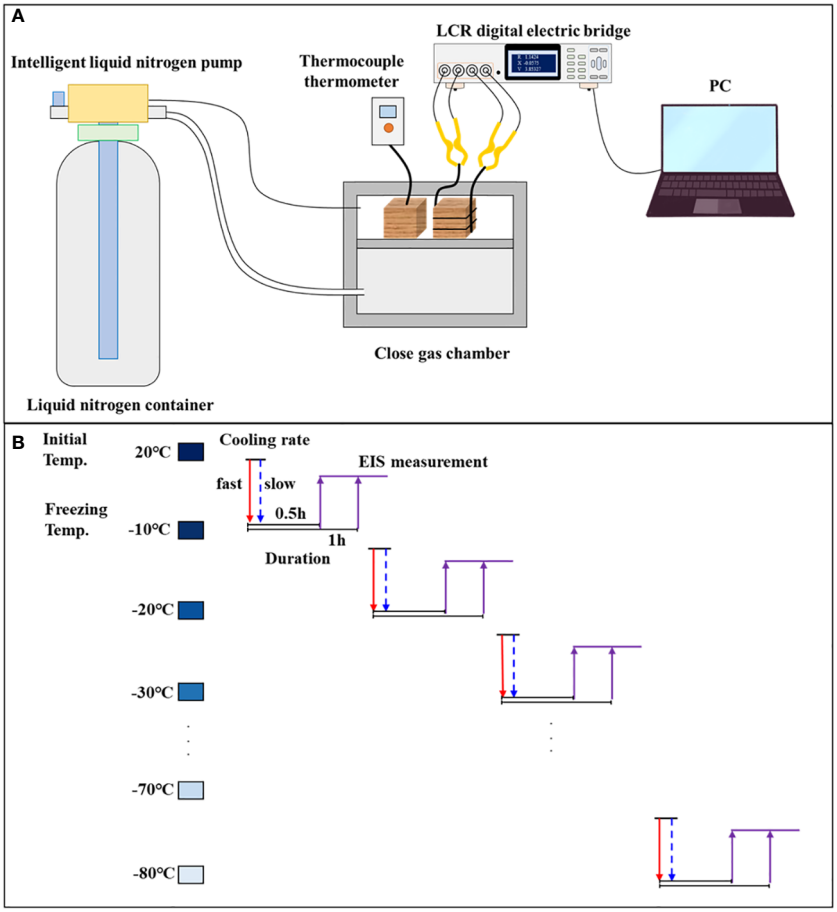

FIGURE 3
(A) Schematic diagram of the freezing system and **(B)** the flowchart of EIS measurement.

Equation 4:

$$R_I = R_\infty(1 + \frac{R_\infty}{R}) \quad (4)$$

The resistance parameter ($R_{e/i}$) was normalized to the cross-sectional area (A, m^2) and length (l, m) of the sample to obtain the corresponding specific resistance value ($r_{e/i}$) using Equation 5:

$$r_{e/i} = \frac{A}{l} \times R_{e/i} \quad (5)$$

2.6 LT50 estimation based on parameter r_e

Specific extracellular resistance (r_e) is a measure of the leaching of symplastic electrolytes to the apoplastic space as a result of cell membrane injuries (Repo et al., 2021). To estimate the LT50 value, the specific extracellular resistance (r_e) was modeled using a logistic sigmoid function with respect to the exposure temperature, using the MATLABR2021 software (Equation 6).

$$y = \frac{A}{(1 + e)^{B(C-x)}} + D \quad (6)$$

In this equation, y represents parameter r_e, and x corresponds to the exposure temperature. The parameters A and D define the asymptotes of the sigmoid curve, and parameter B represents the slope of the curve at inflection point C. The temperature corresponding to the inflection point C of the sigmoid curve obtained from Equation 6 is considered the LT50 value (Repo et al., 1994; Sutinen et al., 1992; Wu et al., 2019; Zhang et al., 2010). The initial values of A, B, C, and D in Equation 6 were fitted using the 1stopt5.0 software (First Optimization, 7D-Soft High Technology Inc., Beijing, China). The LT50 value (parameter C in Equation 6) of the wood sample was determined through measured electrical impedance spectroscopy using nonlinear regression analysis.

2.7 Statistical analysis

The interactive effects of temperature, MC, cooling rate, and freezing duration on EIS characteristics values and LT50 were statistically analyzed and evaluated using software SPSS (IBM, Armonk, NY, USA). A repeated measures one-way ANOVA was used to test significant differences in EIS characteristics (r_e, r_i, τ) and LT50 between MC, cooling rate, and freezing duration. The

significance differences of LT50 between MC were compared using Duncan Multiple Range Test at a significance level of $p < 0.05$.

3 Results

3.1 Thermal behaviors and transitions of sapwood during the freezing process

The DSC curves of wood samples during the freezing process are shown in Figure 4. HTEs occurred at a temperature of approximately −10°C for all wood samples of both species with MC over 70%. Samples with MC below fiber saturation point (FSP) exhibited rounded curves, indicating the transition of free water (extracellular) in the sapwood tissue from liquid to solid starting at −10°C. However, no LTEs were detected in any of the wood samples. This finding suggests that the DSC method is not suitable for detecting the transition of the intracellular water or changes in the membrane structure of wood cells.

3.2 EIS profiles during the freezing process

3.2.1 Observation of extracellular resistance

The extracellular resistance r_e of both species at different MC levels and temperature points is illustrated in Figure 5. It is evident that r_e exhibits an overall increasing trend at all subzero temperature points compared to −10°C, and the extent of increase varies with the MC. In the groups subjected to slow cooling rates, the maximum increase in r_e for pine (PiS group) (Figure 5A) mainly occurred at −40°C for 80% MC and at −70 to −80°C for MC over 100%. The r_e value for 30% and 10% MC were relatively smaller, with the maximum increase occurring at approximately −40°C. Poplar (PoS group) (Figure 5C) showed a trend similar to that observed for pine for all MC levels, with −40°C and −70 to -80°C serving as turning points for the impedance measured at slow cooling rates.

In the groups subjected to fast cooling rates, the maximum increase in r_e for pine (Pif group) (Figure 5B) mainly occurred at approximately −40°C for all MC levels, followed by temperatures of −70 to −80°C for MC over 80%. Poplar and pine (PoF group) (Figure 5D) showed similar trends to those observed for the slow cooling rate groups for all MC levels.

In the present study, the measured intracellular resistance (r_i) was only 10^{-6} to 10^{-7} times that of the r_e and the transition temperature of r_i was not clearly observed for either species under all freezing conditions. Therefore, intracellular resistance was not deemed suitable for estimating the LT50.

3.2.2 Observation of the relaxation time of wood

The relaxation time (τ) of both species generally increased as the temperature decreased for all MC levels at fast and slow cooling rates (Figure 6). The behavior of τ differed for pine with different MC levels in the PiS group. A greater increase in τ occurred around −70 to -80°C, followed by −40 to −50°C for slow cooling rates. In the PiF group, tissues with MC ≥ 30% showed significant changes in τ in the ranges of −40 to −50° and −60 to −70°. Tissues with MC of 10% showed a significant increase in τ only at −40°. These results indicate that the r_e and τ varied significantly with changes in the temperature and MC.

The ANOVA results revealed that subzero temperature, MC, and cooling rate exhibited highly significant effects on r_e, r_i, and τ of the sapwood tissues (Table 1). Additionally, their interactions also had a significant effect on the r_e, r_i, and τ of the sapwood tissues.

3.2.3 Interactive effects of freezing factors and MC on LT50 based on r_e

The corresponding LT50 values of sapwood at each transition temperature for both species were estimated based on the parameter r_e using Equation 6. The LT50 values at different MC levels and cooling rates were calculated and are presented in Table 2.

For a freezing duration of 0.5 h, the LT50 values for pine at a slow cooling rate (PiS group) and poplar at a slow cooling rate (PoS group) ranged from −30 to −60°C, decreasing as the MC increased

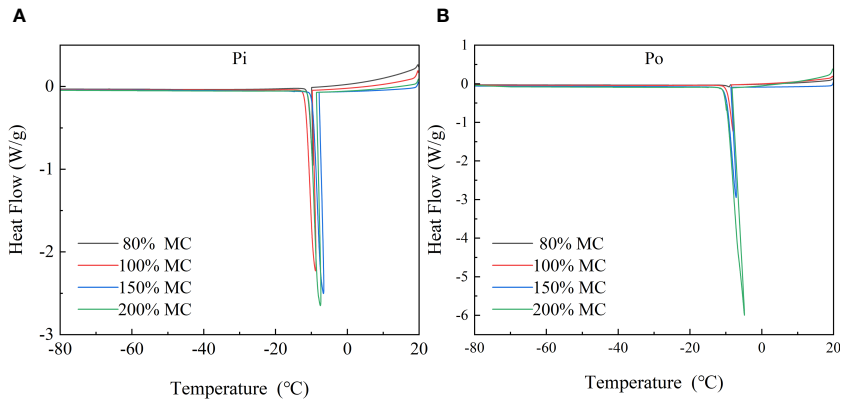

FIGURE 4
DSC curves of pine and poplar wood during controlled cooling at a rate of 2°C/min (from 20°C to −80°C). **(A)** DSC curves of pine and **(B)** DSC curves of poplar.

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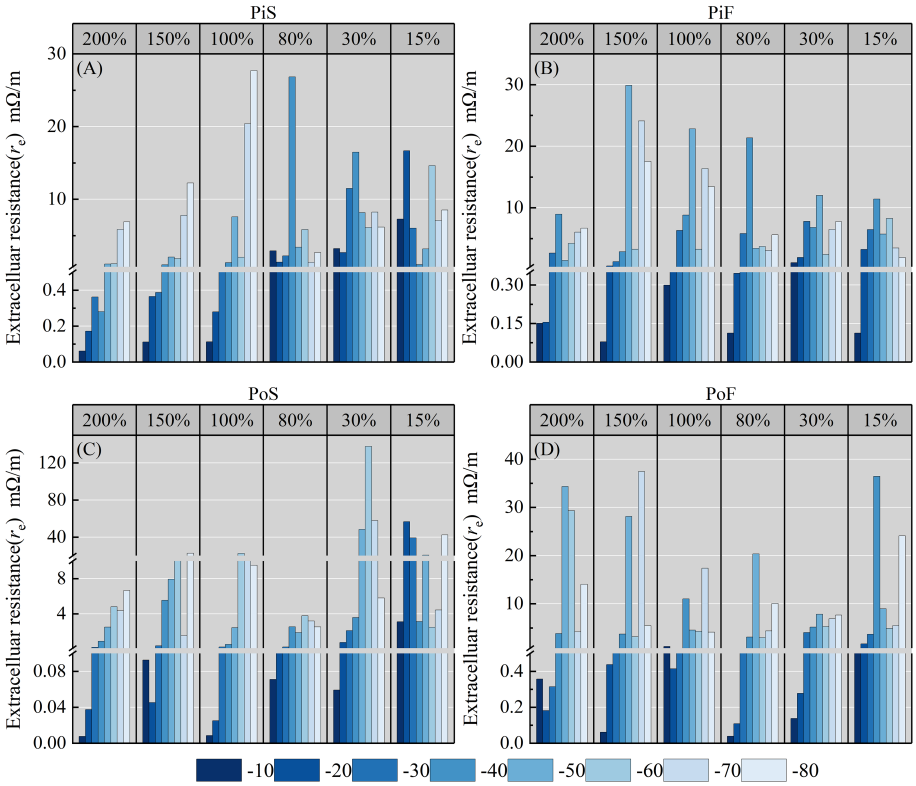

FIGURE 5
Variation in r_e with temperature for different moisture content levels and cooling rates during the freezing process. **(A)** pine–slow cooling rate, **(B)** poplar–slow cooling rate, **(C)** pine–fast cooling rate, and **(D)** poplar–fast cooling rate.

from 80% to 200% when the MC exceeded the FSP. The LT50 values for pine at a fast-cooling rate (PiF group) ranged from −30 to −40°C and for poplar at a fast growing rate (PoF group), they ranged from −30 to −50°C. The FSP did not behave as a distinct point for changes in LT50 change with different MC levels. For a freezing duration of 1 h, LT50 values for pine at both slow and fast cooling rates ranged from −20 to −40°C, decreasing with increasing MC levels. The LT50 values for poplar ranged from −30 to −50°C at a slow cooling rate and from −30 to −40°C at a fast rate. The LT50 values from pine wood samples frozen for 1 h were approximately 30% higher on average than those frozen for 0.5 h, with a maximum increase of approximately 53%. For poplar wood, the LT50 values for a 1-h freezing duration were 8% higher on average than those for a 0.5-h freezing duration, with a maximum increase of approximately 15.61% for 10% MC. The increased LT50 values with longer freezing duration indicates that the longer trees survive at certain subzero temperatures, the earlier the LT50 appears for wood tissue. The difference in LT50 between the MC levels in the PiS–0.5 group was minimal. Furthermore, the FSP did not act as a distinct point for changes in LT50 with different MC levels.

A comparison of LT50 values between the slow and fast cooling rate groups revealed that the LT50 values for both species at a slow cooling rate were slightly lower than those at a fast rate for freezing durations of both 0.5 h and 1 h, except the LT50 value for samples with 80% MC, which was slightly higher than that in the fast-cooling rate group.

The results revealed that the MC, freezing duration, and cooling rate had significant effects on the LT50 values of sapwood tissues (Table 3). The ANOVA results confirmed the significant influence of the MC, freezing duration, and cooling rate, and their interactions on the LT50 values of sapwood tissues ($p< 0.0001$) (Table 3).

3.2.4 Prediction of LT50 based on the MC and freezing factors

The LT50 values decreased with an increase in the MC to values above the FSP, indicating that the higher water content in the saturated sapwood led to a lower freezing damage temperature (LT50) (Figure 7). However, for MC below the FSP or at MC below 30%, no significant changes in LT50 were observed for both cooling rate and freezing duration. The decreasing rate in LT50 of the sapwood tissues was slow when the MC was over 100% for both species. In the slow cooling rate group, the LT50 values of wood frozen for 1 h concentrated around −40°C for pine and around −55°C for poplar, whereas the LT50 values for a freezing duration of 0.5 h concentrated around −60°C for both species. In the fast-cooling rate group, the LT50 values for sapwood tissues with MC over 80% showed approximate agreement for both species, with values LT50 concentrated around −30°C for pine and around −45°C for poplar after 1 h of freezing. For a freezing duration of 0.5 h, the LT50 values stayed around −40°C for pine and around −50°C for poplar. A significant difference of approximately 20°C was observed between the LT50 values of sapwood samples frozen for 1 h (samples with

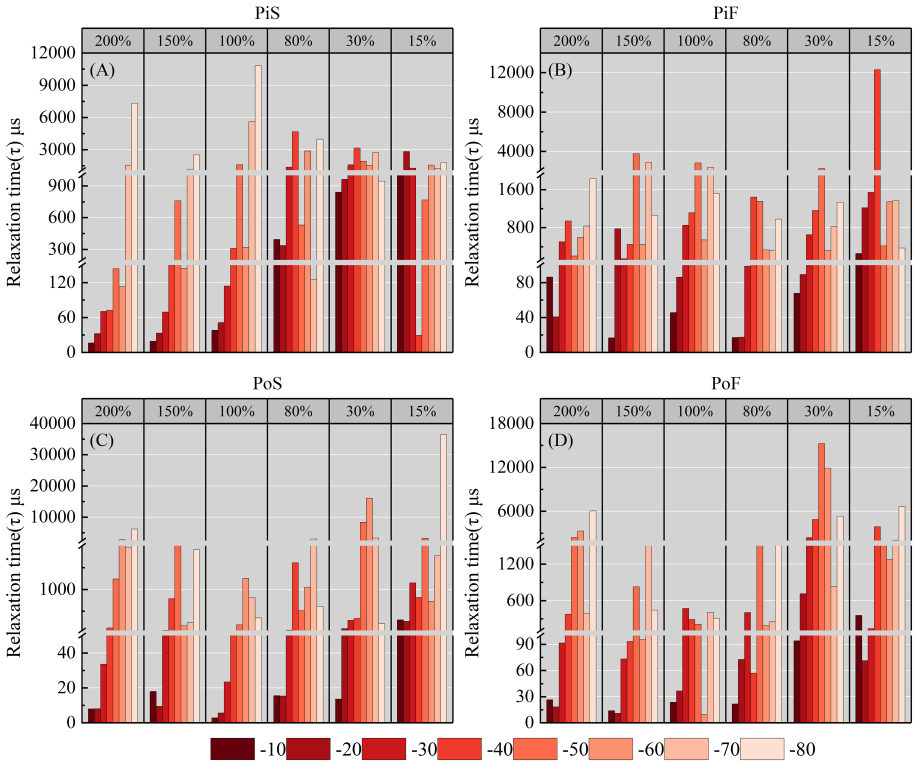

FIGURE 6
Variation of τ in sapwood tissues with different moisture content with changing temperatures. **(A)** pine–slow cooling rate, **(B)** poplar–slow cooling rate, **(C)** pine–fast cooling rate, and **(D)** poplar–fast cooling rate.

MC levels of 200% and 15%); the LT50 increased considerably when the MC was below 80% in the slow group.

A comparison of the LT50 values between wood exposed to 0.5 h and 1 h of freezing revealed that at an MC above 30%, the LT50 of pine sapwood tissue decreased by over 10°C after 1 h of freezing compared to the case after 0.5 h of freezing at both the cooling rates, whereas the LT50 of poplar sapwood tissue decreased by approximately 5°C after 1 h of freezing compared to the case after 0.5 h of freezing. However, when the MC was below 30%, the difference in LT50 between 0.5 h of freezing and 1 h of freezing was over 20°C for *Pinus* at both the cooling rates, but only about 5°C for poplar at a slow cooling rate. When the MC exceeded the FSP (>80% MC), the LT50 values remained relatively constant at a fast-cooling rate (Figures 7C, D), but slightly decreased at a slow cooling rate (Figures 7A, B).

Random forest (RF) and support vector machine (SVM) models were used to model the relationships among the MC, cooling rate, freezing duration, and LT50 values. The coefficients of determination were examined to evaluate the reliability of the

TABLE 1 A repeated measures ANOVA of MC, freezing duration and cooling rate on EIS characteristics (r_e, r_i, τ).

Source of variation	Pi						Po					
	r_e		r_i		τ		r_e		r_i		τ	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
MC	1670.199	0.000**	98.549	0.000**	524.827	0.000**	335.501	0.000**	45.7390	0.000**	908.567	0.000**
T	4159.020	0.000**	98.507	0.000**	1895.976	0.000**	513.056	0.000**	16.035	0.000**	795.155	0.000**
FR	1355.403	0.000**	434.501	0.000**	634.760	0.000**	213.267	0.000**	116.688	0.000**	84.245	0.000**
MC/T	1614.349	0.000**	35.328	0.000**	647.100	0.000**	239.823	0.000**	10.685	0.000**	461.684	0.000**
MC/CR	2051.950	0.000**	74.786	0.000**	705.423	0.000**	195.802	0.000**	35.415	0.000**	206.478	0.000**
T/CR	1679.400	0.000**	94.150	0.000**	1185.106	0.000**	149.030	0.000**	8.771	0.000**	154.432	0.000**
MC/T/CR	1802.738	0.000**	56.226	0.000**	597.734	0.000**	224.383	0.000**	10.352	0.000**	207.052	0.000**

**$P< 0.01$.
F-values and *p*-values for source of variation in extracellular resistivity (r_e), intracellular resistivity (r_i) and relaxation time (τ) estimates of pine (Pi) and poplar (Po) sapwood tissues based on the moisture content (MC), temperature (T), cooling rate (CR), and their statistically significant interactions.

TABLE 2 One-way ANOVA of moisture content (MC) on LT50. LT50 values of sapwood tissues from pine (Pi) and poplar (Po) with different moisture contents after freezing at two rates for 0.5 h and 1 h.

TS	FR	MC	Duration		TS	FR	MC	Duration	
			0.5 h	1 h				0.5 h	1 h
Pi	Slow	200%	−60.50 ± 1.55a	−43.86 ± 2.91a	Po	Slow	200%	−57.23 ± 1.36a	−54.86 ± 1.34a
		150%	−57.31 ± 1.29b	−41.29 ± 2.27a			150%	−58.25 ± 1.59a	−54.34 ± 2.76ab
		100%	−52.83 ± 2.37c	−40.68 ± 2.03a			100%	−56.78 ± 3.10a	−51.27 ± 1.53b
		80%	−51.62 ± 1.67cd	−32.13 ± 2.34b			80%	−41.58 ± 1.50c	−36.49 ± 2.39d
		30%	−49.44 ± 1.52d	−25.13 ± 1.75c			30%	−50.98 ± 1.40b	−44.16 ± 1.41c
		10%	−50.30 ± 1.71cd	−23.88 ± 1.90c			10%	−40.81 ± 1.78c	−33.77 ± 1.66d
	Fast	200%	−41.02 ± 1.49ab	−30.35 ± 1.56a		Fast	200%	−47.61 ± 1.59a	−41.98 ± 1.49ab
		150%	−42.61 ± 1.24a	−31.20 ± 1.44a			150%	−49.93 ± 1.99a	−42.41 ± 1.95ab
		100%	−43.02 ± 1.76a	−31.82 ± 1.48a			100%	−48.02 ± 1.74a	−43.16 ± 0.91a
		80%	−39.09 ± 1.55b	−29.78 ± 1.90a			80%	−47.80 ± 1.32a	−39.81 ± 1.51b
		30%	−40.02 ± 1.99ab	−21.05 ± 1.93b			30%	−44.00 ± 1.94b	−32.14 ± 1.59c
		10%	−38.10 ± 1.28b	−20.00 ± 1.97b			10%	−44.36 ± 1.58b	−30.60 ± 1.52c

Different letters indicate significant differences between subspecies ($P< 0.05$).

models. The analysis results were plotted using Origin2022 (OriginLab, Northampton, MA, USA) (Figure 8). The contributions of MC, cooling rate, and freezing duration were tested using the RF and SVM models. The root mean squared error (RMSE) and R^2 values of the RF model were 0.9945 and 0.8941, respectively (Figure 8A). Among the three variables, freezing duration and MC were found to be the dominant factors influencing the LT50 values, whereas the impact of cooling rate was relatively lower. As shown in Figure 8B, the penalty coefficient (c) and parameter g of the SVM model were 0.7071 and 45.2548, respectively, and the accuracy of the model was 86.21%.

4 Discussion

Whether the intracellular liquid allows cells to recover mainly depends on the speed and intensity of the freezing conditions (Ochandio Fernández et al., 2019). If the exposure temperature is sufficiently low and the freezing duration is sufficiently long, larger ice crystals are formed, causing cell wall rupture and cell death with no chance of recovery. Therefore, the objective of this study was to investigate the semi-lethal temperature for two tree species using the EIS method and the factors influencing this temperature, such as exposure temperature, cooling rate, and cooling duration, through a controlled laboratory experiment. The EIS profiles of frozen Korean pine and Simon poplar sapwood were obtained and the electrical parameters (r_e, r_i, and τ) were characterized.

As plant tissues have passive electrical properties, the impedance of biological tissues is primarily related to cellular ion content, cell membrane structure, and viscosity (Jócsák et al., 2019). As the water in plant tissues acts as the conductor of electrical currents, any changes in the state and content of water within the tissues directly affect ion migration, membrane permeability, and

TABLE 3 A repeated measures ANOVA of MC, freezing duration and cooling rate on LT50 for Pine and Poplar.

Source of variation	Pi		Po	
	F	*P*	*F*	*P*
Moisture Content (MC)	67.32	0.000**	126.22	0.000**
Duration (D)	1515.73	0.000**	269.72	0.000**
Cooing Rate (CR)	483.39	0.000**	187.70	0.000**
MC/D	27.77	0.000**	5.91	0.000**
MC/CR	16.78	0.000**	41.37	0.000**
D/CR	30.07	0.000**	17.31	0.000**
MC/D/CR	2.57	0.038	1.44	0.224

**$P< 0.01$.

F-values and *p*-values for the LT50 estimates for MC, freezing duration, cooling rate, and their statistically significant interactions.

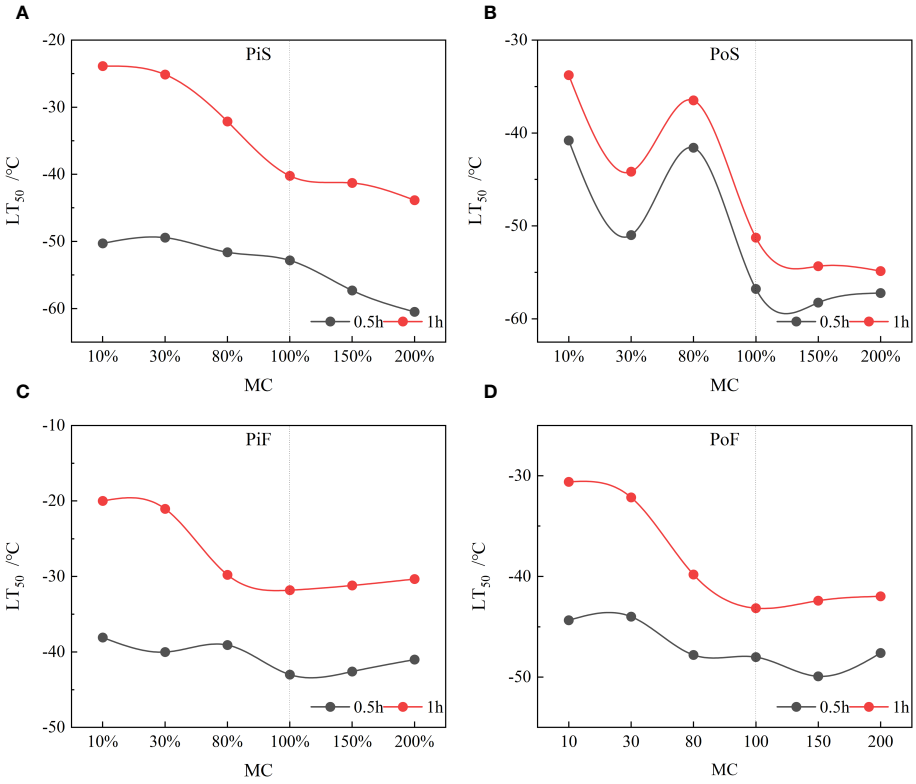

FIGURE 7
Changing trend of LT50 with MC at all freezing conditions. **(A)** pine–slow cooling rate, **(B)** poplar–slow cooling rate, **(C)** pine–fast cooling rate, and **(D)** poplar–fast cooling rate.

other factors, resulting in corresponding changes in tissue impedance (Repo et al., 1997; Ando et al., 2014). Therefore, the significant changes observed in impedance properties of sapwood tissues at freezing temperatures, especially in the range of −40 to −80°C, are reasonable (Figures 5, 6A-D).

The rapid increase in r_e at temperatures around −40°C (Figure 5) was primarily caused by the difference in chemical potential between the extracellular ice and intracellular solution. This difference in chemical potential can lead to cellular dehydration and a consistent increase in the volume of ice crystals, which punctures the plasma membrane, resulting in electrolyte leakage (Imaizumi et al., 2015; Ando et al., 2019). In contrast, the second change in r_e around −70°C is likely the result of extensive rupture of plasma and organelle membranes, resulting in tissue damage and significant cell death.

The cellular structure of plant tissues can be altered due to changes in water content when exposed to freezing conditions (Mahajan and Tuteja, 2005), along with other factors, such as the

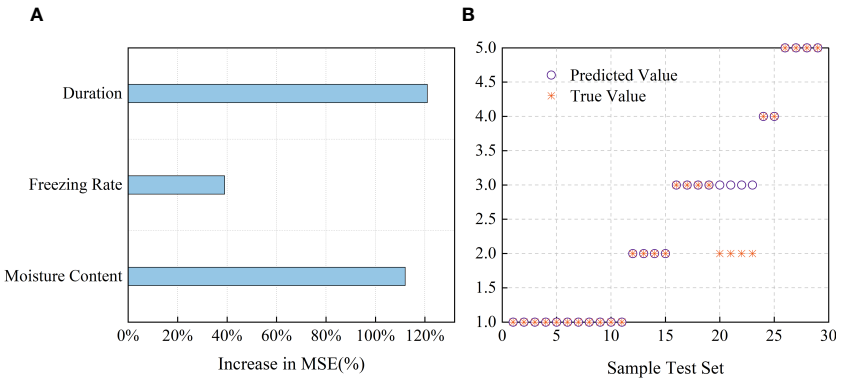

FIGURE 8
Driven correlations of LT50 with MC, cooling rate and freezing duration based on RF and SVM model simulation. **(A)** Contributions of each factor LT50 based on random forest (RF). Increases in the MSE of variables were used to estimate the importance of each factor, and higher MSE% values imply more important factors. **(B)** Comparison of predicted value and true value for the test set results based on SVM model.

species and types of cells and the rate of decrease of the environmental temperature.

HTEs usually occur due to extracellular freezing of water in the ectoplasmic space, xylem conduits, and non-living cells (Neuner et al., 2010; Yu et al., 2017; Hincha and Zuther, 2020). In the DSC curves, HTEs were observed in the sapwood tissues of Korean pine and Simon poplar between −7 and −12°C (Figure). Conversely, LTEs generally occur in xylem parenchyma cells (XPCs) and serve as a strategy in many woody plants to avoid freezing damage through deep supercooling (Neuner et al., 2010; Repo et al., 2022). However, LTEs were not detected in the DSC curves obtained in this study. This does not imply that deep supercooling or intraprotoplasmic freezing does not occur in sapwood tissues. XPCs in tree trunk tissues do not freeze simultaneously during freezing but freeze in small clusters at random locations in the xylem, resulting in only a minimal amount of exothermic release (Neuner et al., 2010). As the sapwood samples used in this study had a lower proportion of XPCs for deep supercooling, LTEs could not be detected using the DSC method.

Although freezing damage in trees is commonly observed at a temperature of approximately −40°C (Repo et al., 2022), the appearance of freezing in XPCs is not solely dependent on environmental temperature but is also influenced by factors such as embolism, cavitation, ion contents of xylem sap, vessel size, cell wall rigidity, degree of lignification, and cell maturation (Wu et al., 2019). Freezing damage in plants primarily occurs as a result of injuries to the cell membranes caused by water freezing into ice crystals and cell dehydration (Pearce, 2001). The electrical properties of plant tissues are significantly affected by their water content across most frequency ranges of EIS (Tiitta et al., 2009; Tomppo et al., 2011; Zelinka et al., 2007). In the present study, the impedance characteristics differed considerably between the different MC groups at the same temperature, especially in terms of the extracellular resistance (r_e), where the difference between MCs above the FSP and below the FSP was at least 100-fold (Figure 5). This result aligns with those of previous studies that observed differences in electrical impedance between 20% MC and 12% MC in Southern pine (*Pinus* spp.) (Zelinka et al., 2008).

Extracellular resistance (r_e) can be modeled using a logistic sigmoid function to obtain the LT50 value (Equation 5), allowing EIS to be used as a method to estimate cell membrane injuries in wood tissue (Repo et al., 2021). Maintaining the structural and functional stability of cell membranes during freezing is crucial for the resistance of tissues against mechanical and dehydration stresses and for overcoming complex frost damage (Lenz et al., 2013; Markovskaya and Shibaeva, 2017; Takahashi et al., 2018). The LT50 values of sapwood tissues from Korean pine and Simon poplar with MC above the FSP (approximately 30% MC), and particularly, above 80% MC, were generally lower by approximately 5–15°C than the case for the tissues with MC below the FSP (Figure 7). These findings suggest that sapwood tissues have a better ability to supercool (deep supercool) or tolerate freezing-induced cytosolic dehydration when the internal MC is relatively higher. In nature, as free water in cells is susceptible to freezing, some less cold-tolerant plants undergo dehydration during cold acclimation to adapt to freezing temperatures (Ameglio et al., 2015; Karlson et al., 2003; Rajashekar and Panda, 2014; Ouyang et al., 2019b). Even when plant cells are severely dehydrated due to freezing, injury to the cell membrane is reduced because water molecules bound to the cell membrane surface remain spatially separated from the inner cell membrane (Takahashi et al., 2018). Numerous studies have shown that plant tissues have evolved structural adaptations to accommodate the formation of large ice crystals in specific locations within the tissue (Wisniewski et al., 2009). This implies that despite the high-water contents within the tissue, the freezing of water always occurs at specific locations, and the formation of large ice crystals does not harm the tissues for a certain period of time (Wisniewski et al., 2014b). Guy (2003) proposed the control of MC to prevent intracellular freezing; however, this approach is complex and depends on factors such as the amount of liquid state water in the cell, cooling rate, volume ratio, and membrane permeability (Guy, 2003).

In nature, different types of plants typically freeze at relatively slow rates (−1 to 3°C/h) (Arora, 2018). In contrast, freezing of intracellular water under natural episodic frosts is uncommon, but can occur under artificial conditions that involve rapid freezing. For the *Pinus* samples evaluated in this study, the LT50 values were generally higher, ranging from 15 to 20°C, at a fast-cooling rate of 10°C/min than at a slow cooling rate of 2°C/min. The cooling rate had a significant effect on the LT50 values with $p<0.01$ for both species (Table 3). This result indicates that freezing damage occurs earlier when the environmental temperature drops at a faster rate.

Sapwood tissues typically employ two contrasting strategies to cope with freezing stress. When the cooling rate is slow, ice crystals first appear in the extracellular solution, creating a chemical potential difference between the osmotically active water within the cell and the extracellular ice. In contrast, when at fast cooling rates, the sapwood tissue typically resists freezing stresses through supercooling and deep supercooling, which helps prevent the formation of ice crystals (Levitt, 1980). However, at excessively low temperatures, the supercooled metastable liquid can freeze resulting in intracellular freezing that damages the membrane systems and inevitably leads to cell death. In the present study, supercooling and deep supercooling may not have occurred in the sapwood tissues of Korean pine and Simon poplar, as LTEs were not detected (Gusta et al., 1983). This suggests that the chemical potential imbalance induced by freezing stress was responsible for the different LT50 values observed in the two groups subjected to different cooling rates. Furthermore, the rate of cooling that leads to intracellular freezing is influenced by various factors, including the physiological state of the tissue, amount of frozen water in the cell, and permeability of the membrane to water (Arora, 2018). These results further highlight that freezing in cells may be attributed to the interplay of external freezing stress, tissue MC and tissue structure. From the findings of this study, it is evident that even at relatively fast cooling rates of 2°C/min and 10°C/min, some sapwood tissues of Korean pine and Simon poplar were able to survive at temperatures below −40°C. The differences in freezing

tolerance exhibited by these tissues is reflected in the LT50 values, as shown in Table 2. Weiser (1970) found that at cooling rates of 8 to 10°C/min, leaves already experienced frost damage at −10°C. This suggests that even at very high cooling rates, there may still be subtle differences in the freezing tolerance mechanisms of different plant tissues.

Different durations of freezing can result in varying levels of structural damage. When freezing persists for an extended period, tissue death can occur even if the temperature is considered "non-injurious" (Gusta et al., 1997). The LT50 values were significantly influenced by the duration of freezing (Table 3). Generally, for a 1 h freezing duration, the LT50 values were higher by approximately 10–15°C with an MC over 80% and 10–20°C with an MC below 80%, compared to a freezing duration of 0.5 h. These results suggest that tree tissues can tolerate short-versus extreme dehydration during freezing, but not prolonged dehydration (Gusta et al., 1997). Longer durations of freezing lead to the premature death of sapwood tissues, indicating the existence of different freezing tolerance mechanisms for short-versus and long-duration freezes. Min et al. (2014) observed a progressive increase in injury with longer freezing durations in spinach leaves. The findings of this study support the conclusions of a study by Min et al. (2014) and imply that cell injury is not solely influenced by the duration of freezing but is also related to the amount of water exuded during freezing.

Statistical analysis revealed significant interactions between freezing duration and cooling rate (Table 3), confirming that cell injury and death are influenced by the interplay of cooling rate and duration (Arora et al., 1992; Waalen et al., 2011). Although extracellular freezing is widely considered the main cause of injury due to cell dehydration, fatal damage resulting from freezing stress and prolonged freezing cannot solely be attributed to dehydration stress. This is likely due to a synergistic effect between different lesions. For example, an increased duration of mechanical stress and strain caused by extracellular ice and cell collapse, leading to shearing at the adhesion sites between the cell membrane and cell wall, can amplify the injurious effects of cellular dehydration over time (Min et al., 2014). Furthermore, the results of the present study suggest that the effects of short-duration and long-duration freezing vary across tree species. The occurrence of deep supercooling and extracellular freezing in the xylem is primarily influenced by the ring porous structure of the tissue and the cell wall thickness, with more elastic cell walls being susceptible to dehydration reactions during freezing. The ability of cells to resist collapse during freezing in response to dehydration is also related to the cell wall thickness (Gusta et al., 1983; Gusta et al., 1997).

Theoretically, the freezing of water in plant tissues cannot occur below −40°C except for the case of intracellular freezing, as the homogeneous freezing temperature of pure water is approximately −38.5°C (Pearce, 2001; Wisniewski et al., 2014a). However, non-homogeneous ice nucleation always occurs in plant tissues due to the presence of ice nucleating active substances in the extracellular space (Wisniewski et al., 2014b). This means that in nature, frost damage occurs earlier in plant tissues. Previous studies have also shown that the LT50 values in stem and seedling tissues of most tree species are concentrated around −40°C (Repo et al., 2022; Wu et al., 2019; Yu et al., 2017). In contrast, in the present study, the extreme LT50 values of sapwood tissues with an MC over 200% for Korean pine and Simon poplar, at slow cooling rates, were concentrated around −60°C (Figures 7A, B). This result may be attributed to the presence of dissolved electrolytes in the cells and structural differences between sapwood tissues and other plant tissues, as plant tissues with phloem, wood parenchyma, and meristematic tissue typically have a relatively low LT50 value when subjected to freezing (Lenz et al., 2013).

However, it is noteworthy that −60°C is very close to the extreme temperature for deep supercooling in plant tissues. Deep supercooling in plants can occur anywhere between −15 and −60°C, and −60°C is usually close to the extreme temperature in xylem tissues (Quamme, 1991; Peter, 2012, Wisniewski, 1995; Wisniewski et al., 2014b). In this study, LTEs were not detected during freezing, likely due to prolonged freezing within a certain temperature range, causing all the freezable water within the cells to escape from the xylem cells to distal sites. In contrast, LTEs were detected upon thawing in other species such as red ash (*Fraxinus pennsylvanica* Marsh.) (Gusta, 1983). However, determining the nucleation temperature and supercooling point is not a straightforward task due to its stochastic nature, and estimating these parameters requires 200–300 measurements on individual samples. This is not practical when studying whole plants, as continuous freezing and thawing alter tissue properties (Peter, 2012).

Therefore, the occurrence of freezing damage to plant tissues is influenced by various factors. such as the physiological state and structure of the tissues, MC, carbohydrate composition, cooling rate, and duration of freezing, among others. However, these factors are complex and often do not follow the assumption of a normal distribution and are challenging to effectively correlate with the degree of frost damage. From the relationships established between freezing factors and LT50 of sapwood tissue, it is evident that the MC and freezing duration played a more significant role in determining the change in the LT50 value of wood tissue, whereas the cooling rate had a lesser impact. Although the cooling rates of 2°C/min and 10°C/min are relatively rapid, their effect on the LT50 was different but minimal. However, it is important to consider the interactive effects of the MC, cooling rate, and freezing duration on the temperature at which freezing damage occurs. The R^2 of the RF model and the accuracy of the SVM model suggest that estimating the occurrence of freezing-induced damage to sapwood tissue based on the MC, cooling rate, and freezing duration is feasible.

5 Conclusions

Through the controlled laboratory experiment, the mechanism of freezing injury occurring in trees was investigated. We found that the freezing duration at subzero temperature had the greatest impact on the LT50 of wood tissue, followed by the MC, while the cooling rate had a minimal impact on LT50. Slow cooling combined with long-duration freezing at subzero temperatures led to earlier and more severe freezing damage. The findings of this study could aid the establishment of measures for preventing

damage and protecting trees and serve as important references for developing non-destructive and reliable electrical impedance-based methods for detecting freezing damage in trees.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

XS: Conceptualization, Data curation, Formal Analysis, Writing – original draft, Writing – review & editing. TG: Conceptualization, Methodology, Software, Writing – review & editing. MA: Data curation, Methodology, Resources, Software, Writing – review & editing. SG: Funding acquisition, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Whole-transcriptome profiling and identification of cold tolerance-related ceRNA networks in *japonica* rice varieties

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Introduction: Low-temperature stress negatively impacts rice yield, posing a significant risk to food security. While previous studies have explored the physiological and linear gene expression alterations in rice under low-temperature conditions, the changes in competing endogenous RNA (ceRNA) networks remain largely unexamined.

Methods: We conducted RNA sequencing on two *japonica* rice varieties with differing cold-tolerance capabilities to establish ceRNA networks. This enabled us to investigate the transcriptional regulatory network and molecular mechanisms that rice employs in response to low-temperature stress.

Results: We identified 364 differentially expressed circular RNAs (circRNAs), 224 differentially expressed microRNAs (miRNAs), and 12,183 differentially expressed messenger RNAs (mRNAs). *WRKY* family was the most prominent transcription factor family involved in cold tolerance. Based on the expression patterns and targeted relationships of these differentially expressed RNAs, we discerned five potential ceRNA networks related to low-temperature stress in rice: osa-miR166j-5p from the miR166 family was associated with cold tolerance; osa-miR528-3p and osa-miR156j-3p were linked to stress response; and osa-miR156j-3p was involved in the antioxidant system. In addition, *Os03g0152000* in the antioxidant system, as well as *Os12g0491800* and *Os05g0381400*, correlated with the corresponding stress response and circRNAs in the network. A gene sequence difference analysis and phenotypic validation of *Os11g0685700* (*OsWRKY61*) within the *WRKY* family suggested its potential role in regulating cold tolerance in rice.

Discussion and conclusion: We identified *Os11g0685700* (*OsWRKY61*) as a promising candidate gene for enhancing cold tolerance in *japonica* rice. The candidate miRNAs, mRNAs, and circRNAs uncovered in this study are valuable

targets for researchers and breeders. Our findings will facilitate the development of cold-tolerant rice varieties from multiple angles and provide critical directions for future research into the functions of cold-tolerance-related miRNAs, mRNAs, and circRNAs in rice.

KEYWORDS

rice, low-temperature, competing endogenous RNA, mRNA, miRNA

1 Introduction

Heilongjiang Province in China is a major hub for the production of high-quality *japonica* rice (Song et al., 2020). Cold damage is a global issue that particularly affects rice-producing countries like Japan, Korea, and China. The problem is exacerbated by the high latitude and low temperature in Heilongjiang Province, where rice seedlings are growing in April, with temperatures ranging from 3 to 15°C. Rice crops prefer warm conditions (Liu et al., 2020a; Liu et al., 2022). The capacity of rice to withstand cold temperatures is commonly referred to as cold tolerance or cold resistance. Typically, rice thrives at temperatures between 15 and 33°C. When temperature drops below this range, it can disrupt the plant's water metabolic balance by reducing its water-absorption capacity and transpiration rates, eventually causing cellular water loss. Low temperatures also reduce the enzymatic activity of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) in rice. This results in a sharp rise in levels of reactive oxygen species (ROS), which subsequently leads to lipid peroxidation in cell membranes, oxidative protein degradation, nucleic acid damage, and enzyme inactivation, thereby triggering programmed cell death (Wang et al., 2021; Xu et al., 2022b). During the seed germination phase, low temperatures can substantially reduce germination rates. In the seedling stage, they can cause symptoms such as leaf curling, seedling stiffness, wilting, reduced dry matter weight, stunted growth, and even plant death in extreme cases. These conditions can also adversely affect the late tillering stage of the plant's life cycle (Shimono, 2011). A significant yield loss or even complete crop failure can occur in case of early snowfall (Jia et al., 2019). Furthermore, low temperatures during the reproductive growth stage can cause pollen sterility and a substantial reduction in fruit set. It is estimated that cold damage reduces China's annual rice production by 3 to 5 billion kg. The vulnerability of Heilongjiang to cold inversion during spring seasons poses a significant risk to the growth and development of rice seedlings in the region.

circRNAs are non-coding RNA molecules that form a closed loop consisting of one or more exons connected by a spliceosome complex (Xu et al., 2022a). Unlike typical linear RNAs, circRNAs lack a 5' cap structure and 3' polyadenylation, making them resistant to nucleic acid exonucleases. As a result, they are relatively stable and conserved (Li et al., 2016). A single gene can generate multiple types of circRNAs, some of which are expressed at higher levels than their corresponding linear RNAs and function as miRNA sponges (Wu et al., 2020). Previously, circRNAs were believed to be the byproducts of splicing errors until the 1970s, when Sanger et al. identified these closed-loop RNA molecules in plant viruses and confirmed their presence in eukaryotic cells (Sanger et al., 1976). The advent of high-throughput sequencing technology allowed the identification of plant circRNAs in *Arabidopsis thaliana* in 2014 (Zhao et al., 2019). Similar to their animal counterparts, plants produce diverse types of circRNAs through selective back-splicing (Romero-Barrios et al., 2018). circRNA-related studies in a variety of plants, including maize (Han et al., 2020), rice (Lu et al., 2015), wheat (Xu et al., 2019), barley (Darbani et al., 2016), and soybean (Wang et al., 2020) have revealed that circRNAs can originate from exons, introns, or intergenic regions (Liu et al., 2020b). They have also shown that circRNA expression patterns often vary across different tissues and developmental stages (Huang et al., 2020). Furthermore, evidence suggests that circRNAs are more stable than linear RNAs (Zhong et al., 2018) and may play roles in various plant functions through specialized mechanisms, including regulating chlorophyll metabolism, hormone signaling, flower development, fruit ripening, and leaf senescence (Zhang et al., 2017). Additionally, they modulate distinct responses to different stress conditions.

circRNAs can serve as ceRNA or miRNA sponges, playing a pivotal role in various biological processes. A study identified 31 differentially expressed circRNAs, 47 differentially expressed miRNAs, and 4,779 differentially expressed mRNAs in rice. Using Cytoscape software, the researchers constructed miRNA-mediated regulatory and ceRNA networks. Their findings indicated that the circRNAs A02:23507399|23531438 are post-transcriptionally significant and regulate anther development (Liang et al., 2019). Additionally, senescence-associated circRNAs are implicated in flag leaf senescence via parental gene expression and ceRNA network regulation (Huang et al., 2021). However, the literature is scant concerning the role of circRNAs in regulating low-temperature responses in rice. To date, none of the studies have explored ceRNA networks specifically related to cold tolerance in rice. Consequently, it remains uncertain whether circRNA–miRNA–mRNA targeting interaction networks participate in rice's response to low-temperature stress.

The objective of this study was to construct a ceRNA network to understand how rice responds to low-temperature stress. Our findings shed light on the functional roles of *japonica* rice and lay the groundwork for future research into circRNAs, as well as offer insights into the regulatory mechanisms governing cold tolerance in this rice variety.

2 Materials and methods

2.1 Plant materials and cold treatments

The study was carried out in November 2021 at Northeast Agricultural University in Harbin, Heilongjiang Province, China (longitude: 126°22'-126°50'; latitude: 45°34'-45°46'N). Two rice varieties were selected for the experiment: JL (Jilin Sunset), which is cold-tolerant, and JH (Jinhe), which has low cold tolerance. For seed preparation, rice seeds were sequentially sterilized using a 75% ethanol solution for 2 min and a 2% H2O2 solution for 30 min. The seeds were then rinsed three times with sterile water and allowed to germinate for 3 days. Subsequently, one germinated seed was placed in each hole of a hydroponic setup. The nutrient solution for the hydroponic system was formulated based on the International Rice Research Institute's (IRRI) standard nutrient solution guidelines. The seedlings were incubated in an intelligent artificial climate chamber (model FYS-20, Nanjing, China) under controlled conditions that practically simulated the seedling growth conditions of Heilongjiang rice: 20°C day/18°C night temperature, 12-h light/12-h dark cycle, and 50% relative humidity. When the seedlings reached the "three leaves and one heart" developmental stage, they were transferred to the same climate chamber but set at 4°C for cold treatment (Huang et al., 2021).

2.2 Sample collection and mRNA extraction for sequencing and quality control

Leaves from the JL and JH rice varieties were harvested at 0, 4, 12, 24, and 48 h following the initiation of cold treatment. Each time point had three replicates for each variety. Each replicate was composed of a pooled sample of leaves from three individual seedlings, resulting in a total of 30 samples collected across both varieties (2 varieties × 5 time points × 3 replicates). These samples were immediately frozen in liquid nitrogen and stored at -80°C until further analysis. Total RNA was extracted from the leaf samples using an RNA extraction kit (Tiangen Biotech, Beijing, China). The concentration and integrity of the RNA was assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Residual rRNA was removed from the total RNA using an rRNA Removal Kit (Epicentre Technologies, Madison, WI, USA). An aliquot of the purified RNA underwent first-strand cDNA synthesis, followed by second-strand cDNA synthesis, end repair, and 3' end addition.

After sequencing, the raw data were subjected to quality analysis, and low-quality sequences and junctions were removed. The remaining sequences were aligned to the Nipponbare reference genome (*Oryza sativa*/IRGSP_1.0_release_62) using the default parameter of HISAT2 (v2.2.1.0) (Kim et al., 2015), which was made available through the Ensemble Plants database (https://plants.ensembl.org/index.html). Post-alignment, StringTie (version 2.2.0) is used to assemble and quantify the sequence according to the default parameters (Pertea et al., 2016). Raw gene expression levels were determined based on read counts for each gene and were subsequently normalized using Fragments Per Kilobase Million (FPKM). Differential gene expression analysis between sample groups was conducted using DESeq2 (version 1.28). Genes were considered differentially expressed if they met the following criteria: an absolute $\log_2$ fold-change ($|\log_2^{FC}|$) greater than or equal to 1 and a false discovery rate (FDR) < 0.01. The FDR was calculated by adjusting the p-value for the significance of differences between groups.

2.3 Sequencing and differential analysis of circRNA

An aliquot of the extracted RNA was treated with RNaseR (Epicentre, Madison, WI, USA) to selectively remove linear RNA and construct a circRNA-seq library. This library was sequenced by Biomarker Ltd. (Beijing, China) on the Illumina NovaSeq 6000 platform. Low-quality reads, defined as those with > 5% unknown ('N') bases, splice sequences, or > 50% bases with a low-quality score ($Q \leq 20$), were excluded from the raw data. The remaining high-quality reads were aligned to the rice reference genome using the HISAT software (Kim et al., 2015). circRNAs were identified using the Find_circ software (version1.1) with default parameters (Memczak et al., 2013). The expression levels of the identified circRNAs were normalized following a previously established method (Zhu et al., 2019). Differential expression analysis was conducted using DESeq software, with a screening criterion for differentially expressed circRNAs set at an absolute $\log_2$ fold change ($|\log_2^{FC}|$) > 1 and P-values < 0.05. Genes giving rise to differentially expressed circRNAs were subjected to Gene Ontology (GO) (Version2.10.0) enrichment analysis (Consortium, 2004). GO terms with $P < 0.05$ were considered significantly enriched. Additionally, Kyoto Gene and Genome Encyclopedia (KEGG) R package (version1.4.0) pathway enrichment analysis was performed using the R package (Kanehisa et al., 2016); pathways with a $P < 0.05$ were deemed significantly enriched.

2.4 miRNA library construction, sequencing, and quality control

Total RNA was isolated from each sample using TRIzol reagent (Invitrogen, USA) and miRNA was extracted from the total RNA through size fractionation on 15% PAGE gels. RNA molecules ranging from 18 to 30 nucleotide (nt) in length were purified from these gels. Adapters were ligated to both the 5′ and 3′ ends of the miRNA molecules, followed by reverse transcription and PCR amplification, resulting in the creation of 30 miRNA libraries.

These libraries were then sequenced on an Illumina HiSeq 2500 platform.

Upon sequencing, the raw reads underwent initial quality control steps. This involved the removal of sequences < 18 nt or > 30 nt, sequences with unidentified bases making up ≥ 10% of the read, and sequences lacking 3′ end splice sites. The 3′ end splice sequences were subsequently trimmed to generate clean reads, which were then aligned against various databases, including Silva (Version138) (Quast et al., 2012), GtRNAdb (version 3.0) (Chan and Lowe, 2016), Repbase (version21.01) (Jurka et al., 2005), and Rfam (version12.0) (Nawrocki et al., 2015). Following this, unannotated reads containing miRNAs were identified by filtering out snoRNA, tRNA, snRNA, rRNA, and other non-coding RNAs (ncRNAs), as well as repetitive sequences. These filtered, unannotated reads were then aligned to the rice reference genome (*Oryza sativa*, MSU_v7.0) using Bowtie software (version 1.1.2) (Langmead and Salzberg, 2012) with default parameters to examine their expression and distribution. Differential expression analysis was subsequently conducted using DESeq2 (version 1.28), with screening criteria set at an absolute log-fold change ($|\log_{1.5}^{FC}|$) of ≥ 1 and P-values ≤ 0.05.

2.5 Analysis of the ceRNA network construction

To construct the circRNA-miRNA-mRNA network, we initially selected differentially expressed circRNAs, differentially expressed miRNAs, and differentially expressed mRNAs in accordance with ceRNA theory. TargetFinder (Version 2.0) (Bo and Wang, 2005) was employed to predict the target interactions between circRNAs and miRNAs, while psRNATarget (Version2017) (Dai and Zhao, 2011) was used for predicting the target interactions between miRNAs and mRNAs. We list the detailed parameters used by both software. TargetFinder: with parameters as -c (Prediction score cutoff value) 4 were used to predict target genes. Mismatches, single-nucleotide gaps or single-nucleotide bulges are assessed with a penalty of +1. G:U base pairs are assessed a penalty of +0.5. Penalty scores are doubled at positions 2-13 relative to the 5' end of the small RNA query sequence. Duplexes are rejected if they: have more than one single-nucleotide bulge or gap; have more than seven total mismatches, G:U base pairs, bulges and gaps; have more than four total mismatches or four total G:U base pairs. PsRNAtarget:Max Expectation cutoff: 5.0; HSP length for scoring: 19; Penalty for GU pair: 0.5; Penalty for other mismatch: 1.0; Penalty for opening gap: 2.0 Penalty for extending gap: 0.5. RNAhybrid is used to calculate RNA : RNA interaction regions with the default parameters, according to the thermodynamics of RNA structure formation and several "targeted mimicry rules" (Krüger and Rehmsmeier, 2006); psRobot was used to analyze the targeting relationship between circRNA:miRNA and miRNA:mRNA and the correlation between them according to the default parameters (Wu et al., 2012). A p-value < 0.05 was used to select statistically significant RNA : RNA and target:ncRNA interactions predicted with the four programs, in addition to: a "maximum expectation score" of 5 for psRNATarget, a "maximum prediction score" of 6.5 for TargetFinder, a low minimum free energy (MFE) value for RNAhydrid, and "low target scores" between a threshold of 0 and 5 for psRobot. These relationships were further refined based on the correlations between their expression levels. Finally, ceRNA networks relevant to cold tolerance regulation were visualized using Cytoscape software (Version3.2.1) (Shannon et al., 2003).

2.6 RNA extraction and qRT-PCR

Rice leaves were subjected to cold treatment at 4°C for 0, 4, 12, 24, and 48 h. Total RNA was extracted using the TranZol-UpRNA kit (Tiangen Biotechnology, Beijing, China). The reverse transcription process was conducted using the HiFiScript cDNA synthesis kit (Cwbio, Beijing, China). qRT-PCR was carried out on a Roche LightCycler 2.10 system, with each sample having three technical replicates and three biological replicates. The housekeeping gene *Actin1* was used for expression normalization. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The correlation between RNA-Seq and qRT-PCR data was assessed using Pearson's correlation, based on absolute log-fold change ($|\log_{10}^{FC}|$), and was analyzed using SPSS software (SPSS Inc., Chicago, IL, USA). The primer sequences used for the PCR reactions are listed in Supplementary Table 1.

2.7 Sequencing and sequence alignment analysis of *OsWRKY61*

The coding sequence (CDS) region of the *OsWRKY61* gene was cloned and sequenced from both the JH and JL rice varieties using PCR. Sequence alignment was conducted using DNAMAN software (version 5.0), with the genes from the Nipponbare genome serving as a reference.

2.8 Construction of *OsWRKY61* overexpression plants

For *OsWRKY61* overexpression, cDNA from the JH rice variety was used as a template. The full-length *OsWRKY61* coding sequence was cloned into the pUbi:1390-3Flag plant expression vector, which was subsequently transformed into *Agrobacterium tumefaciens* strain EHA105. For the rice transformation, mature rice seeds were dehusked and sterilized in a 3% sodium hypochlorite solution for 30 min. The seeds were then rinsed three times with sterile distilled water and placed on MS basal medium supplemented with 500 mg/L proline, 300 mg/L casein hydrolysate, 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and 30 g/L sucrose. Finally, the seeds were incubated for one week. Freshly induced calli were inoculated with the prepared Agrobacterium culture and transferred to NB medium composed of N6 macro-elements and B5 micro-elements, along with 500 mg/L proline, 300 mg/L casein hydrolysate, and 30 g/L sucrose. After two days in these conditions (minimal to no light exposure, 28°C), the calli were transferred to fresh NB medium containing 2 mg/L 2,4-D, 50 mg/L hygromycin, and 500 mg/L cefotaxime. Subsequently, resistant calli were subcultured on fresh plates at two-week

intervals over a period of four weeks. These were then transferred to MS medium supplemented with 0.2 mg/L α-naphthalene acetic acid (NAA), 3 mg/L 6-benzylaminopurine (6-BA), and 30 mg/L hygromycin until shoots regenerated. For the final rooting and further growth, shoots were transferred to 1/2 MS medium containing 0.5 mg/L NAA. Eventually, the plantlets were transferred to a greenhouse for further growth.

2.9 Determination of cold tolerance in *OsWRKY61* overexpression plants

The overexpression and wild-type (WT) plants that were previously screened were cultivated together in the "Three leaves and one heart" stage under the following conditions: 20°C day/18°C night temperature, 12-h light/12-h dark cycle, and 50% relative humidity. These plants then underwent cold treatment at 4°C and subsequently samples were collected at 0, 12, 24, and 48 h following the initiation of cold treatment. Commercial kits from Suzhou Grace Biotechnology Co., Ltd. (Jiangsu, China) were employed to quantify the levels of SOD, POD, PRO, and MDA, in accordance with the manufacturer's guidelines.

3 Results

3.1 High-throughput sequencing of mRNA and circRNA

To comprehensively assess the expression profiles of circRNAs and mRNAs related to cold tolerance in *japonica* rice seedlings, we performed whole-transcriptome RNA-seq analyses. These analyses targeted the leaves of control and cold-treated seedlings from two *japonica* rice varieties, JH and JL, using an Illumina sequencer. Each sample set, consisting of two varieties at five time points with three replicates each, was analyzed in three independent biological replicates. After filtering out low-quality and contaminated reads, the aggregated data from both JH and JL yielded approximately 3.3 billion high-quality reads. These high-quality reads mapped to between 95.97% and 97.86% of the rice reference genome. Furthermore, all samples displayed Q30 values above 93.64% and their GC contents ranged from 46.55% to 48.21% (Supplementary Table 2), thereby confirming the reliability of the RNA-seq data. Our results generated a robust dataset for the subsequent examination of mRNA and circRNA expression profiles.

3.2 mRNA identification and differential analysis

Clean reads from each sample were aligned to the reference genome, yielding 36,850 mRNAs. Based on the screening criteria of an absolute $|\log_2^{FC}|$ of ≥ 1 and FDR < 0.01, we identified 12,817 and 15,806 differentially expressed mRNAs in the JH and JL varieties, respectively. Compared to the control at 0 h, JH exhibited 8,088 significantly upregulated genes and 4,729 significantly downregulated genes, whereas JL showed 10,446 upregulated and 5,363 downregulated genes. A comparative analysis between the two varieties at identical time points revealed 3,102 genes significantly upregulated and 3,028 genes downregulated in JL compared to JH (Figure 1A). Genomic coordinates and expression values of all differential mRNAs are organized in Supplementary Table 3. Our interspecies comparison identified a considerable number of differential transcription factors, with the *WRKY* family containing the highest count at 13 (Figure 1B), and notably, previous research has demonstrated the importance of WRKY in various abiotic stress response pathways, including those for drought, salinity, temperature, and ultraviolet radiation (Li et al., 2020).

3.3 Functionality of *OsWRKY63* in cold tolerance

The gene *OsWRKY63* has been determined to play a role in cold regulation (Zhang et al., 2022). Under normal conditions, the overexpression strain of *Os11g0686250* (*OsWRKY63*) from the *WRKY* family displayed no significant differences compared to the wild type. However, under cold stress conditions, the overexpression strain showed both a significantly lower fruit set rate and reduced seedling survival rate compared to the wild type. In contrast, CRISPR/Cas9-mediated knockdown of *OsWRKY63* led to enhanced cold tolerance (Zhang et al., 2022). Importantly, among the differentially expressed *WRKY* transcription factors identified, *Os11g0685700* (*OsWRKY61*) exhibited a similar response pattern compared to *OsWRKY63*. It showed high expression exclusively in the JH variety across all five time points, while its expression in the JL variety was virtually negligible, displaying a substantial fold change at each time point. Further, our cold-response sequencing data indicated that *OsWRKY61* had a higher ploidy level compared to *OsWRKY63* (Figure 1C). Based on these findings, we hypothesize that *OsWRKY61* likely possesses a regulatory function similar to that of *OsWRKY63* in rice, and we have initiated experiments involving its overexpression.

3.4 Identification and differential analysis of circRNA

A comprehensive set of 3,387 circRNAs was identified from the transcriptome data of 30 samples (Supplementary Table 4), using CIRI2 (version1.2) with default parameters, a circRNA prediction software. The expression levels of these circRNAs, measured in FPKM pair values, predominantly ranged from -1 to 2 (Supplementary Figure 1). These identified circRNAs were categorized into three distinct groups based on their expression in the rice varieties: 526 circRNAs were unique to JH, 351 were exclusive to JL, and 2,510 were expressed in both varieties (Figure 2A). Further classification based on genomic location showed that 234 circRNAs were in intergenic regions, 503 in intronic regions, 2,591 in exonic regions, and 59 in antisense strands of genes (Figure 2B). Chromosome distribution analysis

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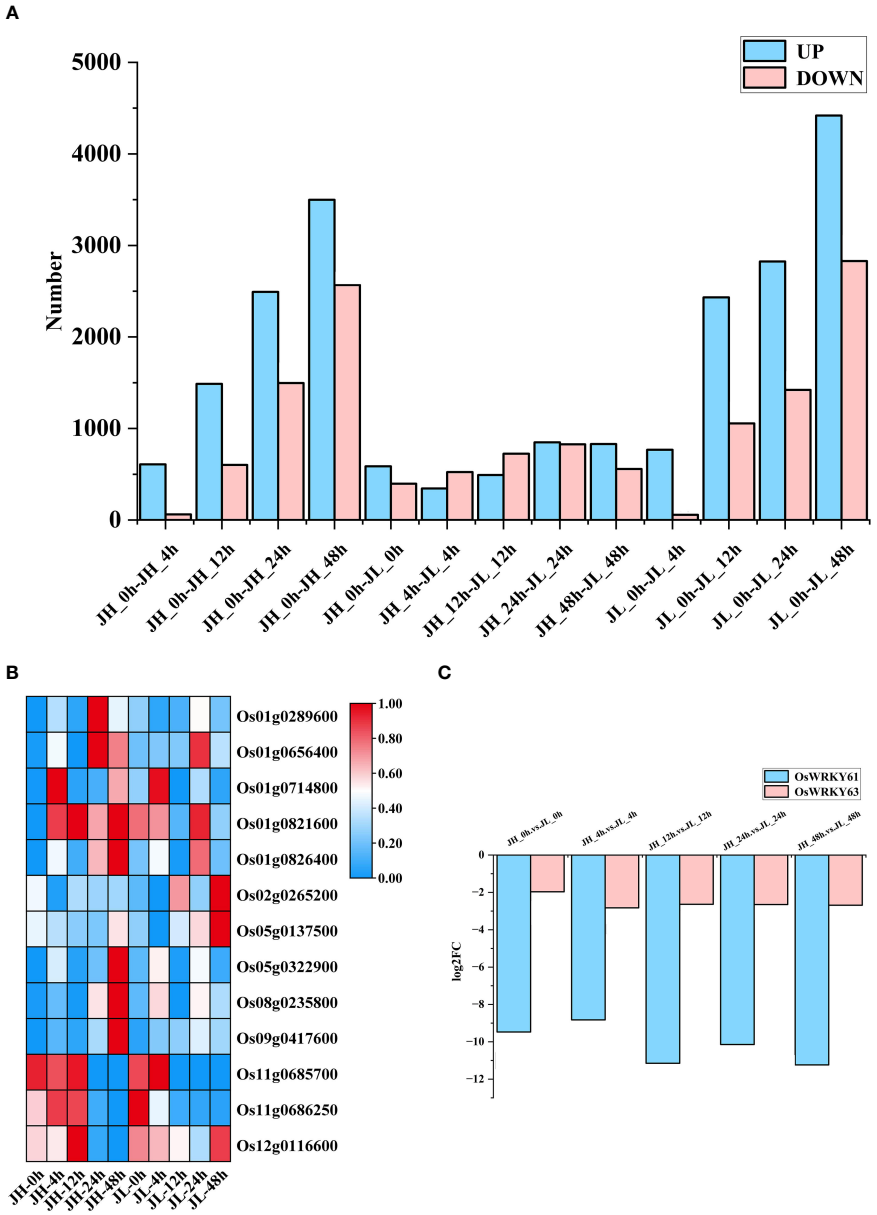

FIGURE 1
Differential analysis of mRNA expression in rice seedling leaves. **(A)** Number of differentially expressed genes in JH and JL varieties at various time points following cold treatment. **(B)** Representation of WRKY family members among differentially expressed transcription factors. **(C)** Temporal expression patterns of *OsWRKY61* and *OsWRKY63* based on transcriptome data after cold treatment.

indicated that a majority of the circRNAs were primarily located on chromosomes 1, 2, and 3 (Figure 2C). Differential expression analysis of the circRNAs was conducted using the edgeR v3.24.3 analytical model. Applying stringent screening criteria of $|\log_2^{FC}| > 1$ and a P-value < 0.05, we identified 364 differentially expressed circRNAs. Specifically, 154 differentially expressed circRNAs were observed in JH and 142 in JL, compared to their respective controls at 0 h. Additionally, a comparative analysis between the two varieties at the same time point revealed 68 differentially expressed circRNAs in relation to JH (Figure 2D).

GO enrichment analysis of the parental genes for all differentially expressed circRNAs revealed significant involvement in various biological processes. These included cellular metabolic compound salvage, reductive pentose-phosphate cycle, photosynthesis (encompassing both dark reaction and photorespiration), carbon fixation, protein autophosphorylation, sulfur amino acid biosynthetic processes, and Golgi localization (Supplementary Table 5). Concurrently, KEGG enrichment analysis identified several significantly enriched pathways for these differentially expressed circRNAs. Notable pathways included glyoxylate and dicarboxylate metabolism, taurine and hypotaurine metabolism, carbon fixation in photosynthetic organisms, arachidonic acid metabolism, ubiquinone and other terpenoid-quinone biosynthesis, selenocompound metabolism, biosynthesis of secondary metabolites, isoquinoline alkaloid biosynthesis, and tropane, piperidine, and pyridine alkaloid biosynthesis (Supplementary Table 6) (Figure 2E).

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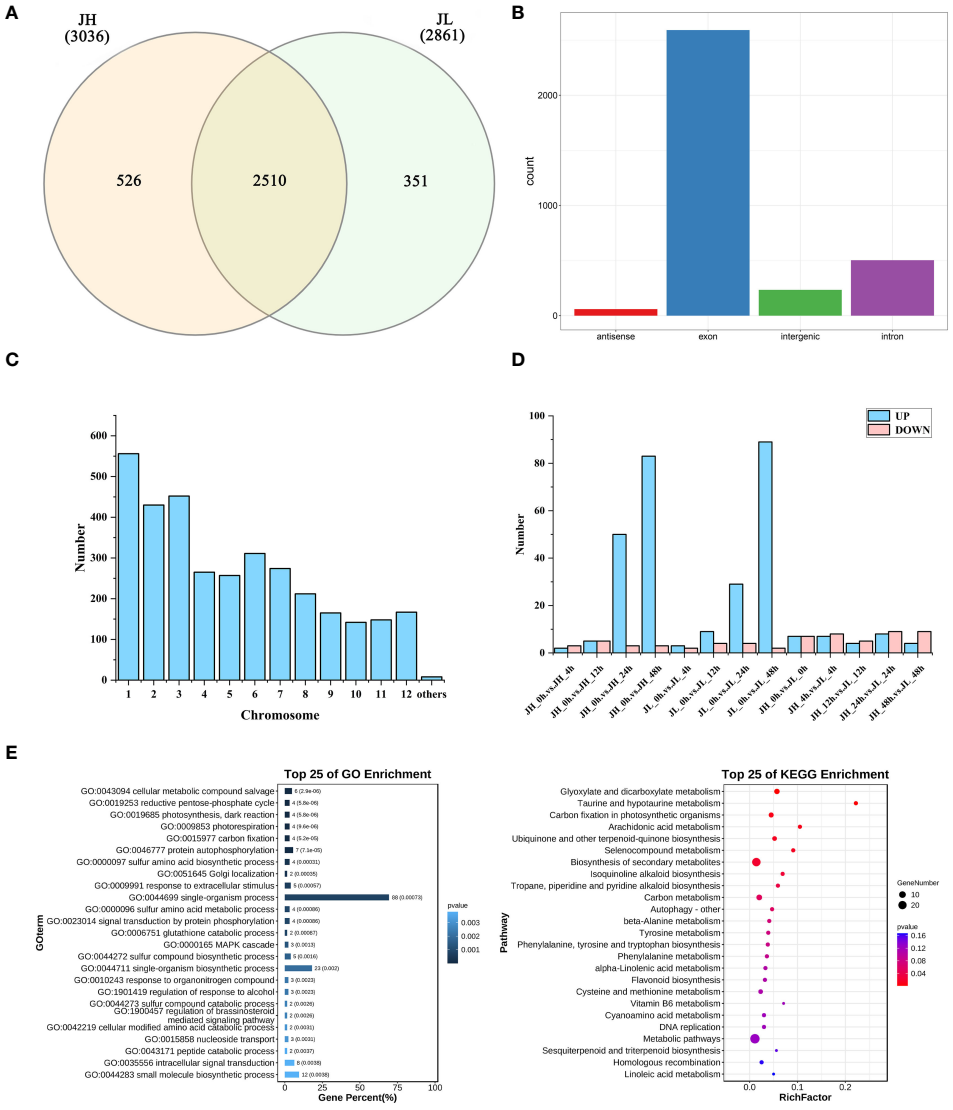

FIGURE 2
Identification and differential analysis of circRNAs in rice seedling leaves. **(A)** Count of differentially expressed genes in both JH and JL varieties after cold treatment. **(B)** Categorization of circRNAs based on their genomic position. **(C)** Chromosomal distribution of identified circRNAs. **(D)** Number of differentially expressed genes in JH and JL at each time point following cold treatment. **(E)** GO and KEGG enrichment analyses of parental genes for all differentially expressed circRNAs.

3.5 Identification and differential analysis of miRNAs

miRNA sequencing was performed on all samples, generating 409,055,593 clean reads. Using Bowtie software (version 1.1.2), we identified 542 miRNAs (Supplementary Table 7); 409 of these were previously known, while 113 were newly discovered. The majority of identified miRNAs had lengths of either 21 nucleotides (nt) (49.82% miRNAs) or 22 nt (20.30% miRNAs) (Figure 3A). Utilizing edgeR, we conducted a differential expression analysis of miRNAs based on the criteria of $|\log_{1.5}^{FC}| \geq 1$ and $P \leq 0.05$. For the JH and JL varieties, differentially expressed miRNAs were identified at 0 h vs 4 h, 0 h vs 12 h, 0 h vs 24 h, and 0 h vs 48 h time points, with the counts being (41, 32, 55, 24) and (33, 34, 27, 36), respectively. A comparative analysis between JH and JL at these time points revealed the presence of 78, 86, 37, 78, 64 miRNAs that were differentially expressed in both varieties (Figure 3B).

3.6 Construction of ceRNA networks

The regulatory network involving circRNAs, miRNAs, and mRNAs functioning in response to low-temperature stress in rice was investigated. Differentially expressed miRNAs that appeared more than five times in each comparison were filtered and eventually the following five key miRNAs were identified: osa-miR528-3p, osa-miR166j-5p, osa-miR159a.2, osa-miR156j-3p, and osa-miR1428e-5p. Target genes for these miRNAs were predicted using psRNATarget software (Version2017) (Supplementary Table 8). Among the significantly differentially expressed mRNAs,

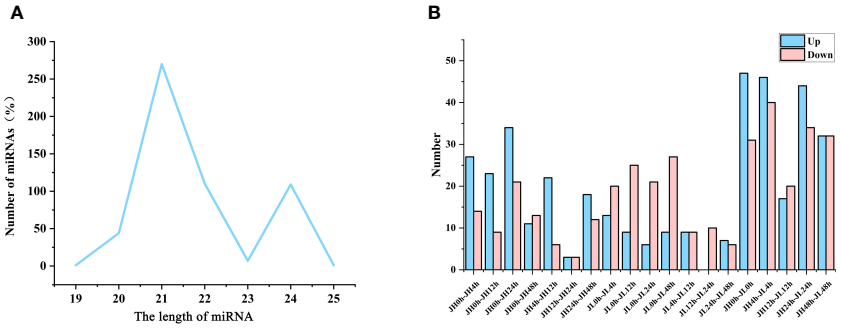

FIGURE 3
Identification and differential analysis of miRNAs in rice seedling leaves. **(A)** Length distribution of the identified miRNAs. **(B)** Count of differentially expressed genes in JH and JL varieties at various time points following cold treatment.

those showing inverse expression patterns relative to the miRNAs were selected, yielding a total of 25 relevant target genes. The circRNA-miRNA interaction pairs were forecasted using TargetFinder (Version 2.0) software (Supplementary Table 9), and 16 differentially expressed circRNAs were identified using a similar methodology. For these predicted relationship pairs, RNAhybrid and psRobot software were used to conduct thermodynamic calculation of RNA structure formation for RNA : RNA interaction region analysis, further confirming the reliability of the results obtained by psRNATarget and TargetFinder software (Supplementary Tables 10-13). Subsequently, five ceRNA networks were established, elucidating the reciprocal relationships among these circRNAs, miRNAs, and mRNAs (Supplementary Table 14) (Figure 4).

3.7 Validation of differentially expressed genes

To validate our RNA-Seq findings, we performed qPCR analysis on selected candidate genes from the ceRNA network. We compared the log-fold changes for these 10 selected differentially expressed genes between the RNA-Seq and qRT-PCR datasets. Each sample for qRT-PCR was analyzed using three technical and three

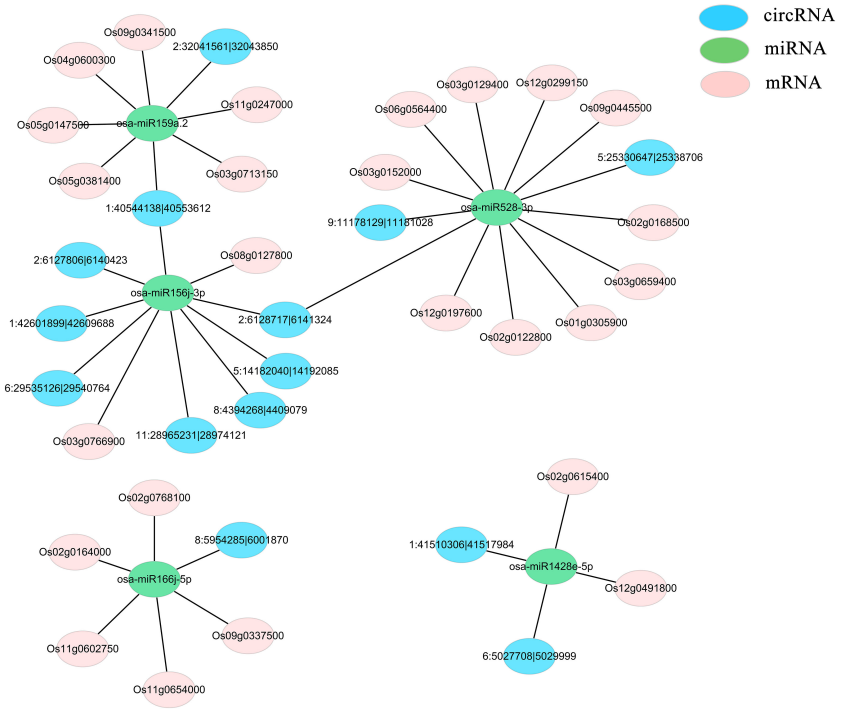

FIGURE 4
CeRNA network comprising significantly differentially expressed mRNAs, miRNAs, and circRNAs. Blue represents circRNAs, pink denotes mRNAs, and green indicates miRNAs.

biological replicates. A correlation coefficient (R^2) of 0.8795 was obtained (Supplementary Figure 2), demonstrating strong agreement between the qRT-PCR and RNA-Seq data. This result reinforces the reliability of our RNA-Seq findings.

3.8 Sequence variation analysis of *OsWRKY61*

To further investigate the role of *OsWRKY61* in modulating cold tolerance in rice, we amplified the coding sequence (CDS) of *OsWRKY61* from JH and JL rice varieties. The PCR was carried out using the Nipponbare genome as a reference sequence, followed by separate sequencing of each variety. In comparison to the Nipponbare sequence, the CDS of JH was identical. However, an SNP was identified in the CDS sequence of JL when compared to the Nipponbare reference (Figure 5); specifically, where Nipponbare has an alanine at this base, JL features an arginine.

3.9 Phenotype identification of *OsWRKY61* overexpression plants

Initial DNA-level identification was performed on both T0 and T1 generations. Subsequent analysis focused on the T2 generation, where PCR and qRT-PCR methods were employed to evaluate plants with *OsWRKY61* overexpression. Firstly, DNA samples were extracted from *OsWRKY61* overexpression plants (labeled as WRKYOE-1 and WRKYOE-2) and the WT plants. These DNA samples served as templates for PCR amplification using expression vector primers, whose sequences are provided in Supplementary Table 1. No bands were detected in the WT samples, whereas distinct bands with consistent PCR product lengths were observed in the *OsWRKY61* overexpression plants (WRKYOE-1 and WRKYOE-2). This confirmed that the vector was absent in the WT plants. Next, RNA was extracted from both the *OsWRKY61* overexpression plants (WRKYOE-1 and WRKYOE-2) and the WT plants and reverse-transcribed into cDNA. This cDNA served as the template for the RT-qPCR reaction, carried out according to the previously established RT-qPCR protocol. The data indicated that the expression levels of *OsWRKY61* in WRKYOE-1 and WRKYOE-2 were significantly higher than those in the WT plants (Supplementary Figure 3). This provides evidence that *OsWRKY61* was overexpressed in the WRKYOE-1 and WRKYOE-2 plants.

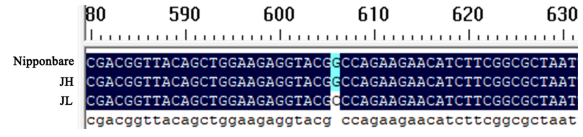

FIGURE 5
Comparative analysis of CDS differences in *OsWRKY61* between the two rice varieties. The Nipponbare genome serves as the reference sequence.

SOD, POD, PRO, and MDA are vital indicators for assessing cellular physiological responses to cold stress. After 12 and 48 h cold treatment, both wild-type and overexpression plants showed elevated levels of SOD, POD, PRO, and MDA compared to the levels of the control group at 0 h. While the PRO content was comparable between the wild-type and overexpression plants at 48 h, the overexpression plants demonstrated lower levels of SOD, POD, PRO, and MDA throughout the study compared to the wild-type plants (Figure 6A). The discrepancies in these physiological indicators between the wild-type and overexpression plants became more pronounced as the duration of cold treatment increased. Furthermore, the overexpression plants displayed heightened phenotypic vulnerability to low temperatures in comparison to their wild-type counterparts (Figure 6B). These results suggest that *OsWRKY61* overexpression reduces cold tolerance in rice seedlings.

4 Discussion

4.1 Identification and characterization of circRNAs in rice

circRNAs, a subclass of non-coding RNAs, have increasingly gained attention for their roles in various biological processes such as stress responses, growth, and development (Zhu et al., 2019). Advances in high-throughput sequencing and bioinformatics have enabled the identification of circRNAs associated with a range of stress conditions, including high temperatures (Zhao et al., 2020), salinity (Dong et al., 2022), and senescence (Huang et al., 2021). Nonetheless, the role of the ceRNA network in rice's response to low-temperature stress remains largely unexplored.

In the current study, we identified a total of 3,387 circRNAs, consisting of 3,036 circRNAs in the cold-sensitive JH variety and 2,861 circRNAs in the cold-tolerant JL variety. A comparative analysis between the two varieties revealed that 2,510 circRNAs were expressed in both types (Figure 2A), emphasizing the unique, variety-specific expression patterns of circRNAs in rice seedlings.

4.2 Rice circRNAs and their role in low-temperature stress response

The response of rice to low-temperature stress is a multifaceted process, involving the activation of low-temperature-responsive genes and signaling pathways. In our study, we identified 364 circRNAs that exhibited significant differential expression under conditions of low-temperature stress (Figure 2D). Specifically, 154 and 142 differentially expressed circRNAs were identified in the cold-sensitive JH variety and the cold-tolerant JL variety, respectively, in comparison to the control group at 0 h. Additionally, a side-by-side comparison of the two varieties at the same time point revealed 68 differentially expressed circRNAs in JH relative to those in JL. GO and KEGG pathway enrichment analyses of the host genes for these differentially expressed circRNAs indicated their involvement in a diverse set of metabolic

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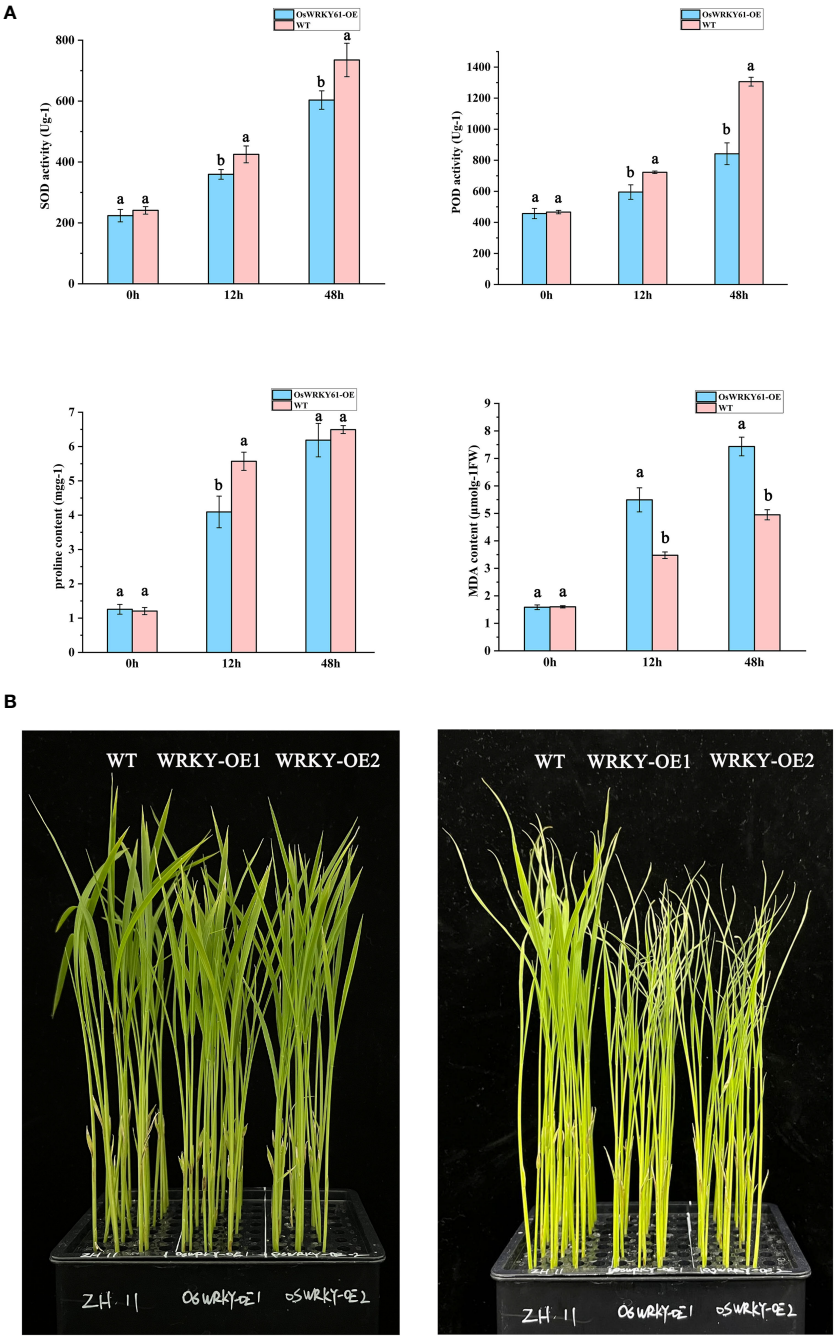

FIGURE 6
Physiological characterization of *OsWRKY61* overexpression plants following cold treatment. **(A)** Changes in SOD, POD, PRO, and MDA content levels in both overexpression and wild-type (WT) strains after low-temperature exposure. Labels "a" and "b" indicate statistical significance between varieties at the 0.05 level. **(B)** Phenotypic evaluation of overexpression and WT strains before and after both normothermic and low-temperature treatments. The left panel displays phenotypes before the initiation of low-temperature treatment, while the right panel shows phenotypes after 5 days of low-temperature exposure.

pathways. Notably, these host genes were enriched in pathways related to cellular metabolic compound salvage, the reductive pentose-phosphate cycle, various aspects of photosynthesis (including dark reactions and photorespiration), carbon fixation, protein autophosphorylation, glyoxylate and dicarboxylate metabolism, carbon fixation in photosynthetic organisms, arachidonic acid metabolism, and terpenoid-quinone biosynthesis (Figure 2E). Significantly, enhanced activity in the pentose-phosphate cycle has been linked to increased NADPH content, which is known to improve a plant's ability to neutralize ROS and thereby enhance cold tolerance (Tian et al., 2021).

Protein phosphorylation pathways play critical roles in responses to various stressors (Tian et al., 2021). A proteomic analysis of winter turnip rape under cold stress revealed

significant enrichment in similar metabolic pathways, including those involved in photosynthesis and carbon metabolism (Xu et al., 2018). Consistently, our study also identified a significant number of pathways related to photosynthesis and carbon metabolism, such as photosynthesis, dark reaction, photorespiration, carbon fixation, glyoxylate and dicarboxylate metabolism, and carbon fixation in photosynthetic organisms. This leads us to hypothesize that low-temperature conditions have a considerable impact on photosynthesis and carbon metabolism in plants, which will be a key area for our future research endeavors.

4.3 Potential circRNA-miRNA-mRNA regulatory network in rice in response to low-temperature stress

circRNAs modulate the expression of miRNA target genes by competitively binding to miRNAs, thereby suppressing latter's silencing effect on these target genes. In this study, we investigated the circRNA-miRNA-mRNA regulatory network relevant to low-temperature stress in rice. We constructed five circRNA-miRNA-mRNA regulatory modules potentially associated with rice's response to such stress conditions (Figure 4). By examining the functions of these differentially expressed target genes, we aim to elucidate the potential roles of these circRNA-miRNA-mRNA regulatory modules. In previous research, osa-miR528-3p was found to be significantly upregulated under cadmium stress in two rice varieties (Liu et al., 2020). However, our present study revealed that this gene was markedly downregulated during 4, 12, 24, and 48 h cold treatment in the JL variety, as well as at 4, 12, and 24 h when comparing the JL variety to the JH variety. miR166 enhances the resistance to abiotic stress in rice by mitigating oxidative damage. Additionally, miR166k and miR166h are implicated in regulating disease resistance in rice (Ding et al., 2018; Salvador-Guirao et al., 2018; Zhang et al., 2018). Furthermore, members of the miR166 family, including osa-miR166h-3p, osa-miR166g-3p, osa-miR166j-5p, osa-miR166k-3p, and osa-miR166l-3p, were differentially expressed in rice variety 9311 under low-temperature stress (Zhao et al., 2022).

In this study, osa-miR166j-5p exhibited significant downregulation at 4, 12, 24, and 48 h of cold treatment in the cold-tolerant JL variety. It was also significantly upregulated at 48 h compared to its expression in the cold-sensitive JH variety. These findings further underscore osa-miR166j-5p's critical role in regulating cold tolerance in rice. In a separate study on salt-treated rice seedlings, osa-miR156j-3p was notably downregulated (Goswami et al., 2017). Osa-miR156 is implicated in regulating multiple abiotic stress responses and demonstrates contrasting expression patterns when exposed to different abiotic stressors. For example, while osa-miR156 exhibited increased expression upon radiation exposure, it showed the opposite trend under low-temperature or drought conditions (Zhang et al., 2013).

In our study, this gene was notably upregulated at 4, 12, 24, and 48 h of cold treatment in the JH variety. This suggests that osa-miR156 serves as a stress regulator with distinct expression behaviors under different stress conditions. Among the 25 differentially expressed mRNAs, *Os03g0152000*, which is part of the antioxidant system, showed significant upregulation in the cold-tolerant JL variety at 4, 12, 24, and 48 h of cold treatment. This gene features a structural domain encoding a redox-active, copper-linked protein. This domain is also found in enzymes like laccase, SOD, and multi-copper oxidase, which catalyze a variety of cellular processes. Previous research (da Maia et al., 2016) also demonstrated that this gene was upregulated in cold-tolerant (Oro) varieties and downregulated in cold-sensitive (Tio Taka) varieties. *Os12g0491800*, previously implicated in diterpenoid biosynthesis studies on low-temperature tolerance in rice, exhibited differential expression according to both transcriptomic and proteomic data. When compared to the JH variety, this gene was notably downregulated at 4, 12, 24, and 48 h. *Os05g0381400*, known to respond to heat stress in rice, was significantly downregulated at the 24-hour mark in comparison with the JH variety in this study. This gene may be responsive to both low and high temperature stress conditions in rice. Collectively, these genes are promising candidate genes for stress responses in rice and offer valuable reference points. Our findings lay the groundwork for further exploration of the circRNA-miRNA-mRNA regulatory network in rice under low-temperature stress conditions.

The prediction of competing endogenous RNA (ceRNA) networks, while a significant advancement in understanding RNA interactions and gene regulation, comes with limitations that necessitate additional bioinformatic and experimental work for validation caused by complex post-transcriptional regulation mechanisms. Computational models often rely on simplified assumptions about RNA interactions, which may not fully capture the intricacies of miRNA binding and the nuances of molecular competition. Additionally, the transient and context-specific nature of ceRNA interactions makes it difficult to generalize findings across different cell types or physiological conditions. To address these limitations, experimental validation is equally essential to confirm ceRNA predictions. Techniques like luciferase reporter assays, RNA immunoprecipitation, and gene knockdown or overexpression studies can provide direct evidence of ceRNA interactions and their functional consequences. In summary, ceRNA network prediction requires a combination of sophisticated bioinformatic modeling and rigorous experimental validation to overcome its inherent limitations and prove the existence and functional significance of the predicted ceRNA candidates.

4.4 Functional validation of *OsWRKY61*

We subsequently analyzed the CDS of *OsWRKY61* and found that its sequence in the cold-sensitive JH variety was consistent with that in the Nipponbare variety. In contrast, the cold-tolerant JL variety had one SNP in the CDS compared to Nipponbare (Figure 5). This single-base variation, resulting in a non-synonymous amino acid substitution, may account for the significant difference in cold tolerance between the two varieties. To explore this, we conducted physiological and phenotypic assessments of *OsWRKY61*-overexpressing plants under low-temperature stress. Our observations revealed that the overexpression plants were notably less cold-tolerant than their wild-type counterparts (Figure 6B), shedding light on the role of this particular transcription factor in cold tolerance.

OsWRKY71 transgenic rice lines, OX12 and OX21, have demonstrated superior cold tolerance in terms of survival rate, photosynthetic capacity, fresh weight, and dry weight when subjected to cold treatment at 4°C (Kim et al., 2016). Under normal conditions, the OsWRKY63 overexpression strain showed no significant differences compared to the wild type. However, under cold stress, the strain had a significantly reduced fruit set rate and lower seedling cold-stress survival than the wild type. Intriguingly, the use of CRISPR/Cas9 technology to knock down OsWRKY63 expression led to enhanced cold tolerance (Zhang et al., 2022). Similarly, OsWRKY45-1 and OsWRKY45-2 have been found to negatively regulate cold tolerance in rice; overexpressing these genes resulted in a markedly lower survival rate after cold treatment compared to wild-type plants (Tao et al., 2011; Zhang et al., 2021). While these findings provide valuable insights, additional research is needed to confirm these conclusions. Future work should focus on the construction of *OsWRKY61* mutants and the use of RNA interference techniques to further clarify the role of this candidate gene in the signaling pathways associated with low-temperature stress.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Bioproject accession number: PRJNA1071886 and PRJNA1068025.

Author contributions

HW: Writing – original draft, Writing – review & editing. YJ: Resources, Funding acquisition, Writing – original draft. XB: Writing – original draft, Data curation. JW: Writing – original draft, Conceptualization. GL: Writing – original draft, Formal analysis. HXW: Writing – original draft, Methodology. YW: Writing – original draft, Project administration. JX: Writing – original draft, Software. HM: Writing – original draft, Supervision. ZL: Writing – original draft, Validation. DZ: Visualization, Writing – review & editing. HZ: Resources, Funding acquisition, Writing – original draft, Writing – review & editing.

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Conflict of interest

Author HW was employed by the company Bei Da Huang Kenfeng Seed Limited Company.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2024.1260591/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Logarithmic values of identified circRNAFPKM.

SUPPLEMENTARY FIGURE 2
Correlation coefficient (R^2) plots comparing differential gene expression data from RNA-Seq and qRT-PCR analyses.

SUPPLEMENTARY FIGURE 3
Construction and molecular characterization of *OsWRKY61* overexpression plants. **(A)** PCR-based identification of WT along with OsWRKYOE-1 and OsWRKYOE-2 overexpression plants at the DNA level. Labels "a" and "b" indicate statistical significance between varieties at the 0.05 level. **(B)** RNA-level characterization using RT-qPCR for WT, OsWRKYOE-1, and OsWRKYOE-2.

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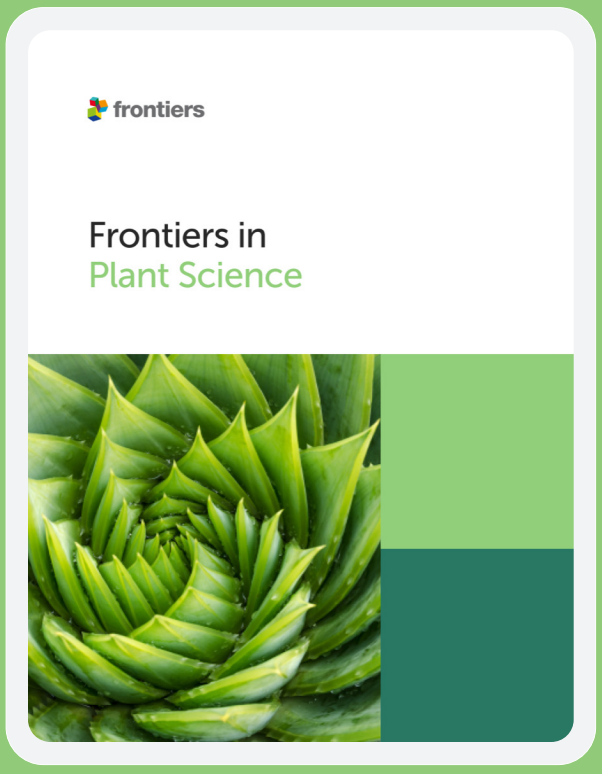

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