MOLECULAR AND GENETIC PERSPECTIVES OF COLD TOLERANCE IN PLANTS

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MOLECULAR AND GENETIC PERSPECTIVES OF COLD TOLERANCE IN PLANTS

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Table of Contents

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Integrated Analysis of Metabolome and Transcriptome Reveals Insights for Cold Tolerance in Rapeseed (*Brassica napus* L.)

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Rapeseed (*Brassica napus* L.) is an important oilseed crop in the world. Its productivity is significantly influenced by numerous abiotic stresses, including cold stress (CS). Consequently, enhancement in CS tolerance is becoming an important area for agricultural investigation and crop improvement. Therefore, the current study aimed to identify the stress-responsive genes, metabolites, and metabolic pathways based on a combined transcriptome and metabolome analysis to understand the CS responses and tolerance mechanisms in the cold-tolerant (C18) and cold-sensitive (C6) rapeseed varieties. Based on the metabolome analysis, 31 differentially accumulated metabolites (DAMs) were identified between different comparisons of both varieties at the same time points. From the transcriptome analysis, 2,845, 3,358, and 2,819 differentially expressed genes (DEGs) were detected from the comparison of C6-0 vs. C18-0, C6-1 vs. C18-1, and C6-7 vs. C18-7. By combining the transcriptome and metabolome data sets, we found that numerous DAMs were strongly correlated with several differentially expressed genes (DEGs). A functional enrichment analysis of the DAMs and the correlated DEGs specified that most DEGs and DAMs were mainly enriched in diverse carbohydrates and amino acid metabolisms. Among them, starch and sucrose metabolism and phenylalanine metabolism were significantly enriched and played a vital role in the CS adaption of rapeseed. Six candidate genes were selected from the two pathways for controlling the adaption to low temperature. In a further validation, the T-DNA insertion mutants of their *Arabidopsis* homologous, including *4cl3*, *cel5*, *fruct4*, *ugp1*, *axs1*, and *bam2/9*, were characterized and six lines differed significantly in levels of freezing tolerance. The outcome of the current study provided new prospects for the understanding of the molecular basis of CS responses and tolerance mechanisms in rapeseed and present a set of candidate genes for use in improving CS adaptability in the same plant.

Keywords: abiotic stress, carbohydrate metabolism, metabolites, phenylalanine metabolism, omics

INTRODUCTION

Rapeseed (*Brassica napus* L.) is an essential oilseed crop. Due to the high oil content in its seeds, it is considered to account for one-third of the total edible oils throughout the world, which helps in the mass production of animal feed and vegetable oil (Jiang et al., 2014). Rapeseed is sensitive to several abiotic stresses, such as drought, salinity, flood, and cold, severely reducing the growth and production of the crop, thus resulting in agricultural economic loss and production risks (Raza, 2020a). Among the different abiotic stresses, cold stress (CS) is one of the critical abiotic stresses in China, as it limits crop species, growth, yield, and quality (Yang et al., 2015). Cold tolerance is an essential trait for crops to withstand low temperatures, especially for overwintering crops (Sun et al., 2010; He et al., 2021). In general, CS thermodynamically decreases the kinetics of the various physiological and metabolic processes occurring in plants (Ruelland et al., 2009), which, accordingly, cause cell membrane rigorousness, protein complex uncertainty, and photosynthesis weakness (Ruelland et al., 2009). Subsequently, CS seriously reduces the rate and uniformity of germination, hampers seedling vigor, and delays plant development, resulting in serious crop yield losses (He et al., 2021; Raza et al., 2021b). For instance, severe winter freezing temperatures affected 77.8% of the rapeseed growing area and caused significant production and yield loss in China (Zhang et al., 2008). Furthermore, the late sowing of winter rapeseed reduced the rapeseed yield, and early flowering was also significantly affected by CS. Moreover, intracellular ice crystals are formed due to CS, distorting the sensitive plant organs (Lardon and Triboi-Blondel, 1995; Raza, 2020a; Shafighi et al., 2020). New seedlings, flowering, and seed formation are also mainly affected by CS (Lardon and Triboi-Blondel, 1995; Shafighi et al., 2020). In recent studies, it was found that CS significantly reduced survival rate by impairing different physio-biochemical (reduced antioxidant enzyme activities, osmolyte content, and phytohormone levels) and molecular (reduced transcript levels of CS-responsive marker and antioxidant genes) mechanisms (Yan et al., 2019; He et al., 2021). Furthermore, CS also greatly affects the photosynthetic rate, stomatal conductance, transpiration rate, and chlorophyll fluorescence parameters in rapeseed (Mehmood et al., 2021). Therefore, it is urgent to develop CS-tolerant rapeseed varieties to ensure yield under such unfavorable conditions.

Several plants showed improved chilling tolerance after an experience with chilling stress in a mechanism called cold acclimation (Ruelland et al., 2009) which includes a collection of physiological and biochemical adjustments. These changed responses involve deviations in gene expression trends *via* the ICE-CBF-COR regulatory pathway (Shi et al., 2018). The C-repeat binding factors (CBF) transcription factors, also recognized as dehydration responsive element binding factor 1 (DREB1) proteins belonging to the AP2/ERF domain family, are meaningfully induced under CS (Chinnusamy et al., 2010; Akhtar et al., 2012; Shi et al., 2018). Other than regulatory networks, plants have modulated their biochemical ways in response to CS. For instance, carbohydrates, lipids, and amino acids have been extensively described to be elaborated in multiple abiotic stress responses, not only for protein amalgamation procedure, but also as pioneers of further key metabolites under stress conditions (Zhao et al., 2007; Bhandari et al., 2016; Zhang et al., 2017). However, more CS-related metabolites need to be comprehensively explained in future research.

Nowadays, high-throughput omics approaches, including genomics, transcriptomics, proteomics, and metabolomics, have been widely used by plant researchers to study different abiotic stresses and deepen their understanding of diverse biological pathways (Raza et al., 2021a,b). Recently, two groups used the integrative comparative analysis of metabolite and transcript profiles to dissect the CS-tolerance mechanisms in tomatoes (*Solanum lycopersicum* L.) (Zhang W.-F. et al., 2019) and wheat (*Triticum aestivum* L.) (Zhao et al., 2019) under CS conditions. These studies identify different amino acids, sugars, organic acids, and lipid-related compounds and metabolic pathways under CS conditions. In another study, the complex molecular mechanisms of metabolites and candidate genes involved in the salt response of sesame have been explored (Zhang Y. et al., 2019). Overall, these integrated studies have expanded our knowledge and understanding of the complex regulatory mechanisms that are used to respond to stressful environments. To date, significant progress has been made toward the conjoint analysis of metabolome and transcriptome in response to different abiotic stresses in *Arabidopsis*, Ethiopian mustard (*B. carinata*), oat (*Avena sativa*), sorghum (*Sorghum bicolor*), and Chinese woad (*Isatis indigotica* Fort.) (Hirai et al., 2004; Li et al., 2009, 2019; Zhou et al., 2015; Wang et al., 2018a). However, studies on the molecular mechanisms of CS response in *Brassica* crops, mainly in rapeseed under CS conditions, have made few advances. Comparative approaches, therefore, should be used in the research of complex molecular mechanisms responses to CS in rapeseed. Nevertheless, the integrated omics analysis (transcriptome and metabolome) of rapeseed under CS could be considered a fascinating approach to revealing key CS-responsive mechanisms at the molecular and metabolic levels.

In the present study, the transcriptome and metabolome profiles of the seedlings of a cold-tolerant and a sensitive rapeseed variety were performed in the early phase of CS. Conjoint analysis of metabolome and transcriptome permitted us to identify putative metabolites, genes, metabolic pathways, and their interactions in response to CS. To the of our best knowledge, this is the first time that a correlation analysis between identified genes and metabolites from the combined data set under CS, mainly in rapeseed, was carried out. This study provides novel insights into the mechanisms underlying rapeseed CS adaptation, thus facilitating the development of CS-tolerant rapeseed varieties.

MATERIALS AND METHODS

Plant Material, Stress Treatments, and Evaluation of Some Physiological Indexes

Two rapeseed varieties, C18 (cold tolerant; CT-C18) and C6 (cold-sensitive; CS-C6), were used to analyze the defense

mechanisms against CS. The seeds of both varieties were obtained from the Oil Crops Research Institute, Chinese Academy of Agricultural Science (CAAS), Wuhan, China. The rapeseed seeds were sterilized with distilled sterilized water for 10 min. Then, the seeds were germinated on two wet filter papers in a Petri dish under normal conditions for 8 days. After that the uniform seedlings were transferred to small pots containing soil in a growth chamber with a 16/8-h light/dark cycle at 25°C. Twenty-one-day-old seedlings were then treated at 8/4°C day/night for 7 days. Samples were collected at 0 (control/CK), 1, and 7 days after the treatment. Our findings were parallel with a recent report (Tian et al., 2017), which found that rapeseed CK plants did not show any significant difference in morphological attributes when harvested under different temperatures and light intensities. Thus, only one CK for the entire analysis was kept. Three biological replicates were harvested for transcriptomic and metabolomic analysis, which was performed in different comparisons (**Supplementary Table 1**). All the samples were immediately frozen in liquid nitrogen and stored at −80°C for further use.

The above-mentioned harvested samples were used for evaluating some physiological indices in both varieties. The activities of superoxide dismutase (SOD, EC 1.15.1.1; cat no. G0101F), and peroxidase (POD, EC 1.11.1.7; cat no. G0107F), the contents of malondialdehyde (MDA, cat no. G0109F), soluble sugar (cat no. G0501W), soluble protein (cat no. G0418W), and proline (cat no. G0111W) were measured using the kits provided by Suzhou Grace Biotechnology Co., Ltd. (Suzhou, Jiangsu, China). All the parameters were measured using three technical replications and a spectrophotometer microplate reader (Epoch, BioTek, Instruments, Inc., Winooski, VT, USA).

Total RNA Isolation and Transcriptome Sequencing

Total RNA was isolated from each sample using the HiPure Plant RNA Mini Kit (Magen R4151-03, Zhengzhou, China) according to the instructions of the manufacturer. The RNA concentration was measured using the NanoDrop 2000 (Thermo Scientific, USA). The RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 Bioanalyzer (Agilent Technologies, CA, USA). For RNA-seq/transcriptome and metabolome analysis, the three biological samples were sent to BioMarker Technologies (Beijing, China). Lastly, RNA-seq was conducted on the Illumina HiSeq high throughput sequencing platform (HiSeq 2000, SanDiego, CA, USA).

Transcriptome Assembly, Annotation, and Differential Expression Analysis of the Genes

Raw data (raw reads) of the fastq format were first processed through in-house perl scripts. In this step, clean data (clean reads) were attained by removing reads comprising adapter, reads containing ploy-N, and low-quality reads from raw data. Simultaneously, Q20, Q30, GC-content, and sequence duplication levels of the clean data were calculated. All the downstream analyses were based on clean data with high quality. The adaptor sequences and low-quality sequence reads were removed from the data sets. The clean reads were then mapped to the reference genome sequence "Darmor-bzh" (http://www.genoscope.cns.fr/brassicanapus/) (Chalhoub et al., 2014). Only reads with a perfect match or one mismatch were further analyzed and annotated based on the reference genome using the Hisat2 V2.1.0 tool.

Gene function was annotated based on the following databases: Pfam (Protein family) (https://pfam.xfam.org/); Swiss- Prot (A manually annotated and reviewed protein sequence database) (https://www.uniprot.org/); KO [Kyoto Encyclopedia of Genes and Genomes (KEGG) Ortholog database] (https://www.genome.jp/kegg/ko.html); GO (Gene Ontology) (http://www.geneontology.org). The quantification of the gene expression levels was projected by fragments per kilobase of transcript (FPKM) per million fragments mapped.

The differential expression analysis of two conditions or groups was completed using the R package DEseq. The resulting *p*-values were adjusted using the approach of Benjamini and Hochberg for controlling the false discovery rate (FDR). Genes with $\log_2$FC > 4, adjusted $p < 0.001$, and FDR < 0.001 were assigned as differentially expressed genes (DEGs).

Metabolite Extraction

The above-mentioned samples were also considered for metabolome analysis. Briefly, 50 mg of the sample were taken and placed in an EP tube, then added with 1,000 μl extraction solvent containing an internal target (*V* methanol: *V* acetonitrile: *V* water = 2:2:1, containing internal standard 2 μg ml^{-1}). They were then homogenized in a ball mill for 4 min at 45 Hz, then ultrasound treated for 5 min (incubated in ice water). After homogenizing 3 times, they were incubated for 1 h at −20°C to precipitate proteins. They were then centrifuged at 12,000 rpm for 15 min at 4°C. After which, the supernatant (500 μl) was transferred fresh into the EP tubes. The extracts were dried in a vacuum concentrator without heating and added with 100 μl extraction solvent (*V* acetonitrile: *V* water = 1:1) reconstitution. The samples were then vortexed for 30 s, sonicated for 10 min (4°C water bath), and centrifuged for 15 min at 12,000 rpm, 4°C. Afterward, the supernatants (60 μl) were transferred into a fresh 2-ml LC/MS glass vial. Lastly, 10 μl from each sample were then taken and pooled as QC samples and 60 μl of supernatant were taken for an ultra-high-performance liquid tandem chromatography-quadrupole time of flight-mass spectrometry (UHPLC-QTOF-MS) analysis.

Metabolite Detection

The LC-MS/MS analyses were performed using a UHPLC system (1290, Agilent Technologies) with a UPLC BEH Amide column (1.7 μm 2.1 * 100 mm, Waters) coupled to TripleTOF 6600 (Q-TOF, AB Sciex). The mobile phase consisted of 25 mM NH_4Ac and 25 mM NH_4OH in water (pH = 9.75) (A) and acetonitrile (B) was carried with elution gradient as follows: 0 min, 95% B; 0.5 min, 95% B; 7 min, 65% B; 8 min, 40% B; 9 min, 40% B; 9.1 min, 95% B; 12 min, 95% B, delivered at 0.5 ml min^{-1}. The injection volume was pos:1.5 μl, neg: 1 μl. The Triple TOF mass spectrometer was used for its ability to acquire MS/MS spectra

on an information-dependent basis (IDA) during an LC/MS experiment. In this mode, the acquisition software (Analyst TF 1.7, AB Sciex) continuously evaluates the full scan survey MS data as it collects and triggers the acquisition of MS/MS spectra depending on preselected criteria. In each cycle, 12 precursor ions with intensities <100 were chosen for fragmentation at a collision energy (CE) of 30 V (15 MS/MS events with product ion accumulation time of 50 ms each). The ESI source conditions were set as follows: ion source gas 1 as 60 psi, ion source gas 2 as 60 psi, curtain gas as 35 psi, source temperature at 650°C, and Ion Spray Voltage Floating (ISVF) 5,000 or −4,000 V in positive or negative modes, respectively.

Bioinformatics Analysis of Combined Data

The metabolome data were normalized before analysis through the normalizing of the total peak area by dividing each metabolite in the sample by the total peak area of the sample. PCA, cluster heatmap analysis, correlation analysis between samples, and Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) were carried out for classification and discrimination between the samples using R package ropls (R 3.3.2.; http://bioconductor.org/packages/release/bioc/html/ropls.html) (Thevenot, 2016). We combined the multivariate statistical analysis of the VIP value of OPLS-DA and the univariate statistical analysis of the *t*-test *P*-value to screen differentially accumulated metabolites (DAMs) among different comparison groups. The screening criteria FC > 1.5, $P < 0.05$, and VIP > 1 were considered to determine the DAMs between samples.

Gene ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was executed by the GOseq R package (https://bioconductor.org/packages/release/bioc/html/goseq.html) based on the Wallenius non-central hyper-geometric distribution, which can adjust for gene length bias in DEGs (Anders and Huber, 2010). The KEGG pathway enrichment analysis of the DEGs and DAMs was carried out using the KOBAS software (Mao et al., 2005). The heatmap was created using the TBtool V0.66839 software (Chen et al., 2020).

Co-expression Network Visualization

The gene expression data (log_2FC) of the targeted metabolites were selected and imported to Cytoscape (Version 3.5.1. https://cytoscape.org/) for differential metabolites and gene correlation network visualization (Shannon et al., 2003). Firstly, log_2 conversion was performed on the data uniformly before analysis. For the joint analysis between metabolome and transcriptome, the Pearson Correlation Coefficient (PCC) and the corresponding *P*-value were used for screening, and the screening criteria were set at PCC > 0.8.

MapMan Pathway Visualization and Enrichment Analysis

The MapMan pathway annotator (version 3.6.0 RC1, https://mapman.gabipd.org/mapman/) was used to display the graphical overview of metabolism pathways (Thimm et al., 2004). Release Genome of *Brassica napus* annotation v5 was used as the mapping reference data to group and display metabolism pathways. *B. napus* gene IDs and their log_2FC values were imported to MapMan as an experimental data set. The Wilcoxon rank-sum test with Benjamini–Hochberg corrected was used to analyze which bins/pathways were differentially enriched between two accessions.

qRT-PCR Analysis for DEGs

Total RNA was extracted using the RNAprep Pure Plant Kit (Tiangen) according to the instructions of the manufacturer. The first-strand cDNA was reverse transcribed from 1 μg of the total RNA using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The quantitative real-time PCR (qRT-PCR) was carried out as described previously (Wang et al., 2014). The qRT-PCR reaction was carried out using the StepOnePlusReal-Time PCR System (Applied Biosystems, US) on the *Power*SYBR® Green PCR Master Mix (Applied Biosystems, US). The total volume for each reaction was 10 μl, comprising 0.4 μl specific primers, 1 μl cDNA, 5 μl SYBR mixture, and 3.6 μl double-distilled H_2O. The cycling program were as follows: 10 min at 95°C, followed by denaturation at 95°C for 15 s, annealing, and elongation at 60°C for 1 min (40 cycles). The *BnActin* gene was used as an endo control. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer used for randomly selected genes is listed in **Supplementary Table 2**.

Cold-Induced Alteration of Some Metabolite

To evaluate whether CS reconfigures the metabolite contents in leaves, we analyzed L-Kynurenine, L-Tyrosine, N-Acetyl-L-Phe, salicylic acid, and succinic acid contents. The concentrations of these compounds were calculated using the calibration curves generated from corresponding standard solutions, and the detailed protocol is described in **Supplementary File 1**.

Functional Validation of Candidate Genes

For the functional validation of candidate genes, the rapeseed gene IDs were used to search the *Arabidopsis* homolog in the rapeseed genome database (Genoscope; https://www.genoscope.cns.fr/brassicanapus/). The resultant *Arabidopsis* genes were used to search the sequence similarities and annotation in the TAIR database using blastP. Based on the annotation and sequence results, the most suitable candidates were selected for functional validation and had their mutation status checked in the SIGnal database (T-DNA Express: *Arabidopsis* Gene Mapping Tool; http://signal.salk.edu/cgi-bin/tdnaexpress). After PCR screening, only homozygous lines were chosen for further analysis. The sequence alignment between respective rapeseed and *Arabidopsis* T-DNA lines was performed using the MegAlign Pro software (https://www.dnastar.com/software/lasergene/megalign-pro/). Furthermore, *Arabidopsis* homozygous mutants *4cl3* (SALK 014297C), *cel5* (SALK 079921C), *fruct4* (SALK 011312C), *ugp1* (SALK 100183C), *axs1* (SALK 000016C), and *bam2/9* (SALK 020838C) were obtained from the Arashare database, China (https://www.arashare.cn/index/), which were genotyped *via* PCR sequencing to check the homozygous status. Moreover, the relative expression of T-DNA mutants was checked in T-DNA lines and wild-type (WT) plants to confirm their knockout status. The primer used for PCR and qRT-PCR analysis are listed

in **Supplementary Table 2**. The *Arabidopsis* ecotype Columbia-0 was used as the WT. All *Arabidopsis* seeds were germinated and grown for 4 weeks in controlled greenhouse conditions under short-day conditions (10 h of light) at a constant temperature of 22°C. The 4-week-old seedlings of all mutants and the WT were exposed to freezing stress (−5°C for 8 h) in a growth chamber followed by recovery at 25°C for 2 days. The phenotype and survival rate of the leaves were observed, and samples were harvested before and after exposure to freezing stress. The survival rate was measured using the following equation:

$$\text{Survival rate } (\%) = \frac{\text{Number of survived plants}}{\text{Number of originally planted plants}} \text{ x } 100$$

After being exposed to freezing stress, most WT plants survived and could continuously grow under normal conditions after recovery, while the mutant plants wilted severely under freezing treatment and even died after recovery. For each mutant, 12 pots (4 plants pot^{-1}) were assessed, and the experiment was completed with three replications. The leaves were harvested before stress (CK), after 8 h of freezing stress, and after 2 days of recovery to measure the osmoprotectant substances [soluble sugar (G0501W), soluble protein (G0418W), and proline (G0111W)] using the kits provided by Suzhou Grace Biotechnology Co., Ltd. (Suzhou, Jiangsu, China). All the parameters were measured using three biological replications and a spectrophotometer microplate reader (Epoch, BioTek, Instruments, Inc., Winooski, VT, USA).

Statistical Analysis

The statistical analysis was performed using GraphPad Prism 9 (https://www.graphpad.com/) (Swift 1997). The experiments were performed with three biological replicates, and plant materials from three seedlings were pooled for each biological replicate. The statistical significance was determined through a one- or two-way ANOVA and Tukey's test. Error bars represent SD (n = 3). The difference was statistically significant as $^{****}P < 0.0001$, $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, and $^{*}P \leq 0.05$.

RESULTS

Physiological Responses and Metabolic Profiling of C6 and C18 Varieties Under CS

To examine the variations of rapeseed during CS, two rapeseed varieties were selected. According to previous studies, zhongshuang 6 (C6) was a common transgenic receptor that exhibited susceptibility to CS, whereas C18 was an excellent breeding line that exhibited cold tolerance (Yan et al., 2019). The phenotypic result showed that C6 displayed severe wilting and chlorosis at 7 days of CS compared to C18 (**Figure 1A**). At physiological levels, CT-C18 performed better than CS-C6 (**Figures 1B–G**). For instance, the activities of antioxidant enzymes such as SOD and POD were found to be higher in CT-C18 under CS conditions, mainly at 1 and 7 days (**Figures 1B,C**). The MDA is interpreted as an end product of lipid peroxidation, and regulation of MDA content indicates the oxidative stress levels in plants (Gaweł et al., 2004). Thus, the MDA content was considerably higher in CS-C6 compared to CT-C18 (**Figure 1D**), indicating that CT-C18 had a better capacity to cope with oxidative stress than CS-C6. Moreover, osmoprotectant content, including soluble sugar, soluble protein, and proline contents, was significantly higher in CT-C18 under CS conditions (**Figures 1E–G**). These findings showed that CT-C18 is a more cold-tolerant variety than CS-C6 and could mitigate the adverse effects of CS by increasing the activities of antioxidant defense enzymes and content of osmoprotectants and reducing oxidative stress (Yan et al., 2019).

To construct a systematical profile of metabolic changes that occur in response to CS, an untargeted metabolome analysis was performed between cold-sensitive (CS-C6) and cold-tolerant (CT-C18) varieties. In total, 3,368 metabolites were detected; out of these, 626 were known metabolites (**Supplementary Table 3**, **Supplementary Figure 1**). These known metabolites belonged to 81 classes, with majority of these metabolites belonging to carboxylic acids and derivatives, organooxygen compounds, fatty acyls, benzene and substituted derivatives, organonitrogen compounds, prenol lipids, purine nucleosides, etc. (**Supplementary Table 3**, **Supplementary Figure 1**). To get a better view of the metabolic profiles in response to CS, the unsupervised hierarchical cluster was built, indicating that metabolic data from the early stage of CS (0–1 day) were clearly separated from the late-stage stress (7 days) (**Figure 1H**). Similarly, the PCA explained 47.29% of the total variance (27.78 and 19.51% for PC1 and PC2, respectively). The PCA showed the precise isolation between the different time points by PC1. The isolation of genotypes from biological replications can be distinguished by PC2 (**Figure 1I**), which showed a good correlation between replications (Iezzoni and Pritts, 1991; Ringnér, 2008). These results indicated that differential metabolic response between both varieties could be the basis of their different tolerances to CS.

DAMs Involved in Cold Response Between Two Rapeseed Varieties

Differentially accumulated metabolites (DAMs) were screened with a screening standard of FC > 1.5, $P < 0.05$, and VIP > 1. To identify the DAMs of rapeseed under CS, we compared the metabolite numbers from the control condition to their stress level at each time point. Out of the total (3,368), we identified 156 DAMs from the comparison of C6-0 vs. C6-1, 357 DAMs from C6-0 vs. C6-7, 358 DAMs from C6-1 vs. C6-7, respectively (**Figures 2A–C** and **Supplementary Table 4**). For the CT-C18 variety, 182 DAMs from C18-0 vs. C18-1, 755 DAMs from C18-0 vs. C18-7, and 469 DAMs from C18-1 vs. C18-7 were detected, respectively (**Figures 2E–G** and **Supplementary Table 4**). Furthermore, we also analyze the DAMs by comparing both varieties at each time point. From these comparisons, we identified 429 DAMs from C6-0 vs. C18-0, 366 DAMs from C6-1 v C18-1, and 403 DAMs from C6-7 vs. C18-7 that were either upregulated or downregulated (**Figures 2I–K** and **Supplementary Table 4**).

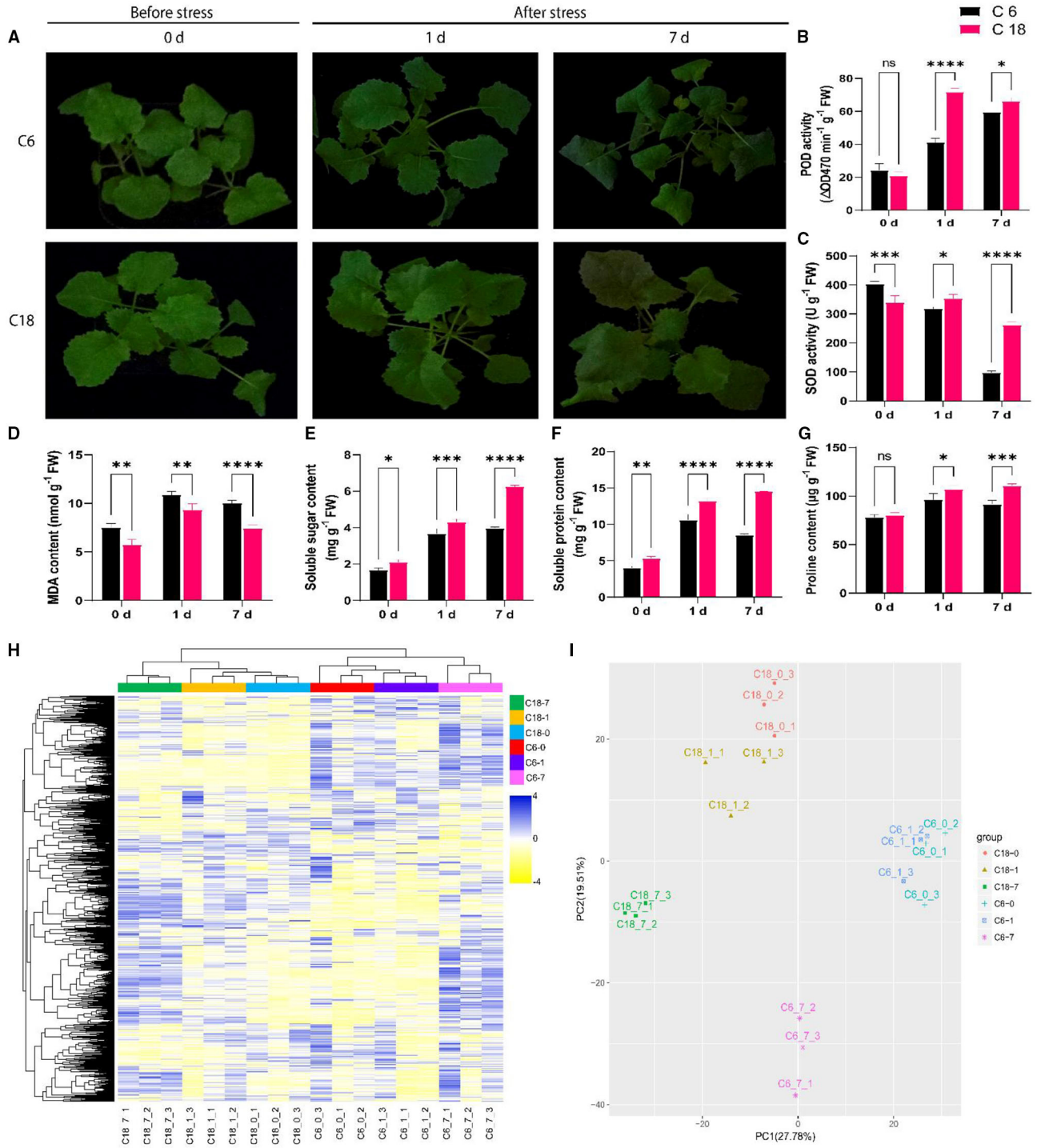

FIGURE 1 | Physiological and metabolic profiling of cold-tolerant (C18) and cold-sensitive (C6) rapeseed varieties responding to cold stress (4°C) at 0 days (CK), 1 day after stress, and 7 days after stress. **(A)** Phenotypic evaluation of C18 and C6; **(B–G)** Physiological responses including **(B)** peroxidase (POD) activity, **(C)** superoxide dismutase (SOD) activity, **(D)** malondialdehyde (MDA) content, **(E)** soluble sugar content, **(F)** soluble protein content, and **(G)** proline content. Error bars represent SD ($n = 3$). The statistical significance was determined *via* a two-way ANOVA and Dunnett's multiple comparisons test with $^{****}p < 0.0001$, $^{***}p \leq 0.001$, $^{**}p \leq 0.01$, $^{*}p \leq 0.05$, and ns mean non-significant. **(H)** The unsupervised hierarchical cluster analysis for untargeted metabolomic profiles. The dendrogram was built based on the fold change ($P < 0.05$, VIP > 1 and $\log_2$FoldChange > 1.5) heatmap. The rows in the heatmap indicate metabolites, and the columns represent groups/samples. The colors of the heatmap cells represent the scaled expression level of metabolites among the different groups. The color gradient, ranging from blue to yellow through white, indicates the low, high, and middle values of metabolite expression. The colors of the different groups represent the biological replicates at three different time points for two genotypes, while 0 (CK), 1, and 7 days represent the sample collection time points after the treatment. **(I)** Principal component analysis (PCA) based on metabolomic data. The X-axis represents the first principle component (PC1), and the Y-axis represents the second principal component (PC2).

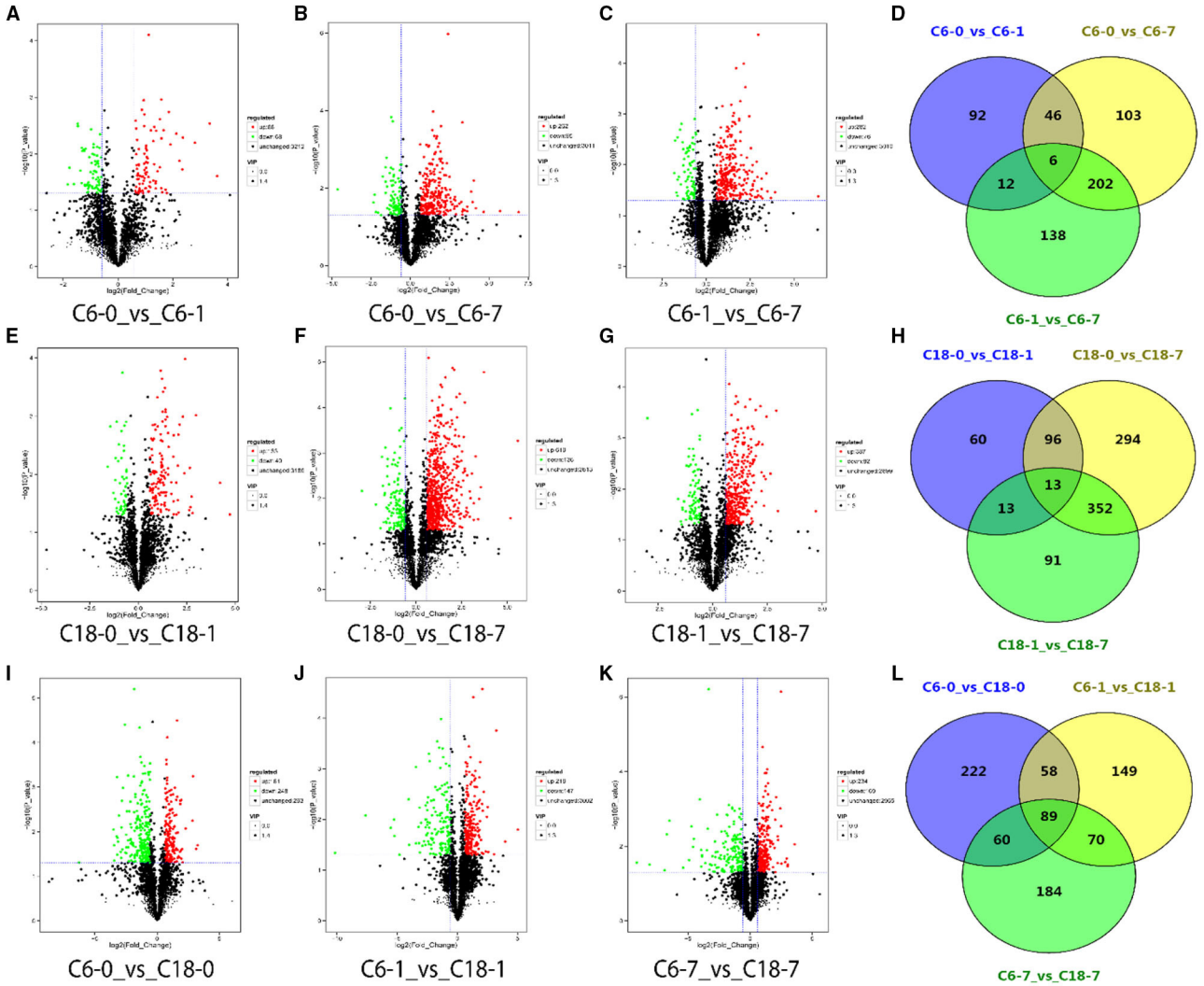

FIGURE 2 | Volcano plots for differentially accumulated metabolites (DAMs) with the comparison of control with their time points. **(A–C)** DAMs from the cold-sensitive (C6) variety; **(E–G)** DAMs from the cold-tolerant (C18) variety; **(I–K)** DAMs between C6 and C18 at the same time points. Venn diagrams showing the shared and common DAMs for all time points **(D)** between the different time points in C6; **(H)** between the different time points in C18; **(L)** between the same time points in C6 and C18.

Notably, we observed an increased number of DAMs over the stress time point in both varieties, which showed the active adaptation of the metabolites in response to CS (**Supplementary Figure 2**). Nevertheless, after 7 days of CS, more DAMs were detected in CT-C18 than CS-C6, which indicated the strong metabolic disturbance experienced by CT-C18. By analyzing the types of DAMs (either upregulated or downregulated) in both varieties at the different time points, it was observed that the CT-C18 variety responded rapidly to the stress based on the number of DAMs (**Supplementary Figure 2A**). However, when we compared the same time points of both varieties, after 1 day, the DAMs were notably higher at 7 days of stress (**Supplementary Figure 2B**). Furthermore, as the stress time increased, more DAMs were upregulated in CT-C18 than CS-C6, while a similar trend was observed for downregulated DAMs for both varieties (**Supplementary Figure 2C**). The comparison between CS-C6 and CT-C18 at the same time points indicated that, with prolonged treatment, an increasing number of upregulated DAMs were observed. Furthermore, a decreasing number of downregulated DAMs was detected after 1 day of treatment (**Supplementary Figure 2D**). In total, CS-C6 failed to accumulate the optimum number of DAMs, which may be related to CS sensitivity. On the contrary, CT-C18 performed better by accumulating a larger number and more upregulated DAMs throughout the stress conditions. This data also supports our finding that CS-C6 was sensitive to CS and CT-C18 showed significant tolerance to CS at metabolome levels.

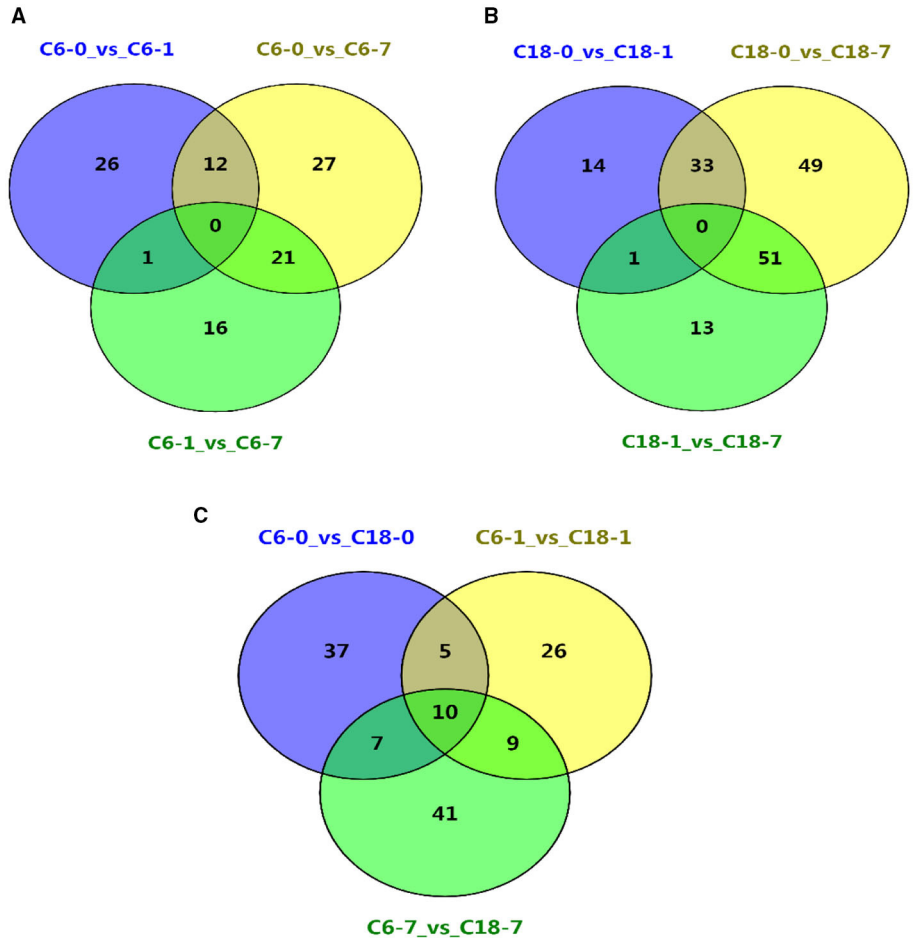

FIGURE 3 | Venn diagrams showing the shared and commonly known DAMs for all time points **(A)** between the different time points in C6; **(B)** between the different time points in C18; **(C)** between the same time points in C6 and C18.

The Core Metabolites Responded to CS in Rapeseed Varieties

The common and specific DAMs were identified at different time points for both varieties under CS conditions (**Figures 2D,H,L** and **Supplementary Table 5**). However, some of these DAMs were known metabolites. Therefore, the named metabolites were compared using different comparisons. A considerable number of DAMs were commonly or specifically detected no matter if they were in comparison with one variety at a different time point (**Figures 3A,B**) or in comparison with both varieties at the same time point (**Figure 3C** and **Supplementary Table 6**). Interestingly, these results showed that CT-C18 accumulated a greater number of known and annotated DAMs compared to CS-C6 during the comparison of both varieties at the same time points (**Figure 3C** and **Supplementary Table 6**). The KEGG analysis of these DAMs disclosed that these metabolites mainly belonged to the following classes: amino acid metabolism, carbohydrate metabolism, biosynthesis of other secondary metabolites, membrane transport, lipid metabolism, metabolism of terpenoids and polyketides, metabolism of cofactors and vitamins, nucleotide metabolism, and energy metabolism (**Supplementary Figures 3–5**). Interestingly, there were no significantly enriched pathways in the comparisons of C6 1 vs. C6 7, C18 1 vs. C18 7, and C6 1 vs. C18 1 (**Supplementary Figure 6**).

Based on the above-discussed findings, the present study focused on the common DAMs (annotated) detected in comparing both varieties at the same time point (**Figure 3C**). The summary of these core DAMs is presented in **Table 1**. Interestingly, more upregulated metabolites were detected in the CT-C18 variety than CS-C6, indicating the positive role of such metabolites in CS tolerance and adaptation in rapeseed. Based on the KEGG annotation, 31 DAMs were involved in 19 pathways, including biosynthesis pathways, amino acid metabolism, carbohydrate metabolism, nucleotide metabolism, membrane transport, and energy metabolism (**Supplementary Table 7**).

Analysis Based on Transcriptome Data of C6 and C18 Under CS Conditions

To dissect how the two rapeseed varieties differed in their response to CS at the transcript level, the global transcriptome profiles of CT-C18 and CS-C6 were constructed using a deep

TABLE 1 | Information about important known DAMs with their compound ID, molecular formula, log_2FC values, *p*-values, and type of regulations along with ko-ID identified by OPLS-DA model.

S. no.	Metabolite ID	Metabolite name	Compound ID	Molecular formula	OPLS-DA model's VIP values	log_2FC	*P*-value	Regulated	ko-ID
(G1) 10 common known DAMs in "C6-0_vs_C18-0," "C6-1_vs_C18-1," and "C6-7_vs_C18-7"									
1	Meta 665	3-Methylglutaric acid	C03761	$C_6H_{10}O_5$	1.59/1.57/1.50	−2.58/−2.39/−2.88	0.0295/0.0309/0.0488	Down	N/A
2	Meta 1457	gamma-Tocotrienol	C14155	$C_{28}H_{42}O_2$	1.39/1.49/1.47	0.84/1.10/1.11	0.0469/0.0119/0.0320	Up	ko00130
3	Meta 56	Imidazoleacetic acid	C02835	$C_5H_6N_2O_2$	1.55/1.53/1.60	−1.38/−1.47/−2.09	0.0207/0.0083/0.0106	Down	ko00340
4	Meta 1057	L-Gulonic gamma-lactone	C01040	$C_6H_{10}O_6$	1.64/1.48/1.64	0.80/1.09/1.03	0.0016/0.0352/0.0018	Up	N/A
5	Meta 190	L-Kynurenine	C00328	$C_{10}H_{12}N_2O_3$	1.41/1.62/1.58	0.75/1.20/1.36	0.0382/0.0013/0.0034	Up	ko00380
6	Meta 1297	N,N'-Diacetylchitobiose	C01674	$C_{16}H_{28}N_2O_{11}$	1.67/1.63/1.61	−3.09/−2.32/−2.64	0.0043/0.0117/0.0141	Down	ko00520, ko02010
7	Meta 1529	Oxethazaine	C12552	$C_{28}H_{41}N_3O_3$	1.67/1.56/1.52	−1.88/−1.78/−1.94	0.0005/0.0199/0.0322	Down	N/A
8	Meta 958	Phenyllactic acid	C01207	$C_9H_{10}O_5$	1.54/1.52/1.64	1.03/1.46/1.35	0.0328/0.0203/8.73E-05	Up	N/A
9	Meta 1678	Squalene	C00751	$C_{30}H_{50}$	1.65/1.61/1.61	−2.89/−3.24/−2.79	0.0080/0.0142/0.0145	Down	ko00100, ko00909
10	Meta 908	Trehalose	C01083	$C_{12}H_{22}O_{11}$	1.56/1.56/1.65	1.20/1.26/1.27	0.0268/0.0121/0.0001	Up	ko00500, ko02010
(G2) 7 common known DAMs in "C6-0_vs_C18-0" and "C6-7_vs_C18-7"									
11	Meta 217	4-Hydroxyphenylpyruvate	C01179	$C_9H_8O_4$	1.38/1.37	−0.66/−1.01	0.0423/0.0452	Down	ko00400, ko01210, ko00261, ko00130, ko00950, ko01230, ko00350
12	Meta 1149	4-Methylumbelliferyl beta-D-glucuronide	91553[a]	$C_{16}H_{16}O_9$	1.66/1.54	−1.45/−1.01	0.0053/0.0270	Down	N/A
13	Meta 1043	Adenosine 3′,5′-cyclic phosphate (cAMP)	6076[a]	$C_{10}H_{12}N_5O_6P$	1.54/1.64	1.92/1.76	0.0402/0.0010	Up	N/A
14	Meta 1197	Aminopterin	169371[a]	$C_{19}H_{20}N_8O_5$	1.45/1.39	−0.59/0.66	0.0336/0.0368	Down	N/A
15	Meta 1762	Doxorubicin	C01661	$C_{27}H_{29}NO_{11}$	1.60/1.56	−1.19/−1.01	0.0066/0.0049	Down	N/A
16	Meta 338	N-.alpha.-Acetyl-L-arginine	67427[a]	$C_8H_{16}N_4O_3$	1.59/1.46	−0.79/−0.64	0.019302849/0.026369916	Down	N/A
17	Meta 800	N-Acetylaspartylglutamate (NAAG)	188803[a]	$C_{11}H_{16}N_2O_8$	1.64/1.55	0.66/0.79	0.0013/0.0229	Up	N/A
(G3) 5 common known DAMs in "C6-0_vs_C18-0" and "C6-1_vs_C18-1"									
18	Meta 1215	Acetylcarnitine	7045767[a]	$C_9H_{17}NO_4$	1.56/1.48	0.59/1.50	0.0241/0.0127	Up	N/A
19	Meta 291	Alpha-D-Glucose	C00267	$C_6H_{12}O_6$	1.62/1.49	1.82/0.76	0.0024/0.0176	Up	ko00520, ko00052, ko00051, ko01200, ko00010
20	meta 319	Altretamine	D02841	$C_9H_{18}N_6$	1.68/1.60	−3.24/−2.36	0.0005/0.0145	Down	N/A
21	Meta 481	Deoxyadenosine	C00559	$C_{10}H_{13}N_5O_3$	1.44/1.52	1.14/1.34	0.0372/0.0099	Up	ko00230
22	Meta 292	Leu-Ala	6992295[a]	$C_9H_{18}N_2O_3$	1.63/1.62	−1.39/−0.88	0.0139/0.0019	Down	N/A
(G4) 9 common known DAMs in "C6-1_vs_C18-1" and "C6-7_vs_C18-7"									
23	Meta 959	Cyclopentolate	C06932	$C_{17}H_{25}NO_3$	1.62/1.54	−3.17/−3.95	0.0059/0.0352	Down	N/A

(Continued)

TABLE 1 | Continued

S. no.	Metabolite ID	Metabolite name	Compound ID	Molecular formula	OPLS-DA model's VIP values	log_2FC	*P*-value	Regulated	ko-ID
24	Meta 1522	Cytidine 5'-diphosphocholine (CDP-choline)	25202509[a]	$C_{14}H_{25}N_4O_{11}P_2^-$	1.52/1.56	0.80/0.64	0.0133/0.0051	Up	N/A
25	Meta 722	Dimethyl 4,4-o-Phenylene-Bis (3-Thiophanate)	3032791[a]	$C_{12}H_{14}N_4O_4S_2$	1.60/1.54	0.87/0.75	0.0032/0.0074	Up	N/A
26	Meta 216	DL-Vanillylmandelic acid	C05584	$C_9H_{10}O_5$	1.56/1.55	0.95/1.22	0.0107/0.0092	Up	ko00350
27	Meta 26	Larixinic Acid	8369[a]	$C_6H_6O_3$	1.59/1.49	0.96/0.89	0.0229/0.0158	Up	N/A
28	Meta 1611	Malvidin 3-O-glucoside cation	C12140	$C_{23}H_{25}O_{12}$	1.60/1.47	1.68/1.39	0.0173/0.0207	Up	N/A
29	Meta 390	N-Acetyl-L-phenylalanine	C03519	$C_{11}H_{13}NO_3$	1.57/1.55	0.86/1.24	0.0108/0.0154	Up	ko00360
30	Meta 1900	Pristimerin	C08633	$C_{30}H_{40}O_4$	1.52/1.53	−1.34/−1.19	0.0390/0.0189	Down	N/A
31	Meta 983	Tyr-Asp	CHEBI:141455[b]	$C_{13}H_{16}N_2O_6$	1.54/1.57	1.04/1.32	0.0351/0.0067	Up	N/A

OPLS-DA, orthogonal projections to latent structures-discriminant analysis; VIP, variable importance in projection; log_2FC, log_2 of fold change values; P-value, p-value of the student's t-test. [a]PubChem CID; [b]ChEBI ID.

RNA-seq strategy (BioProject ID. PRJNA596550). Samples were obtained after 0, 1, and 7 days of exposure to CS. The same was done with the samples for the metabolome analysis. The standards of $log_2FC > 4$, adjusted $P < 0.001$, and FDR < 0.001 were used to recognize the DEGs in response to CS. From the transcriptome analysis, we detected 2,845 DEGs from the comparison of C6-0 vs. C18-0, 3,358 DEG from C6-1 vs. C18-1, and 2,819 DEG from C6-7 vs. C18-7 (**Figure 4A** and **Supplementary Table 8**). To predict the functional insights of all DEGs, the MapMan pathway annotator was used for the metabolism overview of the DEGs. It was found that rapeseed responded to CS depending on the genetic background (**Figures 4B–D**), which is supported by previous reports (Lei et al., 2018; Zhang C. et al., 2019; Wrucke et al., 2020). A similar expression trend for up- and downregulated DEGs was mainly detected in the secondary metabolism, amino acids, lipids, cell wall, minor CHO, and light reaction pathways during the three comparisons. More genes were enriched in the secondary metabolism, amino acids, lipids, and cell wall pathways (**Figures 4B–D**). Interestingly, most of the pathways were found to be enriched with upregulated genes at 1 and 7 days of stress (**Figures 4C,D**). Mainly, secondary metabolism, amino acids, lipids, and cell wall pathways exhibited a greater number of DEGs. These outcomes indicate that these DEGs and associated metabolic pathways could help rapeseed adapt to CS conditions. The results here showed that the step-wise overview of metabolism pathways in rapeseed gave a clear presentation of the specific pathways in rapeseed that functioned at different time points upon CS treatment (**Figure 4**).

Network Analysis of DAMs and DEGs in Response to CS

To understand the regulatory networks of DAMs between the CS-C6 and CT-C18 varieties, we carried out a correlation test between the core DAMs and DEGs in the three groups (C6-0 vs. C18-0, C6-1 vs. C18-1, and C6-7 vs. C18-7) (**Table 1**). These DEGs were subjected to PCC analysis, and screening criteria were PCC > 0.8. Interaction networks were organized between the DAMs that were annotated by the KEGG database. Therefore, to get insights into the type and strength of correlation among DAMs and DEGs, an interaction network was performed among four different groups at different time points in both varieties (see **Table 1** for groups setting; G1-G4).

The interaction network for G1 was performed between 6 DAMs and 41 DEGs from C6-0 vs. C18-0 (**Figure 5A**), 6 DAMs and 67 DEGs from C6-1 vs. C18-1 (**Figure 5B**), and 5 DAMs and 44 DEGs from C6-7 vs. C18-7 (**Figure 5C**, and **Supplementary Table 9**). The network showed that meta_908 (Trehalose) and meta_1297 (N,N'-Diacetylchitobiose) were clustered together and shared 19 common DEGs. However, these common DEGs showed diverse expression patterns in the three comparisons. Meanwhile, meta_56 (Imidazoleacetic acid), meta_190 (L-Kynurenine), meta_1457 (gamma-Tocotrienol), and meta_1678 (Squalene) were clustered separately (**Figure 5A**). The interaction network for G2 was carried out between 1 DAM and 19 DEGs from C6-0 vs. C18-0 (**Figure 6A**) and

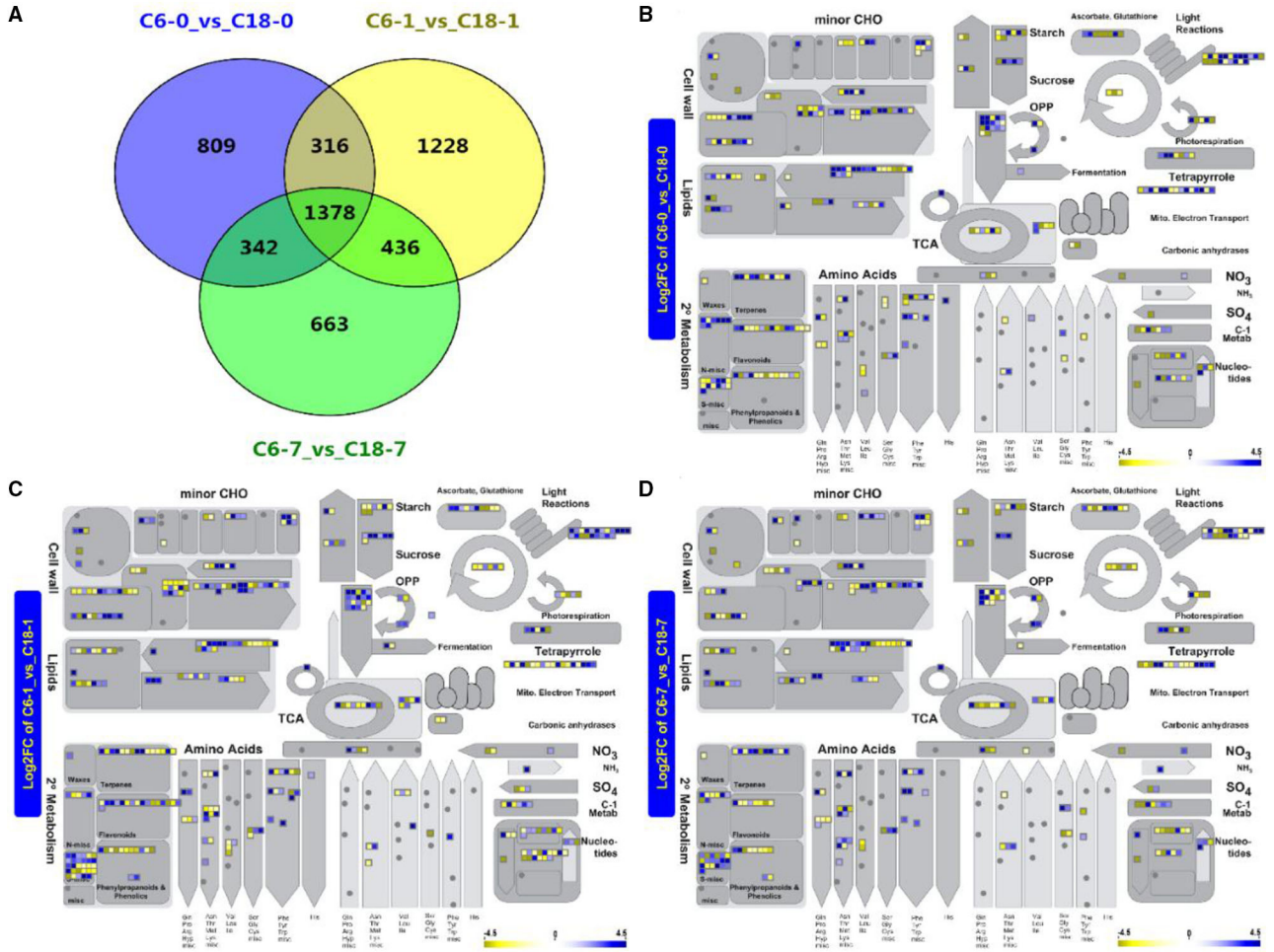

FIGURE 4 | (A) Venn diagram showing the shared and common differentially expressed genes (DEGs) for the same time points between C6 and C18 varieties. **(B–D)** Metabolism overview of all DEGs at 0 (CK), 1, and 7 days of stress between both cultivars revealed by the MapMan pathway annotator, respectively. **(B)** A total of 2,725 of the 2,845 data points were mapped (some of the data points may be mapped multiple times to different bins), and 363 genes are visible in data points; **(C)** A total of 3,297 of the 3,358 data points were mapped (some of the data points may be mapped multiple times to different bins) and 449 genes are visible in data points; **(D)** A total of 2,713 of the 2,829 data points were mapped (some of the data points may be mapped multiple times to different bins) and 360 genes are visible in data points. Boxes in a blue or yellow color mean significantly up- and downregulated genes, respectively. CHO, carbohydrate; OPP, oxidative pentose phosphate; TCA, tricarboxylic acid.

23 DEGs from C6-7 vs. C18-7 (**Figure 6B**). There were 5 common DEGs (**Supplementary Table 10**). The results showed that meta_217 (4-Hydroxypheny lpyruvate) was correlated with DEGs at 0 and 7 days of CS. The interaction network for G3 was visualized between 2 DAMs and 59 DEGs for C6-0 vs. C18-0 (**Figure 7A**) and 85 DEGs for C6-1 vs. C18-1 (**Figure 7B**). There were 39 DEGs shared between these two networks. Meta_291 (Alpha-D-Glucose) and meta_481 (Deoxyadenosin) were correlated with more DEGs at 1 day of stress than that of 0 days (**Supplementary Table 11**). Interaction network for G4 was carried out among 2 DAMs and 18 DEGs for C6-1 vs. C18-1 (**Figure 8A**) and 9 DEGs for C6-7 vs. C18-7 (**Figure 8B**); among which, 5 DEGs were common between these two groups. The network showed that meta_216 (DLVanillylmandelic acid) and meta_390 (N-Acetyl-L-phenylalanine) were clustered and associated with common and specific DEGs (**Supplementary Table 11**). Overall, our results showed strong and positive correlations between different DAMs and DEGs (involved in the same metabolic pathways), thus, contributing to CS tolerance and adaption in rapeseed.

GO and KEGG Analysis of DEGs Detected by Correlation Analysis

Differentially expressed genes correlated with G1-G4 (see **Table 1** for group settings) were further subjected to GO-biological process (BP) category analysis to determine the specific GO-BP terms related to DAMs under CS conditions. As shown in **Figure 5D**, the highly enriched DEGs are associated with the organic substance metabolic process (GO:0071704),

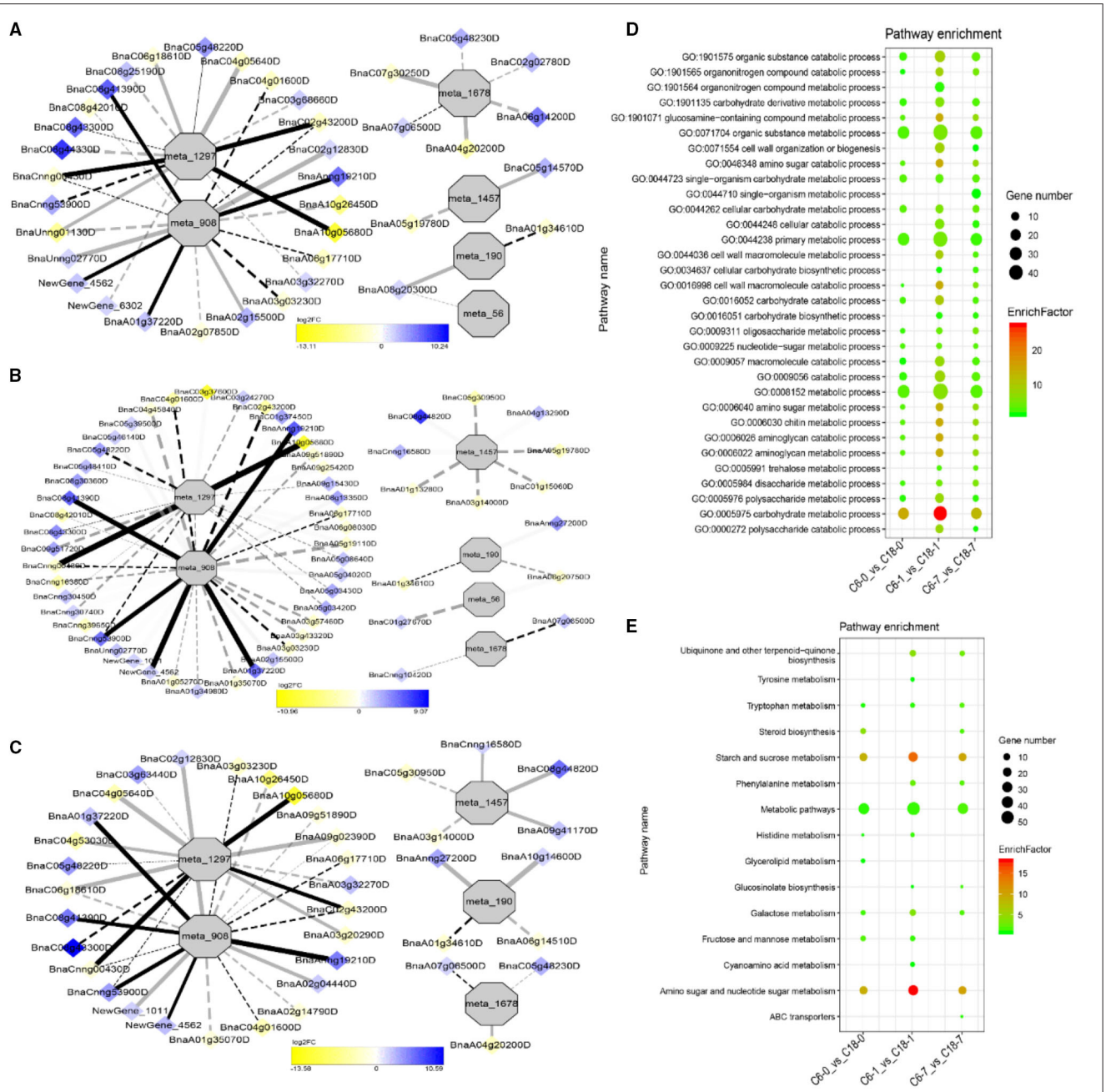

FIGURE 5 | Correlation network analysis between DAMs and regulatory genes related to cold stress (CS). **(A)** Analysis of C6-0 vs. C18-0, **(B)** analysis of C6-1 vs. C18-1, and **(C)** analysis of C6-7 vs. C18-7. Log_2FC of genes, in response to CS, are indicated in heatmap colors ranging from yellow (downregulated) to blue (upregulated); log_2FC values are indicated in the legend; the width of the connecting lines indicating the strength of the correlation; solid lines indicating the positive correlation and dashed lines indicating the negative correlation; black lines indicating the common genes and gray lines indicating the specific genes accumulated at that time point. **(D,E)** Scatter plots for the gene ontology-biological process (GO-BP) enrichment analysis and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DEGs from G1, respectively. Only the highly enriched and most relevant terms and pathways are shown in the plots ($Q < 0.05$.) $-log^{10}$ (Q) for enrichment factor.

primary metabolic process (GO:0044238), and metabolic process (GO:0008152). Most of the terms existed throughout the stress and only a few terms were specific to one time point. For instance, the carbohydrate metabolic process (GO:0005975) was highly significant and enriched at 1 day as compared to 0 and 7 days of CS. In comparison, some terms were explicitly enriched at 1 and 7 days, like the organonitrogen compound metabolic process (GO:1901564) and the single-organism metabolic process

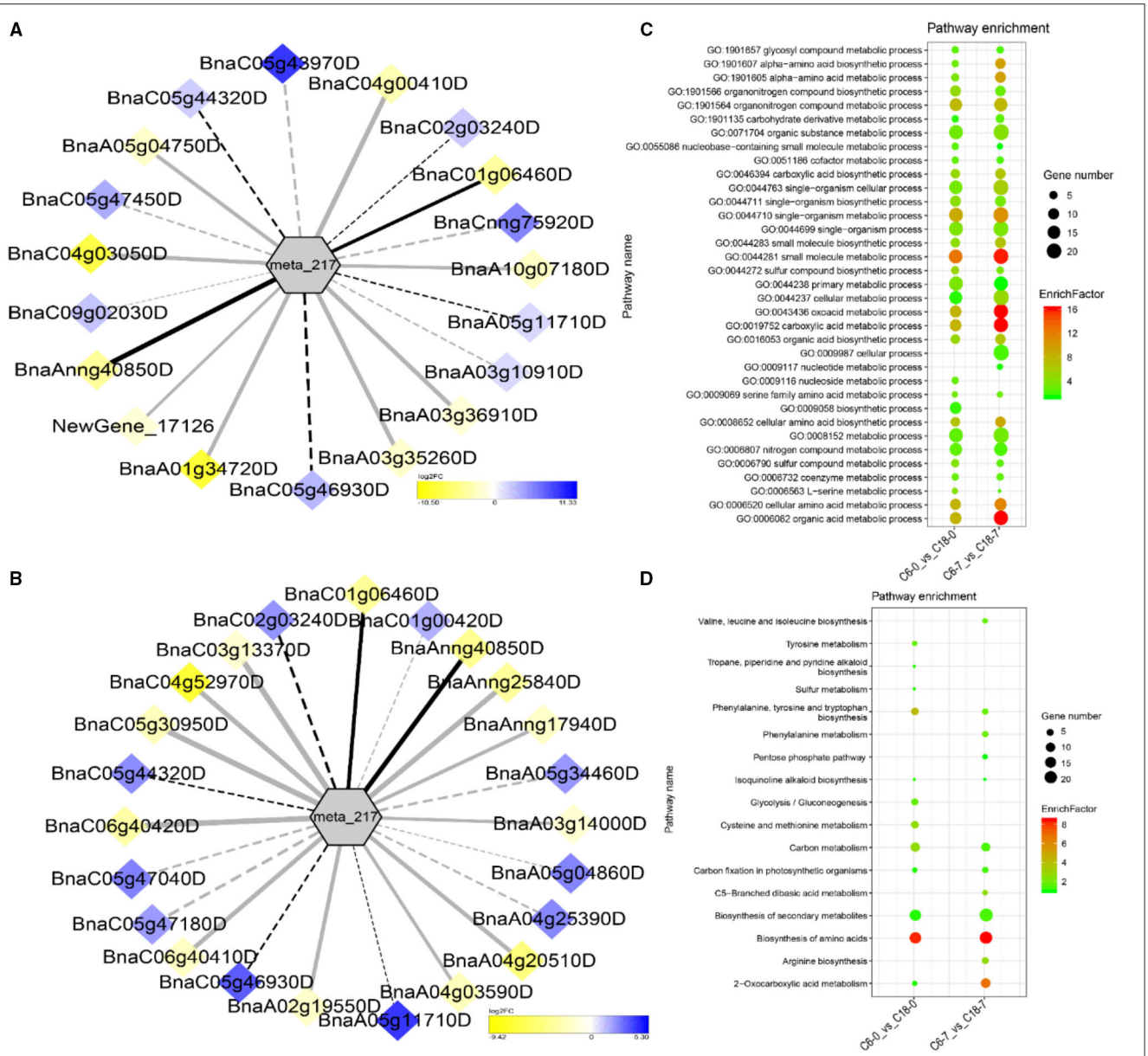

FIGURE 6 | Correlation network analysis between DAMs and regulatory genes related to CS. **(A)** Analysis of C6-0 vs. C18-0 and **(B)** analysis of C6-7 vs. C18-7. Log_2FC of genes, in response to CS, are indicated in heatmap colors ranging from yellow (downregulated) to blue (u-regulated); log_2FC values are indicated in the legend; the width of the connecting lines indicating the strength of the correlation; solid lines indicating the positive correlation and dashed lines indicating the negative correlation; black lines indicating the common genes and gray lines indicating the specific genes accumulated at that time point. **(C,D)** Scatter plots for the GO-BP enrichment analysis and KEGG pathway enrichment analysis of DEGs from G2, respectively. Only the highly enriched and most relevant terms and pathways are shown in the plots ($Q < 0.05$.) $-log^{10}$ (Q) for enrichment factor.

(GO:0044710). Similarly, DEGs correlated with meta_217 (**Table 1**, G2) were highly enriched in the same terms at 0 or 7 days of CS. Among these, the oxoacid metabolic process (GO:0043436), carboxylic acid metabolic process (GO:0019752), and organic acid metabolic process (GO:0006082) were more highly enriched at 7 days than that of 1 day (**Figure 6C**). The findings of G3 indicated that the carbohydrate metabolic process (GO:0005975) was significantly enriched at 1 day of CS treatment (**Figure 7C**). However, the enriched terms of DEGs from the two comparisons (C6-1 vs. C18-1 and C6-7 vs. C18-7) were totally different. Only the cellular amino acid metabolic process (GO:0006520) was highly enriched at 1 day (**Figure 8C**). Given the above observation, it was discovered that the enriched GO-BP terms were mainly the carbohydrate metabolic process, cellular amino acid metabolic process, organic acid metabolic process, carboxylic acid metabolic process, and

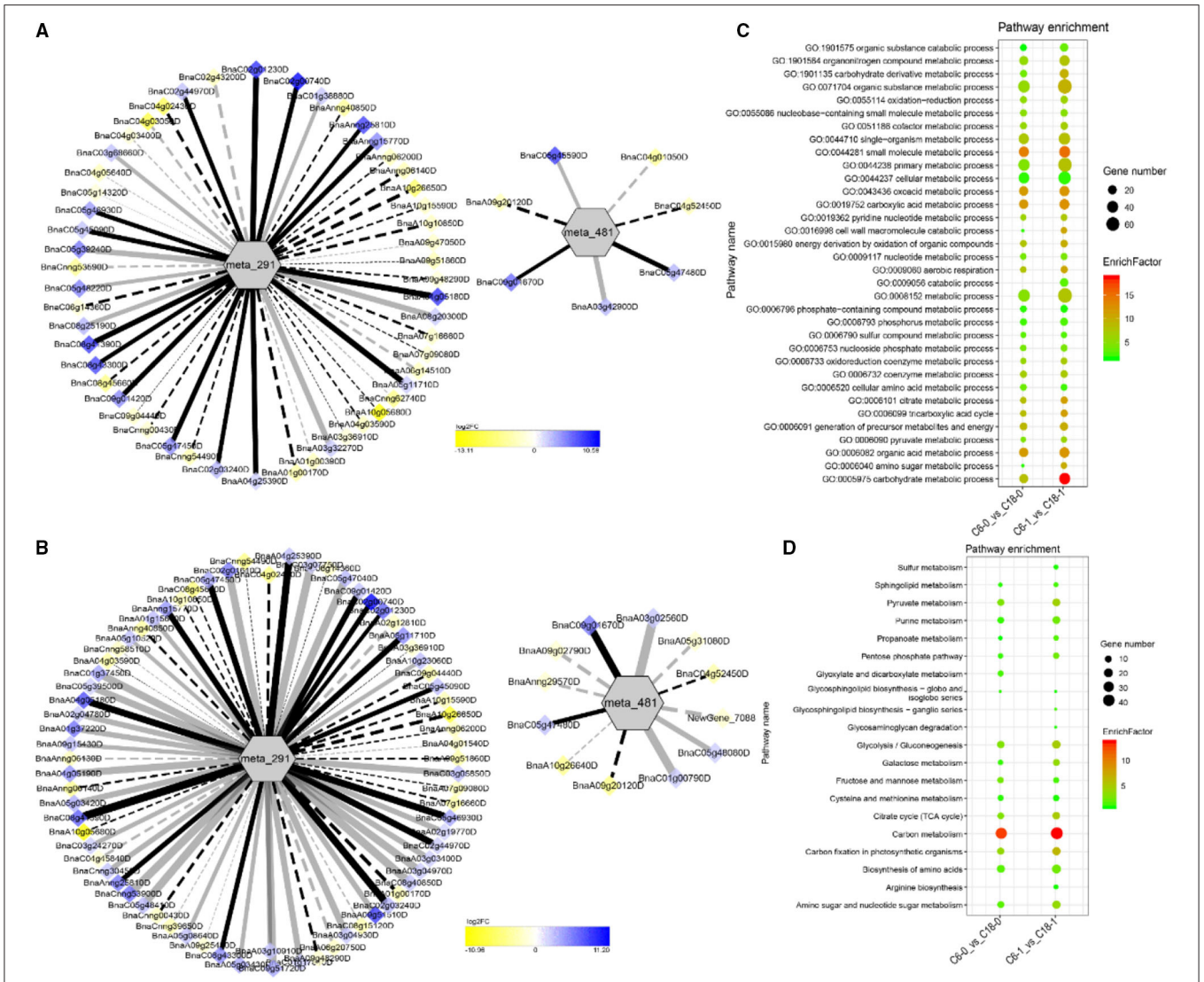

FIGURE 7 | Correlation network analysis between DAMs and regulatory genes related to CS. **(A)** Analysis of C6-0 vs. C18-0 and **(B)** analysis of C6-1 vs. C18-1. Log_2FC of genes, in response to CS, are indicated in heatmap colors ranging from yellow (downregulated) to blue (upregulated); log_2FC values are indicated in the legend; the width of the connecting lines indicating the strength of the correlation; solid lines indicating the positive correlation and dashed lines indicating the negative correlation; black lines indicating the common genes and gray lines indicating the specific genes accumulated at that time point. **(C,D)** Scatter plots for the GO-BP enrichment analysis and KEGG pathway enrichment analysis of DEGs from G3, respectively. Only the highly enriched and most relevant terms and pathways are shown in the plots ($Q < 0.05$.) $-log^{10}$ (Q) for enrichment factor.

oxoacid metabolic process. Taken together, the GO enrichment analysis in this study provided important guidance to identify DEGs that functioned in key pathways that may directly or indirectly involve in the CS response of rapeseed.

To further exploit the enriched metabolic pathways related to CS tolerance and adaptation in rapeseed, DEGs from G1 to G4 (**Table 1**) were subjected to KEGG enrichment analysis. The results of G1 showed that amino sugar and nucleotide sugar metabolism was mostly enriched throughout the CS at 0, 1, and 7 days. A similar trend was observed for starch and sucrose metabolism (**Figure 5E**). The results of G2 revealed that the biosynthesis of amino acids was the most significant and enriched pathway existing in two time points, i.e., 1 and 7 days of CS. Notably, 2–Oxocarboxylic acid metabolism was specifically enriched at 7 days of CS (**Figure 6D**). The results of G3 indicated that carbon metabolism was enriched at 0 or 1 day of CS, which was consistent with the corresponding GO-BP analysis (**Figure 7D**). Similarly, phenylalanine metabolism, tyrosine metabolism, isoquinoline alkaloid biosynthesis, tropane, piperidine, and pyridine alkaloid biosynthesis were enriched terms in G4 at 1 day of CS (**Figure 8D**). As a result, we discovered that the KEGG and GO-BP analyses were mainly matched with

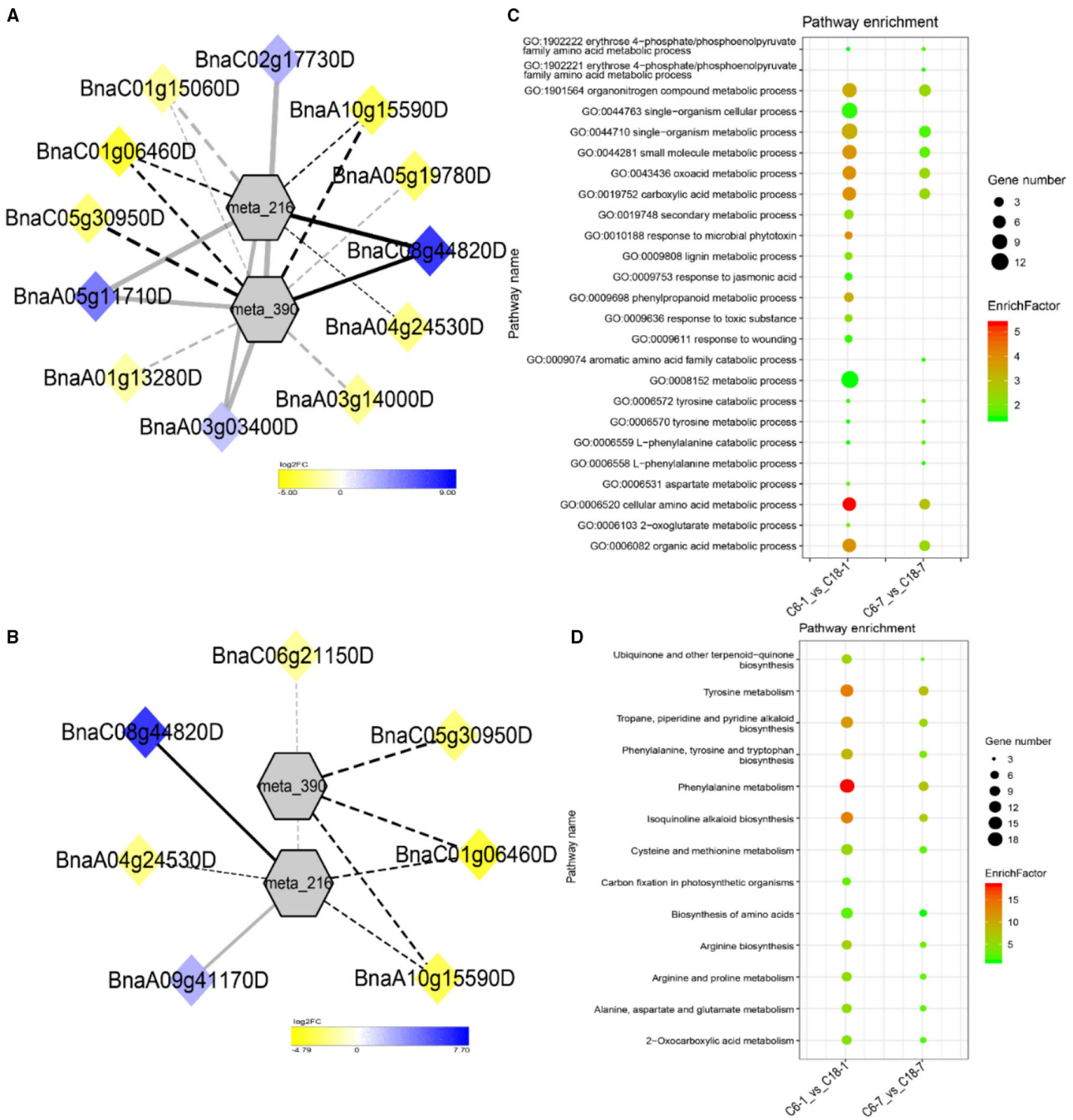

FIGURE 8 | Correlation network analysis between DAMs and regulatory genes related to CS. **(A)** Analysis of C6-1 vs. C18-1 and **(B)** analysis of C6-7 vs. C18-7. Log_2FC of genes, in response to CS, are indicated in heatmap colors ranging from yellow (downregulated) to blue (upregulated); log_2FC values are indicated in the legend; the width of the connecting lines indicating the strength of the correlation; solid lines indicating the positive correlation and dashed lines indicating the negative correlation; black lines indicating the common genes and gray lines indicating the specific genes accumulated at that time point. **(C,D)** Scatter plots for the GO-BP enrichment analysis and KEGG pathway enrichment analysis of DEGs from G4, respectively. Only the highly enriched and most relevant terms and pathways are shown in the plots ($Q < 0.05$.) $-log^{10}$ (Q) for enrichment factor.

each other, generating two terms (amino acid metabolisms and carbohydrate metabolisms) associated with CS responses in both rapeseed varieties. These comparable findings of GO-BP and KEGG enrichment analyses showed that mainly amino acid and carbohydrate metabolisms could play a crucial role in the CS tolerance and adaptation of rapeseed.

Starch and Sucrose Metabolism in Response to CS

Based on the GO and KEGG enrichment analyses, many DAMs and DEGs of rapeseed were correlated with starch and sucrose metabolism under CS conditions. In total, 5 DAMs were correlated with 19, 34, and 19 DEGs from the comparisons of C6-0 vs. C18-0, C6-1 vs. C18-1, and C6-7 vs. C18-7, respectively. **Figure 9A** shows the proposed general layout of starch and sucrose metabolism and the relative changes of 5 DAMs during CS treatment based on the three comparisons. Trehalose (meta_908) was the only metabolite that accumulated throughout the stress, followed by cellobiose (meta_1009), which accumulated only at 1 day of treatment, suggesting that CS-C6 should keep a higher level of trehalose and cellobiose than that of CT-C18. Meanwhile, glycogen (meta_2272), D-glucose-6P (meta_514), and D-fructose-6P (meta_697) were downregulated at a different time point of the treatment, indicating that CT-C18 should keep a higher level of glycogen, D-glucose-6P, and D-fructose-6P than that of C6. These outcomes indicate that the higher contents of different sugar-related compounds could significantly contribute to CS tolerance in rapeseed.

The expression patterns of the DEGs involved in the starch and sucrose metabolism pathway were summarized in the heatmap (**Figure 9B**). These DEGs were correlated with one to three of the 5 mentioned DAMs and revealed diverse expression patterns in both varieties. For instance, D-glucose-6P was one of the major products of starch and sucrose metabolism that were correlated with 16 DEGs. The three DEGs directly related to the glucose branch showed lower expression in C6, subsequently leading to a lower accumulation of D-glucose-6P in CS-C6. However, the DEGs that functioned in other branches were up- or downregulated in the comparisons. Besides D-glucose-6P, no significant difference was observed for the other final product contents of the starch and sucrose metabolism pathway within the two rapeseed varieties. These results suggested that the glucose branch was strongly improved in CT-C18 and may contribute to the CS tolerance of rapeseed.

Notably, 10 DEGs (common/shared) were detected by three comparisons (**Figure 9B**). Among them, six DEGs exhibited higher expression level in CS-C6, encoding beta-glucosidase (BnaAnng19210D), beta-fructofuranosidase (BnaA01g37220D or BnaC08g41390D), trehalose-6-phosphate phosphatase (newGene_4562), beta-amylase (BnaC02g12830D), and UTP–glucose-1-phosphate uridylyltransferase (BnaCnng53900D) enzymes. Furthermore, 4 DEGs exhibited higher expression level in C18, which encoded 4-alpha-glucanotransferase (BnaC04g01600D), sucrose-phosphate synthase (BnaA03g03230D), glycogen phosphorylase (BnaA06g17710D), and glucose-1-phosphate adenylyltransferase (BnaCnng00430D) enzymes. These enzymes led to the strong CS-induced accumulation of different forms of glucose, fructose, trehalose, and maltose. Most of the genes responsible for the biosynthesis of these carbohydrates were upregulated in both varieties under CS and showed greater changes in CT-C18 than CS-C6 at 1 or 7 days after exposure to CS (**Figure 9B**). This suggests that CT-C18 had performed better in accumulating some carbohydrates and, thus, showed an improved CS tolerance level compared to the CS-C6 variety. On the contrary, the lower level of these molecules might lead to sensitivity under CS conditions.

Phenylalanine Metabolism in Response to CS

It was found that phenylalanine metabolism was significantly enriched in both metabolome and transcriptome data. In total, 6 DAMs were correlated with 7, 11, and 8 DEGs detected from C6-0 vs. C18-0, C6-1 vs. C18-1, and C6-7 vs. C18-7, respectively. **Figure 10A** shows the schematic layout of phenylalanine metabolism and the relative changes of the six DAMs in both varieties under CS conditions. Notably, only two DAMs, i.e., 2-Phenylacetamide and N-Acetyl-L-phenylalanine, were accumulated at 0 and 1 day while 2-Hydroxyphenylacetic acid, N-Acetyl-L-phenylalanine, 3-Phenylpropanoic acid, and salicylic acid were upregulated, and succinate was downregulated at 7 days (**Figure 10**).

The expression patterns of DEGs involved in the phenylalanine metabolism pathway were presented in the heatmap (**Figure 10B**). Particularly, 5 DEGs (common) were detected by three comparisons (**Figure 8B**). Among these, two genes were upregulated and encoding 4-hydroxyphenylpyruvate dioxygenase (BnaC08g44820D) and aspartate aminotransferase (BnaA05g11710D)-mitochondrial chloroplastic/cytoplasmic enzymes, whereas three genes were downregulated, among which two genes were encoding aspartate aminotransferase (BnaC01g06460D and BnaA10g15590D)-chloroplastic/cytoplasmic and one gene was encoding trans-cinnamate 4-monooxygenase (BnaA03g14000D) enzymes. The three mentioned genes were responsible for the biosynthesis of phenylalanine or phenylpyruvate and trans-4-hydroxycinnamate, respectively.

Overall, most of the genes involved in the pathways leading to the accumulation of phenylacetamide (meta_741), succinate (meta_211), 2-Hydroxyphenylacetic acid (meta_266), N-Acetyl-L-phenylalanine (meta_390), and 3-Phenylpropanoic acid (meta_75), while salicylic acid (meta_92) was downregulated. A few genes were upregulated based on the three comparisons (**Figure 10A**), suggesting the DEGs were expressed at a higher level in CT-C18. However, other final or intermediate products of phenylalanine metabolism exhibited no difference between the two varieties except for the three DAMs. These findings suggested that rapeseed responded to CS by normalizing amino acid accumulation through phenylalanine metabolism. Nonetheless, having higher contents of these DAMs, mainly in CT-C18, may be associated with CS tolerance in rapeseed.

Validation of Transcriptome and Metabolome Data

Six DEGs were randomly selected (involved in the starch and sucrose and phenylalanine metabolisms) to validate the RNA-seq data with qRT-PCR. As demonstrated in **Supplementary Figure 7**, all the selected genes display

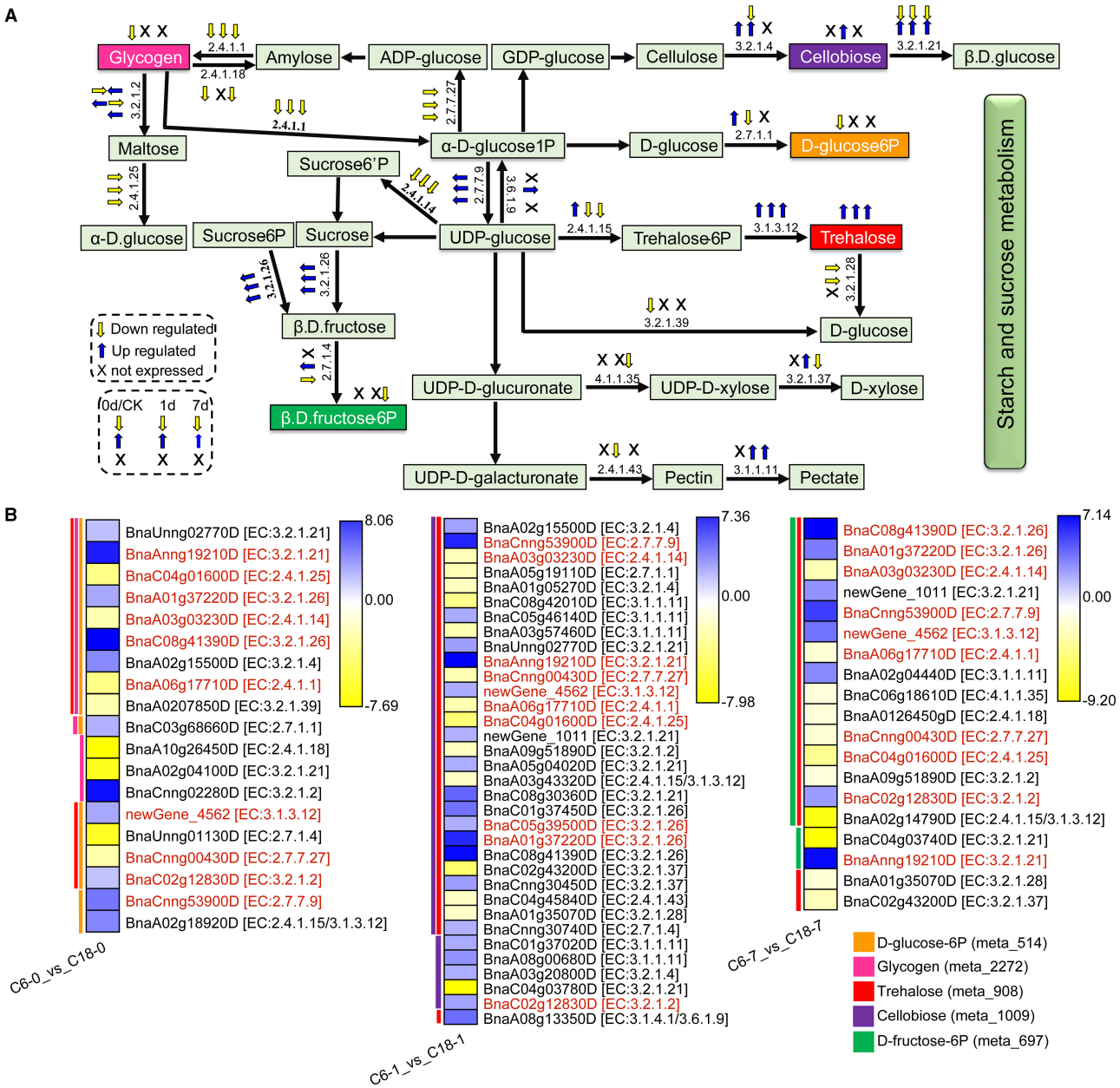

FIGURE 9 | The proposed layout of the starch and sucrose metabolism pathway in response to CS in C6 and C18 cultivars. **(A)** Layout showing the DEGs involved in starch and sucrose metabolism. Colored boxes indicate the metabolites observed in this pathway. This pathway shows only the DEGs that have been correlated with the metabolites from the combined analysis of transcriptomes and metabolomes. The yellow and blue arrows indicate the expression pattern (up-/downregulated) of DEGs based on the three comparisons, i.e., C6-0 vs. C18-0, C6-1 vs. C18-1, and C6-7 vs. C18-7, whereas "X" indicates not expressed at specific time points. **(B)** Heatmap showing the expression of DEGs corresponding to "A." Red text color for gene names indicating the shared/common DEGs. Colored bars on the left side of each heatmap showing the metabolites correlated with DEGs based on Pearson correlation coefficient (PCC) analysis. The enzyme commission (EC) numbers are as follows: 2.4.1.1, glycogen phosphorylase; 2.4.1.14, sucrose-phosphate synthase; 2.4.1.15/3.1.3.12, trehalose 6-phosphate synthase/phosphatase; 2.4.1.18, 1,4-alpha-glucan branching enzyme 2-2; 2.4.1.25, 4-alpha-glucanotransferase; 2.4.1.43, alpha-1,4-galacturonosyltransferase; 2.7.1.1, hexokinase; 2.7.1.4, fructokinase; 2.7.7.9, UTP–glucose-1-phosphate uridylyltransferase; 2.7.7.27, glucose-1-phosphate adenylyltransferase; 3.1.1.11, pectinesterase; 3.1.3.12, trehalose 6-phosphate phosphatase; 3.2.1.2, beta-amylase; 3.2.1.4, endoglucanase; 3.2.1.21, beta-glucosidase; 3.2.1.26, beta-fructofuranosidase; 3.2.1.28, alpha, alpha-trehalase; 3.2.1.37, beta-D-xylosidase 4; 3.2.1.39, glucan endo-1,3-beta-glucosidase 5/6; 3.1.4.1/3.6.1.9, ectonucleotide pyrophosphatase/phosphodiesterase family member 1/3; 4.1.1.35, UDP-glucuronate decarboxylase.

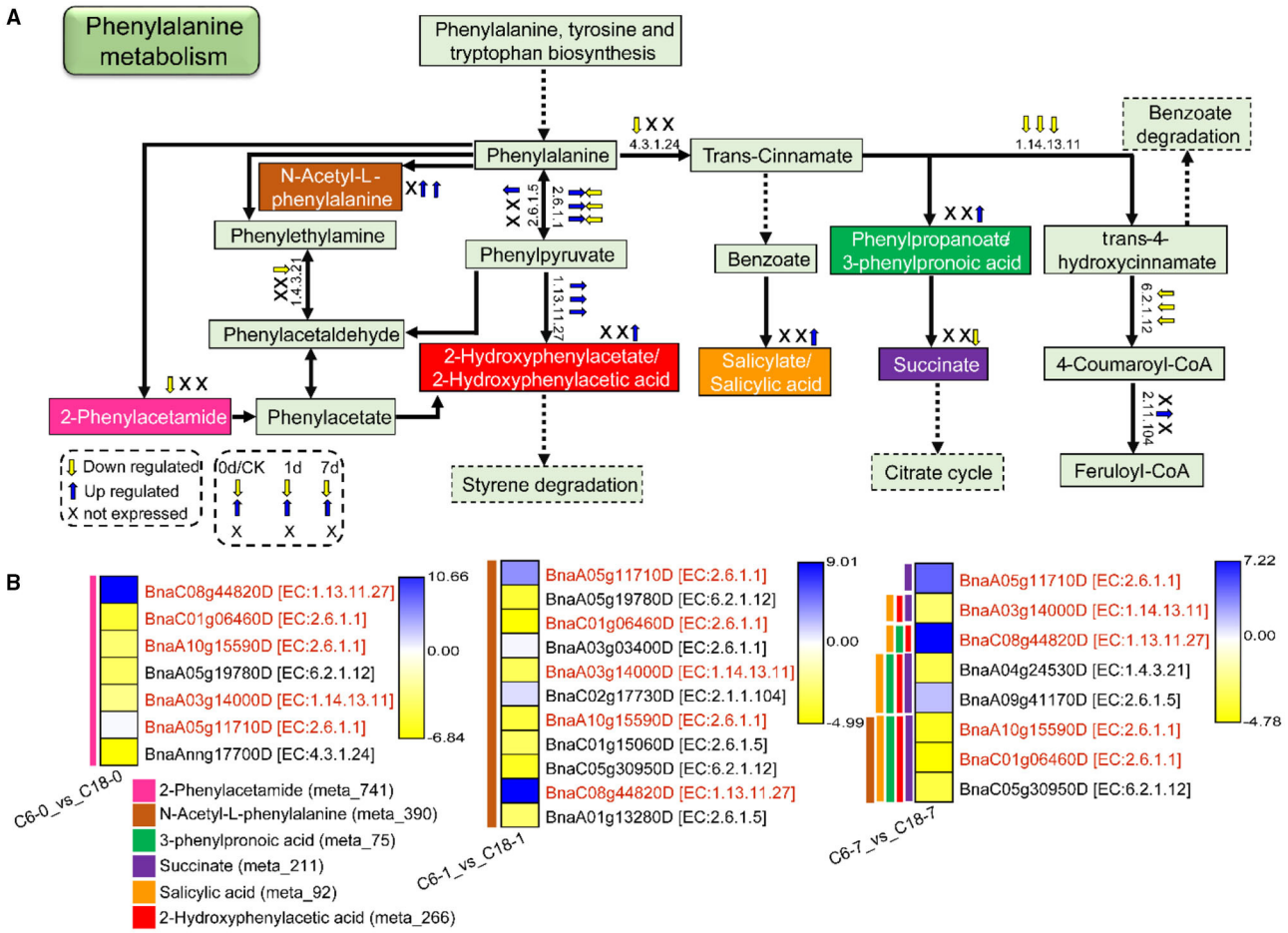

FIGURE 10 | The proposed layout of the phenylalanine metabolism pathway in response to CS in C6 and C18 cultivars. **(A)** Layout showing the DEGs involved in the phenylalanine metabolism. Colored boxes are indicating the metabolites observed in this pathway. This pathway shows only the DEGs that have been correlated with the metabolites from the combined analysis of transcriptomes and metabolomes. Dotted lines show the omission of some reactions. The yellow and blue arrows indicate the expression pattern (up-/downregulated) of DEGs based on the three comparisons, i.e., C6-0 vs. C18-0, C6-1 vs. C18-1, and C6-7 vs. C18-7, whereas "X" indicates not expressed at specific time points. **(B)** Heatmap showing the expression of DEGs corresponding to "A." Red text color for gene names indicating the shared/common DEGs. Colored bars on the left side of each heatmap showing the metabolites correlated with DEGs based on PCC analysis. The EC numbers are as follows: 1.4.3.21, primary-amine oxidase; 1.13.11.27, 4-hydroxyphenylpyruvate dioxygenase; 1.14.13.11, trans-cinnamate 4-monooxygenase; 2.6.1.1, aspartate aminotransferase, chloroplastic/cytoplasmic; 2.6.1.5, tyrosine aminotransferase; 2.1.1.104, caffeoyl-CoA O-methyltransferase; 4.3.1.24, phenylalanine ammonia-lyase; 6.2.1.12, coumarate–CoA ligase.

similar expression patterns to those generated from RNA-seq data, thus indicating the reliability of the RNA-seq data. Likewise, five DAMs were also validated by the targeted metabolite detection method, and the L-Tyrosine contents were significantly increased at 1 and 7 days of stress in both varieties. Similarly, L-Kynurenine contents were significantly increased at 7 days rather than 0 and 1 day. On the other hand, the succinic acid contents were reduced at 7 days in both varieties, while the N-Acetyl-L-Phe content was notably increased at 7 days in the CT-C18 variety than CS-C6. Similarly, the salicylic acid content was increased at 7 days in the CT-C18 variety compared to 0 and 1 day of CS treatments (**Supplementary Figure 8**). The noticed accumulation trends were consistent for selected metabolites, indicating the reliability of the metabolome data.

Functional Validation of Candidate Genes

For functional validation, some candidate genes were selected from the starch and sucrose metabolism and phenylalanine metabolism pathways. Previous studies showed that the use of *Arabidopsis* homologues T-DNA insertion mutants is a very suitable approach for functional validation, which has functional elements of targeted genes and can provide insights into the key role of a particular gene under a given environment (Jia et al., 2016; Melencion et al., 2017; Yu et al., 2020). Our selections of genes were based on the hypothesis that mutations (lack-of-function) of these candidate genes would affect CS tolerance levels in rapeseed. To date, the homologues of selected rapeseed genes, including *Bn4CL3* (BnaC05g30950D), *BnCEL5* (BnaA02g15500D), *BnUGP1* (BnaCnng53900D), *BnFRUCT4* (BnaC08g41390D), *BnAXS1* (BnaC08g43300D),

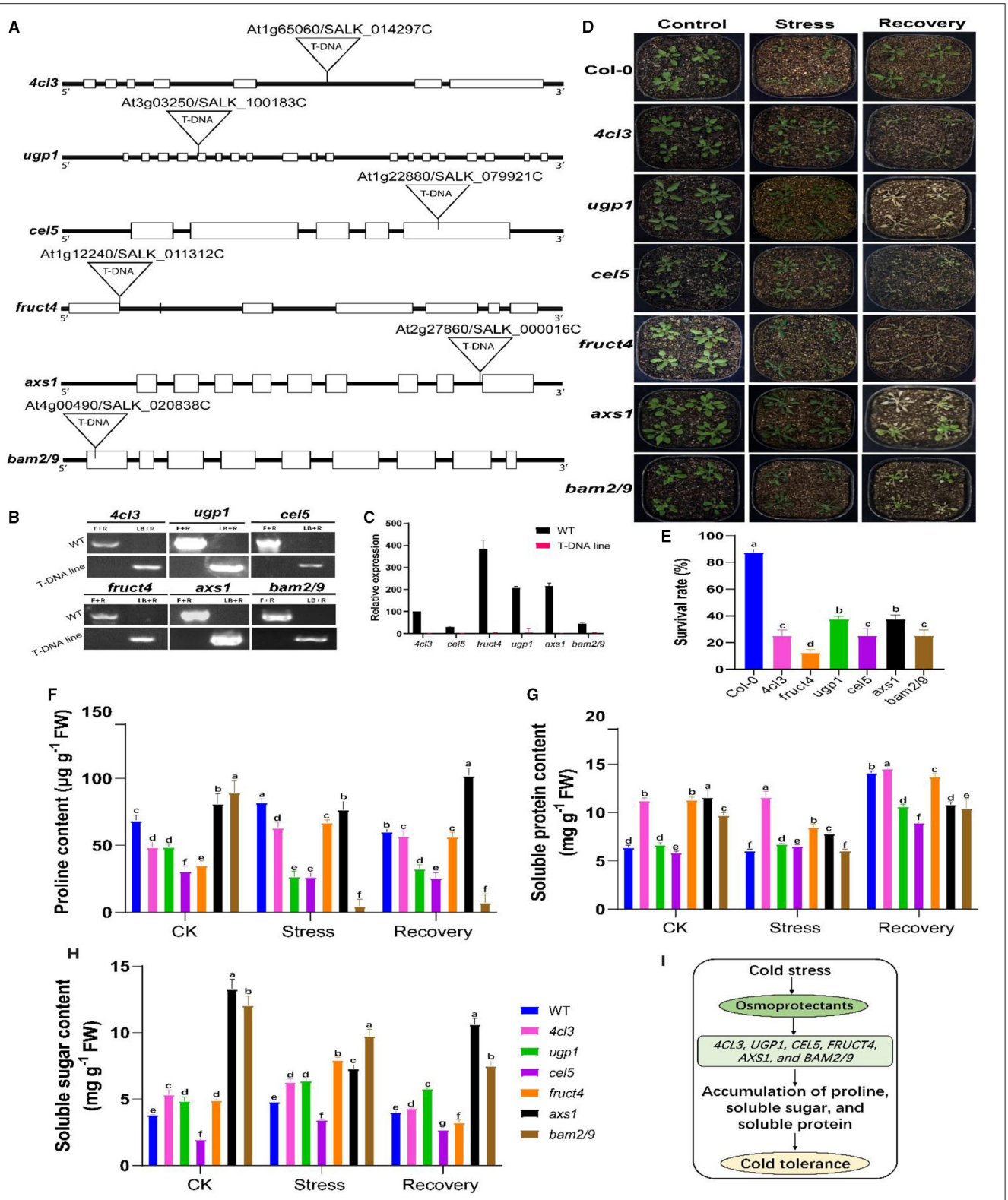

FIGURE 11 | Functional validation of selected candidate genes using *Arabidopsis* mutants. **(A)** Schematic diagram of the gene structure of *4cl3* (4-coumarate coenzyme A ligase 3), *ugp1* (UDP-glucose pyrophosphorylase 1), *cel5* (CELLULASE 5), *fruct4* (beta-fructosidase 4), *axs1* (UDP-D-xylose synthase 1/UDP-D-apiose), and *bam2/9* (beta-amylase 2/9). Exons and introns are indicated in black boxes and black lines, respectively. **(B)** PCR-based genotyping of these mutants. **(C)**

(Continued)

FIGURE 11 | Relative expression of mutant genes in wild-type (WT) and mutant plants. **(D)** Phenotypic evaluation before stress [control (CK) at 25°C], 8 h of freezing stress (−5°C), and after 2 days of the recovery phase (recovery at 25°C). **(E)** Analysis of survival rates in the leaves of the above-mentioned mutants and WT plants during the freezing treatment. **(F–H)** Analysis of the osmotic substances (proline, soluble protein, and soluble sugar) in selected *Arabidopsis* mutants before stress (CK), after stress (−5°C for 8 h), and after 2 days of recovery at 25°C. **(I)** A working proposed model for the role of osmoprotectants in mutants for cold tolerance. Upon exposure to cold stress, the accumulation of osmotic substances helps improve cold tolerance. Error bars represent SD ($n = 3$) of the three biological replicates in **(D)** and the mean of the three technical replicates **(E–G)**. The statistical significance was determined *via* one- or two-way ANOVA and Dunnett's multiple comparisons test. Different lowercase letters on the error bars indicate significant differences at $p \leq 0.05$.

and *BnBAM2/9* (BnaCnng02280D/BnaA09g51890D) have not been characterized. The sequence similarity between respective rapeseed and *Arabidopsis* lines were ranged from 69 to 96% (**Supplementary Figure 9**). Hence, T-DNA insertion mutants for the *Arabidopsis* homologues including *4cl3* (SALK 014297C), *cel5* (SALK 079921C), *ugp1* (SALK 100183C), *fruct4* (SALK 011312C), *axs1* (SALK 000016C), and *bam2/9* (SALK 020838C) were functionally validated under freezing stress conditions (**Figure 11**). The phenotypic evaluation results showed that the leaves of the mentioned mutants concurrently suffered severe freezing injuries and existed with low survival rates compared to the control plants after the freezing treatment, whereas the mutants of the homologues of *4cl3*, *cel5*, *fruct4*, *ugp1*, *axs1*, and *bam2/9* were sensitive to freezing stress (**Figures 11E,F**). These outcomes support our hypothesis that the lack-of-function of these genes could cause sensitivity to CS in rapeseed. On the other hand, these overexpression of these genes (in future works) could significantly improve CS tolerance in rapeseed plants by regulating sugar and amino acid contents in a particular metabolism.

According to our hypothesis, to get further mechanistic insights into the mechanisms behind CS tolerance, we evaluated osmoprotectant substances, including proline (Pro), soluble sugar (SS), and soluble protein (SP), and their contents in *Arabidopsis* mutants (**Figures 11F–H**). Results showed that all the mutants greatly differed in Pro, SS, and SP contents before and after stress and after recovery at 25°C. For example, *4cl3* showed a higher accumulation of Pro, SP, and SS than CK (**Figures 11F–H**), while, in other mutants, it was noticed that the contents of osmoprotectants were increased after stress, and, in some cases, a slight reduction was observed at the recovery stage compared to CK. However, this was not the case in all mutants. For instance, *cel5* did not significantly differ in the osmoprotectant content; whereas, in *bam2/9*, the SS, SP, and Pro, contents were decreased after stress treatment compared to CK. These results agreed with our hypothesis that CS leads to the accumulation of osmoprotectants, which helps plants minimize the adverse effect of CS (**Figure 11I**). Overall, it can be concluded that these candidate genes were expected to play a significant role in CS response and tolerance in rapeseed. In the future, genetic analysis can be carried out in rapeseed to understand the key role of these genes. Hence, these results confirmed the influence of conjoint analysis in revealing the genetic mechanisms of CS response and presented a set of candidate genes for improving CS tolerance in rapeseed.

DISCUSSION

Cold stress is one of the crucial environmental factors affecting the growth and development of plant species (Hu et al., 2017; He et al., 2021). Plants have evolved governing mechanisms that can respond to CS, including alterations in gene expression and metabolic profiles (Raza, 2020a; Mehmood et al., 2021; Raza et al., 2021b). Different genotypes have revealed different abilities to sustain growth and production. Therefore, it is important to use high-throughput omics approaches to identify CS-responsive genes or metabolites (Raza et al., 2021a,b). In the current study, an integrated analysis of transcriptomes and metabolomes during CS treatment was carried out in the CT-C18 and CS-C6 varieties, which allowed us to gain insights into the key metabolites, genes, and metabolic pathways involved in CS adaptation and tolerance in rapeseed.

General Metabolome Analysis Revealed Stress Responses and Tolerance Mechanisms in Rapeseed

The plant kingdom comprises ∼200,000 metabolites, majority of which are still unknown, reflecting their complicated roles in the natural life of plants. Metabolites are the final products of metabolism, playing substantial physiological and biochemical roles in several plant species under various environmental stresses (Foito and Stewart, 2018; Raza, 2020b). It has been reported that different sugars and amino acids such as trehalose, glucose, fructose, inositol, galactinol, raffinose, sucrose, putrescine, ascorbate, phenylalanine, and alanine would accumulate after CS in tobacco (Jin et al., 2017). However, the variation of metabolites in response to CS in rapeseed was yet to be reported. Thus, the current research also confirmed the fluctuation in the levels of the different metabolites in rapeseed varieties during CS treatment and the significant difference of DAMs belonging to carbohydrates, sugars, and amino acids between the two rapeseed varieties (**Table 1**). However, a huge number of metabolites that respond to multiple abiotic stresses, including CS in rapeseed and other major crop plants is unknown, unannotated, and yet to be reported.

For example, the adenosine 3′,5′-cyclic phosphate (cAMP; meta_1043) works as a secondary messenger upon binding activities protein kinase A (PKA), allowing the phosphorylation of protein substrates (Alqurashi et al., 2016). According to a previous report, cAMP shortage harmfully disturbs cell proliferation (Sabetta et al., 2016). In *Arabidopsis*, a cAMP-based proteome revealed the vital role of cAMP under salinity

and CS conditions, suggesting that cAMP is crucial to induce multifaceted variations in cellular energy homeostasis (Alqurashi et al., 2016). In our study, the increased accumulation of cAMP was detected in C6-0 vs. C18-0 and C6-7 vs. C18-7 (**Table 1**, G2). Likewise, acetylcarnitine, alpha-D-glucose, and deoxyadenosine had a higher expression in short-term stress responses (**Table 1**, G3). The high quantities of cAMP and acetylcarnitine have also been reported by Djami-Tchatchou et al. (2019) in chili pepper inoculated with *Pectobacterium carotovorum*. Some ammonium molecules such as acetylcarnitine can be abundant in some soils and are thus taken up as intact compounds by plants. These ammonium compounds play a vital role in stress responses and rely on nitrogen cycling and plant nutrition (Warren, 2013). The detection of acetylcarnitine is becoming increasingly common in plants under normal and stressful conditions, e.g., plant developmental processes (Nguyen et al., 2016) and recovery from salinity stress (Charrier et al., 2012). Further, another important metabolite, trehalose, is composed of two alpha-D-glucose molecules. These molecules play a key role in stress tolerance and different developmental processes by the hydrolysis of trehalose. Hence, Liu et al. (2017) found the upregulation of alpha-D-glucose in okra metabolome analysis during postharvest senescence, confirming the vital role of trehalose in senescence.

Senescence plays a significant role in mobilizing nutrients from the roots to other plant parts for proper growth and development. In this regard, sugar molecules act as the main signaling molecules and help the movement of nutrients to cope with the current stress environment (Sami et al., 2016). In metabolome analysis, the upregulation of deoxyadenosine has been noted in maize seedlings when exposed to salinity stress (Yue et al., 2020) and in Tibetan hulless barley under CS (Yang et al., 2020).

Cytidine 5′-diphosphocholine (CDP-choline) is a nucleotide consisting of choline, cytosine, ribose, and pyrophosphate molecules and was upregulated in C6-1 vs. C18-1 and C6-7 vs. C18-7 comparisons. Several metabolome studies found the upregulation of CDP-choline, for instance, in halophilic microalga under salinity stress (Jiang et al., 2019). In another study, Sawada et al. (2017) reported the higher expression pattern of CDP-choline in a single-grain-based metabolome profiling of *Arabidopsis* seeds. Zhang et al. (2020) suggested that choline-mediated lipid reprogramming could lead to a salinity tolerance mechanism in non-glycine betaine accumulating Kentucky bluegrass (*Poa pratensis*). An untargeted metabolome profiling between japonica and indica rice cultivars revealed the upregulation of gamma-tocotrienol (a type of vitamin E) during network analysis (Hu et al., 2014). Recently, the metabolic profiling of DREB-overexpressing transgenic wheat seeds also indicated the upregulation of gamma-tocotrienol under different abiotic stress conditions (Niu et al., 2020). Tocotrienols arise in photosynthetic plants in variable quantities, and vegetable oils like sunflower, corn, safflower, and cottonseed deliver a valuable basis for these vitamin E forms (Ahsan et al., 2015).

Another metabolite, N-Acetyl-L-phenylalanine, had a higher expression in a long-term stress response (**Table 1**, G4). This metabolite has also been upregulated in *Atriplex halimus* metabolic profiling when exposed to salinity and drought stress (Alla et al., 2012). Moreover, the comparative metabolome and transcriptome analysis of different tissues of wheat plants documented the vital role of the upregulation of N-Acetyl-L-phenylalanine in stamen and pistil growth (Yu et al., 2019). These findings suggest that N-Acetyl-L-phenylalanine is another vital metabolite responsible for stress tolerance and several plant developmental stages.

The organic compounds Tyr-Asp were upregulated in a long-term stress response (**Table 1**, G4). Several metabolome-based investigations found the higher expression of this compound under different stress conditions. For instance, Sun et al. (2015) suggested that the upregulation of Tyr-Asp plays an important role in plant stress physiology and alters the plant metabolome profiling in wheat plants under drought and salt stress. In addition, squalene was down-regulated throughout the stress at all time points (**Table 1**, G1). A similar trend was also reported by Lu et al. (2016) under CS in *Nitrosomonas europaea*, as ammonia uptake patterns were altered.

In this study, trehalose, L-Kynurenine, gamma-tocotrienol, phenyllactic acid, and L-Gulonic gamma-lactone had increased contents throughout the stress in both varieties (**Table 1**, G1), suggesting the vital role of these DAMs in CS tolerance and adaptation. According to previous investigations, L-kynurenine has been characterized for auxin biosynthesis *via* the TAR/YUC pathways in plants (Brumos et al., 2018; Wang et al., 2018b). Under ammonium stress, the exogenous application of L-kynurenine inhibits root growth in rice, increases sensitivity to ammonium, and helps rice plants improve auxin biosynthesis under stress conditions (Di et al., 2018). Notably, gamma-tocotrienol, phenyllactic acid, and L-Gulonic gamma-lactone are actively involved in a wide range of biotic stresses (He et al., 2017; Fan and Song, 2018; Tóth et al., 2018; Dao et al., 2019). In the current study, these DAMs were upregulated and accumulated throughout the stress in both sensitive and tolerant varieties. Among these DAMs, trehalose, a naturally occurring sugar present in numerous organisms of plants, and several studies have demonstrated the beneficial role of trehalose against several abiotic stresses such as cold tolerance in rice (Fu et al., 2020), chickpeas (Farooq et al., 2017), and tomatoes (Liu et al., 2020). The overexpression of the trehalose-encoding gene, trehalose 6-phosphate synthase 11 (*TaTPS11*), improves the freezing tolerance in *Arabidopsis* (Liu et al., 2019). Notwithstanding the insights provided by these investigations, some of the identified metabolites are yet to be studied under various abiotic stresses. Furthermore, resolving some bottlenecks such as mistakes in the identification and annotation tools would lead to the precise identification of a huge number of metabolites under adverse environmental stress conditions.

Roles of Enriched Pathways Identified by the Combined Analysis

In this study, the enrichment analyses (KEGG, BP-GO, and MapMan) of DEGs correlated with DAMs were significantly associated with amino acid and carbohydrate metabolisms (**Figure 4** and **Supplementary Table 7**). As previous studies

showed, amino acid and carbohydrate metabolic pathways play significant roles in the salinity stress tolerance of tomatoes (Zhang et al., 2017). Several amino acid pathways have been observed after drought stresses in chickpea plants (Khan et al., 2019). Similar observations (tryptophan, phenylalanine, and histidine metabolisms) have also been detected in a combined metabolome and phenome analysis of maize under drought stress conditions (Witt et al., 2012). The three essential metabolisms, such as phenylalanine, tryptophan, and tyrosine, are well-thought-out in plant metabolisms (Galili and Höfgen, 2002). To date, many combined-omics studies have detected the vital role of these three metabolites and metabolic pathways, such as in *Pinus radiata* under high-temperature stress conditions (Escandón et al., 2018), in chickpea under drought stress conditions (Khan et al., 2019), in tobacco under CS conditions (Zhou et al., 2019), and in *Sargassum fusiforme* under heat stress conditions (Xie et al., 2019). Interestingly, the high accumulation of several carbohydrates provides energy to plants and helps them to cope with multiple abiotic stresses (Gupta and Kaur, 2005; Zhang et al., 2017). In *Salvadora persica*, a similar trend of galactose metabolism, starch and sucrose metabolism, and phenylalanine metabolism were observed after water deficit stress (Rangani et al., 2020). In rice, fructose and mannose metabolism and glutathione metabolism were significantly enriched after drought stress (Ma et al., 2016). Additionally, carbohydrate metabolism has been reported to be critical for heat and drought stress in soybeans (Das et al., 2017), for drought stress in poplar (Jia et al., 2020), and for salinity stress in sesame (Zhang Y. et al., 2019). These observations provide further evidence for the significant role of amino acid and carbohydrate metabolic pathways in response to various abiotic stresses.

The ABC transporters were one of the major enriched biological pathways detected during CS, mainly in CS-C6 (**Supplementary Figure 3B**), which was consistent with previous reports. In tall fescue, ABC transporters have been identified in response to nitric oxide-modulated cadmium stress tolerance by a combined transcriptome and metabolome analysis (Zhu et al., 2020). The ABA transporters and/or proteins are mainly involved in translocating several substances such as carbohydrates, ions, lipids, xenobiotics, antibiotics, drugs, heavy metals, etc., within the plants (Theodoulou, 2000; Rogers et al., 2001; Martinoia et al., 2002; Jungwirth and Kuchler, 2006). These findings suggest that ABC transporters play a significant role in translocation and help plants to cope with stressful environments. However, the excessive accumulation of substances may also affect plant growth and productivity and make the plant susceptible to stress conditions. Overall, it can be concluded that most of the identified pathways were reported under different abiotic stress conditions. Nevertheless, their significant role in CS tolerance in different crop plants needs more investigation.

The Crucial Role of Starch and Sucrose Metabolism in Stress Tolerance

In starch and sucrose metabolism, several DEGs were correlated with the five DAMs (**Figure 9**). In the subsequent part, we discussed the vital role of some important genes. For instance, beta-glucosidase 16 (BnaAnng19210D) was upregulated and associated with five DAMs (**Figure 9**). Beta-glucosidases are largely present in the vacuole and play a vital role in plant carbohydrate metabolisms, including cell wall modification, defense, plant hormone signaling, and secondary metabolism (Cairns et al., 2015). It also works in the hydrolysis of disaccharides, i.e., cellulose hydrolysis, by translating cellobiose to glucose (Singhania et al., 2013). Consequently, the tolerant genotype seemed to be maintaining disaccharides under CS conditions. In rice, beta-glucosidase was upregulated when exposed to ABA, MeJA, submergence, and salinity stress (Opassiri et al., 2007). The loss of beta-glucosidase in *Arabidopsis* (*AtBG1*) gives rise to the drought-sensitive phenotype, while overexpression of *AtBG1* led to improved drought tolerance in *Arabidopsis* (Lee et al., 2006).

The expression level of beta-amylase (BnaC02g12830D) was upregulated and correlated with four DAMs in both varieties (**Figure 9**). Glucans produced from starch pellets are hydrolyzed through beta-amylase to maltose (Smith et al., 2005), and starch is an ample storage carbohydrate formed in plants (Feike et al., 2016). In *Arabidopsis*, beta-amylases were upregulated under drought, salinity, and cold stresses (Seki et al., 2002). Furthermore, higher beta-amylase content was crucial for the persistence of rice seedlings throughout the premature growth phase and following seedling growth with exposure to flooding stress (Ella et al., 2010). In another study, Wang X. et al. (2017) reported that beta-amylase and beta-glucosidase activities were significantly increased by modifying carbohydrate metabolism. These discoveries advise that beta-amylase is elaborated in starch degradation in rapeseed exposed to CS. This enzyme might normalize carbohydrate utilization to upsurge energy facilities in the rapeseed seedlings. Moreover, the beta-fructofuranosidase gene (BnaA01g37220D/BnaC08g41390D) was upregulated and associated with five DAMs found in starch and sucrose metabolism (**Figure 9**). In Bambara groundnut landraces, Khan et al. (2017) also found the upregulation of the beta-fructofuranosidase gene in two genotypes grown under water-limited and water-sufficient conditions. Notably, beta-fructofuranosidase hydrolyzes the sucrose to produce additional glucose, thereafter playing an important role in osmoprotection and energy production in plants under stressful environments (Khan et al., 2017). The UTP-glucose-1-phosphate uridylyltransferase (BnaCnng53900D) showed higher expression and correlated with four DAMs in both varieties (**Figure 9**). As an important enzyme of carbohydrate metabolism and cell wall biosynthesis, it can catalyze the adjustable reaction among glucose-1-phosphate and UDP-glucose (Munoz-Bertomeu et al., 2010). As a glycosyl giver in cells, UDP-glucose can be elaborated in the glycosylation of several compounds in plant cells (Munoz-Bertomeu et al., 2010). In a combined root proteomic and metabolic study, the upregulation of UTP-glucose-1-phosphate uridylyltransferase was strongly linked to the drought stress responses in grapevine tolerant rootstocks (Prinsi et al., 2018). Under CS, Wang T. et al. (2017) observed that this enzyme plays a vital role in developing cold tolerance in *Anabasis aphylla* seedlings. Considering this, it can be concluded that the starch

and sucrose metabolism was improved for contributing to CS tolerance in rapeseed.

The Crucial Role of Phenylalanine Metabolism in Stress Tolerance

In phenylalanine metabolism, numerous DEGs were correlated with six DAMs (**Figure 10A**). Concisely, the biosynthesis of phenylalanine, tyrosine, and tryptophan begins with phenylalanine, which leads to the biosynthesis of other metabolites. Here, the 4-Hydroxyphenylpyruvate dioxygenase (HPPD)-encoding gene (BnaC08g44820D) was upregulated and correlated with five DAMs (**Figure 10**). The HPPD is an enzyme involved in photosynthesis regulation and catalyzes the conversion of 4-hydroxyphenylpyruvic acid (HPPA) into homogentisic acid (HGA) (Ndikuryayo et al., 2017). The HPPD enzyme-encoding genes play an essential role in pyomelanin synthesis (pigment produced by microbes) (Ahmad et al., 2016). Recently, the mutation analysis of pyomelanin production revealed the protective role of the pigment against oxidative stress tolerance in *Ralstonia solanacearum* (Ahmad et al., 2016). According to Kohlhase et al. (2018), waterhemp populations were found to be resistant to HPPD-inhibitor herbicides. In the future, the isolation of such a pigment from several bacteria grown in harsh environmental conditions may serve as a useful molecule for abiotic stress tolerance in plants.

Furthermore, aspartate aminotransferase (AAT)-encoding genes (BnaA05g11710D, BnaC01g06460D, and BnaA10g15590D) were correlated with 2-phenylacetamide, N-Acetyl-L-phenylalanine, and succinate DAMs (**Figure 10**). It has been reported that AAT catalyzes a changeable reaction using coenzyme pyridoxal-5-phosphate (PLP), which subsequently creates aspartate and 2-oxoglutarate and vice versa (Toney, 2014). Recently, it was noticed that the upregulation of *AAT* (LOC105167001) in the salinity-tolerant soybean variety was stronger than that in the sensitive variety (Zhang Y. et al., 2019). Consistently, it also showed a significantly higher expression level in the CT-C18 variety. Moreover, one AAT-encoding gene (*AlaAT*) from *Medicago sativa* was overexpressed in rapeseed, and the transgenic rapeseed lines presented promising improvement in biomass under differently fertilized conditions (McAllister et al., 2016). Thus, even genes functioning in producing vital compounds were found to be downregulated in both varieties. However, members of these compounds exhibited higher expression levels in CT-C18 like AAT, which might positively impact the stress tolerance of rapeseed. The current study also detected the downregulation of two genes encoding AAT (chloroplastic/cytoplasmic) in CS-C6, indicating that absence or low expression may give rise to the stress-sensitive genotype.

Additionally, the trans-cinnamate 4-monooxygenase (BnaA03g14000D) gene was downregulated and correlated with five DAMs in both varieties (**Figure 10**). In plants, phenylalanine ammonia-lyase (PAL) catalyzes phenylalanine and tyrosine to produce cinnamic acid and *p*-cinnamic acid, respectively. Cinnamic acid can be catalyzed by trans-cinnamate 4-monooxygenase (C4H) to produce *p*-cinnamic acid (Tohge et al., 2017). Monooxygenase reactions are important in the biosynthesis of diverse metabolites, including fatty acids, phenylpropanoids, alkaloids, and terpenoids, in plants (Tohge et al., 2017). Recently, a *BnGC4H* gene was characterized from cultivated *Boehmeria nivea*, with results showing that it was strongly expressed in the xylem during the maturity stage, which indicated its crucial role in the developmental process. Thus, these discoveries advised that CS tolerance of rapeseed could be related to normalizing amino acid accumulation and/or a breakdown in phenylalanine metabolism.

CONCLUSION

The present study performed a conjoint analysis based on transcriptomes and metabolomes to identify the key genes, metabolites, and metabolic pathways related to CS response in tolerant and sensitive rapeseed varieties. It provided a total of 3,368 DAMs, among which 626 have already been annotated. Combining the transcriptome data, a total of 2,845, 3,358, and 2,819 DEGs from the comparison of C6-0 vs. C18-0, C6-1 vs. C18-1, and C6-7 vs. C18-7 were detected, respectively. Further analysis of these DAMs and DEGs displayed that carbohydrate and amino acid metabolisms, specifically starch and sucrose metabolism and phenylalanine metabolism, might be involved in the complex response or regulation of CS tolerance in rapeseed. Thus, a functional validation based on the *Arabidopsis* T-DNA insertion mutants revealed that *Bn4CL3*, *BnCEL5*, *BnFRUCT4*, *BnUGP1*, *BnAXS1*, and *BnBAM2/9* could be considered as strong candidates in adapting to CS in rapeseed. Therefore, these candidate genes could be used for future rapeseed genetic improvement programs. The combined analysis expanded our understanding of the critical metabolic pathways that influence rapeseed adaption to CS stress. Lastly, the DAMs or DEGs identified here may be exploited for agronomic improvement under cold stress conditions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found at: National Center for Biotechnology Information (NCBI) BioProject database under accession number PRJNA596550.

AUTHOR CONTRIBUTIONS

AR and YL conceived the idea and designed the experiment. AR performed the experiments, analyzed the data, and wrote the manuscript. WS, MAH, and SSM participated in the experiments and analysis. YC, YL, XZh, and XZo supervised, proofread, and edited the manuscript. All authors have read and approved the final version of the manuscript.

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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.2021.721681/full#supplementary-material

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Super Rice With High Sink Activities Has Superior Adaptability to Low Filling Stage Temperature

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To investigate the differential responses of super rice grain filling to low filling stage temperature (LT) and the regulative effect of nitrogen panicle fertilizer (NPF), physiological and molecular experiments were conducted with two super rice varieties (Nanjing 7th: N7 and Nanjing 9108th: N9108) on two different filling stage temperature treatments implemented by applying two sowing dates [Normal filling stage temperature (CK): Sowed on May 30, T_{mean} = 24.7°C and low filling stage temperature (LT): Sowed on July 1, T_{mean} = 20.3°C], and two NPF levels (0 and 150 kg N ha^{-1}). In this study, LT, NPF, and simultaneous LT and NPF treatments suppressed the grain filling in all varieties with different levels. Under LT or NPF treatments, the reduction of grain weight, seed setting rate, and filling rate were closely associated with suppressed starch biosynthesis rate in inferior seeds, suggesting that reduced starch biosynthesis rate, expression, and activities of enzymes encoded by related genes, Floury endosperm-4 (*FLO4*), Starch branching enzyme-I (*SBE1*), and Starch phosphorylase-L (*PHO-l*), were responsible for the grain filling reduction. Under LT or NPF treatments, significantly higher grain filling rates and lower variance were found in N9108 compared to that in N7, which were closely related to their higher starch biosynthesis ability, related gene expression, and enzymes activities. One of the probable explanations of the grain filling difference was the variation in the relative amount of key regulative hormones, Abscisic acid (ABA) and 1-aminocyclopropane-1-carboxylic acid (ACC). These results raise a possibility that super rice with higher sink activities has superior adaptability to LT and NPF due to their higher sink activities.

Keywords: super rice, grain filling, temperature, sowing date, sink activity

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INTRODUCTION

Rice (*Oryza sativa* L.) is one of the staple food crops in China. It has made a great contribution to China's national economy (Khush, 2013; National Bureau of Statistics of China, 2015). Rapid population growth and economic development are growing pressures for an increase in food production (Zhang et al., 2007). However, the increase in rice yield has declined since the mid-1980s, as reflected by the sharp drop in the rate of annual yield increase from 2.7% in the 1980s to 1.1% in the 1990s (Horie et al., 2005). To further increase yield, breeding efforts have expanded the yield sink capacity (the maximum size of sink organs to be harvested) mainly by increasing the number of spikelets per panicle (Kato et al., 2007). As a result, cultivars with large panicles or extra-heavy panicle types with numerous spikelets per panicle have

become available, such as hybrid rice and "super" rice or "super" hybrid rice in China (Cheng et al., 2007; Peng et al., 2008). However, some past research showed that these varieties can hardly realize their yield potential due to their poor grain filling in the field (Peng et al., 1999; Yang et al., 2002; Ao et al., 2008; Yang, 2010). Different grain filling and yield performance were found, in some super rice varieties, with similar panicle architecture and growth duration, suggesting that the panicle architecture and growth duration were not the major limitations of poor grain filling (Wang et al., 2002; Cheng et al., 2007; Zhang et al., 2007). The sink activity, which is represented by starch synthesis ability, could be the limiting factor of rice grain filling and final yield establishment (Venkateswarlu and Visperas, 1987; Ho, 1988; Liang et al., 2001).

Slow grain-filling rate and low grain weight were proved to be attributed to two major factors: the limitation of carbohydrate supplement and suppression of sink activities (Yang and Zhang, 2010). Previous studies showed that the supply of carbohydrates could be altered by abiotic stress conditions and was also closely related to the sucrose-starch conversion by controlling the concentration of the major substrate of the conversion (Yang and Zhang, 2010). Lower concentrations of soluble carbohydrate in the grain were found in response to abiotic stresses conditions and could be responsible for the final grain weight reduction (Yang and Zhang, 2010). The starch synthesis ability of rice grains was also positively correlated with the grain filling rate, and further controls the final grain weight (Wang et al., 2008; Yang and Zhang, 2010). The starch synthesis can be controlled by several factors, such as Floury endosperm-4 (*FLO4*), Starch branching enzyme-I (*SBE1*), and Starch phosphorylase-L (*PHO-l*) (Yoshida and Hara, 1977; Yang et al., 2001a; Zhao et al., 2004; Zhu et al., 2004; Duan and Sun, 2005). Therefore, the difference in the sink activity of rice grains could be explained by exploring the activity and expression of the target enzyme in the grain filling stage. Previous studies always focused on the gap between superior and inferior seeds of different varieties (Yang et al., 2008). However, the difference in filling rates of superior and inferior seeds is also a major contributor in grain yield and weight establishment.

With the rapid development of agriculture and the change of planting structure, the traditional manual transplanting mode has been replaced by mechanic transplanting (Peng, 2016; Zou and Huang, 2018). This can be observed in the middle and lower reaches of the Yangtze River, known as the major rice-wheat rotation cropping region where lower sowing dates have occurred due to traditional manual transplanting mode being gradually replaced by mechanized transplanting (Xing et al., 2016). As a result, the late sowing date postpones the rice growth duration, which leads to a lower temperature, suppressed rice filling, and reduced grain weight, which consequently produces a reduced grain yield (Qiu et al., 2016). Low temperature stress in the reproductive stage was correlated to the suppression of gene expression related to starch accumulation (Sipaseuth et al., 2007; Jacobs and Pearson, 2010). It was also correlated to further reducing the biosynthesis of starch in different varieties. However, the response of rice, especially "super" rice, and underlying physiological mechanism were still not clear. Sowing time is always given great importance in fields to ensure that rice plants avoid low-temperature stress and in completing the grain filling process earlier (Van Loon et al., 2014). Many previous studies have investigated changes in grain filling in response to different temperatures during the reproductive stage. However, most of those studies were carried out in a controlled greenhouse. Greenhouse studies are unlikely to accurately imitate the long-term cultivation in the field conditions and, therefore, show discrepant results from field studies (Patindol et al., 2015). Some previous studies also adopted this method to mimic low temperature treatment in the field. Wang et al. (2015) performed a field study to investigate the effects of environmental temperature on rice starch accumulation. On the other hand, many previous studies showed that grain filling was mainly controlled by reproductive stage temperature, while light treatment only slightly suppressed the grain filling (Yoshida and Hara, 1977). Thus, we adopted similar sowing dates to mimic the variation of reproductive stage LT stress. The plant hormones that mediate spikelet development, especially ethylene and abscisic acid (ABA), play important roles in regulating grain filling. A higher rate of ethylene evolution in developing seeds suppresses the expression of most starch-synthesis genes and inhibits the activities of starch synthesis-related enzymes which thus leads to a low grain-filling rate (Yang et al., 2006; Zhu et al., 2011; Panda et al., 2018). The 1-aminocyclopropane-1-carboxylic acid (ACC), as the precursor of ethylene biosynthesis, was found to be closely correlated with ethylene content, and could significantly downregulate the grain filling rate (Yang et al., 2006). ABA acts as a sensitive signal during abiotic stress in plants, and its role in grain filling is complicated (Wang et al., 2019). It is notable that ABA plays a key role in grain filling by regulating the sink activity. In addition, it functions in a dose-dependent manner (Wang et al., 2015). An appropriate concentration of ABA can enhance the activities of enzymes involved in sucrose cleaving and starch synthesis and increase the expression of genes related to starch metabolism (Wang et al., 2015). Davies (1995) proposed that plant hormones can act either synergistically or antagonistically and is the balance between promoting and inhibiting agents that ultimately determines the path of plant growth and development. Yang et al. (2006) found that the grain filling rate was not only correlated with the concentration of ABA and ethylene, but also with the ratio of ABA and ACC. In this study, the metabolite content dynamics, ACC and ABA, were also measured to find out their regulative mechanism under low temperature condition.

Nitrogen was always adopted as one of the most important regulators of rice growth, grain yield, and quality (Kirk et al., 1997). Nitrogen panicle fertilizer (NPF) is always necessary for the field conditions to enhance the spikelet number, whereas the seed setting rate was suppressed (Ding et al., 2003, 2010, 2014; Zhang et al., 2007). Some studies showed that rational utilization of nitrogen fertilizer improved the activity of grain starch synthesis (Zhang et al., 2008). Other studies showed poor grain filling and no increase in the number of grains per panicle simultaneously (Mae, 1997; Yang A. et al., 2004; Samonte et al., 2006). Moreover, high nitrogen levels in grains reduced the carbohydrate accumulation in plant source organs,

TABLE 1 | The mean daily temperature (T_{mean}, °C), minimum temperature (T_{min}, °C), maximum temperature (T_{max}, °C), and accumulated temperature ($T_{accumulated}$, °C) in response to CK and LT treatments of 2 varieties (N7 and N9108) in 2016 and 2017.

Stage	Parameters	Year							
		2016				2017			
		CK		LT		CK		LT	
		N9108	N7	N9108	N7	N9108	N7	N9108	N7
Transplanting	T_{mean} (°C)	23.37	23.37	26.76	26.76	24.25	24.25	27.12	27.12
	T_{max} (°C)	29.32	29.32	30.08	30.08	28.77	28.77	31.95	31.95
	T_{min} (°C)	20.02	20.02	23.99	23.99	19.19	19.19	23.17	23.17
	$T_{accumulated}$ (°C)	253.33	253.33	321.07	321.07	285.01	285.01	342.45	342.45
Tillering	T_{mean} (°C)	26.38	26.98	30.99	31.32	27.67	27.97	31.52	31.54
	T_{max} (°C)	33.98	33.98	33.98	33.98	34	34.22	34.22	34.22
	T_{min} (°C)	20.37	20.37	27.23	27.23	21.29	21.29	28.12	28.12
	$T_{accumulated}$ (°C)	612.33	633.79	573.08	555.12	671.27	718.73	602.42	581.5
Panicle initiation	T_{mean} (°C)	31.51	30.53	27	27.23	30.59	29.64	26.21	26.44
	T_{max} (°C)	35.25	35.25	33.6	33.6	34.22	34.22	32.62	32.62
	T_{min} (°C)	26.52	24.16	22.6	22.6	25.75	23.46	21.94	21.94
	$T_{accumulated}$ (°C)	660.52	670.65	549.35	557.1	638.18	647.97	534.91	542.45
Grain filling	T_{mean} (°C)	23.12	22.89	17.75	19.28	22.12	21.9	16.99	18.45
	T_{max} (°C)	28.48	28.48	28.29	28.29	27.25	27.25	27.07	27.07
	T_{min} (°C)	11.19	1.52	10.5	10.36	11.3	15.86	10.26	10.26
	$T_{accumulated}$ (°C)	794.79	727.12	484.93	501.97	760.56	695.81	464.05	480.35

and carbohydrate translocation from source organs to grains would eventually cause poor grain filling (Fu et al., 2019). Unlike in regular conditions, nitrogen fertilizer application could reduce the grain yield in low temperature treatment because of the reduced filling rates (Cao et al., 2018; Jia et al., 2019). Moreover, nitrogen panicle fertilizer has different effects on different cultivars (Fageria and Santos, 2015; Ding et al., 2020). Thus, the differential responses of super rice cultivars and LT in the filling stage to NPF were discovered in this study. Three experimental factors, including filling stage temperatures, NPF levels, and varieties were used in this study to clarify the mechanism of super rice varieties with different sink activities response to LT and NPF, which is critical to the accurate underlying mechanism of the effect of nitrogen fertilizer on super rice varieties with LT condition.

MATERIALS AND METHODS

Experimental Sites

The field experiments were conducted in 2016 and 2017 in the subtropical environment of Danyang City, Jiangsu Province, China (32°0′ N, 119°70′ E, 51 m altitude). Weeds, pests, and diseases were intensively controlled to avoid yield loss. The climate data regarding daily radiation and air temperature were measured at a meteorological station located within 1 km of the experimental site. The daily solar radiation and temperature were measured by a silicon pyranometer (LI-200, LI–COR Inc., Lincoln, NE, USA) and a temperature/RH probe (HMP45C, Vaisala Inc., Helsinki, Finland), respectively. The meteorological data of the two rice growing seasons were shown in **Table 1**.

Experimental Design

The experiments were randomized in a complete block design with three replications. Two conventional japonica varieties, namely, Nanjing 9108th (N9108) and Ningjing 7th (N7), with two grain filling temperature, namely, LT (implement by setting late sowing date, average temperature = 20.3°C) and CK (implement by setting early sowing date, average temperature = 24.7°C), were used. Two different nitrogen panicle fertilizer (NPF) levels were adopted in this study, namely, no NPF (N0), and 150 kg N ha^{-1} at panicle initiation (N150), and were applied in panicle initiation. The whole experimental field was applied with the same amount of 450 kg P ha^{-1} (Calcium superphosphate) + 150 kg K ha^{-1} at transplanting, and 150 kg K $ha–^{1}$ at panicle initiation stage (Potassium chloride) + 330 kg N ha^{-1} as carbamide at transplanting in 2016 and 2017. The soil properties of the topsoil layer (0–20 cm) before transplanting were measured in both years as follows: 1 kg soil contains 1.25 g total N, 6.8 mg NH_4^+, 0.9 mg NO_3^-, 27.9 mg Olsens-P and 168 mg NH_4OAc-K, and pH = 6.3. Seedlings were sowed on May 30 and July 1, and machine-transplanted on June 20 (CK) and July 20 (LT) in 2016 and 2017, respectively, with the hill spacing for 14 cm × 30 cm. Machine-transplantation was performed using a rice trans-planter (PZ640, Iseki Agricultural Machinery Co., Ltd., Japan).

Observations and Measurements

Development Stage

The dates of sowing, panicle initiation, heading, and maturity were recorded for determining growth duration. Panicle initiation was defined as the day when 80% of stems in a plot

presented a white feathery cone inside the leaf sheath of the rice plant. Heading was the date when 80% of the stems in a plot started anthesis. Maturity was the date when 95% of grains turned yellow.

Yield and Yield Components

At panicle initiation, heading, and maturity stages, all the plants within an area of 0.5 m^2 in each plot were sampled for the growth analysis. After recording the plant height, the numbers of stems (main stems plus tillers), and panicles (when presented), the plant samples were separated into leaves, stems, and panicles. At maturity, the panicles were hand-threshed, and the filled spikelets were separated from the unfilled spikelets by submerging them into tap water. The empty spikelets were separated from the half-filled spikelet by winnowing. Three sub-samples of the filled (30 g), the half-filled (6 g), and the empty (3 g) spikelets were taken to count the spikelets number. The dry weights of the rachis, filled, half-filled, and empty spikelets were measured after oven drying at 70°C to a constant weight. The total dry weight at maturity was the sum of the dry weights of the straw (leaves plus stems), rachis, and filled, half-filled and empty spikelets. The spikelets per panicle (spikelets m^{-2}/panicles m^{-2}), and grain filling percentage (100 × filled spikelets m^{-2}/spikelets m^{-2}) were calculated. The grain yield was determined from a 5 m^2 area in the center of each plot and was adjusted to 14% moisture content.

Grain Filling Characteristics

We selected 400 panicles that headed on the same day in each treatment, which were tagged to give an accurate record of the flowering date and the position of the spikelets. Superior grains (located on apical primary branches, SS) that flowered on the first 2 days of anthesis (DAA) and inferior grains (located on proximal secondary branches, IS) that flowered on the last 2 days were separated from the panicles (Chen et al., 2013). Thirty tagged panicles from each plot were sampled at every 5 DAA till 45 DAA. The sampled panicles were divided into three groups (10 panicles each) as three replicates. Then, superior and inferior spikelets were separated from the panicles for RNA extraction and measurement of soluble sugars and starch. All the sampled grains were deshelled and immersed in liquid nitrogen and then kept in a −80°C freezer for further analysis. Further 30 tagged panicles (10 panicles formed a subsample) from each treatment were sampled to measure the dry weight of superior and inferior grains at final harvest. The sampled grains were dried at 70°C to constant weight, dehulled, and weighed.

RNA Extraction and qRT-PCR

Total RNA was extracted from 0.1 g of inferior seeds (IS) and superior seeds (SS) of rice plants at 5, 10, 15, and 20 DAA using RNeasy Plant Mini Kit (Qiagen, German) following the method described previously (Wang et al., 2017). First-strand cDNA was synthesized from RNA using the RNAprep Pure (Tiangen, Beijing, China). Relative expression levels of target genes, such as *FLO4*, *SBE1*, and *PHO-l*, were detected by the Real-time PCR System (Takara, Dalian, China). Primers were designed based on the anticipated size of the amplification products (150–250 bp) as listed in **Supplementary Table 1**. Primer Premier 5.0 software (Premier, Ottawa, ON, Canada) was used to complete the design work. Three biological replicates were used. Expression levels were normalized to the reference genes: *Actin* (OSNPB_110163100). The primers were designed as shown in **Supplementary Table 1**.

Assays of Grain Weight, Non-structural Soluble Carbohydrates, and Starch Content

To investigate how sowing date affected the grain-filling process, we measured the weight of superior and inferior grains, and the contents of soluble sugars and starch of the inferior grains under the two water supply treatments. A total of 100 SS and IS grains each was used for the measurement of grain dry weight. The samples used for measuring the starch and non-structural soluble carbohydrate (NSC) contents were ground into fine powder, and 500 mg ethanol (v/v) was added. The tubes were kept in a water bath at 80°C for 30 min. After cooling the tubes in water, they were centrifuged at 5,000 × g for 10 min. The supernatant was collected, and the extraction was repeated three times. The sugar extract was then diluted to 50 ml with distilled water and the sucrose content was measured as described by Yang et al. (2001a,c). The residues left in the centrifuge tubes after extracting sugars were dried at 80°C for starch extraction using $HClO_4$ following the method described by Yang et al. (2001c). Amylose and amylopectin contents were determined according to He (1985) with minor changes. Amylose content was quantified at 620 and 479 nm, while amylopectin content was quantified at 556 and 737 nm, respectively.

Metabolite Extraction and Quantification

Fifty milligrams of plant sample was weighed into a 2 ml plastic microtube and frozen in liquid nitrogen, dissolved in 1 ml methanol/water/formic acid solution (15:4:1, v/v/v). Ten microliters of internal standard mixed solution (100 ng/ml) was added into the extract as internal standards for the quantification. The mixture was vortexed for 10 min, and centrifuged for 5 min (12,000 rpm/min, and 4°C), and the supernatant was then transferred to clean plastic microtubes, followed by evaporation to dryness and dissolved in 100 μl 80% methanol (v/v), and filtered through a 0.22 μm membrane filter for further LC-MS/MS analysis. ABA and ACC contents were detected by MetWare (http://www.metware.cn/) based on the AB Sciex QTRAP 6500 LC-MS/MS platform (Wuhan Metware Biotechnology Co., Ltd., Wuhan, China). The quantification of ACC and ABA were analyzed using an UPLC-ESI-MS/MS system (UPLC'ExionLC™ AD' https://sciex.com.cn/; MS'Applied Biosystems 6500 Triple Quadrupole, https://sciex.com.cn/). The content of ACC and ABA was determined using the external standard method and is expressed as ng/g fresh weight (FW). Three biological replications were performed.

Statistics Analyses

Analysis of variance was performed using SPSS version 20.0 (SPSS Statistics, SPSS Inc., Chicago, USA), and the results are expressed as means (±SD) of three biological replicates. The treatment means were compared based on the least significant difference (LSD) at a 0.05 level of probability.

TABLE 2 | Growth duration (dates and days after sowing) of each growth stage in response to CK and LT treatments of two varieties (N7 and N9108) of 2016 and 2017.

Varieties	Treatments	Sowing	Transplanting	Panicle initiation	Heading	Grain weight stable	Mature
N9108	CK	(5.31) 0	(6.20) 21	(7.27) 58	(8.26) 88	(9.30) 123	(10.30) 153
	LT	(7.10) 0	(7.20) 20	(8.16) 47	(9.17) 79	(10.27) 119	–
N7	CK	(5.31) 0	(6.20) 21	(7.29) 60	(8.30) 92	(10.4) 127	(10.27) 150
	LT	(7.1) 0	(7.20) 20	(8.15) 46	(9.16) 78	(10.21) 113	–

TABLE 3 | Yield components of rice varieties (N7 and N9108) in response to fertilizer treatments (N0 and N150) and different filling stage temperatures (CK and LT) in 2016 and 2017.

Year	Varieties	Treatments	Total spikelets number	Seed-setting rate	1,000-grain weight	Grain yield
			(m^{-2})	(%)	(g)	(t/ha)
2017	N9108	N150-CK	48796.8b	85.2ab	30.37a	12.06a
		N0-CK	34233.3c	89.4a	30.50a	9.33c
		N150-LT	53080.3a	76.7c	28.41b	11.42b
		N0-LT	47486.3b	80.8b	29.11ab	10.17b
	N7	N150-CK	51236.7a	75.6ab	27.67ab	10.87a
		N0-CK	40887.7b	82.1a	28.22a	9.47b
		N150-LT	55838.3a	60.1d	27.91ab	9.36b
		N0-LT	52644.0a	66.8c	26.98b	9.20b
2016	N9108	N150-CK	45512.3a	84.1ab	28.86a	11.91a
		N0-CK	27558.7b	90.1a	28.88a	9.23b
		N150-LT	41615.3a	75.1b	27.67ab	9.33b
		N0-LT	44336.5a	82.1ab	26.09b	10.23ab
	N7	N150-CK	55499.0a	78.7b	26.71a	10.93a
		N0-CK	40963.6b	85.6a	25.89b	9.08b
		N150-LT	53182.0a	65.6c	26.36a	9.20b
		N0-LT	46760.4ab	77.3b	26.12ab	9.45b

Letters after the values indicate statistical significance at the P = 0.05 level.

RESULTS

Grain Yield and Yield Components

The present study was carried out to determine the effect of different temperature and sowing dates on the yield and yield related attributes of rice during the rice-growing seasons 2017–18 as presented in **Table 1**. To accomplish the filling stage temperature treatments in the field, two different sowing date treatments were adopted. Lower Mean temperature (Tmean), Day temperature (Tday), and Night temperature (Tnight) and shorter growth duration were found in LT treatment compared to that in CK treatment (**Tables 1**, **2**). The LT and NPF treatments showed a significant effect on total spikelets number, seed setting rate, and 1,000-seed weight that determine the yield of the rice. The total spikelet number could be enhanced by both NPF and LT treatments while seed setting rate and 1,000-seed weight reduced. Moreover, their balancing eventually altered the grain yield (**Table 3**). However, the grain yield responds differentially to NPF between CK and LT treatments. Under CK condition, the grain yields of N9108 and N7 could be significantly improved by NPF for about 12.7 and 9.4%, respectively, mainly due to enhanced population size (total spikelet number). However, under LT conditions, the grain yield of both varieties showed no significant change if NPF was applied or not since significantly lower seed-setting rate and 1,000-seed weight were observed, although their total spikelet number increased at the same time. The higher seed-setting rate (12.3 ± 2.4%) and 1,000-seed weight (7.4 ± 1.3%) of both varieties were found in CK treatment compared to those in LT treatment. Similarly, a lower seed-setting rate (9.1 ± 1.7%) of both varieties could be induced by applying NPF.

Grain Filling Rate

As shown in **Table 4**, the grain filling rate of N9108 is significantly higher than that of N7, and it is more significant under the LT condition (**Figure 1**). Under CK condition, the inferior and superior mean grain filling rate (GR_{mean}) of N9108 were 0.504 ± 0.073 and 0.811 ± 0.053 ($mg{\cdot}grain^{-1} \cdot D^{-1}$), respectively, was about 25 and 43% higher than those of N7 [0.372 ± 0.079 and 0.617 ± 0.033 ($mg{\cdot}grain^{-1} \cdot D^{-1}$)]. However, under LT condition, the GR_{mean} of inferior and superior grains in N9108 were about 0.416 ± 0.075 and 0.803 ± 0.03 ($mg{\cdot}grain^{-1} \cdot D^{-1}$), which were significantly higher than those of N7 [0.324 ± 0.069 and 0.595 ± 0.011 ($mg{\cdot}grain^{-1} \cdot D^{-1}$)], with an increment of about 40 and 67% compared with those of CK condition. In this study, both NPF and LT treatments reduced the grain filling rate of the two varieties, delayed the time to reach the maximum grain filling rate, and increased the initial grain filling potential (**Table 4**). When NPF was applied, the inferior grain

TABLE 4 | Grain filling characteristics of different grain positions (SS and IS) of rice varieties (N7 and N9108) in different fertilizer treatments (N150, applying NPF treatment; N0, no NPF treatment) and temperature treatments (CK and LT).

Materials	Treatment and position	R_0	GR_{max} ($mg \cdot grain^{-1} \cdot D^{-1}$)	T_{max} (d)	W_{max} ($mg \cdot grain^{-1}$)	GR_{mean} ($mg \cdot grain^{-1} \cdot D^{-1}$)	D (d)
N9108	N150-CK-SS	0.70a	1.5ab	11.58c	28.48a	0.74ab	38.94bc
	N0-CK-SS	0.15d	1.71a	15.04b	28.13a	0.85a	33.08c
	N150-LT-SS	0.43b	1.58a	9.64d	25.49b	0.78ab	32.64c
	N0-LT-SS	0.31c	1.60a	11.98c	26.53b	0.80a	32.05c
	N150-CK-IS	0.20cd	0.72c	22.27a	22.57c	0.36c	56.51a
	N0-CK-IS	0.09e	1.21b	21.66a	24.27bc	0.60b	40.46
	N150-LT-IS	0.31c	0.67c	13.73bc	17.41d	0.34c	56.3a
	N0-LT-IS	0.29c	0.86c	14.05bc	19.17cd	0.43bc	46.68b
N7	N150-CK-SS	0.11ab	1.17b	16.18c	24.74a	0.58b	42.63b
	N0-CK-SS	0.07b	1.51a	22.72a	23.96a	0.75a	31.66c
	N150-LT-SS	0.19a	1.11b	13.45d	22.48ab	0.56b	43.08b
	N0-LT-SS	0.19a	1.09b	14.57cd	21.12b	0.50bc	39.28bc
	N150-CK-IS	0.11ab	0.59d	20.58ab	15.12bc	0.29d	65.21a
	N0-CK-IS	0.08b	0.87bc	22.27a	19.88b	0.44c	46.31b
	N150-LT-IS	0.09b	0.77c	18.83b	13.08c	0.39c	41.86b
	N0-LT-IS	0.07b	0.83bc	19.22b	15.03bc	0.41c	38.29bc

R_0, Initial grain filling potential; GR_{max}, Maximum grain filling rate; T_{max}, Time reaching the maximum grain filling rate; W_{max}, Weight of a kernel at the time of maximum grain filling rate; GR_{mean}, Mean grain filling rate; D, The time of grain filling reaching 99%.
Letters after the value indicate statistical significance at the P = 0.05 level.

filling rate of N9108 and N7 decreased by about 16 ± 2.7% and 27 ± 8.3%, respectively. The time to reach the maximum filling rate was delayed by about 13 ± 3.3% and 21 ± 7.1%, and the initial grain filling potential decreased by about 13 ± 5.4% and 15 ± 7.8%. In addition, the covariance (CV) of filling rates significantly differed by varieties and seed positions, indicating their differential stability. The CV of GR_{mean}, max grain filling rate (GR_{max}), and max grain weight (W_{max}) in inferior seeds of N7 were 0.33, 0.21, and 0.31, respectively. The CV of GR_{mean}, GR_{max}, and W_{max} in superior seeds of N7 were 0.12, 0.09, and 0.13, respectively. Differently, the CV of GR_{mean}, GR_{max}, and W_{max} in inferior seeds of N9108 were 0.22, 0.14, and 0.21, respectively. The CV of GR_{mean}, GR_{max}, and W_{max} in superior seeds of N9108 were 0.07, 0.04, and 0.04, respectively. It is obvious that the stability of superior and inferior grain filling levels of N9108 was significantly higher than those of N7. The stability of the superior grain filling level of both varieties was higher than that of inferior grain filling, which indicated that grain filling of inferior seeds could be more regulated by NPF and LT treatments.

Starch Accumulation

The starch accumulation pattern was consistent with the grain filling rates results as shown in **Figure 2**. In **Figure 2**, the starch accumulation of both inferior and superior seeds in N9018 is significantly higher than that in N7. Compared to superior seeds, the starch accumulation and filling rates were significantly higher than that of inferior seeds (**Figures 1**, **2**). Unlike inferior seeds, the starch accumulation of superior seeds was not significantly regulated by temperature and fertilizer treatments. Under CK conditions, the total amount of starch accumulation in inferior seeds was suppressed by NPF. The LT treatment showed a lower inferior seeds starch accumulation compared to that of CK, and the application of NPF had no significant effect under LT conditions. The suppression effect of NPF on starch accumulation in CK condition was higher than that in the LT condition, which was consistent with grain filling results (**Figure 2**). On the other hand, the effect of temperature and fertilizer treatment on the starch accumulation of N7 was significantly higher than those of N9108 (**Figure 2**).

To further provide metabolism level clues about changing the biosynthesis of starch, we measured the key intermediate metabolite, amylose, and amylopectin content. We found that, similar to the changing pattern of total starch accumulation, the amylose and amylopectin contents of both varieties were increased rapidly in all measured periods (**Figure 5**). The content of amylose and amylopectin was significantly reduced by LT and NPF treatments in both varieties. On the other hand, the dynamics of amylose and amylopectin were lower in N7 compared to that in N9108, which is consistent with gene expression data (**Figure 3**).

Gene Expression

Since the grain filling difference of superior seeds in both varieties among different treatments was not significant, it could be concluded that the difference of grain weight was mainly derived from inferior seeds starch biosynthesis among all treatments. Thus, the relative expression levels of genes related to starch accumulation in inferior seeds were verified in this study (**Figure 3**). Due to starch, synthesis could be controlled

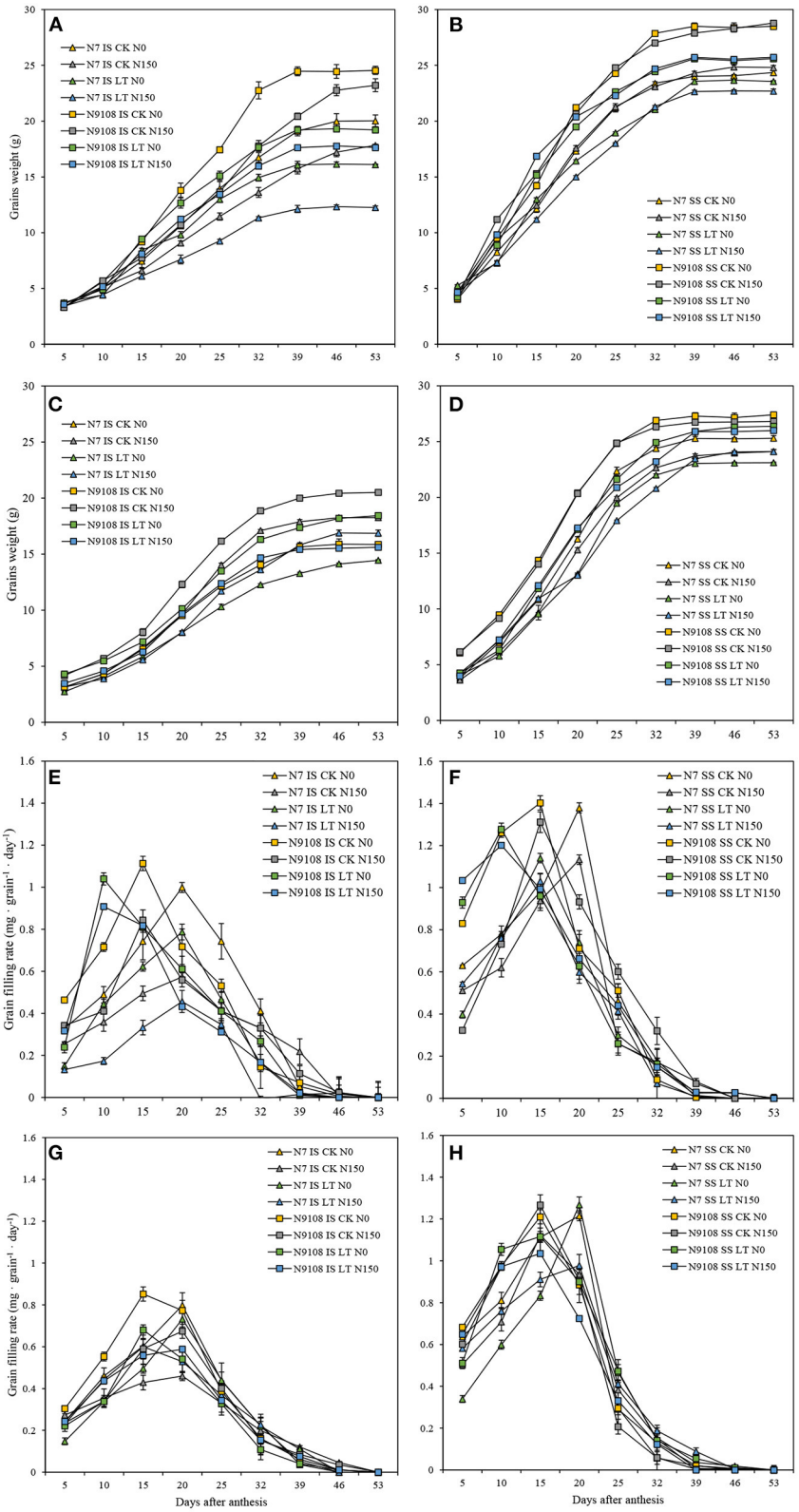

FIGURE 1 | Grain weight and filling rate dynamics of superior (SS) and inferior (IS) in two varieties [Nanjing 7th (N7) and Nanjing 9108th (N9108)] in response to different fertilizer treatments [N150, Applying nitrogen panicle fertilizer (NPF) treatment; N0, No NPF treatment] in filling stage temperature treatments [Normal

(Continued)

FIGURE 1 | temperature (CK) and Low temperature (LT) of 2016 and 2017]. **(A)** Grain weight dynamics of IS in 2016; **(B)** Grain weight dynamics of SS in 2016; **(C)** Grain weight dynamics of IS in 2017; **(D)** Grain weight dynamics of SS in 2017. **(E)** Grain filling rate dynamics of IS in 2016; **(F)** Grain filling rate dynamics of SS in 2016; **(G)** Grain filling rate dynamics of IS in 2017; **(H)** Grain filling rate dynamics of SS in 2017.

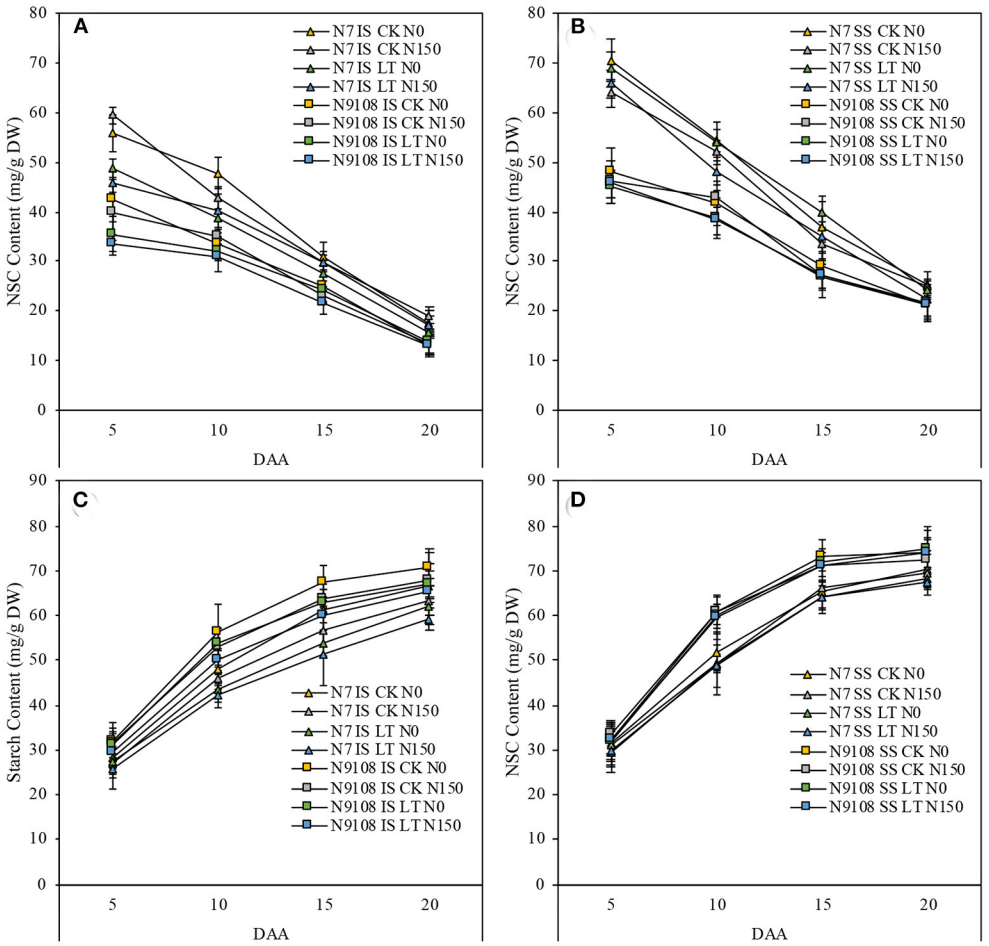

FIGURE 2 | Effect of different nitrogen panicle fertilizer levels (N150: Applying NPF treatment; N0, No NPF treatment) on starch and non-structural soluble carbohydrate (NSC) accumulation dynamics under different filling stage temperature treatments (CK and LT) in IS and SS. DW, dry weight. Values are means (±SD) of three replicates. Samples were taken at 5 days after anthesis (DAA), 10 DAA, 15 DAA, and 20 DAA. **(A)** NSC dynamics of IS in N7 and N9108, respectively; **(B)** NSC dynamics of SS in N7 and N9108, respectively; **(C)** Starch dynamics of IS in N7 and N9108, respectively; **(D)** Starch dynamics of SS in N7 and N9108.

by several key enzymes including starch synthase (SSS, encoded by *FLO4*), starch branching enzyme (SBE, encoded by *SBE* I), and starch phosphorylase (SPS, encoded by *PHO-l*) (Yoshida and Hara, 1977; Yang et al., 2001a; Zhao et al., 2004; Zhu et al., 2004; Duan and Sun, 2005). The expression pattern of the above genes was measured by qRT-PCR and was consistent with the physiological results (**Figure 2**). *FLO4* gene expression was high and kept stable from 5 DAA (**Figure 3**). *FLO4* expression, in both varieties under CK condition, was higher than those under LT condition, but there was no significant difference among different NPF application treatments. The expression of *SBE1* in superior and inferior grains increased continuously, while the expression of *PHO-l* in superior and inferior grains increased continuously from 0 to 15 DAA and then decreased slightly since 15 DAA. The expression of *SBE1* and *PHO-l* were significantly suppressed by LT and NPF treatments. Overall, the expression levels of N9108 were significantly higher than those in N7, which indicated a higher starch biosynthesis ability (**Figure 3**). The gene expression data was consistent with the enzymatic activities data in **Figure 4**. The encoded gene expression levels and activities of *SBE*, *SPS*, and *SS* were significantly suppressed by LT and NPF treatments, which also indicated suppressed starch biosynthesis ability.

Contents of Hormones Related to Grain Filling

In this study, we measured the content of ABA and ACC of inferior seeds among different treatments at three grain-filling stages. The ABA concentration was low at the early

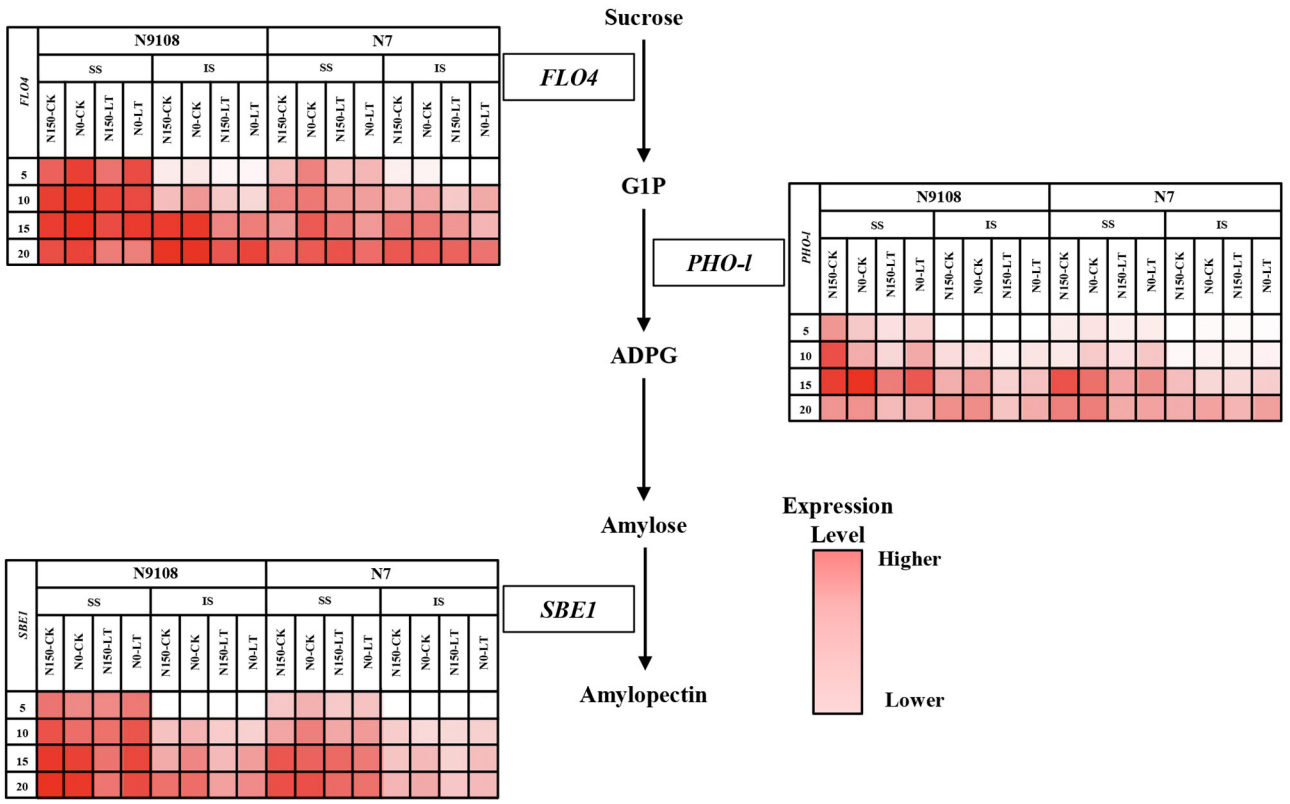

FIGURE 3 | Heat maps of gene expression level involved in the starch biosynthesis pathway. The maps were plotted using the expression level of each gene in the different samples: white indicates low expression level, red indicates high expression level. Two rice varieties (N7 and N9108) were used under different nitrogen panicle fertilizer (N150, Applying nitrogen panicle fertilizer treatment; N0, No nitrogen panicle fertilizer treatment) and subjected to different filling stage temperature treatments (CK and LT). Samples were taken at 5 DAA, 10 DAA, 15 DAA, and 20 DAA. Three genes expression level were measured: *FLO4* (encoding starch synthase), *PHO-l* (encoding starch phosphorylase), and *SBE1* (encoding starch branching enzyme).

grain filling stage, increased from 10 DAA, reached a peak at 15 DAA, and declined at 20 DAA. In all treatments, the concentration of ABA was significantly decreased by LT and NPF treatments compared to CK and no NPF treatments, respectively (**Figure 5**). The reduction was more severe in additive LT and NPF treatment compared to other treatments. In contrast to ABA, the concentration of ACC was high at the early grain filling stage (**Figure 6**) but continuously declined until 20 DAA. Throughout the grain filling period, the concentration of ACC was largely enhanced by LT and NPF treatments compared to CK and no NPF treatments, respectively. Similar to ABA changing pattern, we found a large increment of ACC concentration in additive LT and NPF treatment compared to other treatments. The variance of ACC responding to NPF was more pronounced than that of LT. The ABA concentration of N9108 among all duration was significantly higher than that of N7, while ACC concentration showed no significant difference.

DISCUSSION

NPF and LT Affects Grain Yield

Nitrogen is one of the most important elements for plant growth, and the application of nitrogen fertilizers plays an important role in increasing rice yield due to their increment on total spikelet number (Fu et al., 2019; Wang et al., 2019). However, previous studies also proved that inappropriate nitrogen panicle fertilizer could reduce the grain filling of rice in the field (Ohnishi et al., 1999; Yang A. et al., 2004; Singh et al., 2011; Jiang et al., 2016; Zhang et al., 2021). The grain yield of both varieties could be enhanced by applying NPF since their larger population size. NPF increased grain yield for 11.7 ± 3.3% and 1.6 ± 0.9% under CK and LT conditions, respectively. This meant that the effect of NPF on grain yield was not significant under LT condition (**Table 3**). Under LT condition, the NPF induced enhancement of the population size and could exaggerate the filling issue similar to how a previous study (Fu et al., 2019) reported. This study explained that under abiotic stress in the filling stage, the grain yield of high nitrogen level was even lower than that of low nitrogen level due to insufficient grain filling. Unlike the responding pattern of the grain yield, both NPF and LT suppressed the grain filling while NPF enhanced grain yield as in previous studies since the increment on total spikelet number (Ding et al., 2003; Wang et al., 2015; Peng, 2016; Fu et al., 2019). Since 20 ± 4.9% higher total spikelet number and 9.7 ± 2.1% lower grain yield was found in N7 compared to those in N9108, it could be concluded that the major difference of the grain

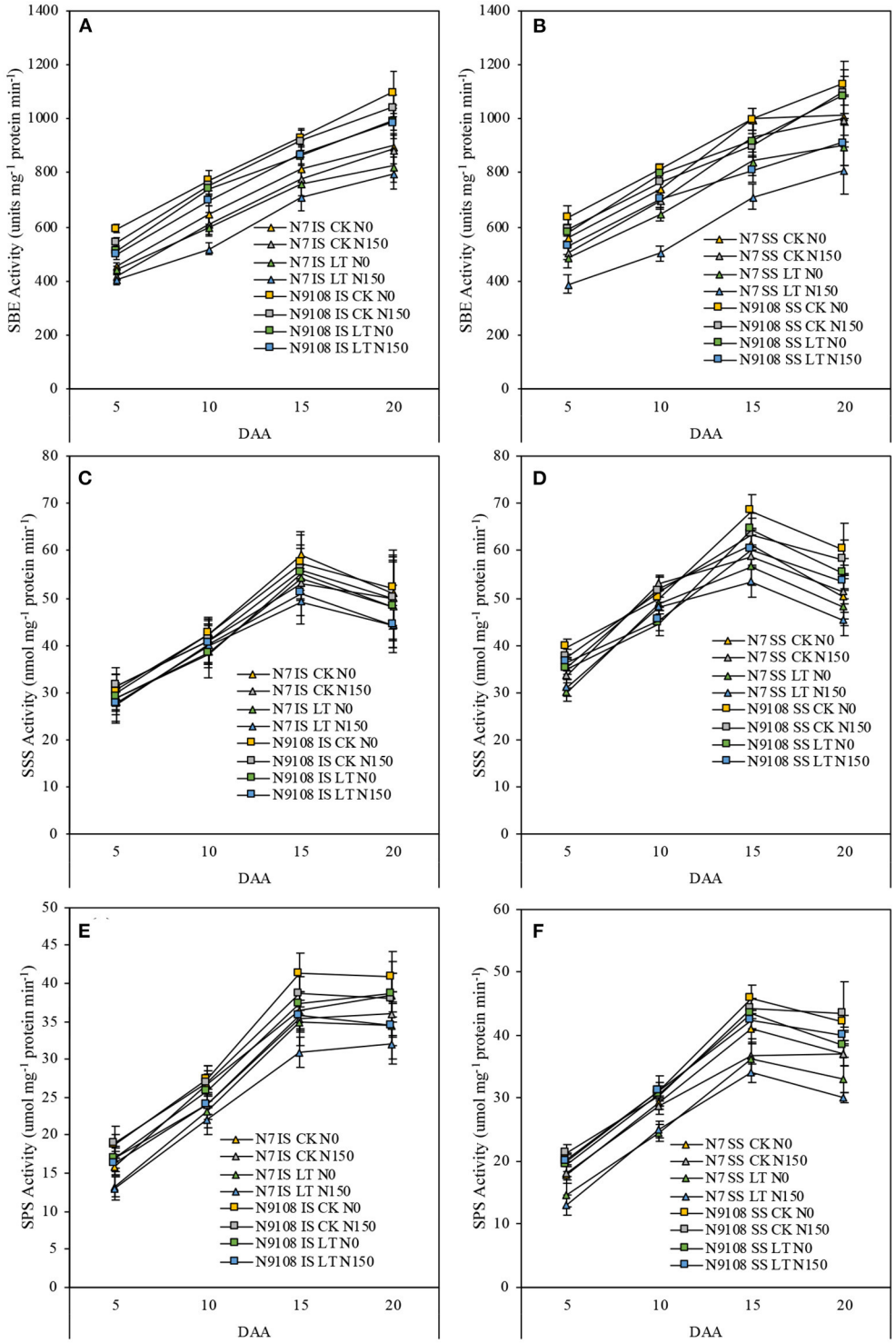

FIGURE 4 | Starch-biosynthesis related enzymes activities (starch branching enzymes (SBE): encoded by *SBE1*, starch synthase (SSS): encoded by *FLO4* and starch phosphorylase (SPS): encoded by *PHO-I*) in IS and SS of two rice varieties (N7 and N9108) under different nitrogen panicle fertilizer (N150, Applying NPF treatment; N0, No NPF treatment) and filling stage temperature treatments (CK and LT). Samples were taken at 5 DAA, 10 DAA, 15 DAA, and 20 DAA Values are means (±SD) of three replicates. **(A)** SBE activities of IS in N7 and N9108, respectively; **(B)** SBE activities of SS in N7 and N9108, respectively; **(C)** SSS activities of IS in N7 and N9108, respectively; **(D)** SSS activities of SS in N7 and N9108, respectively; **(E)** SPS activities of IS in N7 and N9108, respectively; **(F)** SPS activities of SS in N7 and N9108, respectively.

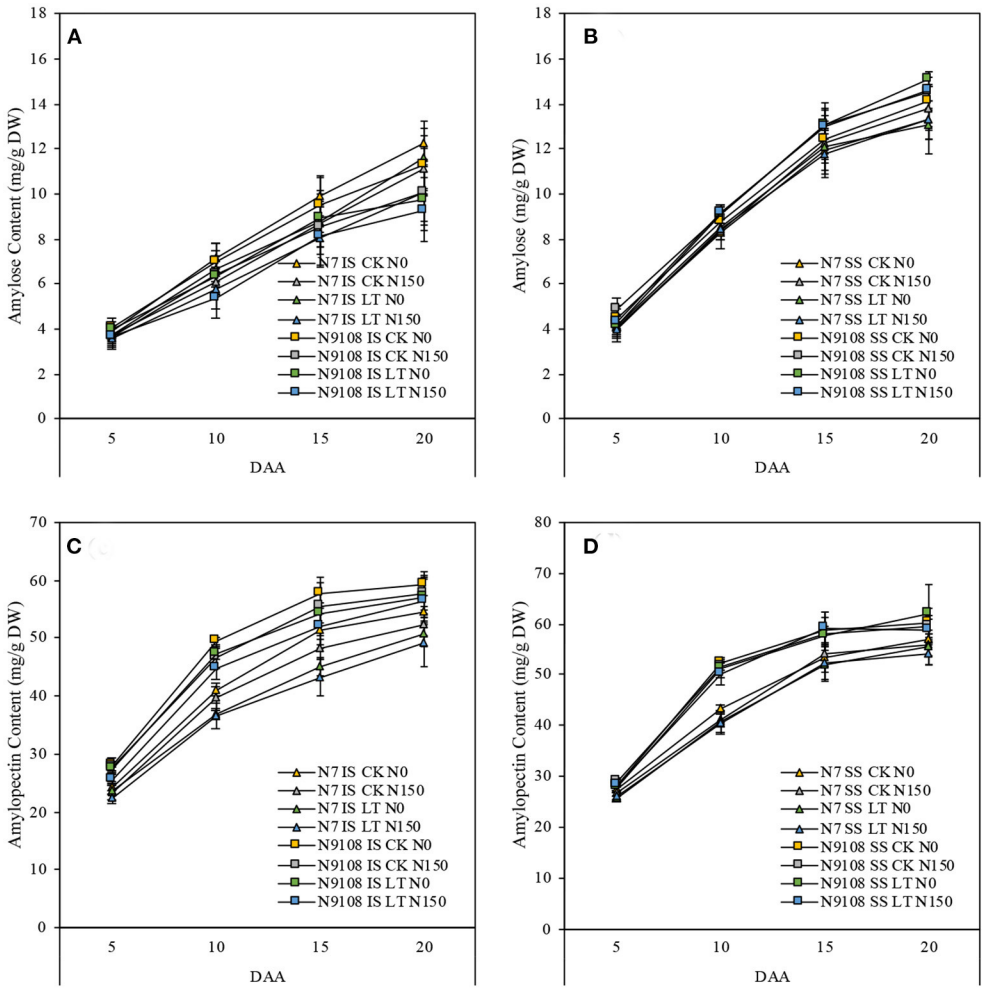

FIGURE 5 | Amylose and amylopectin accumulation dynamics from 5 DAA to 20 DAA in IS and SS of two varieties (N7 and N9108) in response to different fertilizer treatments (N150, Applying nitrogen panicle fertilizer treatment; N0, Without nitrogen panicle fertilizer treatment) and filling stage temperature treatments (CK and LT). Values are means (±SD) of three replicates. **(A)** Amylose content dynamics of IS in N7 and N9108, respectively; **(B)** Amylose content dynamics of SS in N7 and N9108, respectively; **(C)** Amylopectin content dynamics of IS in N7 and N9108, respectively; **(D)** Amylopectin content dynamics of SS in N7 and N9108, respectively.

yield of the two varieties was mainly derived from the grain weight which was closely related to their filling level instead of their population size. The variance of grain weight of N9108 was significantly lower than that of N7 indicated their higher filling stability (**Table 3**). To further investigate the underlying mechanism of the different sensitivities of grain weight, the filling rate was examined in this study, and the results were consistent with the grain yield (**Table 4**).

Abiotic stress, like temperature, suppressed the grain filling rate by slowing enzymes activities, which further reduce the starch biosynthesis (Wang et al., 2021). In the present study, grain filling rate was suppressed by LT but, on the other hand, the late sowing of rice increases the spikelet numbers that are in line with the previous study, which in turn enhances the vegetative stage temperature and rice population size (Wang et al., 2021). As shown in the result, both NPF and LT suppressed the grain filling rate while they both enhanced the spikelet number (**Table 3**). It could be concluded that increasing grain filling could be a more effective method to increase grain yield in the field rather than increasing population size, since the rice population size has been already enlarged a lot due to the popularization of "super rice" varieties. The grain yield was determined by the balancing between grain filling reduction and spikelet number enhancement. Therefore, the variety selection of late sowing conditions in the middle and lower reaches of the Yangtze River could be guided by strong sink activity rather than sink capacity. The varieties with higher sink activities showed higher LT and NPF adaptivity than low sink activities varieties in this study. The reduction of grain filling level in response to NPF and LT was further proved by lower sugar-to-starch conversion and starch biosynthesis level. Low starch content was found to be produced under LT and NPF conditions, mainly due to lower expression levels of starch biosynthesis correlated with gene expression (*FLO4*, *PHO-l*, and *SBE1*). A

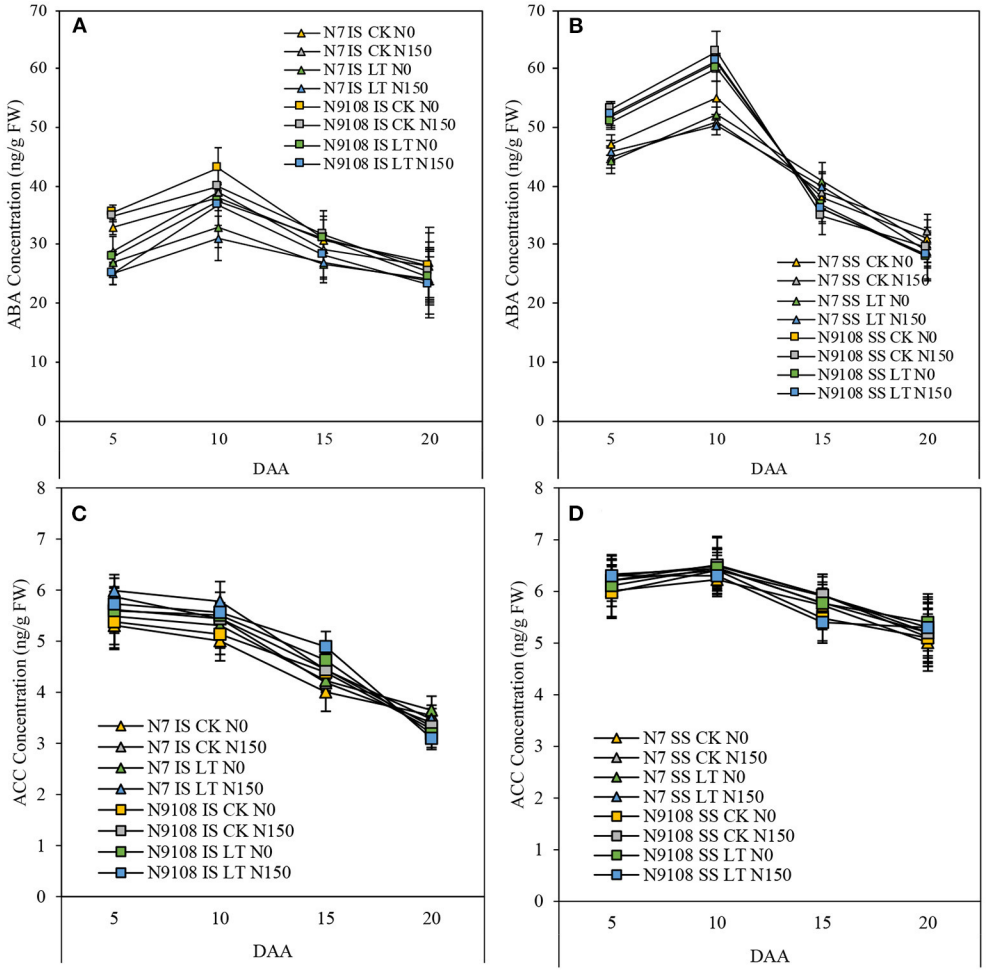

FIGURE 6 | Key hormones related to grain filling such as: Abscisic acid (ABA) and 1-aminocyclopropane-1-carboxylic acid (ACC) in IS and SS of two rice varieties (N7 and N9108) under different nitrogen panicle fertilizer (N150, Applying NPF treatment; N0, without applying NPF treatment) and filling stage temperature treatments (CK and LT). Values are means (±SD) of three replicates. Samples were taken at 5 DAA, 10 DAA, 15 DAA, and 20 DAA. **(A)** ABA concentration dynamics of IS in N7 and N9108, respectively; **(B)** ABA concentration dynamics of SS in N7 and N9108, respectively; **(C)** ACC content dynamics of IS in N7 and N9108, respectively; **(D)** ACC content dynamics of SS in N7 and N9108, respectively.

previous study also showed that abiotic stresses affected the grain filling level by controlling starch biosynthesis (Wang et al., 2019). Compared to LT treatment, grain yield could be more significantly regulated by the NPF application in CK condition due to their insufficient filling rate (**Table 3**). The grain filling rate was reduced by individual LT treatment (15 ± 2.9%). Moreover, the grain filling rate was further reduced (23 ± 4.6%) by applying NPF simultaneously (**Table 4** and **Figures 2**, **3**). It could be concluded that higher NPF's yield increasing effect was found in CK condition compared to that in LT condition, which indicated that the appropriate NPF should be selected in LT condition.

Sink Activity Controls Rice Grain Filling Rate

The grain-filling issue in inferior spikelets proved to be more serious in the newly bred "super rice" cultivars, although they generally show 8–20% higher yield potential than other conventional rice cultivars due to their large sink size (Kato, 2004; Cheng et al., 2007; Zhang et al., 2007). For example, 12 "super rice" cultivars in the lower Yangtze River basin were investigated in 2006 and 2007, and it was found that the average grain weight and filling proportion of inferior spikelets were 20.9 and 20.7%, respectively, lower than those of superior spikelets, while for three conventional cultivars, on average, it was only 10.5 and 6.3%, respectively. In this study, analyzing the balance between the positive and negative effects of NPF and LT on yield formation indicated that their negative effect on grain weight formation could be more serious than the reduction of grain yield (**Table 4** and **Figure 1**). Thus, we further investigated the grain filling rate among all treatments (**Figure 1** and **Table 4**). The major limitation factor of different grain-filling of super rice varieties with similar sink capacity was assumed as their differential sink activities and carbohydrate supply (Yang, 2010; Yang and Zhang, 2010). In this study, significantly lower sucrose to starch conversion was found in both LT and NPF treatments, which means that sink activity could be the major controlling factor of rice grain weight (**Figure 2**), similar to a previous

study (Yoshida, 1972; Kato and Takeda, 1996; Liang et al., 2001; Ishimaru et al., 2003; Yang A. et al., 2004). The sink activity could be represented by the starch biosynthesis efficiency in rice grains (Kato et al., 2007). In this study, the reduction of grain filling level in response to NPF or LT treatments were only found in the inferior seed rather than in both inferior and superior seeds, similar to the result of Wang et al. (2019). However, unlike that of environmental factors (filling stage temperature or fertilizer), the major controlling factor of grain weight between different varieties was their grain filling rate of both inferior and superior (**Table 4**). The grain filling rates of N9108 with superior and inferior grains were 15 ± 4.3 and 33 ± 11.6% faster than that of N7, respectively, due to its higher sink activity. Unlike previous study, higher gene expression and starch accumulation was found in both inferior and superior seeds of N7 compared to that of N9108 (**Table 4** and **Figures 2**, **3**) which is closely related to higher filling rate and weight in N9108 (**Table 4**). Therefore, it can be concluded that (1) The sink activities of superior and inferior seeds are the major limiting factors among different varieties' grain filling levels, and (2) the environmental factors (like NPF and LT in this study) mainly affects inferior seeds.

NPF and LT Suppressed Grain Filling Related Characteristics

In the process of starch accumulation, more than 30 major enzymes participate in the metabolism of carbohydrates during endosperm development in rice. Among them, several enzymes play important roles in this process, namely, ADP-glucose pyrophosphorylase, granule bound starch synthetase, soluble starch synthase, starch branching enzyme, and starch phosphorylase (Yang et al., 2001a,b; Yang J. et al., 2004; Hannah and James, 2008; Chen and Bao, 2017). The activities of these enzymes are closely related to total starch, amylose, and amylopectin accumulation in rice endosperm. To clarify the underlying regulating molecular mechanism, the genes expression of *FLO4*, *SBE1*, and *PHO-1* were measured and demonstrated as heatmap in this study (**Figure 3**). Enzymes involved in starch accumulation are not only affected by genotype but also by the growing environment of rice (Yang, 2001; Yang J. et al., 2004; Halford et al., 2015; Mayer et al., 2016). Many previous studies found that abiotic factors could affect enzyme activity and subsequently, change total starch, amylose, and amylopectin contents (Pan, 1999; Sun et al., 2018; Cheng et al., 2019; Prathap et al., 2019). Among these factors, enzyme activities are sensitive to environmental factors (like nitrogen fertilizer and temperature), and they can, consequently, affect starch accumulation (Cao et al., 2015; Fu et al., 2019), which is consistent with our results in which the gene expression was suppressed by LT and NPF (**Figure 3**). In this study, the lower gene expression of enzymes related to starch biosynthesis under NPF and LT treatments further reduced the amount of starch synthesis (**Figures 1–3** and **Table 4**) which resulted in the reduced grain yield of both varieties (**Table 3**). Application of individual NPF and LT treatments decreased the inferior grain weight for about 13 and 21%, respectively, of both varieties, which is similar to previous studies (Mae, 1997; Samonte et al., 2006), while synergistic NPF and LT treatment decreased the inferior grain weight for about 27% (**Table 3**). The additive and negative effects of NPF and LT treatments could be concluded based on the above results.

Many studies have demonstrated that hormonal changes at the whole-plant level can regulate senescence and nutrient remobilization (Davies, 1995; Lee and Masclaux-Daubresse, 2021). Abiotic stresses also affect hormonal levels, potentially regulating seed development and nutrient mobility (Ober et al., 1991; Davies, 1995; Wang et al., 2006). To further investigate the variation of grain filling and related characteristics, regulative phytohormones content was measured (**Figure 6**). Ethylene and ABA are two of the major phytohormones induced in response to stress, and their content showed complex changing pattern to biotic and abiotic stresses (Davies and Zhang, 1991; Gazzarrini and Mccourt, 2001; Yang et al., 2001b, 2006; Davies et al., 2002; Wilkinson and Davies, 2002; Cheng and Lur, 2010). It was found that activities of three key enzymes involved in the sucrose-to-starch pathway in the grains. SuSase, AGPase, and SSSase (Hawker and Jenner, 1993; Ahmadi and Baker, 2001; Hurkman et al., 2003), were significantly enhanced by the application of ABA, while ethylene played an inhibitive role in grain filling, since ethylene proved to be a negative regulator of ABA action in the seed (Ghassemian et al., 2000). Many previous studies have also described the interaction between ABA and ethylene in inferior seeds and the relationship between this interaction and starch accumulation pathway (Mohapatra et al., 1993; Kato et al., 2007; Zhu et al., 2011; Zhang et al., 2012; Wang et al., 2015). It was proved by Yang et al. (2006) that higher ABA concentration and lower ACC concentration could improve the grain-filling. In this study, we measured the concentration of ABA and ACC and found that the concentration of ABA was reduced by LT and NPF, while ACC was mainly enhanced by NPF. The result showed that the ratio of ABA and ACC was reduced by LT and NPF, which plays a negatively regulative role in the grain filling process and is consistent with grain filling changing pattern (**Figure 6**). One of the probable explanations of reduced grain filling in this study is the ratio of ABA and ACC. However, the biosynthesis and catabolism of ABA and ethylene in responding to LT and NPF are still unclear. Hence, the molecular studies on the hormones are necessary to dissect the underlying mechanism.

CONCLUSION

The result led to the conclusion that higher sink activities of super rice, namely, higher grain filling rates, which was regulated by the elevated ratio of ABA and ACC, are the major contributor of higher adaptivity to NPF and LT conditions. Moreover, the negative and additive effect of LT and NPF was also found on grain filling events of both varieties. Higher sensitivities of grain filling in response to NPF were found under LT compared to that under CK. This study provides basic knowledge about the mechanism of grain filling of different super rice cultivars with different sink activities in response to LT and NPF.

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 author/s.

AUTHOR CONTRIBUTIONS

GL contributed to conception and design of the study. CX performed organization of the database, statistical analysis, and manuscript writing. All authors contributed to manuscript revision, read, and approved the submitted version.

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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.2021.729021/full#supplementary-material

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Identification and Functional Verification of Cold Tolerance Genes in Spring Maize Seedlings Based on a Genome-Wide Association Study and Quantitative Trait Locus Mapping

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Maize (*Zea mays* L.) is a tropical crop, and low temperature has become one of the main abiotic stresses for maize growth and development, affecting many maize growth processes. The main area of maize production in China, Jilin province, often suffers from varying degrees of cold damage in spring, which seriously affects the quality and yield of maize. In the face of global climate change and food security concerns, discovering cold tolerance genes, developing cold tolerance molecular markers, and creating cold-tolerant germplasm have become urgent for improving maize resilience against these conditions and obtaining an increase in overall yield. In this study, whole-genome sequencing and genotyping by sequencing were used to perform genome-wide association analysis (GWAS) and quantitative trait locus (QTL) mapping of the two populations, respectively. Overall, four single-nucleotide polymorphisms (SNPs) and 12 QTLs were found to be significantly associated with cold tolerance. Through joint analysis, an intersection of GWAS and QTL mapping was found on chromosome 3, on which the *Zm00001d002729* gene was identified as a potential factor in cold tolerance. We verified the function of this target gene through overexpression, suppression of expression, and genetic transformation into maize. We found that *Zm00001d002729* overexpression resulted in better cold tolerance in this crop. The identification of genes associated with cold tolerance contributes to the clarification of the underlying mechanism of this trait in maize and provides a foundation for the adaptation of maize to colder environments in the future, to ensure food security.

Keywords: cold tolerance, GWAS, QTL mapping, gene cloning, functional annotation

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INTRODUCTION

Various abiotic stresses, such as low temperature, drought, and high salinity, significantly affect the normal growth and yield of plants. Of these, cold stress has had an impact on the growth, yield, and spatial distribution of crops, including maize (*Zea mays* L.). Cold tolerance in maize is a complex quantitative hereditary trait, controlled by multiple genes; different periods of this crop are controlled by different genetic mechanisms, which are easily affected by environmental factors (Hodges et al., 1997; Gao et al., 2009). Jilin Province is one of the most important sites of maize cultivation in China; however, it is extremely prone to cold damage, resulting in reduced maize production (Jena et al., 2012; Ma et al., 2018; Yan et al., 2018).

Several reports identifying molecular markers and quantitative trait locus (QTLs) associated with cold tolerance have been published, but their practical application in maize breeding to improve resistance to cold conditions has so far been limited (Revilla et al., 2014). Many studies have shown that cold tolerance at the germination and seedling stages of maize is a quantitative trait, controlled by multiple genes (Trzcinska-Danielewicz et al., 2009); studies have also shown that epistasis and additive and dominant gene effects in maize germination under low temperature significantly affect tolerance (Ma et al., 2007).

Rodríguez et al. (2008) used a recombinant inbred line (RIL) constructed using a cross between the inbred lines B73 × Mo17 to locate QTLs affecting maize leaf color under low temperatures; they found that the major QTL that control maize leaf color under these conditions is located at the chromosome positions bin 3.01 and bin 6.03. Jompuk et al. (2005) used the F_2 population ETH-DH7 × ETH-DL3 to identify the main QTL governing the operation of the photosynthetic system at low temperatures during the seedling stage of maize, which was found to be distributed along chromosome 6. Leipner et al. (2008) also used this F_2 population ETH-DH7 × ETH-DL3 to assess cold tolerance in the maize seedling photosynthetic system under low temperature stress and detected that the major QTL for this trait was distributed along chromosome 3. Pimentel et al. (2005) used the constructed F_2 population W6786 × IL731A to identify the QTL controlling the degree of photoinhibition in maize under low temperature stress; three QTLs on chromosomes 3, 4, and 8 were detected in plants cultivated under controlled indoor and outdoor conditions. The QTL in bin 3.09 on chromosome 3 was detected in both indoor and outdoor plants; the other two QTLs were only detected in indoor environments. Li et al. (2018) used two cold-tolerant inbred lines, 220 and P9-10, and two cold-sensitive inbred lines, Y1518 and PH4CV, to construct three $F_{2:3}$ populations; and using the germination rate as the phenotype to QTL mapping, they identified 43 QTLs that each explained 0.62–39.44% of the phenotypic variation among plants in this population. Seven of these QTLs together explained more than 10% of the total phenotypic variation. Hu et al. (2016) identified a total of six QTLs governing seed germination under low temperature conditions, on chromosomes 4, 5, 6, 7, and 9, under different degrees of cold stress, which were found to explain 3.39–11.29% of phenotypic variation.

Strigens et al. (2013) conducted a genome-wide association study (GWAS) in maize, locating 19 single-nucleotide polymorphisms (SNPs) related to cold tolerance, which explained 5.7–52.5% of the phenotypic genetic variation in maize seedlings. Meanwhile, a number of candidate cold tolerance genes were discovered in the Strigens' study. Hund et al. (2004) used the $F_{2:3}$ population, Lo964 × Lo1016, to identify QTL controlling cold-related traits of maize seedlings under cold stress. A major QTL was found on chromosome 5 that could explain 14% of the total phenotypic variation of root diameters. Fracheboud et al. (2002, 2004) chlorophyll fluorescence parameters, CO2 exchange rate, leaf greenness, shoot dry matter, and shoot nitrogen content as phenotype in an $F_{2:3}$ population under cold stress and found that a major QTL located on chromosome 6 explained 37.4% of the phenotypic variance in the chronic photoinhibition at low temperature. Rodríguez et al. (2014) used the $F_{2:3}$ population, EP42 and A661, to map QTLs using four cold tolerance-related traits, number of survival plants, dry weight, quantum yield of photosystem II, and total anthocyanin content. Four QTLs associated with cold stress were identified three genomic regions in chromosomes 2, 4, and 8.

In our study, we used GWAS and QTL mapping to identify major SNPs and QTL in two populations of maize. We used an integrative analysis approach, combining the results of the GWAS and QTL mapping, to further identify key candidate genes for cold tolerance. We then performed a functional annotation and cloning into *Z. mays* for functional verification of these candidate genes. Using these methods, we aimed to provide the basis for developing possible strategies for breeding new maize with improved cold tolerance, thus improving food security.

MATERIALS AND METHODS

Plant Materials and Experimental Design

There were two genetic populations in our study: the first (population 1) was a natural population consisting of 80 backbone inbred lines. These lines were selected from numerous maize materials based on their frequency and genetic diversity in maize breeding in Jilin Province, China (**Supplementary File 1**). The second population (population 2) was an F_2 population of 210 offspring, with parents derived from population 1 (W72 × W10). Two lines W10 (chilling-tolerant) and W72 (chilling-sensitive) were used as parents to produce a segregating F_2 population. To obtain F_2 hybrids, we self-crossed F_1 individuals, and then self-crossed F_2 individuals to obtain $F_{2:3}$ seeds. The above experimental materials were provided by the Biotechnology Center of Jilin Agricultural University, China.

Analysis of Phenotypic Data and Peroxidase Activity

We measured the peroxidase (POD) activity of each maize inbred line using the guaiacol method (Javadian et al., 2010) at seedling stage. Three consecutive batches of tests were

performed on each inbred line at a low temperature of 6°C for 24 h. Ten strains of the same line were tested in each test, and average values of all 30 strains were obtained for subsequent analysis, which was conducted using SPSS 23.0 software (Pallant, 2013).

The formula for calculating POD activity was as:

$$\mathrm{POD}\left(\mathrm{U}/\mathrm{g}\times\mathrm{min}\right)=\frac{\mathrm{A470}\bullet\mathrm{Vt}}{0.01\times\mathrm{W}\bullet\mathrm{Vs}\bullet\mathrm{t}}$$

where A470 was the change in absorbance within the reaction time; *W* was the fresh weight (g); *Vt* was the total volume of extracted enzyme solution (ml); *Vs* was the volume of enzyme solution during measurement (ml); and *t* was time (min).

DNA Extraction for Sequencing in Populations 1 and 2

The leaves of populations 1 and 2 were collected and flash frozen in liquid nitrogen. The DNA was extracted using the NuClean Plant Genomic DNA Kit (CWBio, Jiangsu, China); the concentration and purity of the extracted DNA were determined using a Nano Drop 2000 micro spectrophotometer (Thermo Fisher, Waltham, MA, United States). The integrity of the extracted DNA was confirmed using agarose gel electrophoresis (agarose concentration 1%, voltage 200 V, time 25 min; Zhao et al., 2018).

Whole-Genome Sequencing and SNP Identification in Population 1

We used the NuClean Plant Genomic DNA Kit (CWBio, Jiangsu, China) to extract genomic DNA from the 80 inbred maize lines in population 1 at seedling stage. DNA quality was assessed using 1% agarose gel electrophoresis under 150 volts 70 milliampere, 30 min and a NanoDrop 2000 spectrophotometer (NanoDrop, Wilmington, DE, United States). Qualified DNA samples with clear bands were used to construct a genomic library. Sequencing was performed using an Illumina HiSeq PE150 (Illumina Inc., San Diego, CA, United States) to obtain raw data. Then, read pairs containing the linker sequence were filtered out; paired reads with N content in a single-end sequencing read exceeding 10% of the length of the read, and those with a number of low-quality bases (defined as quality value $Q \leq 5$) in a single-end sequencing read exceeding 50% of the length of the read, were removed. Finally, the sequencing data were left high-quality, clean genomic data.

Maize B73 RefGen_v4 was the data mining resource of the Maize Genetics and Genome Database (MaizeGDB).[1] We compared these clean data to the reference genome, Maize B73 RefGen_v4 (Shamimuzzaman et al., 2020), using the Burrows-Wheeler Aligner (BWA) software (Jia et al., 2013), and SAMtools software (Zhou et al., 2015) was used to remove duplicates (command: rmdup). Then, we used a Bayesian model to detect polymorphic sites in the population, and filtered and screened high-quality SNPs using a minor allele frequency (MAF) > 0.05 and call rate >80%. Finally, we used ANNOVAR software (Yang and Wang, 2015) to functionally annotate the identified SNPs.

Population Structure, Linkage Disequilibrium, GWAS, and Candidate Gene Annotation in Population 1

The phylogenetic tree was inferred using TreeBeST[2] using the *p*-distances genetic distance estimation model and the identified SNPs. The Principal Component Analysis (PCA) was analyzed by Genome-wide Complex Trait Analysis (GCTA; Yang et al., 2011).[3]

We used Haploview (Barrett et al., 2005) to calculate linkage disequilibrium (LD) in population 1 and FarmCPU to perform a GWAS to identify associations between genetic loci and phenotypic traits (Kaler et al., 2020). Research in recent years had shown that the SNPs at which the maximum value of R^2 (corresponding to the LD) decays to half are linked. When R^2 decayed to half, the corresponding LD was about 5.0 kb. Therefore, we chose a LD of 5.0 kb to scan for functional genes.

All identified functional genes were annotated using the SWISS-PROT,[4] COG,[5] GO,[6] KEGG,[7] and NCBI[8] databases; possible candidate cold tolerance genes were screened based on previous studies.

Genotyping by Sequencing in Population 2

Given that the parental line of population 2 had already been sequenced among population 1, we used GBS to sequence population 2 only. We used qualified samples with clear bands in electrophoresis for GBS and quality analysis of SNPs. Sequencing was completed using Illumina NovaSeq 6,000 (Illumina Inc., San Diego, CA, United States).

The GBS data were filtered to obtain clean data, using the same criteria as for population 1, detailed in section "Whole-Genome Sequencing and SNP Identification in Population 1."

We used BWA software (Jia et al., 2013) to compare the clean data with the reference genome (Maize B73 RefGen_v4) and used SAMtools to convert the compared result format into SAM/BAM files; in addition, the Perl script was used to calculate the mapping rate and coverage, and SAMtools "sort" command was used to detect mutations. Polymorphic tags of parental line were genotyped to facilitate subsequent genetic analysis. Because the parents were homozygous inbred lines with aa and bb genotypes, SNPs with the segregating pattern of aa × bb were screened (Zhang et al., 2016a). Three standards were used for the screening of SNP: first, in the offspring typing, there may be a few base types that did not appear in the parents, and we considered them as deletions; second, included genotypes covered at least 80% of SNPs in the offspring;

[1]https://www.maizegdb.org/genome/assembly/Zm-B73-REFERENCE-GRAMENE-4.0

[2]http://treesoft.sourceforge.net/

[3]https://yanglab.westlake.edu.cn/software/gcta/#Overview

[4]http://www.uniprot.org/

[5]http://clovr.org/docs/clusters-of-orthologous-groups-cogs/

[6]http://geneontology.org/

[7]https://www.genome.jp/kegg/

[8]https://www.ncbi.nlm.nih.gov/

and third, segregation distortion. We used the chi-square test to assess offspring with a significance threshold of $p < 0.001$.

Construction and Evaluation of the Genetic Linkage Map

We used Join Map 4.1(Stam, 1993) software to construct a genetic linkage map of high-quality SNPs obtained after screening. First, the linkage group was divided into chromosomes; second, a maximum likelihood method was used to sort the markers in each linkage group. That was, A and B were linked, B and C were linked, and then, A and C were linked; otherwise, B and C were not linked, then A and C were not linked. Third, the genetic distance between markers was calculated using Kosambi mapping function. Finally, we used the Perl SVG module to draw the genetic linkage map.

To ensure the quality of the genetic linkage map, we created a map of the haploid source of population 2 in 10 linkage groups, and possible double crossover sites were identified; we also evaluated linkage relationships between each of the markers in the same linkage group on the map (Zhao et al., 2016); and finally, we performed a collinearity analysis by the R programming language using marker positions on the genome and the genetic map to perform collinearity analysis (Zhang et al., 2016b).

Screening QTLs Associated With POD

QTLs were detected using composite interval mapping (CIM) in WinQTL Cart 2.5 software (Jiang et al., 2018) utilizing the phenotype and genotype data from population 2. The Kosambi function was used with the scan step set as 1 cM, and the maximum value of p for entering variables in a stepwise regression of residual phenotype on marker variables (PIN) was set as 0.001. LOD (=0.217 likelihood ratio) threshold for declaring a putative QTL for each trait, data set, and model was defined by 1,000 permutations. Finally, we selected a QTL with a threshold logarithm of the odds (LOD) > 2.5 (Meng et al., 2015). Fracheboud et al. (2002) found the presence of a QTL was declared significant if the LOD value was >2.5 for a single trait analysis.

Integrated GWAS and QTL Mapping Analysis

We integrated the results of the GWAS and QTL mapping in populations 1 and 2 to identify SNPs that were significantly associated with the POD phenotype. We used an LD of 5.0 kb to scan for functional genes.

Expression Analysis of the Candidate Cold Tolerance Gene *Zm00001d002729*

We selected 10 strains in each of population 1 and 2 for expression analysis of the candidate gene *Zm00001d002729*; in population 1, we selected the extremely cold-resistant strains W10, W18, and W26; general cold-resistant strains W1, W15, W36, and W52; and extremely cold-sensitive strains W43, W48, and W72. In population 2, we selected extremely cold-resistant strains J15, J75, and J144; general cold-resistant strains J8, J98, J183, and J191; and extremely cold-sensitive strains J107, J123, and J200. We extracted RNA from leaves of each inbred line as a template at seedling stage and measured the relative expression of *Zm00001d002729* in each line using quantitative real-time PCR (qRT-PCR) and the fluorescent dye SYBR Green (Takara Biomedical Technology Co., Ltd., China). The internal reference gene we chose was *Actin-1* (*Act*; Accession: J01238). The primers used are given in **Supplementary File 2**.

Total RNA was extracted using the Plant RNA Kit R6827-02 (Omega Bio-tek, United States), and then reverse-transcribed into cDNA using the All-in One™ First-Strand cDNA Synthesis Kit (GeneCopoeia Inc., United States); qRT-PCR primers were designed using an online tool (https://sg.idtdna.com/PrimerQuest/Home/Index). qRT-PCR was performed using the All-in One™ qPCR Mix (GeneCopoeia Inc., United States) and the Stratagene Mx3000P PCR system (Agilent Technologies, United States) with three repetitions, and the relative expression level of the genes was calculated using the $2^{-\Delta\Delta CT}$ method (Lu et al., 2018).

Cloning of *Zm00001d002729* and Vector Construction

We used Primer Premier 5.0 (Wei et al., 2009) to design specific primers for cloning *Zm00001d002729* by RT-PCR, using cDNA from leaf tissue of maize strain Dan 340 at seedling stage as the template, and annealing temperature was about 55.0°C (**Supplementary File 2**). Then, we constructed the pMD-18T-*Zm00001d002729* cloning vector and sequenced it to confirm successful cloning. We used the Seamless Assembly Cloning Kit (Taihe Biotechnology Co., Ltd., China) to construct the pCAMBIA3301-*Zm00001d002729* expression vector. We used the CRISPR-P Web site[9] to design small guide RNA (sgRNA). We selected sgRNA with high-target scores, low miss-target rates, and used Seamless cloning to construct a pCXB053-*Zm00001d002729*-CRISPR/Cas9 vector with a single gene and dual targets. Meanwhile, we used U6 as a promoter to construct a CRISPR/Cas9 gene editing vector.

Genetic Transformation of *Zm00001d002729* Into *Z. mays* H299 and Functional Verification

In order to verify the function of Zm00001d002729, we transformed Zm00001d002729 into strain H299 using an Agrobacterium-mediated method (Frame et al., 2006). In this study, Agrobacterium-mediated methods were used to transfer the constructed expression vector to the recipient. After embryoculture, subculture, pre-culture, co-culture, screening culture, elongation culture, and rooting culture, we obtained T0 maize and cultivated it to the T2 generation. We performed test strip detection for *bar* gene by GeneTureTM BAR Test Kit (Artron Laboratories Inc.) and qRT-PCR detection on the transformed maize. Finally, we cultivated the T2 generation containing pCAMBIA3301-Zm00001d002729 or CRISPR/

[9]http://crispr.hzau.edu.cn/CRISPR2/

Cas9-Zm00001d002729, and three wild-type maize plants in total, to under conditions of cold stress at 6°C for 24 h at seedling stage, monitoring their morphology and measuring POD activity, malondialdehyde (MDA) content, and relative conductivity of seedlings leaves.

RESULTS

Analysis of Phenotypic Data and POD Activity

The POD activity phenotype data of two population were shown in **Supplementary File 3**. The average POD activity in population 1 was 46.95 U/g × min, with a range of 22.97–100.75 U/g × min, and a coefficient of variation of 0.30. The average POD activity in population 2 was 55.19 U/g × min, with a range of 27.44–83.37 U/g × min, and a coefficient of variation of 0.28. These data were found to be normally distributed according to the Kolmogorov-Smirnov test ($p = 0.20$) of normality (**Figure 1**).

Whole-Genome Sequencing and SNP Identification in Population 1

The quality of DNA extracted from the sample was high and the fragments were clear (**Figure 2**), thus meeting the requirements of DNA library construction for sequencing.

We performed WGS on population 1 and obtained 1.58 TB of clean data after quality control. Comparison with the maize inbred line reference genome showed that the mapping rate of the population samples was 98.72%. The average sequencing depth was 17.10×, and the average genome coverage was 89.16%. The similarity between each sample and the reference genome met the requirements of WGS analysis and had a very good sequencing depth and coverage. We obtained a total of 24,860,241 high-quality SNPs, of which 85.4% were intergenic. The results of SNP genotyping are shown in **Supplementary File 4**.

Population Structure and Linkage Disequilibrium in Population 1

We constructed a phylogenetic tree for population 1, based on genotype data of 80 inbred lines (**Figure 3A**). The results

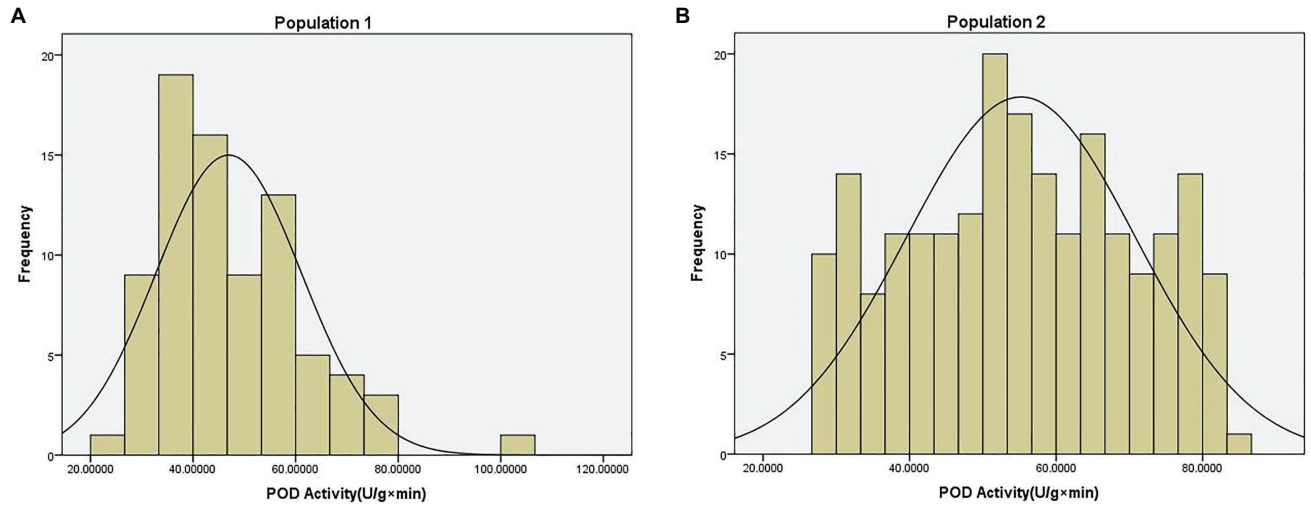

FIGURE 1 | Phenotype distribution of peroxidase (POD) activity traits in **(A)** population 1 and **(B)** population 2.

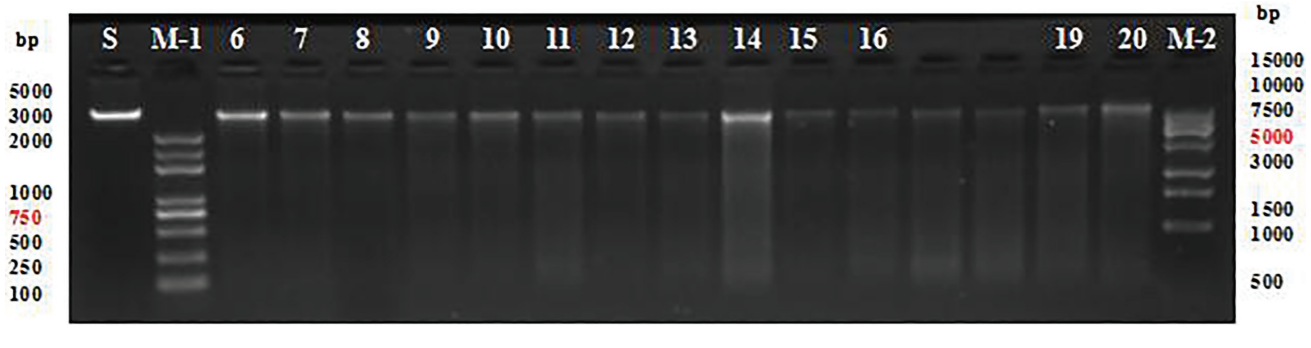

FIGURE 2 | Agarose gel electrophoresis of DNA fragments from populations 1 and 2. S, standard sample; M-1, trans 5k DNA marker; M-2, trans 15k DNA marker; and 6–20, partial maize genome.

showed three subpopulations: subpopulation 1 (in red) contains lines mainly from Tang Si Ping Tou and Lv Da Red Bone; subpopulation 2 (in blue) contains lines mainly from Lancaster; and subpopulation 3 (in yellow) contains lines mainly from Reid. The PCA results were shown in **Figure 3B** and **Supplementary File 5**. The results showed the distributions of inbred lines, and they were separated accordingly.

The distribution of LD on chromosomes is described in the LD attenuation diagram (**Figure 3C**). The abscissa represents the distance at which LD occurs, and the ordinate is the correlation coefficient of LD, a way of displaying the LD value.

The attenuation distance was 3.7 kb in chromosome 1; 3.6 kb in chromosome 2; 3.5 kb in chromosome 3; 3.5 kb in chromosome 4; 3.1 kb in chromosome 5; 3.1 kb in chromosome 6; 2.6 kb in chromosome 7; 3.2 kb in chromosome 8; 3.8 kb in chromosome 9; and 3.2 kb in chromosome 10. The average attenuation distance of the whole genome was 3.4 kb.

GWAS to Identify Genes Associated With POD

The Manhattan chart and Q-Q chart for the GWAS for POD activity are shown in **Figure 4**. We used $p < 0.000001$ as the threshold; our analysis detected four SNPs that were significantly related to POD activity. These SNPs were all located on chromosome 3 (**Table 1**).

GBS, Development, Encoding, and Screening of SNPs in Population 2

The two parents from population 1 (whose genomes were sequenced using WGS) and 210 offspring from population 2 (GBS) were successfully sequenced with an average sequencing depth of 30× and 10×, respectively. Our results yielded a total of 261.005 GB raw data from the offspring in population 2, with an average of 1,243 GB of raw data for each sample, and the filtered clean data was 260.879 GB, with 1.242 GB on average in each sample. The sequencing quality for the offspring was very high (Q20 ≥ 94% and Q30 ≥ 85%), and the sample GC distribution was normal. The database was successfully constructed and sequenced.

On comparison with the maize reference genome, 9,141,219 and 6,127,709 SNPs were identified in the parent plants, W72 and W10, respectively. A total of 5,759,868 SNPs were identified in population 2 compared with the reference genome, and 1,847,696 polymorphic SNPs were assigned to the parental aa × bb segregation pattern (**Supplementary File 6**). After

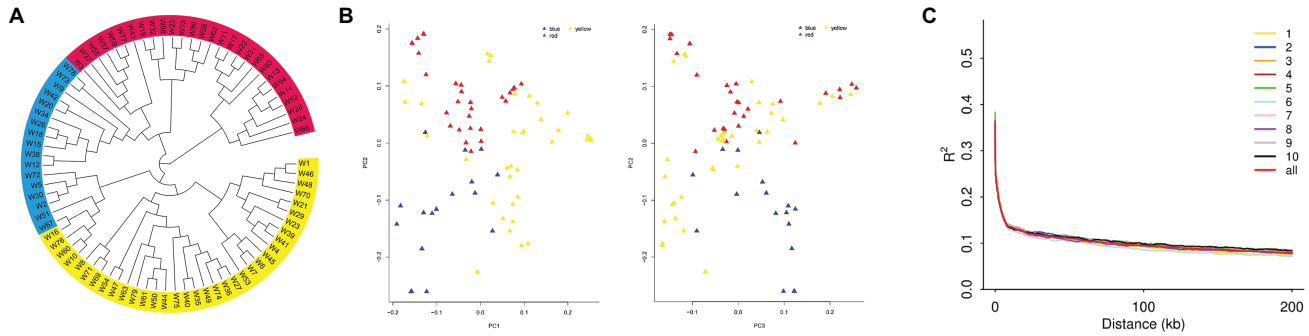

FIGURE 3 | (A) The population structure, **(B)** Principal Component Analysis (PCA), and **(C)** linkage disequilibrium (LD) diagram for population 1.

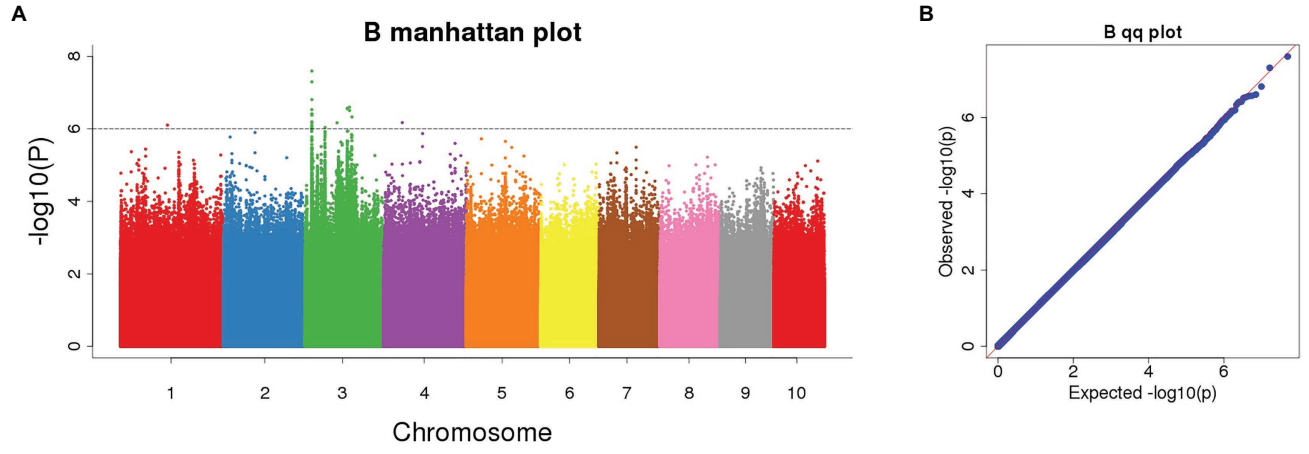

FIGURE 4 | (A) Manhattan plot of significant SNPs correlating with POD activity using the FarmCPU model; **(B)** Quantile-Quantile (Q-Q) plot.

filtering, 26,693 SNPs remained. We used these SNPs for genetic linkage map construction. SNPs were screened and adjusted iteratively to optimize the map following its initial construction.

Construction and Evaluation of the Genetic Linkage Map

Each chromosome constituted a linkage group. A total of 8,213 SNPs were shown on the map, with a total genetic distance of 6516.35 cM. The average genetic distance between two adjacent markers was 0.79 cM. There were 1,163 markers in the first linkage group covering a genetic distance of 1114.99 cM: the longest genetic distance among the 10 linkage groups. On the other hand, there were 619 markers in the fifth linkage group over a genetic distance of 414.49 cM, covering the shortest genetic distance among the 10 linkage groups (**Supplementary File 7**). We also analyzed the distribution of gaps in each linkage group (**Supplementary File 8**); most gaps were less than 5 cM.

We used the Perl SVG module to draw a genetic linkage map (**Supplementary File 9**) and evaluated the quality of the map by constructing a map of the haploid source with all markers in each linkage group from population 2. The haploid source of the large intervals observed in every offspring was one of the parents, and the proportion of double crossing-over was less than 3%, indicating high quality of the linkage map (**Supplementary File 9**). We constructed a heat map of linkage relationships between each marker in the same linkage group from the genetic linkage map. The linkage relationship between adjacent markers in each group was very strong. With increasing distance, the linkage relationship between markers weakened gradually, indicating that the order of markers in the map was correct (**Supplementary File 9**). Finally, we analyzed the collinearity of the markers on the genome and the genetic map. The collinearity map can not only reflect the collinearity of the position of the markers on the genome and genetic map, but also reflect the distribution of the markers on the genetic map mapped to the chromosome in general. It can also reflect the relationship of each marker on the genome and genetic map. It can be seen from this figure that the distribution of markers on the chromosomes is relatively uniform, most of the marker sequences on each linkage group are basically consistent with the genome, the collinearity is good, and the calculation accuracy of the genetic recombination rate is high (**Supplementary File 9**).

Screening of QTLs Associated With POD

We detected a total of 12 QTLs related to POD. Among them, 11 QTLs were located on chromosome 2, and 1 QTL was located on chromosome 3. The QTL qPOD2b was located on chromosome 2, which had the largest contribution to POD activity of all QTLs, at 6.27%, with an additive effect of 52.80 U/g × min and a dominant effect of 6.51 U/g × min. qPOD3 was located on chromosome 3, with a contribution to the POD phenotype of 5.60%, an additive effect of 1.17, and a dominant effect of 4.52 (**Figure 5**; **Table 2**).

Annotation and Screening of Cold Tolerance Candidate Genes in Populations 1 and 2

We integrated the results of the GWAS and QTL mapping in two populations to identify SNPs and QTLs that were significantly associated with the POD activity phenotype. Then, we used an LD of 5.0 kb to scan for functional genes. Our integrated GWAS and QTL mapping analysis revealed that the SNP (sPOD-20,592,562) was located in the QTL qPOD3 and that this SNP was associated with the gene *Zm00001d002729*. It meant that *Zm00001d002729* has been identified in both populations, which greatly increased the credibility of this gene. Our functional annotation indicated that this gene was an acyl-acyl carrier protein thioesterase.

We used Cell-PLoc 2.0[10] to predict the subcellular localization of the Zm00001d002729 protein (Chou and Shen, 2008). The result showed that the predicted location was chloroplast and nuclear.

[10]http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/

TABLE 1 | Single-nucleotide polymorphisms (SNPs) significantly associated with POD activity ($p < 0.000001$).

Name	Start (bp)	End (bp)	peakPOS (bp)	chr	ref	alt	peak_value	Candidate gene	LD
sPOD-20,592,562	20,587,562	20,597,562	20,592,562	3	A	G	6.10	*Zm00001d002729*	0
sPOD-96,084,381	96,079,381	96,089,381	96,084,381	3	C	T	6.17	*Zm00001d041082*	3,183
sPOD-133,504,984	133,499,956	133,509,984	133,504,984	3	T	A	6.60	*Zm00001d041691*	3,551
sPOD-140,877,969	140,872,969	140,882,969	14,087,7,969	3	T	C	6.33	*Zm00001d041853*	1,384

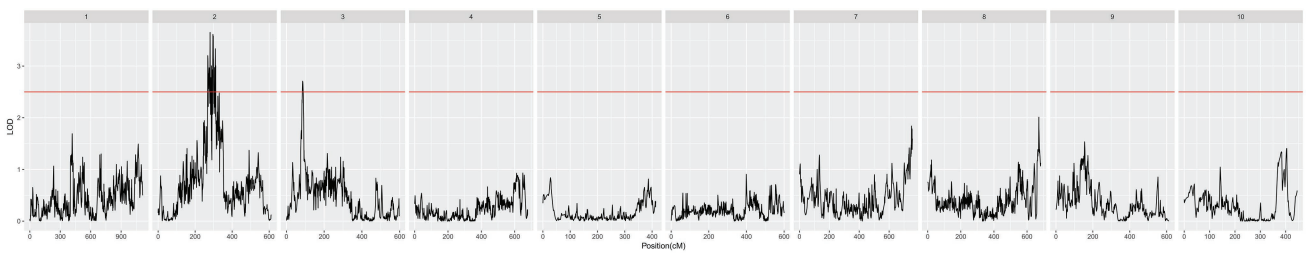

FIGURE 5 | Quantitative trait locus (QTL) mapping of POD activity.

Expression Analysis of Cold Tolerance Candidate Gene *Zm00001d002729*

We analyzed the relative expression levels of the candidate gene *Zm00001d002729* seedling stage in the two populations. The results showed that the expression level of this cold tolerance candidate gene in maize strains with better cold tolerance (W10, W18, W26, J15, J75, and J144) was significantly ($p < 0.05$) higher than that in cold-sensitive maize strains (W43, W48, W72, J107, J123, and J200). From this we concluded that higher expression levels of *Zm00001d002729* in maize result in greater cold tolerance (**Figure 6**).

Cloning and Vector Construction of *Zm00001d002729*

We successfully obtained *Zm00001d002729* cDNA by RT-PCR method by using RNA extracted from the leaves seedling stage Dan 340 as the template. The full length of *Zm00001d002729* coding sequence was 690 bp.

After construction of the cloning vector, we used Seamless cloning technology to construct the plant over expression vector and CRISPR/Cas9 vector. Firstly, we used pCAMBIA3301 as the vector backbone and constructed pCAMBIA3301-*Zm00001d002729*. Meanwhile, we used U6 as a promoter to construct a CRISPR/Cas9 gene editing vector. Two carrier structures are shown in **Supplementary File 10**.

Functional Verification of *Zm00001d002729*

We transformed *Zm00001d002729* into *Z. mays* H299 *via* Agrobacterium-mediated method; the growth process is shown in **Supplementary File 11**. After testing to confirm successful transformation using the GeneTure™ BAR Test Kit (Artron Laboratories Inc.; **Supplementary File 12**), we cultivated the genetically modified maize to the T2 generation.

The relative expression levels of *Zm00001d002729* gene in the roots, stems, and leaves of each maize strain are shown in **Figure 7**. When the target gene was overexpressed, *Zm00001d002729* mRNA levels increased by an average of 274% in roots, 144% in stems, and 128% in leaves. After gene editing, the target gene dropped by an average of 82% in roots, 93% in stems, and 92% in leaves.

We also measured the POD activity, MDA content, and relative conductivity of these nine inbred maize lines after 24 h at 6°C (**Figure 7**). We found that in maize with overexpression of the target gene, the POD activity was significantly increased, the MDA content significantly decreased, and the relative conductivity was also significantly decreased (all $p < 0.05$); while in maize where the target gene was silenced, POD activity was decreased, and the MDA content and relative conductivity significantly increased. This indicates that *Zm00001d002729* is a positive regulator of cold resistance in maize. The higher the expression of *Zm00001d002729*, which could increase cold tolerance of maize.

TABLE 2 | Quantitative trait locus (QTLs) associated with POD activity.

Name	chr	Left marker	Right marker	Left position (bp)	Right position (bp)	LOD	Additive	Dominance	PVE (%)
qPOD2a	2	mk-2-411	mk-2-1,108	73,584,577	143,233,161	3.14	−57.66	6.30	5.41
qPOD2b	2	mk-2-1,107	mk-2-561	143,233,142	92,502,399	2.75	−52.80	6.51	6.27
qPOD2c	2	mk-2-561	mk-2-801	92,502,399	123,536,831	3.11	−55.91	6.18	5.63
qPOD2d	2	mk-2-801	mk-2-950	123,536,831	133,026,652	2.75	−58.22	6.45	5.47
qPOD2e	2	mk-2-972	mk-2-1,062	133,419,121	137,369,789	2.82	−119.36	5.84	5.94
qPOD2f	2	mk-2-947	mk-2-1,133	132,958,156	143,771,596	2.93	−119.36	5.80	5.62
qPOD2g	2	mk-2-1,133	mk-2-731	143,771,596	115,063,134	3.15	−119.36	6.19	5.77
qPOD2h	2	mk-2-731	mk-2-1,152	115,063,134	144,527,652	2.53	−119.36	5.89	5.90
qPOD2i	2	mk-2-1,157	mk-2-1113	144,789,539	143,268,023	2.97	−30.49	5.11	5.90
qPOD2j	2	mk-2-1,113	mk-2-998	143,268,023	133,848,401	2.81	−120.31	5.75	6.12
qPOD2k	2	mk-2-998	mk-2-1,174	133,848,401	146,341,832	2.77	−119.36	5.99	5.43
qPOD3	3	mk-3-75	mk-3-78	20,191,382	23,118,526	2.61	1.17	4.52	5.60

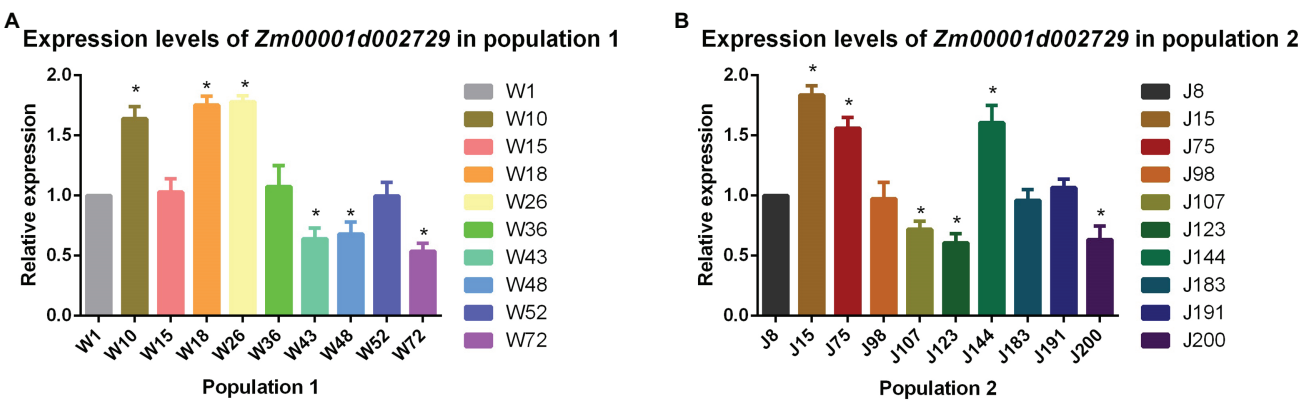

FIGURE 6 | **(A)** Relative expression levels of *Zm00001d002729* in population 1 and **(B)** population 2.

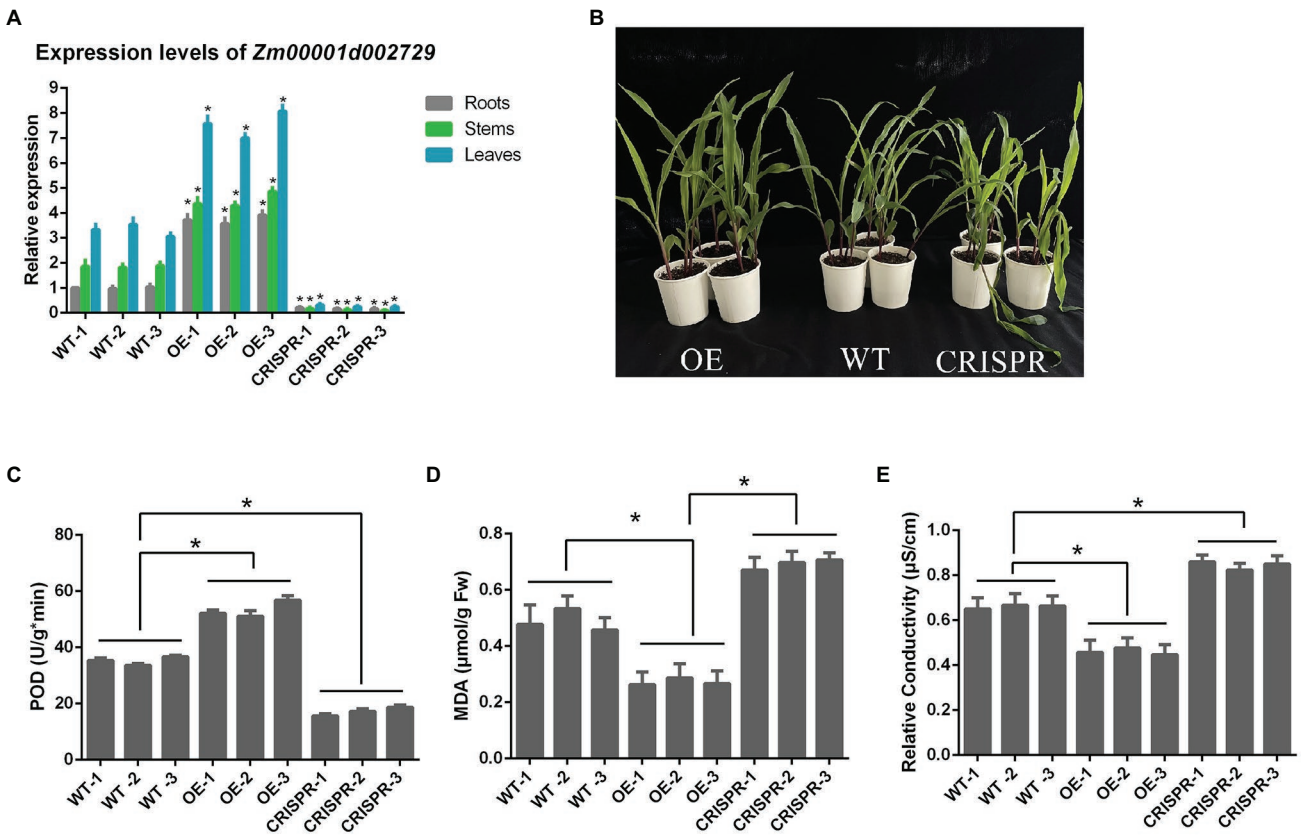

FIGURE 7 | (A) Relative expression levels of *Zm00001d002729* in different maize tissues; **(B)** Comparison of maize morphology after exposure to 6°C cold stress for 24 h; **(C)** POD activity; **(D)** malondialdehyde (MDA) content; and **(E)** relative conductivity. $^{*}p < 0.05$.

DISCUSSION

POD Activity and Maize Cold Resistance

In our study, we demonstrated that cold-tolerant maize had higher POD activity than cold-sensitive maize. The maize antioxidant stress pathway is a known important mechanism underlying cold tolerance (Özlem et al., 2013; Zhang et al., 2020; Yu et al., 2021). Most research on antioxidant enzymes has so far focused on maize catalase, which removes excess H_2O_2 from mitochondria and protects plants from cold stress (Prasad, 1997). Studies had shown that in the early stages of cold stress, POD activity in maize seedlings declines and then rises. With increased time and enhanced intensity of cold stress, POD activity has been shown to increased significantly (Guy et al., 1997; Roncatto and Pascholati, 1998; Šukalović et al., 2010; Ao et al., 2013). POD activity has been shown in previous studies to be higher in cold-tolerant than cold-sensitive varieties (Hodges et al., 1996; Wang et al., 2015), which is consistent with our results.

QTLs Associated With Maize Cold Tolerance

The QTL we found to be associated with cold tolerance was on chromosome 3 in maize, located in bin 3.04. Fracheboud et al. (2002, 2004) studied the effect of low temperature on the photosynthetic mechanism of maize and analyzed five QTLs related to this function; one of these QTLs was located near the mmc0022 marker on chromosome 3, and explained 28% of the phenotypic variation, believed to be related to photosynthetic function at low temperatures in maize. The relevant gene is likely to be located on chromosome 3, close to the centromere; this is very close to bin 3.04. Interestingly, there are previous reports of other biotic or abiotic stress-related QTLs located near the QTL we identified as: Messmer et al. (2009) detected three drought-related stable QTLs located on chromosome 3 bin 3.04, and their contributions of phenotypic variation in this trait were 8.9, 10.8, and 6.3%. Balint-Kurti identified QTLs related to southern leaf blight resistance in two RIL populations (H99 × B73 and B73 × B52), also located in bin 3.04 (Balint-Kurti et al., 2008). Based on a genetic map of 246 polymorphic simple sequence repeat markers with an average genetic distance of 9.1 cM, Ding et al. (2008) used the compound interval mapping method (CIM) for QTL mapping to analyze associations with maize fusarium ear rot under four environments in a RIL population. Two QTLs, both located at bin 3.04 of chromosome 3, were detected in all environments, and these QTLs were located on either side of the markers umc1025 and umc1742, respectively; the QTL we identified in this study was very close to umc1742. Guan et al. (2012) found that a *Ragged leaves 1*

(*Rg1*) maize mutant is susceptible to disease caused by abiotic stress, resulting in the extensive accumulation of H_2O_2 in diseased tissues and lesions on the leaves, sheaths, and bracts of maize. Interestingly, this is similar to the mechanism by which plants are subjected to cold stress (Weig et al., 1997). However, *Rg1* is located on chromosome 3 bin 3.04. In summary, for the QTL we discovered, there were almost no previous reports of association with maize cold tolerance traits, though there were some with tolerance to other stresses. This indicates that we have discovered a new QTL related to maize cold tolerance.

Analysis of *Zm00001d002729* Associated With Maize Cold Tolerance

Functional annotation suggested that *Zm00001d002729* is an acyl-acyl carrier protein thioesterase. We found that overexpression of *Zm00001d002729* increases the cold tolerance of maize, while inhibiting the expression of this gene increases sensitivity to low temperatures. In previous studies, acyl-acyl carrier protein thioesterase is the key enzyme in the regulation of fatty acid carbon chain elongation during fatty acid synthesis. It can catalyze the hydrolysis of thioester bonds and terminate the synthesis of fatty acids. Its activity thus affects the content of various fatty acids in plants (Nieto et al., 2007). However, there are relatively few functional verifications of this activity in plant resistance to stress (Dormann et al., 2000; Wu et al., 2009). In a study by Raffaele et al. (2008), it was shown that mutation in acyl-acyl carrier protein thioesterase in *Arabidopsis thaliana* leads to serious defects in the fatty acid supply for long-chain fatty acid biosynthesis. *Arabidopsis thaliana* also lost its hypersensitive response to pathogen attack. Zhang et al. (2012) found that the overexpression of acyl-acyl carrier protein thioesterase in tobacco improves drought tolerance. The phase transition hypothesis of cold stress damage in plants states that when temperate plants suffer low temperature damage below a certain threshold, the biological membrane will first undergo a phase transformation of membrane lipids, from a liquid crystal phase to a gel phase. The fatty acid chains in the membrane lipids change from a disorderly to an orderly arrangement; the fluidity of the membrane is greatly reduced, and pores or cracks form in the membrane, increasing the permeability of the membrane and changing the structure of membrane-bound proteins. The efflux of a large amount of materials in the membrane destroys the ion balance between the inside and outside of the cell, leading to changes in physiological metabolism and functional disorders in plant cells (Lyons and Raison, 1970). We quoted the expression levels of *Zm00001d002729* from MaizeGDB (**Supplementary File 13**). We found that RNA-seq showed that the expression of target genes in V1 and V3 phases was extremely low in B73 (Winter et al., 2007; Makarevitch et al., 2015; Hoopes et al., 2019). In previous studies, it was found that B73 belong to cold-sensitive inbred line (Joets et al., 2018; Grzybowski et al., 2019; Jonczyk et al., 2020). Therefore, we speculated that the more cold-sensitive maize, the lower the expression levels of *Zm00001d002729*. It is consistent with the conclusions we got before. We do not yet understand the mechanism by which *Zm00001d002729* confers greater cold tolerance, and how this might fit with the phase transition hypothesis; however, our identification of the general function of this gene has laid the foundation for elucidating and exploiting this mechanism through genetic engineering in crops.

In conclusion, we successfully used GWAS and QTL mapping to successfully identify *Zm00001d002729*, a gene on maize chromosome 3 that is significantly related to POD activity. We used genetic transformation to overexpress or inhibit the expression of *Zm00001d002729* in maize, verifying its function as a cold tolerance gene. We have shown for the first time that *Zm00001d002729* positively regulates cold resistance in maize and may be a potentially useful tool to generate cold-resistant crops to ensure food security in the face of climate change.

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 at: https://www.ncbi.nlm.nih.gov/, PRJNA495031 and PRJNA763107.

AUTHOR CONTRIBUTIONS

YJ drafted the original manuscript. ZZ reviewed and edited the manuscript. YX and ZY conducted the experiments and performed the analysis. ZX, SG, and JQ performed the phenotyping. PW conducted formal analysis. RZ supervised the experiments. All authors contributed to the article and approved the submitted version.

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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.2021.776972/full#supplementary-material

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Melatonin Induced Cold Tolerance in Plants: Physiological and Molecular Responses

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Cold stress is one of the most limiting factors for plant growth and development. Cold stress adversely affects plant physiology, molecular and biochemical processes by determining oxidative stress, poor nutrient and water uptake, disorganization of cellular membranes and reduced photosynthetic efficiency. Therefore, to recover impaired plant functions under cold stress, the application of bio-stimulants can be considered a suitable approach. Melatonin (MT) is a critical bio-stimulant that has often shown to enhance plant performance under cold stress. Melatonin application improved plant growth and tolerance to cold stress by maintaining membrane integrity, plant water content, stomatal opening, photosynthetic efficiency, nutrient and water uptake, redox homeostasis, accumulation of osmolytes, hormones and secondary metabolites, and the scavenging of reactive oxygen species (ROS) through improved antioxidant activities and increase in expression of stress-responsive genes. Thus, it is essential to understand the mechanisms of MT induced cold tolerance and identify the diverse research gaps necessitating to be addressed in future research programs. This review discusses MT involvement in the control of various physiological and molecular responses for inducing cold tolerance. We also shed light on engineering MT biosynthesis for improving the cold tolerance in plants. Moreover, we highlighted areas where future research is needed to make MT a vital antioxidant conferring cold tolerance to plants.

Keywords: antioxidants, cold stress, gene expression, melatonin, oxidative stress, photosynthesis

INTRODUCTION

Cold stress is a severe abiotic stress that significantly limits crop growth and productivity, particularly in temperate areas (Aazami et al., 2021; Feng et al., 2021). Cold stress induces severe alterations in plant physiological, biochemical, metabolic and molecular processes, resulting in a significant reduction in crop productivity (Hu et al., 2016; Repkina et al., 2021). The plasma

membrane is considered the first place affected by cold stress (Barrero et al., 2017). Exposure to cold stress substantially alters lipid composition and increases fatty acid saturation (Lado et al., 2016). Low temperature reduces water uptake, and inadequate moisture in aboveground organs leads to drought stress (Aroca et al., 2012; Hussain et al., 2018). In turn, the onset of drought owing to cold stress causes a significant reduction in root growth, nutrient, and water uptake (Nezhadahmadi et al., 2013; Hassan et al., 2017). Low temperature also induces the production of reactive oxygen species (ROS) (Hu et al., 2016; Dey et al., 2021) that damage the proteins, lipids and resultantly inhibit plant growth and, eventually, its productivity (Hassan et al., 2019, 2020, 2021). However, plants have an excellent antioxidant system to cope with ROS under stress conditions (Chattha et al., 2021; Dustgeer et al., 2021; Imran et al., 2021; Seleiman et al., 2021; Sultan et al., 2021). Additionally, plants can neutralize the impact of cold stress by accumulating various osmolytes such as proline, glycine betaine, soluble sugars, and proteins (Erdal, 2012; Ghosh et al., 2021). However, the accumulation of these osmolytes varies depending on crop species and stress conditions (Erdal, 2012). The accumulation of these osmolytes protects the membranes and ensures better growth and production in cold stress (Sun et al., 2020; Ghosh et al., 2021).

Melatonin (*N*-acetyl-5-methoxytryptamine) (MT) is an imperious endogenous molecule that possesses excellent antioxidant properties (Arnao and Hernández-Ruiz, 2015a; Kołodziejczyk et al., 2021). MT is involved in different processes ranging from root growth (Zhang et al., 2014), flower development, fruit ripening, photosynthesis (Tan et al., 2012), leaf senescence (Byeon et al., 2012; Wang et al., 2013), and alleviation of stress-induced oxidative damage (Wang et al., 2014; Arnao and Hernández-Ruiz, 2015b; Shi et al., 2015a; Kołodziejczyk et al., 2021). The application of MT improves antioxidant activities performing ROS scavenging and conferring cold tolerance to plants (Zhang H. et al., 2021). Melatonin improves gene expression, which regulates the antioxidant activities and redox status under cold stress (Wang et al., 2017; Li et al., 2018a). MT reduces cold-induced inhibition in photosynthesis and photosystem-II (PS-II) activities by increasing antioxidant activities (Han et al., 2017). Moreover, MT also improves the cold tolerance by degradation of starch and increasing the electron transport and antioxidant activities (Li et al., 2018b). In recent years, many functions of MT have been identified, among which contributing to stress tolerance. Therefore, in this review we systematically discussed the potential regulatory mechanism of MT to induce cold tolerance. Further, we also focused on the future directions to make MT an essential antioxidant for cold tolerance.

MELATONIN BIOSYNTHESIS IN PLANTS

Tryptophan (TP) is considered a precursor of MT. The conversion of TP into MT involves four enzymatic reactions (**Figure 1**). The first step consists of converting TP into tryptamine by tryptophan decarboxylase (TDC). Tryptamine is converted into serotonin (ST) by the action of an enzyme named tryptamine 5-hydroxylase (T5H) (Posmyk and Janas, 2009). Later on, ST is converted into *N*-acetyl-serotonin by means of *N*-acetyltransferase (SNAT) or arylalkylamine *N*-acetyltransferase (AANAT). Afterward, *N*-acetyl-serotonin is converted into MT by *N*-acetyl-serotonin methyltransferase (ASMT) or hydroxyindole-O-methyltransferase (HIOMT) (Zuo et al., 2014). In parallel to this, ST is also converted by HIOMT into the 5-methoxytryptamine, which in turn is converted by SNAT into the final product, MT (Tan et al., 2016). A recent study also identified the reverse pathway for MT biosynthesis, in which *N*-acetyl-serotonin deacetylase catalyzes *N*-acetyl-serotonin into serotonin (Lee et al., 2017). As a precursor of MT, tryptophan is also a precursor of indole-3-acetic-acid (IAA). Tryptamine pathway is one of the pathways of IAA synthesis, in which TP is converted into tryptamine, and then tryptamine is converted into IAA by indole-3-acetaldehyde (Wang J. et al., 2012; Wang Y. Y. et al., 2012). This similarity explains why MT has effects similar to those of IAA, as it has been reported that MT improves vegetative growth to an extent comparable with IAA (Hernandez-Ruiz et al., 2004).

ENDOGENOUS MELATONIN BIOSYNTHESIS IN PLANTS

Plant chloroplast and mitochondria are considered important sites of MT biosynthesis (Tan et al., 2013). MT biosynthesis has been reported in many plants, including fruit trees, herbs, and crops (Byeon et al., 2012). The levels of MT synthesis in plants are subjected to seasons and circadian rhythms (Beilby et al., 2015). Additionally, MT concentrations vary among plant species, organs, and growth stages (Hernández-Ruiz and Arnao, 2008). For instance, in morning glory, the MT concentration was significantly increased during the maturation period (Van-Tassel et al., 2001). Lastly, environmental conditions significantly affect MT synthesis in plants; for instance, MT concentration was significantly higher in field grown rice compared to the growth chamber (Byeon et al., 2012). Similarly, MT levels were also significantly higher in grapevine plants grown under illumination than under darkness, indicating that light signals induce MT synthesis (Boccalandro et al., 2011). In contrast to this, another source reports that MT synthesis in grapevine was significantly higher during the night compared to the day, which indicates that light inhibits the MT biosynthesis in these species (Arnao and Hernández-Ruiz, 2013b).

ABIOTIC STRESS INDUCED MELATONIN BIOSYNTHESIS IN PLANTS

Melatonin, an excellent antioxidant, interacts with ROS and reduces ROS production and its damaging effects under stress conditions (Arnao and Hernández-Ruiz, 2013a). Therefore, in stress conditions, the increases in MT synthesis is linked with an increase in ROS (Arnao and Hernández-Ruiz, 2013b). The concentration of MT in grapevine and barley was significantly increased in stress conditions, and the level of MT was

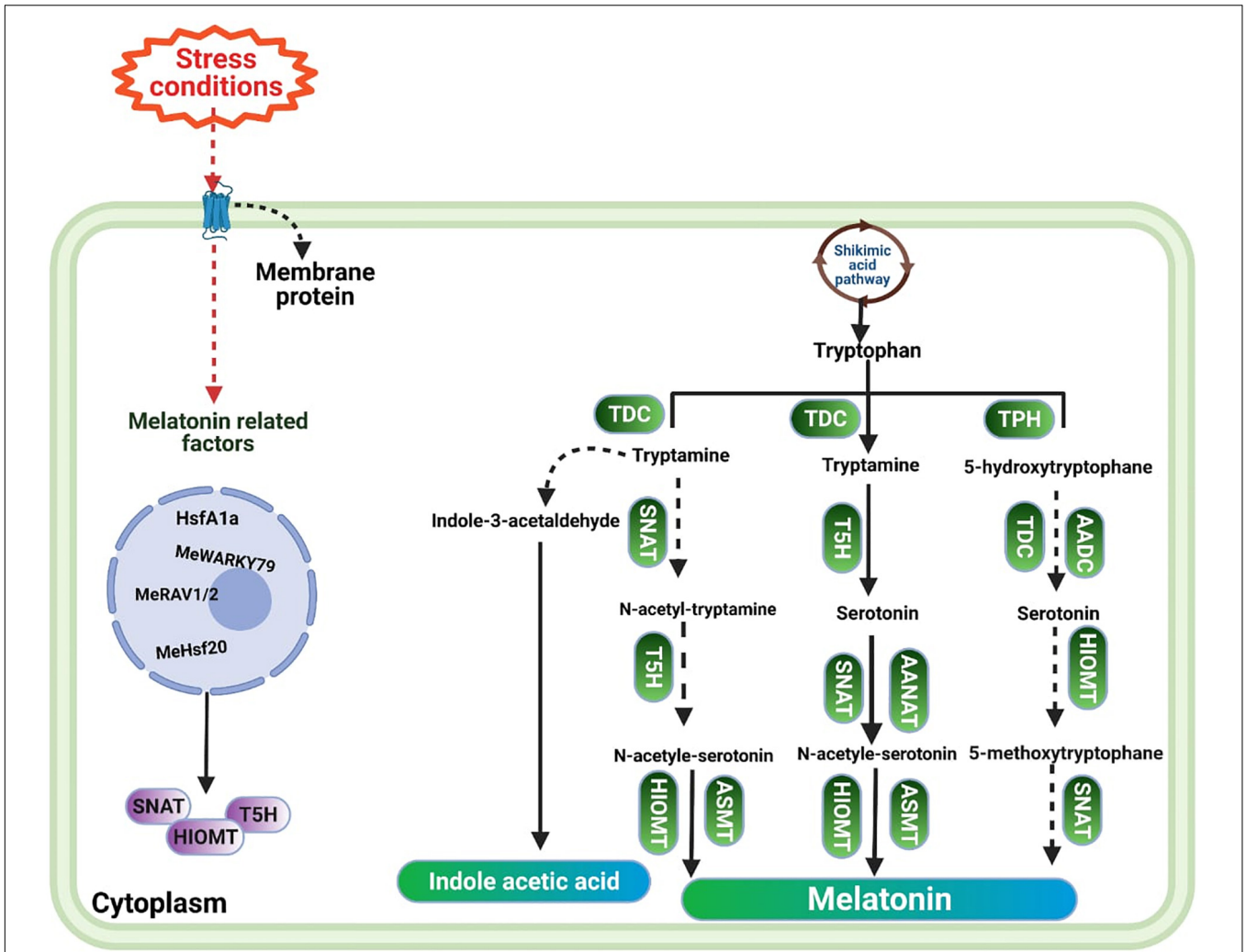

FIGURE 1 | The pathway of melatonin biosynthesis in plants.

further enhanced by increasing the stress intensity (Arnao and Hernández-Ruiz, 2009). Moreover, MT synthesis in rice seedlings was also significantly increased under heat stress (Byeon and Back, 2013). MT biosynthesis considerately increased on exposure to stress, proving that MT plays an imperative role in plants' response to different stresses (Hardeland, 2016).

Melatonin biosynthesis in plants is related to gene expression and enzymatic activities responsible for MT biosynthesis. For instance, an increase in genes expression (TDC: tryptophan decarboxylase: TDC, T5H: tyrosine gene) significantly increased MT synthesis in rice seedlings grown under cadmium stress (Byeon et al., 2015). Moreover, an increase in MT in rice was also linked with SNAT and ASMT under high temperatures (Byeon and Back, 2013). Generally, the concentration of MT in plants is strongly correlated with the availability of its precursors (Byeon et al., 2015), and ST plays a crucial role to improve cold tolerance (Kang et al., 2010). Moreover, a higher level of 2-hydroxymelatonin under cold and drought stress in rice indicates its role in plant resistance to these stresses (Lee and Back, 2016). Additionally, in the tomato crop, the concentration of MT was significantly increased by direct binding of a transcription factor (HsfA1a) to the caffeic acid O-methyltransferase 1 (COMT1) gene promoter under Cd stress (Cai et al., 2017).

EFFECT OF COLD STRESS ON PLANTS

Cold stress induces several morphological alterations in plants and causes a reduction in growth and productivity (Equiza et al., 2001). Cold stress determines leaf chlorosis and wilting, leading to necrosis and stunted growth (Janowiak et al., 2002). Cold stress delayed and reduced wheat germination, reducing stand establishment and final productivity (Jame and Cutforth, 2004). Cold stress limits root proliferation, growth and surface areas (**Figure 2**), leading to a substantial reduction in nutrient and water uptake (Hussain et al., 2018; Kul et al., 2020). The reproductive stage of plant life is also susceptible to cold stress (Thakur et al., 2010). For instance, cold stress causes shedding of

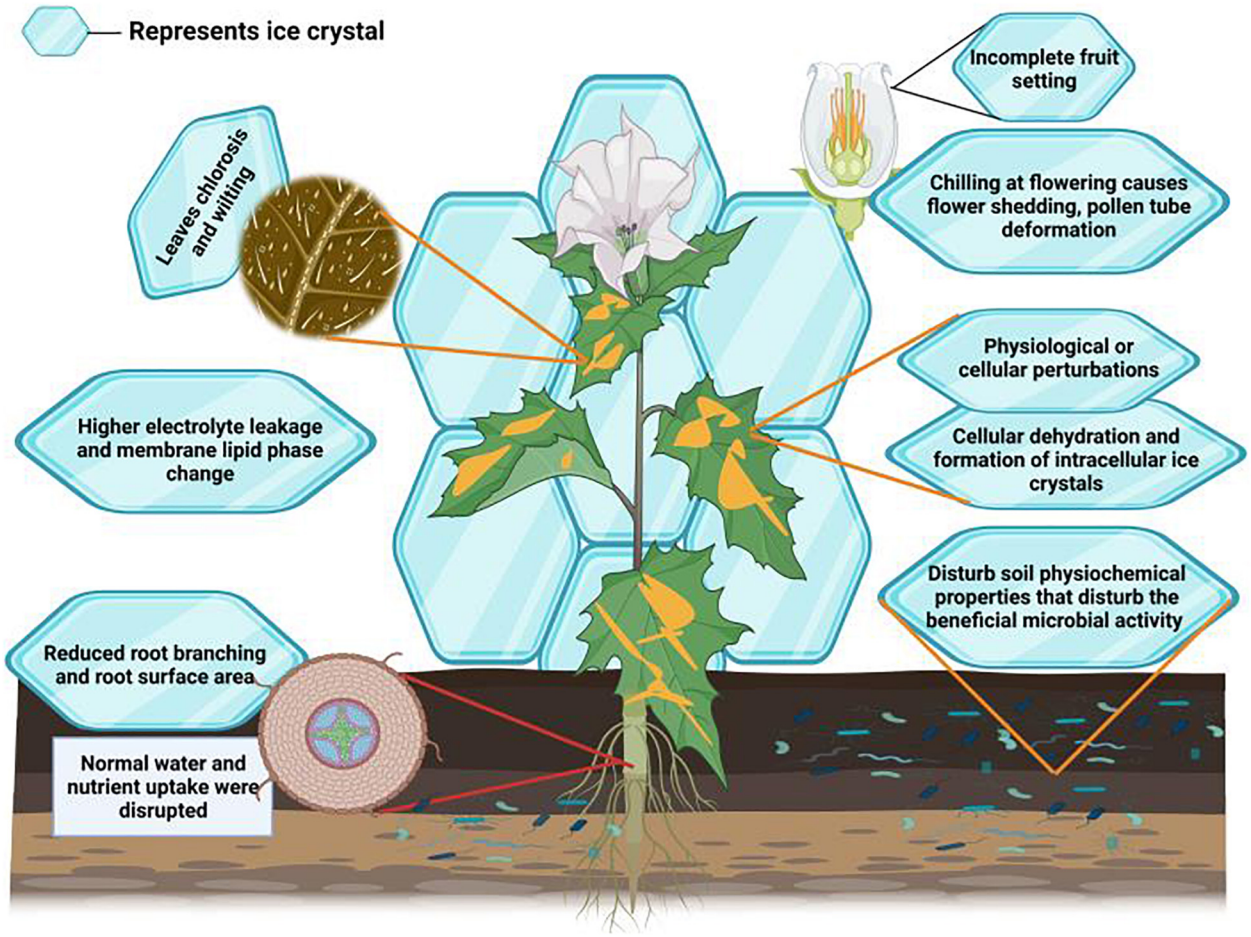

FIGURE 2 | Effect of cold stress on plants. Cold stress induces the formation of crystal which reduces membrane integrity, causes electrolyte leakage and lipid saturation, reduces root growth which in turn decreases the water and nutrient uptake. Moreover, cold stress also causes leaf wilting and chlorosis and disturbs photosynthetic performance and microbial activities, and induces flowering shedding, deformation of pollen tube, incomplete fruit setting and results in significant growth and yield losses.

flowers, deforms pollen tubes (Chakrabarti et al., 2011), induces pollen sterility (Ji et al., 2017), and disrupts grain development (Barton et al., 2014), consequently causing a reduction in final productivity (Hussain et al., 2018).

Cold stress severely alters plant physiological processes. Plants need to maintain membrane stability in stress conditions; however, cold stress reduces membrane stability (**Table 1**) and protein structures (Chen et al., 2018). Cold stress induces the formation of ice crystals in plant tissues (Puhakainen, 2004), which reduces apoplastic water potential and results in the flow of water from cells. Thus, cold stress at the cellular level, often followed by drought stress, seriously reduces growth and productivity (Hassan et al., 2021). This onset of drought stress reduces root growth (**Table 1**), root hydraulic conductivity and turgidity of plant leaves (Siddique et al., 2000). Resultantly, plant water and nutrient uptake and carbohydrate metabolism are seriously disrupted, involving significant yield losses (Hassan et al., 2021). Besides this, lower temperature also disturbs soil microbial activities, affecting plant nutrient relationships (Massenssini et al., 2015). Ice crystals' formation also increases electrolyte leakage and causes lipid peroxidation (Hassan et al., 2021). Ice crystals can also puncture the cells, resulting in cytosol outflow and causing plant death (Zhang F. et al., 2011; Demidchik et al., 2014; Sun et al., 2019). Thus, preventing the formation of ice crystals is considered an essential cold tolerance mechanism in plants. Plants accumulate various cryoprotective polypeptides (e.g., COR15a) and osmolytes to cope with the damaging effects of cold stress (Ritonga and Chen, 2020).

Photosynthesis is a major source of grain production, and this process is seriously affected by cold stress (Rinalducci et al., 2011; Khan et al., 2017). Cold stress causes the over-excitation of PS-II, which increases the energy loss by non-radiative reactions (Venzhik et al., 2011; Cvetkovic et al., 2017). Further, cold stress reduces chlorophyll synthesis, photosynthetic efficiency, Rubisco activity, electron transport, stomatal conductivity, which reduce the assimilates production and cause severe yield losses (Bota et al., 2004; Yamori et al., 2009; Hussain et al., 2018). Moreover, cold stress also damages mitochondria's structure,

disturbs enzymatic activities, and slows down the flow of kinetic energy, consequently diminishing the respiration rate (Ikkonen et al., 2020).

Reactive oxygen species increase under cold stress improved the oxygenation response in plant chloroplast and increased glycolate accumulation. This glycolate is converted to glyoxylate in plant peroxisomes, accompanied by accumulation of hydrogen peroxide (H_2O_2) (Hassan et al., 2021). However, plants have an excellent antioxidant defense system to scavenge these ROS (Ritonga and Chen, 2020). The response of plants to cold stress consists of different steps, including dictation of stress followed by signal perception, transduction, and increased expression of stress-responsive genes (Ganeshan et al., 2008). Many genes have been identified in plants that initiate a cascade of transcriptional, biochemical, and physiological processes crucial to chilling tolerance (Kosová et al., 2008). Moreover, plants accumulate various osmolytes, reduce water content, scavenge ROS and maintain carbon metabolism to counter the effects of cold stress (Ruelland and Zachowski, 2010; Thakur and Nayyar, 2013; Hassan et al., 2021). Plants also accumulate various soluble sugars that stabilize the cellular membrane on exposure to cold stress (Yokota et al., 2015). Moreover, the accumulation of osmolytes and sugars also decrease the ROS and malondialdehyde (MDA) contents under cold stress by improving catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) activities (Sun et al., 2019).

MELATONIN IMPROVES GROWTH AND YIELD UNDER COLD STRESS

Cold stress is a severe abiotic stress that substantially limits crop productivity by imposing serious alterations in plant physiological and metabolic processes, and hormonal imbalance, nutritional disorders, poor photosynthetic efficiency, and production of ROS (Turk and Genisel, 2020). MT is an major signaling molecule that promotes plant growth (**Table 2**) and development, and protects against abiotic stresses (Posmyk and Janas, 2009). Low temperature inhibits plant growth and development, in response to which, MT possesses excellent potential to counter cold influence (**Table 2**; Bajwa et al., 2014). Cold stress induces reduction in photosynthetic pigments; however, MT application (100 μM) significantly increases the synthesis of photosynthetic pigments, and therefore maintains plant growth under cold stress (Han et al., 2017). Cold stress induces a marked increase in MDA accumulation, lipid peroxidation and electrolyte leakage (Hulya et al., 2014; Hu et al., 2016). However, MT supplementation was shown to markedly reduce MDA accumulation and ROS deleterious impact on cellular membranes of rice seedlings, which in turn resulted in appreciably improved plant growth under cold stress (Han et al., 2017).

In the same experiment (Han et al., 2017), MT reduced the cold-induced inhibition in plant photosynthetic activities, and protected the photosynthetic apparatus by improving the antioxidant activities; all this determined better plant performance under cold stress. However, MT mediated improvement in plant growth largely depends on methods and rate of MT application under cold stress (Han et al., 2017). MT supplementation maintains higher Fv/Fm and plant water relationships, while it reduces MDA and H_2O_2 by improving antioxidant activities (**Table 2**: ascorbate peroxidase: APX, CAT, POD, and SOD), enhancing plants tolerance to cold stress (Li et al., 2018a). MT supplementation also protects the photosynthetic machinery, maintains the redox homeostasis, and enhances gene expression so as to mitigate the deleterious impacts of cold stress and improve plant growth (Li et al., 2018a). Exogenous application of MT improved plant defense to counter the harmful effects of cold stress in Bermudagrass (Fan et al., 2015). MT application also improved osmolyte accumulation, nutrient and water uptake, hormonal accumulation and enzymatic activities, which countered the effects of cold stress by strengthening the anti-oxidant defense system and improving plant growth (Irshad et al., 2021). In addition, MT supplementation also improved carbon assimilation, osmotic potential, enhanced plant water content and photosynthetic efficiency, resulting in substantial growth improvement and unconstrained development under freezing temperature (Irshad et al., 2021).

MELATONIN MAINTAINS MEMBRANE STABILITY AND IMPROVES PLANT WATER RELATIONS UNDER COLD STRESS

Membrane stability is a major damage in plants caused due to cold stress. Cold stress decreases membrane fluidity and changes the balance between transpiration and water uptake, and cause water dehydration in plant shoots (Turk et al., 2014). Eventually, it also affects the stomata movements and substantially decreases the photosynthetic rate (Hassan et al., 2021). However, MT application protects membranes and improves membrane stability by scavenging ROS through enhanced antioxidant activities (Turk et al., 2014). The foliar and seed priming with MT appreciably improved the membrane stability linked with reduced MDA and H_2O_2 (**Table 3**) accumulation (Sun et al., 2018). The increase in membrane stability reduced the EL and loss of osmolytes and conferred the cold tolerance in plants (Sun et al., 2018). Additionally, exogenous MT also improved the enzymatic and non-enzymatic antioxidant activities, maintaining the membrane integrity and conferring cold tolerance with corresponding lower electrolyte leakage (EL), MDA, and H_2O_2 accumulation (**Table 3**; Fan et al., 2015). The regulation of plant water relationships is plants are linked with plants adaptation to cold stress (Turk et al., 2014). Cold stress significantly decreased the plant relative water contents (RWC); however, MT application reduces the negative impacts of cold stress and maintains higher RWC (Pu et al., 2021). The larger leaf surface area with MT treatment may be associated with improved water contents under cold stress (Turk et al., 2014). Moreover, MT also protects the plant membranes,

TABLE 1 | Effect of cold stress on growth, physiological attributes, and anti-oxidant activities.

Crop	Stress conditions	Effects	References
Soybean	17/13°C DNT	Cold stress reduced the plant height, nodes production, stem biomass, pods production, biomass, and seed production.	Staniak et al., 2021
Maize	4°C	Chilling stress reduced the seedling growth, biomass production, RWC, and increased the MDA contents membrane permeability, proline accumulation, and APX, CAT, POD, and SOD activities.	Zhang Q. et al., 2021
Wheat	6°C	Cold stress reduced the root and shoot growth and biomass production, and increased the MDA and H_2O_2 accumulation, proline, glycine betaine accumulation, and EL.	Golizadeh and Kumleh, 2019
Stevia	5°C	Cold stress reduced efficiency of PS-II, chlorophyll contents, photosynthetic rate, and water use efficiency.	Hajihashemi et al., 2018
Chickpea	4°C	Cold stress increased EL, MDA, and H_2O_2 accumulation. However, cold stress also resulted in increase in activities of APX, CAT, and SOD.	Karami et al., 2018
Sunflower	−3°C	Cold stress increased the EL, reduced the chlorophyll fluorescence, osmotic potential of sunflower plants.	Helena et al., 2017
Sugarcane	4°C	Cold stress reduced the root growth, root biomass, root vigor, activities POD and SOD, MDA, proline, and soluble sugars accumulation.	Sun et al., 2017
Barley	−8°C	The cold stress increased the lipid per-oxidation, MDA and H_2O_2 accumulation, CAP and POD activities and decreased the membrane stability.	Valizadeh et al., 2018
Wheat	4°C	Cold stress reduced the leaf moisture contents, RWC, dry matter contents, photosynthetic, and transpiration rates of wheat crop.	Bibi et al., 2017

DNT, day/night temperature.

TABLE 2 | Effect of melatonin application on growth and physiological and molecular attributes under cold stress.

Crop	Cold stress	MT application	Effects	References
Barley	5°C	1 μM	MT supplementation increases the germination, seedling growth, endogenous MT concentration, chlorophyll and caroteniod contents, proline and soluble proteins accumulation, and expression of HvCCA1 and HvTOC1 genes.	Chang T. et al., 2021
Watermelon	4°C	150 μM	The application of MT improves the endogenous MT contents and accumulation of MeJA, chlorophyll fluoresces, expression of ClCBF1 and ClCBF2 genes.	Li et al., 2021
Pepper	25/20°C DNT	5 μM	MT foliar spray improves the leaf area, photosynthetic rate, stomatal conductance, biomass production, water potential, proline contents, and fruit yield.	Korkmaz et al., 2021
Pistachio	25/20°C DNT	0.5 μM	MT supplementation improves the growth, chlorophyll and caroteniod and phenolic contents, carbohydrate, proline, and GABA accumulation.	Barand et al., 2020
Wheat	20°C	1 μM	MT treatment improves the plant biomass production, root/shoot ratio, nitrogen uptake and activities of nitrate reductase and glutamine synthetase.	Qiao et al., 2019
Wheat	10/4°C DNT	1 mM	MT application improves the stomatal conductance, photosynthetic efficiency and expression of Cu/Zn SOD to confer cold tolerance.	Sun et al., 2018
Tea	25/20°C DNT	500 μM	MT foliar spray increases the photosynthetic rate, efficiency of PS-II, chlorophyll contents and expression of stress proteins.	Li et al., 2018a
Bermudagrass	4°C	100 μM	MT supplementation increases the chlorophyll fluoresce and endogenous MT contents to confer the cold tolerance.	Hu et al., 2016
Bermudagrass	4°C	100 μM	MT treatment increases the chlorophyll contents, chlorophyll fluoresce, endogenous MT contents.	Fan et al., 2015
Maize	27/25°C DNT	1 mM	MT application improves the growth, chlorophyll contents, RWC and increased the concentration of Fe, Mg, K, S, B, and Zn.	Turk and Erdal, 2015

DNT, day/night temperature.

reducing water loss and maintaining the higher RWC under cold stress (Turk et al., 2014).

MELATONIN IMPROVES WATER AND NUTRIENT UPTAKE UNDER COLD STRESS

The potential water reduction is considered the fastest effect of chilling stress. Cold stress diminishes the water influx through plants roots due to increased water viscosity and a decrease in membrane fluidity, which reduces the cell turgor pressure (Turk et al., 2014). However, MT application improves the plant water uptake under cold stress, which indicates that MT can reduce the negative impacts of cold stress (Turk et al., 2014; Hussain et al., 2018). Exogenous MT application increases the vapor pressure deficit between the plant leaf surface and atmosphere, enabling the plant roots to improve the water uptake (Pu et al., 2021).

Cold stress alters membrane structure by disturbing various physiological and biochemical properties, disturbing multiple processes, including nutrient and water uptake (Nayyar et al., 2005). Optimum nutrient uptake and transportation is necessary

for plants to maintain their physiological processes and structural integrity under cold stress (Dumlupinar et al., 2011). MT application significantly improved the nutrient uptake under cold stress. Likewise, MT application causes a significant increase in calcium (Ca) uptake under cold stress, which shows that MT achieved its protective role on membranes under cold by increasing the Ca uptake. Moreover, increased Ca uptake following MT application protects the membranes and reduces electrolyte leakage and MDA accumulation under cold stress (Turk and Erdal, 2015). MT application also maintained higher uptake of potassium (K), phosphorus (P), sulfar (S), boron (B), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), and zinc (Zn), which improved the plant performance and confer the cold tolerance (Turk and Erdal, 2015).

Cold stress also decreased the Mg uptake which in turn decreased the chlorophyll synthesis owing to fact Mg is important constituent of chlorophyll. However, MT treatment improves the Mg uptake and ensures the better chlorophyll synthesis and subsequent photosynthetic performance under cold stress (Turk and Erdal, 2015). MT application also improved the N uptake and maintained higher N contents in plant shoot under cold stress. The increase in N uptake following MT application is attributed to higher activities of nitrate reductase (NR) and glutamine synthetase (GS) and resultantly improve the plant growth and productivity (Qiao et al., 2019). Cold stress substantially reduced the NPK however, MT application improved the NPK under cold stress (Irshad et al., 2021). Cold stress reduced the N uptake by reducing the root activities (Feng et al., 2011) nonetheless, exogenous MT application upregulates nutrient uptake by increasing root activity and enzymatic activities under cold stress (Turk and Erdal, 2015; Irshad et al., 2021).

MELATONIN IMPROVES HORMONES AND OSMO-LYTES ACCUMULATION TO CONFER COLD TOLERANCE

Osmo-lytes accumulation is one of the most important mechanisms used by plants to improve the stress tolerance (Hassan et al., 2021). The formation of viscous among cells is imperious to improve the cold tolerance; however, this formation largely depends on carbohydrate contents. The application of MT improved the carbohydrate contents which in turn improve the cold tolerance in plants (Sarropoulou et al., 2012; Turk et al., 2014). Amino acids and proteins also play an imperative role in plants tolerance to cold stress. The application MT substantially increased the MT accumulation in plants which in turns improve the plant anti-oxidant performance and confer the cold tolerance (Turk et al., 2014). Melatonin application also maintained higher proline contents under cold stress that keeps cell water contents, maintain membrane stability and increases the anti-oxidant activities to confer cold stress in plants (Turk et al., 2014). The application of MT appreciably improved the synthesis of proline enzymes including the 1-pyrroline-5-carboxylate syntheses (P5CS) and ornithine aminotransferase (OAT) which in turn improve the proline synthesis under cold stress and confer the cold tolerance were (Madebo et al., 2021). Melatonin application also improved the endogenous MT, glycine betaine and soluble sugars accumulation and resulting in substantial increase in anti-oxidant activities and subsequently in cold stress (Irshad et al., 2021).

Different hormones including, auxins (IAA), abscisic acid (ABA), gibberellins (GA3), and cytokinins (CK) play a significant role in chilling tolerance (Khan et al., 2017). The response of plants to various stresses are depends on the cross talk among the hormonal signaling pathways (Verma et al., 2016). The exogenous MT supplementation improved the IAA and GA3 concentration while MT application reduced the ABA accumulation under cold stress (Pu et al., 2021). ABA induces stomata closing and reduced the photosynthetic rate under cold stress (Lata and Prasad, 2011). Cold stress significantly increases the ABA contents (Zhang et al., 2014), however, MT application markedly reduced the ABA accumulation in cold stress (Zhao et al., 2016). The reduction in ABA accumulation under cold stress is attributed to re-opening of stomata following MT application (Pu et al., 2021). Melatonin application also induced significant increase in methyl jasmonate (MeJA) that leads to an increase in H_2O_2 accumulation and cold tolerance (Li et al., 2021). Nitric oxide (NO) maintains cellular homeostasis under stress conditions by repairing the stress induced oxidative damages (Zhao et al., 2007; Kaya et al., 2020). The increase in NO following MT initiate the signaling processes involved in maintenance of cellular redox homeostasis that neutralize the adverse impacts of ROS and provide NO induced defense against oxidative by improving anti-oxidant activities, carotenoid contents and electron transport under cold stress (Irshad et al., 2021).

MELATONIN IMPROVES PHOTOSYNTHETIC PERFORMANCE UNDER COLD STRESS

Photosynthesis is an imperative physiological process that occurs in plants and it is considered as a basis of biological world, however, this process is considered to be very sensitive to cold stress (Dalal and Tripathy, 2012). Cold stress decreases the plant photosynthetic pigments, destroy chloroplasts structure, close stomata, and decreases photosynthetic rate and stomata conductance (Fan et al., 2015; Cai et al., 2016; Han et al., 2017). Melatonin supply alleviate the cold induce inhibition in plant photosynthetic efficiency, maintain lower non-photochemical quenching (NPQ) and protect the photosynthetic apparatus from cold stress (Han et al., 2017). MT application also improves the chlorophyll synthesis (**Figure 3**) by improving the anti-oxidant activities and protecting the photosynthetic apparatus (Irshad et al., 2021). MT supplementation improved the endogenous MT contents that decreased the expression of oxygenase (PAO) gene that is involved in chlorophyll degradation and senescence-related hexokinase-1 (HXK1) gene (Wang et al., 2013; Weeda et al., 2014). MT supply also improved the stomata conductance and improve the plant photosynthetic efficiency by increasing the carbon dioxide (CO_2) absorption (Zhong et al., 2020).

TABLE 3 | Effect of melatonin supplementation on different oxidative stress markers under cold stress.

Crop	Cold stress	MT application	Effects	References
Common sage	20/15°C DNT	200 μM	MT application significantly reduced the MDA and H_2O_2 accumulation under cold stress.	Bidabadi et al., 2020
Sapota fruit	8°C	90 μM	MT application decreased electrolyte leakage, MDA contents, and production of H_2O_2, and O^{2-}.	Mirshekari et al., 2020
Tea	4°C	100 μM	MT application reduced the H_2O_2, and O^{2-} and MDA accumulation under cold stress.	Li et al., 2019
Peach	4°C	100 μM	MT foliar spray reduced the MDA accumulation and production of H_2O_2, and O^{2-}.	Cao et al., 2018
Tomato	5°C	200 μM	MT supplementation reduced the chilling injury, ion leakage, MDA accumulation and H_2O_2, and O^{2-}production.	Azadshahraki et al., 2018
Tomato	4°C	100 μM	Exogenous MT reduced the electrolyte leakage, MDA H_2O_2, and O^{2-} accumulation under cold stress.	Ding et al., 2017
Pepper	15°C	25 μM	MT improved membrane stability and reduced the MDA and H_2O_2 accumulation.	Korkmaz et al., 2017
Melon	12/6°C DNT	400 μM	MT application reduced the MDA contents and ROS production.	Zhang et al., 2017
Bermudagrass	4°C	100 μM	Foliar MT supplementation reduced the electrolyte leakage, MDA accumulation and ROS production.	Shi et al., 2015a

DNT, day night temperature.

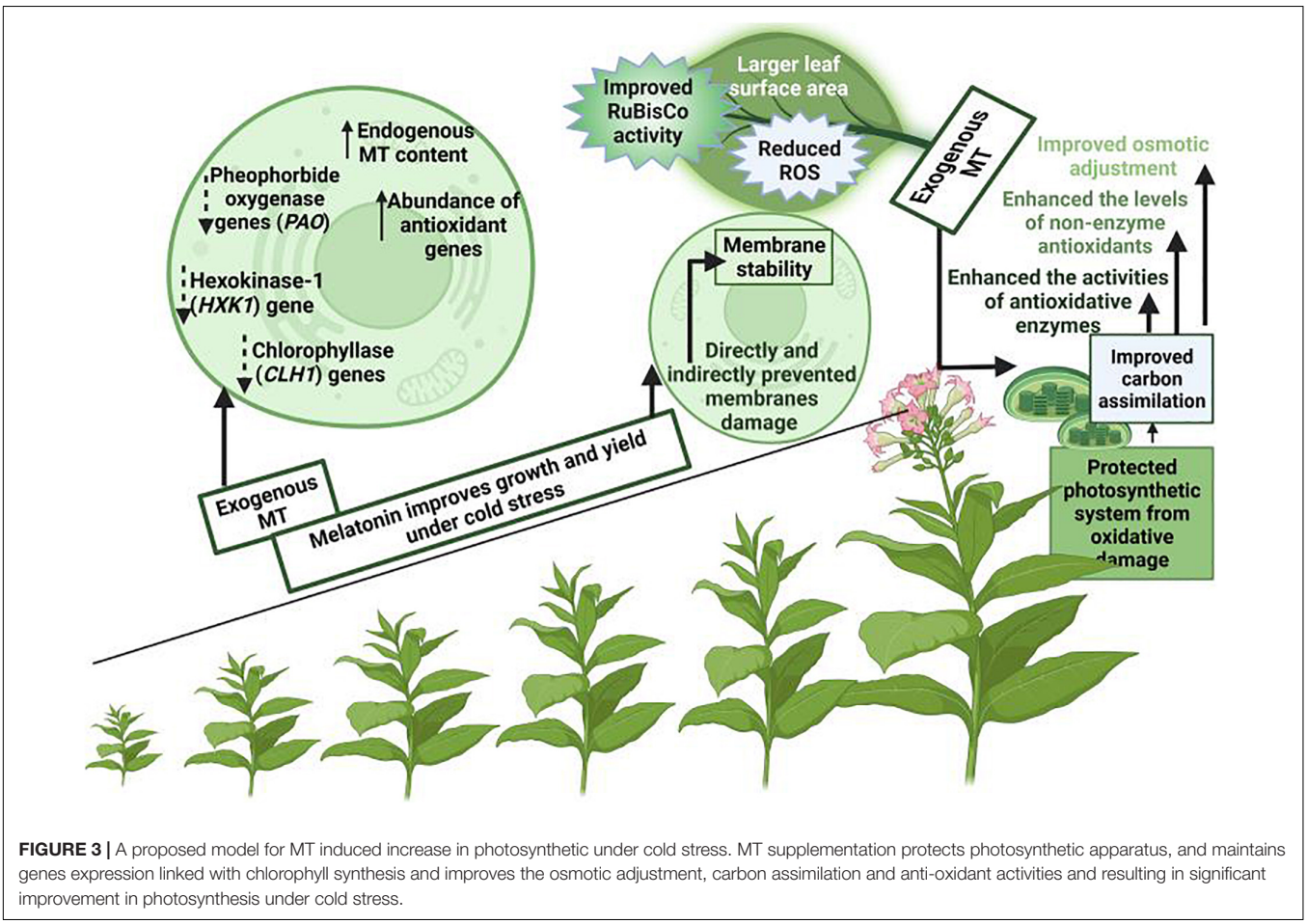

FIGURE 3 | A proposed model for MT induced increase in photosynthetic under cold stress. MT supplementation protects photosynthetic apparatus, and maintains genes expression linked with chlorophyll synthesis and improves the osmotic adjustment, carbon assimilation and anti-oxidant activities and resulting in significant improvement in photosynthesis under cold stress.

Cold stress decreases the activities of enzymes involved in photosynthesis and RuBisCo is considered as a most important enzyme of photosynthetic process (Turk et al., 2014). Cold stress inhibited RuBisCo activity, however; MT maintained the higher RuBisCo activity and improves the photosynthetic efficiency under cold stress owing to reduced ROS production (Turk et al., 2014). The improvement in photosynthetic efficiency with MT application under cold stress is attributed to decreased ROS production, increase in light perception and RuBisCo activity (Turk et al., 2014; Erland et al., 2018; Yang et al., 2018). Additionally, MT also protects the chlorophyll degradation addition, and delays the leaf senesces

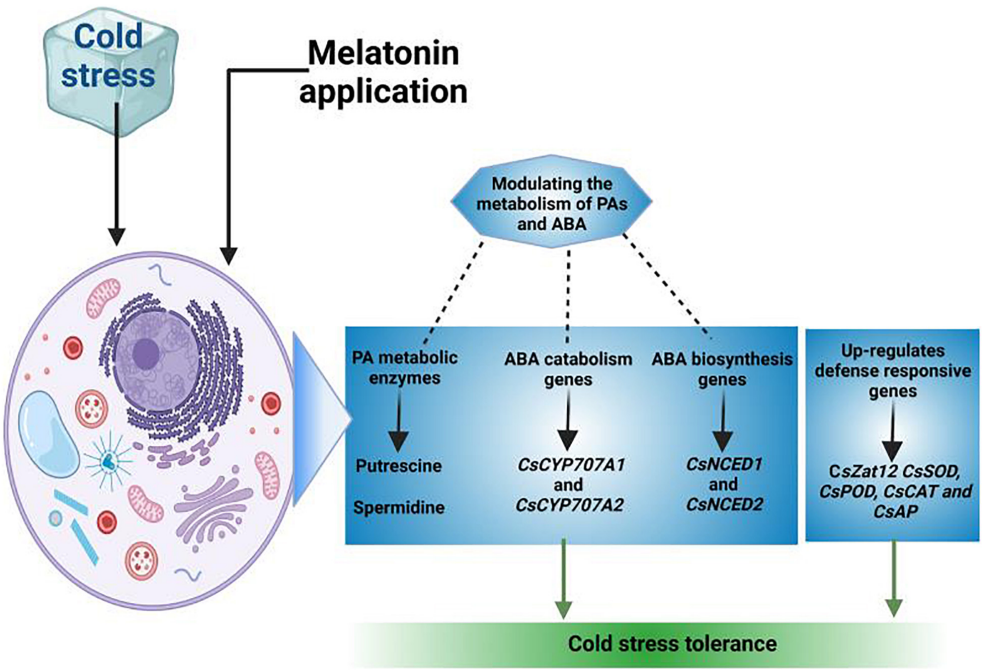

FIGURE 4 | A proposed model of MT medicated polyamines accumulation for improving cold tolerance in plants. MT application upregulates genes expression linked with polyamines accumulation and genes linked with ABA synthesis and ABA catabolism. The application of MT improves polyamines accumulation and increase the genes expression to for catabolism of ABA and resulting in significant improvement in photosynthetic efficiency and cold tolerance in plants.

which also leads to marked improvement in photosynthetic efficiency under cold stress (Han et al., 2017; Ye et al., 2020).

The exogenous application of MT also maintains the higher Fv/Fm and reduced the MDA and H_2O_2 accumulation which favors an increase in photosynthetic efficiency (Tan et al., 2012; Bajwa et al., 2014; Li et al., 2018a). The effect of MT on photosynthesis is concentration dependent. Since the endogenous MT varies among the species, therefore, different concentrations of exogenous MT may exert different effects on plant photosynthetic efficiency (Lazar et al., 2013). The photo-inhibition of photosystem-I (PS-I) is considered to be more dangerous as compared to PS-II, however, MT application protect the thylakoid membranes and recover the photo-inhibition of PS-1 and PS-II and maintain the higher photosynthetic efficiency under cold stress (Yang et al., 2018).

MELATONIN IMPROVES ACCUMULATION OF SECONDARY METABOLITES IN COLD STRESS

Phenolic compounds possess excellent anti-oxidant properties and they accumulate in plants in response to different stress conditions (Agati et al., 2007). Cold stress increased the levels of phenolic compounds while exogenous application of MT further enhanced the phenolic contents to confer the cold tolerance (Szafranska et al., 2012; Turk et al., 2014). Polyamines maintain enzymatic activities, membrane integrity and protein structures by scavenging ROS and phospholipid binding capacity (Aghdam et al., 2019). MT application improves the cold stress defense mechanism by increasing the concentration of polyamines (**Figure 4**) (Put, Spd, and Spm) (Cao et al., 2016). Moreover, increased expression of LeARG1 and LeARG2 encoding arginase genes, arginine decarboxylase (LeADC) and ornithine decarboxylase (LeODC) improved the chilling tolerance in plants (Zhang X. et al., 2011). Additionally, MT pre-treatments increased the accumulation of spermine, spermidine, and putrescine by regulating the S-adenosylmethionine decarboxylase (SAMDC) and tranglutaminase (TGase) activities and resulting in increase in cold tolerance (Du et al., 2021).

The increase in zinc finger protein (Zat12) gene expression involved in putrescine accumulation is also upregulated by expression of ADC1 and ADC2 genes following MT application that improved the cold tolerance in plants (Zhao et al., 2017). Moreover, MT supplementation also increased the enzymatic activities and encoding genes (CsADC and CsODC) expression level which in turn improved the polyamines accumulation and improved the cold tolerance by increasing anti-oxidant activities (Madebo et al., 2021). Gamma-aminobutyric acid (GABA) is a non-protein amino acid which is found in most of organisms (Madebo et al., 2021). The MT treatment improved the upregulation of PpGAD expression and increases ascertain of GABA in chilling stress (Cao et al., 2016). The increase in GABA accumulation following MT application serves as H_2O_2 scavenger which protects the membranes and improved

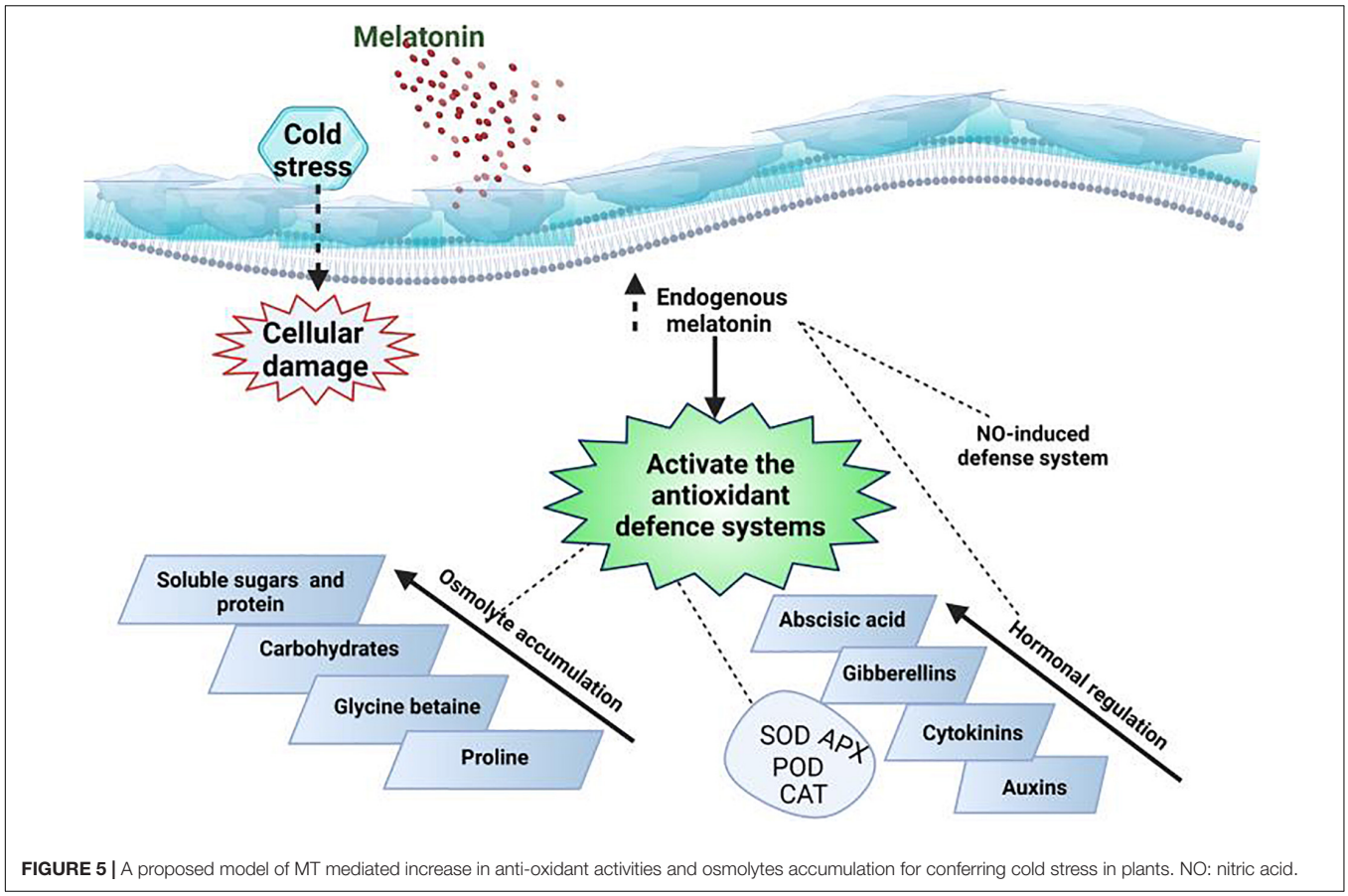

FIGURE 5 | A proposed model of MT mediated increase in anti-oxidant activities and osmolytes accumulation for conferring cold stress in plants. NO: nitric acid.

the plant performance under cold stress (Cao et al., 2016; Wang et al., 2016).

MELATONIN STRENGTHENS ANTIOXIDANT DEFENSE ACTIVITIES TO CONFER COLD TOLERANCE

Cold stress induces certain changes in plant anti-oxidant activities and these alterations are considered as mechanisms to alleviate the adverse impacts of ROS (Barand et al., 2020). MT application scavenges the ROS directly or indirectly by raising the activities of anti-oxidant (**Figure 5**) enzymes (Li et al., 2012; Arnao and Hernández-Ruiz, 2015a). APX, CAT and glutathione peroxidase (GPX) are considered as (**Table 4**) essential enzymes responsible for breaking the H_2O_2 into H_2O in plant cells (Mittler et al., 2004; Aamer et al., 2018). MT application enhances the activities of aforementioned enzymes and counters the deleterious impacts of various abiotic stresses (Fan et al., 2015). MT application appreciably increased the APX, CAT, POD, and SOD (**Table 4**) activities which is attributed to drop in leaf temperature and increase in ROS accumulation in plant leaves (Li et al., 2018c). The reduction in ROS production by MT improved the plant performance under cold stress (Li et al., 2018c). MT seed priming and foliar application upregulated the APX and SOD activities which reduced the ROS production and conferred the cold tolerance in barley (Li et al., 2016). The exogenous MT application increased the expression of anti-oxidant genes including Cu/Zn-SOD and Fe-SOD that increased the SOD activities and improved the cold tolerance in MT treated plants (Sun et al., 2018).

Melatonin also induced the accumulation of anti-oxidant metabolism-related proteins and increases the potential of anti-oxidant system to scavenge the ROS under cold stress (Tan et al., 2012; Turk et al., 2014; Shi et al., 2015b). MT application also increases the stress tolerance in different plant species by inducing H_2O_2 as defense signaling (Shi et al., 2015b; Li et al., 2016). Plants also have to maintain optimum cellular redox homeostasis to continue normal functioning under stress conditions (Kocsy et al., 2001). Glutathione being a redox active compound maintains cellular homeostasis by affecting the different biological pathways and maintain plant performance under cold stress (Suzuki et al., 2012). MT pretreatment maintains higher GSH:GSSG ratio and reduce the ROS production under cold stress (Li et al., 2018a). Moreover, MT application also improved the AsA activity in cold stress, additionally, MT also improved GSH content by improving the activity of c-glutamylcysteine enzymes involved in glutathione (GSH) synthesis (Xu et al., 2010; Li et al., 2018a). All these findings finding indicated that MT application upregulates the activities both enzymatic and non-enzymatic anti-oxidant to confer cold tolerance in plants.

TABLE 4 | Effect of melatonin application on enzymatic and non-enzymatic activities under cold stress.

Plant species	Stress conditions	MT application	Impact on anti-oxidant activities	References
Maize	5°C	50 μM	↑ APX, CAT, GPX, and SOD	Kołodziejczyk et al., 2021
Wheat	12°C	2000 μM	↑ APX and SOD	Zhang H. et al., 2021
Pepper	10/5°C DNT	5 μM	↑ CAT, POD, and SOD	Korkmaz et al., 2021
Litchi Fruit	4°C	0.5 mM	↑ APX, CAT, DHAR, MDHAR, GR, POD, and SOD	Liu et al., 2021
Soybean	12°C	5 μM	↑ APX, CAT, POD, and SOD	Bawa et al., 2020
Pepper	20°C	100 μM	↑ APX, CAT, POD, and SOD	Kong et al., 2020
Tomato	4°C	100 μM	↑ AsA, CAT, GSH, and SOD	Wang et al., 2020
Maize	13°C	100 μM	↑ APX, CAT, POD, and SOD	Cao et al., 2019
Wheat	2°C	1 mM	↑ APX, CAT, and SOD	Sun et al., 2018
Tea	−5°C	500 μM	↑ APX, CAT, GR, and SOD	Li et al., 2018b
Tomato	25/20°C DNT	100 μM	↑ APX, CAT, GSH, POD, and SOD	Ding et al., 2017
Bermudagrass	4°C	1 mM	↑ APX, GPX, GST, and POD and SOD	Hu et al., 2016
Cucumber	15/8°C DNT	200 μM	↑ APX, AsA, GR, and MDHAR	Zhao et al., 2016
Cucumber	10°C	500 μM	↑ CAT, POD, GR, and SOD	Marta et al., 2016
Wheat	5/2°C DNT	1 mM	↑ APX, CAT, GPX, GR, and SOD	Turk et al., 2014

DNT, day night temperature.

MELATONIN INCREASES THE EXPRESSION OF STRESS RESPONSIVE GENES TO CONFER COLD TOLERANCE

The increase in genes expression plays an imperious role to mitigate the adverse impacts of cold stress. MT treatment appreciably improved the genes expression and improved the plant tolerance to cold stress. Likewise, MT seed treatment and foliar spray considerably increased the expression of Cu/Zn SOD, Fe/SOD gene and CAT genes which in turn improves overall plant performance and anti-oxidant activities under cold stress (Sun et al., 2018). MT markedly upregulate the expression of C-repeat-binding factors (CBFs)/drought response element binding factors (DREBs) and different cold responsive genes (COR15a and CAMTA1) and anti-oxidant genes (ZAT10 and ZAT12) that contributes to improved growth and cold tolerance in plants (Bajwa et al., 2014). MT application increased the IAA and jasmonic acid levels, however, it decreased the ABA concentration in cold stress. This indicates that MT works synergistically with IAA and jasmonic acid (JA) and anta-agonistically with ABA to regulate the plant responses to cold stress (Chang et al., 2020).

Cold stress also increased the expression of fatty acid desaturase (FAD2), conversely MT treatment lower the FAD2 expression and consequently reduced the lipid per-oxidation under cold stress (García et al., 2014; Barand et al., 2020). MT treatment upregulates stress responsive gene (CsZat12) and increases the accumulation of polyamines (Put, Spm, Spd) by altering the activity of polyamine metabolic enzymes. Moreover, MT also modulates the expression of ABA synthesis genes (CsNCED1 and CsNCED2) and ABA catabolism genes (CsCYP707A1 and CsCYP707A2) to confer cold tolerance in plants (Zhao et al., 2017). The application of MT also induces the RBOHD-dependent H_2O_2 generation in cold stress and increase in H_2O_2 promotes Ca^{2+} accumulation that sends signals for anti-oxidant activities and improve the cold tolerance (Chang T. et al., 2021). MT application also upregulate the expression of anti-oxidant genes (CsSOD, CsPOD, CsCAT, and CsAPX) that increases the anti-oxidant activities of and resultantly increased the ROS scavenging (Li et al., 2019).

ENGINEERING MELATONIN BIOSYNTHESIS IMPROVES COLD TOLERANCE

The efforts are being made to develop the transgenic plants with improved MT bio-synthesis for ensuring the cold tolerance in plants. For instance higher SNA (Serotonin *N*-acetyltransferase) specific enzyme activities were noticed in transgenic plants, and higher expression of SNA induces a significant increase in MT biosynthesis and subsequent cold tolerance (Kang et al., 2010). Likewise, over-expression of SNAT2 in rice lines increased the MT biosynthesis, which improved plant tolerance to cold stress (Hwang and Back, 2019). The *oAANAT* gene's over-expression plays a significant role in MT biosynthesis under stress conditions. The increase in expression of the oAANAT gene enhanced the MT contents and promoted the plant growth and spike length of switchgrass under cold stress (Yuan et al., 2016). In cotton crops, over-expression of *GhM2H* gene improved the tolerance against heat and cold stress by increasing endogenous MT contents and antioxidant activities and reducing ABA accumulation (Zhang Y. et al., 2021).

The insertion of ClCOMT1 in transgenic watermelon plants significantly increased the MT bio-synthesis. ClCOMT1 expression in watermelon was also substantially increased under cold, drought, and salt stress following increased MT accumulation. Therefore, ClCOMT1 over-expression is considered a positive plant growth regulator in response to heat, cold and drought stresses (Chang J. et al., 2021). Another group of researchers identified that inserted the *ASMT* genes apple plant. They noted that ASMT genes were significantly upregulated

under cold, drought and heat stress. The expression of these genes appreciably increased MT biosynthesis, which increased the plant tolerance to cold, drought, and heat stresses (Wang et al., 2022).

CONCLUSION AND FUTURE PERSPECTIVES

Melatonin application effectively modulates plant growth and confers cold tolerance in plants. The exogenous MT application improved the synthesis of photosynthetic pigments and maintains membrane stability, plant water status, increasing the nutrient and water uptake, which improved plant growth under cold stress. Melatonin supplementation also alleviates the cold-induced osmotic imbalance by increasing the accumulation of different osmolytes, endogenous MT, hormones, and secondary metabolites. Moreover, exogenous MT supply also helps the cold-induced deleterious impacts by increasing the expression of different defensive genes responsible for the higher antioxidant activities under cold stress. The genes manipulation associated with enhanced MT biosynthesis also appreciably improved the cold tolerance in plants by favoring the antioxidant activities, photosynthetic performance and accumulation of different osmolytes.

Still, the role of MT in cold tolerance is not fully explored, and more research is direly needed to uncover its potential benefits under cold tolerance. The exact position of MT biosynthesis in plants requires further investigation. MT is also an unstable molecule; therefore, its transportation in plants organs under cold stress must also be studied in future research programs. The role of MT in improving root growth under cold stress is well studied; however, its role in nutrient uptake is poorly studied. Therefore, the role of MT in nutrient uptake and transportation must be explored in future research studies.

Moreover, increased endogenous MT level in plants under cold stress occurs by upregulation of MT bio-synthesis genes or MT absorption from the exogenous MT application; both mechanisms need more investigation to ensure better MT biosynthesis in plants. The role of MT on pollen viability, abscission and crop quality under cold stress must be explored at the field level. Further studies are also direly needed to identify the interaction of MT with other osmolytes and hormones under cold stress. Recent improvements in genetic engineering have provided clues to diverse complex gene-protein interactions and interconnected networks. Therefore, genetic engineering will enable us to understand better the interaction of MT with other hormones under cold stress. The role of MT in stomatal signaling under cold stress is also unknown; therefore, future research direction on this aspect would also fascinate. Plant chloroplast and mitochondria are e significant sites of ROS production. MT works as signaling molecules; therefore, it would be fascinating to explore the inter-organelle MT signaling under cold stress. Additionally, molecular mechanisms of MT in increasing the expression of antioxidant as stress-responsive genes must also be examined under cold pressure in future research studies. All these efforts will increase our understanding of the roles of MT as a potential antioxidant to be used in cold stress conditions.

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 author.

AUTHOR CONTRIBUTIONS

SHQ and MUH conceived the idea and wrote the original draft. MUC, AM, MS, MaN, MuN, LB, MAA, and MA helped with organization and editing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Fine-Tuning Cold Stress Response Through Regulated Cellular Abundance and Mechanistic Actions of Transcription Factors

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Inflictions caused by cold stress can result in disastrous effects on the productivity and survival of plants. Cold stress response in plants requires crosstalk between multiple signaling pathways including cold, heat, and reactive oxygen species (ROS) signaling networks. CBF, MYB, bHLH, and WRKY families are among the TFs that function as key players in the regulation of cold stress response at the molecular level. This review discusses some of the latest understanding on the regulation of expression and the mechanistic actions of plant TFs to address cold stress response. It was shown that the plant response consists of early and late responses as well as memory reprogramming for long-term protection against cold stress. The regulatory network can be differentiated into CBF-dependent and independent pathways involving different sets of TFs. Post-transcriptional regulation by miRNAs, control during ribosomal translation process, and post-translational regulation involving 26S proteosomic degradation are processes that affect the cellular abundance of key regulatory TFs, which is an important aspect of the regulation for cold acclimation. Therefore, fine-tuning of the regulation by TFs for adjusting to the cold stress condition involving the dynamic action of protein kinases, membrane ion channels, adapters, and modifiers is emphasized in this review.

Keywords: cold stress, transcription factors, CBF-dependent pathway, ICE1, chromatin modification, miRNA, REIL, ubiquitin proteosomic degradation

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INTRODUCTION

Abiotic stresses such as low temperature, drought, and high salinity are complex quantitative traits where numerous stress-responsive genes including transcription factors (TFs) take part to ensure the survival of plants. The low temperature adversely affects plant growth and yield and significantly reduces crop efficiency. The TFs play a pivotal role in regulating eukaryotic gene expression, and several TFs families in plants are involved in regulating the expression of cold stress-responsive genes (Wang et al., 2020a). These TFs bind to the promoter regions of these genes to achieve a coordinated and effective response for cold acclimation. Depending on the types of TFs, different

mechanisms are employed, thus affecting the selectivity and expression levels of the genes being regulated.

Plants respond differently for tolerance to low temperatures (0–15 degrees Celsius) and frost (below zero degrees Celsius). Adaptation to low temperatures is achieved by exposure to short-term cold, a process known as cold tolerance. Therefore, the cold tolerance can be defined as the ability of a plant to withstand low temperatures to prevent the stress from damaging its tissues (Ding et al., 2019). This process is associated with biochemical and physiological changes that will eventually lead to dramatic changes in gene expression, membrane lipid status, cell water uptake, membrane fluidity, and small molecules accumulation. Cold adaptation will increase the plant's tolerance to physical and physicochemical changes when exposed to freezing temperatures. Plants in the tropical and subtropical regions however lack cold resistance mechanisms and they will suffer irreversible damage in the face of low temperatures (Knight and Knight, 2012). Plant resistance to low temperatures has a complex mechanism that involves different metabolic pathways in diverse cellular segments. Several genes have been identified to be involved in cold stress response, including genes that encode TFs, phosphatases, and kinases (Sun et al., 2018).

Plants show different reactions to cold stress where stress signaling pathways play a key role at the molecular and cellular levels. Signal detection, signal transmission, and stress response are three stages of signal transduction in plants when they receive stress stimuli. Possible mechanisms for coping with cold stress include binding of TFs to the cis-element in the promoter of cold stress-responsive genes, interactions of TFs with auxiliary proteins to facilitate their functions, regulation of reactive oxygen species (ROS), and signal transduction (Kumar et al., 2021). The stress response phase begins with phosphorylation and dephosphorylation events regulating the expression of these TF genes for protection at the cellular level. In addition, the expression of the TFs is regulated by microRNAs (miRNA) and their presence in the cells are subjected to translational and post-translational regulation. The different mechanisms will be described and discussed in the different sections of this review.

The TFs that are key players in plant response to cold stress including C-repeat Binding Factor (CBF)/Dehydration-responsive element-binding protein (DREB), Myeloblastosis (MYB), Basic helix–loop–helix (bHLH), NAC (NAM-ATAF1,2-CUC2), WRKY, and Basic leucine zipper (bZIP) gene families (Zhao et al., 2018) are shown in **Table 1**. The structures and activities of CBF/DREB, MYB, bHLH, and WRKY as well as their roles in regulating cold stress response are described in greater details as these TFs are mainly covered in this review.

C-Repeat Binding Factor/ Dehydration-Responsive Element-Binding Protein

Plant stress TFs can control a set of genes by binding specifically to the cis-regulatory elements in the target gene promoters. The products of these genes act as stress response proteins, thereby increasing the plant's tolerance to stress. CBF/DREB TFs are a large subset of the AP2/ERF family that play a key role in the expression of stress-responsive genes in the ABA-independent pathway. This family of TFs plays a vital role in regulating plant growth and response to external environmental stresses (Chen et al., 2013). At low temperatures, CBF activates the expression of cold-regulated (*COR*) genes which encode key enzymes for osmolyte biosynthesis and other cold stress-responsive genes by binding to the DRE/CRT (A/GCCGAC) cis-regulatory element (Vazquez-Hernandez et al., 2017). This enhances freezing tolerance through the accumulation of cryoprotective proteins and soluble sugars that repair cold-hardened membranes and stabilize cellular osmotic potential (Shi et al., 2018b).

The most well-known pathways for understanding and responding to cold stress in plants are the ICE-CBF-COR pathway involving 12% of all cold-responsive genes. This pathway is stimulated by low temperature, and with the induction of multiple intermediates step by step, eventually increases the expression of downstream genes regulated by CBF (Wang et al., 2020a). *ICE* (Inducer of CBF Expression) genes are at the forefront of the cold adaptation process, which induce the expression of *CBF* genes. When plants experience non-freezing low temperatures, the *CBF* genes are activated rapidly, and subsequently, the expression of downstream target *COR* genes referred to as regulons of CBF is induced. Thus far, there are four CBFs found in plants, and they are known as CBF1, CBF2, CBF3, and CBF4 representing DREB1B, DREB1C, DREB1A, and DREB1D (Alves et al., 2017; Liu et al., 2019; Xie et al., 2021a). The CBF1, CBF2, and CBF3 involved in cold response are sequentially located on chromosome four of Arabidopsis (Shi et al., 2018b). Mutations made on the *CBF* genes through the CRISPR/Cas9 system demonstrated the triple CBF mutants were more at risk of freezing (Zhao et al., 2016). Thus, indicates *CBF* genes are critical for cold adaptation. Therefore, further studies on CBF/DREB TFs will increase our understanding of applying beneficial strategies to improve plant tolerance to cold stress.

Myeloblastosis

The MYB family is one of the most widespread plant TF families, containing above 100 members in Arabidopsis and rice involved in low temperature stress response. These TFs are divided into four different subtypes based on the structure of the DNA binding domain, which include 1R-MYB, R2R3-MYB, R1R2R3-MYB, and 4R-MYB (Su et al., 2014). This super family of proteins is involved in various processes such as cell cycle control, flower and seed development, primary and secondary metabolites regulation, hormonal signals, and biotic and abiotic stress responses (Li et al., 2015). Many studies have shown that the expression of *MYB* genes in response to cold and frost stress is dependent on the CBF/DREB pathway (Mehrotra et al., 2020). Transgenic plants that over-expressed *MYB* show enhanced expression of *CBF* genes with corresponding increase in tolerance to freezing stress before and after cold adaptation (Wang et al., 2014a).

TABLE 1 | Transcription factor, cis-acting elements, and the corresponding responsive genes that are involved in plant abiotic stress.

No	TF families	Cis-acting element (Core Sequence)	Gene containing Cis-acting element	Species	Phenotypic changes in transgenic plant after induction of cold stress in comparison with wild type	References
1	CBF/DREB	DRE/CRT (A/GCCGAC)	*COR* genes	Sweet potato (*Ipomoea batatas* [L.] Lam)	Slight wilting, lower damaged leaves, little changed in the photosynthetic efficiency in transgenic sweet potato	Jin et al., 2017
2	NAC	CATGTG	Abiotic stress-responsive genes such as: *RD26*	*Arabidopsis thaliana*	Slightly chlorotic leaves with a larger leaf blade and shorter petiole than the wild-type plants	Nakashima et al., 2012
3	MYB	Type I Myb recognition sequences CNGTT(A/G) or type II G(G/T)T(A/T) GTT(A/G) and type IIG G(G/T)T(A/T)GGT(A/G)	Abiotic stress-responsive genes including *COR* genes	*Arabidopsis thaliana*	Induces darker green phenotypes in transgenic tobacco Enhances the contents of anthocyanins, phenolics, flavonoids, and soluble solids and, aroma volatiles in transgenic tomato	Agarwal et al., 2006; Yang et al., 2021; Ma et al., 2022
4	WRKY	W-box TTGACC/T	*RD29B*/*RD29A*/*COR6.6*/*DREB2A*	wild oat (*Avena fatua*)	Higher survival rates, increases in proline, lower electrolyte leakage in transgenic cucumber	Rushton et al., 2010; Zhang et al., 2016
5	bHLH	E-box (CANNTG) G-box (CACGTG)	*COR* and anthocyanin biosynthetic genes	Sweet potato (*Ipomoea batatas* [L.] Lam)	Slight damage under cold stress and returned to a normal state after recovery treatment in transgenic sweet potato	Jin et al., 2021; Shen et al., 2021
6	bZIP	ABRE (PyACGTG/TC)	*RD29A*, *RD29B*, *COR15A*, *COR47*	*Arabidopsis thaliana*	Lower ion leakages and higher survival in transgenic Arabidopsis	Wang et al., 2016
7	AP2 (ERF)	GCC-box (AGCCGCC)	Several abiotic stress-responsive genes	*Arabidopsis thaliana*	Greater survival rates of transgenic birch (*Betula platyphylla*) compared to wild type plant	Zhu et al., 2010; Lv et al., 2020

MYB15 can be found in vegetative and reproductive organs of plants and plays a special role in regulating cold and salinity stress (Ding et al., 2009; Lindemose et al., 2013). MYB15 belongs to R2R3-MYB family of TFs in Arabidopsis that negatively regulates frost tolerance, which is brought about by its ability to repress the levels of expression of the *CBF* genes. MYB proteins are also involved in the interactions between cold, drought, and salinity stress responses (Li et al., 2015). Therefore, MYB proteins can be considered as an important regulator in plant responses to multiple abiotic stresses that can simultaneously control these abiotic stresses in higher plants. Current interest focuses on molecular mechanism associated with plant response to multiple stresses rather than focusing on a single type in isolation.

Basic Helix–Loop–Helix

One of the important families of TFs in eukaryotes is bHLH, which has completely different functions in animals and plants. In plants, this TF family is active in regulating the expression of genes involved in hormonal and optical signals, stomata development, flowering, flavonoid biosynthesis, and response to biotic and abiotic stresses, including cold stress (Mehrotra et al., 2020). The protein sequence of this TF shows the presence of two functional domains, the HLH and basic domains, which together contain 60 conserved amino acids. The HLH domain with two alpha helices separated by a loop located at the C-terminal of the TF allows the interaction with other subunits to form homodimer and heterodimer. The basic domain with 15 amino acids at the N-terminal facilitates in binding to the G-box and E-box in gene promoters (Jin et al., 2021).

Studies in Arabidopsis and rice demonstrated the presence of 167 and 177 *bHLH* genes, respectively (Mao et al., 2017). Although limited information is available on this protein family in plants, studies have shown the involvement of bHLH in responding to abiotic stresses, including cold tolerance. *ICE1* and *ICE2* encode MYC-type bHLH TFs consisting of 496 amino acid residues. ICE1 binds to the MYC cis-element (CANNTG) at the promoter of *CBF1*/*2*/*3* to promote *CBF* genes transcription (Mehrotra et al., 2020; Sharma et al., 2020). NtbHLH123 transcription factor in tobacco is also a transcriptional activator that controls the expression of genes involved in ROS clearance, which led to the increase in cold tolerance (Zhao et al., 2018). In a study on sweet cherries, 66 *bHLH* genes were identified and most members of the PavbHLH family are associated with various processes in response to cold stress (Shen et al., 2021). The *IbbHLH* gene in sweet potato may also play a role in regulating cold stress, and *IbbHLH79* was identified as a potential candidate for plant molecular breeding to increase cold tolerance in sweet potatoes (Jin et al., 2021). These findings show the importance of bHLH in cold stress response. However, more studies are needed to reveal cold and ROS signaling pathways regulated by bHLH in plants.

WRKY

The WRKY TF family in plants responds to a variety of abiotic stresses such as drought, salinity, and abnormal temperature.

This family has undergone significant changes in higher plants during evolution, and the function of their genes has changed greatly from the original ancestral gene. Segmental and tandem duplications have also played a vital role in the development of this gene family. Previous reports indicate the essential role of WRKY TFs in managing low temperature stress. In tomatoes, for example, fragmentary duplication significantly contributed to the development of *WRKY* genes, and the expression of ten TFs from the WRKY family doubled during the period of cold stress (Chen et al., 2015). In a study on cucumber, over-expression of *CsWRKY6* increased cold tolerance as well as sensitivity to ABA and proline accumulation (Zhang et al., 2016). Recently, transcriptomic analysis in *Brassica napus* identified several genes of the WRKY family that play a vital role in cold resilience (Ke et al., 2020). Studies involving cold-resistant and cold-sensitive peanuts suggested that members from the NAC, MYB, and WRKY TF families were jointly involved in cold tolerance, indicating the importance of crosstalk between signaling pathways involving different TF families in response to cold stress (Jiang et al., 2020). The findings show WRKY enhances plant defense response to cold stress through ABA signaling pathway. It would be valuable to delineate the mechanism for cooperative involvement of the different TFs in cold stress response.

Many of the TFs involved in regulating cold stress response also play a role in other abiotic stress response pathways such as heat and drought. This is expected as most of the stress conditions lead to similar cellular disturbance especially excessive accumulation of ROS with damaging effects on macromolecules including carbohydrates, proteins, lipids, and DNA. Each TF recognizes a specific regulatory motif found on the promoters of different stress-responsive genes enabling simultaneous alteration in their expression (either up- or downregulation). The detection of adverse environmental condition is conveyed through plant signaling cascades to the TFs in order to achieve the desired cellular responses to overcome the damaging effects from the external environment as elaborated in the following sections.

TRANSCRIPTIONAL REPROGRAMMING INVOLVING CHROMATIN MODIFICATION AND HEAT SHOCK FACTORS

In response to cold stress, the production of ROS and NO mediates the alterations in chromatin structure and coordinates modification of histone and DNA methylation to activate the expression of stress-responsive genes (Kim, 2021; Tanpure et al., 2021). In eukaryotes, chromatin is organized as repeating subunits called nucleosome consisting of 147 bp DNA wrapped around a histone octamer containing four different histone proteins, H2A, H2B, H3, and H4 present in pairs. Loosening of the chromatin structure at a particular gene locus increases the accessibility of the transcriptional machinery and enhancing the transcriptional activity, which can be heritable and stably maintained between cell generations (Xie et al., 2021b). Histone acetyltransferase GENERAL CONTROL NON-REPRESSED PROTEIN5 (GCN5) catalyzes the acetylation of H3K9 and H3K14, which improves thermotolerance by activating heat stress-responsive genes, such as HEAT SHOCK TRANSCRIPTION FACTOR A3 (HSFA3), UV-HYPERSENSITIVE6 (UVH6), CHOLINE TRANSPORTER-LIKE 1 (CTL1), POLYGALACTURONASE INVOLVED IN EXPANSION3 (PGX3), and MYB54 (Hu et al., 2015; Zheng et al., 2019). The bivalent H3K4me3 and H3K27me3 marks found in active cold stress-responsive genes represent enhanced chromatin accessibility, which potentially allow access of proteins involved in transcriptional gene regulation (Zeng et al., 2019). The HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE15 (HOS15)-mediated chromatin modifications enable recruitment of CBF to *COR* gene promoters (Park et al., 2018).

The involvement of heat shock proteins (HSPs) in cold stress is still unclear compared to their action in heat stress. However, recent findings showed the role played by the HSPs (HSP90, HSP70, and smHSP) in controlling the intervention between heat and cold stresses in plants. Among HSPs, the HSP90 acts as a regulator of signal transfer to the nucleus, which is believed to be involved in reprogramming of transcriptional regulation of both stresses (Sohrabi et al., 2022). The regulation of HSPs expression is controlled by heat shock factors (HSFs). In plants, HSFs are categorized into three groups, the HSFA, HSFB, and HSFC. Among the three HSFs, the HSFA is involved in regulating HSPs expression and therefore activates multi-chaperone network. Under normal condition, the HSFA activity is negatively regulated by HSP90 while when responding to abiotic stress, the HSP90 is dissociated from the phospho-protein complex allowing interaction between HSFA and heat stress element (HSE) in the gene promoter region of many genes including those encoding antioxidants enzymes (Haq et al., 2019). The HSP90 was also reported to regulate cold stress response through resistance (R) proteins when pathogen attacks occurred under cold condition. This can be observed in Arabidopsis wherein HSP90 was found to be involved in the activation of RECOGNITION OF PERONOSPORA PARASITICA 4 (*RPP4*), one of the *R* genes in plants through SALICYLIC ACID GLUCOSYLTRANSFERASE 1-REQUIRED FOR Mla12 RESISTANCE 1-HEAT SHOCK PROTEIN 90 (SGT1-RAR1-HSP90) chaperone complex. The activity of SGT1-RAR1-HSP90 complex activated ENHANCED DISEASE SUSCEPTIBILITY 1 (EDSI)- and WRKY70-dependent cell death and defense response under cold condition (Bao et al., 2014). The findings show significant function of HSP90 in regulating cold signaling pathway even though under combined stresses of biotic and temperature stresses. In cold stress alone, increased HSP90 transcript was observed in *Brassica napus* (Krishna et al., 1995). However, in *Glycine max*, the downregulation of HSP90 was observed under cold condition compared to heat and salinity stress (Xu et al., 2013). This suggests the strong involvement of HSP90 in dehydration acclimation in *G. max* compared to cold stress. Recent findings in lentil further showed the involvement of other HSPs, the HSP70, HSP83, and HSP21 in mediating the cold response. Interestingly, these HSPs are also involved in heat stress response (Sohrabi et al., 2022). As cold and heat stress conditions do not occur concurrently in the plant, regulation of a specific HSP by HSF under cold and

heat stress is potentially two independents events that assist plants to cope with abnormal temperature.

Stress memory programming at the transcriptional level is also important to enhance acclimation process toward cold stress in plants. This process involves the ability of primed plants to remember previous stress experience and therefore acquire them to enhance tolerance to similar or different stresses. It is a long-term effect that enables the plants to be protected from various stresses based on a previous encounter. In this process, the epigenetic modifications take place to mediate transcriptional memory, thus increasing plant adaptation to adverse temperatures (Xie et al., 2021b). As reported in Arabidopsis, the repetitive cold stress treatments reduced COR15A in resistance-improved plants. The reduction suggests the acclimation process, which could involve establishment of stress-induced H3K4me3 histone modification (Leuendorf et al., 2020). Increase in the expression of HISTONE DEACETYLASE 6 (*HDA6*) under cold stress in Arabidopsis had positively regulated freezing tolerance (Luo et al., 2017). Maize histone deacetylases (HDACs) may directly activate *ZmDREB1* gene expression and histone hyperacetylation under cold stress (Yu et al., 2018; Ding et al., 2019). Meanwhile, the regulation of expression of *COR* genes (*COR47* and *COR15A*) was reported to be regulated by HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 15 (HOS15) and HISTONE DEACETYLASE 2C (HD2C) through direct binding to *COR* gene promoters (Park et al., 2018).

EARLY AND LATE COLD STRESS RESPONSE BY TRANSCRIPTION FACTORS

Modification of membrane proteins and activation of Ca^{2+} ion channels are plants primary responses when they sense cold stress from the environment. The chilling tolerance divergence 1 (COLD1) localized on plasma membrane and endoplasmic reticulum is the cold signal sensor that is responsible for receiving the cold signal from the environment (**Figure 1**). *COLD1* gene encodes a regulator of G-protein signaling, which causes the G-protein α subunit to activate the guanosine triphosphatase (GTPase) activity of rice G-protein A subunit 1 (RGA1; Guo et al., 2018). Ma et al., 2015 showed that over-expression of japonica rice *COLD1* enhanced chilling tolerance in rice compared to that of rice lines with downregulated expression of *COLD* 1. Once COLD1 receives the cold signal, elevation of cytosolic and nuclear Ca^{2+} occurs, and the plants activate calcium Ca^{2+} signaling pathway. Transportation of extracellular Ca^{2+} across plasma membrane through Ca^{2+} channels and/or Ca^{2+} pumps is triggered, increasing Ca^{2+} influx across plasma membrane (Yuan et al., 2018). The intracellular Ca^{2+} signal is then transduced through Ca^{2+} sensors, such as calmodulins (CaMs), CaM-like proteins (CMLs), Ca^{2+}-dependent protein kinases (CPKs/CDPKs), and calcineurin B-like proteins (CBLs; Shi et al., 2018a). The Ca^{2+} sensors such as CaMs convey cold signal to TFs such as Calmodulin-Binding Transcription Activators (CAMTA). The promoter sequence of *CAMTA* genes possesses multiple cis-acting elements responsible for stress responses such as ABRE, SARE, G-box, W-box, AuXRE, DRE, and others (Noman et al., 2021), suggesting the potential interaction of cold-stressed responsive TFs with cis-acting elements of *CAMTA*. Upregulation of *CBF2* in Arabidopsis was reported due to interaction of Arabidopsis AtCAMTA3 to CM2 motif located in the *CBF2* promoter (Doherty et al., 2009). Kim et al., 2013 further reported the involvement of salicylic acid in inducing interaction between CAMTA1, CAMTA2, and CAMTA3 that further activated CBF1, CBF2, and CBF3 under cold stress. The decrement of salicylic acids was observed when the plants were exposed to warm temperature. Thus, CAMTAs play a critical role in the cold stress signaling cascade through binding to specific promoter motifs, which consequently influence the expression of *CBF* genes. However, based on the availability of multiple stress-responsive motifs located at *CAMTAs* promoters, multiples stress-responsive TFs can induce *CAMTAs* expression; therefore, accumulation of CAMTAs protein can enhance cold stress response through CBF-dependent signaling pathway. It further suggests the regulation of *CBFs* expression through CAMTAs other than that of ICE1 regulatory pathway.

Recently, Ca^{2+}-permeable transporter ANNEXIN1 (AtANN1) localized in plasma membrane was reported to be involved in mediating the accumulation of cytosolic Ca^{2+} in response to accumulation of ROS. In this study, the research group found that the activated protein kinase, Open Stomatal 1 (OST1), phosphorylated AtANN1 at Ser289, and therefore enhances the Ca^{2+} transport activity and amplifies the Ca^{2+} signaling to activate the expression of *CBFs* and *CORs* in the nucleus (Liu et al., 2021). Moreover, the Ca^{2+} signal transduction through calcium/calmodulin-regulated receptor-like kinases (CRLK) activates the perception of downstream regulatory and signaling pathway through phosphorylation and activation of mitogen-activated protein kinases (MAPKs; Shi et al., 2018a). Two MAPKs are recognized in Arabidopsis, the MEKK1-MKK2-MPK4-CBFs and MKK5-MPK3/6-CBFs. Initiation of MEKK1 activity activates signal transmission to MPK4 through MKK1/2 by phosphorylation. Further, the activated MKK2 phosphorylates MPK4 and MPK6, and consequently activates physio-biochemical responses in cold-stressed plants (Chen et al., 2022). The MAPKs regulate the expression of cold-responsive genes including TFs, where their involvement in cold stress response can be distinguished as early (e.g., changes of membrane proteins and activation of ion channels) and late responses (i.e., cold acclimation; Wang et al., 2020d). Recently, studies by Shu et al. (2022) showed the involvement of ferulic acid in enhancing cold stress response in tomato. The exogenous application of ferulic acid decreased the severity of cold injury and upregulated the expression of *SlMAPK3*, *SlCBF1,* and *SlICE1*. Knockout mutant of *SlMAPK3* showed decrement of *CBF* expression in cold-stressed tomato suggesting the role of MAPK3 in *CBF* regulation.

The CBF coordinates cold signaling pathway through CBF-dependent regulatory pathway and CBF-independent signaling pathway (Park et al., 2018; Liu et al., 2019). In

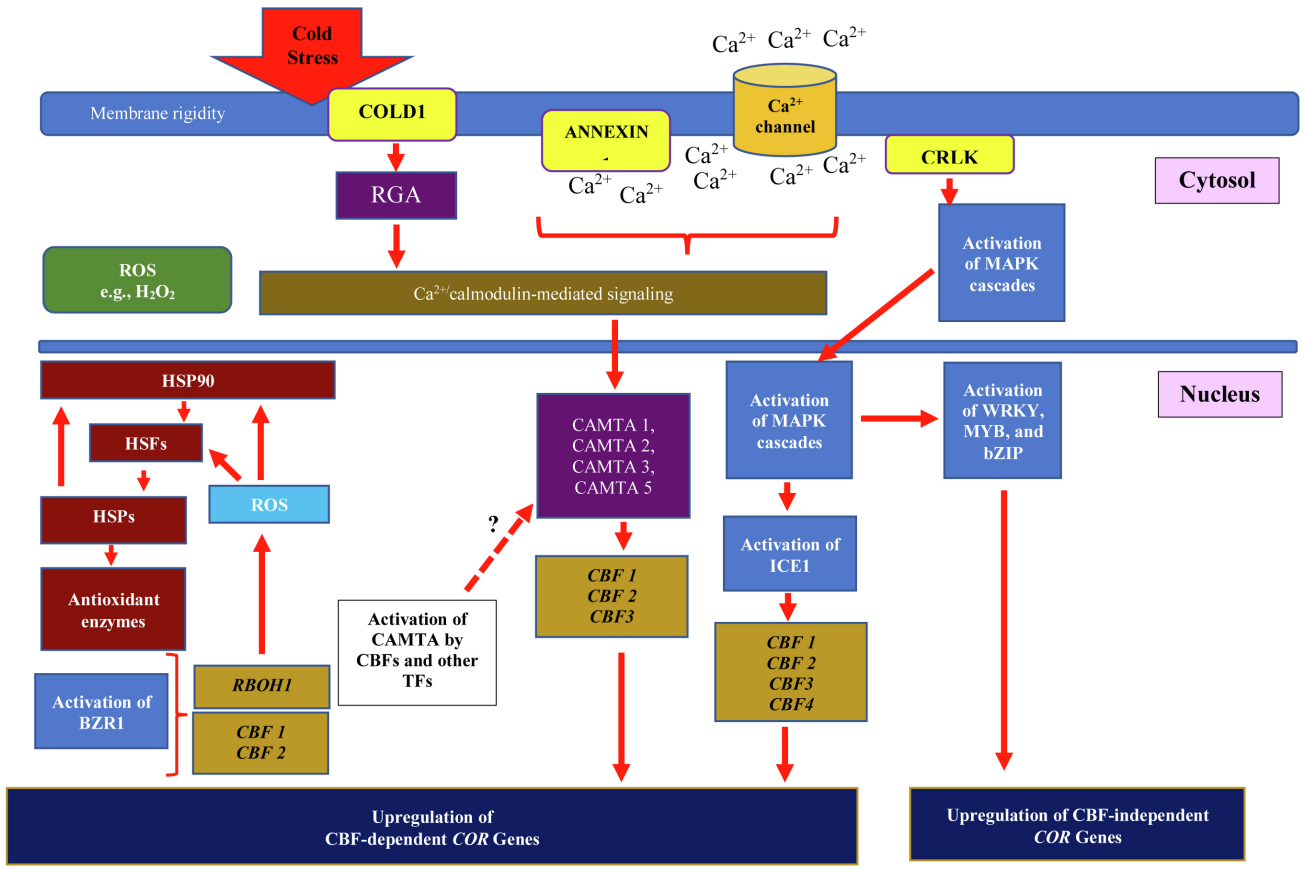

FIGURE 1 | Integration of cold, heat, and ROS signaling in CBF-dependent and independent pathways for regulating plant cold stress response. Cold stress triggers changes in membrane fluidity and rigidity, and activates the expression of cold-regulated genes (*CORs*) through CBF-dependent and independent pathways. The chilling tolerance divergence 1 (COLD1) receives external cold stress signal and stimulates rice G-protein A subunit 1 (RGA1) activity. This further activates ANNEXIN1, the Ca^{2+} channels that transport Ca^{2+} into the cell. The COLD1/RGA activity and Ca^{2+} transmit cold stress signal to Ca^{2+}/calmodulin-mediated signaling and Ca^{2+}-dependent protein kinases (CDPKs) located in the cytosol. The CDPKs activities further convey cold stress signal through cytosolic and nuclear MAPKs signaling pathways, which leads to induction of ICE1 activity. ICE1 binds DRE/CRT motif in the promoter of *CBF 1*, *2*, *3*, and *4* and upregulates their expression. Integration of cold signal through Ca^{2+}/calmodulin-mediated signaling activates interaction between CAMTAs and CM2 motif located in CBF promoter, which further upregulates *COR* genes expression. The presence of ABRE, SARE, G-box, W-box, AuXRE, and DRE motifs in *CAMTAs* promoters suggests the potential regulation of CAMTA by CBFs and other stress-responsive transcription factors (TFs). Accumulation of brassinosteroids promotes binding of BZR1 to E-box found in *CBF1* or *CBF2* genes and activates the expression of *COR* genes. Through CBF-independent pathway, the expression of CBF-independent *COR* genes is regulated through interaction of other TFs such as WRKY, MYB, and bZIP. The expression of CBF-independent *COR* genes could also be regulated through ROS signaling pathway, involving ROS produced by RESPIRATORY BURST OXIDASE HOMOLOG 1 (RBOH1). The ROS further regulate heat shock factors (HSFs) and heat shock protein 90 (HSP90) and hence develop multi-chaperone network that controls production of antioxidant enzymes. The multi-chaperone network is also involved in controlling HSP90 activity.

response to cold stress, the abscisic acid (ABA), jasmonic acid (JA), and indole acetic acid (IAA) are among the phytohormones involved in activating CBF-dependent signaling pathway (Wang et al., 2021; Zhou et al., 2021; Fu et al., 2022). The interaction between CBF and CRT/DRE motif regulates the downstream expression of an array of *COR* genes, which belong to *KIN* (cold induced), *RD* (responsive to desiccation), *LTI* (low temperature induced), and *ERD* (early dehydration inducible; Wani et al., 2021). The regulated products of *COR* genes include osmo-protectants biosynthetic enzymes, late embryogenesis abundant (LEA) proteins, TFs, protein kinases, proteins associated with lipid metabolism, proteins for hormone responses, cell wall modifiers, and chloroplast proteins (Liu et al., 2019). Discovery of an array of *COR* genes in Arabidopsis such as *COR6.6* (Gilmour et al., 1996), *COR15* (Lin and Thomashow, 1992), *COR47* (Gilmour et al., 1992), *COR78* (Horvath et al., 1993), and *COR413* (Breton et al., 2003) shows the important function of these genes in plant growth, development, and response to abiotic stresses.

In the early response to cold stress, the regulation of membrane fluidity of chloroplast is important for photosynthesis. The COR413 was reported to be involved in the regulation process. The COR413 TFs have been categorized into two groups, the Cor413-plasma membrane (COR413pm) and COR413-inner membrane (COR413im) proteins. The COR413im plays a role in stabilizing chloroplast membrane under cold

stress. Meanwhile, over-expression of *Phlox subulata* PsCor413pm2 in Arabidopsis showed increased influx of Ca^{2+} in transgenic Arabidopsis root responding to cold shock. In addition, under cold stress, the increment of cytosolic Ca^{2+} in P*sCor413pm2* transgenic Arabidopsis increased the expression of five *AtCOR* (*AtCor6.6/AtKIN2*, *AtCor15A*, *AtCor15B*, *AtCor47*, and *AtCor78/AtRD29*) and two *AtCBF* (*AtCBF2* and *AtCBF3*; Zhou et al., 2018). Studies by Su et al. (2018) further revealed the involvement of COR413PM1 in regulating the expression of *COR* regulons in response to cold stress including the fatty acid biosynthesis 1 (*FAB1*), fructokinase 3 (*FRK3*), sucrose phosphate synthase A1 (*SPSA1*), and GLN phosphoribosyl pyrophosphate amidotransferase 2 (*ASE2*) in Arabidopsis leaves. Our previous studies showed that over-expression of oil palm *CBF1*, the *EgDREB1* enhanced tolerance to cold stress in lowland tomato. The EgDREB1 was suggested to control the expression of *COR* regulons such as *LePOD*, *LeAPX*, *LeCAT*, *LeGP*, *LeHSP70*, *LeLEA*, and *LeMET2.* Promoter analysis showed the presence of DRE/CRT motif in the promoter regions of these genes. EgDREB1 is also involved in drought stress signaling pathway as its expression was observed in transgenic tomato seedlings exposed to PEG treatment (Azzeme et al., 2017). These findings were similar with the observation carried out in oil palm, which we found that *EgDREB1* and *COR* regulons were also differentially upregulated in the drought-stressed oil palm (Azzeme et al., 2016). These findings, therefore, further suggest the involvement of EgDREB1 in both cold and drought signaling of oil palm. Similar findings were observed in Arabidopsis, where CBF/DREB1 that was known to be involved only in cold signaling was also induced under drought, ABA treatment, and salinity (Wang et al., 2014c). Lowland tomato over-expressing *EgDREB1* exhibited reduction in seed number, development of parthenocarpic fruits, alteration of leaves morphology, and increment of root biomass (Azzeme et al., 2020). Meanwhile, over-expression of *EgCBF3* increased the expression of antifreeze proteins, the *SlCHI3*, *SlPR1*, *SlPR-P2,* and *SlLAP2* in the lowland tomato. Delayed leaf senescence and flowering, increased chlorophyll content, and abnormal flowering were also recorded (Ebrahimi et al., 2016). These show involvements of CBF1 and CBF3 in plant growth and development, apart from their function as master regulators of abiotic stress.

Differentiating CBF-Dependent and CBF-Independent Signaling Pathways

The expression of *COR* genes and their regulation either through CBF-dependent regulatory pathway or CBF-independent signaling pathway is still controversial. In Arabidopsis, the different alleles of the *CBF* gene were found to be controlling different regulatory mechanisms involving *COR* genes, and therefore resulting in different degrees of freezing tolerance. However, interestingly most of the *COR* genes found in this study were co-regulated by CBF-dependent and CBF-independent pathways (Park et al., 2018). It shows that multiple allelism could control different metabolic pathways in cold-stressed plants due to the presence or absence of CRT/DRE motif in the *COR* gene promoters. The absence of CRT/DRE motif indicates the activation of CBF-independent signaling pathway through other TF proteins like WRKY, MYB, and bZIP families to modulate plant responses to cold stress. For instance, Arabidopsis BRASSINAZOLE-RESISTANT 1 (BZR1) was reported to be involved in regulating the expression of *CBF1* and *CBF2* and other *COR* genes *via* CBF-independent pathway to modulate plant response to cold stress (Li et al., 2017). The regulation of BZR1 under cold stress is controlled by brassinosteroids accumulation. In tomato, BZR1 was found to interact with E-box/BRRE motifs located in *CBF1*, *CBF3*, and RESPIRATORY BURST OXIDASE HOMOLOG 1 (*RBOH1*) promoters and upregulated their expression. The activation of tomato ROS signaling pathway was achieved through the expression of *RBOH1*, in which the gene was found responsible in triggering H_2O_2. The H_2O_2 modulated the redox status in cold-stressed plants and increased BZR1 production and *CBF* genes transcription (Fang et al., 2021). Further, Ca^{2+} influx is also reported to be involved in the activation of *RBOH1*, hence enhancing the production of ROS (Kour et al., 2021). Apart from that, the expression of *CBF* could also be controlled by other TFs like ICE1 that activates *CBF* in response to cold stress. The mutation of ICE1 in Arabidopsis showed inhibition in expression of *CBF1*, *2,* and *3* and reduced resistance to cold stress (Chinnusamy et al., 2003). Conversely, the over-expression of *SlICE1* enhanced the expression of *SlCBF1* and increased the cold tolerance of tomato (Suzuki et al., 2012). The two-hour of cold exposure in transgenic potato over-expressing *SaMKK2* showed significant increase in the expression of *CBF1*, *2,* and *3* (Chen et al., 2022). In addition, a report by Jin et al., 2021 showed the involvement of bHLH (IbbHLH79) in activating sweet potato *IbCBF3*, through recognition of E-box (5′-CANNTG-3′) and G-box (5′-CACGTG-3′) located in the *IbCBF3* promoter, therefore enhancing tolerance to cold stress.

The crosstalk between CBF-dependent and -independent signaling pathways in plant response to cold stress enables a more coordinated regulation by the large network of TFs. Fine-tuning of transcriptional regulation by these TFs is achieved through regulating their cellular abundance and their activities as discussed further in the following sections.

REGULATED EXPRESSION OF TRANSCRIPTION FACTORS BY MIRNA FOR MODULATING COLD STRESS RESPONSE

MicroRNAs (miRNAs) are recognized as a major gene family with an important role in gene regulation acting at the post-transcriptional level. First discovered in 1993, miRNAs are short endogenous single-stranded RNA of 20 to 24 nucleotides in length. These non-coding RNA molecules are derived from the typical stem loop precursors of 70 to 80 nucleotides long through the action of Dicer-like family of enzymes. Suppression of gene expression by miRNAs is achieved through two main mechanisms, which are mRNA cleavage and translation inhibition or combination of both (Megha et al., 2018). These mechanisms

require binding of miRNAs to the target sites in mRNAs through perfect or near perfect base-pairing and there can be multiple mRNA targets for each miRNA. In plants, the target sites are mostly in the open-reading frame (ORFs) but some are found in the 5′-untranslated regions (UTRs) and 3'-UTRs of the mRNAs (German et al., 2008). Many of the miRNAs target genes encode TFs involved in transcriptional regulation of different plant developmental processes and stress responses. This constitutes an effective fine-tuning measure as the effects are extended to the various downstream genes regulated by these TFs.

Regulation by miRNA is critical for achieving the complex temporal and spatial expression profiles of each regulatory TF and the downstream genes being regulated by the TF (Samad et al., 2017; Wang et al., 2019a). The predicted target genes of miRNA under cold stress in winter turnip rape mainly encode for TFs, such as MYB, GAMYB, Teosinte branched 1/Cycloidea/Proliferating cell factor (TCP), bHLH, and SQUAMOSA promoter binding proteins (SBP; Zeng et al., 2018). The differential regulation of the TFs by the miRNA leads to cellular outcome essential for plant to cope with the cold stress condition. For example, integrated small RNA and transcriptome analysis of *Populus simonii* × *P. nigra* subjected to cold stress identified differential expression of miR319, miR159, miR167, miR172, miR395, miR393, miR390, and novel_63 and TFs including *MYB*, *SBP*, *bZIP*, Auxin Response Factors (*ARF*), and LONESOME HIGHWAY (*LHW*, atypical bHLH). These miRNAs and TFs direct or indirectly regulate the expression of Leucine-Rich Repeats (*LRR*) receptor kinase, DnaJ-related photosystem II, *ARF,* and *SPLs* associated with chilling injury (Zhou et al., 2019). In the construction of plant cold-responsive Gene Regulatory Network (GRN), the number of affected targets increased with the inclusion of TFs, which behave as central nodes for relaying information from miRNAs downstream to the TF regulons (Tiwari et al., 2020). Gene ontology enrichment analyses showed over-representation of distinct functional modules such as cold stress, transcription and translation, transport and pentatricopeptide repeat (PPR), cell wall and lignin synthesis, and signaling and protein degradation in the GRN.

Key miRNAs Involved in Regulating Transcription Factors for Cold Acclimation

MiRNAs are conserved among plant species and their expression varies depending on environmental biotic and abiotic conditions. The regulation of expression of targeted TFs by miRNAs is dependent on the miRNAs' tissue-specific expression and the differential effects of the cleavage on the target transcripts (Megha et al., 2018). miR166 and miR319 showed altered expression in the roots of winter turnip rape under cold stress compared to normal condition suggesting their pivotal roles in cold stress response. The strong downregulated expression of miR319 (miR319e-1, miR319a, and miR319–2) and miR166 (miR166e-3p) in the roots of cold tolerant compared to cold-sensitive cultivars has important implication as root tolerance is critical during winter for plants' survival (Zeng et al., 2018). It was found that the miR319 from winter turnip rape targets TCP4-like which controls secondary cell wall formation (Sun et al., 2017) while the miRNA166 regulates HD-ZIP III with a key role in cell wall and cellulose synthesis (Zhang et al., 2018). Significantly higher expression of both TFs demonstrated in the cold-tolerant cultivar compared to cold-sensitive cultivar showed that improvement in cell wall thickness and strength is important for cold acclimation.

Significantly higher expression of *COR*s including *DREB1A/B/C*, *DREB2A*, and *TPP1/2* was observed in rice plants over-expressing Osa-miR319b, which targets GAMYB and TCP TFs. The increase in cold tolerance was evident based on the increase in proline content and survival rate of transgenic plants. It was suggested that miR319 negatively regulated *OsPCF6* and *OsTCP21* expression in rice, which partially increased the ROS scavenging capacity, therefore facilitating rice response under cold stress (Wang et al., 2014b). Over-expression of sha-miR319d from wild tomato silenced GAMYB-like1 and conferred chilling tolerance in cultivated tomato (Shi et al., 2019). Differential expression of key regulatory genes involved in chilling (*CBF1* and *MYB83*), heat stress response (*HSFA1a, HSFA1b,* and *HSP90*), and ROS signaling [zinc-finger proteins (*ZAT12* and *ZAT10*) and scavenging, superoxide dismutase (*SOD*), and catalase (*CAT*)] was observed in the transgenic compared to WT plants. This suggests that sha-miR319d regulates temperature stress in tomato *via* interaction of cold stress, heat stress, and ROS signaling pathways.

In rice, OsmiR156 plays an important role in enhancing tolerance to cold stress. Enhancement in cell viability and growth rate under cold stress was observed in Arabidopsis, pine, and rice over-expressing rice miRNA156 that targets *OsSPL3* involved in upregulating the expression of *OsWRKY71* (Zhou and Tang, 2019). Suppression of *OsWRKY71* enhanced the expression of *OsMYB2* and *OsMYB3R-2*. OsMYB2 is involved in regulating multiple stress responses including cold stress (Yang et al., 2012). OsMYB3R-2 transgenic rice showed enhanced tolerance to chilling stress due to the increase in expression of stress-responsive genes and the alteration in cell cycle (Ma et al., 2009).

However, a contrasting result was reported with OsmiR156k, probably due to the production of a different mature miRNA. OsmiR156k is a precursor miRNA which is differentially processed from the other precursor miRNAs (OsmiR156a-j; Moreal et al., 2016) and this may contribute to its differential accumulation and distinct function in plant tissues. Phenotypic analysis found that transgenic rice lines over-expressing OsmiR156k showed seedling growth inhibition at the very early seedling stage under cold stress. Lower survival rates, as well as reduction in chlorophyll and proline contents resulting from the ectopic expression of OsmiR156k, were observed. Downregulated expression of the *CORs* and *SPL3*, *SPL14*, and *SPL17*, the targets of OsmiR156k, was also detected (Cui et al., 2015).

Clearly, the comparison of miRNA expression between cold-tolerant and cold-sensitive cultivars and the functional studies in transgenic plants provided useful insights on regulatory mechanisms for cold acclimation involving miRNA156, miRNA166, and miRNA319 that play key roles in cold stress response in plants. The findings are summarized in **Table 2**.

TABLE 2 | Functional studies involving miRNA156, miR166, and miR319. The target transcription factors of the miRNAs, effects on the expression of downstream or cold stress-responsive genes regulated by the transcription factors are provided. The observed phenotypic effects of the miRNA regulation under cold stress are also included.

miRNA	Target transcription factors	Observed effects on downstream or other cold stress-responsive genes	Phenotypic observation	References
miR156 (OsmiR156)	*SPL3*	Suppression of *OsWRKY71* leading to enhanced expression of *OsMYB2* and *OsMYB3R-2*.	Enhancement in cell viability, growth rate and reduction in ion leakage under cold stress in Arabidopsis, pine, and rice over-expressing rice OsmiRNA156	Zhou and Tang, 2019
miR156k (Osmi156k)	*SPL3*, *SPL14* and *SPL17*	Suppression of proline synthase and ROS scavenger genes	Seedlings' growth inhibition at the very early stage, lower survival rates, lower ROS scavengers, reduction in chlorophyll and proline contents in rice over-expressing OsmiR156k	Cui et al., 2015
miR166 and miR319	*HD-ZIP III and TCP4-like*	N/A	Downregulated expression of miR166 and miR319 in roots under cold stress led to increase expression of HD-ZIP III and TCP4-like that potentially improve cell wall thickness and strength in the cold-tolerant cultivar.	Zeng et al., 2018
miR319 (Osa-miR319b)	*TCP21*, *PCF5* and *PCF6*	Higher expression of *CBF/DREB1*	Increase in proline, plant survival rate and ROS scavenging capacity under cold stress of transgenic rice over-expressing Osa-miR319b	Wang et al., 2014b
miRNA319 (sha-miR319d)	*GAMYB-like1*	Promote expression of genes involved in chilling (*CBF1* and *MYB83*), heat stress response (*HSFA1a, HSFA1b* and *HSP90*), and ROS signaling (*ZAT12* and *ZAT10*) and scavenging (*SOD CAT*)	Lower relative electrolyte leakage and malondialdehyde concentration, reduced O^{2-} generation and H_2O_2 concentration and higher chlorophyll contents and Fv/Fm values in cultivated tomato cultivar over-expressing sha-miR319d from wild tomato	Shi et al., 2019

CONTROLLED OF TRANSCRIPTION FACTOR LEVELS THROUGH DIFFERENTIAL PROTEIN SYNTHESIS AND DEGRADATION DURING COLD STRESS

Ribosome Biogenesis Factors in Accelerating *de novo* Protein Synthesis in Cold Acclimation

An effective cold stress response is dependent on controlled cellular levels of specific TFs along the cold signaling pathway through mechanisms that regulate their synthesis and degradation. This influences the cascade of events through the cold signaling pathway enabling fine-tuning of the cold stress responses. Protein translation which occurs at the ribosome is an important step in the production of a functional cellular proteins in living organisms. Ribosome assembly is a complex process which requires coordination of the activities of three RNA polymerases and more than 200 transiently associated ribosome biogenesis factors (RBFs; Sáez-Vásquez and Delseny, 2019). Arabidopsis zinc-finger proteins, REILs are cytosolic ribosomal 60S-biogenesis factors with potential role in accelerating ribosome *de novo* synthesis (Wang et al., 2017; Beine-Golovchuk et al., 2018). Studies utilizing Arabidopsis *REIL* mutants provided valuable insights on the factors influencing ribosome biogenesis and function under cold stress. Findings of the study by Cheong et al., 2021 suggested that biosynthesis of specialized ribosomes is required for cold acclimation.

It was demonstrated that *STCH4/REIL2* helps in maintaining rRNA processing and promotes translation of CBF for regulating cold stress response (Yu et al., 2020). Over-expression of *STCH4/REIL2* in Arabidopsis can confer chilling and freezing tolerance possibly through modified association of STCH4 with multiple ribosomal proteins. The reduction in rRNA processing ability exhibited by *stch4* Arabidopsis mutants was further worsened by cold stress treatment. The key observations of the mutants include reduction in the level of CBF and delayed induction of the CBF regulons. Clearly, REILs play a role in enhancing cold stress tolerance through altering ribosomal composition and functions, which promotes translation of proteins essential for growth and survival of plants under the adverse effects of cold stress (Yu et al., 2020; Cheong et al., 2021). Studies involving Arabidopsis double mutants of *REIL* complemented by systems analyses of transcriptome and metabolome of the ribosomal complexes (Wang et al., 2017; Beine-Golovchuk et al., 2018) suggest that REILs likely serve as kinetic modulators of ribosome biogenesis or recycling that may assist in overcoming the initial cold-induced inhibition of translation.

Post-translational Regulation for Selective Degradation of Transcription Factors for Fine-Tuning Cold Stress Response

The 26S proteasome degrades TFs that have been covalently linked to a polyubiquitin chain to enable selective reduction in the abundance of a specific TF. Polyubiquitin chain is covalently attached to the target proteins through three sequential steps involving three enzymes; Ub-activating enzyme (E1), Ub-conjugating enzyme (E2), and Ub ligase (E3). The degradation of selective TFs is controlled strictly by numerous E3 Ub ligases responsible for substrate recognition. CULLIN-REALLY INTERESTING NEW GENE (RING) E3 ligases (CRLs) are the largest group of E3 ligase in plants. The multisubunit CRL

regulates numerous biological processes through targeted ubiquitylation of signaling proteins. The four components of CRL core are the cullin scaffold protein, a RING finger protein that binds to an E2 ubiquitin conjugating enzyme, a receptor that recognizes the target protein, and adaptor proteins linking the receptor to the cullin. Different targets are recruited through binding of a large pool of distinct substrate-receptor modules to the N-termini of cullins (Lydeard et al., 2013; Wang et al., 2020c).

The ability to bind to the respective TFs that serve as substrates is crucial for the E3 Ub ligases activity and it is affected by other post-translational modifications including phosphorylation and SUMOylation (Buetow and Huang, 2016; Ding et al., 2019). Regulating the expression of CBF through controlling 26S proteasomic degradation of upstream TFs that act as regulators in the CBF-dependent signaling appeared as one of the key mechanisms for fine-tuning cold stress responses (Shi et al., 2018b). The increase or decrease in cellular abundance of a TF which acts as a positive or negative regulator influences the outcome of the cold signaling pathway.

In Arabidopsis, it was found that cold activation of ICE1 TF, the positive regulator of CBF3/DRB1A, is negatively affected by ubiquitination by RING E3 ubiquitin ligase HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1) which leads to its degradation through the 26S proteosomic degradation pathway (Ishitani et al., 1998). Similarly, cold tolerance is negatively regulated in banana by MaSINA1 which is an E3 ubiquitin ligase that targets MaICE1 (Fan et al., 2017). OPEN STOMATA 1 (OST1) is an important protein kinase involved in the regulation of plant response to cold stress (Ding et al., 2015). Under cold stress, it interacts with and phosphorylates ICE1, preventing it from interacting with HOS1. Stabilization of ICE1 promotes the transcriptional activity of CBF for *COR* induction. While under prolonged cold stress, activation of BRASSINOSTEROID-INSENSITIVE 2 (BIN2) kinase leads to phosphorylation of ICE1 which promotes ICE1 interaction with HOS1 and its subsequent degradation. This suppresses CBF activity and expression of *COR* genes (Ye et al., 2019). Thus, both kinases which act at different phases following cold stress encounter serve as partial regulators of *CBF* indirectly through ICE1. It is also believed that fine-tuning of *CBF* expression by BIN2 is important for balancing cold tolerance and plant growth (Ye et al., 2019). Thus, the regulation of the cellular abundance and activities of the CBF is important for plant adaptation and survival.

U-box E3 ligases (PUB25 and PUB26) from Arabidopsis target the upstream negative regulator of CBF/DREB1, MYB15. Phosphorylation of PUB25 and PUB26 by OST1 enhances their activities and the reduction in MYB15 level leads to increase expression of *CBF* genes which enhances the plant cold stress response (Wang et al., 2019b). Anthocyanin is essential for the cold acclimation response. Increase in anthocyanin improves antioxidant capability resulting in an increase in plant tolerance to low temperature (Naing et al., 2018). MYB TFs interact with bHLH TFs in regulating plant growth and development (Wang et al., 2020b). Apple MdbHLH33 activates the expression of *MdCBF2* and for regulating cold tolerance and anthocyanin accumulation. An et al. (2020) reported on MdMYB308L interaction with MdbHLH33 for enhancing binding to *MdCBF2* and *MdDFR* promoters. However, MYB30-INTERACTING E3 LIGASE 1 (MdMIEL1), an apple RING E3 ubiquitin ligase, was found to be interacting with MdMYB308L. The interaction which promotes degradation of MdMYB308L suppresses cold-tolerant response and anthocyanin accumulation in apple.

Other Mechanisms Modulating 26S Proteosomic Degradation of Cold Stress-Responsive Transcription Factors

SUMOylation can modulate the activity of TFs through regulating the localization and abundance of TFs as well as by influencing their interaction with chromatin. SUMOylation protects conjugated protein from degradation by blocking the lysine residues that can be ubiquitinated (Roy and Sadanandom, 2021). Thus, SUMOylation of ICE1 by SAP AND MIZ1 DOMAIN-CONTAINING LIGASE 1 (SIZ1; Jmii and Cappadocia, 2021) protects ICE1 from degradation through the 26S proteosomic degradation pathway. Expression of *CBF/DREB1* and the downstream *COR* genes was increased while the expression of *MYB15* was suppressed due to SIZ1 conjugation of ICE1, enhancing the tolerance to low temperature (Miura et al., 2007). The fine-tuning of the CBF/DREB1 signaling pathway through post-translational SUMOylation and ubiquitination events involving the positive regulator ICE1-like was also observed in apple. The apple *MdCIbHLH1* which encodes an ICE1-like protein showed induced expression under cold stress. It can be modified through ubiquitination and SUMOylation pathways and binds to MdCBF2 for enhancing cold tolerance *via* the CBF signaling pathway. The ability of MdCIbHLH1 in maintaining its functionality in distantly related species, *Nicotiana tabacum* (tobacco; Feng et al., 2012), may suggest a universal mechanism for cold stress response in plants.

Long Hypocotyl 5 (HY5), a bZIP TF which acts as a positive regulator of cold acclimation, has been shown to activate about 10% of all cold-induced transcripts including anthocyanin biosynthetic genes, facilitating in the complete development of cold acclimation in Arabidopsis. Recent report showed that HY5 can directly regulate the expression of *CBF 1*, *2,* and *3,* or indirectly *via* MYB15 as it was shown to bind to *MYB15* promoter region (Zhang et al., 2020). It has been reported that HY5 is subjected to post-translational control by COP1 E3 ubiquitin ligase (Catalá et al., 2011). Chaperonins are molecular chaperons involved in protein assembly, folding, trafficking, and degradation, with critical role in cellular development (Hartl et al., 2011). Prefoldin is a hexameric molecular chaperone belonging to group II chaperonins (Cao, 2016). Perea-Resa et al. (2017)demonstrated negative regulation of cold stress response in Arabidopsis by prefoldin which destabilizes HY5. Interaction of prefoldin, which accumulates in the nucleus with HY5, triggers ubiquitination and HY5 subsequent degradation through 26S proteosomic degradation pathway. This attenuated anthocyanin biosynthesis, which helped ensure accurate development of cold acclimation. Interestingly, the study showed that degradation of HY5 occurred in

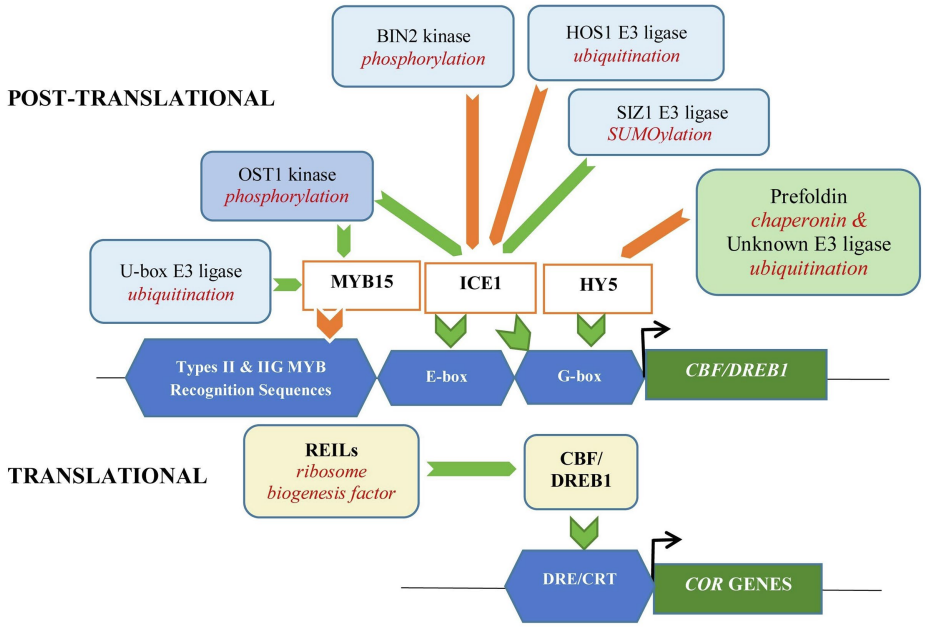

FIGURE 2 | Translational and post-translational modifications of transcription factors affecting CBF-dependent signaling for cold acclimation in *Arabidopsis thaliana*. Translational regulation by ribosome biogenesis factor, REILs increase rRNA processing and CBF levels, positively affecting cold acclimation. Post-translational regulation through ubiquitination for proteosomic degradation of ICE1 (positive regulator of CBF) is suppressed by SUMOylation involving SIZ1 E3 ligase. Phosphorylation of ICE1 by OST1 disrupts its interaction with HOS1 E3 ligase preventing degradation of ICE1 and enhancing cold tolerance. While under prolonged cold stress, phosphorylation of ICE1 by BIN2 kinase facilitates interaction of ICE1 and HOS1 and subsequent degradation of ICE1. Phosphorylation of U-box E3 ligase (PUB25 and PUB26) by OST1 increases ubiquitination and degradation of the negative regulator MYB15 in Arabidopsis, enhancing the expression of CBF and its regulons, *COR* GENES. In contrast, promotion of ubiquitination of the positive regulator, HY5 through interaction with prefoldin which acts as a molecular chaperon, suppresses *CBF* expression. Green and orange arrows represent reactions/interactions that provide positive and negative effects, respectively, on the signaling pathway and expression of *COR* GENES.

COP1-independent manner (Perea-Resa et al., 2017). Based on the findings, it is anticipated that prefoldin contributes indirectly through participation in cellular proteostasis to regulate the stability of TFs or complexes involved in different stages of gene expression (Blanco-Touriñán et al., 2021). **Figure 2** provides a schematic diagram on translational and post-translational modifications affecting CBF-dependent signaling for cold acclimation that has been discussed.

SUMMARY AND FUTURE PERSPECTIVE

The crosstalk between multiple signaling pathways in plant response to cold stress enables access to a large network of TFs as transcriptional regulators. Fine-tuning through regulation of the cellular abundance and activities of the TFs in the GRN is important for plant adaptation and survival. The expression of TFs is affected by intracellular accumulation of secondary messengers like Ca^{2+}, ROS, NO, and phytohormones. Activities of the secondary messengers that regulate rearrangement of chromatin and coordination of histone modification and DNA methylation allow plants to express specific TFs during cold stress. The spatial and temporal regulation by highly conserved miRNAs such as miRNA156, miRNA166, and miRNA319 that modulates the expression of target TFs is important for fine-tuning the cold stress response across plant species. In Arabidopsis, the production of CBF, the key TF for regulating the expression of COR genes is enhanced during cold stress through interaction of REILs, the ribosome biogenesis factors with multiple ribosomal proteins that influence ribosome composition and RNA processing. Other TFs like ICE, HY5, and MYB15 that serve as negative or positive regulators of ICE-CBF-COR signaling cascades are selectively degraded through the proteosomic degradation pathway. This provides the essential cellular metabolic balance for a proper cold stress response. It also helps overcome retardation in plant growth associated with CBF expression. Post-translational modifications of these TFs including through phosphorylation and SUMOylation events or attachment of molecular chaperons, which block or promote binding of the respective E3 ubiquitin ligases, regulate their degradation, with major consequence on the expression of COR genes.

The CBF-independent BZR1 signaling cascades involving other TF families such as WRKY regulate the induced production of specific sets of COR genes independent of the DRE/CRT motif, the recognition site for CBF. BZR1 and HSFA play a central role in linking the cold stress signaling with ROS signaling and heat stress signaling pathways, respectively, through a complex mechanism involving other TFs, HSPs, and ROS

scavenger genes. While CAMTAs which possess multiple cis-elements recognized by a variety of stress-responsive TFs serve central position in the cold stress signaling cascades. CAMTAs integrate inputs from these TFs and convey them downstream for a coordinated expression of the different COR genes. Together, the CBF- and CBF-independent pathways allow plants to adjust to the varying severity of cold stress. The plants could also enhance their defense response by memory reprogramming at the transcriptional level based on previous cold stress encounters.

In general, plants have developed fine-tuning mechanisms for understanding temperature fluctuations to increase their chances of survival. Comprehensive understanding of the fine-tuning of spatial and temporal expression of genes and their translational and post-translational regulation associated with cold stress is critical to produce stress-resistant plants through genetic engineering or genome-assisted breeding strategies for increasing crop yield. It is also of critical importance for extending geographical distribution of crops and for survival under extreme seasonal conditions due to the effects of climate change. Studies on REILs, which are still confined to the model plant, *Arabidopsis thaliana*, can be expanded to economically important crops through powerful multi-omics strategy and functional studies utilizing gene editing technology. Functional analysis of the different TFs could benefit from CRISPR/dCas9 activation and suppression systems to unravel the complex GRN in endogenous system which could provide valuable additional information compared to utilizing model plant systems.

AUTHOR CONTRIBUTIONS

SA produced the overall concept of the review, led the manuscript preparation, carried out the overall editing of the content, and she finalized the write-up. SA, AA, and KY contributed in writing and producing the tables and figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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Recent Advances in the Analysis of Cold Tolerance in Maize

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Maize (*Zea mays* L.) is an annual grass that originated in tropical and subtropical regions of the New World. Maize is highly sensitive to cold stress during seed gemination and the seedling phase, which can lead to reductions in plant vigor and grain production. There are large differences in the morphological and physiological changes caused by cold stress among maize varieties. In general, cold tolerant varieties have a stronger ability to maintain such changes in traits related to seed germination, root phenotypes, and shoot photosynthesis. These morphological and physiological characteristics have been widely used to evaluate the cold tolerance of maize varieties in genetic analyses. In recent years, considerable progress has been made in elucidating the mechanisms of maize in response to cold tolerance. Several QTL, GWAS, and transcriptomic analyses have been conducted on various maize genotypes and populations that show large variations in cold tolerance, resulting in the discovery of hundreds of candidate cold regulation genes. Nevertheless, only a few candidate genes have been functionally characterized. In the present review, we summarize recent progress in molecular, physiological, genetic, and genomic analyses of cold tolerance in maize. We address the advantages of joint analyses that combine multiple genetic and genomic approaches to improve the accuracy of identifying cold regulated genes that can be further used in molecular breeding. We also discuss the involvement of long-distance signaling in plant cold tolerance. These novel insights will provide a better mechanistic understanding of cold tolerance in maize.

Keywords: maize, cold tolerance, QTL, GWAS, transcriptome, long-distance signaling

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INTRODUCTION

Maize (*Zea mays* L.) is one of the world's most important cereal crops for food, economy, and feed (Wang et al., 2020a). Maize is particularly susceptible to cold injury as it is a crop that requires high temperatures due to its tropical/subtropical origins (yyGreaves, 1996). Although studies have shown that maize is susceptible to the effects of low temperature during grain filling (Chen and Tang, 2012), cold stress mainly affects seed germination, seedling development, and growth at the seedling phase, eventually leading to reduced grain production (Li et al., 2019b). Cold damage in early spring is one of the main meteorological disasters that can befall maize production, especially in northern regions and high altitude areas in mountainous regions (Zhang, 2014).

Cold reduces both the seed germination rate and seedling vigor (Zhang et al., 2020). When maize seeds are exposed to low temperatures during the water absorption phase (imbibition), the permeability of cell membranes is impaired, resulting in the loss of cellular components (Hussain et al., 2018). Cold may also damage the ultrastructure of the embryonic root meristem cells and impair root development (Farooq et al., 2009). Maize seedling growth slows down considerably when the temperature falls below 10°C, and it ceases at temperatures between 6 and 8°C (Peng et al., 2016). The cells and tissues of the seedling can be irreversibly damaged at lower temperatures. Low temperatures during the seedling phase can reduce plant height, root length, the ability of the roots to absorb mineral nutrients, leaf chlorophyll content, and the net photosynthetic rate in seedlings. This will eventually result in growth inhibition, leaf yellowing, wilting, and necrosis, or even death of the seedlings (Chen and Tang, 2012; Yang et al., 2018). Cold stress can not only reduce the emergence rate and seedling vitality of maize directly, but can also affect plant health indirectly by increasing the chances of infection by soil bacteria (Juurakko et al., 2021). These direct and indirect impacts can both acutely reduce the yield and quality of the maize crop. Improving cold tolerance in maize will allow early sowing to withstand cold snaps that occur in early spring. Early sowing can result in an extended vegetative period, thus allowing the plants to accumulate additional biomass (Aydinoglu, 2020).

Detailed cold tolerance mechanisms have been investigated in other plant species, such as *Arabidopsis* [*Arabidopsis thaliana* (L.) Heynh.] and rice (*Oryza sativa* L.; Farhangi-Abriz and Torabian, 2017; Ding et al., 2019; Ritonga and Chen, 2020). In recent years, considerable progress has been made in elucidating the mechanisms by which maize responds to cold stress. Although three very recent reviews have discussed the efficacy of genetic and genomic approaches used to assess maize cold tolerance (Frascaroli and Revilla, 2018; Sowiński et al., 2020; Gillani et al., 2021), a comprehensive review of this subject is still lacking. Here, we have summarized the recent progress in molecular, physiological, genetic, and genomic analyses of cold tolerance in maize in order to provide a theoretical basis for molecular breeding of cold tolerance in maize.

THE COLD-RESPONSIVE MOLECULAR NETWORK IN *ARABIDOPSIS*

The cold-responsive pathways of *Arabidopsis* have been the subject of intensive studies. Briefly, when *Arabidopsis* plants are exposed to cold stress, some cold-responsive inorganic substances activate transcription factors (TFs) through signal transduction pathways. Activated TFs bind to cis-elements present in downstream cold-responsive genes to activate their expression and induce cold tolerance in the plants (Li et al., 2019a). The signal pathway with CBF (C-repeat-binding factor) transcription factors as the core elements is mainly involved in regulation of the cold response (Rihan et al., 2017; shown in **Figure 1A**). There are three members of the CBF gene family in *Arabidopsis*: *CBF1/DREB1B* (*dehydration responsive element binding factor 1B*), *CBF2/DREB1C*, and *CBF3/DREB1A* (Liu et al., 2018). Overexpression of *AtCBF1*, *AtCBF2*, and *AtCBF3* in *Arabidopsis* and other plant species is reported to significantly improve plant cold tolerance (Liu et al., 2018, 2020; Ding et al., 2019). The *CBF* genes have been cloned from other plant species such as rice, *Brassica rapa*, wheat and maize, indicating that the *CBF* genes show an important association with the cold response in plants (Liu et al., 2018, 2020; Ding et al., 2019).

Overexpression of *CBF* genes significantly induces the expression of *COR* (*Cold-regulated*) genes in plants (light green ellipses in **Figure 1A**). *COR* refers to a class of genes regulated by cold stress, such as *LTI* (*LOW TEMPERATURE INDUCED*) and *KIN* (*COLD INDUCIBLE*). Some of these genes encode key enzymes involved in synthesizing osmotic substances, and are associated with the accumulation of cryoprotective proteins and soluble sugars to normalize cellular osmotic pressure and provide protection from freezing damage (Shi et al., 2018; Ding et al., 2019; small dots in **Figure 1A**). The CBF protein recognizes CRT/DRE (C-repeat/ Dehydration Responsive Element) motifs in the promoters of *COR* genes and induces their expression to enhance cold tolerance (Lu et al., 2017). The removal of the CBF-CRT/DRE regulatory module of AtCBF2 protein reduces the level of cold resistance in plants (Park et al., 2015).

The expression of *CBF* genes is regulated by both transcriptional activators and repressors (indicated by arrows and lines with a bar in **Figure 1A**). The positive regulatory genes include *AtICE1* (*inducer of CBF expression 1*), *AtICE2*, and *CAMTA* (*calmodulin-binding* transcription *activator*; Chinnusamy et al., 2007; Ding et al., 2015). The transcriptional inhibitors which are directly involved in inhibiting cold-induced CBF activation include AtMYB15 and the AtPIFs (phytochrome interacting factors; Shi et al., 2018; red lines with a bar in **Figure 1A**). The AtICE1 protein interacts with the MYC-binding sequences present in the promoters of *CBF* genes to increase their expression during cold stress (Chinnusamy et al., 2003). Over-expression of *AtICE2* has been shown to improve cold tolerance and the expression of *AtCBFs* in *Arabidopsis* (Fursova et al., 2009). The CAMTA3 protein binds to a CAMTA DNA regulatory motif, vCGCGb, located in a region of the promoter of *AtCBF* genes and functions together with CAMTA1 and CAMTA2 to promote the expression of *CBF* genes and cold tolerance in plants (Doherty et al., 2009; Kim et al., 2013). AtMYB15 and AtICE1 form a protein complex that binds to MYB recognition sites in the promoters of *CBF* genes. Overexpression of *AtMYB15* results in reduced expression of *CBF* genes, whereas loss-of-function of *AtMYB15* leads to increased expression of *CBF* genes (Agarwal et al., 2006). Furthermore, it has been recently discovered that redox changes mediated by cold stress can induce structural transformations and functional activation of CBF proteins (Lee et al., 2021).

At present, the ICE-CBF-COR cold responsive pathway is a well-accepted defense mechanism to cope with cold stress, but only some of the *COR* genes are regulated by CBF (Park et al., 2015). Some other transcription factors, including HSFC1 (Heat shock transcription factor C1), AtZAT10, and AtZAT12, are also capable of inducing *COR* gene expression under cold stress, and may co-regulate cold signal transduction with CBF

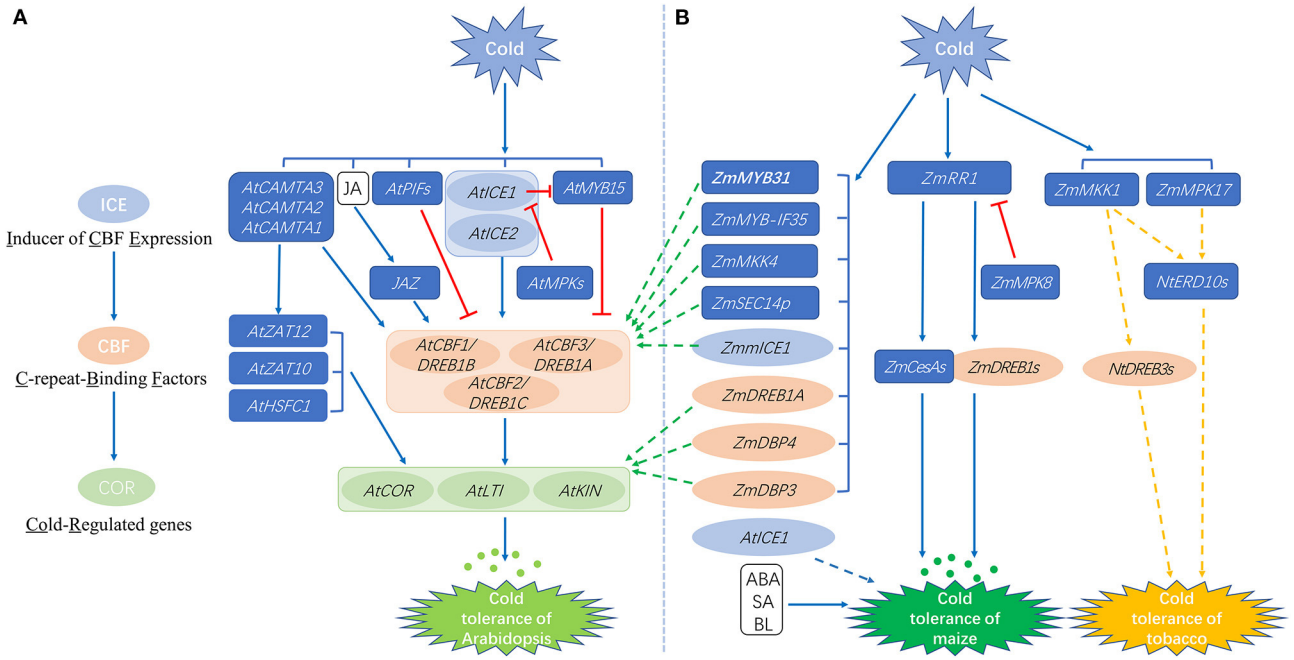

FIGURE 1 | Schematic diagram of the cold-responsive molecular networks in Arabidopsis **(A)** and maize **(B)**. The eight green dashed arrows that point to **(A)** from **(B)** indicate that the maize genes were functionally verified in transgenic *Arabidopsis* plants. The orange dashed arrows in **(B)** indicate that the maize genes were functionally studied in tobacco. The blue dashed arrow in **(B)** indicates that the Arabidopsis gene *AtICE1* was transformed into forage maize. The colored ellipses represent molecular elements that belong to the ICE-CBF-COR pathway. Small dots represent osmotic substances. Brackets encompass genes with the same induction level. Straight and dashed arrows represent positive regulation, whereas lines ending with a bar represent negative regulation. At, *Arabidopsis thaliana* L.; Zm, *Zea mays* L.; Zmm, *Zea mays* ssp. mexicana L.; Nt: *Nicotiana tabacum* L.; CAMTA, Calmodulin-binding transcription activator; ZAT, Zinc-finger transcription factor; HSFC, Heat shock transcription factor C; JAZ, Jasmonate ZIM-domain; DREB, Dehydration responsive element binding factor; LTI, Low temperature induced; KIN, Cold inducible; MPK, Mitogen-activated protein kinase; SEC14P, Sec14-like protein; RR, Response regulator; DBP, Dehydration responsive element binding protein; CesA, Cellulose synthase; MKK, Mitogen-activated protein kinase kinase; ERD, Early response to dehydration; JA, Jasmonic acid; ABA, abscisic acid; SA, Salicylic acid; BL, Brassinolide.

(Park et al., 2015). Plant hormone JA (jasmonic acid) also positively regulates the CBF signal by mediating the interaction between the JAZ1/4 protein and ICE1/2, thereby regulating the transcriptional activity of ICE proteins and the expression of *CBF1-3* (Hu et al., 2013). Taken together, these findings indicate that the cold regulatory network is very complicated in its functioning and needs to be seen in a broader sense so that it can be better understood.

THE COLD-RESPONSIVE MOLECULAR NETWORK IN MAIZE

The ICE-CBF-COR pathway has also been investigated in maize, in order to understand its involvement in cold tolerance in maize (as shown by ellipses in **Figure 1B**). However, the functional verification of most of these maize genes, as well as other cold-responsive genes, were determined by expression in heterologous species such as *Arabidopsis* or tobacco, and not in maize (green and orange arrows in **Figure 1B**). Four maize *CBF* TF genes, including*ZmDREB1A, ZmDREB2A*, *ZmDBP3*, and *ZmDBP4*, are induced by cold stress in maize. Overexpression of *ZmDREB1A*, *ZmDBP3*, and *ZmDBP4* in *Arabidopsis* induced the overexpression of cold-inducible genes, resulting in enhanced cold tolerance (Qin et al., 2004; Wang and Dong, 2009; Wang et al., 2011). The overexpression of *ZmDREB2A* was found to enhance thermotolerance, but not cold tolerance (Qin et al., 2007). A low temperature-associated gene, *ZmmICE1*, has been isolated from *Zea mays ssp. mexicana* L., a close relative of maize, and ectopic expression of *ZmmICE1* in the *Arabidopsis ice1-2* mutant was observed to be associated with enhancing plant cold tolerance (Lu et al., 2017). When transformed with the *Arabidopsis AtCBF1* gene, plants of the forage maize line SAUMZ1 showed reduced relative electrolyte leakage compared to wild type plants, and this resulted in improved cold tolerance (Xiang et al., 2012; blue dashed arrow in **Figure 1B**). These studies indicate that ICE-CBF-COR is a conserved pathway in various plant species. Other than the *ICE* and *CBF* genes reviewed above, there are many other regulators, such as transcription factors and protein kinases related to cold tolerance, that have been functionally characterized (Weckwerth et al., 2015; Erpen et al., 2018; Kimotho et al., 2019; Wang and Fu, 2019).

ZmMYB Family Genes

The MYB family is one of the largest TF families in plants, and it was named for the conserved MYB domain that is present in all eukaryotic MYB TFs (Katiyar et al., 2012). MYB TFs play important roles in the tolerance to various stresses in plants, including cold stress (Wu et al., 2019). The *Arabidopsis CBF* gene promoters contain a MYB recognition sequence and can be activated by MYB TFs (Li et al., 2019a). Unlike *AtMYB15* in Arabidopsis, which is a negative regular of *CBF* gene transcription, two characterized maize MYBs are positive regulators of *CBF* genes (**Figure 1B**). Expression of the maize *ZmMYB31* gene, which encodes an R2R3-MYB transcription factor, is induced at low temperature. Overexpression of *ZmMYB31* in *Arabidopsis* was found to upregulate the expression of *Arabidopsis CBF* genes, thereby enhancing the resistance of transgenic *Arabidopsis* plants to low temperature and oxidative stress (Li et al., 2019a). Meng and Sui (2019) isolated another nucleus-located R2R3-MYB transcription factor, ZmMYB-IF35. Low temperature induced the expression of *ZmMYB-IF35* in the cold-tolerant maize inbred line M54. Transgenic *Arabidopsis* plants overexpressing *ZmMYB-IF35* showed improved cold tolerance, higher antioxidant enzyme activity, reduced levels of reactive oxygen species (ROS), and less ion leakage. *ZmMYB-IF35* expression also positively regulated the expression of stress-related genes such as *AtCBF2*, *AtCBF3*, *AtCOR1*, and *AtCOR2*. Similar to the functions of maize *ZmMYB* genes, overexpression of a rice R2R3-MYB gene, *OsMYB2,* was found to improve cold tolerance, indicating that OsMYB2 is also a positive regulator of cold tolerance in rice (Yang et al., 2012). Further analysis of the expression patterns of 46 *ZmMYB* genes under different abiotic stress showed that 22 of these genes respond to different stress treatments. Among them, there were six *ZmMYB* genes that responded to cold treatment, but only *ZmMYB53* expression was exclusively induced by cold stress (Chen et al., 2018).

Protein Kinase Family Genes

MAPK or MPK (mitogen-activated protein kinases) proteins, a family of serine/threonine protein kinases, are involved in many important processes including stress signal transduction and development (Kong et al., 2013). The MAPK cascade pathway is a ubiquitous signal transduction module in eukaryotes that transmits biological signals from receptors to target molecules through a variety of intracellular and extracellular routes (Moustafa, 2014). The MAPK cascade component involves a three-kinase module, namely MAPK, MAPKK (MAPK kinase), and MAPKKK (MAPKK kinase; Xiang et al., 2021). The MAP kinase cascade can phosphorylate AtICE1 to promote its degradation, and thus is involved in the regulation of cold tolerance in *Arabidopsis* (Zhao et al., 2017; **Figure 1A**). In maize leaves, *ZmMPK5* was found to be involved in the recovery of plants from cold stress (Berberich et al., 1999). Kong et al. (2011) isolated *ZmMKK4*, a group C MAPKK gene, from the root system of maize variety "Zhengdan 958," and expression of the *ZmMKK4* transcript was found to be up-regulated by low temperature exposure. Overexpression of *ZmMKK4* in *Arabidopsis* increased the plants' cold tolerance, which showed that *ZmMKK4* is a positive regulator of cold tolerance in maize (indicated by the green arrow in **Figure 1B**). Pan et al. (2012) identified *ZmMPK17*, a group D MAPK gene, that was induced by cold stress. The overexpression of *ZmMPK17* enhanced cold tolerance in tobacco (*Nicotiana tabacum* L.) plants by affecting the antioxidant defense system. Cai et al. (2014) isolated and identified *ZmMKK1*, a group A MAPKK gene, from "Zhengdan 958". Ectopic expression of *ZmMKK1* in tobacco enhanced its cold tolerance, suggesting that *ZmMKK1* is also involved in the response of plants to low temperature.

A very recent study identified a new cold regulation pathway, ZmMPK8-ZmRR1-ZmDREB1.10/ZmCesA2, in maize (Zeng et al., 2021; **Figure 1B**). The *ZmRR1* (*type-A Response Regulator 1*) transcript level is slightly decreased, whereas the ZmRR1 protein level is increased by cold treatment of maize seedlings at 4°C. Overexpression of *ZmRR1* leads to enhanced cold tolerance by accumulating and inducing the expression of the downstream genes, *ZmDREB1.10* and *ZmCesA2* (*Cellulose synthase 2*), suggesting that *ZmRR1* acts as a positive regulator of maize cold tolerance. The ZmMPK8 protein, a negative regulator of cold tolerance, phosphorylates ZmRR1 at Ser15. A natural variation of *ZmRR1* with a 45-bp deletion that encompasses Ser15 prevents its phosphorylation by ZmMPK8. At present, the ZmMPK8-ZmRR1-ZmDREB1.10/ZmCesA2 pathway is the best characterized cold regulation pathway in maize, and it provides an in-depth understanding of the molecular mechanism underlying cold tolerance in maize.

To date, functional investigations of many cold responsive genes have been performed in maize, but the functions of many of these genes were studied by overexpression in model plant species. As shown by the green and orange dashed arrows in **Figure 1B**, a total of eight maize genes were transformed into *Arabidopsis*, and two maize genes were transformed into tobacco. Only one study investigated the molecular function of *ZmRR1*, *ZmMPK8*, and their downstream genes *ZmDREB1.10* and *ZmCesA2*, by either mutating or over-expressing the corresponding genes in maize. Transformation of maize genes into maize plants not only provides stronger evidence for gene functions, but also enables the straightforward elucidation of molecular pathways to provide in-depth biological insights. Furthermore, such native transformation would provide potential genetic resources for improving cold tolerance in maize.

PHYSIOLOGICAL ACCLIMATION AND SEED TREATMENTS TO RELIEVE DAMAGE IN MAIZE DUE TO COLD STRESS

Cold stress can induce a series of physiological responses in maize, such as the adjustment of osmotic substances, accumulation of ROS, disruption of hormonal homeostasis, impaired uptake of mineral nutrients, and a decrease in photosynthesis. To survive under such unfavorable conditions, plants need to maintain cellular function and integrity by stabilizing the cell membranes and biologically active proteins in order to sustain basic physiological activities (Ritonga and Chen, 2020). Even though plants have developed mechanisms by which they can to acclimate to cold temperatures, several

strategies have been developed to reduce the effects of cold stress on maize seedlings.

Adjustment of Osmotic Substances

Osmotic adjustment is one of the most important physiological mechanisms employed by plants to cope with many types of stresses. Under adverse conditions, osmotic adjustment substances help to maintain cell turgor and the capacity of tissues to retain water, and the contents of osmotic adjustment substances are positively correlated with plant stress resistance (Farhangi-Abriz and Torabian, 2017). The accumulation of osmotic adjustment substances can reduce the water potential in plant cells, prevent water outflow, and at the same time protect the structure of macromolecules in the cell (Li et al., 2013). The main osmotic adjustment substances in plants include soluble sugars, soluble proteins, and free proline (Zhang, 2014).

Soluble sugars are strongly hydrophilic, and they can reduce the stress damage to plant cells by reducing the water potential and maintaining the activity of some biological macromolecules (Guan et al., 2009). Soluble proteins can scavenge ROS (reactive oxygen species) and stabilize cell membrane structure (Nadarajah, 2020). Proline is a hydrophilic amino acid, and free proline functions to stabilize the metabolic process in the cytoplasm (Guan et al., 2009). Ma et al. (2015a) found that cold stress induces the expression of *ZmP5CS1*, a key gene for proline synthesis in immature maize embryos. Some studies have reported that low temperature treatments increase the contents of soluble sugars, proline, and soluble proteins in the seedlings of many maize varieties. The relative increases were larger in the varieties with strong cold tolerance compared to the varieties with relatively weaker cold tolerance. The lower the temperature, the more significant the difference between varieties (Li and Fang, 2018; Zhao et al., 2020). In conclusion, cold tolerant maize can maintain the water absorption capacity of cells and can reduce cold damage by accumulating higher levels of osmotic adjustment substances in response to cold stress.

Antioxidant Enzyme System and MDA Production

Cold stress can destroy cellular homeostasis and cause the accumulation of high levels of ROS in plant cells. Excessive levels of ROS can be harmful to the cell membrane system because of lipid peroxidation (Nadarajah, 2020). Plant cells can induce the expression of a protective system to resist the threat posed by ROS. The protective enzymes within a plant cell, such as SOD (superoxide dismutase), CAT (catalase), POD (peroxidase), and APX (ascorbate peroxidase), cooperate with one another to scavenge the reactive oxygen free radicals so as to maintain normal physiological metabolic activities as much as possible to avoid damage to cell components (Nadarajah, 2020). Maize cells can stabilize the membrane structures and reduce cell damage by constantly oxidizing the reactive oxygen free radicals. Many studies have shown that cold stress enhances the activities of SOD, POD, and CAT in maize. The activities of the three main antioxidant enzymes and the relative expression of related genes are positively correlated with the cold tolerance of inbred lines (Wei et al., 2014; Yang et al., 2016; Li and Fang, 2018). For example, an early study showed that low temperature induces the expression of the *APX* gene, and the APX activity in cold tolerant maize was found to be higher than in cold sensitive maize (Pinhero et al., 1997). One study showed that the activities of protective enzymes decreased in the three cold-tolerant and three cold-sensitive maize inbred lines under cold stress, but the relative decrease in the cold-tolerant inbred lines was less than that in the cold-sensitive inbred lines (Peng et al., 2016). These differences could be due to many reasons, but we can conclude that the antioxidant enzyme system in cold-resistant maize lines is generally stronger than that of cold-sensitive lines.

MDA (malondialdehyde) is the main product of peroxidation of polyunsaturated cell membrane lipids and is a marker for oxidative stress. MDA is a highly reactive compound that can restrain the activity of cell protective enzymes and aggravate membrane peroxidation. When the cell membrane is damaged, a large number of electrolytes flow out of the cell, resulting in a surge of electrolytes (Wang et al., 2006). Therefore, relative conductivity and MDA levels are negatively correlated with cold tolerance in maize lines (Zhang, 2014). Several studies have reported that with increasing time of exposure to cold, the relative conductivity and MDA content increases. The cell membranes of cold tolerant varieties were less damaged, and the relative conductivity and MDA contents increased to a lesser extent in maize lines that show strong cold tolerance (Zhang, 2014; Peng et al., 2016).

Plant Hormones

Plants tightly regulate the levels of some hormones to cope with the changing environment (Lamers et al., 2020). ABA (abscisic acid) is an abiotic stress hormone that can improve the stress resistance of plants, and it participates in the regulatory response for a variety of abiotic stresses, including cold (Qin et al., 2021). The ABA contents in the roots of cold-tolerant lines were significantly higher than in the roots of cold-sensitive lines. Exogenous ABA treatment can promote the ability of maize seeds to germinate at low temperature (Zhang, 2011). GA (gibberellic acid) is also considered to play a role in plant cold resistance, but its effect is not as obvious as that of ABA (Eremina et al., 2016; Rihan et al., 2017). Studies have shown that the GA and IAA (indole-3-acetic acid; auxin) contents in the roots of maize seedlings decrease under cold stress, while the ABA content gradually increased (Janowiak et al., 2002; Zhang, 2011; Wei et al., 2014).

SA (salicylic acid) is another important plant hormone that is involved in cold tolerance in several plant species including maize, and its direct physiological effect is the change in the antioxidant enzyme activity of plants (Farooq et al., 2009). At low temperature, the increase in endogenous SA biosynthesis is closely related to the increase in antioxidant enzyme activity in maize seeds and during seedling growth (Wang et al., 2013). Cold stress in maize seedlings was not moderated by treating the seeds with SA (Gómez-Muñoz et al., 2018), but SA+H_2O_2 treatment at low temperature shortened seed germination time, improved seedling vigor, reduced cold damage to maize seeds, increased the activities of antioxidant enzymes and the expression levels of the corresponding genes, and thus improved cold tolerance (Li et al.,

2017). Exogenous application of BL (brassinolide) also increased the germination rate, reduced cold damage to maize seedlings and caused an increase antioxidant enzyme activity, all of which resulted in an increase in plant biomass (Sun et al., 2020). Thus, manipulation of plant hormones appears to be an efficient way to alleviate the damage caused by cold temperature and to ensure the growth of seedlings at later stages of development.

Mineral Nutrients

Cold tolerance in plants is highly correlated with mineral nutrient levels (Waraich et al., 2012). The inhibition of growth in maize from cold stress is at least partly caused by indirect damage due to the decreased uptake of nutrients from the soil (Gómez-Muñoz et al., 2018). For example, the absorbance of K and Pi (orthophosphate) by maize roots is particularly affected by cold soil temperature (Bravo et al., 1981). Seed treatment with Mn/Zn can mitigate the negative effects of cold stress, and it resulted in increased biomass production in high-P soil but not in low-P soil, indicating that the absorbance of P is relieved by Mn/Zn treatment (Gómez-Muñoz et al., 2018). In addition, both Mn and Zn are key co-factors of several enzymes that are involved in the detoxification ROS caused by cold stress. Application of Si (silicon) is also a useful strategy to improve cold tolerance in maize seedlings during the early growth stage. The beneficial effects of Si seed treatments included the restoration of hormonal balances that were disrupted by cold stress and maintaining homeostasis of other micronutrients (Moradtalab et al., 2018). Therefore, appropriate plant nutrition is a useful strategy to alleviate the cold stress.

Photosynthesis

Cold stress significantly decreases photosynthesis in maize (Fracheboud et al., 2002). This decrease in photosynthesis may result from many factors, such as impaired chloroplast development, changes to the pigment composition, damage of the PSII reaction centers, and reduced activity of carbon cycle enzymes. The reduced photosynthetic ability at low temperatures also reflects the cold resistance of maize (Hund et al., 2008). Fracheboud et al. (2002) found that the chlorophyll a/b ratio in the cold tolerant lines was higher than in cold sensitive lines. Duran Garzon et al. (2020) showed that cold tolerance is related to higher chlorophyll content, higher G6PD (glucose-6-phosphate dehydrogenase) activity, and a higher sucrose-to-starch ratio. Many studies have used photosynthesis-related traits to quantify cold tolerance in maize. These studies are discussed below and are given in **Tables 1**, **2**.

In general, cold tolerant maize may have stronger ability to accumulate more osmotic adjusting substances, stronger antioxidant enzyme system to stabilized ROS, stronger ability to maintain hormonal homeostasis, higher absorbance of mineral nutrient, higher photosynthetic ability, etc.

GENETIC AND GENOMIC APPROACHES TO DISSECT COLD TOLERANCE IN MAIZE

Cold tolerance in maize is a quantitative agronomic trait that is controlled by multiple genes (Turk et al., 2019). In the past few years, a number of genetic and genomic approaches have been applied to dissecting the components of this complex trait.

Quantitative Genetics

Quantitative genetic analyses of cold tolerance in maize have so far shown that heterosis, general and specific combining abilities, reciprocal maternal and non-maternal effects, additive effects, genotype, growth stage, and environmental factors are all involved in the expression of cold tolerance in maize. For example, Hodges et al. conducted two studies to explore the cold tolerance of maize at both germination and seedling stages using twelve hybrids derived from a complete diallel of four inbred lines (Hodges et al., 1997a,b). The results showed that the seed germination rate, the activities of CAT and APX in leaves at the 3-leaf stage, and the general and the special combining ability of the dry matter weight of leaves at the 4-leaf stage were significantly different. Revilla et al. (2000) proposed that cold tolerance has additive, dominant, and maternal effects. The experiment of Yan et al. (2017) showed that there was significant heterosis in maize cold tolerance at the seedling stage. Neta et al. (2020) used three cold-tolerant lines and three cold-sensitive lines to carry out partial diallel cross experiments to analyze the heterosis, general and specific combining abilities, reciprocal maternal and non-maternal effects, as well as the expression of CAT, APX, SOD and other genes. Results showed that there was heterosis and a reciprocal effect for germination under cold stress, and that the non-additive genes were more important. The genes that control cold tolerance depend on the particular materials used and the traits studied (Frascaroli and Revilla, 2018). Each growth stage of maize, including germination, emergence, and early seedling growth, is controlled by an independent genetic model; therefore, cold tolerance might also be regulated independently in the different growth stages (Frascaroli and Revilla, 2018). To make the situation more complicated, some studies have also reported that there are interactions between genes and the environment in the expression of cold tolerance in maize (Presterl et al., 2007).

The studies cited above show that the genetics of cold tolerance in maize is very complicated. However, with the broad application of genomic tools, considerable progress has been made in recent years in the identification of genetic loci and genes that are associated with maize cold tolerance. The information from these studies will facilitate a deeper understanding of the genetic mechanisms that control maize cold tolerance at the molecular level.

QTL Mapping

QTL (quantitative trait locus) mapping is a powerful tool for the identification and manipulation of loci underlying important and complex traits in agricultural crops. Recent studies have used different mapping populations, such as $F_{2:3}$ families, RILs (recombinant inbred lines), and BC (back cross) populations, to study the QTLs that control cold tolerance in maize (**Table 1**). Most of these segregating populations were constructed by crossing cold-sensitive and cold-tolerant inbred lines in order to increase the relative degree of variation in the cold-response phenotypes. Several cold responsive physiological traits, such as seed germination rate, root phenotypes, and

TABLE 1 | Quantitative trait loci (QTLs) for traits related to cold tolerance in maize.

References	Population	Number of families or inbred lines	Traits or indicators	Number of QTLs	Chr.
Fracheboud et al. (2002)	RILs (Ac7643 × Ac7729/TZ)	233	Fv/Fm, ΦPSII, SR, etc.	18	1, 2, 3, 4, 6, 7, 9
Fracheboud et al. (2004)	F2:3 (ETH-DH7 × ETH-DL3)	266	CFP, SDW, SPAD, etc.	19	1, 2, 3, 4, 6, 8
Hund et al. (2004)	F2:4 (Lo964 × Lo1016)	168	GI, Fv/Fm, RL, etc.	60	1, 2, 3, 4, 5, 6, 7, 8, 9, 10
Jompuk et al. (2005)	F2:3 (ETH-DH7 × ETH-DL3)	226	CFP, SPAD, LA, etc.	29	1, 2, 3, 4, 5, 6, 7, 8, 9, 10
Presterl et al. (2007)	DH (SL × TH)	720	LC', LP, and FD	18	1, 2, 3, 4, 5, 6, 7, 8, 9, 10
Rodríguez et al. (2008)	RILs (B73 × Mo17)	302	LC	2	3, 6
Guerra-Peraza et al. (2012)	RILs (B73 × Mo17)	295	Fq'/Fm', Fv/Fm, SPAD, etc.	19	4, 5, 6, 7, 8, 9
Rodríguez et al. (2014)	F2:3 (EP42 × A661)	210	DW, ΦPSII, TAC, etc.	4	2, 4, 8
Shi et al. (2016)	RILs (Yu82 × Shen137 and Yu537A × Shen137)	420	Gp, GI, MGT, etc.	26	1, 2, 3, 4, 5, 6, 7, 8, 9
Hu et al. (2016)	RILs (B73 × Mo17)	243	LTGR and LTPRL	12	4, 5, 6, 7, 9
Yan et al. (2017)	F2:3 (K932 × Mei C)	207	LRD, WCS, RRS, etc.	7	1, 2, 3
Li et al. (2018)	F2:3 (220 × PH4CV, 220 × Y1518 and P9-10 × PH4CV)	650	ER, GI, RL, etc.	43	1, 2, 3, 4, 5, 8, 9, 10
Yi et al. (2020)	RILs (MAGIC)	406	RLCC, Fv/Fm, SDW, etc.	62	1, 2, 3, 4, 5, 6, 7, 8, 9, 10
Goering et al. (2021)	RILs (B73 × Mo17)	97	CC, LC, and TD	2	1, 5
Jin et al. (2021)	Two genetic populations	290	PA	12	2, 3

MAGIC, Multi-parent advanced generation intercross population; DH, Doubled haploid; Fv/Fm, Maximum quantum efficiency of photosystem II; ΦPSII, Quantum yield of photosystem II; SR, stomatal resistance; CFP, Chlorophyll fluorescence parameters; SDW, Shoot dry weight; SPAD, leaf greenness; GI, Germination index; RL, Root length; LA, Leaf area; LC', Leaf chlorosis; LP, Leaf purpling; FD, Frost damage; LC, Leaf color; Fq'/Fm', Operating quantum efficiency of photosystem II; DW, Dry weight; TAC, Total anthocyanin content; Gp, Germination percentage; MGT, Mean germination time; LTGR, Low-temperature germination rate; LTPRL, Low-temperature primary root length; LRD, Leaf rolling degree; WCS, Water content in shoots and leaves; RRS, Ratio of root-to-shoot; ER, Emergence rate; RLCC, relative leaf chlorophyll content; CC, chlorophyll concentration; TD, Tissue damage; PA, Peroxidase activity; Chr, chromosome number.

TABLE 2 | SNPs linked to traits related to the bud and seedling stages in maize.

References	Population	Number of families or inbred lines	Traits or indicators genotyped SNPs	Number of	Number of SNPs	Chr.
Strigens et al. (2013)	Diversity panel	375	Fv/Fm, SPAD, LA, etc.	56,110	19	1, 4, 5, 6, 7, 10
Huang et al. (2013)	Association panel	125	RLN, RSL, RFSW, etc.	56,110	43	1, 2, 3, 4, 5, 6, 7
Yan et al. (2017)	TAMP(S-Mo17)	338	LRD, WCS, RRS, etc.	556,809	19	1, 2, 3, 4, 6, 10
Hu et al. (2017)	Association panel	282	RDT50, RGI, RGR, etc.	2,271,584	17	1, 2, 4, 6, 7, 9
Zhang et al. (2020)	Association panel	222	RGR, RGL, RRL, etc.	40,697	30	1, 2, 3, 4, 5, 6, 7, 8, 9, 10
Yi et al. (2021)	Association panel	836	DE, EV, RLCC, etc.	156,164	32	1, 2, 3, 4, 5, 6, 7, 8, 9, 10
Zhang et al. (2021)c	Association panel	300	FG, RL, RRS, etc.	43,943	15	1, 2, 3, 4, 5, 7, 8, 10
Jin et al. (2021)	Two genetic populations	290	PA	24,860,241 + 5,759,868	4	3

TAMP, Testcrossing association mapping population; Fv/Fm, Maximum quantum efficiency of photosystem II; SPAD, leaf greenness; LA, Leaf area; RLN, Relative leaf number; RSL, Relative shoot length; RFSW, Relative fresh shoot weight; LRD, Leaf rolling degree; WCS, Water content in shoots and leaves; RRS, Ratio of root-to-shoot; RDT50, Relative days to 50% Germination; RGI, Relative germination index; RGR, Relative germination rate; RGL, Relative germ length; RRL, Relative radicle length; DE, Days to emergence; EV, Early vigor; RLCC, relative leaf chlorophyll content; FG, Germination rate at 5 d; RL, Root length at 10 d; PA, Peroxidase activity; Chr, chromosome number.

seedling photosynthesis, were measured under both normal and cold conditions (**Table 1**). These studies identified many reliable molecular marker loci associated with QTLs or genes that regulate cold tolerance and could be further used for breeding of cold tolerant inbred lines or hybrids.

Fracheboud et al. (2002) performed a QTL analysis of five traits related to the function of the photosynthetic apparatus at low temperature using a set of 233 maize RILs. They identified 18 QTLs that were significantly correlated with the target traits, of which the main QTL for leaf development at low temperature was the main QTL for pigment composition. Fracheboud et al. (2004) used an $F_{2:3}$ population derived from the cross ETH-DH7×ETH-DL3 and detected 19 QTLs controlling chlorophyll fluorescence at low temperature. The major QTL for photosynthetic cold tolerance in maize seedlings is located on chromosome 6. Using the same population, Jompuk et al. (2005) detected a major QTL for cold tolerance located on chromosome 6 that corresponded to chlorophyll fluorescence and chlorophyll

content. Hund et al. (2004) used an $F_{2:3}$ population constructed from the cross Lo964×Lo1016 to map QTLs for root and shoot development in maize seedlings under cold conditions, and found a dominant QTL located on chromosome 5. Presterl et al. (2007) performed QTL mapping on 720 DH (doubled haploid) lines and found a total of 18 QTLs associated with leaf chlorosis, leaf purpling (anthocyanin), and frost damage at low temperature. Rodríguez et al. (2008) performed QTL analysis by using an IBM (intermated B73×Mo17) population. None of the QTLs was identified under normal growth conditions, but two QTLs significantly correlated with leaf color at low temperature were located on chromosomes 3 and 6, and these two OTLs explained 14.2% of the phenotypic variation and 28.2% of the genetic variation. Guerra-Peraza et al. (2012) used the IBM302 population for QTL analysis and discovered a major QTL for photosynthesis-related traits on chromosome 5. The favorable allele of this QTL was contributed by Mo17 and appeared to be the major factor that explained the differential response of B73 and Mo17 to changes in the temperature at night. Rodríguez et al. (2013) used an $F_{2:3}$ population derived from the cross of a cold susceptible (A661) with a cold tolerant (EP42) inbred line to detected genomic regions related to the cold-induced albino phenotype. A major QTL on chromosome 2 was identified that explained 14% of the phenotypic variation. Using the same $F_{2:3}$ population, 10 QTLs related to photosystem traits were identified, with six of them at normal temperatures and four under cold conditions. A parallel meta-QTL analysis identified three genomic regions that regulate the development of maize seedlings at low temperatures (Rodríguez et al., 2014). Hu et al. (2016) performed a QTL analysis using 243 IBM Syn4 RILs and detected six QTLs associated with the low-temperature germination rate and six QTLs correlated with low-temperature primary root length. Of these, four pairs of QTLs were located in the same genomic regions. Yan et al. (2017) performed a QTL analysis with seedlings from an $F_{2:3}$ population (207 lines) obtained by crossing a cold sensitive line (K932) with a cold tolerant (Mei C) inbred line. Their analysis resulted in the detection of seven QTLs controlling four cold-related traits, and one of the QTLs explained 10.55–25.29% of the phenotypic variation.

While the above-cited QTL analyses used "regular" populations, several studies have used "advanced" populations to improve the accuracy of the study. For example, Shi et al. (2016) constructed two connected RIL populations that shared one parental line and were able to identify 26 QTLs associated with seed vigor traits under low temperature conditions. Fourteen of these QTLs were further integrated into five mQTL regions through a meta-analysis. Two of the mQTL regions located on chromosomes 2 and 9 had R^2 values >10% and were previously identified as QTLs for seed vigor traits. Using two cold-tolerant and two cold-susceptible inbred lines, Li et al. (2018) generated three connected $F_{2:3}$ populations to detect QTLs related to seed germination ability at low temperature. A total of 43 QTLs and three mQTL regions were detected. Yi et al. (2020) used 406 lines from a multi-parent advanced generation intercross (MAGIC) population and found 858 SNPs grouped in 148 QTLs that were significantly associated with cold tolerance-related traits, and most of the QTLs were located in specific regions, particularly bin 10.04.

Several studies combined QTL analyses with other genetic or genomic approaches such as GWAS (genome-wide association study) or transcriptome analysis to efficiently uncover further biological insights. From the same population used by Hu et al. (2016), Goering et al. (2021) selected a panel of 97 lines for QTL analysis of traits including chlorophyll concentration, leaf color, and tissue damage at low temperature. Two cold-related QTLs with high additive impact were detected. These authors further verified the two QTLs using transcriptome data and identified 13 candidate genes likely to be involved in controlling the cold responses. Recently, Jin et al. (2021) studied cold responses in maize using a joint analyses that combined QTL and GWAS. They first performed QTL mapping for POD activity using an $F_{2:3}$ population (210 lines) derived from cold-tolerant (W10) and cold-sensitive (W72) lines, and detected 12 QTLs significantly associated with POD activity and cold tolerance. They then conducted GWAS on a natural population consisting of 80 backbone inbred lines and found that four SNPs were significantly associated with POD activity at low temperature (**Table 2**). Using a joint analysis of the QTL and GWAS results, *Zm00001d002729* was determined to be a potential cold tolerance gene. Overexpression of *Zm00001d002729* increased the cold tolerance of maize seedlings by increasing POD activity and decreasing the MDA content and relative conductivity (ion leakage).

Table 1 shows that the genomic loci involved in the regulation of cold tolerance are distributed on almost all of the maize chromosomes, but most of these QTLs have not been further fine mapped or functionally characterized as cold tolerant genes, which prevents more detailed studies of cold tolerant mechanism in maize. One major reason for this may be the huge amount of work involved in gene mapping in large populations. In recent years, joint analysis using advanced populations or genomic tools has become increasingly popular to narrow down the search for candidate genes. Such an approach was recently used successfully to identify the major-effect cold regulating gene *Zm00001d002729* (Jin et al., 2021), suggesting that it can be more widely applied in other studies.

GWAS

Compared to biparental segregating populations, natural populations consisting of several hundred maize inbred lines have also been used for the genetic analyses of cold tolerance in GWAS (Frascaroli and Revilla, 2018). Although most of the cold-responsive physiological traits used in GWAS were similar to those in QTL analyses, including leaf color, root length, and seed germination-related traits (**Tables 1**, **2**), GWAS offers increased mapping resolution and accuracy due to the higher level of genetic diversity in the mapping populations (Lipka et al., 2015).

Strigens et al. (2013) conducted the first GWAS in maize to dissect traits related to cold tolerance. Using a maize germplasm collection of 375 inbred genotypes with 56K SNPs (single nucleotide polymorphisms) GWAS identified 19 significant association signals that explained between 5.7 and 52.5% of the

phenotypic variance for cold-related traits such as early growth and chlorophyll fluorescence. Huang et al. (2013) used 125 maize inbred lines to perform GWAS on 10 traits related to cold tolerance at the germination and seedling stages. A total of 43 SNPs were detected that were associated with cold tolerance and 40 candidate genes were predicted based on 31 of these SNPs. Yan et al. (2017) crossed the cytoplasmic male sterile parental line S-Mo17 with 338 different inbred lines to generate a test cross association mapping population. GWAS was performed on these 338 test crosses, and 19 significant SNPs associated with cold tolerance-related traits were detected. Hu et al. (2017) conducted GWAS on seed germination traits using 282 inbred lines of maize under normal and low temperature conditions. A total of 17 associated SNPs related to cold tolerance were identified, and seven of the SNPs were located in candidate genes. In a population of 222 maize inbred lines, Zhang et al. (2020) used GWAS to identify 30 SNPs related to cold tolerance during maize seed germination. Fourteen candidate genes directly related to the SNPs were found and further verified by gene expression analysis. Zhang et al. (2021) studied germination-related traits in 300 inbred lines under low temperature conditions. GWAS analysis revealed a total of 15 significant SNPs, and three genomic loci were repeatedly associated with multiple traits. Yi et al. (2021) evaluated a large panel of 836 maize inbreds, and GWAS analysis uncovered a total of 187 significant SNPs that could be integrated into 159 genomic regions that controlled seed emergence and traits related to early growth.

Despite its higher genetic mapping resolution, GWAS has not been without controversy. In particular, many of the cold-associated SNP markers identified in these studies were found to be located in the non-coding regions and generally thought to function in the regulation of gene expression. But which gene(s) do they regulate? There are large structural variations present in the genomes of different maize inbred lines, and the genetic loci predicted in the B73 genome may not represent all the genetic loci in other inbred lines. Hence, there may be some other unpredicted genes near or even located on cold-associated SNP markers, leading to inaccurate results.

Transcriptomic Analyses

Transcriptome analyses are also widely used to understand the molecular responses of maize to cold stress and to mine for cold tolerant genes. Li et al. (2016) used RNA-seq analyses to compare the transcriptomes of a freezing tolerant (KR701) and a freezing sensitive line (Hei8834) before and after cold treatment at the seedling stage and identified 948 DEGs (differentially expressed genes). GO (Gene Ontology) analysis revealed that the terms "binding functions," "protein kinase," and "peptidase activity" were over-represented in the DEGs. Li et al. (2019b) used RNA-seq to analyze the gene expression of cold tolerant (M54) and cold sensitive (753F) inbred lines under cold stress at the seedling stage. More DEGs were found in M54 than in 753F after both 4 and 24 h of cold treatment, indicating that the cold-responsive signaling networks were more active in the cold tolerant line. Li et al. (2020) analyzed the transcriptome of maize B73 seedlings under different low temperature conditions. In this study, 5,358, 5,485, and 5,312 DEGs were detected in response to cold stresses of 4, 10, and 16°C, respectively, and the expression of five genes including Zm*DERB1* was significantly up regulated. Frey et al. (2020) selected 21 DH lines from a DH population (flint landrace "Petkuser Ferdinand rot") based on their cold tolerance; 11 lines were cold resistant and 10 were cold sensitive. The transcriptomes of the 21 DH lines were analyzed after control and cold treatments. Here, 148, 3,254, and 563 DEGs were found to be related to cold treatment, cold tolerance, and growth rate at low temperature, respectively. Zhang et al. (2020) used RNA-seq to verify the correlation between the candidate genes and low-temperature tolerance and found 10 DEGs that were located in the linkage disequilibrium region of a GWAS analysis. Of these genes, two of them appear to regulate cold signal transduction and cell membrane fluidity. Li et al. (2021) analyzed transcriptomic changes in seeds of three sweet corn NILs and their parents under cold stress. A total of 20 DEGs were found to be highly related to low-temperature germination, and a gene encoding UDP-glucosyltransferase was hypothesized to be essential to cold germination in sweet corn. Waititu et al. (2021) conducted a comparative analysis of the transcriptomes of seedlings of 24 cold-tolerant and 22 cold-sensitive inbred lines under cold stress. A total of 2,237 DEGs were identified, which included 147 TFs belonging to 32 families such as MYB, ERF, NAC, WRKY, bHLH, MIKC MADS, and C2H2. Yu et al. (2021) studied the leaf transcriptomic response of two maize inbred lines with contrasting cold tolerance levels under a time series cold treatment. The results showed that cold tolerance in line B144 is due to active mediation of stomatal opening and protection of photosystem II from photooxidation by upregulating the expression of genes for D1 proteins, while the sensitive line Q319 was unable to close its stomata in response to cold.

In addition to RNA-seq analyses, other genomic tools have also been used to study the transcriptomic changes in maize to cold. Rodríguez et al. (2013) used microarray hybridization on the chlorophyll-less and chlorophyll-containing sections of leaves of maize inbred line A661 which shows a cold-induced albino phenotype. A total of 1,002 differentially expressed transcripts were identified between the two sections, and these DEGs were classified into 23 categories including genes in the tetrapyrrole biosynthesis pathway and photosynthesis. Di Fenza et al. (2017) conducted microarray analysis with four varieties, with two cold-tolerant and two less cold-tolerant lines, to identify genes that were differentially expressed under chilling conditions. A total number of 64 DEGs were identified in the two chilling-tolerant varieties, while no significant changes in expression were observed in less cold-tolerant lines. Another study used cDNA-AFLP to analyze the gene expression changes in response to cold stress and identified three maize genes, *ZmMAPKKK*, *ZmCLC-D*, and *ZmRLK*, that were possibly involved in the cold response (Yang et al., 2011).

To investigate the regulatory roles of miRNAs (microRNAs) in cold tolerance, Aydinoglu (2020) studied the miRNome (miRNA microarray) in seedlings of the maize hybrid ADA313 that were treated with cold temperature. In this study, 24, 6, and 20 miRNAs were specifically differentially regulated in the meristem, the elongation zone, and the mature zone by cold stress, respectively. This study highlighted the importance of

miRNAs in the maize response to cold stress. Combined with large-scale bioinformatic analysis, Zhou et al. (2022) examined the transcriptome changes in seedlings of the inbred lines B73, Mo17, and W22 and the F_1 hybrids with all three combinations (B73xMo17, W22xB73, and W22xMo17) in response to cold or heat stress. They identified many stress-related DEGs among the different maize genotypes and assigned these expression changes to cis-or trans-regulatory mechanisms using the F_1 hybrid data. Their study answered the question of how sequence differences in cis-elements of different genotypes impact a gene's responsiveness to stress, and shed light on the prediction of a plant response to stress by implementing a more sophisticated model construction (convolutional neural networks).

Above transcriptomic analyses indentified tens to thousands of DEGs in individual studies. This astonishing variaiton in the numbers of DEGs indicates that a vast majority of the DEGs can not overlap to each other, leaving an almost impossible task to mine the conserved overlaping genes. In deed, when Sowiński et al. (2020) surveyed 13 independent studies addressing the transcriptomic changes in response to cold stress in maize seedlings at the V2-V5 stages, they found <500 DEGs were reported in more than one study and the rest are specific to individual studies. Furthermore, among the 13 independent studies survied, four of these 13 studies used moderate low temperature stress and the other nine used severe cold stress. By comparing the DEGs, it was found that the transcriptomic changes that occur in response to moderate low tempreture and severe cold stress are fundamentally different (Sowiński et al., 2020). Taken together, the variation in gene expression in different studies can be caused by the genotypes, tissue, developmental stage, experimental design and the strength of cold. Although the results of individual studies varied considerably, the "common genes" shared by several independent projects after carefully eliminate the differences can be attractive candidates for further functional studies.

LOCAL AND LONG-DISTANCE TRANSMITANCE OF COLD SIGNALS

All of the above studies extensively investigated the "responses" of maize to cold, but how does maize actively perceive cold and transmit these signals to other parts of the plant? In rice, the OsCOLD1 protein was found to localize to the endoplasmic reticulum and plasma membrane and promote GTPase activity by interacting with a G-protein. In response to cold stress, COLD1 changes membrane fluidity to mediate extracellular Ca^{2+} influx and cytosolic Ca^{2+} concentration, and the altered Ca^{2+} concentration acts as a secondary messenger to regulate downstream genes (Ma et al., 2015b). Thus, COLD1 is thought to be the first cold sensor identified in plants (Shi and Yang, 2015). In maize, ZmSEC14p, a Sec14-like protein, was found to regulate the expression of phosphoinositide-specific phospholipase C in the phosphoinositide signal transduction pathway, which generates the second messengers inositol 1,4,5-trisphosphate and 1,2- diacylglycerol for downstream signal transduction (Wang et al., 2016). Over-expression of *ZmSEC14p* results in the up regulation of some cold-responsive genes such as *CBF3*, suggesting its important role in cold signal transduction.

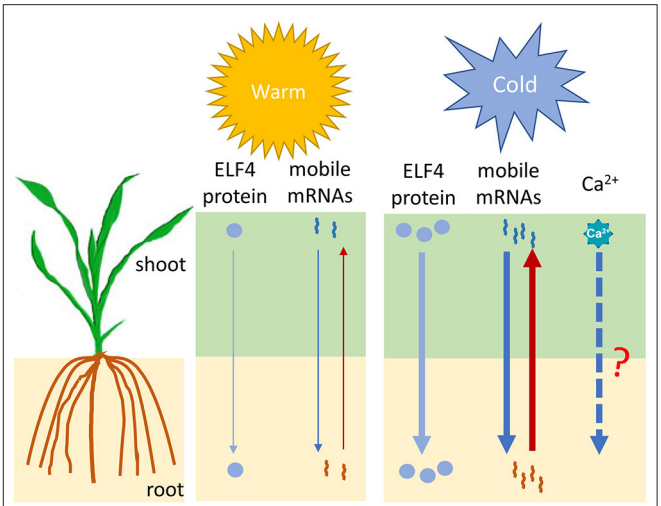

FIGURE 2 | A proposed model for the long-distance regulation of cold signaling by the ELF4 protein and mobile mRNAs. Full and dashed arrows indicate known and potential long-distance signaling molecules, respectively. The thickness of the arrows indicates the relative strength of the signal. ELF4, Early flowering 4.

The above studies, together with the findings in the ICE-CBF-COR pathways, lay important foundations for understanding the cold signal transduction pathway within a local cell; however, the way in which plants transmit cold signals to other parts of the plant is largely unknown. One of the possibilities involves the long-distance signaling system. The phloem sap contains plant hormones, small peptides, transcription factors, and various types of RNA molecules (mRNAs, miRNAs, and tRNAs) that can travel long distances to the other parts of the plant. These molecules, as potential signaling factors, regulate plant growth and development and the stress response (Xia et al., 2018). Wang et al. (2020b) developed a watermelon–bottle gourd heterografting system to identify the mobile mRNAs under both control and cold conditions. Their results showed that cold stress significantly enhances the mobility of mRNAs in the phloem (**Figure 2**). In particular, some of the scion-delivered mobile mRNAs in the rootstock are derived from some well-known genes related to osmotic adjustment and cold tolerance, while mRNA that moves in the opposite direction includes transcripts from genes related to ABA-activated signaling. In another study, Chen et al. (2020) found that the *Arabidopsis* clock component ELF4 (EARLY FLOWERING 4) protein showed long-distance movement from the shoot to the root through the vascular system to control the root clock in a temperature-dependent manner, and low temperatures favored ELF4 mobility (**Figure 2**). Hence, ELF4 has been suggested to a mobile long-distance cold signal that establishes a shoot-to-root communication for temperature information. In addition to ELF4 protein and mobile mRNAs, Ca^{2+} has also been implicated as a long-distance signal for water

trafficking and defense signaling (Shkolnik et al., 2018; Toyota et al., 2018). A previous study showned that the AtMIZ1 (Mizu-Kussey 1) protein regulates AtECA1 (ER-type CA^{2+}-ATPase 1) to generate the long-distance phloem-mobile Ca^{2+} signal for water trafficking (Shkolnik et al., 2018). Interestingly, both *AtMIZ1* and *AtECA1* were found to be responsive to cold treatment (He et al., 2016; Sharma et al., 2018). Despite the involvement of Ca^{2+} in local cold signaling, it is unknown whether Ca^{2+} also functions as a long-distance cold signal through the MIZ1-ECA1- Ca^{2+} pathway (**Figure 2**). These studies clearly show that long-distance signaling is involved in the response to cold. One very recent study shows that monocotyledonous plants are also capable of being grafted (Reeves et al., 2021), and discoveries related to cold-induced long-distance signaling in maize can be expected soon through use of this powerful method.

CONCLUSION

Cold tolerance is an important breeding objective in the pursuit of high productivity and better environmental compliance. Cold tolerance research in maize is of great significance to stabilize yield and enhance food security by broadening the geographical regions in which maize can be cultivated. Cold tolerance in maize is a complex trait and is a cumulative function of many physiological and molecular pathways. Significant achievements have made in studying the physiology of maize cold tolerance, but much remains to be done to solve the problems that result from cold damage to maize plants. The molecular analyses of maize cold tolerance mechanisms are still insufficient, and many of the current studies do not contribute much to maize biology compared to those in *Arabidopsis* and rice. This could be due in part to the lack of discovery of novel genes that may regulate cold tolerance in maize.

There are large differences in cold tolerance among various maize varieties. Cold tolerant varieties usually have a stronger ability to maintain osmotic pressure, the ROS balance, hormonal homeostasis, mineral nutrient absorbance, and photosynthesis. These physiological characteristics have been widely used to evaluate the cold tolerance of maize varieties in QTL and GWAS analyses. Many of the QTL and GWAS analyses were based on various maize genotypes and populations that express large variations in cold tolerance, but only a few candidate genes have been identified. Further fine mapping and map-based cloning of genes in the genomic regions identified *via* QTL and GWAS analysis is required, and more candidate genes will provide a basis for further understanding the molecular and genetic mechanism of cold tolerance in maize. Furthermore, using QTL mapping and GWAS, molecular markers closely linked to cold tolerance genes can also be identified as resources for MAS (marker assisted selection) of cold tolerant varieties. In addition, with the aid of data used for GWAS analysis, future work can also use GS (genomic selection) to predict cold tolerant varieties in large maize populations.

Transcriptome analysis is a powerful tool that has been used in many studies for the identification of cold-responsive genes. Comparisons of the DEGs detected in different studies have shown little overlap with each other. A few studies integrated QTL, GWAS, RNA-seq, and other methods, which significantly reduced the number of candidate genes for selection, partly because joint analyses in the same study can eliminate the differences that arise from the genotypes used or the experimental design. Future research that integrates more methods, such as phenomic, proteomic, metabolomic, and bioinformatic approaches, may greatly improve the accuracy of identifying cold-regulated genes and provide better candidates that can be further used in molecular breeding.

AUTHOR CONTRIBUTIONS

CX and HL conceived the work. XZ drafted the manuscript. HL, CX, and IM made comments and revisions. All authors have read and approved the manuscript.

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REVIEW
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Calcium Mediated Cold Acclimation in Plants: Underlying Signaling and Molecular Mechanisms

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Exposure of plants to low temperatures adversely affects plant growth, development, and productivity. Plant response to cold stress is an intricate process that involves the orchestration of various physiological, signaling, biochemical, and molecular pathways. Calcium (Ca^{2+}) signaling plays a crucial role in the acquisition of several stress responses, including cold. Upon perception of cold stress, Ca^{2+} channels and/or Ca^{2+} pumps are activated, which induces the Ca^{2+} signatures in plant cells. The Ca^{2+} signatures spatially and temporally act inside a plant cell and are eventually decoded by specific Ca^{2+} sensors. This series of events results in the molecular regulation of several transcription factors (TFs), leading to downstream gene expression and withdrawal of an appropriate response by the plant. In this context, calmodulin binding transcription activators (CAMTAs) constitute a group of TFs that regulate plant cold stress responses in a Ca^{2+} dependent manner. The present review provides a catalog of the recent progress made in comprehending the Ca^{2+} mediated cold acclimation in plants.

Keywords: calcium, calmodulin, CAMTA, cold stress, transcription factor

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INTRODUCTION

Plants sense and respond to distinct environmental and developmental cues *via* intricate signal transduction pathways. The signal transduction pathways comprise various protein and non-protein elements. The protein elements encompass various enzymes, receptors, and TFs, while the non-protein elements include second messengers such as Ca^{2+}, cyclic AMP, cyclic GMP, inositol triphosphate, diacylglycerol, lipids, and hydrogen ions. Amongst all the reported second messenger molecules, Ca^{2+} is considered central to several signal transduction pathways (Stael et al., 2012; Sarwat et al., 2013; Kudla et al., 2018). Ca^{2+} is an essential plant macro-nutrient that is pivotal for maintaining the structural integrity of cell walls, regulating stomatal guard cells movement, growth of pollen tubes, and elongation of root hairs (Sanders et al., 2002; White and Broadley, 2003; Dodd et al., 2010). Ca^{2+} signals are elicited when a plant experiences any environmental and developmental stimuli, leading to spatial and temporal changes in Ca^{2+} ion concentration in cells. Several reviews have extensively covered different aspects of plant Ca^{2+} signaling (Costa et al., 2018; Kudla et al., 2018; Thor, 2019; Tian et al., 2020; Iqbal et al., 2021a; Pirayesh et al., 2021). Briefly, under control conditions, the levels of Ca^{2+} ions in the cell are usually low (ranging from 100 to 200 nm), but upon receiving signals to respond, the Ca^{2+} channels are transiently opened, resulting in the rapid influx of Ca^{2+} ions inside the cell. This eventually leads to an increase in cytosolic

Ca^{2+} ($[Ca^{2+}]$cyt) levels. The levels of Ca^{2+} ion inside the cell fluctuates either due to Ca^{2+} influx *via* dedicated channels or Ca^{2+} efflux *via* specific pumps (Xiong et al., 2006; Tuteja and Mahajan, 2007). In *Arabidopsis thaliana,* plasma membrane-bound Ca^{2+}-permeable channels are categorized into four main families, namely, cyclic nucleotide-gated channels (CNGCs), glutamate receptor-like channels (GLRs), stretch-activated Ca^{2+} channels (OSCAs), and the MID1-complementing activity (MCA) (Romola, 2002; Kurusu et al., 2013; Jha et al., 2016; Liu X. et al., 2018). Several other Ca^{2+} channels are localized in organelles, such as endoplasmic reticulum, mitochondria, golgi body, and plant vacuole (Costa et al., 2018; Thor, 2019; He et al., 2021; Pandey and Sanyal, 2021). These include autoinhibited Ca^{2+}-ATPases (ACAs), ER-type Ca^{2+}-ATPases (ECAs), mitochondrial Ca^{2+} uniporter (MCU), P1-ATPases (e.g., HMA1), Ca^{2+} exchangers (CAX), two-pore channel (TPC), 1,4,5-trisphosphate receptor-like channel ($InsP_3R$), 1,4,5-trisphosphate (IP_3), cyclic ADP-ribose (cADPR)-activator ryanodine receptor-like channel (RyR), slow-activating vacuolar channel (SV), and sodium–calcium exchanger (NCX).

The stimuli triggered by environmental or developmental signals generates discrete Ca^{2+} signatures that are sensed and recognized by specific Ca^{2+} sensors. This cascade of events eventually results in transcriptional and metabolic responses (Perochon et al., 2011). Ca^{2+} signals are recognized by most of the Ca^{2+} sensors *via* the elongation factor hand (EF-hand) motif. Multiple EF-hand containing proteins are present in plants, and Ca^{2+} sensors represent just one of the many that translate chemical signals into an appropriate biochemical response. The EF-hand motif is represented by a conserved helix–loop–helix structure that binds to one Ca^{2+} ion. They occur in pairs as distinct domain, hence, the majority of Ca^{2+} sensors harbor two, four, or six EF-hands (Gifford et al., 2007; Perochon et al., 2011). The pairing in certain cases is generally co-operative, consequently minimizing the required Ca^{2+} signal for protein saturation. Conformational changes occur upon binding of Ca^{2+} ion to appropriate Ca^{2+} sensor. These structural changes prompt the interaction between the sensor and its target protein (TP). Three major classes of Ca^{2+} sensor families have been recognized in plants, namely, (i) Calmodulins (CaMs) and calmodulin-like proteins (CMLs), (ii) calcineurin B-like proteins (CBLs), and (iii) Ca^{2+}-dependent protein kinases (CDPKs) (Hrabak et al., 2003; Batistič and Kudla, 2012). CaMs are highly conserved in eukaryotes, while CMLs, CBLs, and CDPKs had only been reported in plants and protists (Day et al., 2002; Reddy and Reddy, 2004). CaMs, CMLs, and CBLs are small protein molecules possessing a Ca^{2+} sensing domain, thereby, acting as sensor relays. They tend to bind to the downstream effector molecules in a Ca^{2+} concentration-dependent manner (Luan et al., 2002). Different from the aforementioned Ca^{2+} sensors, CDPKs possess an effector domain (serine/threonine protein kinase catalytic domain) along with the Ca^{2+} sensing domain. Accordingly, CDPKs act as sensor responders to directly activate and regulate their TPs upon sensing Ca^{2+} signals (Hashimoto and Kudla, 2011). Thus, the series of events: perception of stress, the opening of Ca^{2+} channels, transient changes in Ca^{2+} levels, sensing of Ca^{2+} signals by appropriate Ca^{2+} sensor, and subsequent activation of TFs for downstream molecular and biochemical outputs generates specific responses by the plant to combat the cold stress condition. One such TF is CAMTA that regulates plant responses toward cold stress in a Ca^{2+} dependent manner (Iqbal et al., 2020b). The CAMTA protein is characterized by the presence of five functional domains: CG- DNA binding motif, TAD- transcriptional activation domain, TIG- for non-specific DNA interaction, Ankyrin repeats- protein–protein interaction, CAMBD- for CaM binding. Concisely, when a plant is exposed to cold stress, the Ca^{2+} channels are opened leading to a rapid and transient influx of Ca^{2+} inside the cell. This results in an increase in ($[Ca^{2+}]_{cyt}$), which is sensed by Ca^{2+} sensor—CaM. Eventually, CaM in a Ca^{2+} dependent manner regulates the transcriptional activity of the *CAMTA* gene, withdrawing an appropriate response by the plant against cold stress. The present review summarizes the progress made in the recent years to comprehend the involvement of Ca^{2+}signaling in cold stress tolerance (**Figure 1**).

CALCIUM SENSING NETWORK UNDER COLD STRESS

Low temperatures lead to intricate cellular and molecular mechanisms inside plant cells *via* key components of Ca^{2+} signaling (Yuan et al., 2018b). Ca^{2+} channels play critical roles in low-temperature acclimatization of chilling-tolerant *A. thaliana* and root hair development (Hong-Bo et al., 2008). It has been proposed that Ca^{2+}-permeable mechanosensitive channels MCA1 and MCA2 regulate cold-induced $[Ca^{2+}]$cyt increase, cold tolerance, and *CBF/DREB1*-independent cold signaling. The cold-induced $[Ca^{2+}]$cyt was lower in *mca1* and *mca2* mutants than control plants. The *mca1 mca2* double mutant compared to control were more sensitive to chilling and freezing stress (Mori et al., 2018). Additionally, vesicle membrane Ca^{2+}/H^+ antiporter, *A. thaliana calcium exchanger 1* (*AtCAX1*) is implicated in an accurate development of the cold-acclimation response by regulating the induction of *CBF/DREB1* and downstream genes (Catalá et al., 2003). Recently, Ca^{2+}/cation antiporter (CaCA) superfamily proteins have been identified in *Saccharum* to play pivotal roles in environmental stresses, including cold (Su et al., 2021). Likewise, CNGC is a family of non-selective cation-conducting channels primarily localized to the plasma membrane (Zelman et al., 2012). They are implicated in thermal sensing and thermotolerance in *Arabidopsis thaliana* and mosses (Finka et al., 2012). CNGCs have been reported to play crucial roles in regulating cold tolerance in plants. *Oryza sativa OsCNGC9* transcriptional activation and phosphorylation confers enhanced chilling tolerance in rice (Wang et al., 2021). *OsCNGC9* overexpression provides increased cold tolerance, while its mutation leads to defects in cold-induced Ca^{2+} influx. Rice OsDREB1A TF is responsible for the activation of *OsCNGC9* transcription. In crux, *OsCNGC9* increases chilling tolerance by regulating cold-induced Ca^{2+} influx and $[Ca^{2+}]$cyt elevation (Wang et al., 2021). Additionally, CNGC family has been characterized in *Chinese jujube (Ziziphus jujuba Mill.)*,

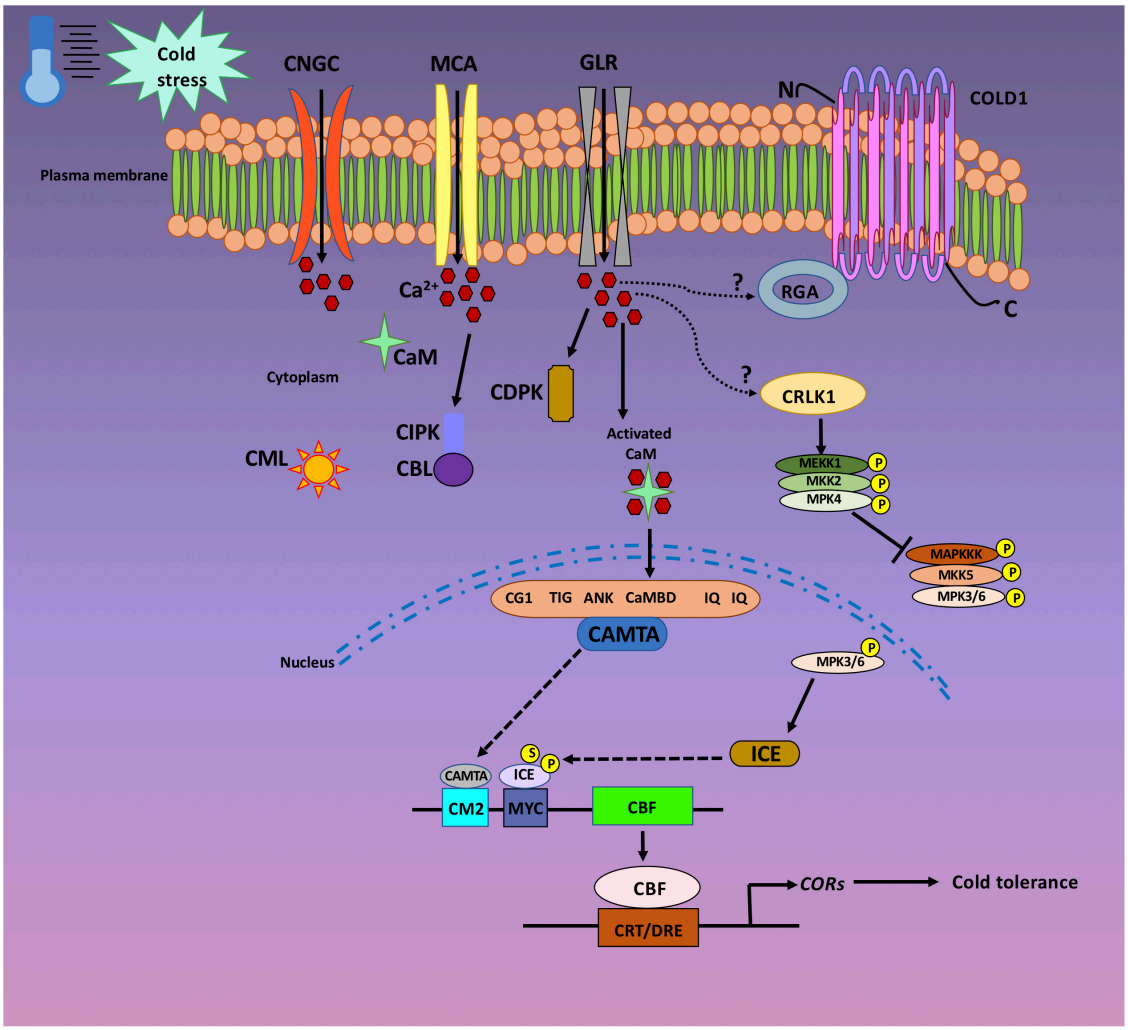

FIGURE 1 | Cold stress signaling in a plant cell. The plasma membrane is considered as one of the primary target for cold sensing and eventual transmission of Ca^{2+} signals into the plant cell nuclei. Cyclic nucleotide-gated channels (CNGCs), glutamate receptor-like channels (GLRs), and the MID1-complementing activity (MCA) channels are the main plasma membrane Ca^{2+} channels that allow the entry of Ca^{2+} ions into the cytoplasm. Once the Ca^{2+} ion enters the plant cell, they are sensed by Calmodulins (CaMs) and Calmodulin-like proteins (CMLs), Calcineurin B-like proteins (CBLs), and Ca^{2+}-dependent protein kinases (CDPKs). Upon cold exposure, plasma membrane associated cold sensor chilling-tolerance divergence 1 (COLD1) interacts with G protein a subunit (RGA). Ca^{2+}/CaM regulated receptor-like kinase (CRLK) positively regulate cold triggered gene expression by inducing the MEKK1–MKK2–MPK4 pathway. CRLK suppress cold-induced activation of MPK3/6 and is necessary for inducer of CBF expression (ICE) accumulation. ICE proteins are stabilized by either phosphorylation (P) or sumolyation (S). Calmodulin binding transcription activators (CAMTAs) activate *C-repeat binding factor* (CBF) expression through the CM2 (CCGCGT) promoter motif. CBF proteins eventually activate the expression of various *cold-responsive (COR) genes* which confers cold tolerance in plants.

and *ZjCNGC2* was reported to regulate signaling cascades in response to cold stress (Wang et al., 2020b). Further, it was shown that rice *CNGC14* and *CNGC16* are involved in promoting tolerance toward heat and chilling stresses, and are regulators of Ca^{2+} signals in response to temperature stress (Cui et al., 2020). Their homologs in *A. thaliana* (*AtCNGC2* and *AtCNGC4*) are also implicated in tolerance toward low temperature (Cui et al., 2020). CNGCs had also been implicated in modulating cold stress responses along with other biological stresses *via* Ca^{2+} signals in *Brassica oleracea* (Kakar et al., 2017), *O. sativa* (Nawaz et al., 2014), and *Nicotiana tabacum* (Nawaz et al., 2019).

The endoplasmic reticulum and plasma-membrane localized G-protein regulator CHILLING TOLERANCE DIVERGENCE1 (COLD1) coupled with RICE G-PROTEIN α SUBUNIT1 (RGA1) was reported in cold stress signaling *via* Ca^{2+} signals and electrophysiological responses in *O. sativa* (Ma Y. et al., 2015). The COLD1-RGA1 complex regulates the cold stress-driven influx of intracellular Ca^{2+}, eventually resulting in the activation of *COR* (*cold regulated*) genes. It remains a subject of further evaluation whether COLD1 plays a role as a Ca^{2+}-permeable channel or as a mediator promoting Ca^{2+}-permeable channel activity. Taking into account another plasma membrane-bound Ca^{2+} channel—GLR—mediate Ca^{2+} fluxes across membranes

and is responsive to an array of exogenous and endogenous signals in plants. *AtGLR3.4* localizes to the plasma membrane and is stimulated by cold stress in a Ca^{2+}-dependent manner (Meyerhoff et al., 2005; Weiland et al., 2015). *AtGLR1.2* and *AtGLR1.3* were reported to positively regulate cold tolerance by modulating jasmonate signaling in *A. thaliana* (Zheng et al., 2018). The cold sensitivity of *glr1.2* and *glr1.3* mutants was attenuated by exogenous jasmonate treatment, while the over-expression of *GLR1.2* or *GLR1.3* led to elevated cold tolerance by enhancing endogenous jasmonate levels. Additionally, under cold stress, the expression of genes in the *CBF/DREB1* signaling pathway were lowered in *glr1.2* and *glr1.3* mutants, whereas higher in *GLR1.2* and *GLR1.3* over-expression lines (Zheng et al., 2018). Similar to the above finding, tomato *GLR3.3* and *GLR3.5* were reported to regulate cold acclimation-induced chilling tolerance by modulating apoplastic H_2O_2 production and redox homeostasis (Li H. et al., 2019). Next, annexins are Ca^{2+} permeable transporters that mediate the accumulation of $[Ca^{2+}]$cyt in responses to abiotic stresses (Lee et al., 2004; Laohavisit et al., 2012; Richards et al., 2014). Recently, ANNEXIN1 was reported to regulate cold-induced Ca^{2+} influx and freezing tolerance in *A. thaliana* (Liu et al., 2021). The mutation of *AtANN1* decreased freeing tolerance, impaired cold triggered $[Ca^{2+}]$cyt increase, and upregulated cold-responsive *CBF* and *COR* genes. The study revealed that *AtANN1* acts downstream of *OST1* in responses to cold stress (Liu et al., 2021). Furthermore, the organellar Ca^{2+} channel, *GhCAX3* gene from *Gossypium hirsutum* was characterized under various abiotic stresses, including cold. Transgenics compared to control plants were more sensitive to cold stress during seed germination. Over-expression of *GhCAX3* led to the transcript enrichment of some of the abscisic acid (ABA)-and cold-responsive genes. The study concluded that *GhCAX3* plays an imperative part in the cross-talk of cold and ABA signal transduction (Xu et al., 2013). Likewise, IP3 was reported to mediate nitric oxide (NO) triggered chilling tolerance in postharvest peach fruit (Jiao et al., 2019).

ROLE OF CALCIUM SENSORS IN COLD STRESS

Calmodulin and Calmodulin-Like Protein Mediated Responses Toward Cold Stress

Calmodulins and calmodulin-like protein are widely studied Ca^{2+} sensors that sense and decode rapid and transient fluctuations in the intracellular Ca^{2+} levels in response to environmental cues. In plants, CaMs and CMLs have been reported to play pivotal roles in developmental and stress biology (Zeng et al., 2015; Ranty et al., 2016; Aldon et al., 2018; Gao et al., 2019). *CaMs* and *CMLs* transcripts are induced or suppressed in response to a variety of abiotic stresses (Zeng et al., 2017; Li C. et al., 2019). Initial studies revealed that *CaM3* overexpressing lines had reduced levels of *COR* transcripts, suggestive of the fact that CaM might act as a negative regulator of cold stress (Townley and Knight, 2002). In a similar vein, *AtCaM4* had been reported to negatively regulate freezing tolerance in *A. thaliana*. The *cam4* mutants exhibited increased tolerance to freezing stress. *AtCaM4* might regulate freezing tolerance in a CBF-independent manner (Chu et al., 2018). In an interesting study, the germination of developing immature *cml39* seeds in comparison to control seeds was not sensitive to cold-stratification. Hence, it was reported that CML39 has a role in stratification-dependant seed dormancy (Midhat et al., 2018). Lately, the effect of cold stress along with other abiotic stresses was assessed for the expression of CaMs and CMLs in wild-growing grapevine *Vitis amurensis*. *VaCaM8* and *VaCaM10* showed significant differential expression under cold stress (4°C). Incubation at 4°C or 10°C induced the expression of six *CML* genes (*VaCML21*, *VaCML44*, *VaCML61*, *VaCML78*, *VaCML86*, and *VaCML89;* while reduced the expression of eight *CML* genes (*VaCML9a*, *VaCML48*, *VaCML57*, *VaCML75*, *VaCML82*, *VaCML85*, *VaCML92*, and *VaCML107*) (Dubrovina et al., 2019). The same group reported four alternatively spliced mRNA forms of the grapevine *CML21* gene (*CML21v1*, *CML21v2*, *CML21v3*, and *CML21v4*). All the four splice variants were highly induced under cold stress. Heterologous expression of *CML21v2* and *VaCML21v4* in *A. thaliana* increased the survival percentage of the transgenics upon freezing. Cold stress-responsive marker genes: dehydration-responsive element-binding, *AtDREB1A* and *AtDREB2A* were induced in *VaCML21v2* overexpression lines, while *AtCOR47*, *AtRD29A*, *AtRD29B*, and *AtKIN1* genes were induced in *VaCML21v4* overexpression lines after freezing stress in the transgenic *Arabidopsis* plants. Thus, it was established that *CML21* acts as a positive regulator of cold stress (Aleynova et al., 2020). Likewise, *Medicago sativa*, *MsCML46* gene encoding calmodulin-like protein confers tolerance to cold and other abiotic stress in tobacco. The *MsCML46* was upregulated in the leaves and roots after exposure to cold stress. The expression peaked after 1 h in leaves, while in roots, the expression peaked at 3 h (Du et al., 2021). In a similar vein, five *Camellia sinensis-CsCML* genes (*CsCML16*, *CsCML18-1*, *CsCML18-2*, *CsCML38,* and *CsCML42*) were functionally characterized under various environmental stresses. The transcript levels of *CsCML16*, *18-2,* and *42* were significantly induced by low temperature and salt stress (Ma Q. et al., 2019). Previously, *Solanum habrochaites* (cold-tolerant wild tomato) *ShCML44* gene was functionally characterized under a variety of environmental stresses, including cold stress. The *ShCML44* overexpressed plants had higher antioxidant enzymes activity, better gas exchange and water retention capacity, lower malondialdehyde (MDA) accumulation and membrane damage, reduced reactive oxygen species (ROS), and higher relative water contents (Munir et al., 2016). Very recently, *Solanum lycopersicum SlCML37* has been shown to interact with proteasome maturation factor SlUMP1 and has been reported in tomato fruit chilling stress tolerance (Tang et al., 2021). Additionally, *Medicago truncatula MtCML42* has been reported to regulate cold tolerance and flowering time (Sun et al., 2021). Further, in rice, six new putative interacting partners of OsCML16 were identified (OsLRK5a, OsDCNL2, OsWD40-139, OsGDH1, OsCIP, and OsERD2). The *in vitro* peptide-binding assays suggested that OsERD2 could bind both OsCaM1 and OsCML16, while the other five TPs specifically binded

to OsCML16. Moreover, Ca^{2+} and trifluoperazine (TFP)—CaM antagonist were involved in ABA-induced transcription of *OsCML16* and its target genes. *OsCML16* and its target genes were triggered by salt, drought, and low-temperature stress (Yang et al., 2020).

Calcium/Calmodulins-regulated receptor-like kinases 1 (CRLK1) encoding a plasma membrane-associated serine/threonine kinase has been reported to play a crucial role in cold stress responses (Yang et al., 2010a,b; Furuya et al., 2013, 2014). The *crlk1* mutants compared to control plants are sensitive to freezing temperatures. The expression of cold-responsive genes, such as, *CBF1*, *RD29A*, and *COR15a* was suppressed in *crlk1* mutants, making them more susceptible to cold stress than control plants. CRLK1 protein expression is induced upon low temperature (4°C) exposures and oxidative stress (H_2O_2). Thus, CRLK1 is considered a positive regulator of cold stress responses in *A. thaliana*. Additionally, the Ca^{2+}/CaM complex is a requisite for triggering CRLK1 kinase. It has been reported previously that an increase in CaM levels in the presence of Ca^{2+} elevates the activity of CRLK1 kinase. On the contrary, chlorpromazine (CPZ)—CaM antagonist blocked the CaM mediated CRLK1 kinase activity (Yang et al., 2010a). Explicitly, the presence of CaM-binding domain at the C-termini of CRLK1 is essential for CaM-modulated kinase activity (Yang et al., 2010a). Besides, the inducer of CBF expression 1 (ICE1) is a transcription activator and a major component of the cold response pathway as it binds with the promoters of the *C-repeat binding factor (CBF)* and *COR* genes (Tang et al., 2020). CRLK1 and CRLK2 suppress cold-induced activation of MPK3/6 and are necessary for ICE1 accumulation (Zhao et al., 2017). Hence, there exists a Ca^{2+} signaling-mediated cold-responsive pathway which is regulated by CRLK1 (Yang et al., 2010a,b).

Calcineurin B-Like Proteins Mediated Responses Toward Cold Stress

Calcineurin B-like proteins represent a major class of Ca^{2+} binding proteins and are considered imperative relays in plant Ca^{2+} signaling pathways. CBL and CBL-interacting protein kinase (CIPK) complex are central to Ca^{2+} signaling. This complex had been reported to be implicated in a plethora of external stress signals (Kolukisaoglu et al., 2004; Yu et al., 2014; Mohanta et al., 2015). In this context, *CBL9* had been shown to negatively regulate cold tolerance *via* Ca^{2+} signaling in *A. thaliana* (Gao and Zhang, 2019). *cbl9* mutants showed enhanced freezing tolerance under cold-acclimating and non-acclimating conditions. Exposure to cold stress increased $[Ca^{2+}]$cyt in *cbl9* mutants compared to wild type. Contrarily, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)—Ca^{2+} chelator and lanthanum chloride—Ca^{2+} channel blocker significantly altered $[Ca^{2+}]$cyt in *cbl9* mutants (Gao and Zhang, 2019). Lately, in *Camellia sinensis* (tea plant), it was shown that *CsCBL9* and *CsCIPK4/6a/6b/7/11/14b/19/20* were upregulated in both mature leaves and young shoots upon cold stress. Results of yeast two-hybrid assay demonstrated that CsCBL1 potentially interacted with CsCIPK1/10b/12 but not with CsCIPK6a/7/11/14b/20. Similarly, CsCBL9 interacted with CsCIPK1/10b/12/14b but not with CsCIPK6a/7/11/20. Thus, the study proposed distinct responses to cold stress mediated by CBL–CIPK complexes (Wang et al., 2020a). In addition, CIPKs had also been functionally characterized in *Triticum aestivum* (Deng et al., 2013), *Capsicum annuum* (Ma X. et al., 2019), *Manihot esculenta* (Mo et al., 2018), *Malus domestica* (Wang et al., 2012; Niu et al., 2018), and *Brachypodium distachyon* (Luo et al., 2018) under different environmental cues, including cold stress. *TaCIPK29* transcript increased after cold treatment (Deng et al., 2013), while *CaCIPK1* expression changed in response to cold stress (Ma X. et al., 2019). The expression of *MeCIPK7* significantly increased in roots upon cold treatment. The transcript levels of *MeCIPK10* and *13* in roots, whereas transcript levels *MeCIPK12* and *16* in leaves were also altered upon cold treatment (Mo et al., 2018). This study by Mo et al. (2018) suggested that cassava (*Manihot esculenta*) CBL–CIPK signal networks function in responses to abiotic stresses. *MdCIPK6L* ectopic expression significantly enhanced chilling tolerance in transgenic tomatoes (Wang et al., 2012), whereas the ectopic expression of *BdCIPK31* renders increased low-temperature tolerance in transgenic tobacco (Luo et al., 2018). Likewise, CBLs had been molecularly characterized under a variety of environmental stresses, including cold in *Brassica napus* (Zhang H. et al., 2014), *Brassica rapa* (Jung et al., 2017), *Stipa purpurea* (Zhou et al., 2016), and *Pyrus betulifolia Bunge* (Xu Y. et al., 2015). For *Brassica napus*, *BnaCBL1* transcripts significantly increased at 6 h of cold treatment; however, it was downregulated at 24 h. At 24 h of cold treatment, only *BnaCBL10* was slightly upregulated, and transcripts of *BnaCBL2*, *-3*, *-4* were downregulated (Zhang H. et al., 2014). For *Brassica rapa*, *BrCBL1-1* transcript levels were highly elevated (∼30-fold upregulation) after 4 h of cold treatment in one of the in-bred lines of *Brassica rapa* (Chiifu) (Jung et al., 2017). Further, overexpression of *SpCBL6* from *Stipa purpurea* increased cold tolerance and decreased drought tolerance in transgenic *A. thaliana* (Zhou et al., 2016). On similar grounds, *PbCBL1* responded to alterations in the intracellular Ca^{2+} concentrations and was induced by cold stress (Xu Y. et al., 2015).

Calcium-Dependent Protein Kinases Mediated Responses Toward Cold Stress

Calcium-dependent protein kinases comprise a multi-gene kinase family in plants and are major regulators of developmental and stress responses in plants (Cheng et al., 2002; Valmonte et al., 2014). As already stated, CDPKs function as direct sensor responders to decode the Ca^{2+} signals (Hashimoto and Kudla, 2011). Upon sensing Ca^{2+} signals, CDPKs activate and regulate the TPs directly. Several CDPK-encoding genes are differentially expressed upon cold stress; however, their underlying molecular mechanisms remain elusive. In rice, *OsCPK17* targets the sucrose–phosphate synthase and plasma membrane intrinsic proteins and was reported in cold stress response (Almadanim et al., 2017). Additionally, *OsCPK24* inhibits glutaredoxin (*OsGrx10*), thereby, sustaining higher glutathione levels and phosphorylation. *OsCPK24* has been shown to positively regulate cold stress tolerance (Liu Y. et al., 2018). In yet another

monocot plant—banana, *MaCDPK7* was shown to regulate the fruit ripening process and chilling resistance induced by heat treatment (Wang et al., 2017). Later, the *CDPK* gene family was characterized in banana for their involvement in the development, fruit ripening, and abiotic stress responses, including cold (Li et al., 2020). Genome-wide identification of the *CDPK* gene family in *Medicago truncatula* also revealed that *MtCDPK4*, *8*, *15*, *16,* and *22* transcripts were quickly elevated after 2 h of cold treatment (Zhao et al., 2021). Previously, the *CDPK* gene family had been identified and assessed for its involvement under abiotic stress conditions, including cold in *Solanum lycopersicum* (tomato; Hu et al., 2016), *Cucumis melo* (melon; Zhang et al., 2017), *Cucumis sativus* (cucumber; Xu X. et al., 2015), *zea mays* (maize; Kong et al., 2013). Moreover, in *Populus euphratica*, *PeCPK10* confers cold and drought stress tolerance. Precisely, overexpression of *PeCPK10* increased freezing tolerance in the transgenics. The expression of ABA and stress-responsive genes such as *RD29B* and *COR15A* were induced by constitutive expression of *PeCPK10* (Chen et al., 2013). In an interesting study, the roles of *VaCPK16*, *VaCPK25*, *VaCPK30*, and *VaCPK32* in secondary metabolites biosynthesis and stress resistance was studied in *V. amurensis* (grapevine) (Dubrovina et al., 2018). Overexpressing the *VaCPK30* gene conferred enhanced resistance to cold and salt stress in transgenics, whereas overexpressing *VaCPK16*, *VaCPK25*, and *VaCPK32* did not influence temperature and salt stress tolerance. Instead, the overexpression of *VaCPK16* and *VaCPK32* enhanced stilbene accumulation in *V. amurensis* cell cultures (Dubrovina et al., 2018). Earlier the same group had reported the involvement of *VaCPK20* in cold and drought stress response pathways (Dubrovina et al., 2015). On similar lines in *Zea mays*, *ZmCPK1* was reported as a negative regulator of cold stress signaling in maize (Weckwerth et al., 2015). *ZmCPK1* displayed Ca^{2+}-independent protein kinase activity. The expression of *ZmCPK1* increased, while the expression of *ZmCPK25* decreased upon cold stress (Weckwerth et al., 2015). Recently, *Malus domestica* (apple) *MdCPK1a* gene was reported to enhance tobacco cold resistance *via* scavenging ROS accumulation (Dong et al., 2020). The underlying mechanism of cold resistance through the involvement of *MdCPK1a* was further investigated. The *MdCPK1a* tobacco transgenics had a better survival ratio and root length when subjected to cold stress. The superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activities were higher, while electrolyte leakages (EL), MDA content, and ROS were lower. This was suggestive of the fact that the transgenics underwent less chilling injury than control plants (Dong et al., 2020). Thus, Ca^{2+} signaling plays a pivotal part in cold acclimation in plants (**Table 1**).

AtSR/CAMTA Regulated Transcription Under Cold Stress

Upon perception of cold stress, Ca^{2+} signals are elevated, which might direct Ca^{2+} to either repress or activate the activity of Ca^{2+} responding protein. Similarly, the interaction of Ca^{2+} with Ca^{2+} sensors either suppresses or enhances the binding to a TF. Depending upon whether the TF itself is a repressor or activator, the transcription of the target gene is repressed or activated. Ca^{2+}/CaM dependent TFs relay cold-induced Ca^{2+} transients to transcriptional reprograming. CAMTAs are one such group of TFs that regulate plant cold stress responses in a Ca^{2+}-dependent manner. CAMTA proteins have been stipulated to play a direct link between Ca^{2+} signals and cold acclimation (Eckardt, 2009). CAMTAs also known as signal responsive (SR) protein (Yang and Poovaiah, 2000) or EICBP (ethylene-induced CaM-binding proteins) (Reddy et al., 2000) is a well-characterized CaM dependent TF that regulates gene expression by binding to the signature "CGCG" DNA motif (Galon et al., 2008; Du et al., 2009; Yuan et al., 2018a). Furthermore, CBF cold response pathway plays a pivotal role in cold acclimation (Shi et al., 2018). It is characterized by rapid cold induction of genes encoding the *CBF1-3* TFs, followed by the expression of the *CBF* gene regulon. The CRT/DRE *cis*-element is recognized by the CBF protein and is characterized by the presence of a conserved CCGAC sequence. The CCGAC sequence is present in the 1000 bp upstream region of a subset of *COR* genes (Stockinger et al., 1997; Gilmour et al., 1998; Shi et al., 2018; Liu et al., 2019). The *cis* and *trans*-acting factors implicated in the expression of *CBF2* were studied by Doherty et al. (2009). Seven conserved DNA motifs (CM1 to 7) were identified in the promoters of *CBF2* and *ZAT12* (cold-induced genes). CM4 and CM6 have negative regulatory activity, while CM2 has both negative and positive activity. The study also revealed that CAMTA3 binds to the CM2 motif and is a positive regulator of *CBF2* expression. Moreover, *camta1 camta3* double mutant plants were impaired in freezing tolerance. This study exhibited a novel role of CAMTA in cold acclimation and provided a plausible link of low-temperature Ca^{2+} and CaM signaling with cold-regulated gene expression (Doherty et al., 2009). Later, CAMTA3 and CAMTA5 were reported to respond to a rapid decrease in temperature and induce the expression of *DREB1s* (Kidokoro et al., 2017). Additionally, contrary to *circadian clock associated1* and *late elongated hypocotyl* genes that modulate *DREB1* expression only during the day, CAMTA3 and CAMTA5 function both during the day and night (Kidokoro et al., 2017).

Salicylic acid (SA) has a central role in transcriptional machinery at low temperatures (Scott et al., 2004). However, accumulated SA did not influence cold tolerance in *atsr1* (also referred as CAMTA3) (Kim et al., 2013). CAMTA1 and CAMTA2 in combination with CAMTA3 induced transcripts of *CBF1*, *CBF2,* and *CBF3* at 2 h and enhanced plant freezing tolerance. Additionally, CAMTA1, CAMTA2, and CAMTA3 work simultaneously to inhibit SA biosynthesis at warm temperatures (22°C). However, the SA levels increased in plants exposed to low-temperatures for more than one week. The study revealed that the isochorismate synthase (ICS) pathway is involved in chilling-induced SA biosynthesis. The accumulation of *ICS1*, *CBP60g,* and *SARD1* transcripts were suppressed at warm temperatures by these three CAMTAs, but not at low temperatures (Kim et al., 2013). The analysis of upstream regions to the transcription start site (TSS) in wound-induced genes indicated the presence of rapid stress response DNA element

TABLE 1 | Calcium signaling components in cold stress acclimation in plants.

Gene	Ca^{2+} component	Species	References
MCA1 and MCA2	Ca^{2+} channel	*Arabidopsis thaliana*	Mori et al., 2018
AtCAX1	Ca^{2+} channel	*Arabidopsis thaliana*	Catalá et al., 2003
Ca^{2+}/cation antiporter	Ca^{2+} channel	*Saccharum*	Su et al., 2021
CNGC9	Ca^{2+} channel	*Oryza sativa*	Wang et al., 2021
ZjCNGC2	Ca^{2+} channel	*Ziziphus jujuba Mill*	Wang et al., 2020b
CNGC14 and CNGC16	Ca^{2+} channel	*Oryza sativa*	Cui et al., 2020
AtCNGC2 and AtCNGC4	Ca^{2+} channel	*Arabidopsis thaliana*	Cui et al., 2020
AtGLR3.4	Ca^{2+} channel	*Arabidopsis thaliana*	Meyerhoff et al., 2005; Weiland et al., 2015
AtGLR1.2 and AtGLR1.3	Ca^{2+} channel	*Arabidopsis thaliana*	Zheng et al., 2018
GLR3.3 and GLR3.5	Ca^{2+} channel	*Solanum*	Li H. et al., 2019
ANNEXIN1	Ca^{2+} channel	*Arabidopsis thaliana*	Liu et al., 2021
GhCAX3	Ca^{2+} channel	*Gossypium hirsutum*	Xu et al., 2013
CaM3	Ca^{2+} sensor	*Arabidopsis thaliana*	Townley and Knight, 2002
CaM4	Ca^{2+} sensor	*Arabidopsis thaliana*	Chu et al., 2018
CML39	Ca^{2+} sensor	*Arabidopsis thaliana*	Midhat et al., 2018
VaCaM8 and VaCaM10	Ca^{2+} sensor	*Vitis amurensis*	Dubrovina et al., 2019
VaCML21, *VaCML44*, *VaCML61*, *VaCML78*, *VaCML86*, *and VaCML89*	Ca^{2+} sensor	*Vitis amurensis*	Dubrovina et al., 2019
CML21v1, *CML21v2*, *CML21v3*, *and CML21v4*	Ca^{2+} sensor	*Vitis amurensis*	Aleynova et al., 2020
MsCML46	Ca^{2+} sensor	*Medicago sativa*	Du et al., 2021
CsCML16, *18-2*, *and 42*	Ca^{2+} sensor	*Camellia sinensis*	Ma Q. et al., 2019
ShCML44	Ca^{2+} sensor	*Solanum habrochaites*	Munir et al., 2016
SlCML37	Ca^{2+} sensor	*Solanum lycopersicum*	Tang et al., 2021
MtCML42	Ca^{2+} sensor	*Medicago truncatula*	Sun et al., 2021
CRLK1	Ca^{2+} sensor	*Arabidopsis thaliana*	Yang et al., 2010a,b
CBL9	Ca^{2+} sensor	*Arabidopsis thaliana*	Gao and Zhang, 2019
CsCBL9 and CsCIPK4/6a/6b/7/11/14b/19/20	Ca^{2+} sensor	*Camellia sinensis*	Wang et al., 2020a
TaCIPK29	Ca^{2+} sensor	*Triticum aestivum*	Deng et al., 2013
CaCIPK1	Ca^{2+} sensor	*Capsicum annuum*	Ma X. et al., 2019
MeCIPK7	Ca^{2+} sensor	*Manihot esculenta*	Mo et al., 2018
MdCIPK6L	Ca^{2+} sensor	*Malus domestica*	Wang et al., 2012
BdCIPK31	Ca^{2+} sensor	*Brachypodium distachyon*	Luo et al., 2018
BnaCBL	Ca^{2+} sensor	*Brassica napus*	Zhang H. et al., 2014
BrCBL1-1	Ca^{2+} sensor	*Brassica rapa*	Jung et al., 2017
SpCBL6	Ca^{2+} sensor	*Stipa purpurea*	Zhou et al., 2016
PbCBL1	Ca^{2+} sensor	*Pyrus betulifolia Bunge*	Xu Y. et al., 2015
OsCPK17	Ca^{2+} sensor	*Oryza sativa*	Almadanim et al., 2017
OsCPK24	Ca^{2+} sensor	*Oryza sativa*	Liu Y. et al., 2018
MaCDPK7	Ca^{2+} sensor	*Musa acuminata cv.Cavendish*	Wang et al., 2017; Li et al., 2020
MtCDPK4, *8*, *15*, *16*, *and 22*	Ca^{2+} sensor	*Medicago truncatula*	Zhao et al., 2021
PeCPK10	Ca^{2+} sensor	*Populus euphratica*	Chen et al., 2013
VaCPK30	Ca^{2+} sensor	*Vitis amurensis*	Dubrovina et al., 2018
VaCPK20	Ca^{2+} sensor	*Vitis amurensis*	Dubrovina et al., 2015
ZmCPK1	Ca^{2+} sensor	*Zea mays*	Weckwerth et al., 2015
MdCPK1a	Ca^{2+} sensor	*Malus domestica*	Dong et al., 2020
CAMTA3	TF	*Arabidopsis thaliana*	Doherty et al., 2009; Kim et al., 2013, Kidokoro et al., 2017; Kim et al., 2017
CAMTA5	TF	*Arabidopsis thaliana*	Kidokoro et al., 2017

(RSRE), CGCGTT. Moreover, promoter activity assay depicted that luciferase activity level induced by cold stress was lower in *camta3* mutants than control plants (Benn et al., 2014). The study revealed that CAMTA3 modulates cold tolerance in *A. thaliana via* the regulation of genes that harbor RSRE elements in their promoters (Benn et al., 2014). Another interesting study found that heptahelical protein 2 (HHP2) interacts with CBF upstream regulators, such as ICE1, ICE2, and CAMTA3 (Lee and Seo, 2015). At low-temperatures, MYB96 (R2R3-type MYB TF) induced the *HHP* genes (Lee and Seo, 2015). This suggests

that a cross-wired mesh of pathways exist that incorporates Ca^{2+} signaling to regulate cold stress tolerance through CAMTA3. Kim et al. (2017) revealed that the IQ motifs in *AtCAMTA3* (residues 850–875) are necessary for its activity (Kim et al., 2017). Post-translational modifications (phosphorylation or dephosphorylation) play imperative part in *AtCAMTA3* mediated response to environmental cues. S454 and S964 were identified as two putative phosphorylation sites in AtCAMTA3 protein (Jones et al., 2009). The *camta1 camta3* double mutants complemented with mutated AtCAMTA3 protein, S454A and S964A (phosphorylation sites of AtCAMTA3) were partially restored to control plants. Moreso, the suppression of SA biosynthesis in the mutants was compromised, suggestive of the fact that phosphorylation is necessary for the full functionality of *AtCAMTA3* (Kim et al., 2017). It is well reported that CAMTA3 is a defense repressor. CAMTA3 is degraded to trigger SA-mediated immune response during pathogen incursion (Galon et al., 2008; Poovaiah et al., 2013; Zhang L. et al., 2014; Fromm and Finkler, 2015; Kim et al., 2017). Intriguingly, SA-mediated signaling pathways also cross-talk with pathways implicated in long-term cold treatments (4°C, 2 weeks) (Kurepin et al., 2013; Miura and Tada, 2014). Nonetheless, AtCAMTA3 protein is also accumulated at low temperatures (Kim et al., 2017). These observations suggest that a complex mesh of networks intersect with each other to overcome the *AtCAMTA*3 suppression of the SA signaling pathway. Very recently, evolution analyses of CAMTA genes in 112 plant species were performed to study its enhancing effect on cold tolerance (Xiao et al., 2021). Thus, CAMTAs *via* Ca^{2+}/CaM signaling has an intersecting role in imparting cold tolerance to plants.

CONCLUSION AND FUTURE PERSPECTIVE

The underpinning mechanisms of cold signaling pathways and genes implicated in cold stress have been extensively studied in the past few years. Different signaling pathways converge to allow plants cope with cold stress. Perception of cold stress by the plant is contemplated to be the first event for the induction of Ca^{2+} transients (Ma Y. et al., 2015). The cold stress-triggered Ca^{2+} transients are generated *via* a number of Ca^{2+} channels and/or Ca^{2+} pumps. These Ca^{2+} transients are relayed and decoded by a variety of Ca^{2+} sensors to regulate gene expression and subsequently confer cold tolerance to plants (Ma Y. et al., 2015; Mori et al., 2018). Considerable advancements have been made to comprehend the underlying components of the Ca^{2+} signaling network, such as, Ca^{2+}–CBL–CIPK, CDPK, and Ca^{2+}–CaM–CAMTA (Weckwerth et al., 2015; Kidokoro et al., 2017; Wang et al., 2020a). Moreover, plant cold tolerance is an intricate process involving dissecting signal transduction pathways. It remains elusive how other signaling pathways intersect with Ca^{2+} signaling pathways to confer cold tolerance in plants. It is still a challenge to deeply decipher the role of Ca^{2+} signals in the cold stress tolerance mechanism and to ascertain whether cold stress-triggered Ca^{2+} transients exist in the cell nucleus. Cutting edge techniques such as multi-omics (Iqbal et al., 2021b), CRISPR/cas9 gene-editing systems (Iqbal et al., 2020a), and sensitive Ca^{2+} imaging (Grenzi et al., 2021) can prove to be potent tools to determine the un-discovered aspects of Ca^{2+} signaling pathways. Thus, future research should focus on deciphering the key converging and diverging pathways pivotal to Ca^{2+} mediated cold signaling. Further, gaining in-depth insights as to how Ca^{2+} signatures are induced and decoded in response to cold stress can help better comprehend the involvement of Ca^{2+} ion in cold stress signaling. Nonetheless, efforts should be made to identify low-temperature sensors using biological methods in combination with biochemical and biophysical approaches.

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ZI drafted and wrote the manuscript. AGM and AA critically revised the manuscript for consistency and content. MSI conceptualized the idea and reviewed the manuscript. All authors reviewed and approved the final version of the manuscript.

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Plant Hormone Response to Low-Temperature Stress in Cold-Tolerant and Cold-Sensitive Varieties of *Zanthoxylum bungeanum* Maxim

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Plant growth and survival in nature, its growth process, will be affected by various factors from the environment, among which temperature has a greater impact. In recent years, extreme weather has frequently appeared, and the growth of crops has been increasingly affected by the environment. As an important flavoring and Chinese herbal medicine crop, *Zanthoxylum bungeanum* is also facing the harm of low-temperature stress. Plant hormones play a vital role in the response of plants to low temperatures. In this study, ultra-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to determine the hormone components of cold-tolerant and cold-sensitive varieties of *Z. bungeanum*. Combined with chemometric analysis and weighted gene co-expression network analysis (WGCNA), the hormone component differences and hormone response strategies of *Z. bungeanum* under low-temperature stress were comprehensively studied. The results showed that 45 hormones were detected in *Z. bungeanum*. Among them, there were 7 kinds of components with high content and were detected in both two varieties. At the late stage of low-temperature stress, the contents of abscisic acid (ABA) and ABA-glucosyl ester (ABA-GE) in Fuguhuajiao (FG) were significantly increased, and the latter served as the storage of the former to supplement the active ABA. Orthogonal partial least squares discriminant analysis (OPLS-DA) found that indole-3-carboxylic acid (ICA), indole-3-carboxaldehyde (ICAld), meta-Topolin riboside (mTR), cis-Zeatin-O-glucoside riboside (cZROG), and N6-isopentenyladenosine (IPR) in FG were the upregulated important difference components, and IPR and 2-methylthio-cis-zeatin riboside (2MeScZR) in Fengxiandahongpao (FX) were the upregulated important difference components. There were common crossing points and independent response pathways in response to low temperature in two varieties. WGCNA analysis found that the main hormone components were associated with multiple metabolic pathways including carbon, fatty acid, amino acid, and sugar metabolism, indicating that hormone regulation plays an important role

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in the response of *Z. bungeanum* to low temperature. This study clarified the hormone response mechanism of *Z. bungeanum* under low-temperature stress and provided a reference and basis for further improving the cold resistance of *Z. bungeanum* and cultivating new varieties.

Keywords: *Zanthoxylum bungeanum*, cold stress, plant hormone, chemometric analysis, WGCNA

INTRODUCTION

Zanthoxylum bungeanum is a shrub plant of Rutaceae, which is distributed in many Asian countries and has abundant resources in China (Feng et al., 2020). The pericarp of *Z. bungeanum* has become an irreplaceable condiment because of its unique numb flavor, especially in the popular Sichuan hot pot. In addition, the pericarps and leaves can be used in traditional Chinese medicine to treat arthritis, hyperlipidemia, thrombosis, and other diseases, which have a long history in China (Yang et al., 2014; Lei et al., 2016; Alam et al., 2018). At present, the genome of *Z. bungeanum* has been sequenced, further research will be carried out on the numb taste and aroma characteristics (Feng et al., 2021). However, in practical cultivation and production, *Z. bungeanum* plants are often affected by a low-temperature environment, resulting in damage to buds, leaves, and flowers. There are still few studies on the resistance of *Z. bungeanum,* and the response mechanism of low temperature is not clear, which is not conducive to the cultivation of excellent varieties.

To adapt to the adverse low-temperature environment, plants have gradually evolved complex mechanisms to avoid and tolerate cold stress. Among them, the hormone system is an important strategy for plants to deal with low-temperature stress. Many studies have shown that plant hormones play important roles in the low-temperature response. Abscisic acid (ABA) is the most studied and can affect plant growth and development by regulating plant signals. One study showed that the ABA content of *Carpobrotus edulis* increased under cold stress (Fenollosa et al., 2018). Exogenous application of ABA can increase the freezing tolerance of grapevines (Wang et al., 2020). Under low-temperature stress, the expression pathways of response genes in plants were divided into ABA-dependent and ABA-independent pathways, which depended on ABA/AREB and DREB/CBF. Auxin is mainly involved in organogenesis and morphogenesis, which are related to the accumulation and polarity distribution of auxin in plant tissues (Rahman, 2013). Shibasaki confirmed that cold stress mainly affected the transport of auxin in cells, and cold blocked the asymmetric distribution of auxin efflux carrier component 3 (PIN3) (Shibasaki et al., 2009). Low temperature can inhibit the gravitropism of auxin in plants. Therefore, the intracellular auxin response is related to the developmental regulation of plant growth under cold stress. As an important growth regulator regulating plant physiological and biochemical characteristics under abiotic stress, salicylic acid (SA) can reduce stress damage by increasing the responses of proline, antioxidants, heat shock protein, secondary metabolism, and sugar, thus improving the tolerance of plants to stress (Ahmad et al., 2019; Arif et al., 2020). It was found that low-temperature stress increased the content of endogenous SA in cucumber, and the expression of COR genes in grafted cucumber (*Cucumis sativus* L.) was significantly upregulated after the application of exogenous SA (Fu et al., 2021). Other hormones such as ethylene have also been reported to enhance cold resistance by increasing the activity of the antioxidant enzyme system (Ohme-Takagi and Shinshi, 1995). Cytokinin response factors *CRF2* and *CRF3* have been reported to regulate lateral root development in *Arabidopsis thaliana* under low-temperature stress (Jeon et al., 2016). Jasmonic acid (JA) was found to be a key upstream signal of the ICE-CBF/DREB1 pathway positively regulating cold tolerance in *A. thaliana* (Hu et al., 2013). Lange found that the gene encoding gibberellin-related catabolic enzyme *AtGA2ox9* increased the transcriptional level after low-temperature treatment in *A. thaliana* and helped to improve cold tolerance (Lange et al., 2020). The hormone response of plants under low-temperature stress is not regulated by a single hormone, but coordinated or antagonistic. In this way, the dynamic balance network of the plant hormone system is formed, which effectively helps plants maintain normal growth and development requirements under stress. At present, studies on hormone metabolism and regulation of *Z. bungeanum* under low-temperature stress have not been reported, and the co-regulation strategy of multiple hormone components in *Z. bungeanum* is not clear.

In this study, cold-tolerant and cold-sensitive varieties of *Z. bungeanum* were selected, and the hormone components were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Chemometric analysis was employed to analyze the differences and changes in hormone components of *Z. bungeanum* under low temperatures. Using weighted co-expression network analysis (WGCNA) combined with hormone component content and transcriptome sequencing analysis, we explored the pathways and genes related to hormone responses of *Z. bungeanum* under cold stress. The hormone response characteristics and strategies of two *Z. bungeanum* varieties under low-temperature stress were investigated at the metabolome and transcriptome levels, which provided the basis for further study on measures to improve the cold resistance of *Z. bungeanum*.

MATERIALS AND METHODS

Plant Materials and Cold Treatment

Two varieties, "Fuguhuajiao" (FG) and "Fengxiandahongpao" (FX) of *Z. bungeanum*, were selected for the study. The variety FG was from the areas with higher latitude and lower temperatures (38°42′ N∼39°35′ N, 110°22′ E∼111°14′ E, mean temperature 9.1°C). FG plants bloom late in the

spring and have strong stress-resistant growth characteristics. The variety FX was from the areas with relatively high temperatures (33°34′ N∼34°18′ N, 106°24′ E∼107°7′ E, mean temperature 12.1°C). The FX plants germinate earlier in the spring and have cold-sensitive growth characteristics. Seedlings of the cold-tolerant variety "Fuguhuajiao" and the cold-sensitive variety "Fengxiandahongpao" of *Z. bungeanum* were planted in the greenhouse (25–28°C, 16/8 h day/night) of Northwest A&F University. The seeds were collected from the Research Center for Engineering and Technology of *Zanthoxylum*, State Forestry Administration, Northwest A&F University, Fengxian, Shaanxi Province, China. Cold treatments were performed on the 4-month-old seedlings under 4°C for 24 h in a growth chamber. Leaf samples of seedlings were collected after low-temperature stress treatments for 0, 1, 3, 6, 12, and 24 h, and immediately put into liquid nitrogen. The samples were then transferred to the −80°C ultra-low temperature refrigerator for further determination and analysis. "Fuguhuajiao" and "Fengxiandahongpao" were marked as FG (FG1-18, 6 time points ×3 biological replicates) and FX (FX1-18, 6 time points ×3 biological replicates).

Plant Hormone Extraction and Composition Analysis by UPLC-MS/MS

The plant hormones in ground samples (50 mg) were extracted by the mixed solvent containing methanol/water/formic acid (15:4:1, v/v/v) and the internal standard (100 ng/ml). After 10 min of vortexing, the samples were centrifuged at 4°C, 12,000 r/min, and the supernatant was taken to a new centrifuge tube for concentration. The concentrated samples were redissolved with 100 μl of 80% methanol/water solution, filtered, and placed in the sample injection bottles for ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis. The standard solutions of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 200, and 500 ng/ml were prepared to obtain the mass spectrum peak intensity data of the corresponding quantitative signal of each concentration standard. The concentration ratio of external standard to internal standard was the abscissa, and the peak area ratio of external standard to internal standard was the ordinate. The standard curves of different substances were drawn.

The UPLC-MS/MS analysis was performed on the Waters Acquity ExionLCTM AD coupled with QTRAP 6500+ LC-MS/MS system (AB Sciex Pte. Ltd). The UPLS HSS T3 C18 column (1.8 μm, 100 mm × 2.1 mm) was equipped for plant hormone determination. The conditions included were as follows: mobile phase A was water with 0.04% acetic acid, mobile phase B was acetonitrile with 0.04% acetic acid; the flow rate was 0.35 ml/min, and the column temperature was 40°C. The gradient elution procedure was as follows: A/B 95:5 (v/v) from 0 to 1 min, A/B 5:95 (v/v) from 8 to 9 min, and A/B 95:5 (v/v) from 9 to 12 min. The quantitative analysis of plant hormone components was completed by using the multiple reaction monitoring (MRM) of a triple quadrupole mass spectrometer.

TABLE 1 | Identification of plant hormone components in two varieties of *Zanthoxylum bungeanum*.

Plant hormone	Compound name	Class
ABA	Abscisic acid	ABAs
ABA-GE	ABA-glucosyl ester	ABAs
IA	3-Indoleacrylic acid	Auxins
IAA	Indole-3-acetic acid	Auxins
IAA-Ala	N-(3-Indolylacetyl)-L-alanine	Auxins
IAA-Glc	1-O-indol-3-ylacetylglucose	Auxins
IAA-Glu	Indole-3-acetyl glutamic acid	Auxins
IAA-Gly	Indole-3-acetyl glycine	Auxins
IAN	3-Indoleacetonitrile	Auxins
IBA	Indole-3-butyric acid	Auxins
ICA	Indole-3-carboxylic acid	Auxins
ICAld	Indole-3-carboxaldehyde	Auxins
ILA	Indole-3-lactic acid	Auxins
MEIAA	Methyl indole-3-acetate	Auxins
OxIAA	2-oxindole-3-acetic acid	Auxins
TRA	Tryptamine	Auxins
TRP	L-tryptophan	Auxins
tZ	trans-Zeatin	CKs
IP	N6-isopentenyladenine	CKs
mTR	meta-Topolin riboside	CKs
KR	Kinetin riboside	CKs
iP9G	N6-Isopentenyl-adenine-9-glucoside	CKs
cZROG	cis-Zeatin-O-glucoside riboside	CKs
cZR	cis-Zeatin riboside	CKs
cZ9G	cis-Zeatin-9-glucoside	CKs
IPR	N6-isopentenyladenosine	CKs
K9G	Kinetin-9-glucoside	CKs
2CltZ	2-Chloro-trans-zeatin	CKs
tZR	trans-Zeatin riboside	CKs
DHZROG	Dihydrozeatin-O-glucoside riboside	CKs
DHZR	Dihydrozeatin ribonucleoside	CKs
DHZ7G	Dihydrozeatin-7-glucoside	CKs
2MeScZR	2-Methylthio-cis-zeatin riboside	CKs
tZOG	trans-Zeatin-O-glucoside	CKs
ACC	1-Aminocyclopropanecarboxylic acid	ETHs
GA19	Gibberellin A19	GAs
GA9	Gibberellin A9	GAs
JA	Jasmonic acid	JAs
JA-ILE	Jasmonoyl-L-isoleucine	JAs
JA-Val	N-[(-)-Jasmonoyl]-(L)-valine	JAs
H2JA	Dihydrojasmonic acid	JAs
OPDA	cis(+)-12-Oxophytodienoic acid	JAs
SA	Salicylic acid	SAs
SAG	Salicylic acid 2-O-β-glucoside	SAs
5DS	5-Deoxystrigol	SLs

Data Analysis

Statistically significant differences were performed by ANOVA using SPSS version 19.0 (IBM Corp., Armonk, NY, USA). Data standardization and the principal component analysis

(PCA) were conducted using OriginPro 2017C (Originlab, Northampton, USA). The cluster heat map (CHM) was performed using TBtools (Chen et al., 2020). The orthogonal partial least squares discriminant analysis (OPLS-DA), the uniform manifold approximation and projection (UMAP) and the nonmetric multidimensional scaling (NMDS) were performed by using online software (https://www.omicshare.com/tools/Home/Soft/getsoft).

The WGCNA was carried out using the R package (Langfelder and Horvath, 2008). The genes from transcriptomes of 36 cold-treated leaf samples were used as the input data for co-expression network construction. Transcriptome data for WGCNA were derived from our previous work (Tian et al., 2021). The parameters were power = 15, minModuleSize = 30, and mergeCutHeight = 0.25. The connectivity of hub genes was the sum of the weights from all edges of a node. The co-expression network was visualized by Cytoscape 3.7.1 (Shannon et al., 2003).

RESULTS

Identification and Changing Profiles of Plant Hormone Components in Two *Z. bungeanum* Varieties

To obtain the plant hormone effect on *Z. bungeanum* under low-temperature stress, UPLC-MS/MS was operated on cold-tolerant and cold-sensitive varieties of leaves for analyzing the plant hormone components. A total of 45 plant hormone components were detected in two varieties of *Z. bungeanum*: 2 ABAs (abscisic acid, ABA; ABA-glucosyl ester, ABA-GE), 15 auxins (3-indoleacrylic acid, IA; indole-3-acetic acid, IAA; N-(3-indolylacetyl)-L-alanine, IAA-Ala; 1-O-indol-3-ylacetylglucose, IAA-Glc; indole-3-acetyl glutamic acid, IAA-Glu; indole-3-acetyl glycine, IAA-Gly; 3-indoleacetonitrile, IAN; indole-3-butyric acid, IBA; indole-3-carboxylic acid, ICA; indole-3-carboxaldehyde, ICAld; indole-3-lactic acid, ILA; methyl indole-3-acetate, MEIAA; 2-oxindole-3-acetic acid, OxIAA; tryptamine, TRA; L-tryptophan, TRP), 17 CKs (*trans*-zeatin, tZ; N6-isopentenyladenine, IP; meta-topolin riboside, mTR; kinetin riboside, KR; N6-isopentenyl-adenine-9-glucoside, iP9G; *cis*-zeatin-O-glucoside riboside, cZROG; *cis*-zeatin riboside, cZR; *cis*-zeatin-9-glucoside, cZ9G; N6-isopentenyladenosine, IPR; kinetin-9-glucoside, K9G; 2-chloro-*trans*-zeatin, 2ClTZ; *trans*-zeatin riboside, tZR; dihydrozeatin-O-glucoside riboside, DHZROG; dihydrozeatin ribonucleoside, DHZR; dihydrozeatin-7-glucoside, DHZ7G; 2-methylthio-*cis*-zeatin riboside, 2MeScZR; *trans*-zeatin-O-glucoside, tZOG), 1 ETHs (1-aminocyclopropanecarboxylic acid, ACC), 2 GAs (gibberellin A19, GA19; gibberellin A9, GA9), 5 JAs (jasmonic acid, JA; jasmonoyl-L-isoleucine, JA-ILE; N-[(-)-jasmonoyl]-(L)-valine, JA-Val; dihydrojasmonic acid, H2JA; *cis*(+)-12-oxophytodienoic acid, OPDA), 2 SAs (salicylic acid, SA; salicylic acid 2-O-β-glucoside, SAG), and 1 SLs (5-deoxystrigol, 5DS) (**Table 1**). Among them, ABA, ABA-GE, ICAld, TRP, ACC, SA, and SAG were detected in the samples of both cold-tolerant and cold-sensitive varieties with high hormone contents (**Figures 1A–G**).

The changing trends of ABA and ABA-GE in FG and FX under cold stress were different. In FG, the content of ABA decreased from 0 to 3 h, then increased to the peak at 24 h

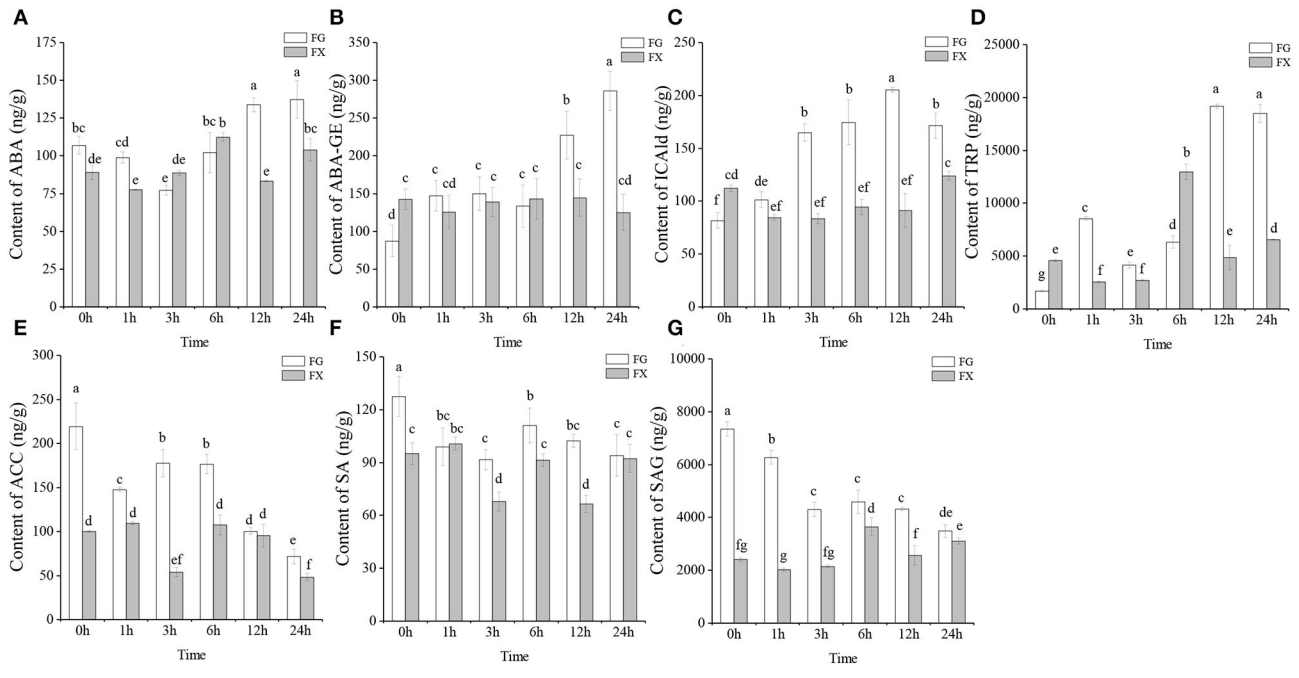

FIGURE 1 | (A–G) The main plant hormone components in cold-tolerant variety FG and cold-sensitive variety FX of *Zanthoxylum bungeanum*.

(137.26 ng/g) (**Figure 1A**). In FX, ABA content basically showed an increasing trend first and then decreased, and reaching the peak value (112.42 ng/g) at 6 h. The content of ABA in FG was significantly higher than that in FX at the early (0–1 h) and late (12–24 h) stage under cold stress. We also noticed that the content of another ABAs component, ABA-GE, increased significantly at the late stage (12–24 h) in FG, and the ABA-GE content at 24 h was 3.29-fold and 1.93-fold compared with that at 0 h in FG and 24 h in FX, respectively (**Figure 1B**). In FX, the content of ABA-GE increased slightly during low-temperature stress, but the change was not obvious. The content of auxin component ICAld in FG showed a gradually increasing trend, which increased significantly at 3 h and reached the peak (205.29 ng/g) at 12 h under cold stress (**Figure 1C**). In FX, the content of ICAld was higher than that of FG only at 0 h and lower than FG in the process of low-temperature stress. The content level of TRP, another auxin component, was the highest among *Z. bungeanum*. The content of TRP was low under the normal growth in FG, but increased significantly and reached the peak at 12 h (19,150.33 ng/g) after cold stress, and maintained a significantly high level at 24 h (18,484.77 ng/g) (**Figure 1D**). In FX, the content of TRP increased to the peak value at 6 h (12,960.14 ng/g), then decreased rapidly. The content of ETH component ACC in *Z. bungeanum* was also at a high level (**Figure 1E**). In FG, the contents of ACC, SA, and SAG all showed a downward trend, and the contents in the samples treated with low temperature were lower than those in the control (0 h). In addition, the contents of the above three hormones in FG were generally higher than those in FX. On the whole, the 7 plant hormone components with high content in *Z. bungeanum* were higher in FG than in FX at the late stage (12–24 h) of low-temperature stress.

Chemometric Analysis of Plant Hormone Components in *Z. bungeanum*

As many hormone components were detected in *Z. bungeanum* under low-temperature stress, the content-changing profile of the two varieties under different treatment times needs to be further analyzed. Therefore, using a variety of chemometric analysis methods, including linear and nonlinear analysis models, to analyze the hormone components under cold stress treatment can make the response of plant hormone in *Z. bungeanum* clearer and screen out the important differential hormone components.

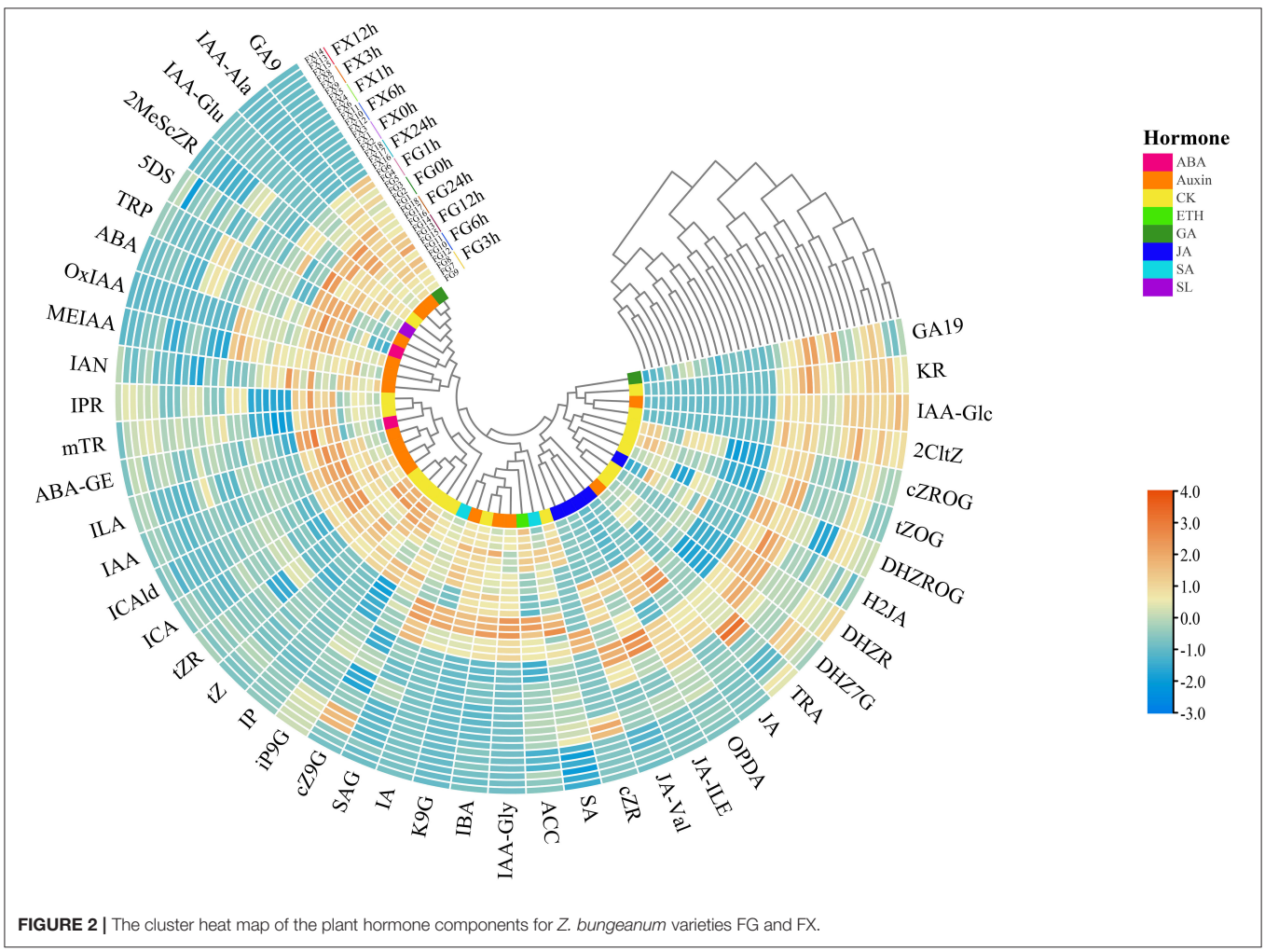

FIGURE 2 | The cluster heat map of the plant hormone components for *Z. bungeanum* varieties FG and FX.

Cluster Heat Map Analysis

Through the cluster heat map analysis of the samples in FG and FX under low-temperature stress treatment, we can preliminarily understand the classification of different samples due to the influence of hormone components, as well as the classification differences and characteristics. The normalized data were used for cluster analysis, and the results showed that 36 samples of *Z. bungeanum* were divided into four groups: FG7-9 (3 h) and FG10-12 (6 h) were the first group, FG13-15 (12 h) and FG16-18 (24 h) were the second group, FG1-3 (0 h) and FG4-6 (1 h) were the separate third group, tall FX samples FX1-18 (0–24 h) were divided into the fourth group (**Figure 2**). In addition, the 45 plant hormone components were grouped into four clusters: the first group included two subgroups, 10 hormone components (GA9, IAA-Ala, IAA-Glu, 2MeScZR, 5DS, TRP, ABA, OxIAA, MEIAA, and IAN) were divided into one subgroup, and 12 (IPR, mTR, ABA-GE, ILA, IAA, ICAld, ICA, tZR, tZ, IP, iP9G, and cZ9G) were divided into another subgroup, 8 components (SAG, IA, K9G, IBA, IAA-Gly, ACC, SA, and cZR) were grouped into the second cluster, 5 components (JA-Val, JA-ILE, OPDA, JA, and TRA) were in the third cluster, the fourth group also included two subgroups, 6 components (DHZ7G, DHZR, H2JA, DHZROG, tZOG, and cZROG) were divided into one subgroup, 4 (2CltZ, IAA-Glc, KR, and GA19) were in another subgroup. The same color in the innermost circle in **Figure 2** represents the same plant hormone class. It was found that some hormone components in the same class were divided into different groups.

Principal Component Analysis

Principal component analysis was used to reduce the dimension of hormone components in *Z. bungeanum* under cold stress, and the components with the highest contribution rates were selected. Through the PCA analysis of 45 hormone components in 36 samples of two *Z. bungeanum* varieties, the eigenvalues and cumulative contribution rates of each component were obtained (**Table 2**). The indicators with an eigenvalue > 1 were selected as the main indicators of principal components. In this result, the eigenvalues of the top eight were >1, with the cumulative contribution rate reaching 92.85%. The results showed that in the first principal component (PC1), IAA-Ala, GA9, K9G, IAA-Glu, and 2MeScZR were the main positive indicators of plant hormone components, and IAA-Glc, 2CltZ, and KR were the main negative indicators (**Figure 3**). In PC2, IPR, mTR, cZROG, iP9G, DHZ7G, and ICA were the main positive indicators, while SAG and ACC were the negative indicators. In addition, PC1 clearly separated the samples of two *Z. bungeanum* varieties, while PC2 divided the samples into FG and FX treated with cold stress at different time points. Therefore, the 16 hormone components with an important cumulative contribution rate screened by PCA were selected as the main indicators in *Z. bungeanum* for further analysis.

TABLE 2 | Eigenvalue and cumulative of plant hormone components.

Principal component number	Eigenvalue	Cumulative (%)
1	17.95	39.88
2	10.09	62.31
3	4.82	73.02
4	2.83	79.29
5	2.03	83.81
6	1.84	87.90
7	1.13	90.40
8	1.11	92.85
9	0.85	94.74
10	0.52	95.91
11	0.47	96.96
12	0.31	97.64
13	0.24	98.19
14	0.16	98.53
15	0.12	98.81
16	0.09	99.02
17	0.07	99.18
18	0.07	99.33
19	0.06	99.47
20	0.04	99.56
21	0.04	99.65
22	0.03	99.72
23	0.03	99.78
24	0.02	99.83
25	0.02	99.87
26	0.01	99.91
27	0.01	99.93
28	0.01	99.95
29	0.01	99.96
30	0.01	99.98
31	0.00	99.98
32	0.00	99.99
33	0.00	100.00
34	0.00	100.00
35	0.00	100.00
36	0.00	100.00

Uniform Manifold Approximation and Projection Analysis

Furthermore, we took the 7 main hormone components with high content and detected in both two varieties and 16 hormone components with important contribution rates screened by PCA as the main hormone indicators for further analysis, including ABA, ABA-GE, IAA-Ala, IAA-Glc, IAA-Glu, ICA, ICAld, TRP, mTR, KR, iP9G, cZROG, IPR, K9G, 2CltZ, DHZ7G, 2MeScZR, ACC, GA9, SA, and SAG.

The nonlinear analysis model uniform manifold approximation and projection analysis (UMAP) was carried out to analyze the above 21 hormone components and project them to a two-dimensional coordinate system to visualize their distribution. The results showed that the distance between FG1-3 (0 h) and FG4-6 (1 h) was close, FG7-9 (3 h) and FG10-12 (6 h) was close, and FG13-15 (12 h) and FG16-18 (24 h) was close (**Figure 4**). This result was consistent with the result of cluster analysis, indicating that the above 21 hormone component indicators can represent the overall hormone response level

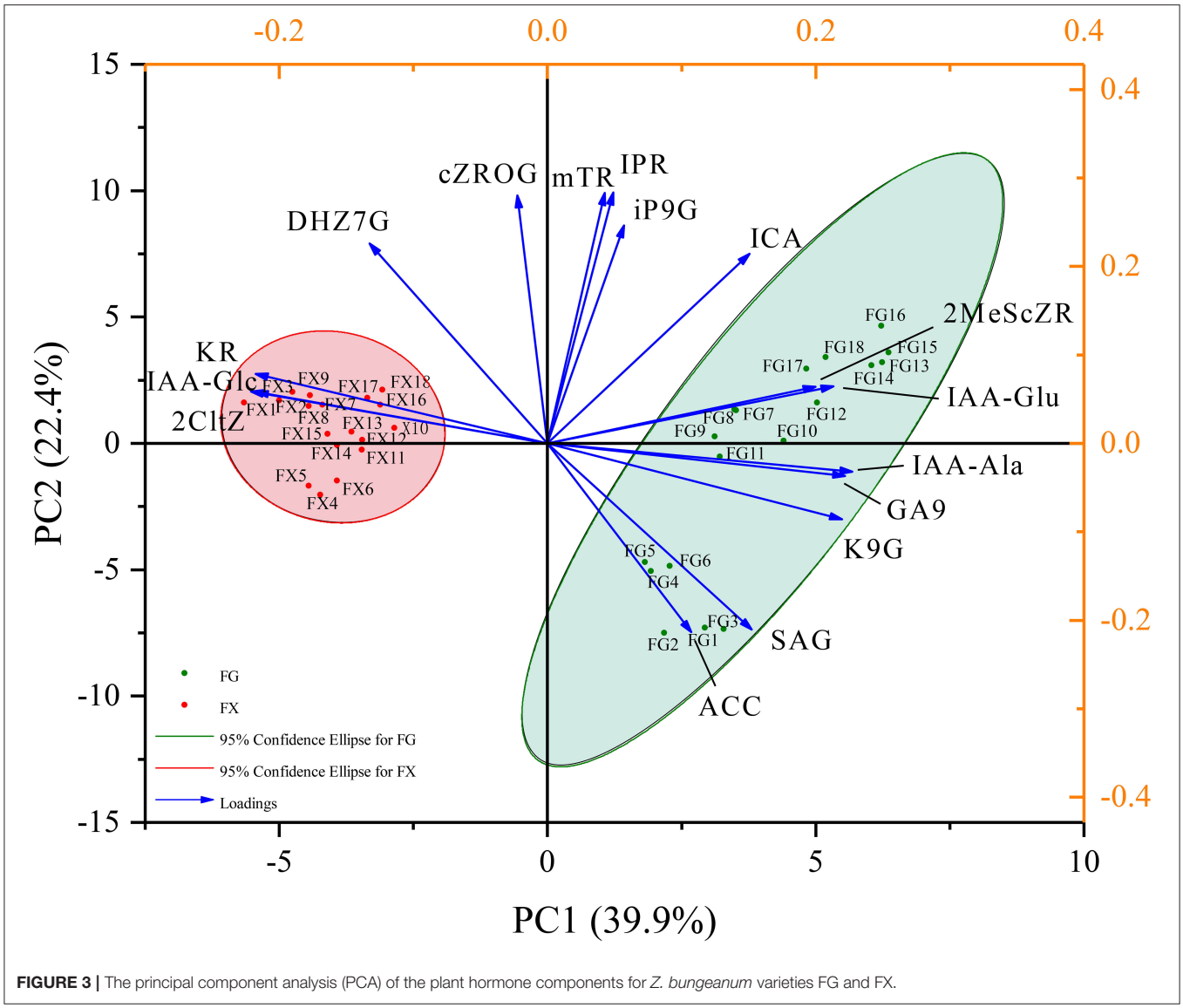

FIGURE 3 | The principal component analysis (PCA) of the plant hormone components for *Z. bungeanum* varieties FG and FX.

in *Z. bungeanum* samples, and the data distribution was also conformed to the nonlinear model, which can be explained and analyzed by the nonlinear model.

Nonmetric Multidimensional Scaling Analysis

Nonmetric multidimensional scaling analysis was used to analyze 21 main plant hormone components in cold-tolerant and cold-sensitive varieties of *Z. bungeanum* samples under cold stress. The results showed that the stress value was 0.023 (<0.05), indicating that the model had good representativeness and the model accurately reflected the real distribution of data sorting (**Figure 5**). NMDS analysis showed that the samples of FG and FX treated with low-temperature stress were obviously separated, and the samples at different treatment time points had certain differences. The plant hormone levels and components of FG in a normal growth state were significantly different from those of other samples. The hormone components in the samples of FX10-12 (6 h) to FG13-15 (12 h) and FG16-18 (24 h) were relatively close.

Orthogonal Partial Least Square Discriminant Analysis

Orthogonal partial least square discriminant analysis was used to determine the differences in hormone components in two varieties and to identify the important differential hormone substances under cold stress. Variable important in projection (VIP) threshold was introduced to measure the influence intensity of different hormone components in low temperature-treated samples. In this study, plant hormone components with VIP > 1 were considered to have a strong influence and explanatory power on the cold stress treatment sample (**Table 3**). OPLS-DA plot showed a clear separation of the two varieties. Results showed that in FG, 15 hormone components, namely, ABA, ABA-GE, ICA, ICAld,

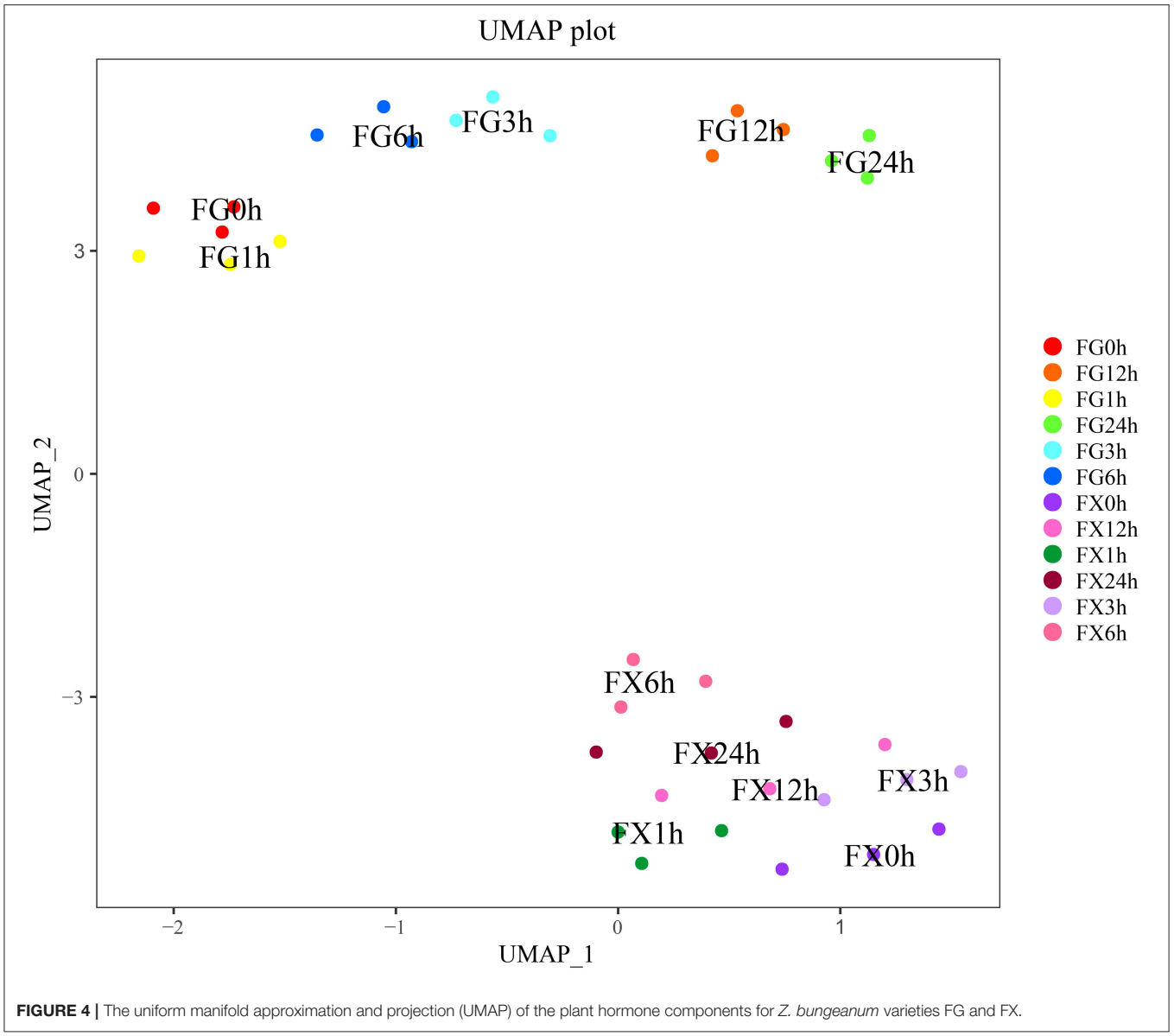

FIGURE 4 | The uniform manifold approximation and projection (UMAP) of the plant hormone components for *Z. bungeanum* varieties FG and FX.

TRP, mTR, iP9G, cZROG, IPR, K9G, DHZ7G, 2MeScZR, ACC, SA, and SAG, were important feature hormone metabolites during the 24 h process of cold stress. Among them, the key hormone components from 3 h of cold stress were ICA, ICAld, mTR, cZROG, and IPR, and all of them were upregulated differential substances. There were also 15 important differential substances in FX, which were ABA, ICA, ICAld, TRP, mTR, KR, iP9G, cZROG, IPR, 2CltZ, DHZ7G, 2MeScZR, ACC, SA, and SAG. Among them, KR and DHZ7G were the key differential hormone components in the process of low-temperature stress and showed a downregulated pattern. We noticed that the pattern of IPR in FX was consistent with FG, both of which were important differentially upregulated hormone components from 3 to 24 h. The results indicated that *Z. bungeanum* mainly responded to low temperature through the important hormone components in the ABA pathway, CK pathway, ACC pathway, IAA pathway, and SA pathway (**Figure 6**).

Analysis of the Co-Expression Network of Important Differential Hormones and Transcriptome in Response to Low Temperature in *Z. bungeanum*

The hormone-related gene network in *Z. bungeanum* was constructed by combining 12 main hormone components with a transcriptome profile by WGCNA. After optimizing and merging the imported data, 16 modules were finally obtained. The results showed that several modules had a high correlation with hormone components (**Figure 7**). Among them, the MEhoneydew module had a high correlation with IAA-Ala, IAA-Glc, IAA-Glu, KR, K9G, 2CltZ, DHZ7G, 2MeScZR, ACC,

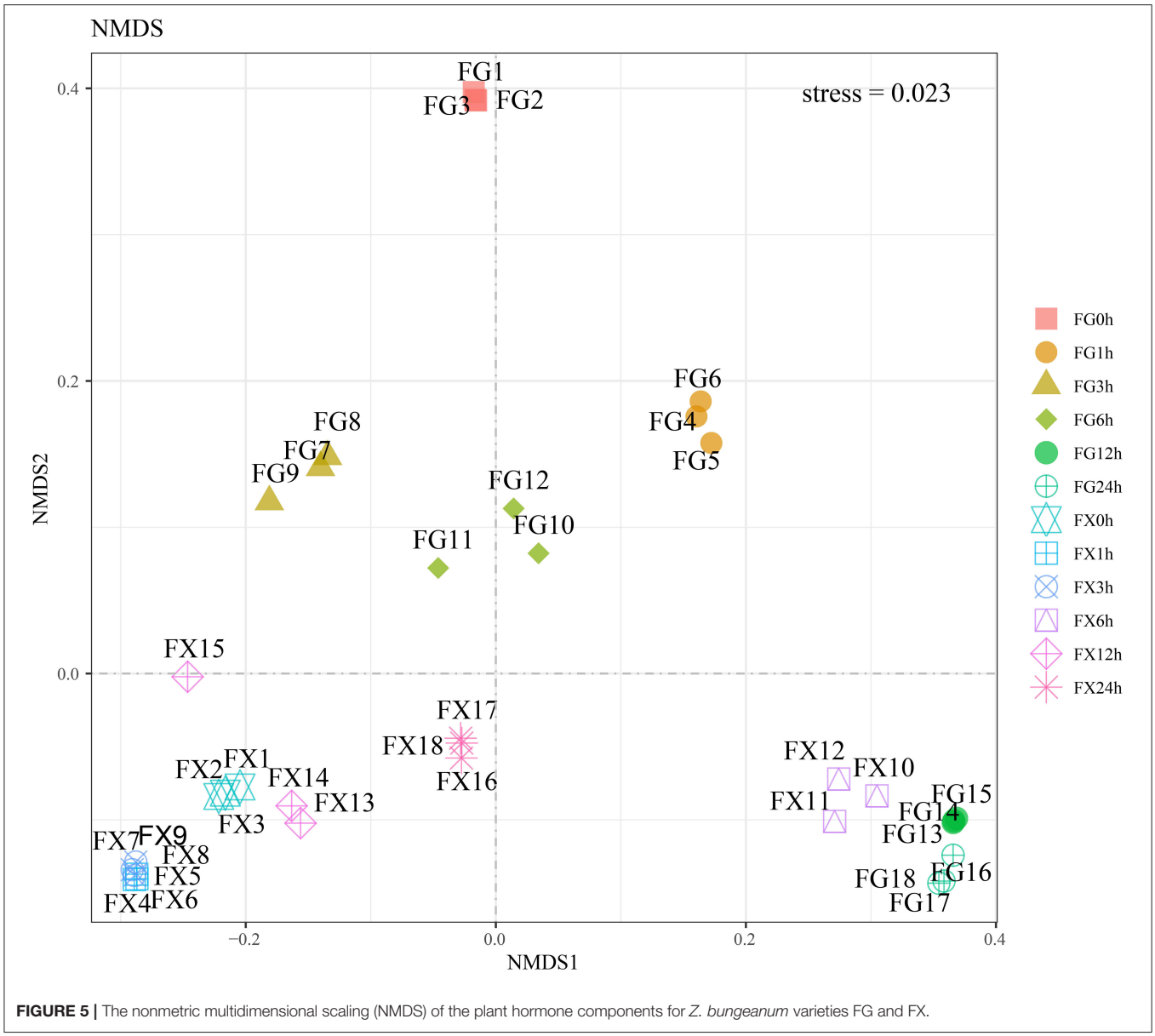

FIGURE 5 | The nonmetric multidimensional scaling (NMDS) of the plant hormone components for *Z. bungeanum* varieties FG and FX.

GA9, and SAG. MEcoral1 module had a high correlation with IAA-Ala, IAA-Glc, IAA-Glu, ICAld, KR, K9G, 2CltZ, DHZ7G, 2MeScZR, GA9, and SAG. As there were a few related hormone components in other modules, or the correlation pattern was similar to the above two modules, we selected MEhoneydew and MEcoral1 for further network model construction and analysis.

Functional annotation and enrichment of Kyoto Encyclopedia of Genes and Genomes (KEGG) genes were carried out on 150 core genes in the MEhoneydew module (**Figure 8**). Results showed that 8 genes were enriched in carbon metabolism-related pathways, namely, *ZB36497* (photosynthesis ko00195); *ZB07173*, *ZB18516*, and *ZB26838* (carbon fixation in photosynthetic organisms ko00710), *ZB07173*, *ZB18516*, *ZB19499*, *ZB20582*, *ZB26838*, and *ZB55699* (carbon metabolism ko01200); 7 genes were enriched in fatty acid metabolism pathways, namely, *ZB20582* and *ZB54028* (fatty acid metabolism ko01212), *ZB00998* and *ZB17366* (linoleic acid metabolism ko00591), *ZB20582* and *ZB54028* (fatty acid degradation ko00071), *ZB53339* (glycerophospholipid metabolism ko00564), *ZB00998*, *ZB17366*, and *ZB54082* (alpha-linolenic acid metabolism ko00592), *ZB16680* and *ZB30550* (glycerolipid metabolism ko00561); 8 genes were enriched in amino acid metabolism pathways, namely, *ZB20582* (lysine degradation ko00310), *ZB07173* and *ZB18516* (biosynthesis of amino acids ko01230), *ZB48222* (beta-alanine metabolism ko00410, glycine, serine, and threonine metabolism ko00260, tyrosine metabolism ko00350, phenylalanine metabolism ko00360), *ZB20582* (glycine, serine, and threonine metabolism ko00260), *ZB19499* and *ZB36273* (cyanoamino acid metabolism ko00460), *ZB20582* and *ZB55699* (tryptophan metabolism ko00380), *ZB39498* (cysteine and

TABLE 3 | Variable important for the projection (VIP) of plant hormone components in orthogonal partial least squares discriminant analysis (OPLS-DA).

	FG1h	FG3h	FG6h	FG12h	FG24h	FX1h	FX3h	FX6h	FX12h	FX24h
ABA	0.68	1.02	0.23	0.81	0.82	0.94	0.05	1.26	0.68	1.15
ABA-GE	1.25	0.84	0.74	1.13	1.33	0.53	0.13	0.01	0.08	0.55
IAA-Ala	0.83	0.38	0.20	0.03	0.31	0.00	0.00	0.00	0.00	0.00
IAA-Glc	0.00	0.00	0.00	0.00	0.00	0.71	0.43	0.97	0.77	0.25
IAA-Glu	0.85	0.01	0.13	0.79	0.83	0.00	0.00	0.00	0.00	0.00
ICA	0.94	1.14	1.36	1.36	1.05	0.95	0.98	0.30	0.09	1.06
ICAld	0.79	1.18	1.37	1.22	1.01	1.04	1.30	0.71	0.90	0.72
TRP	1.37	0.55	0.83	1.24	1.19	0.76	0.90	1.40	0.16	0.83
mTR	0.77	1.27	1.27	1.36	1.23	0.68	0.12	0.78	0.97	1.05
KR	0.00	0.00	0.00	0.00	0.00	1.39	1.11	1.35	1.31	1.53
iP9G	0.76	1.47	1.62	0.94	1.26	1.17	0.43	0.85	0.02	0.96
cZROG	0.66	1.47	1.13	1.18	1.07	1.76	0.93	1.44	1.64	0.64
IPR	0.19	1.17	1.21	1.23	1.23	0.49	1.35	1.05	1.34	1.57
K9G	1.35	0.80	0.62	0.62	0.55	0.00	0.00	0.00	0.00	0.00
2CltZ	0.00	0.00	0.00	0.00	0.00	0.49	0.02	1.03	0.40	0.07
DHZ7G	0.44	1.09	1.28	0.85	0.87	1.84	1.10	1.40	1.96	1.49
2MeScZR	1.37	0.90	1.04	0.83	1.02	1.03	1.23	1.17	0.64	0.72
ACC	1.44	0.66	0.74	1.07	1.17	0.54	1.50	0.29	0.25	1.43
GA9	0.60	0.02	0.50	0.63	0.02	0.00	0.00	0.00	0.00	0.00
SA	1.49	1.19	0.78	0.82	0.92	0.55	1.96	0.35	1.86	0.25
SAG	0.99	1.16	1.20	0.97	1.08	0.62	0.62	1.01	0.28	0.93

methionine metabolism ko00270); 12 genes were enriched in sugar metabolism pathways, namely, *ZB56404* (amino sugar and nucleotide sugar metabolism ko00520), *ZB07173* and *ZB18516* (glycolysis/gluconeogenesis ko00010), *ZB41084* and *ZB55060* (pentose and glucuronate interconversions ko00040), *ZB36689* (starch and sucrose metabolism ko00500), *ZB20582* and *ZB26838* (pyruvate metabolism ko00620), *ZB07173* and *ZB18516* (pentose phosphate pathway ko00030), *ZB07173*, *ZB18516*, *ZB41084*, and *ZB55060* (fructose and mannose metabolism ko00051), *ZB19499*, *ZB20582*, and *ZB55699* (glyoxylate and dicarboxylate metabolism ko00630), *ZB30111*, *ZB34843*, and *ZB56404* (galactose metabolism ko00052); 3 genes were enriched in signaling transduction pathways, namely, *ZB53339* (phosphatidylinositol signaling system ko04070 and inositol phosphate metabolism ko00562), *ZB43608* and *ZB56627* (plant hormone signal transduction ko04075).

The co-expression gene network was constructed with 150 hub genes in the MEhoneydew module, and the gene connectivity ranged from 0.10 to 17.93 (**Figure 9**). In this module, two hormone signaling pathway genes were identified. *ZB43608* was a TGA transcription factor in the salicylate hormone signaling pathway, which connected 14 genes in the network, namely, *ZB00574* (*RPS2*), *ZB06882*, *ZB11428*, *ZB15352* (*AL5*), *ZB16502* (*PUB5*), *ZB20639* (*RNP1*), *ZB23764* (*PUB14*), *ZB24432* (*GALK*), *ZB32169* (*AT4G26450*), *ZB39498*, *ZB39509* (*SRG1*), *ZB50490* (*CYP736A12*), *ZB55060* (*SDH*), and *ZBnew25840* (*Os03g0733400*). *ZB56627* was the two-component response regulator ARR11 in the cytokinine signal transduction pathway, which connected 12 genes in the network, namely, *ZB00574* (*RPS2*), *ZB07755*, *ZB11428*, *ZB15352* (*AL5*), *ZB17764* (*CNGC5*), *ZB21842*, *ZB23764* (*PUB14*), *ZB24432* (*GALK*), *ZB39498*, *ZB39509* (*SRG1*), *ZB55060* (*SDH*), and *ZBnew25840* (*Os03g0733400*). These genes were involved in spliceosome (ko03040), cysteine and methionine metabolism (ko00270), pentose and glucuronate interconversions (ko00040), and fructose and mannose metabolism (ko00051) pathways. Among them, *ZB11428*, *ZB15352*, *ZB06882*, *ZB07755*, *ZB23764*, *ZB00574*, *ZB16502*, and *ZB24432* had high connectivity in the module and were the key genes of the network.

Functional annotation and enrichment of KEGG genes were carried out on 150 core genes in the MEcoral1 module (**Figure 10**). Results showed that 3 genes (*ZB36615* (starch and sucrose metabolism ko00500, amino sugar and nucleotide sugar metabolism ko00520), *ZB05065* and *ZB40450* [fructose and mannose metabolism ko00051, amino sugar and nucleotide sugar metabolism ko00520)] were enriched in the sugar metabolism pathways; 1 gene (*ZB29785*) enriched in the peroxisome ko04146 pathway; 3 genes (*ZB24496*, *ZB29882*, and *ZB54262*) enriched in the endocytosis ko04144 pathway; 1 gene (*ZB50206*) enriched in the tropane, piperidine, and pyridine alkaloid biosynthesis ko00960 pathway; 1 gene (*ZB48959*) enriched in the circadian rhythm-plant ko04712; 1 gene (*ZBnew19225*) enriched in the porphyrin and chlorophyll metabolism ko00860 pathway; and 2 genes (*ZB06550* and *ZB40291*) enriched in the phagosome ko04145 pathway.

The gene connectivity in the MEcoral1 module co-expression network ranged from 0.11 to 18.92 (**Figure 11**). Among them, the gene *ZB11149* had the highest connectivity ($c = 18.92$) and participated in the intracellular protein transport, protein transporter activity and vesicle-mediated transport (GO: 0006886, GO: 0008565, and GO: 0016192) process; gene *ZB06550* ($c = 15.59$) participated in the vacuolar proton-transporting, plasma membrane, chloroplast, integral component of membrane (GO: 0000220, GO: 0005886, GO: 0009507, and GO: 0016021) process; gene *ZB32894* ($c = 13.07$) participated in calcium ion binding (GO: 0005509) and NAD(P) activity (GO: 0032440) process, which were related to signaling transduction; gene *ZB21656* ($c = 11.15$) was a ubiquitin-conjugating enzyme, which had acid-amino acid ligase activity; gene *ZB23593* was Bax1 inhibitor and participated in cellular component process (GO: 0016021). The above genes all had high connectivities and were in the core positions of the co-expression network.

DISCUSSION

Abscisic acid was considered to be an important hormone in plant response to low temperatures. As an important signal factor, ABA regulates plant cold response by regulating various stress pathways. One study showed that cold stress can increase the endogenous ABA levels in plants and then improve their resistance to low temperatures (Li et al., 2017). In our study, ABA was found to be the main hormone component in the two varieties of *Z. bungeanum*, indicating that ABA plays an important role in the cold response of *Z. bungeanum*. However,

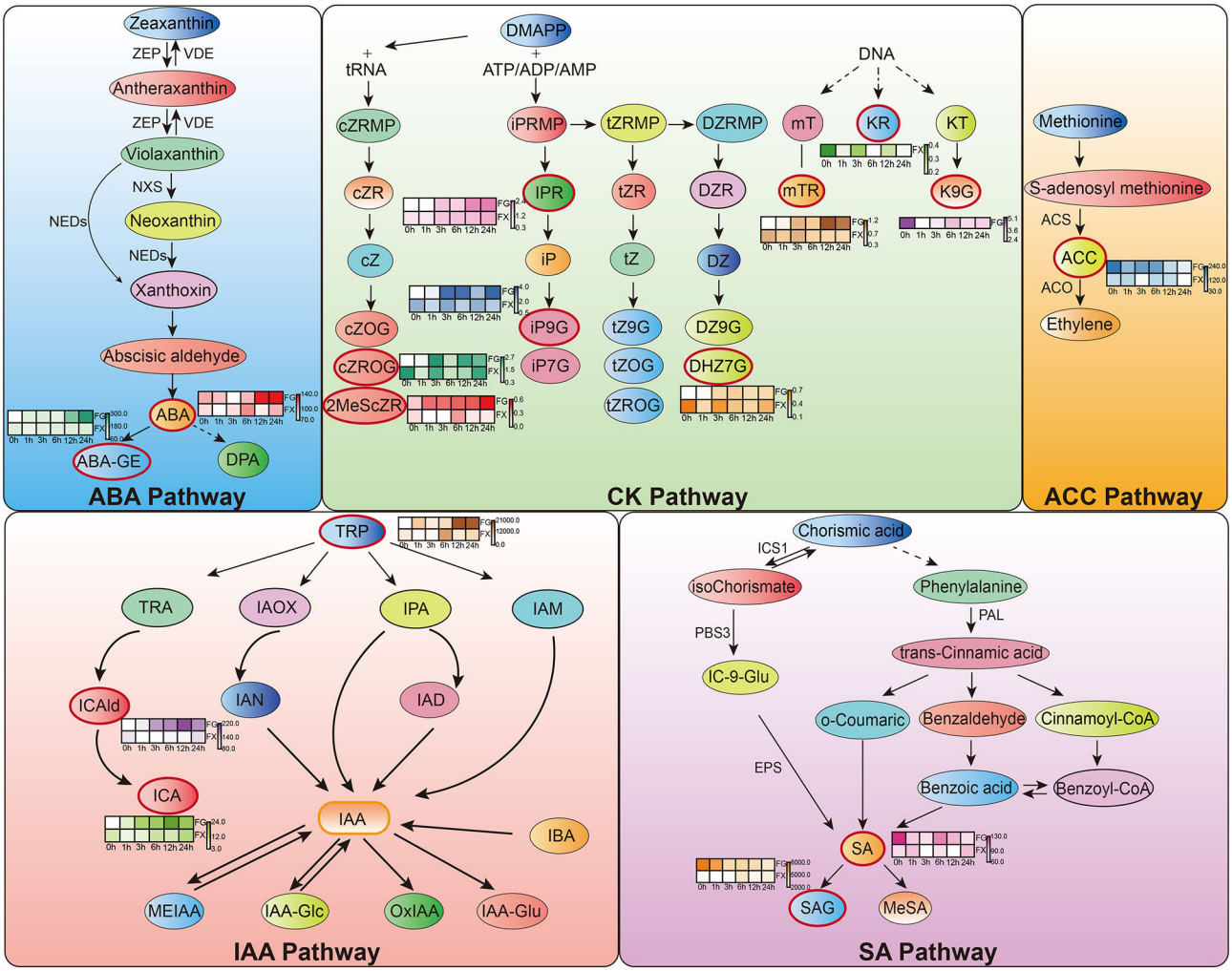

FIGURE 6 | Hormone response pathways in *Z. bungeanum* under cold stress. Red boxes indicate important hormone components identified from orthogonal partial least squares discriminant analysis (OPLS-DA). The heat map graph beside them shows the content change pattern of each important hormone component.

we found that the changing trend of ABA content varied in cold-tolerant and cold-sensitive varieties, indicating that the ABA response patterns of the two varieties were different. The level of ABA content in FG was significantly higher than that in FX at the early (0–1 h) and late stages (12–24 h) under cold stress. It has been reported that a large amount of ABA accumulates during low-temperature stress, and the content of ABA in cold-tolerant plants is higher than that in cold-sensitive plants (Lang et al., 1994; Wang et al., 2009). Under normal growth conditions, the content of ABA in FG was significantly higher than that in FX, which could play a faster role as a signal pathway in the early stage of low-temperature stress, so it made a more rapid response to cold. At the late stage (12–24 h) of low-temperature stress, the content of ABA in FG increased significantly. The level of endogenous ABA can increase temporarily in the initial stage of cold response, while continuous action requires transport and replenishment from other tissue parts (Shi and Yang, 2014). ABA-GE is one of the most abundant forms of ABA couplings. It showed very weak or almost no biological activity. For a long time, ABA-GE has been regarded as a by-product of catabolism that reduces the level of ABA in cells. Studies have shown that ABA-GE acts as an ABA reservoir and can be rapidly hydrolyzed to produce active ABA under the action of glucosidase (Johnson and Ferrell, 1982; Dietz et al., 2000; Sauter et al., 2002). The active ABA produced is very important for plants to adapt to abiotic stress. In addition, ABA-GE can supplement ABA by long-distance transport from the roots up to the leaves (Xu et al., 2014). In this study, we found that the ABA content in leaves of FG increased significantly at 12 and 24 h, while the content of ABA-GE also increased significantly at these time points. Sauter reported that the ABA-GE content increased significantly under stress, which was consistent with our results (Sauter et al., 2002). Moreover, OPLS-DA results showed that ABA-GE was an important differential hormone component in FG samples at 12–24 h. Therefore, we speculate that in the process of cold response in FG, ABA-GE acts as a storage and replenishment

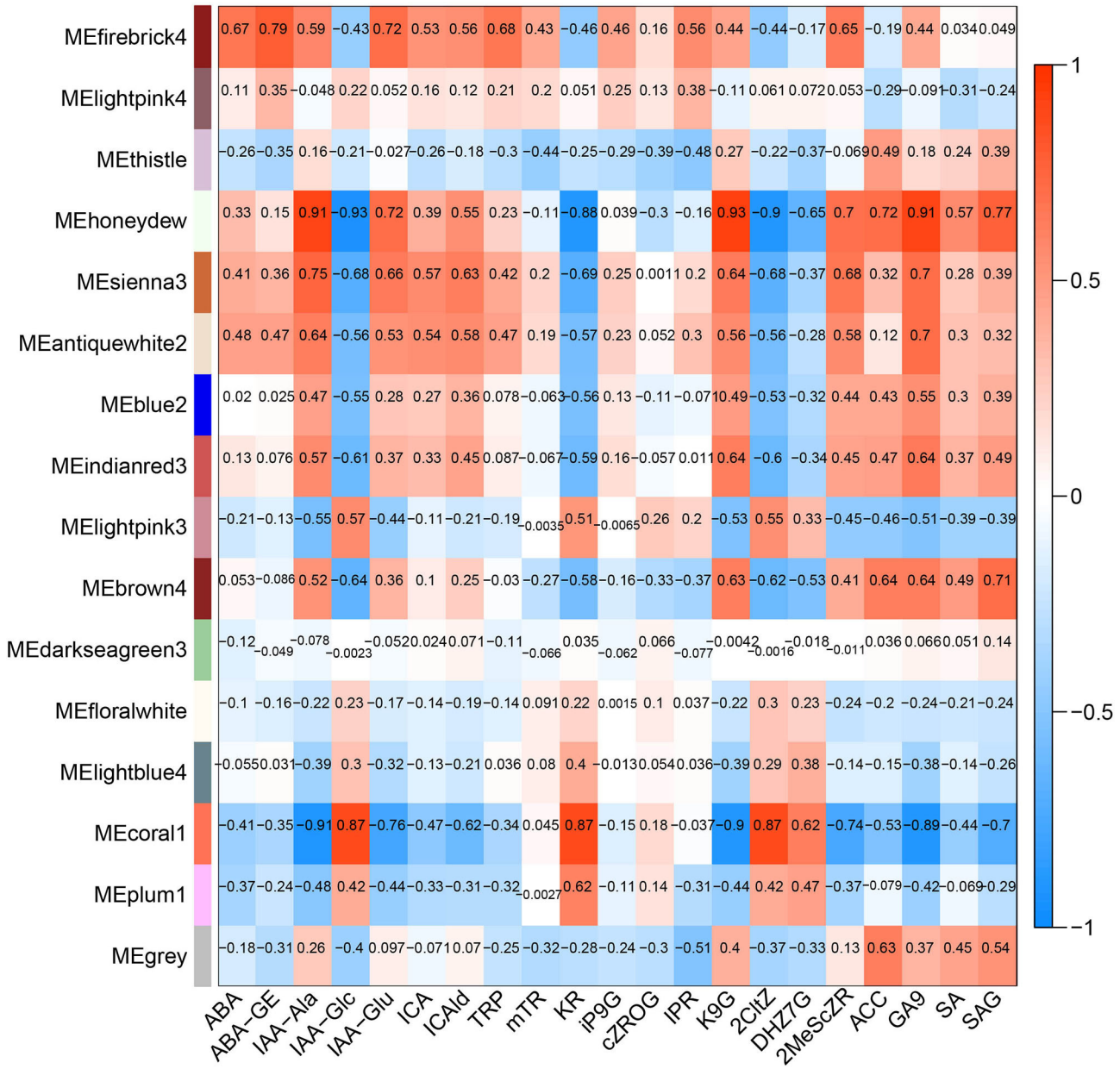

FIGURE 7 | Correlations between modules and dominating plant hormone component traits in *Z. bungeanum*.

repository of active ABA and carries out cold response by long-distance transport from the roots to leaves at cold stimulation sites. This result also adds new evidence to the theory that ABA-GE can be transported over long distances in response to stress. In addition, transmembrane transporters are necessary for long-distance transport of ABA-GE, but the mechanism of it remains unclear. In the WGCNA co-expression module MEcoral1, the gene *ZB1119* with the highest connectivity was annotated to participate in intracellular and vesicle transport, so it was speculated that it may be related to the transmembrane transport of plant hormone. Further experiments and evidence are needed to confirm this.

Cytokinin is the central regulator of plant development, which can promote cell division and participate in biological processes such as plant growth and flowering, leaf senescence, and chloroplast generation (Khan et al., 2017). The effect of low-temperature stress on plants is the opposite. Therefore, a stable level of cytokinin plays a crucial role in the normal growth and development of plants under low temperatures. There is a wide variety of cytokinins in plants, including free state and binding state. It is generally believed that the cytokinin binding state form is more stable and that the two state forms can be converted to each other by the action of related enzymes (Zhang et al., 2003). In our results, cytokinin binding forms,

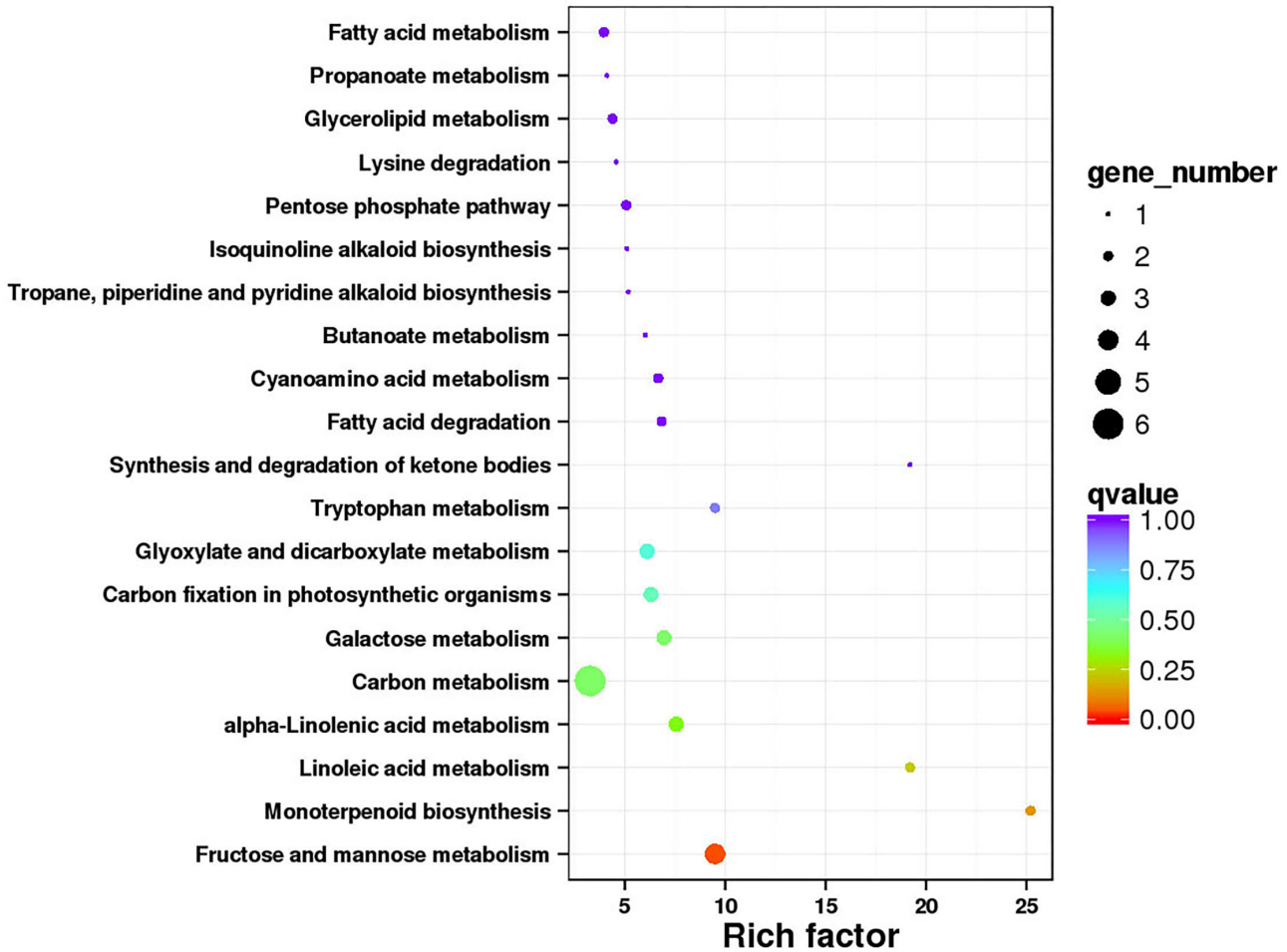

FIGURE 8 | Functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of hub genes in MEhoneydew.

such as cZROG, iP9G, DHZ7G, and K9G, were found to be important components in the low-temperature response pathway of CK in *Z. bungeanum*. It is suggested that the translational replenishment of binding form is the main way of cytokinin pathway regulation in *Z. bungeanum*. In addition, the changes in hormone content revealed that the response time and pattern of these binding state cytokinins differed in cold-tolerant and cold-sensitive varieties. For example, the levels of cZROG and DHZ7G were upregulated in FG, while downregulated in FX. This also provides a basis for the follow-up study on the differential exogenous hormone application between the two varieties.

Auxin plays an important role in the whole plant growth cycle, mainly in regulating cell elongation, organ morphogenesis, and interacting with other hormones. One of the most important characteristics of auxin is polar transport, which mostly depends on the polar distribution of PIN family proteins on the cell membrane (Geldner et al., 2001). One study found that endocytosis and exocytosis in cell activities were involved in the polar distribution of PIN protein on the cell membrane (Dhonukshe et al., 2007). In our previous study, we found that endocytosis played an important role in the cold response network of *Z. bungeanum*, and the endocytosis-related gene *ERD7* was a key gene in the co-expression network, with the highest connection with other genes and located in the center of the network (Tian et al., 2021). In this study, the MEcoral1 module also identified 3 genes (*ZB24496*, *ZB29882*, and *ZB54262*) enriched in the endocytosis (ko04144) pathway. Moreover, TRP, an important initiator in the IAA synthesis pathway, was found to be the most abundant of all hormones in the two varieties of *Z. bungeanum*. These results suggested that the polarity distribution of auxin mediated by endocytosis is an important way and action form of *Z. bungeanum* response to low-temperature stress. In the IAA biosynthesis pathway in *Z. bungeanum*, we also found that ICAld and ICA were the important upregulated hormone components. A study reported that the concentration of ICA in the cell wall increased significantly under pathogen attack and that indolic derivatives may serve as structural scaffolds for cell wall modifications after the attack (Forcat et al., 2010). The plant

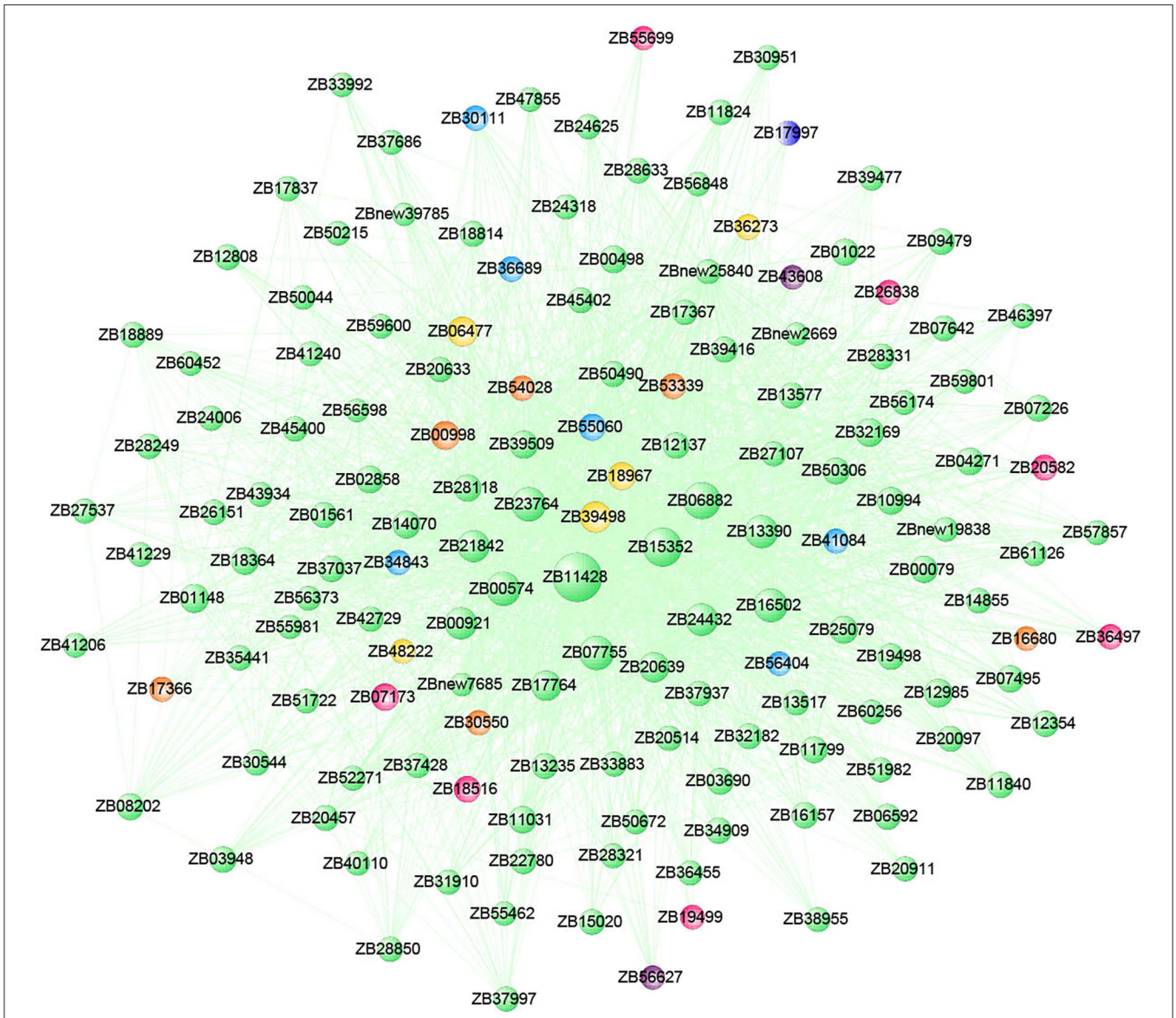

FIGURE 9 | Co-expression network of the top 150 hub genes from the MEhoneydew module. The size of each gene node in the figure represents the connectivity of genes, and the node color represents the category.

cell wall is the first barrier against low temperature. Therefore, we speculated that ICA has the same cell wall scaffolding role in *Z. bungeanum* under cold stress.

The common hormone components, such as ABA, ABA-GE, ICAld, and TRP, were found in the two varieties of *Z. bungeanum*, and the hormone components specifically accumulated in each variety, such as IA, IAA-Glu, and GA9 in FG and IAA-Glc in FX, were also found. This result indicated that the hormone response system of *Z. bungeanum* under low-temperature stress was complex and diverse, and the response patterns varied in cold-tolerant and cold-sensitive varieties. Plant hormone interactions under abiotic stresses are also receiving increasing attention. Strigolactones were found to alleviate low-temperature injury in tomatoes (*Solanum lycopersicum*) through the ABA pathway (Chi et al., 2021). Another study found that ABI5, a transcription factor of the ABA signaling pathway, was involved in regulating the auxin, CK, GA, and IAA hormone signaling pathways in transgenic potatoes (Li, 2019). Various hormones in plants show a dynamic network of physiological effects that promote and antagonize each other, and the dynamic balance between these hormones is very important for maintaining normal growth and development under adversity. The content levels and change patterns of each hormone component obtained in this study provide a basis for subsequent studies of crosstalk among hormones under low-temperature stress.

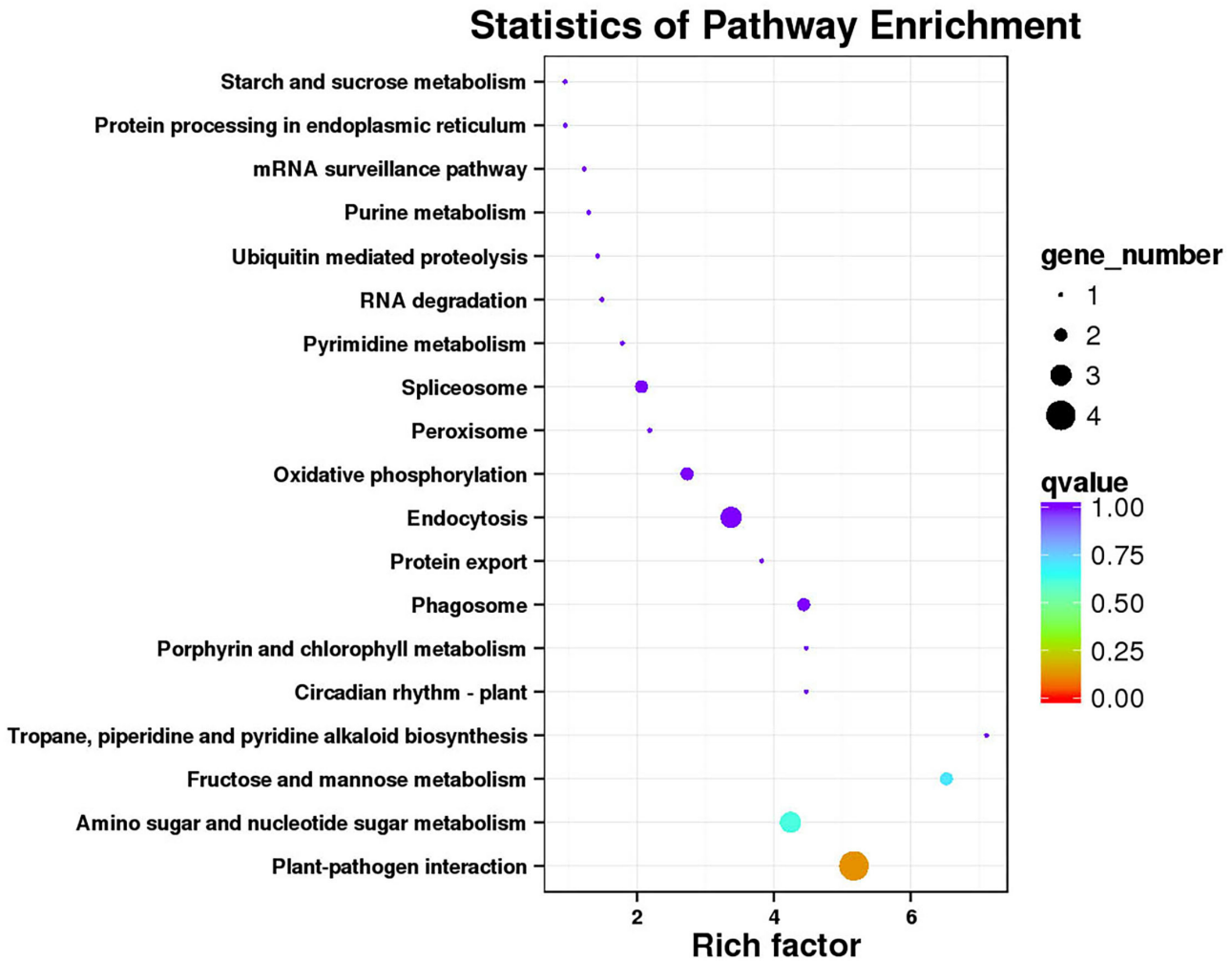

FIGURE 10 | Functional annotation and KEGG pathway enrichment of hub genes in MEcoral1.

The CHM and UMAP analysis of the samples treated with low temperature showed that the samples of 0 and 1 h, 3 and 6 h, and 12 and 24 h in FG were clustered into a group, respectively, indicating that the hormone components and levels of FG were adjusted correspondingly under different time periods of cold stress. NMDS analysis showed that the hormone components in the samples in FX at 6 h were close to the samples in FG at 12 and 24 h, indicating that the hormone response to low-temperature stress was the strongest at 6 h of FX and the ability to resist cold was at a high level. OPLS-DA analysis results showed that in the cold response process of FG and FX, there were not only important differential hormone components with the same expression pattern but also hormone components with the opposite expression pattern. This indicated that with the extension of cold stress treatment time, there were common crossing points and independent response pathways in the two varieties of *Z. bungeanum* under cold stress.

In addition, hormone-related gene modules were constructed by WGCNA, among which the MEhoneydew and MEcoral1 modules were highly correlated with the main hormone components. The response pathway of hormone components in *Z. bungeanum* under low-temperature stress was related to both upstream and downstream metabolic pathways, including carbon, fatty acid, amino acid, and sugar metabolism. These results indicate that hormone regulation plays an important role in the response of *Z. bungeanum* to low temperatures. Moreover, the identified hub genes are located in the center of the hormone response network pathway, and their regulatory effects are more sensitive and faster, which can be used as candidate genes for further study of low-temperature response.

CONCLUSION

The main aim of this study was to investigate the variation in hormone component profiling in *Z. bungeanum* under low-temperature treatment. In the leaves of two *Z. bungeanum* varieties, 45 main plant hormone components were detected, and ABA, ABA-GE, ICAld, TRP, ACC, SA, and SAG were the main

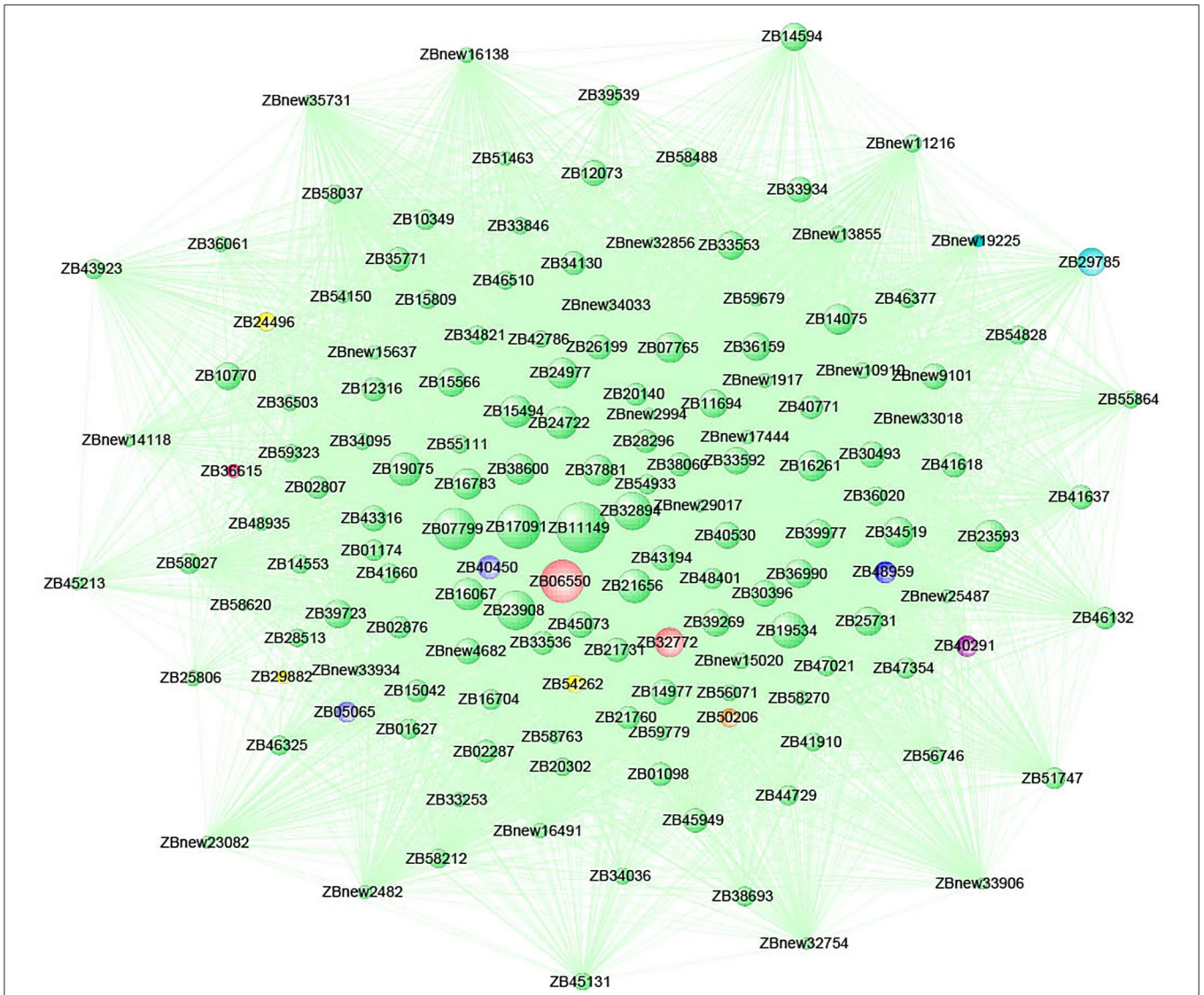

FIGURE 11 | Co-expression network of the top 150 hub genes from the MEcoral1 module.

high-level content of hormones. Low temperature affected the components and contents of plant hormones in *Z. bungeanum*, and the cold response varied in the two varieties. In the low-temperature response process of FG, ABA-GE can serve as the storage and replenishment repository of active ABA and respond to low temperature by long-distance transport from root tissue to leaves at the low-temperature stimulation site, but the same result was not found in FX. The polarity distribution of auxin mediated by endocytosis may be an important way and form of *Z. bungeanum* response to cold stress. OPLS-DA results showed that the important differential hormone components vary in FG and FX, and there were intersections and independent response pathways of plant hormones. WGCNA analysis showed that hormones, as upstream signals of low-temperature stress, were associated with other response pathways in plants, including carbon, fatty acid, sugar, amino acid metabolism, and other signal transduction pathways. Two hormone-related core genes *ZB43608* (a TGA transcription factor in the salicylate hormone signaling pathway) and *ZB56627* (a two-component response regulator ARR11 in the cytokinin signaling pathway) were identified. The results of this study revealed the hormone response strategy of *Z. bungeanum* under low-temperature stress in terms of composition and content level and provided a further reference for improving the cold resistance of *Z. bungeanum* and breeding-resistant varieties.

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/, PRJNA597398.

AUTHOR CONTRIBUTIONS

AW and JT conceived and designed the research. JT performed experiments, analyzed the results, and wrote the manuscript. YM revised the manuscript. YC and XC performed some experiments. All authors reviewed and approved the manuscript.

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Genome-Wide Identification of Wild Soybean Mitochondrial Calcium Uniporter Family Genes and Their Responses to Cold and Carbonate Alkaline Stresses

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The mitochondrial calcium uniporter (MCU), as an important component of the Ca^{2+} channel uniporter complex, plays a regulatory role in intracellular Ca^{2+} signal transduction. However, only a few studies to date have investigated plant MCU genes. In this study, we identified the MCU family genes in wild soybean and investigated their expression under cold and carbonate alkaline stresses. Eleven *Glycine soja* MCU genes (*GsMCUs*) were identified and clustered into two subgroups (subgroups I and II), and subgroup II could be further divided into two branches (MCU5 and MCU6). A total of 21 pairs of *GsMCUs* were characterized as duplicated genes, and displayed a similar exon-intron architecture. All GsMCU proteins contained one conserved MCU domain, within which two transmembrane domains were found. An analysis of the conserved motifs further supported that the GsMCUs showed high conservation in protein sequence and structure. Moreover, we found that all *GsMCUs* were expressed ubiquitously in different tissues and organs, and *GsMCU*s from the same subgroup displayed varied tissue expression profiles. In addition, based on RNA-seq and qRT-PCR assays, six and nine *GsMCUs* were differentially expressed under cold and carbonate alkaline stress, respectively. Promoter analysis also uncovered the existence of two canonical cold-related *cis*-acting elements, LTR and DRE/CRT, as well as stress-related phytohormone-responsive elements. Our results provide valuable information about the MCU family in soybean responses to cold and carbonate alkaline stress, which will be helpful in further characterizing their biological roles in response to abiotic stress.

Keywords: wild soybean, mitochondrial calcium uniporter, cold stress, carbonate alkali stress, expression analysis

INTRODUCTION

In the 1970s, calcium ions were proposed to be important messenger ions in cells. Over the years, a large number of studies have demonstrated that many functions of cells are inextricably linked to Ca^{2+} (Clapham, 2007). Mitochondria absorb Ca^{2+} mainly through mitochondrial calcium uniporters (MCU) located on the inner membrane (Deluca and Engstrom, 1961; Vasington and Murphy, 1962). A typical MCU protein harbors two transmembrane domains within a conserved

MCU domain (PF04678), with the N- and C-terminus facing the mitochondrial matrix. The N-terminal domain is essential for the Ca^{2+} uptake ability of MCU proteins, while the formation of a functional channel requires the pentamerization of transmembrane regions. By interacting with MICU1 (mitochondrial calcium uptake 1), MCUs can transport Ca^{2+} along electrochemical gradients (Perocchi et al., 2010), and play key roles in mitochondrial Ca^{2+} homeostasis, regulation and signaling (Baughman et al., 2011; De Stefani et al., 2011; Drago et al., 2011). In plants, MCUs are important components for sensing environmental stress because they modulate stromal Ca^{2+} in chloroplasts (Teardo et al., 2019). In recent decades, an increasing number of MCUs have been functionally characterized in mammals (Mallilankaraman et al., 2012; Bisbach et al., 2020; Liu et al., 2020), but little has been reported regarding plant MCUs.

Taking advantage of whole genome sequencing and public expression data, researchers could genome-widely identify MCU family genes, and systemically investigate their expression characteristics, which will provide hints to deeply study the function of MCUs in diverse physiological and biological processes. However, compared with other gene families, fewer studies have been reported on MCU family. To date, six, five, seven, and four MCUs have been identified in *Arabidopsis thaliana* (Stael et al., 2012), *Oryza sativa* (Gu, 2020), *Pyrus seratina* (Wang et al., 2018), and *Sorghum bicolor* (Gu, 2020), respectively. Among the six MCU family genes in Arabidopsis, *AtMCU1* and *AtMCU2* were found to regulate pollen tube germination and growth (Selles et al., 2018), while *AtMCU6*, a chloroplast-localized mitochondrial calcium uniporter, negatively modulated resistance to long-term water deficits (Teardo et al., 2019). In pears, MCUs have been suggested to play an important role in fruit ripening and senescence (Wang et al., 2018). However, there have been no reports on soybean MCU proteins.

Compared with cultivated soybean (*Glycine max* L.), wild soybean (*Glycine soja*) exhibits very high adaptability to harsh environments and extensive genetic diversity (Kofsky et al., 2018). It will be of great help to identify the soybean MCU genes, and screen the stress responsive MCUs in wild soybean. Therefore, in this study, we systematically characterized the MCU family genes in wild soybean and investigated their expression profiles in different tissues and under abiotic stress. Our findings suggest the potential regulatory roles of wild soybean MCU genes in response to cold and carbonate saline-alkaline stresses, which will facilitate the molecular breeding of soybean cultivars with higher tolerance to cold and carbonate saline-alkaline stresses.

MATERIALS AND METHODS

Identification of the Mitochondrial Calcium Uniporter Family Genes in the Wild Soybean Genome

To identify the candidate MCU genes in the wild soybean genome, we obtained the MCU proteins sequences which have been identified in *Arabidopsis thaliana* (Stael et al., 2012), *Pyrus seratina* (Wang et al., 2018), *Oryza sativa* and *Sorghum bicolor* (Gu, 2020) from Phytozome database.[1] These protein sequences were used for generating a hidden Markov Model,[2] which was then used to search against the wild soybean (*Glycine soja v1.1*) and cultivated soybean (*Glycine max Wm82.a2.v1*) proteomes. The amino acid sequences of the candidates were manually checked to determine whether they harbored the MCU domain (PF04678) using the NCBI Conserved Domain Database[3] and Pfam[4] program.

Detailed information on the GsMCUs, including their locus ID and genomic and protein sequences, was obtained from the Phytozome database. Isoelectric point values and molecular weights were estimated using ExPASy.[5] The transmembrane (TM) region was predicted by using the TMHMM.[6]

Chromosomal Distribution and Gene Duplication Analysis

The chromosomal locations of the GsMCUs were obtained from the Phytozome database. Duplicated segmental blocks of soybean genome were downloaded from the Plant Genome Duplication Database (PGDD). The criteria for identifying duplicated gene pairs were: (a) the length of the shorter aligned sequence covered > 70% of the longer sequence; and (b) the similarity of the two aligned sequences were > 70%. Orthologous soybean genes were downloaded from Ortho Venn2.[7] The chromosomal locations and syntenic information were visualized using TBtools software. Ka (non-synonymous rate), Ks (synonymous rate), and Ka/Ks ratios were calculated using TBtools according to their coding sequences. The timing of the genome-wide duplication event was calculated using the formula: Time = Ks/2r ($r = 6.98 \times 10^{-9}$).

Phylogenetic Relationship, Exon-Intron Organization and Protein Structure Analysis

For phylogenetic analysis, multiple alignments were performed with full-length amino acid sequences of MCU proteins in rice, Arabidopsis, and soybean using ClustalW. A neighbor-joining phylogenetic tree was constructed using MEGA5.1 software with the following parameters: Poison correction, pairwise deletion, uniform rates, and 1,000 bootstrap replicates.

The exon/intron structure of the GsMCU genes was analyzed using the Gene Structure Display Server (GSDS).[8] Functional domain information was derived from the NCBI Batch-CDD

[1] https://phytozome-next.jgi.doe.gov
[2] http://hmmer.janelia.org/
[3] https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi
[4] http://pfam.sanger.ac.uk/
[5] http://www.expasy.org/
[6] http://www.cbs.dtu.dk/services/TMHMM/
[7] https://orthovenn2.bioinfotoolkits.net/home
[8] http://gsds.gao-lab.org

database.[9] The conserved motifs were predicted using MEME[10] with the following parameters: number of motifs = 15 and zero or one occurrence per sequence. The functional domains and conserved motifs of the GsMCUs were illustrated using TBtools.

Protein Interaction Network Analysis

The protein-interacting network of the GsMCUs was generated using the STRING database.[11]

Cis-Acting Elements Analysis

The 2,000 bp promoter sequences upstream of the transcript start sites of the *GsMCU* genes were obtained from the Phytozome database (see text footnote 1). The *cis*-acting elements were analyzed using New PLACE.[12]

Accession Numbers of Public Expression Data

The accession numbers of the public expression data are as follows: $NaHCO_3$ stress (GSE17883), and cold stress (GSE117686) (Yamasaki and Randall, 2016; Robison et al., 2019).

Plant Growth Conditions and Stress Treatments

Glycine soja (G07256) seeds were treated with 98% sulfuric acid for 10–15 min, and washed five times with distilled water, and then kept in complete darkness with humidity for 1 day to promote germination. The seedlings were transferred and grown in 1/2 Hoagland's nutrient solution at 26–28°C under 16-h light/8-h dark conditions.

To explore the gene expression patterns in different tissues, freshly germinated seeds, roots, stems, and leaves of 21-day-old seedlings and the flowers were harvested. For the cold stress, 2-week-old seedlings were exposed to 4°C. For the carbonate saline-alkaline treatment, 21-day-old seedlings were transferred to a Hoagland's nutrient solution containing 50 mM $NaHCO_3$ (pH 8.5). Equal amounts of seedling roots were harvested at the indicated time points, and the samples were snap-frozen in liquid nitrogen and stored at −80°C.

[9]https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi

[10]http://meme-suite.org/tools/meme

[11]https://cn.string-db.org/cgi/input.pl

[12]https://www.dna.affrc.go.jp/PLACE/?action=newplace

RT-PCR Assays

The total RNA was isolated using the TriPure Isolation Reagent (Roche, United States), and cDNAs were synthesized using HiScript® III RT SuperMix for qPCR (Vazyme, Nanjing, China) according to the manufacturer's instructions. Quantitative real-time PCR assays were performed using *TransStart*® Top Green qPCR SuperMix according to the manufacturer's protocol. The F_box gene in wild soybeans (GlysoPI483463.12G046600) was used as an internal control (Le et al., 2012; Bansal et al., 2015). All experiments were performed using three independent biological replicates for statistical analyses. The primers used are listed in **Supplementary Table 3**.

RESULTS

Identification of Mitochondrial Calcium Uniporter Family Members in Wild Soybean

To investigate the precise information and potential function of *Glycine soja* MCU (*GsMCU*) genes, we carried out systematic genome-wide identification of putative MCU genes in the wild soybean genome. To do this, we generated a hidden Markov model based on protein sequences of the reported Arabidopsis (Stael et al., 2012), *Pyrus seratina* (Wang et al., 2018), and *Sorghum bicolor* (Gu, 2020) MCU proteins, respectively, and then searched for MCU candidates against the soybean proteome. After manually checking protein sequences for to ensure the existence of the MCU domain, a total of 11 genes were identified as GsMCU family members and named according to Arabidopsis orthologs.

Detailed information about wild soybean (*Glycine soja* v1.1) GsMCUs was obtained from the Phytozome database and is listed in **Table 1**. Notably, GsMCU5.2 had only 264 amino acids (aa), and did not possess the N-terminal extension,

TABLE 1 | Detailed information of the MCU family genes in wild soybean.

No.	Gene name	TIGR locus ID	Genomic sequence length (bp)	Protein			TM
				Amino acids (aa)	Isoelectric point	Molecular weight (KD)	
1	*GsMCU1.1*	GlysoPI483463.14G165600	4,011	339	8.8	38.13	2
2	*GsMCU1.2*	GlysoPI483463.02G198500	2,646	346	8.9	38.5	2
3	*GsMCU1.3*	GlysoPI483463.11G188100	3,224	328	9.02	37.09	2
4	*GsMCU1.4*	GlysoPI483463.18G044900	2,681	338	8.65	37.87	2
5	*GsMCU5.1*	GlysoPI483463.14G067700	2,677	348	9.35	39.87	2
6	*GsMCU5.2*	GlysoPI483463.17G212600	3,031	264	9.01	30.79	2
7	*GsMCU5.3*	GlysoPI483463.17G212700	2,645	357	9.24	40.6	2
8	*GsMCU6.1*	GlysoPI483463.01G141000	2,217	325	9.11	36.66	2
9	*GsMCU6.2*	GlysoPI483463.11G058600	2,876	325	9.24	36.81	2
10	*GsMCU6.3*	GlysoPI483463.02G052500	4,547	317	9.51	36.2	2
11	*GsMCU6.4*	GlysoPI483463.16G112200	4,982	328	9.17	37.59	2

compared to other members. The other GsMCU members shared similar protein sequence lengths (from 317 to 357 aa). Correspondingly, the molecular weight of the GsMCU proteins (except GsMCU5.2) varied from 36.2 to 40.6 kDa, and the predicted isoelectric points ranged between 8.65 and 9.51. Furthermore, all GsMCU proteins had two predicted transmembrane regions, with both the N-and C-terminus facing the matrix. These results imply that the GsMCU family possesses a conserved protein structure.

Phylogenetic Relationship and Structure Analysis of *Glycine soja* MCU Genes

To investigate the phylogenetic relationships among the GsMCUs, we generated a neighbor-joining tree using the full-length amino acid sequences of wild soybean, Arabidopsis (Stael et al., 2012) and rice (Gu, 2020) MCU family proteins (**Figure 1**). As shown in **Figure 1**, MCU proteins were clustered into two subgroups: subgroup I and subgroup II. Subgroup I contained nine members, including four GsMCUs, two AtMCUs, and three OsMCUs, whereas subgroup II included seven GsMCUs, four AtMCUs, and two OsMCUs (**Supplementary Table 1**). In each subgroup, the OsMCUs were first separated from the other members and clustered into one branch. This finding implies that the appearance of these two subgroups occurred before the division of monocots and dicots during evolution. Moreover, the soybean possessed more MCU genes than Arabidopsis and rice, and GsMCUs appeared in pairs in the phylogenetic tree, which might be a consequence of whole genome duplication of soybean during evolution. This result also indicates possible gene duplication within the GsMCU family, as well as the conservation of GsMCUs in gene architecture and protein structure.

Gene Duplication and Structure Analysis of *Glycine soja* MCUs

To confirm the existence of gene duplication within the GsMCU family, we performed chromosomal location and collinearity

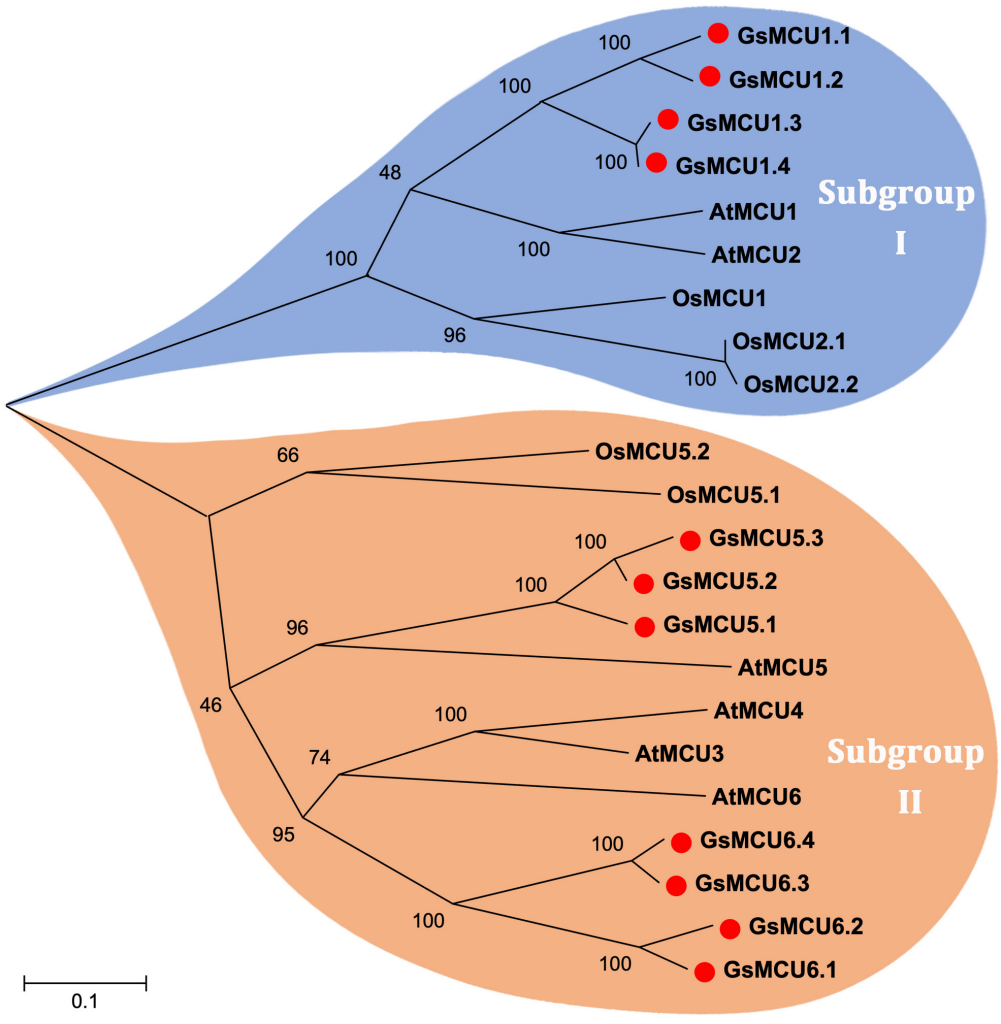

FIGURE 1 | Phylogenetic relationship of MCU proteins from Arabidopsis, rice and soybean. The phylogenetic tree was constructed using MEGA 5.10 software using the neighbor-joining method, and the bootstrap values were set at 1,000.

analyses. As shown in **Figure 2**, 11 *GsMCUs* were randomly distributed on seven chromosomes. Chromosomes 2, 11, 14, and 17 each possessed two *GsMCUs*, whereas chromosomes 1, 16, and 18 each contained one. To obtain the collinearity of GsMCUs, we retrieved duplicated gene pairs of the MCU family from the Plant Genome Duplication Database and identified 21 pairs among 11 GsMCU genes (**Figure 2**). Furthermore, we found that the Ka/Ks values of the duplicated pairs were less than 1, indicating purification of GsMCU genes during evolution (**Supplementary Table 2**). In addition, the duplication of the GsMCUs occurred 10.5246–322.3279 Mya (million years ago).

In generally, duplicated gene pairs are highly conserved in terms of exon-intron organization. As shown in **Figure 3**, all *GsMCUs* contained only two exons of similar length, suggesting a highly conserved gene architecture. Moreover, synteny analysis of MCU genes from different dicot species identified a number of duplicated MCU gene pairs among soybean, alfalfa, and Arabidopsis (**Supplementary Figure 1**). These findings further support the idea that whole-genome duplication was the main contributor to expansion of the MCU family.

Conserved Domains of *Glycine soja* MCU Proteins

To verify the conservation of the GsMCU family, we further analyzed the functional domains and conserved motifs. All GsMCUs harbored one conserved MCU domain (PF04678) (**Figure 4A**). MEME analysis of the conserved motifs also suggested that the GsMCU proteins possessed high similarity in motif organization (**Figure 4B**). Among the 15 motifs,

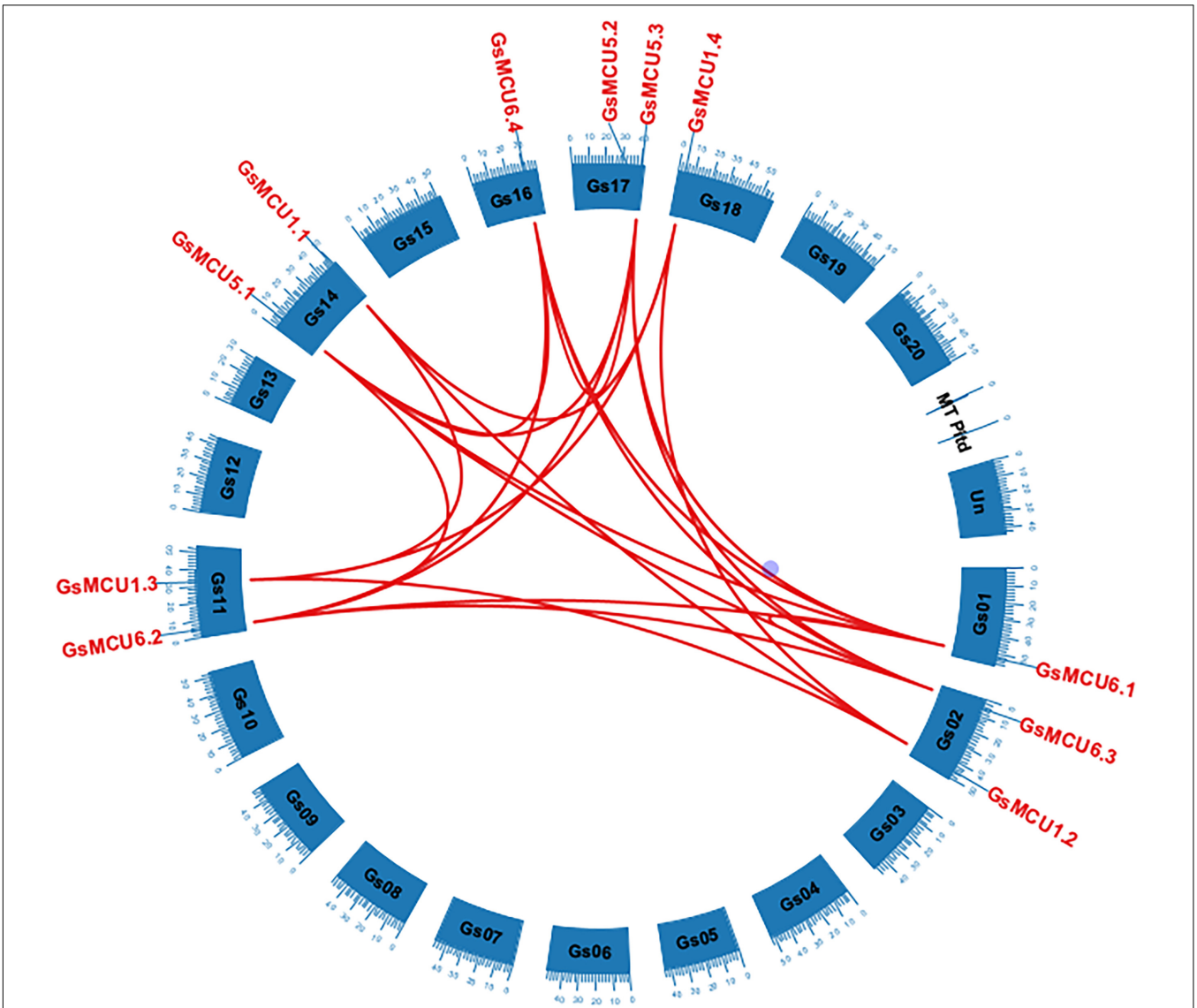

FIGURE 2 | Distribution and duplication of the MCU genes in the wild soybean genome. Duplicated genes among the GsMCUs were downloaded from the PGDD database and are indicated by red lines.

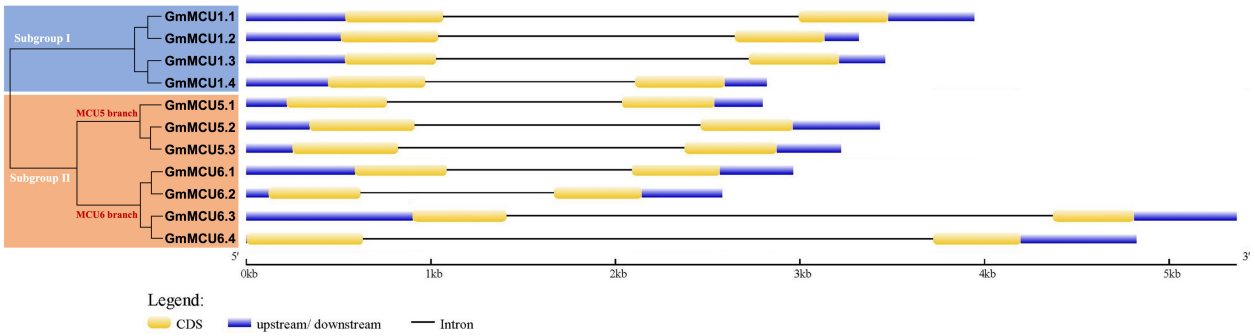

FIGURE 3 | Gene structure analysis of GsMCU family. The position of exons, introns, and untranslated regions (UTRs) are indicated by yellow, black, and blue boxes, respectively. The scale bar at the bottom was used to estimate the sizes of the exons, introns, and untranslated regions.

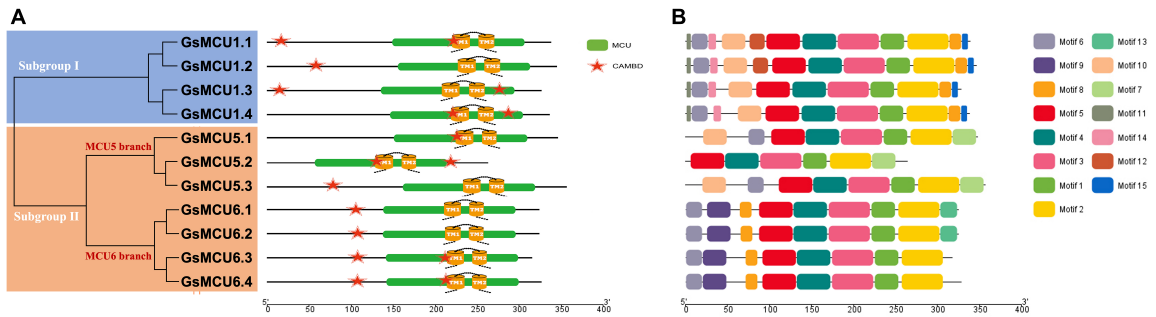

FIGURE 4 | Functional domains and conserved motifs of GsMCU proteins. Functional domains **(A)** and conserved motifs **(B)** are indicated by green boxes and different colored boxes represent different motifs. The five-pointed stars represent the calmodulin-binding sites in the GsMCU proteins. Protein domains and conserved motifs were predicted using a CDD search and MEME.

motifs 1 to 4, representing the MCU domain, were present in all GsMCUs. Multiple alignments illustrated that the MCU domain contained two transmembrane regions (TMs) and displayed a high sequence similarity (**Figure 5**). These results further highlight the high conservation of GsMCUs in terms of protein structure.

Meanwhile, GsMCUs from different subgroups also showed sequence diversity at a certain degree (**Figure 4B**). For example, among the 15 motifs, motifs 11, 14, and 15 were specific to subgroup I GsMCUs. Motif 9 was only observed in the MCU6 branch of subgroup II, whereas motif 7 was observed in the MCU5 branch. Interestingly, motif 8 was located at the C-terminus of subgroup I, but at the N-terminus of the subgroup II MCU6 branch. In addition, one or two calmodulin-binding domains (CAMBD) were found in all GsMCU proteins. However, the location of the CAMBD varied among the different GsMCU proteins (**Figure 4A**). These differences imply possible different regulatory mechanisms of biochemical activity or functional specialization.

Tissues Expression Profiles of *Glycine soja* MCU Genes

Several studies have reported the involvement of plant MCU genes in growth and development (Wang et al., 2018). To explore the potential function of MCU genes in soybean growth, we retrieved the expression profiles of GsMCUs in different tissues from Phytozome and verified these profiles via semiquantitative RT-PCR analysis (**Figure 6**). According to our RT-PCR results, the GsMCU genes were expressed in all detected tissues and organs (**Figures 6B–D**). For the four GsMCUs in subgroup I, two (*GsMCU1.1* and *GsMCU1.2*) were expressed at a very low level, and the other two (*GsMCU1.3* and *GsMCU1.4*) displayed a relatively high expression level, with the highest expression in leaves (**Figure 6A**). The RT-PCR results confirmed this pattern (**Figure 6B**). For members of the MCU5 branch in subgroup II, *GsMCU5.1* was highly expressed in roots, flowers, and seeds, while *GsMCU5.2* and *GsMCU5.3* displayed high expression in seeds (**Figure 6A**). The RT-PCR analysis revealed a relatively high expression of the MCU5 branch genes in seeds and leaves (**Figure 6C**). This finding indicates a possible role for the MCU5 branch in seed development. In the MCU6 branch, GsMCU6.1 displayed the highest expression in roots, while the other three genes showed the highest expression levels in flowers (**Figure 6A**). As shown in **Figure 6D**, *GsMCU6.1* showed a peak expression in leaves, *GsMCU6.2* displayed a ubiquitous expression, with the highest level in the stem, while GsMCU6.3 and *GsMCU6.4* had the highest transcript levels in flowers. In conclusion, these results suggest that *GsMCU*s from the same subgroup display variable

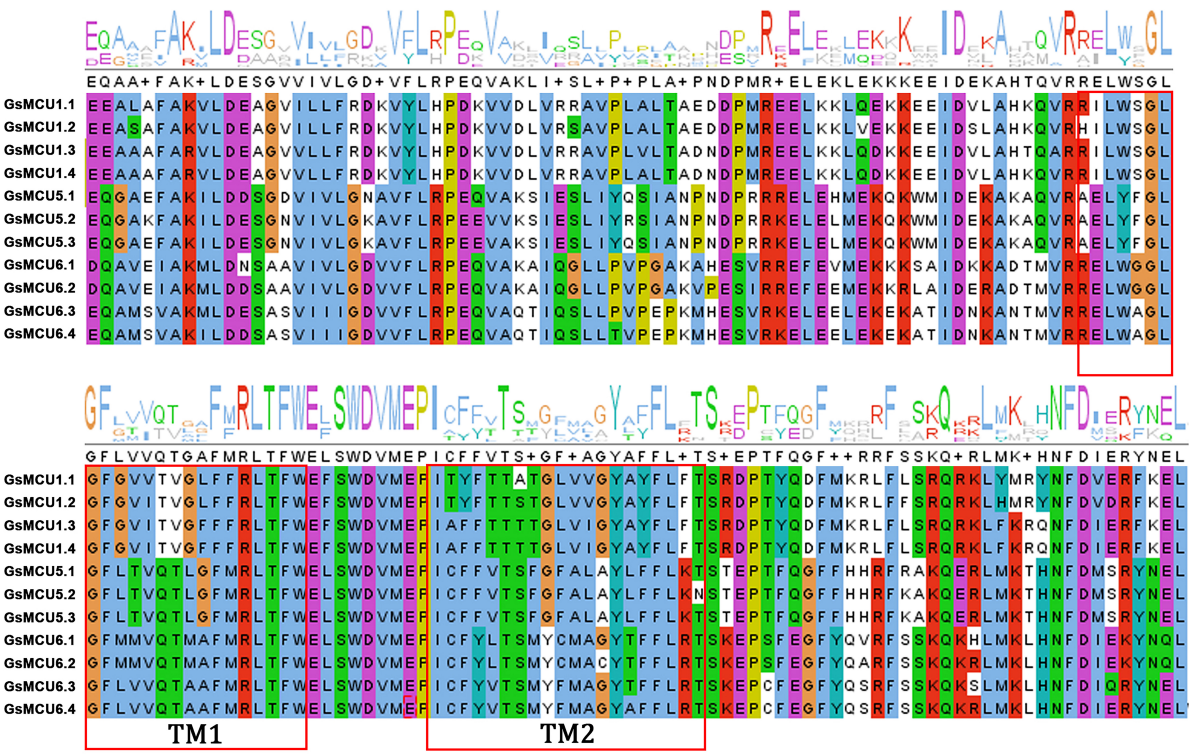

FIGURE 5 | Multiple sequence alignment of the MCU domain within GsMCUs proteins. Two transmembrane regions (TM1 and TM2) are marked by red boxes.

tissue expression profiles, indicating their potentially divergent roles in soybean development.

Expression Patterns of *Glycine soja* MCU Under Cold and Carbonate Alkaline Treatments

To date, few studies have reported the function of plant MCUs in abiotic stress response. Considering the severe effects of low temperature and saline-sodic stress on soybean yields, we further analyzed the expression patterns of GsMCUs under cold and carbonate alkaline treatments to explore their potential roles in these stress responses.

Previous RNA-seq data (Yamasaki and Randall, 2016; Robison et al., 2019) showed that six members of the soybean MCU family genes responded to cold treatment (**Figure 7**). To confirm the differential expression of the *GsMCUs* after cold treatment, we performed quantitative real-time PCR assays and found that the expression of three MCU6 branch genes (*GsMCU6.1*, *−6.3*, *−6.4* in subgroup II) decreased at 1 h and then increased at 24 h after cold stress. However, the expression of *GsMCU5.2* and *GsMCU5.3* from the MCU5 branch in subgroup II was repressed by cold stress. Furthermore, in subgroup I, *GsMCU1.2* expression was greatly increased at 24 h of cold treatment. These expression data imply that these *GsMCUs* may play different roles in cold stress response.

According to our previous RNA-seq data, nine *GsMCUs* showed increased expression after carbonate alkaline treatment (50 mM $NaHCO_3$, pH 8.5). As shown in **Figure 8**, qRT-PCR results showed that the expression of these nine *GsMCUs* was altered by carbonate alkaline treatment. In details, the expression of four genes (*GsMCU6.1*, *−6.2*, *−1.1*, and *−1.3*) was significantly up-regulated at all detected time points after $NaHCO_3$ treatment. *GsMCU5.2* and *GsMCU5.3* displayed decreased expression at 1 h, followed by an increase. These results suggest that *GsMCU* genes may participate in soybean response to carbonate alkaline stress.

To further understand the changes in expression of *GsMCUs* under cold and carbonate alkaline stresses, we predicted stress-related *cis*-acting elements in their promoters (2 kb upstream of the transcription start site) based on the New PLACE database (**Table 2**). We found that two cold-related *cis*-acting elements, LTR (CCGAAA) and DRE/CRT (TACCGACAT), were observed in the promoters of *GsMCU1.1*, *GsMCU5.1*, *GsMCU6.2*, and *GsMCU6.4*. Moreover, phytohormones play well-documented and critical roles in stress responses (Fahad et al., 2015; Manasa et al., 2021). Several *cis*-elements related to phytohormones have also been found in *GsMCU* promoters, such as ABA (ABRE, core sequence ACGTG) (Gusta et al., 2005), JA (CGTCA, core sequence CGTCA) (Kosova et al., 2012), GA (GARE, core sequence TCTGTTG) (Manasa et al., 2021), and SA (TCA, core sequence CCATCTTTTT) (Miura and Ohta, 2010). Notably, *cis*-elements related to ABA and JA, which have been suggested to play key roles in cold stress, were enriched in the *GsMCU* promoters, indicating that *GsMCU*

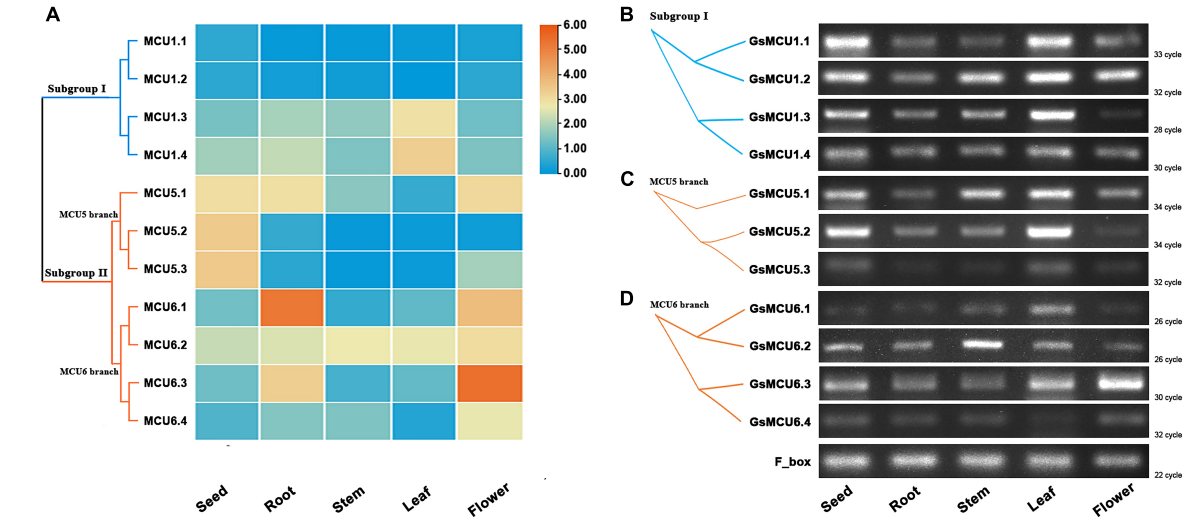

FIGURE 6 | Expression of GsMCU genes in different tissues. **(A)** Heat map showing the expression levels of soybean MCUs in different tissues. The color scale represents the expression values: red indicates high levels and blue represents low levels. **(B–D)** RT-PCR assays for *GsMCUs* expression in different tissues. F_box (GlysoPI483463.12G046600) was used as an internal reference.

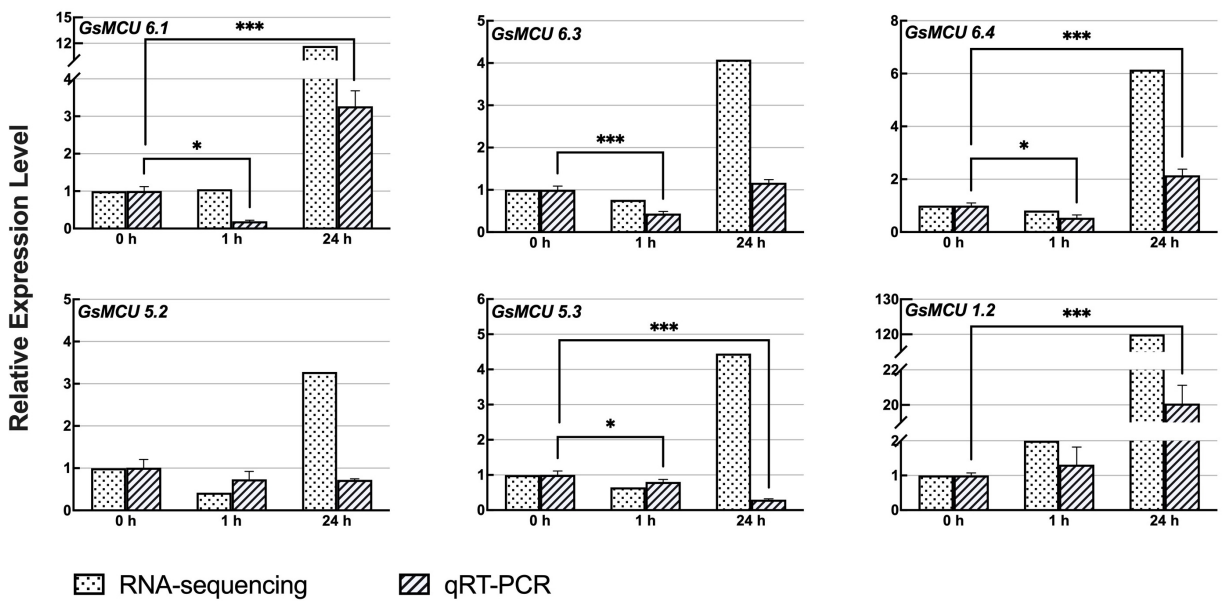

FIGURE 7 | Expression analyses of wild soybean MCU family genes under cold stress. To induce cold stress, 2-week-old wild soybean seedlings were treated at 4°C for 0, 1, and 24 h. GlysoPI483463.12G046600 was used as an internal reference. Mean values (±SE) of the three fully independent biological repeats and three technical repeats are shown. Asterisks indicate significant differences (*$p < 0.05$, ***$p < 0.001$ using Student's *t*-test).

expression may respond to cold stress via ABA and JA signaling pathways.

The Interaction Network of Soybean Mitochondrial Calcium Uniporter Proteins

To explore the potential function of *GsMCUs*, we used the STRING database to generate a protein interaction network for each GsMCU. Remarkably, the predicted interacting proteins of GsMCUs within each subgroup were identical. Therefore, we proposed two networks for the GsMCU family (**Figure 9**): network I (for subgroup I) and network II (for subgroup II). Each network contained 10 interacting partners, among which seven proteins were the same between networks I and II. The same interacting proteins between network I and network II further supported that the GsMCU family was a highly

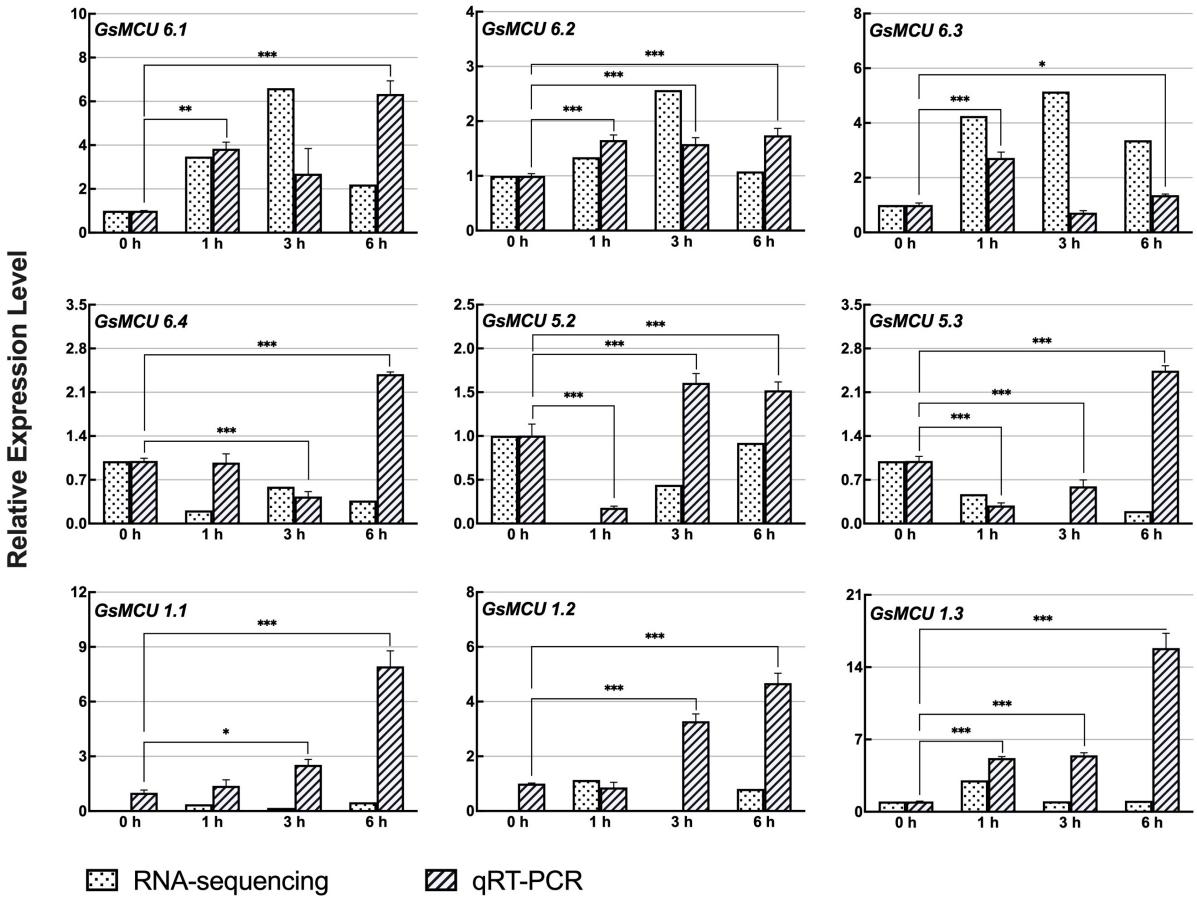

FIGURE 8 | Expression analyses of wild soybean MCU family genes under $NaHCO_3$ stress. For $NaHCO_3$ stress, the 21-day-old wild soybean seedlings were treated with 50 mM $NaHCO_3$ for 0, 1, 3, and 6 h. GlysoPI483463.12G046600 was used as an internal reference. The mean values (±SE) from three fully independent biological repeats and two technical repeats are shown. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by Student's *t*-test).

TABLE 2 | Distribution of *cis*-acting elements in the promoters of *GsMCUs*.

Name	Core sequence	Involved in	MCU 1.1	MCU 1.2	MCU 1.3	MCU 1.4	MCU 5.1	MCU 5.2	MCU 5.3	MCU 6.1	MCU 6.2	MCU 6.3	MCU 6.4
LTR	CCGAAA	Cold	1				1				2		
DRE	TACCGACAT	Dehydration, cold, salt											1
ABRE	ACGTG	ABA	3	5	2	1	1			6	4	4	5
CGTCA	CGTCA	JA	2	1	2	1		2	1		2		2
GARE	TCTGTTG	GA			1		2			1		1	1
P-box	CCTTTTG	GA				1			1				
TCA	CCATCTTTTT	SA	1	1	1	2	2			1	1		1

conserved gene family and possibly shared similar molecular and biological functions.

Notably, among the seven proteins shared by networks I and II are six mitochondrial calcium uptake (MICU) proteins that help transport Ca^{2+} along electrochemical gradients by directly interacting with MCUs. Within network I, *Glyma11g15930* and *Glyma12g29340* encode transport protein Sec61 subunit beta, which forms the Sec61 complex for transporting newly synthesized polypeptides into the ER lumen and allows calcium leakage from the ER into the cytosol (Schmutz et al., 2010). *Glyma10g11020* in network I encodes a calcium-dependent protein kinase that senses Ca^{2+} oscillations and mediates Ca^{2+} signal transduction. Within network II, *Glyma16g01100* and *Glyma07g04515* belong to the Ca^{2+} cation antiporter (CaCA) superfamily, which play a vital role in Ca^{2+} ion homeostasis (**Table 3**). In summary, the investigation of GsMCU-interacting proteins strongly suggests that GsMCUs play a regulatory role in intracellular Ca^{2+} signal transduction.

TABLE 3 | Annotation of MCU interacting protein in soybean.

	Interacting partner	Functional annotation	Arabidopsis homologous	Function	References
Shared by network I/II	Glyma06g06960	Calcium uptake protein	AT4G32060	Calcium uptake protein 1 (MICU)	Wang and Teng, 2018
	Glyma17g32538	Calcium uptake protein			
	Glyma14g13951	Calcium uptake protein			
	Glyma04g06880	Calcium uptake protein			
	Glyma13g23280	Calcium uptake protein			
	Glyma17g11545	Calcium uptake protein			
	Glyma06g01102	DUF1640, Uncharacterized protein	AT2G16460	DUF1640, Uncharacterized protein	Cheng et al., 2017
Network I-specific	Glyma10g11020	Calcium-dependent protein kinase	AT2G38910	Calcium-dependent protein kinase 20 (CPK20)	Shi et al., 2018
	Glyma11g15930	Transport protein Sec61 subunit beta	AT5G60460	transport protein Sec61 subunit beta	
	Glyma12g29340	Transport protein Sec61 subunit beta			Consortium et al., 2011
Network II-specific	Glyma16g24320	DUF1640, Uncharacterized protein	AT2G16460	DUF1640, Uncharacterized protein	Cheng et al., 2017
	Glyma16g01100	Ca^{2+}: cation antiporter (CaCA) family	AT1G54115	Cation calcium exchanger 4 (CCX4)	Fujikura et al., 2020
	Glyma07g04515	Ca^{2+}: cation antiporter (CaCA) family			

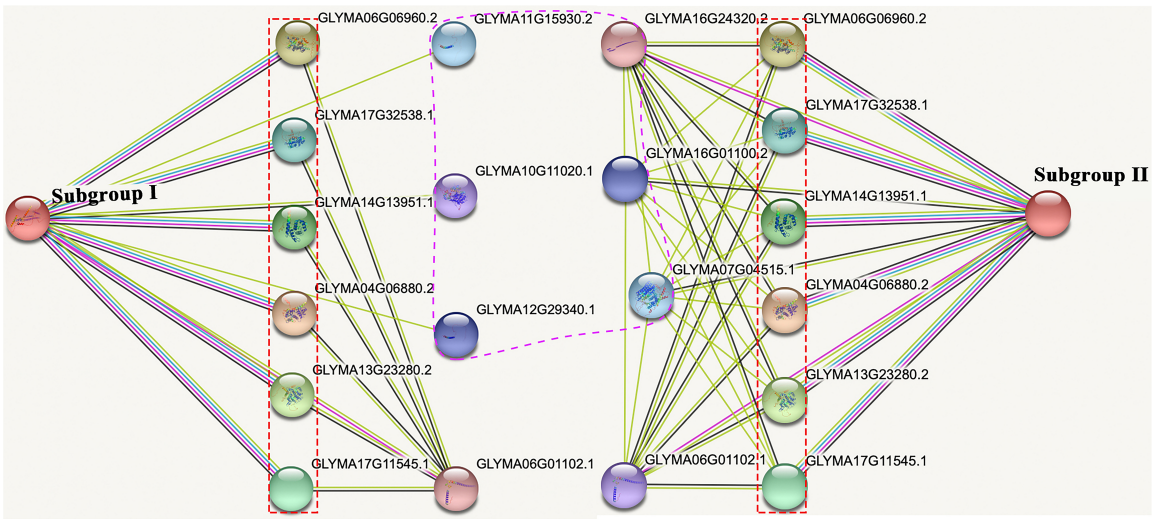

FIGURE 9 | Protein-protein interaction network of soybean MCU proteins. The experimentally determined interacting proteins of the GsMCUs are marked with red boxes. Differences in interacting proteins between the two subgroups are marked by a purple dotted line.

DISCUSSION

It has been suggested that the MCU genes regulate Ca^{2+} signal transduction and participate in diverse physiological processes. Although mammalian MCU genes have been well characterized, the biological functions and regulatory roles of plant MCUs are unknown. Here, we systematically investigated the wild soybean MCU family genes and provided clues regarding their potential function in soybean development and abiotic stress responses.

Soybean Mitochondrial Calcium Uniporter Proteins Are Highly Conserved

In this study, we identified a total of 11 *GsMCU* genes and suggested that the GsMCU family was a highly conserved family. First, all *GsMCUs* contained one long intron at a similar position, indicating a conserved gene structure (**Figure 3**). Secondly, all GsMCU proteins harbored one conserved MCU domain, with two predicted transmembrane regions (**Figure 4A**). It has been suggested that the pentamerization of transmembrane regions is required for the formation of a functional channel (Oxenoid et al., 2016). MEME analysis further showed high similarity in conserved motif organization of the MCU domain, as well as the N- and C-terminal regions (**Figure 4B**), suggesting a conserved protein structure. Previous studies reported that the N-terminal domain was important for the Ca^{2+} uptake activity of the MCU proteins in human (Lee et al., 2015). Further studies are need to identify the function of conserved domains in Ca^{2+} transport activity. Third, based on the PGDD database, we identified a total of 21 duplicated pairs among 11 *GsMCU* genes, possibly due to whole-genome duplication of soybean during evolution (**Figure 2**). Synteny analysis also identified several duplicated MCU gene pairs in soybean, alfalfa, and Arabidopsis (**Supplementary Figure 1**). Similarly, whole-genome duplication is the main driving force for pear MCU family expansion (Wang et al., 2018). Moreover, MCUs from

different plant species were clustered into two subgroups and showed similar phylogenetic relationships (**Figure 1**). In the phylogenetic tree, the GsMCUs appeared in pairs. Previous studies have also reported that MCUs from Arabidopsis and pear were clustered into two subgroups (Stael et al., 2012; Wang et al., 2018). Finally, GsMCUs within each subgroup shared the same interacting proteins in the STRING network prediction (**Figure 9**). In human, MICU1 is suggested to be associated with the mitochondrial inner membrane and is required for high-capacity mitochondrial Ca^{2+} uptake (Perocchi et al., 2010). In Arabidopsis, MICU was demonstrated to regulate mitochondrial Ca^{2+} dynamics in intact plants (Wagner et al., 2015). Therefore, it is important to validate the consensus protein interaction of GsMCU family members. Taken together, our results strongly demonstrate that the GsMCU family is highly evolutionarily conserved.

Mitochondrial Calcium Uniporter Genes Are Involved in Regulating Soybean Development

Although the MCU family is conserved, they display a certain degree of diversity in subcellular localization and tissue-specific expression characteristics. In Arabidopsis, AtMCU1–AtMCU5 localize in the mitochondria (Teardo et al., 2017; Nomura and Shiina, 2021) and AtMCU6 localizes in the chloroplast envelope (Carraretto et al., 2016; Teardo et al., 2019). Therefore, further studies are required to verify the mitochondria localization of GsMCU1s and GsMCU5s, as well as the chloroplast localization of GsMCU6s. Moreover, our studies also showed that *GsMCU6.2* and *GsMCU6.3* from the MCU6 branch were highly expressed in stems and flowers, respectively, while members from the MCU5 branch were highly expressed in seeds and leaves (**Figure 6**). Similarly, *AtMCU* genes also show specific expression characteristics in different tissues (Teardo et al., 2017, 2019). For example, *AtMCU1* displayed the highest expression in roots and an intermediate expression in young leaves, fully expanded leaves, old leaves, and flowers, but no expression (below the detection limit) in seeds (Teardo et al., 2017). *AtMCU4* also showed the highest expression in roots (Teardo et al., 2017). *AtMCU6* is expressed in all tissues, with higher levels in the mature leaves (Teardo et al., 2019). Furthermore, *PbrMCUs* also exhibited different expression profiles in different parts of "Housui" pear fruit (Wang et al., 2018). The diversity of MCUs in terms of subcellular localization and tissue expression might be the main driving force for their potential function specialization in regulating plant growth and development.

Several studies have reported the role of MCU genes in plant development. Interestingly, both *AtMCU1* knockout and overexpression caused shorter primary roots under restrictive growth conditions (Teardo et al., 2017). *AtMCU2* knockout impairs Arabidopsis pollen tube germination and growth (Selles et al., 2018). Arabidopsis plants lacking *AtMCU6* exhibit constitutive stomatal closure (Teardo et al., 2019). Furthermore, three MCU genes are involved in pear ripening (Wang et al., 2018). In future, genetic data regarding *GsMCUs* overexpression or knockout will be of great importance for understanding their biological roles during soybean growth and development.

Expression of *Glycine soja* MCUs Reveals Their Potential Roles in Response to Cold and Carbonate Alkaline Stress

Ca^{2+} signal transduction is of great importance in plant responses to various abiotic stresses including drought, salt and cold. In this study, we identified that six *GsMCUs* responded to cold stress (**Figure 7**), and nine *GsMCUs* showed increased expression under carbonate alkaline treatment (**Figure 8**). In particular, the MCU6 branch genes were upregulated under both cold and carbonate alkaline stresses, while *GsMCU5s* were downregulated. This difference indicates potential functional specialization of subgroup II MCUs. Members within the MCU6 and MCU5 branches, as well as three subgroup I members, showed roughly the same expression pattern under stress treatment, suggestive of possible functional redundancy. A recent study has reported that *AtMCU6* regulates osmotic stress responses (Teardo et al., 2019). *AtMCU6* knockout plants displayed increased resistance to long-term water deficits and improved recovery after re-watering. Therefore, functional validation of *GsMCUs* by using overexpressing or gene-editing soybean is particularly important for investigating their precise biological roles and molecular basis in regulating the cold and carbonate alkaline stress tolerance.

Furthermore, we observed several stress-related *cis*-acting elements in the promoters of *GsMCUs*, including two cold-related *cis*-acting elements, LTR (CCGAAA) and DRE/CRT (TACCGACAT), as well as ABA (ABRE, core sequence ACGTG) and JA (CGTCA, core sequence CGTCA) responsive *cis*-elements (**Table 2**). A previous study showed that *PbrMCU* expression is affected by another type of phytohormone, ethylene (Wang et al., 2018). Hence, more experiments are need to verify the hormone induced expression of *GsMCUs*, and the binding of key transcription factors, for example CBF/DREB1s, to *GsMCUs* promoters. Furthermore, the genetic evidence is also required in future to confirm whether *GsMCUs* participate in cold and carbonate alkaline stress via the ABA and JA signaling pathways.

CONCLUSION

In this study, we identified the MCU family genes in wild soybean and investigated their expression under cold and carbonate alkaline stresses. Eleven *GsMCUs* were identified and clustered into two subgroups. Gene duplication was detected in the *GsMCU* family, and the duplicated pairs displayed a similar exon-intron architecture. All GsMCU proteins contained one conserved MCU domain and two transmembrane helices, showing high conservation in protein sequence and structure. Moreover, all *GsMCUs* were expressed ubiquitously in different tissues and organs, and six and nine GsMCUs were

differentially expressed under cold and carbonate alkaline stress, respectively. Our results provide valuable information about the soybean MCU family, which will facilitate functional analysis of *GsMCUs* in the future.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: The datasets for the RNAseq experiments can be found on NCBI GEO (Accessions # GSE117686 and GSE17883) (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE117686 and https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17883).

AUTHOR CONTRIBUTIONS

XS and KY guided the design of the experiment and revised the manuscript. JL and MS conducted data analysis and manuscript writing. YL finished plant material handling. All authors reviewed and agreed to the published version of the manuscript.

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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.2022.867503/full#supplementary-material

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Analysis of the Expression and Function of Key Genes in Pepper Under Low-Temperature Stress

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The mechanism of resistance of plants to cold temperatures is very complicated, and the molecular mechanism and related gene network in pepper are largely unknown. Here, during cold treatment, we used cluster analysis (*k*-means) to classify all expressed genes into 15 clusters, 3,680 and 2,405 differentially expressed genes (DEGs) were observed in the leaf and root, respectively. The DEGs associated with certain important basic metabolic processes, oxidoreductase activity, and overall membrane compositions were most significantly enriched. In addition, based on the homologous sequence alignment of *Arabidopsis* genes, we identified 14 positive and negative regulators of the ICE-CBF-COR module in pepper, including *CBF* and *ICE*, and compared their levels in different data sets. The correlation matrix constructed based on the expression patterns of whole pepper genes in leaves and roots after exposure to cold stress showed the correlation between 14 ICE-CBF-COR signaling module genes, and provided insight into the relationship between these genes in pepper. These findings not only provide valuable resources for research on cold tolerance, but also lay the foundation for the genetic modification of cold stress regulators, which would help us achieve improved crop tolerance. To our knowledge, this is the first study to demonstrate the relationship between positive and negative regulators related to the ICE-CBF-COR module, which is of great significance to the study of low-temperature adaptive mechanisms in plants.

Keywords: cold stress, ICE-CBF-COR, pepper, gene regulatory network, GO analysis

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INTRODUCTION

Cold stress (at non-freezing temperatures) is one of the main types of abiotic stresses that affects the growth and geographical distribution of plants and causes death in severe cases. Cold stress, including at low (<20°C) and freezing (<0°C) temperatures (Chinnusamy et al., 2010; Sanghera et al., 2011), causes significant adverse effects on the growth and development of plants and restricts

Abbreviations: DEGs, differentially expressed genes; CORs, cold-responsive genes; RNA-seq, RNA sequence; HPT, hours post-treatment; TPM, transcripts per million; FPKM, fragments per kilobase million; HCL, hierarchical clustering; PCA, principal component analysis; FDR, false discovery rate; FC, fold change; GO, gene ontology; BP; biological process; MF, molecular function; CC, cellular component; KEGG, kyoto encyclopedia of genes and genomes; CT, cold tolerance; CS, cold sensitive.

their distribution and productive capability (Chinnusamy et al., 2007; Ding et al., 2019, 2020). A low-temperature environment could induce the expression of cold-responsive genes (CORs) in plants. The function of these genes is to stabilize the membrane, to prevent freezing-induced damage (Thomashow, 1999).

The CBF-dependent low-temperature signaling pathway in plants has been discussed relatively thoroughly. The three CBF genes arranged in tandem on chromosome 4 of the *Arabidopsis thaliana* genome have been successively identified as important transcription factors located upstream of the CORs (Stockinger et al., 1997; Gilmour et al., 1998; Liu et al., 1998). The overexpression of CBF1–3 significantly enhanced the resistance of *Arabidopsis* plants to freezing temperatures, and the RNA sequence (RNA-seq) analysis of the *cbf1*/*cbf2*/*cbf3* mutants of *Arabidopsis* showed that *CBF* mutations affected 10–20% of CORs after their expression (Jia et al., 2016; Zhao et al., 2016). In addition, CBF has been identified in many other plant species, all of which have characteristic cold-induced response generating genes. Therefore, the CBF function is highly conserved in the low-temperature signaling pathway in plants and the relationship between CBFs is complex and deserves further exploration (Shi et al., 2018). The plant ICE-CBF-COR-signaling module was established (Chinnusamy et al., 2003; Hwarari et al., 2022). CBFs play a central role in cold acclimation (Kim Y. S. et al., 2015), and their level of transcription is significantly up-regulated by the CBF expression protein inducer (ICE). They also activate the expression of downstream CORs by binding to cis-elements in their promoters (Lee et al., 2005; Tang et al., 2020).

Capsicum annuum L. is a typical thermophilic vegetable, whose optimal growth occurs at a temperature range of 21–28. It lacks a cold acclimation mechanism and is very sensitive to cold stress during periods of growth. Genes such as *CaDHN4* (Zhang H. F. et al., 2019), *CaMADS* (Chen et al., 2019), and *CaPIF8*, which could increase low-temperature stress, have also been found to occur in peppers (Yang et al., 2021). Low-temperature stress causes a series of abnormal development-related phenomena in pepper, such as metabolic imbalance, reduced nutritional status, hindered female organ development, reduced number of pollen grains, and obstacles in the fertilization process, causing flowers and fruits to stop growing, fruits to be deformed, and even a lack of seeds. These phenomena significantly affect the quality and yield of peppers (Guo et al., 2015; Zhang et al., 2020).

Currently, there is a lack of systematic studies on the identification of CBF genes and CBF-related positive and negative regulatory factors in peppers. This analysis mainly focuses on the roots and leaves of pepper plants at six time points within 0.5–24 h under low-temperature stress conditions at 10°C. The expression of the whole set of genes was compared with that of the control. We constructed a gene regulation network for cold stress in pepper, and based on the CBF gene sequence in *A. thaliana* and currently available research, we identified the important genes that had positive and negative regulatory effects on the CBF genes in the entire pepper genome. We also assessed the expression of CBF signaling pathway-related genes after exposure to cold stress and the relationship between them in the regulatory network. This is the first study to discuss the relationship between positive and negative regulators related to the ICE-CBF-COR module. It is of great significance for the study of the low-temperature stress adaptive mechanism in pepper and discovery of stress resistance genes, which would facilitate the generation of more stress resistant plants for pepper breeding.

MATERIALS AND METHODS

Plant Materials and Data Sources

In the previous study, total RNA was extracted from cold-treated leaves and roots of pepper (Liu et al., 2017). The elite breeding pepper (*Capsicum annuum*) line 6421 was selected from a long red pepper landrace grown widely in the West of the Xiangjiang River, in Hunan Province, China. It is resistant to anthracnose, bacterial spot, and bacterial wilt. Control plants were mock-treated with nutrient solution alone. Leaf and root tissues were collected from both treated and control plants at 0.5, 1, 3, 6, 12, and 24 h post-treatment (HPT).

Differential Gene Expression Analysis

We performed quality analysis using fastp (Chen et al., 2018) and FastQC v0.11.7 (Andrews, 2017), and aligned sequences with the reference genome of pepper (Zunla genome) (Qin et al., 2014). Default mapping parameters (10 mismatches/read; nine multi mapping locations/read) were analyzed using HISAT 2.2.1 (Kim D. et al., 2015). We used v1.20.0 of DESeq2, an R-based software package provided by Bioconductor, for differential gene expression analysis (Love et al., 2014).

The abundance of transcripts was measured as the average normalized count of the reads mapped to the transcript, and the difference in their abundance was examined under two conditions to identify transcripts that are differentially expressed under both conditions (Love et al., 2014). The difference in expression was quantified based on the logarithm (change in the logarithmic multiple) of the average normalized count ratio between the two conditions. The differentially expressed transcripts in our experiment were defined as those with adjusted P-values < 0.01; cut-off threshold, $|\log2\text{fold change (FC)}| \geq 2$ (negative binomial Wald test, followed by the Benjamini-Hochberg correction). Differentially expressed genes (DEGs) were classified as up-regulated or down-regulated genes based on their significant positive or negative logarithmic changes in value.

Venn diagrams were constructed (Lin et al., 2016). Heatmaps were generated using the seaborn heatmap available in python. Statistical sequencing reads have been included in **Supplementary Table 1** and all DEGs genes information in different stages in **Supplementary Table 2**.

Co-expression Cluster Recognition Based on the Expression Level of all Genes in Pepper

Co-expression analysis was performed on samples from 12 control tissues and 12 cold stress-treated tissues using the k-means (Gasch and Eisen, 2002) method in python. The normalized expression value of genes was calculated by dividing

their expression levels in all samples by their maximum observed transcripts per million (TPM); the cluster information for each expressed gene is displayed in **Supplementary Table 3**. Principal component analysis (PCA) and hierarchical clustering (HCL) were performed using the Kernel principal component analysis method in python; it was convenient to graphically explain the correlations between all samples using default settings. Transformed normalized gene expression values with *z*-scores were used for PCA and hierarchical clustering.

Gene Enrichment Analysis

The identified clusters that responded to cold stress were followed up via gene enrichment analysis with GOATOOLS (Klopfenstein et al., 2018), a python package used for gene ontology (GO) enrichment analysis and determination of false discovery rate (FDR) values of statistically significant GO terms (FDR < 0.01). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed by KofamKOALA (Aramaki et al., 2020) to protein sequences by homology search, and the enrichment analysis of the KEGG pathway was carried out R package clusterProfiler (Yu et al., 2012; *P* value Cutoff = 0.05).

Identification of ICE-CBF-COR Signaling Module Genes in Pepper

The *Arabidopsis* genome website contains *Arabidopsis* gene sequence information[1] and enables us to determine the *Arabidopsis* CBF pathway through a comparison of the protein sequence generated by the *Arabidopsis* gene and the protein sequence generated by the pepper genome gene. Related genes corresponded to orthologous genes in pepper. For important genes in the ICE-CBF-COR-signaling module, the pepper genes with the closest corresponding homology relationship that contain specific structural domains were also identified through homology comparison.

Expression Analysis of ICE-CBF-COR Signaling Module Genes in Pepper

Fourteen key genes were identified in the ICE-CBF-COR signaling module in pepper, and their expression under control and cold stress-treated conditions was studied. The TPM value of gene expression was normalized using the *z*-score (**Supplementary Table 4**), and the expression was plotted using a heat map. Then, we used cold tolerance (CT) inbred line A188 and a cold sensitive (CS) inbred line A122 under cold-rewarm treatments and used the RNA-seq data (accession number: PRJNA646356), as described by Miao et al. (2021), to determine the expression levels of the 14 identified genes; then, the *Z*-score was normalized and a heat map was drawn.

Network Analysis of ICE-CBF-COR Signaling Module Genes in Pepper

We performed Pearson correlation analysis of a total of 29,053 genes that were expressed in at least one sample, obtained the correlation matrix between gene pairs, and extracted 14 CBF pathway-related genes and their correlation information. Python NetworkX enabled us to determine the network relationship diagram between 14 genes.

[1]https://www.arabidopsis.org/

RESULTS

Generation of the Pepper Cold Stress Dataset

In order to thoroughly observe the response network of genes in the leaves and roots of pepper plants before and after their exposure to cold stress, we analyzed the complete gene expression in the roots and leaves of control and stress-treated seedlings at six time points, and established a root-based and spatiotemporal dynamic expression data set of whole genes in the leaf tissue. PCA results for the transcriptome showed that the sample exhibited significant tissue specificity (**Figure 1A**). PCA also showed that different samples can be separated by both PC1 (41.76%) and PC2 (10.58%). In the early stage, when leaves were subjected to cold stress, at 0.5–1 h, the untreated leaves were closer together, and were clustered together at 3–24 h, indicating that the CORs in the leaves of peppers were induced to express proteins 3 h after cold stress treatment. Between 0.5 and 24 h of the root tissue being subjected to cold stress, three main clustering modules could be observed according to the time ranges of 0.5–1, 3–6, and 12–24 h. We circled them with the same colors. The difference in the expression at the root tissues of the three of these and the control showed an increasing trend, and a clear distinction was observed between different treatment times (**Figure 1A**). It can also be seen that after pepper was subjected to cold stress, the genes in its leaves and roots had strong tissue specificity with regard to the speed and degree of response to cold stress. The Pearson correlation analysis of datasets displayed a gene expression pattern and significant tissue specificity. The same tissues are clustered together (**Figure 1B**), which is consistent with the phenomenon shown in **Figure 1A**.

Pepper Transcriptomes Are Co-regulated in Fifteen Clusters That Correspond to Different Tissues and Treatment Periods

In order to further analyze whether the dynamic expression of genes in the roots and leaves of peppers after cold stress treatment exhibits a certain trend of differentiation, we used the k-means clustering algorithm to divide them into at least one sample according to the expression pattern of 29,053 genes. After 15 clusters, the clustering information for each gene is shown in **Supplementary Table 3**. Upon analyzing these 15 clusters (**Figure 2**), we found that the expression levels of 1,373 genes in cluster 2 were much higher in the leaf tissues than in the control, 3–24 h after cold stress treatment. The genes in the cluster responded to cold stress. However, the expression levels of genes in cluster 3 were much higher in the leaf tissues under stress at 24 h than those observed in the untreated samples at the same time point when a peak was observed. The expression levels were almost the same at

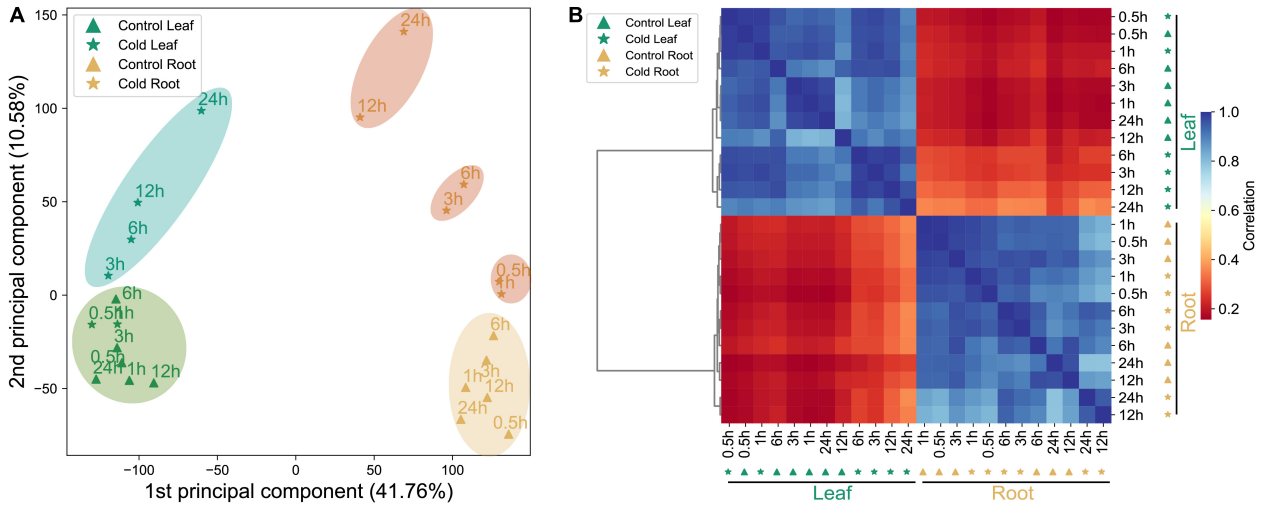

FIGURE 1 | Summary of transcriptome data of the datasets. **(A)** PCA of transcriptome data from the control and cold stress-treated samples (leaf and root). **(B)** The hierarchical clustering analysis of expression profiles of 35,336 genes from 24 samples; the color scale 0–1 represents Pearson's correlation coefficient.

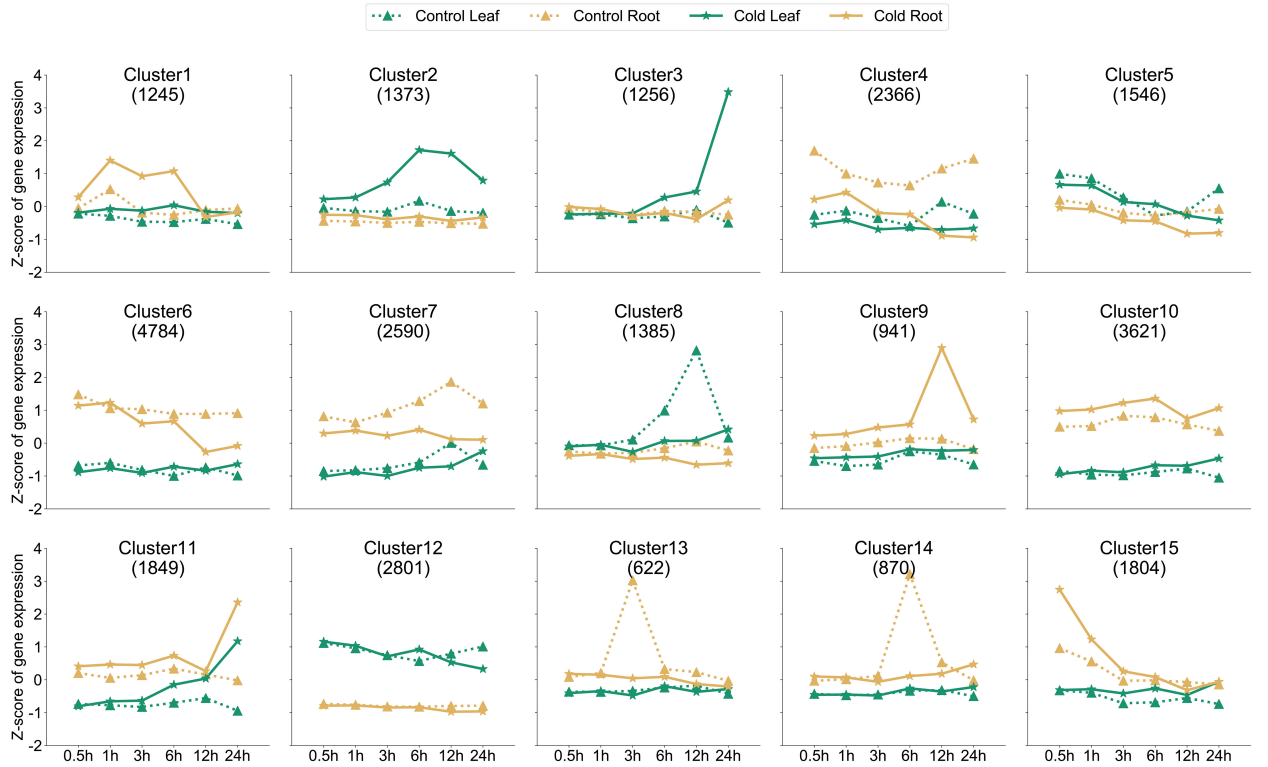

FIGURE 2 | Dynamics of gene expression in all samples. *k*-Means clustering was used to group the expression profiles of the transcriptome into fifteen clusters. The X-axis depicts values at six time points, and the Y-axis depicts the *z*-score standardized per gene. The dotted line represents the roots and leaves of the control, and the solid line represents the roots and leaves of cold stress-treated tissues; different marks represent different organizations. The numbers shown in each box (example: 1,245 genes for cluster 1) indicate the number of genes in that cluster.

other time points. The 15 clusters formed according to the trend of dynamic expression for the entire gene set had different expression patterns, and gene expression patterns in the same cluster were almost the same. We can classify the COR gene response by time and organization, according to the clustering information. However, we simultaneously found that after cold stress treatment, the expression of some genes in clusters did not change significantly at each sampling time point in the

roots and leaves, such as in cluster 5, cluster 10, and cluster 12. Genes may not be sensitive to cold stress, and their expression is always stable.

In order to analyze the functional situation of gene clusters with increased expression after cold stress, the three clusters of the cluster 3, cluster 9, and cluster 15 sets were subjected to a GO term enrichment analysis (**Figures 3A–C**). Those genes in the cluster 3, 9, and 15 were significantly enriched in the "protein-chromophore linkage," "photosynthesis," "peptide biosynthetic process," "amide biosynthetic process," and "phosphatidylinositol phosphate biosynthetic process" biological processes. The top enriched GO terms in "molecular functions" were related to "protein serine/threonine kinase activity," "phosphotransferase activity," "chlorophyll binding," "structural constituent of ribosome," "transferase activity," and "DNA-binding transcription factor activity." In addition, the top enriched GO terms in "cellular component" were "chloroplast thylakoid membrane," "plastid thylakoid membrane," "intracellular non-membrane-bounded organelle," and "ribosome."

Identification of Differentially Expressed Genes Under Cold Stress

In order to gain insight into the response mechanism of peppers to cold stress, we conducted a comparative analysis of the transcriptional differences between the leaves and roots treated at 10°C at six time points (**Figure 4**). DEGs among samples were defined using fold change values, assessed using the expression of assembled transcripts. We have identified 2,306 up-regulated genes and 1,543 down-regulated genes in the leaves of peppers at different processing times. We have also identified 1,551 up-regulated genes and 933 down-regulated genes in the roots; among these, 169 genes exhibited two states of up-regulation and down-regulation in six stress-treated and control leaves. There were 79 genes in the roots in two states, and the remaining DEGs were up-regulated or down-regulated at all periods. Removed genes were identified in both leaves and roots, and the analyzes collectively yielded 4,872 DEGs, constituting ∼16.8% of the expressed genes in the dataset.

In the dynamic situation of gene expression within 0.5–24 h of pepper plants being subjected to cold stress, both tissues had the largest number of DEGs at 24 h. This indicates that a longer duration of exposure to cold stress leads to more profound changes in the pepper transcriptome profile. Currently, there were 1,123 up-regulated genes and 597 down-regulated genes in the roots, and 1,624 up-regulated genes and 1,107 down-regulated genes in the leaves. As shown in **Figure 4**, most of the differentially expressed genes were unique at 24 h. There were 784 unique up-regulated genes and 490 unique down-regulated genes in the roots, and 1,178 unique up-regulated genes and 964 unique up-regulated genes in the leaves. Among the unique down-regulated genes, the longer the pepper plants were subjected to treatment at 10°C, the greater the change in gene expression at the genome-wide level, and the number of differentially expressed genes in the leaves at 24 h was greater than that in the roots, indicating that pepper leaves have a stronger response to cold stress.

Functional Enrichment Analysis of Differentially Expressed Genes in the Leaf and Root

In order to further analyze the possible functions of the identified cold-responsive genes shown in **Figure 4**, the up-regulated and down-regulated differentially expressed genes (DEGs) identified in the leaf (**Figures 5A,B**) and root (**Figures 5C,D**) were used to perform GO enrichment analysis, and Venn diagrams were used to show the aggregation of up-regulated and down-regulated DEGs in the leaf and root, respectively (**Figure 5C**). In the leaf and root (**Figures 5A,B,D,E**), among the "biological process" category, the most significantly enriched terms were "olefinic compound metabolic process," "phototropism," "response to water," and "cell wall organization." The top enriched GO terms in "molecular functions" were related to "DNA-binding transcription factor activity," "monooxygenase activity," "oxidoreductase activity," and "protein serine/threonine kinase activity." In addition, the top enriched GO terms in "cellular component" were "integral component of membrane," "intrinsic component of membrane," "extracellular region," and "nucleus." Low temperatures can slow down the fluidity of cell membranes and stimulate plants to respond to the low temperature.

KEGG pathway enrichment was carried out in order to better understand the biological functions of cold stress DEGs in leaf and root. The enrichment analysis indicated that "MAPK signaling pathway," "Phenylpropanoid biosynthesis," "Cytochrome P450," "Glutathione metabolism," "Zeatin biosynthesis," and "Diterpenoid biosynthesis" were common in leaf and root (**Figure 6**). However, "Plant-pathogen interaction", "Protein kinases," "Photosynthesis proteins," "alpha-Linolenic acid metabolism," "Cutin, suberine and wax biosynthesis" and "Brassinosteroid biosynthesis" were unique in leaf (**Figure 6A**) and "Pentose and glucuronate interconversions" and "Sesquiterpenoid and triterpenoid biosynthesis" were unique in root (**Figure 6B**).

Identification and Functional Analysis of Pepper ICE-CBF-COR Signaling Module Genes

Several studies have focused on ICE-CBF-COR in *Arabidopsis* and some model plants (Thomashow, 1999; Chinnusamy et al., 2003; Kim Y. S. et al., 2015; Barrero-Gil and Salinas, 2017). The ICE-CBF-COR-related genes in peppers have not been systematically identified and functionally analyzed. The expression of ICE-CBF-COR-related genes in peppers under low-temperature stress and their regulatory relationships needed to be examined. Therefore, based on the rich research background associated with ICE-CBF-COR in *Arabidopsis*, and based on the conservation of ICE-CBF-COR in the plant and protein sequence alignment, we identified that pepper contains 14 *CBF1/2/3*-related genes, including *ICE1/2* and *ICE1/2*, and *MYB15*, which have been thoroughly studied in *Arabidopsis* (Agarwal et al., 2006), *SOC1*, positive regulators of *EIN3*, *Brassinazole-resistant1* (*BZR1*) (Li et al., 2017b), *CCA1*, *CESTA*

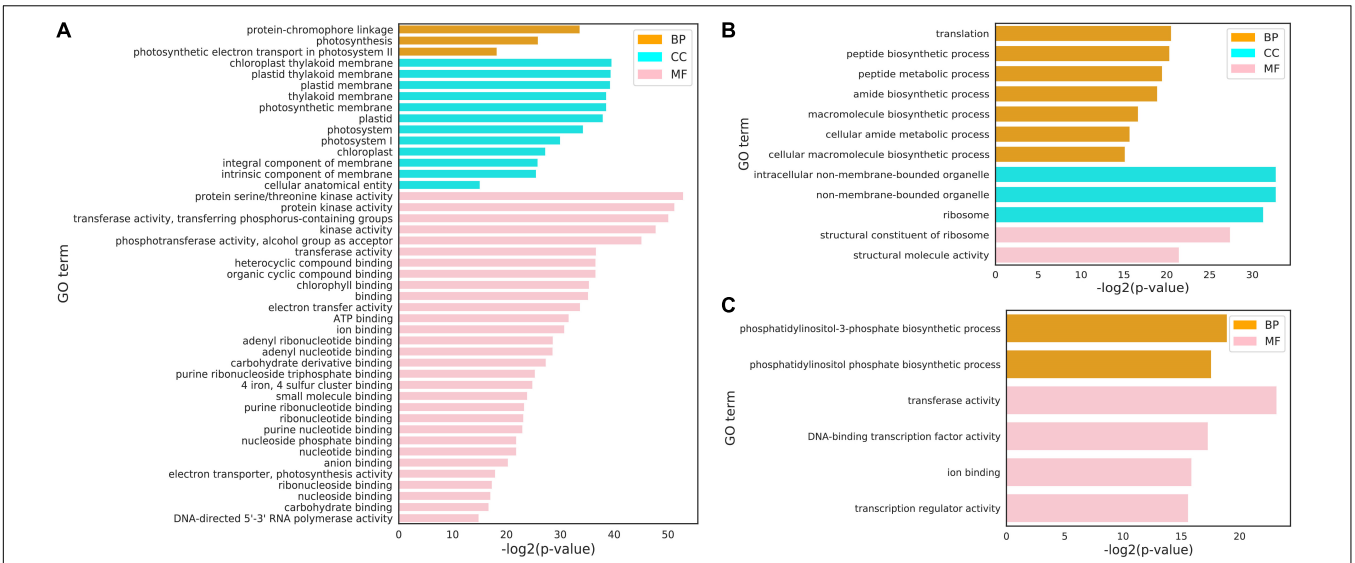

FIGURE 3 | Gene ontology (GO) terms for three clusters under cold stress conditions. **(A–C)** GO terms of cluster 3 (1,256 genes) **(A)**, cluster 9 (941 genes) **(B)** and cluster 15 (1,804 genes) **(C)** all genes at the cellular component (CC), molecular function (MF), and biological process (BP). The complete list of cluster genes and GO terms for enrichment analysis is shown in **Supplementary Tables 5–7**.

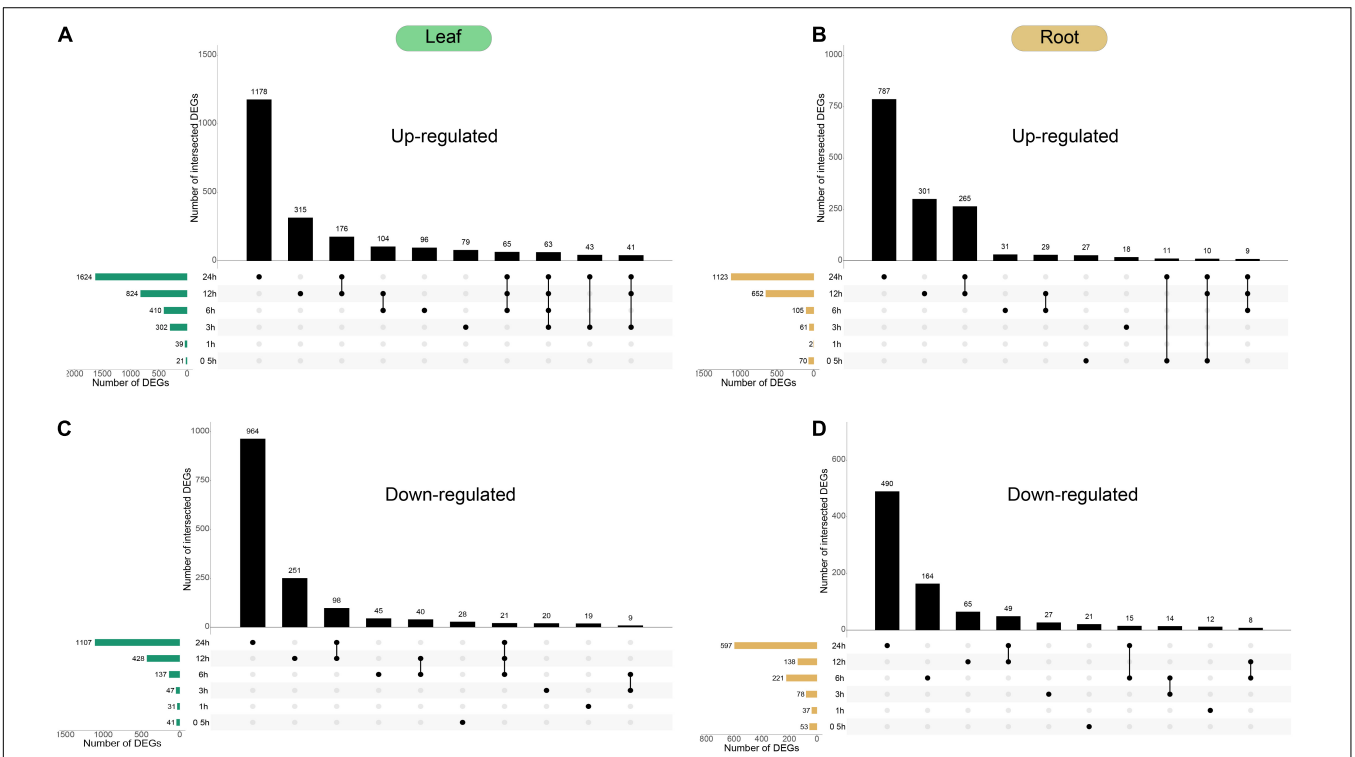

FIGURE 4 | Temporal dynamics of *Capsicum annuum* L. transcriptome during cold treatment. **(A–D)** Forty-day-old *Capsicum annuum* L. plants were subjected to cold stress at 10°C and harvested at the given time points for transcriptome analysis. UpSet plots of the number of up-regulated and down-regulated genes [cut-off threshold, $|\log2(FC)| \geq 2$; FDR < 0.01] demonstrated different temporal expression patterns (top bar graphs). The total numbers of up-regulated and down-regulated genes at each of the time points are shown on the left.

(*CES*) (Eremina et al., 2017), and positive regulators of *LHY* (Seo et al., 2009; Dong et al., 2011; Lee and Thomashow, 2012; Eremina et al., 2016). Notably, the normalized data for the control tissues obtained from the leaves and roots of pepper included that for the 14 orthologous genes in pepper and six time points at which transcript levels observed after cold stress enabled us to

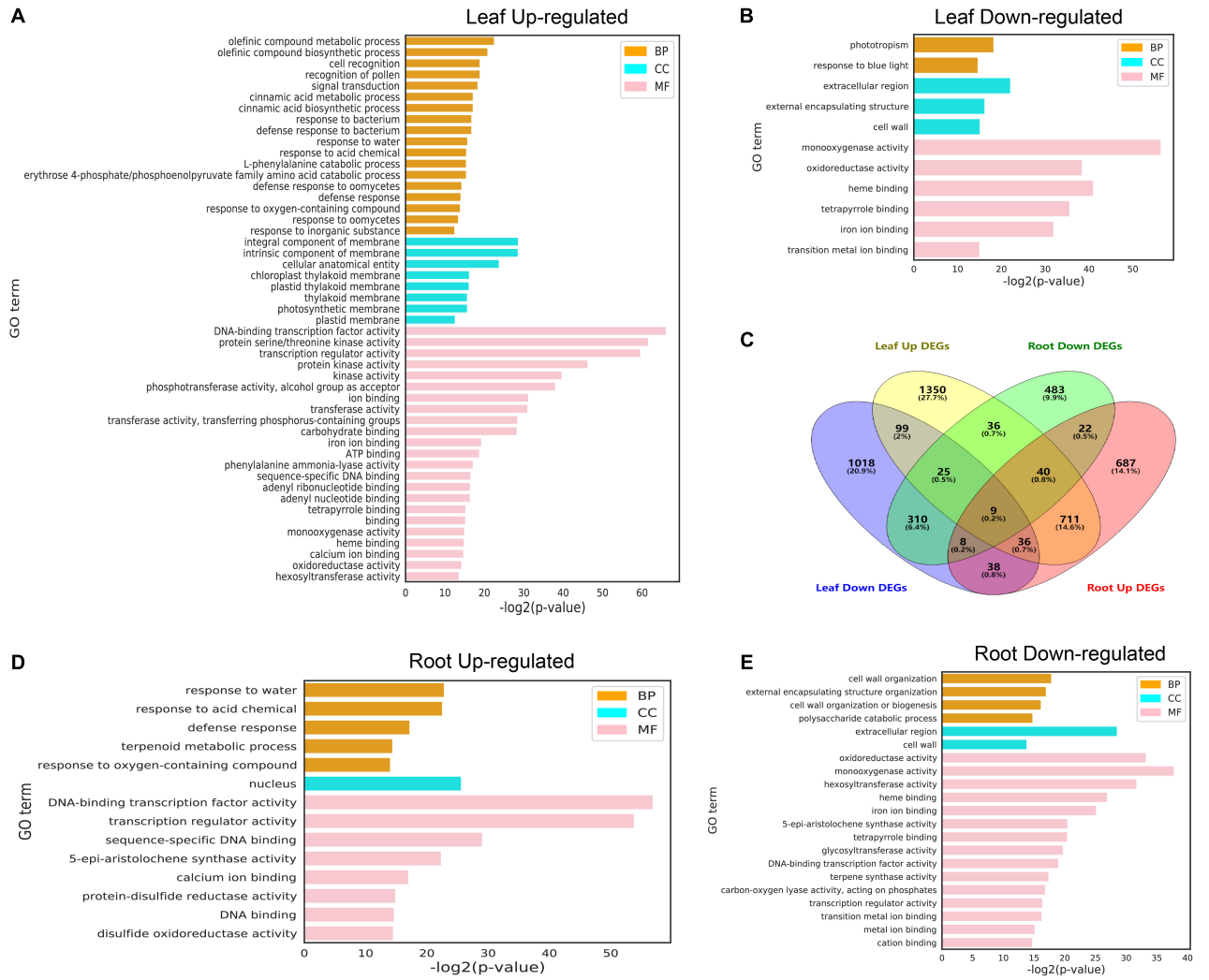

FIGURE 5 | Gene ontology terms for differentially expressed genes (DEGs) under cold stress conditions. **(A,B)** GO terms of upregulated and downregulated DEGs (FDR < 0.01) identified from leaves at different treatment time points. **(C)** Venn diagram of DEGs between the two tissues subjected to cold stress at six different time points. **(D,E)** GO terms of upregulated and downregulated DEGs (FDR < 0.01) identified from roots at different treatment time points. The complete list of DEGs and GO terms for enrichment analysis is shown in **Supplementary Tables 8–11**.

distinguish between negative regulators and positive regulators (**Figure 7A**). In *Arabidopsis*, the expression of *CBF1* and *CBF3* in response to a low temperature precedes that of *CBF2*, and *CBF2* negatively regulates *CBF1* and *CBF3* expression (Novillo et al., 2004). In our pepper dataset, we can also see that *CBF1* and *CBF3* were clustered together, but the distance from *CBF2* was high, indicating that the gene functions of *CBF1/2/3* were conserved in pepper, implying that there is a certain degree of conservation in *A. thaliana*. Genes that are negatively or positively regulated by *CBF* also have similar effects in pepper, which is of great value for the study of the ICE-CBF-COR signaling module in pepper.

We used this data set to establish a co-expression regulatory network of these 14 genes in pepper and visualized the mutual regulatory relationship between these 14 genes. The gray lines represent negative regulatory relationships between genes, and the red lines represent the positive regulatory relationship between genes. Positive regulatory relationships have also been shown. The increased thickness of the line represents a stronger predicted relationship, and the dotted line represents the Pearson coefficient absolute value being less than 0.5. We found that the relationship predicted in pepper is the same as that observed in *A. thaliana* (**Figure 7B**). For example, *MYB15*, *SOC1*, and *EIN3* negatively regulate *ICE1* expression in *Arabidopsis* (Agarwal et al., 2006). The same result is also shown in our network diagram. The co-expression relationship between these 14 genes can provide certain insights into the correlation between related genes in pepper and the ICE-CBF-COR signaling module at low temperatures. Furthermore, we used predecessors in the cold-resistant (A122) and cold-sensitive (A188) varieties of pepper under cold stress (4°C for 0, 1, 2, and 24 h) conditions and after rewarming (28°C for 1 h), and obtained the RNA-seq data (Miao et al., 2021). We analyzed the expression of the 14 genes identified

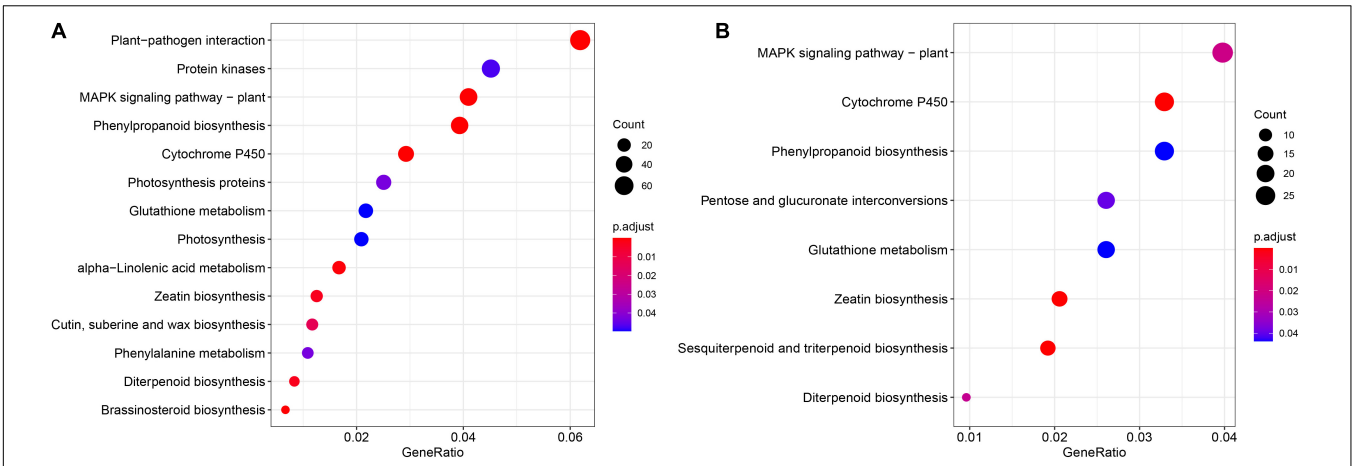

FIGURE 6 | Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis results for the DEGs in leaf **(A)** and root **(B)** among the six different time points. The complete list of DEGs and KEGG pathways for enrichment analysis is shown in **Supplementary Tables 12**, **13**.

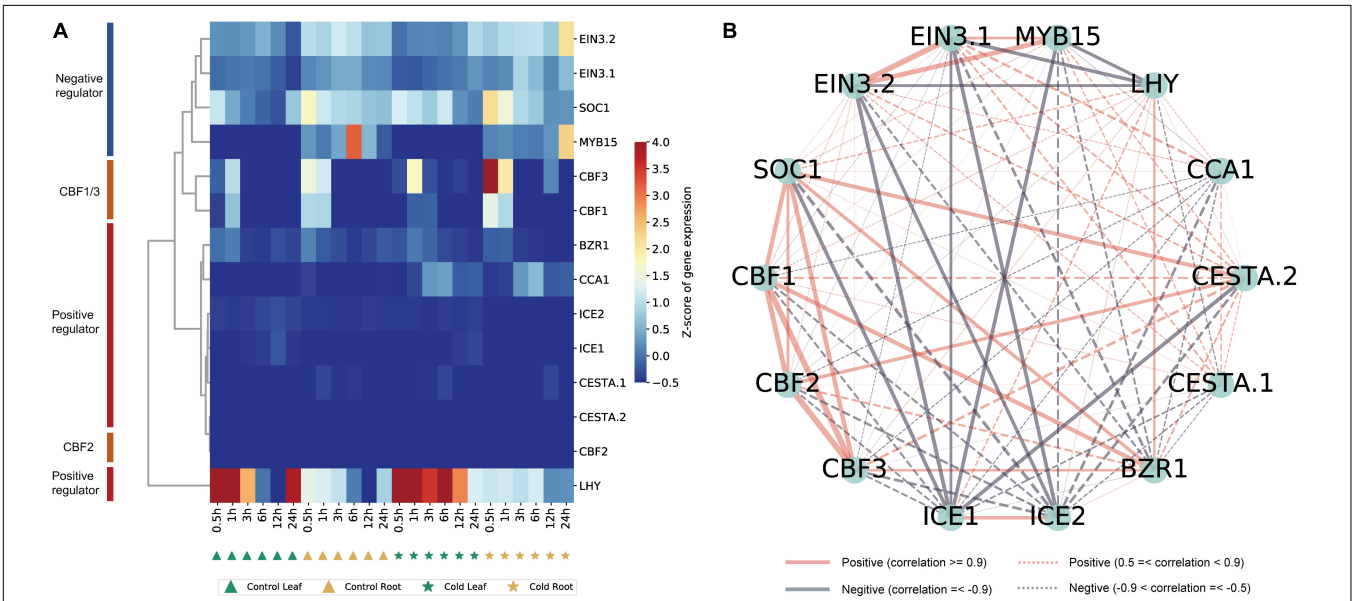

FIGURE 7 | Identification of ICE-CBF-COR genes in pepper and visualization of their co-expression network. **(A)** Hierarchical cluster analysis of 14 ICE-CBF-COR genes. The values in the heatmap represent the *z*-scores of transcripts per million (transcription level) in different samples. The red and blue colors indicate a high and low expression level, respectively. **(B)** Visualization of the 14 ICE-CBF-COR gene co-expression networks using the Python NetworkX package.

in the dataset and found that after the rewarming process, the expression of *EIN3.1* and *EIN3.2* was significantly up-regulated, and the expression of *ICE1/2* in A188 was significantly higher than that in A122 (**Figure 8**).

DISCUSSION

Owing to the completion of the assembly of the whole genome of pepper (Kim et al., 2014; Qin et al., 2014). The whole-genome sequencing of pepper has made it feasible to identify stress resistance genes in the pepper plant, at the genome level. Cold weather often leads to a severe decline in crop yields (Zhang J. et al., 2019). Although the molecular mechanism of cold-induced reprogramming of gene expression has been studied extensively in model plants, only a few of these reports are related to peppers (Liu et al., 2021; Miao et al., 2021). In this study, we tried to determine the regulatory mechanism of the response to cold stress in pepper by subjecting leaves and roots to cold stress at six time points and performing transcriptome analysis of the stress-treated and control tissues at each time point. Cold acclimation involves a cascade of transcriptional events. We divided all samples into 15 clusters based on the expression pattern of 29,053 genes in at least one sample, and found that the expression levels of genes in cluster 2 and cluster 3 in the leaves subjected to cold stress were much higher at different time points than those of

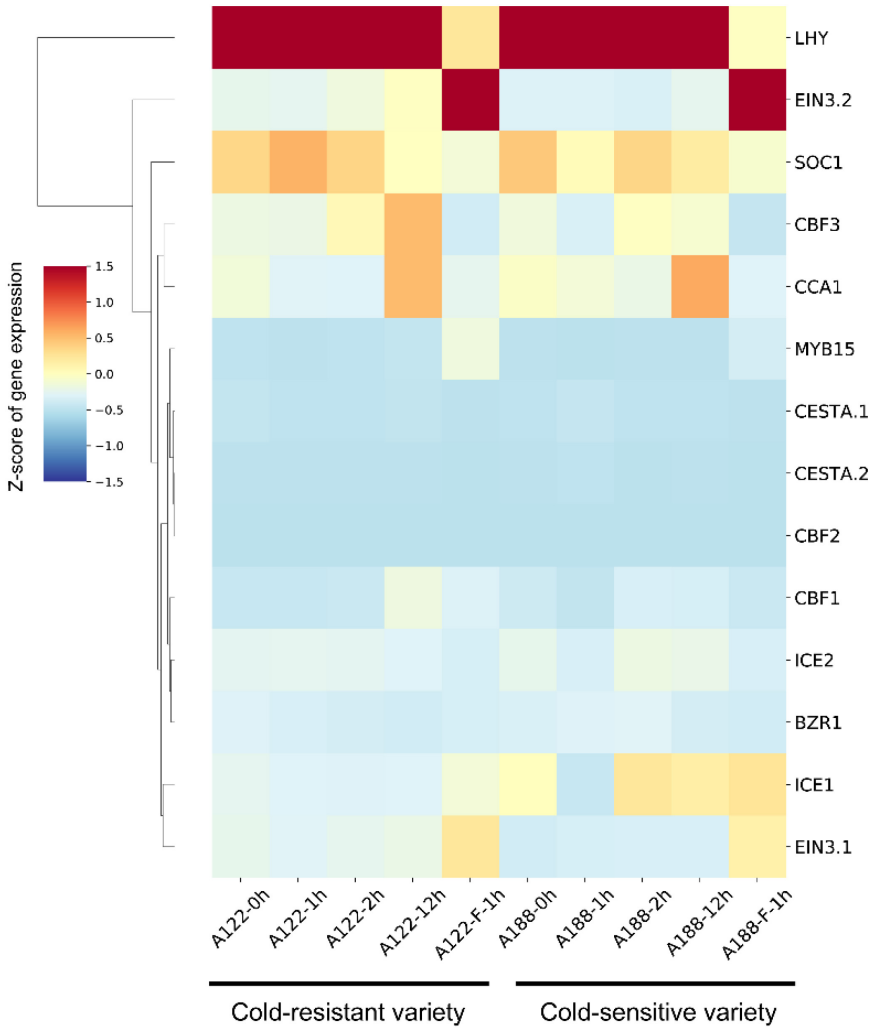

FIGURE 8 | Hierarchical cluster analysis of 14 ICE-CBF-COR genes in A122 and A188. The values in the heatmap represent the *z*-scores of fragments per kilobase million (transcription level) in different samples. The red and blue colors indicate high and low expression levels, respectively.

the control. For example, the expression of 1,373 genes in cluster 2 was higher at 0.5 h, and the peak difference was observed at 6 h, while the expression of 1,256 genes in cluster 3 increased sharply at 24 h. Further, the cluster 3, cluster 9, and cluster 15 were enriched in different GO terms (**Figures 3A–C**). In this study, many tissue-specific DEGs were identified in leaf and root six time points, which may be due to the different effects of cold stress on the leaf and root of pepper seedlings (**Figures 5A–D**). However, these tissue-specific DEGs are also enriched in pathways, such as the "MAPK signaling pathway", suggesting that it plays a key role in the transduction of environmental and developmental signals (**Figures 6A,B**; Jagodzik et al., 2018) and previous studies have reported that *A. thaliana* and rice (*Oryza sativa*) participate in the response to freezing stress through the MAPK cascade pathway (Li et al., 2017a; Zhang et al., 2017; Zhao et al., 2017).

Moreover, we identified 14 genes in the pepper ICE-CBF-COR signaling module, and analyzed the expression and clustering of these genes in different tissues and time points under cold stress (**Figure 7A**). We found that positively and negative regulatory factors in pepper are clustered together, which is consistent with the results of the study of *A. thaliana*, which proves that ICE-CBF-COR is conserved in plants (Kim Y. S. et al., 2015).

We combined the RNA-seq data for the cold-resistant (A122) and cold-sensitive (A188) varieties identified previously after low-temperature treatment and rewarming, to verify the results of our analysis (Miao et al., 2021). We found that the expression level of positive regulators, such as *ICE1/2*, *CBF1*, and *CCA1* was higher in A188 than in A122. After being subjected to cold stress, the stress response in A122 material was stronger, which may be attributable to its significantly lower tolerance to cold stress than A188, as a higher level of expression of these positive regulators was needed to activate the ICE-CBF-COR-signaling module, and activate low-temperature tolerance in the plant more effectively. In addition, we found that the expression levels of *EIN3.1/2*, *MYB15*, and *ICE1* increased in A122 and A188 after rewarming.

In *Arabidopsis*, cold stress up-regulates the expression of *MYB15*, and the *MYB15* protein interacts with *ICE1* and binds to the MYB recognition sequence in the promoter sequence of CBFs (Agarwal et al., 2006). The performance of *MYB15* in pepper is different. In the normal pepper variety 6421, the expression of *MYB15* in the root increases under cold stress at 24 h, but there is no significant response in the leaves (**Figure 7A**). The role of *MYB15* in *A. thaliana* is supported by data showing that *MYB15* overexpression and low expression alter the expression of CBF genes and affect freezing tolerance (Agarwal et al., 2006). The response of *MYB15* in pepper A122 was stronger than that in A188, which also confirmed the situation in *Arabidopsis*.

In this study, we analyzed the transcriptome data for cold stress at six time points in the leaves and roots of the pepper plant, determined the co-expression-based relationship between 14 genes in the pepper ICE-CBF-COR-signaling module, and correlated it with that in A122, which had been identified by previous researchers (Miao et al., 2021). The results of transcriptome analysis of extreme low temperature-tolerant tissues of A188 showed that the expression of these genes occurred in cold-resistant and cold-sensitive varieties after they were subjected to low-temperature stress and rewarming (**Figure 8**). These results provide another perspective regarding the low-temperature reaction mechanism in pepper and *Solanaceae* crops. In this study, we not only identified the key genes in the ICE-CBF-COR-signaling module in pepper, but also constructed a mutual co-expression network of these genes based on the low-temperature stress data set for the leaf and root at six time points. This network needs to be verified via experiments involving genetic transformation in the future, to verify the response mechanism of pepper to low temperatures. In this study, we have used bioinformatics tools to enable follow-up researchers to perform verification in a more effective manner.

CONCLUSION

In this study, we performed a global analysis of 24 samples of leaves and roots of peppers at six time points after subjecting them to low-temperature stress. Control treatments were also performed to assess the dynamic expression of different tissues in peppers at the whole gene level subjected to low temperature stress for different durations. We identified 3,680 and 2,405 DEGs in the leaves and roots, respectively. Fourteen low-temperature-responsive ICE-CBF-COR modular genes were identified in peppers, and a pairwise co-expression network model was constructed between the 14 genes. The expression patterns in different pepper varieties showed that they were resistant to cold stress. The process of collection of data in this study under low-temperature stress would act as a reference for the exploration of a plant ICE-CBF-COR signal model, and contribute to the elucidation of the response mechanism to low temperatures and adaptability to other stress conditions.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: National Genomics Data Center (NGDC), China National Center for Bioinformation (CNCB)/Beijing Institute of Genomics (BIG), Chinese Academy of Sciences (CAS), Genome Sequence Archive (GSA), https://ngdc.cncb.ac.cn/gsa/, PRJCA007952.

AUTHOR CONTRIBUTIONS

LX and XL: conceptualization. BT: data curation, visualization, and writing – original draft. FL, XD, and XZ: funding acquisition. HY and YC: investigation. BT and FL: writing – review and editing. All authors contributed to the article and approved the submitted version.

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Dehydration-Responsive Element Binding Protein 1C, *1E*, and *1G* Promote Stress Tolerance to Chilling, Heat, Drought, and Salt in Rice

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The *dehydration-responsive element binding protein 1* (*DREB1*)/*C-repeat-binding factor* (*CBF*) genes are key regulators of cold acclimation and freezing tolerance in the chilling tolerant *Arabidopsis thaliana*. Here, we investigated the function of three members of the 10 rice *DREB1* genes, *OsDREB1C*, *E*, and *G*, in the chilling sensitive rice plants. Their loss of function (LOF) mutants were each more chilling susceptible compared to the wild type, and the LOF mutants of all three genes, *dreb1ceg*, were more chilling susceptible than any of the single mutants. Strikingly, these mutants were capable of cold acclimation, indicating that these rice *DREB1* genes are important for basal chilling tolerance but not cold acclimation. Transcriptome and physiology analyses suggest that the *OsDREB1C/E/G* genes are involved in reactive oxygen species (ROS) scavenging and cell death regulation under chilling. Furthermore, these three rice *DREB1* genes are found to promote tolerance to other abiotic stresses: the *OsDREB1C/E/G* genes are positive regulators of heat tolerance, *OsDREB1C* and *OsDREB1G* are positive regulators of salt tolerance, and *OsDREB1G* is a positive regulator of drought tolerance. These findings expand our knowledge of the roles of DREB1 proteins in plants, enhance our mechanistic understanding of abiotic stress tolerance and will facilitate the generation of stress-tolerant crop plants.

Keywords: *OsDREB1*, chilling tolerance, cold acclimation, abiotic stress, ROS, cell death, rice

INTRODUCTION

Cold stresses, both freezing and chilling (above freezing), are a major threat to crop production worldwide (Pearce, 2001; Zhang et al., 2019). Rice (*Oryza sativa*), with a tropical and temperate origin, is susceptible to cold stress at all developmental stages (Zhang et al., 2014). A temperature lower than 15°C causes drastic physiological changes and inhibits the growth and development of rice (Jacobs and Pearson, 1999; Aghaee et al., 2011). Chilling stress results in poor germination, stunted seedlings, yellowing or withering of leaves, and reduced tillering in rice (Yadav, 2010). Chilling induces accumulation of reactive oxygen species (ROS) which serves as both a signaling molecule of cold response and a damaging molecule to the cell (Hasanuzzaman et al., 2013;

Devireddy et al., 2021). Excess ROS is harmful to the stability of cell membrane and proteins and leads to growth inhibition or cell death (Mittler, 2002; Mhamdi and Van Breusegem, 2018). ROS-scavenging has an important role in protecting plants against chilling stress, and a correlation of ROS scavenging and chilling tolerance has been reported (Fang et al., 2020; Ge et al., 2020).

Plants have multiple mechanisms to promote tolerance to cold stresses. The most extensively studied process is cold acclimation, where freezing tolerance is enhanced by a prior exposure to low non-freezing temperatures (Thomashow, 1999). The *dehydration-responsive element binding factor 1* (*DREB1*)/*C-repeat binding factor* (*CBF*) genes in the ERF transcription factor family play a critical role in this cold acclimation in *Arabidopsis thaliana*. Their transcripts are rapidly induced by chilling stress (Gilmour et al., 1998), and the CBF/DREB1 proteins recognize and bind to the C-repeat/dehydration-responsive (CRT/DRE) cis-elements in the promoters of *cold-responsive* (*COR*) genes to activate *COR* genes expression for freezing tolerance (Stockinger et al., 1997; Liu et al., 1998; Sakuma et al., 2002). Arabidopsis has six *DREB1* genes, namely *DREB1A*/*CBF3*, *DREB1B*/*CBF1*, *DREB1C*/*CBF2*, *DREB1D*/*CBF4*, *DREB1E*/*DDF2*, and *DREB1F*/*DDF1* (Sakuma et al., 2002). Three of them, *CBF1*, *CBF2*, and *CBF3* genes, are shown to be required for freezing tolerance and cold acclimation by the study of knockout mutant combinations generated by genome editing (Hua, 2016). The *cbf1 cbf2 cbf3* triple mutants were more susceptible to freezing than the wild type (WT) with prior low temperature treatment (Jia et al., 2016; Zhao et al., 2016). Without prior treatment, the *cbf1 cbf2 cbf3* mutants did not exhibit more susceptible to freezing (Jia et al., 2016) or was only slightly more susceptible under one out of eight freezing temperatures tested (Zhao et al., 2016). The three Arabidopsis CBF/DREB1 proteins are predicted to positively regulate expression of a total of 346 and 112 *COR* genes in two independent studies (Jia et al., 2016; Zhao et al., 2016). Recently, 146 Arabidopsis genes are defined as direct CBF/DREB1 targets by chromatin immunoprecipitation (ChIP) combined with a transcriptional analysis, and these genes are involved in abiotic stress responses, hormone signaling, and environmental signaling (Song et al., 2021).

CBF/*DREB1* genes are also implicated in tolerance to other abiotic stresses. Compared to the WT, the Arabidopsis *cbf1 cbf2 cbf3* triple mutant is more susceptible to salt (Zhao et al., 2016), and *cbf4* mutant is more susceptible to drought (Vonapartis et al., 2022). Overexpression of the Arabidopsis *DREB1A*, *DREB1B*, and *DREB1C* enhanced tolerance to freezing, salt, and drought in Arabidopsis (Liu et al., 1998; Kasuga et al., 1999; Gilmour et al., 2004). Overexpression of the Arabidopsis *DREB1D* enhanced drought tolerance in soybean (Guttikonda et al., 2014), while overexpression of the Arabidopsis *DREB1F*/*DDF1* enhanced tolerance to freezing, drought, and heat in Arabidopsis (Kang et al., 2011). The function of *DREB1* genes in other plants have also been investigated. Overexpression of *DREB1* genes from cotton, soybean, and zoysia grass was shown to enhance tolerances to chilling, drought, heat, and salt (Gao et al., 2005; Shan et al., 2007; Kidokoro et al., 2015; Feng et al., 2019; Zhou et al., 2020). These results suggest that DREB1 proteins could regulate tolerance to multiple abiotic stresses.

The roles of rice *DREB1* genes have been investigated mostly by overexpression studies. Overexpression of four rice DREB1 genes is shown to enhance stress tolerance: *OsDREB1A* for freezing and high salt tolerances in Arabidopsis (Dubouzet et al., 2003), *OsDREB1D* for tolerances to freezing and high-salt in Arabidopsis (Zhang et al., 2009), *OsDREB1F* for tolerances to salt, drought, and cold in both Arabidopsis and rice (Wang et al., 2008), and *OsDREB1G* for chilling tolerance in rice (Moon et al., 2019). The only study of *DREB1* function by its loss of function (LOF) mutant is on *OsDREB1A* which was shown to be a positive regulator of cold-induced calcium influx and chilling tolerance in rice (Wang et al., 2021).

In this study, we investigated the roles of three rice *DREB1* genes (*OsDREB1C*, *E*, and *G*) which are the closest homologs of the three Arabidopsis *DREB1* genes (*CBF1*, *CBF2,* and *CBF3*) by generating and analyzing their LOF mutants. These genes are found to be positive regulators of chilling tolerance but do not affect cold acclimation. They are also found to promote tolerance to other abiotic stresses including heat, salt and drought in rice. Transcriptome analysis suggests that these *OsDREB1* genes affect ROS scavenging and cell death. Thus, our study uncovers function of *OsDREB1C*/*E*/*G* in multiple abiotic stress tolerance in rice and reveals potential similar and distinct functions of these genes in rice and Arabidopsis.

MATERIALS AND METHODS

Phylogenetic and Expression Analyses

Protein sequences of *DREB1* genes from Arabidopsis and rice were aligned using ClustalX. A neighbor-joining tree with a bootstrap setting of 1,000 was generated using MEGA7.0.14 (Kumar et al., 2016). Expression data of *OsDREB1* genes in response to abiotic stresses were obtained from GENEVESTIGATOR.[1]

Mutant Generation and Identification

All rice plants in this study are *Oryza sativa* cv. Nipponbare (NIP). Mutants were generated in NIP using CRISPR/Cas9 system (Xing et al., 2014). Specific gRNA targets were designed using CRISPR-PLANT website.[2] The gRNA targets were cloned into the pHUE411 vector (Xing et al., 2014), and the resulting constructs were transformed into NIP by Agrobacterium-mediated transformation (Nishimura et al., 2006). Genomic fragments around the target sites in the mutants were amplified by PCR, and the edited mutations were identified by sequencing the PCR products. The homozygous mutants were selected by PCR based genotyping. All the primers used in this study are listed in **Supplementary Table 1**.

[1]https://www.genevestigator.com/
[2]http://www.genome.arizona.edu/crispr/CRISPRsearch.html

Plant Growth Conditions and Abiotic Stress Treatments

All plants were grown in growth chamber with a photoperiod of 16h-light/8h-dark and at 28°C unless specified (such as for stress treatment). Seeds were sterilized with 5% bleach for 10 min followed by three washes with water. The sterilized seeds were soaked in water at 28°C for 3 days for germination. For hydroponic growth, seeds at a similar germination stage were transferred to a 96-well plate with the bottom cut out. Seedlings were grown hydroponically till the third leaf just emerged, and then cultured in Yoshida nutrient solution (Yoshida et al., 1971). For soil growth, the germinated seeds were transferred from water to soil (nutrient soil:vermiculite = 3:1) and grown in growth chamber.

All stress treatments were carried out in growth chambers. For chilling treatment, the hydroponically grown seedlings at three-leaf stage (when the third leaf was fully expanded) were subject to 6°C treatment for 2.5–4 days and then returned to 28°C for 7 days for recovery growth. For suboptimal low temperature treatment, germinated seeds were transferred to soil and grown under 16°C. For cold acclimation treatment, seedlings at three-leaf stage (grown hydroponically at 28°C) were subject to 1 day of 12°C growth (cold acclimation, CA) or kept at 28°C for 1 day (no acclimation, NA). They were then subject to a 6°C treatment for 3.5–4 days before a 7-day recovery growth at 28°C. For heat treatment, the soil grown seedlings at three-leaf stage were treated at 48°C for 2 days and then returned to 28°C for 7-day recovery growth. For salt treatment, the soil grown seedlings of 3-day-old were treated with 200 mM NaCl for 21–23 days. For drought treatment, the soil-grown seedlings at three-leaf stage were withheld of watering for 10 days and then re-watered for 7 days for recovery growth. For survival analysis, 24 hydroponically grown seedlings or 21 soil growth seedlings were used in a biological repeat. At least three biological replicates were performed for each analysis.

DAB and Trypan Blue Staining

Accumulations of H_2O_2 and cell death were detected by 3,3′-diaminobenzidine (DAB) staining and trypan blue staining, respectively, as previously described (Bach-Pages and Preston, 2018). The second leaves of the soil grown seedlings at three-leaf stage were used.

Transcriptome Analysis

The three-leaf stage seedlings that were soil grown at 28°C were treated at 6°C for 0, 4, and 24 h before all above ground tissue of three biological replicates were collected. Total RNA was extracted from tissues using an RNA extraction kit (RP1002, Bioteke Corporation). RNA sequencing (RNA-seq) was carried out at Berry Genomics.[3] Approximately 6.0 GB of raw reads were generated from each sample. The clean reads were mapped to rice genome,[4] and expression level for each gene was determined by its relative abundance or value of fragments per kilobase of exon model per million mapped (FPKM). Gene expression values in FPKM was used for principal component analysis.[5] Differentially expressed genes (DEGs) were identified using edgeR package (3.32.1) by R Language (R Core Team, 2021). DEGs between two samples were defined by fold change ≥ 2 and false discovery rate (FDR) ≤ 0.01. Heat maps were plotted *via* TBtools or using pheatmap (1.0.12) and ggplot2 (3.3.3) packages by R language (Chen et al., 2020; R Core Team, 2021). Venn diagrams were plotted using Venn Diagram package (1.6.20) by R language (R Core Team, 2021). Gene ontology (GO) enrichment analysis was performed by PANTHER.[6] GO annotation was downloaded from RiceXPro.[7] The promoter analysis was performed by a manual search for the DRE/CRT motif (G/ACCGAC) in the 1.5 kb fragment 5′ to the translation start codon of the candidate gene. The RNA-seq data were submitted to National Center for Biotechnology Information (accession number: PRJNA797855).[8]

RESULTS

Phylogenetic and Expression Analyses of the *OsDREB1* Genes in Rice

The *DREB1* family has six members in Arabidopsis and 10 members (named *OsDREB1A* to *OsDREB1J*) in rice.[9] Analysis of genes from 38 organisms revealed that this gene family diverged before the separation of Arabidopsis and rice, and the *DREB1* genes have independently amplified in two species (**Supplementary Figure 1**). This suggests that the three *CBF* genes in Arabidopsis do not have a one-to-one orthologous *DREB1* genes in rice. Protein sequence homology analysis of the six Arabidopsis and 10 rice *DREB1* genes, however, revealed that *OsDREB1C*, *OsDREB1E*, *OsDREB1F*, and *OsDREB1G* are likely closer to the six Arabidopsis *DREB1* genes in sequence identity (**Figure 1A**).

We further examined the expression patterns of these 10 *OsDREB1* genes (except for *OsDREB1H* which is absent in the database) using the curated high-quality transcriptome data at GENEVESTIGATOR (see Footnote[1]). All these nine *OsDREB1* genes (*1A*, *1B*, *1C*, *1D*, *1E*, *1F*, *1G*, *1I,* and *1J*) have altered expression in response to one or multiple abiotic stresses. Chilling treatment (4°C for 4 h) increased the expression of *1A*, *1B*, *1C*, *1E*, *1F*, *1G* and *1I*. Dehydration treatment (on 3 mm Whatman paper for 3 h) increased the expression of *1A*, *1B*, *1C*, *1E*, *1F,* and *1G*. Drought treatment (no watering till leaves were completely rolled) increased the expression of *1C*. Heat treatment (42°C for 3 h) increased expression of *1I* and repressed the expression *1A* and *1B*. Salt treatment (300 mM NaCl for 5 h) had no significant effect on the expression of any of these nine *OsDREB1* genes. Simulated drought treatment (25% polyethylene glycol6000

[3]http://www.berrygenomics.com/
[4]http://plantgdb.org/OsGDB/
[5]https://www.omicshare.com
[6]http://www.pantherdb.org/help/PANTHERhelp.jsp
[7]https://ricexpro.dna.affrc.go.jp/
[8]https://www.ncbi.nlm.nih.gov/
[9]http://www.phylogenes.org/tree/PTHR31839

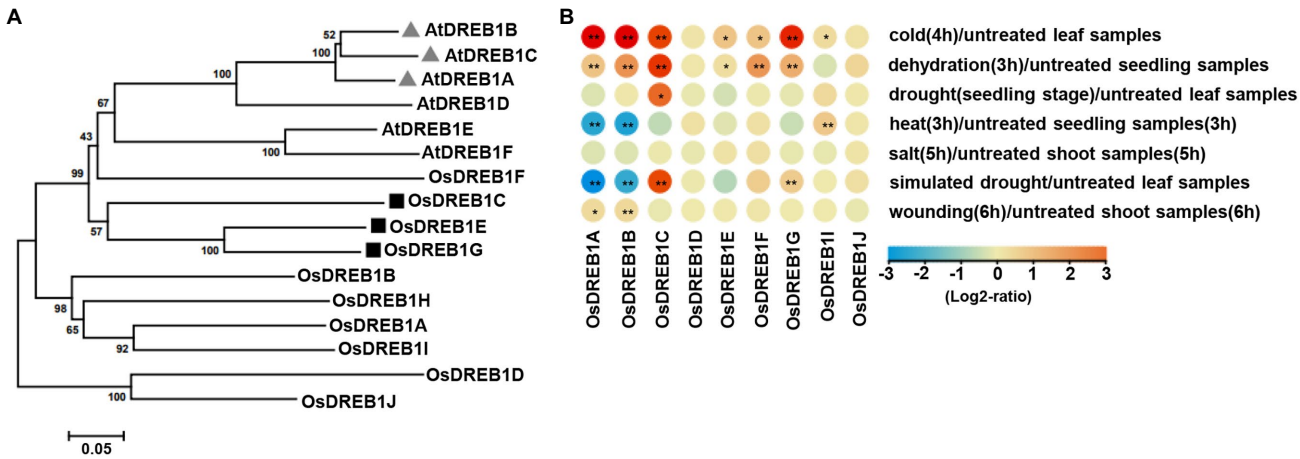

FIGURE 1 | Expression patterns and phylogenetic analyses of *OsDREB1* genes in rice. **(A)** Phylogenetic tree of the OsDREB1 and AtDREB1 proteins. The protein sequences were aligned using ClustatW method, and the neighbor-joining tree with a bootstrap setting of 1,000 was generated using MEGA7.0.14 software. The genes marked with black targets are discussed in this study. The AtDREB1 and OsDREB1 protein sequences were available by TAIR (https://www.arabidopsis.org/) and RiceXPro (https://ricexpro.dna.affrc.go.jp/), respectively. **(B)** Expression patterns of *OsDREB1* genes in response to abiotic stresses. Heat map was plotted *via* Tbtools. Expression levels are given as log2 ratio values. Expression values are obtained from GENEVESTIGATOR (https://genevestigator.com/). * and ** indicate significant differences compared to no treatment at $p < 0.05$ and $p < 0.01$, respectively, by Student's *t*-test.

for 1 h) increased the expression of *1C* and *1G* and repressed the expression *1A* and *1B*. Wounding treatment (needle puncturing 15 times in 6 h) increased expression of *1A* and *1B* (**Figure 1B**). Therefore, most of the *OsDREB1* genes had increased expression in response to chilling and dehydration stresses.

Generation of *dreb1* Mutants in Rice

We selected three *OsDREB1* genes (*OsDREB1C*, *OsDREB1E,* and *OsDREB1G*) for further functional analysis because they are more related to each other and are more closely related to the Arabidopsis *DREB1* genes than six other rice genes (**Figure 1A**). LOF mutants of these three genes were generated in the NIP background by using CRISPR/Cas9 gene editing system (Xing et al., 2014). Two gRNA targets were used to generate single mutants of *dreb1c* (*via* targets C1, C2), *dreb1e* (*via* targets E1, E3), and *dreb1g* (*via* targets G2, G3). A total of four *dreb1c* mutant alleles (*dreb1c-1*, *dreb1c-2*, *dreb1c-3,* and *dreb1c-4*), two *dreb1e* alleles (*dreb1e-1* and *dreb1e-2*), and one *dreb1g* mutant (*dreb1g-1*) were obtained (**Table 1**). A gRNA target (C2) specific for *OsDREB1C* and gRNA targets (E2, G1) for both *OsDREB1E* and *OsDREB1G* was used to generate the *dreb1ceg* triple mutant (**Table 1**).

All these mutants were LOF as predicted by the mutation types. The *dreb1c-1* mutant has an insertion of "C" at position 12 bp (relative to the translation initiation site) and a deletion at 13–279 bp, which are predicted to cause a reading frame shift. The *dreb1c-2* mutant has a deletion of 35–37 bp and an insertion of "A" at 275 bp, leading to a reading frame shift. The *dreb1c-3* mutant has an insertion of "T" and "A" at 38 and 275 bp, respectively, leading to coding termination. The *dreb1c-4* mutant has a deletion at 32–67 bp and an insertion of "A" at 275 bp leading to a reading frame shift. The *dreb1e-1* mutant has an insertion of "TGG" and "T" at 21 and 337 bp, respectively, leading to a reading frame shift. The *dreb1e-2* mutant has an insertion of "G" at 21 bp and a deletion of "G" at 336 bp, leading to protein sequence changes starting from amino acid (aa) positions 8 and 112, respectively. The *dreb1g-1* mutant has an insertion of "A" at 164 bp, leading to coding termination. The *dreb1ceg* mutant has a deletion of "G" at 274 bp in *OsDREB1C*, and an insertion of "A" and "C" at 50 bp in *OsDREB1E* and *OsDREB1G*, respectively, leading to a reading frame shift of *OsDREB1E* and coding terminations of *OsDREB1C* and *OsDREB1G* (**Table 1**). Homozygous single or triple mutants of these three genes were used for further analyses.

OsDREB1C/E/G Genes Promote Basal Chilling Tolerance in Rice at Seedling Stage

The role of *OsDREB1C/E/G* genes in chilling tolerance was assessed by chilling treatment of the single and triple mutants of *dreb1c*, *dreb1e*, and *dreb1g*. Three-leaf-stage seedlings were subjected to 2.5–4 days of chilling treatment at 6°C and allowed to recover for 7 days. All *dreb1* single mutants had significantly lower survival rates than NIP, although the survival rate of the WT differed among different sets of experiments likely due to a slight variation of temperature and duration of chilling treatment (**Figures 2A–F**; **Supplementary Figure 2**). The *dreb1c-1* and *dreb1c-2* mutants had survival rates of 19% and 27%, respectively, lower than the rate of 42% for NIP (**Figure 2A**). The *dreb1c-3* and *dreb1c-4* mutants had survival rates of 55% and 49%, respectively, lower than the rate of 92% for NIP (**Supplementary Figures 2A–C**). The *dreb1e-1* mutant had a survival rate of 36%, lower than the rate of 55% for NIP (**Figure 2B**). The *dreb1e-2* mutant had survival rates of 53%, lower than the rate of 72% for NIP (**Supplementary Figures 2D,E**).

TABLE 1 | Mutations in rice *DREB1c*, *DREB1e*, and *DREB1g* genes generated *via* the CRISPR/Cas9 system.

Gene (mutant)	Nucleic acid mutation		Predicted impact on protein
OsDREB1C (*dreb1c-1*)	ACC - - - - - - - - - -// - - - - - - - - -GCC	1 bp insertion after 11 bp and a deletion from 13 to 279 bp (including targets C1 and C2)	Reading frame shift after amino acid (aa) 4
OsDREB1C (*dreb1c-2*)	GAGTACGCGACGG - - - CGT TGCCTCAACTTCGCCGAACT	3 bp deletion at target C1; 1 bp increase at target C2	Altered at aa 12-13 and reading frame shift after aa 91
OsDREB1C (*dreb1c-3*)	GAGTACGCGACGGTGATCGT TGCCTCAACTTCGCCGAACT	1 bp increase at target C1; 1 bp increase at target C2	Reading frame shift after aa 12 and coding termination after aa 147
OsDREB1C (*dreb1c-4*)	GAGTACGCGA - - - - - // - - -GGA TGCCTCAACTTCGCCGAACT	36 bp deletion at target C1; 1 bp increase at target C2	Deletion of aa 11-22 and reading frame shift after aa 91
OsDREB1E (dreb1e-1)	GAGTGGGCGTACTACGGTGGCAG CTTCGCCGACTCGCCGTCGC	3 bp increase at target E1; 1 bp increase at target E3	1 aa insertion at aa 8 and reading frame shift after aa 112
OsDREB1E (dreb1e-2)	GAGTGGGCGTACTACGGGCAG CTTCGCCGACTCGCC - CGC	1 bp increase at target E1; 1 bp deletion at target E3	Altered sequence from aa 8 to aa 112
OsDREB1G (dreb1g-1)	ACGCGGCACCCCGTGTATCA	1 bp increase at target G2	Reading frame shift after aa 54 and coding termination after aa 134
OsDREB1C (*dreb1ceg*)	TGCCTCAACTTCGCC-ACT	1 bp deletion at target C2	Reading frame shift after aa 91 and coding terminated after aa 146.
OsDREB1E (*dreb1ceg*)	TCGTCGGGGACGCCGTACGC	1 bp increase at target E2	Reading frame shift after aa 16
OsDREB1G (*dreb1ceg*)	TCGTCGGGGACGCCGTCCGC	1 bp increase at target G1	Reading frame shift after aa 17 and coding termination after aa 134

Nucleic acid mutations (colored in red) and the predicted impact on proteins in the dreb1c, dreb1e, dreb1g, and dreb1ceg mutants.

The *dreb1g-1* mutant had a survival rate of 29%, lower than the rate of 56% for NIP (**Figure 2C**). The *dreb1ceg* triple mutant also had lower survival rate than the wild type NIP (27% versus 57%; **Figure 2D**). Chilling tolerance levels among *dreb1* single mutants and the triple mutants were also compared. The survival rates of *dreb1e-1* and *dreb1g-1* were 50% and 59%, respectively, higher than the rate of 23% for *dreb1c-3* (**Figure 2E**). The survival rate of *dreb1c-3* was 63%, higher than the rate of 30% for *dreb1ceg* (**Figure 2F**). These results indicate that all three rice genes, *OsDREB1C*/*E*/*G*, positively regulate chilling tolerance in rice at seedling stage.

We further analyzed H_2O_2 accumulation by DAB staining as ROS accumulation is highly related to chilling tolerance. All *dreb1* mutants had more H_2O_2 accumulation in leaves than NIP after 6°C treatment for 24 h. The *dreb1c-3* mutant had more H_2O_2 accumulation than *dreb1e-1* and *dreb1g-1*, and *dreb1ceg* had the highest H_2O_2 accumulation than all single mutants (**Figure 2G**). Cell death caused by chilling was subsequently analyzed by trypan blue staining. All *dreb1* mutants had more cell death than NIP after chilling treatment. The *dreb1c-3* mutant had more cell death than *dreb1e-1* and *dreb1g-1*, and *dreb1ceg* had the highest cell death than all single mutants (**Figure 2H**). Therefore, H_2O_2 accumulation and cell death are each associated with chilling susceptibility in the rice *dreb1* mutants.

OsDREB1C/*E*/*G* Genes Promote Rice Growth Under Suboptimal Low Temperature

We analyzed the growth phenotypes of the *dreb1* mutants at non-chilling temperatures. Under normal growth temperature (28°C), no growth difference was observed between the *dreb1* mutants and NIP at seedling, tillering, or mature stages (**Supplementary Figure 3**). Under a suboptimal low temperature (16°C), none of the single *dreb1* mutants showed detectable growth difference compared with NIP at the seedling stage (**Figures 3A–C**). In contrast, the *dreb1ceg* triple mutant was significantly shorter than the WT NIP at 16°C, with a height of 7.9 cm compared to 11.5 cm of the WT (**Figure 3D**). These results indicate that the *OsDREB1C*/*E*/*G* genes have an overlapping function in promoting rice growth at suboptimal low temperature.

OsDREB1C/*E*/*G* Genes Do Not Participate in Cold Acclimation in Rice

We further determined whether *OsDREB1C*/*E*/*G* genes regulate cold acclimation. The WT NIP plants exhibited a higher survival rate at 6°C with a prior 12°C treatment of 1 day compared to no prior treatment (**Figure 4**), indicating that rice has cold acclimation for chilling tolerance. All the *dreb1* mutants also exhibited cold acclimation. In one set of experiment, NIP, *dreb1c-1,* and *dreb1c-2* had survival rates of 63%, 42%, and 53%, respectively, with prior cold treatment (cold acclimation, CA), higher than the rates of 37%, 0%, and 0% observed with no prior cold treatment (no acclimation, NA; **Figure 4A**). NIP and *dreb1e-1* had survival rates of 57% and 34%, respectively, with CA, higher than the rates of 28% and 9% with NA (**Figure 4B**). NIP and *dreb1g-1* had survival rates of 75% and 52%, respectively, with CA, higher than the rates of 54% and 25% with NA (**Figure 4C**). NIP and *dreb1ceg* had survival rates of 86% and 72%, respectively, with CA, higher than the rates of 53% and 29% with NA (**Figure 4D**). With CA, all mutants including *dreb1c-1*, *dreb1e-1*, *dreb1g-1*, and *dreb1ceg* plants showed more chilling

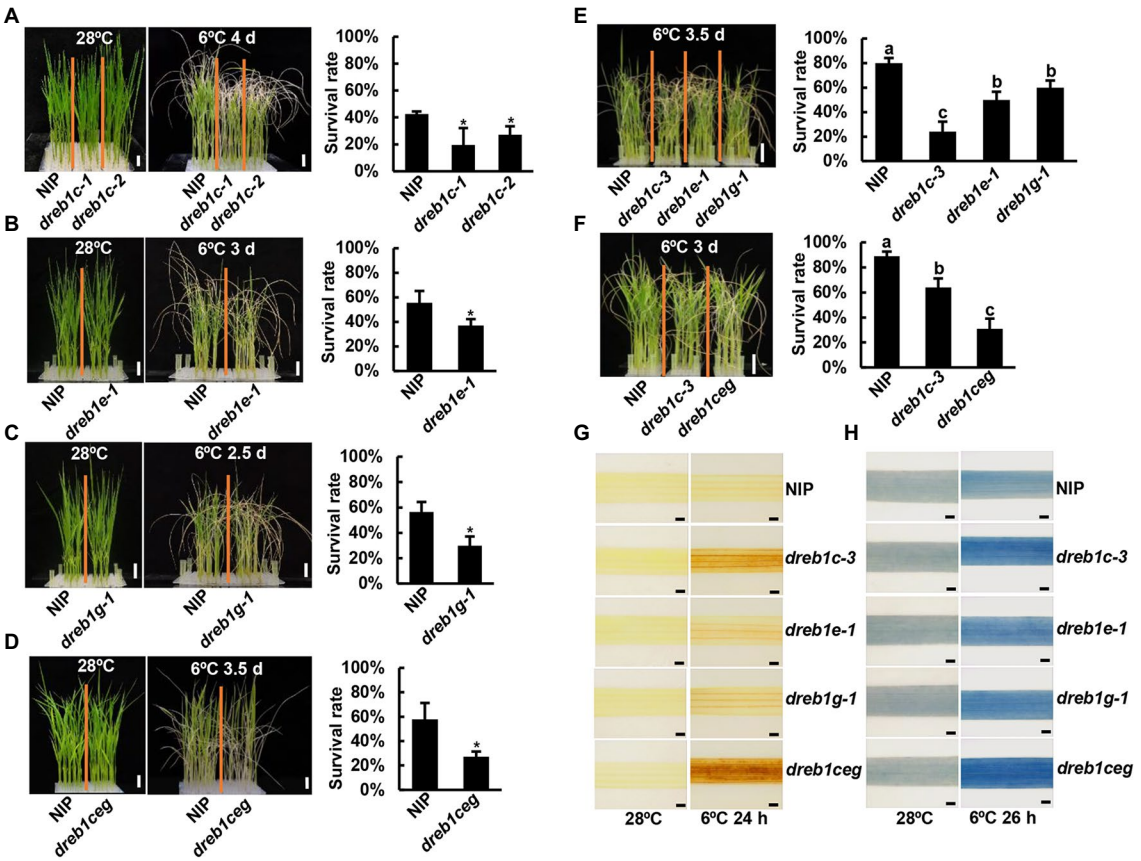

FIGURE 2 | The *dreb1* mutants are more susceptible to chilling at seedling stage. **(A–F)** Growth phenotypes and survival rates of *dreb1c* and WT **(A)**, *dreb1e* and WT **(B)**, *dreb1g* and WT **(C)**, *dreb1ceg* and WT **(D)**, *dreb1c*, *dreb1e*, *dreb1g,* and WT **(E)** as well as *dreb1c*, *dreb1ceg*, and WT **(F)** after chilling treatment and recovery. Seedlings were hydroponically grown at 28°C for 3 weeks, incubated at 6°C (for 2.5–4 days as indicated) and then transferred to normal conditions for 7 days for recovery. Left panels show plant morphology and bars = 2 cm. Right panels show mean values of survival rates from three biological replicates ±SD (standard deviation). Each biological replicate has more than 24 seedlings. Asterisks indicate significant differences compared to NIP (*$p < 0.05$, Student's *t*-test). Lowercase letters above the bars indicate significant differences among samples at $p < 0.05$, by SSR-Test. **(G,H)** DAB **(G)** and trypan blue **(H)** staining of the second leaves of *dreb1c-3*, *dreb1e-1*, *dreb1g-1*, *dreb1ceg*, and NIP plants before and after chilling treatment at 6°C for 24 h (DAB) or 26 h (trypan blue). Bars = 1 mm.

susceptibility compared with WT NIP (**Figures 4A–D**). The only exception was *dreb1c-2* which had a lower survival rate compared with NIP, but the difference was not significant (**Figure 4A**). This indicates that *OsDREB1C/E/G* genes do not affect cold acclimation in rice. We further analyzed the genotype by environment (G x E) interaction between each genotype pair (WT versus the *dreb1* mutants) and cold acclimation (NA versus CA) on survival rates. No significant interaction was found for any of the *OsDREB1* genotype and CA, indicating that the *dreb1* mutants have a cold acclimation-independent chilling susceptibility (**Figure 4**). These results indicate that *OsDREB1C/E/G* are not important for cold acclimation in rice, or they have overlapping functions with other *OsDREB1* genes in cold acclimation.

Transcriptome Analysis of the *dreb1* Mutants

To further understand how *OsDREB1* regulate chilling tolerance in rice, we carried out RNA-seq analysis on the WT, *dreb1c-2*, *dreb1g-1,* and *dreb1ceg* plants at 3-leaf-stage before and after a chilling treatment of 6°C. Principal component analysis (PCA) revealed that the WT and the *dreb1* mutants were similar to each other at any time points in the first two principal components, and chilling treatment was the most important factor in differentiating the samples (**Supplementary Figure 4**). This PCA showed that chilling treatment induced a similar global transcriptional change in WT and *dreb1* mutants. A total of 747 chilling up-regulated and 406 chilling down-regulated DEGs were shared among the WT, *dreb1c-2*, *dreb1g-1*, and *dreb1ceg* at 4 h of 6°C treatment, accounting for 69%, 56%, 56%, and 70% of up-regulated DEGs as well as 52%, 45%, 42%, and 52% of down-regulated DEGs in the WT, *dreb1c*, *dreb1g*, and *dreb1ceg*, respectively (**Supplementary Figure 5**). Moreover, 2,774 up-regulated and 2,148 down-regulated DEGs were shared among the WT and three *dreb1* mutants at 24 h, accounting for 77%, 84%, 71%, and 71% of up-regulated DEGs as well as 64%, 65%, 59%, and 57% of down-regulated DEGs in the WT, *dreb1c*, *dreb1g*, and *dreb1ceg*, respectively (**Supplementary Figure 5**). These genes represent the majority

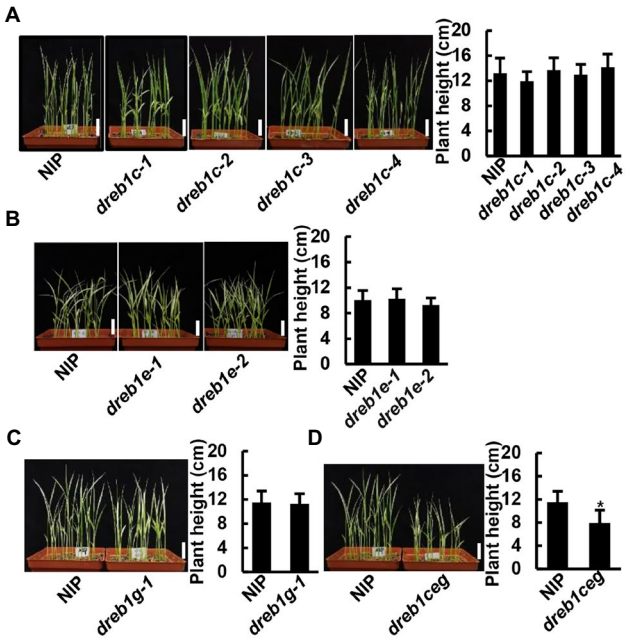

FIGURE 3 | The *dreb1ceg* mutant had reduced growth at seedling stage under suboptimal low temperature. Growth phenotypes and quantification of plant heights of *dreb1c* **(A)**, *dreb1e* **(B)**, *dreb1g* **(C)** and *dreb1ceg* **(D)** grown at 16°C for 32 days. Seeds were germinated at 28°C for 3 days, and then transferred to 16°C for 32 days. Shown is the mean value ±SD of 24 seedlings. Similar results were obtained from two additional biological repeats. Each replicate has 24 seedlings. Asterisks indicate significant differences compared to NIP (*$p < 0.05$, Student's *t*-test). Bars in plant images represent 2 cm.

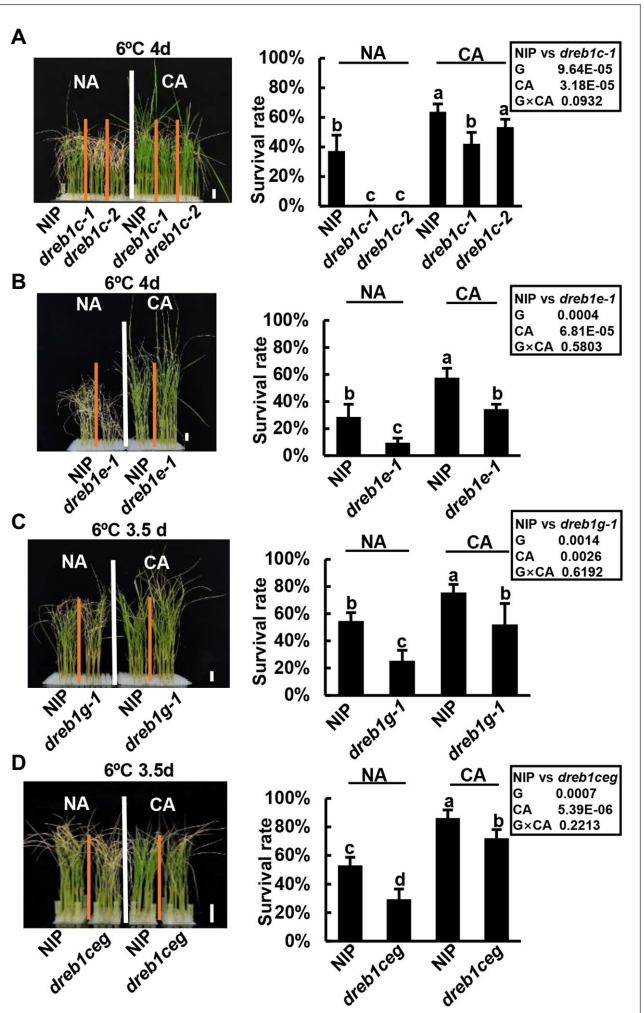

FIGURE 4 | *OsDREB1C/E/G* genes do not affect cold acclimation in rice. Growth phenotypes and survival rates of *dreb1c* **(A)**, *dreb1e* **(B)**, *dreb1g* **(C)**, and *dreb1ceg* **(D)** after chilling treatment and recovery growth with cold acclimation (CA) and without cold acclimation (NA). Seedlings were hydroponically grown at 28°C for 3 weeks till three-leaf stage, treated 1 day at 12°C (CA) or 1 day at 28°C (NA), before treated at 6°C for 3.5 or 4 days followed by a recovery growth at 28°C for 7 days. Left panels show plant morphology after recovery. Bars in plant images represent 2 cm. Right panels show survival rates expressed as mean ± SD from three biological replicates each with 24 seedlings. Lowercase letters above the bars indicate significant differences among samples at $p < 0.05$, by SSR test. Insert shows multiway-ANOVA analysis of genotype (NIP versus *dreb1* mutants) and cold acclimation (NA versus CA).

of the DEGs in WT or *dreb1* mutants, indicating that the *dreb1* mutations do not drastically alter chilling-induced or chilling-repressed DEGs. At 4 h, 895 up-regulated and 565 down-regulated DEGs were identified in both *dreb1g* and *dreb1ceg*, and 874 up-regulated and 506 down-regulated DEGs were identified in both *dreb1c* and *dreb1ceg* (**Supplementary Figure 5**). At 24 h, 3,419 up-regulated and 2,960 down-regulated DEGs were identified in both *dreb1g* and *dreb1ceg*, and 2,962 up-regulated and 2,570 down-regulated DEGs were identified in both *dreb1c* and *dreb1ceg* (**Supplementary Figure 5**). The *dreb1g* mutant shared more DEGs with *dreb1ceg* than *dreb1c*, suggesting that *OsDREB1G* has a larger functional contribution than *OsDREB1C* to gene expression changes under chilling.

Considering that *OsDREB1* genes are likely transcriptional activators, we examined genes that had a reduced expression in the *dreb1ceg* mutant under chilling because they are potentially regulatory target genes of *OsDREB1C/E/G*. These include chilling up-regulated DEGs in WT but not in the triple mutant as well as chilling down-regulated DEGs in *dreb1ceg* but not in WT at 4 h and/or 24 h. These DEGs were analyzed for enrichment of Gene Ontology (GO) terms defined by FDR < 0.05. No enriched GO terms were identified for the 428 up-regulated DEGs that appeared in WT but not in *dreb1ceg* (**Supplementary Figure 6A**). The 1,092 down-regulated DEGs that appeared in *dreb1ceg* but not in WT were enriched for the GO terms "plant-type hypersensitive response", "protein maturation", and "peptidyl-amino acid modification", with "plant-type hypersensitive response" having the highest fold enrichment of 24 (**Supplementary Figures 6B,C**). Under the GO term "plant-type hypersensitive response" were four genes that had a lower expression at 24 h in *dreb1ceg* compared to the WT (**Supplementary Figure 6D**). These four genes code for a transporter, a ubiquitin-protein ligase, and two MACPF domain-containing protein. One MACPF domain containing protein CAD1 is a negative regulator of the cell death in Arabidopsis, and its mutant exhibited a hypersensitive response-like cell

death (Morita-Yamamuro et al., 2005). The ubiquitin-protein ligase *OsNLA1* modulates phosphate accumulation in rice, and the loss of its function leads to lethality (Zhong et al., 2017). This suggests that the LOF of *OsDREB1C/E/G* leads to reduced expression of potential negative regulators of cell death, which might contribute to the increased cell death in the *dreb1ceg* mutant under chilling.

Chilling downregulated 3,568, 3,534, 4,006, and 4,006 genes in WT, *dreb1c*, *dreb1g*, and *dreb1ceg*, respectively (**Supplementary Figure 7A**). GO analysis on downregulated DEGs in WT and the three *dreb1* mutants was performed to identify differential GO terms between WT and the mutants. One GO term "hydrogen peroxide catabolic process" was present in all *dreb1* mutants but not in WT (**Supplementary Figure 7B**). Under this enriched GO term were 18 chilling downregulated DEGs (1 peroxiredoxin-2E-1, 17 peroxidase) which had a lower expression in both WT and triple mutant at 24 h compared to 0 h. Under this GO term were also 10 chilling downregulated genes (one peroxiredoxin-2E-1, nine peroxidase) that had a lower expression in *dreb1ceg* compared to the WT at 24 h (**Supplementary Figure 7C**). Therefore, more genes that are involved in ROS scavenging have a lower expression in the *dreb1* mutant compared to the wild type at 24 h. Plant peroxidase scavenges ROS and protects host cells from damages made by excessive ROS (Liu et al., 2021a). These results suggest that *OsDREB1C/E/G* may promote ROS scavenging under chilling.

Prediction of Potential Target Genes of OsDREB1C/E/G

As transcriptional factors, OsDREB1 proteins are expected to bind to specific cis-element motifs in the promoter region of their target genes and affect their gene expression. Because AtDREB1s mainly function as transcriptional activators (Song et al., 2021), we searched among genes with reduced expression in the *dreb1ceg* mutant compared to the WT for potential direct regulatory targets of OsDREB1C/E/G by the criterion of having DRE/CRT element(s) in their promoter region. We first examined the genes under GO terms uniquely enriched in the mutant but not the WT for chilling down regulated DEGs. Among the four genes under the GO term "plant-type hypersensitive response", one gene (LOC_Os07g07194, MACPF domain-containing protein) contained a DRE/CRT (G/ACCGAC) element in its promoter region. Among the 18 genes under the term "hydrogen peroxide catabolic process", three genes (LOC_Os03g22020, LOC_Os06g42000, and LOC_Os11g43980) contained one DRE/CRT element in their promoter regions. Therefore, these four genes are potential direct targets of OsDREB1C/E/G.

In addition, we examined genes with reduced expression in *dreb1ceg* compared to the WT at each of the three time points (0, 4, and 24 h) of chilling treatment. With 21, 38, and 145 downregulated DEGs (mutant versus WT) at 0, 4, and 24 h, respectively, the *dreb1ceg* mutant had a total of 168 downregulated DEGs compared to the WT with three time points combined (**Figure 5A**). These genes were defined as potential OsDREB1C/E/G target genes.

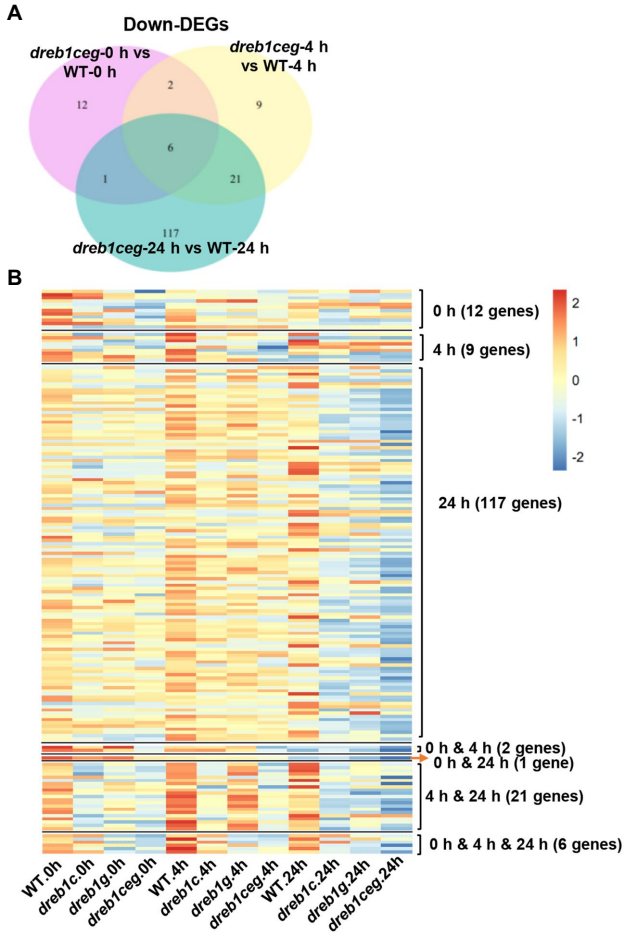

FIGURE 5 | Transcriptome analysis of the *dreb1* mutants. **(A)** Venn diagram indicates the number of downregulated DEGs (fold change ≥ 2, FDR ≤ 0.01) of *dreb1ceg* versus the WT at 0, 4, and 24 h after chilling treatment at 6°C. **(B)** Heat map of expression levels of 168 OsDREB1C/E/G-activated genes in the WT and the *dreb1* mutants at 0, 4, and 24 h after chilling treatment. The OsDREB1C/E/G-activated genes are defined by a reduced expression in *dreb1ceg* compared to the WT (fold reduction ≥ 2, FDR ≤ 0.01) at any of the three time points. Heat map is plotted using pheatmap package (1.0.12) and ggplot2 (3.3.3) by R language. Venn diagram is plotted using VennDiagram package (1.6.20) by R language.

The potential function of these 168 potential OsDREB1C/E/G target genes was subject to GO analysis. Among the 95 genes that had a GO annotation in the biological process, no enrichment of GO terms was identified for these genes. However, many of these genes have shared GO terms. Seventy genes had the GO term "metabolic process", including carbohydrate metabolism, protein metabolism, lipid metabolism, photosynthesis, organic acid metabolism, protein modification process and nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process (**Supplementary Figure 8**). A total of 33, 16, 37, 7, and 14 genes had the GO terms of "response to stimulus", "transport", "cellular process", "signal transduction", and "developmental process", respectively (**Supplementary Figure 8**). Sixteen and 26 genes had the GO terms of "response to abiotic stimulus",

and "response to stress", respectively. Two genes were under the term "cell death," which is a related term of "plant-type hypersensitive response".

We further examined the expression pattern of these 168 potential OsDREB1C/E/G target genes to determine whether their regulation by *OsDREB1C/E/G* genes is time-point specific. Heat map analysis revealed that these genes, although identified from a specific time point, mostly had a reduced expression at all three time points (**Figure 5B**). For the 12 DEGs of *dreb1ceg* that are unique at 0 h, almost all also had a lower expression in *dreb1c* (11 genes) and *dreb1g* (12 genes) than in WT. Ten of these genes had a lower expression in the *dreb1c*, *dreb1g*, and *dreb1ceg* mutants compared to WT at 4 h, and most of them (nine for *dreb1c*, seven for *dreb1g,* and five for *dreb1ceg*) had a lower expression at 24 h. A similar low specificity for time points was also observed for DEGs at 4 and 24 h where they had lower expression at the other two time points in the mutants compared to the WT. In sum, the majority of these genes had decreased expression in the *dreb1ceg* mutants at all three time points although the fold change is less than 2 and were therefore were not identified as DEGs at all time points.

Next, we search for the presence of sequence motif G/ACCGAC, the DRE/CRT element, in the promoter region of the potential OsDREB1C/E/G target genes. Because some genes do not have defined transcription initiation sites, we used the 1.5 kb fragment 5′ to the translation start codon of these genes as a proxy for promoter fragment. Among the 168 genes, 53 genes contained at least one DRE/CRT element in their promoter region, with 15 genes containing more than one element (**Supplementary Table 2**). Eight genes have only one element within the 100 bp region 5′ to the translation start codon (**Supplementary Table 2**), and whether those are in the 5' UTR region needs further investigation. Function of ten out of these 53 genes have been previously studied. Four of them, *OsHAN1*, *OsDREB1F*, *OsNAP,* and *OsGNA1*, have been shown to be involved in chilling tolerance or growth. The *OsHAN1* gene codes for an oxidase, and it LOF mutant had enhanced chilling tolerance (Mao et al., 2019). *OsDREB1F* is member of DREB1 family, and its overexpression has been shown to enhance chilling tolerance in rice (Wang et al., 2008). It is interesting that *OsDREB1F* is a potential target of the OsDREB1C/E/G proteins in the same family. *OsNAP* encodes a NAC transcription factor, and its overexpression also enhances chilling tolerance rice (Chen et al., 2014). *OsGNA1*, encoding a glucosamine-6-phosphate acetyltransferase, and its LOF mutant has a temperature-sensitive defect in root elongation (Jiang et al., 2005). In addition, *OsGRX2* encodes glutaredoxin that is involved in detoxification processes (Jeong et al., 2018) and could contribute to stress tolerance. Five other genes are involved in growth and developmental processes. *OsDof18* is involved in growth and ammonium transport regulation (Wu et al., 2017), *OsDjA7/8* is essential for chloroplast development (Zhu et al., 2015), *OsNIA2*, encoding a nitrate reductase, increases nitrogen uptake capacity (Sun et al., 2015), *OsMYB103L* is involved in GA-mediated regulation of secondary wall biosynthesis (Ye et al., 2015), and *OsSPW1* controls floral organ identity (Nagasawa et al., 2003). Thus, these potential target genes may contribute to chilling tolerance and chilling growth mediated by OsDREB1C/E/G.

OsDREB1C/E/G Genes Promote Tolerance to Heat, Salt and Drought at Seedling Stage

To determine whether these *OsDREB1C/E/G* genes also play a role in tolerance to other abiotic stresses in rice, the *dreb1* mutants were subjected to heat, salt, and drought treatments. The survival rates for the WT NIP and the mutant *dreb1* plants grown in the same pot were compared in the same pot to control variability of stress treatment.

For heat stress, 10-day-old seedlings grown on soil were treated at 48°C for 2 days, followed by a recovery growth. The NIP had a survival rate of 36%, while the *dreb1ceg* mutant plants all died (**Figures 6A,B**). These data indicate that *OsDREB1C/E/G* genes are positive regulators of tolerance to heat in rice.

For salt stress, 3-day-old seedlings were treated with 200 mM NaCl for 21 or 23 days. The survival rates for the four *dreb1c* mutants were 76%, 71%, 57%, and 61%, respectively, lower than the rate of 92% for NIP (**Supplementary Figure 9A**). No significant difference in salt tolerance was observed between *dreb1e* and NIP (**Supplementary Figure 9B**). The survival rate for *dreb1g-1* was 74%, lower than the rate of 96% for NIP (**Supplementary Figure 9C**). The survival rate for *dreb1ceg* was 58%, lower than the rate of 93% for NIP (**Figure 6C**). We further compared salt tolerance of the single and triple mutants. The survival rates for *dreb1c-1*, *dreb1g-1*, and *dreb1ceg* were 42%, 41%, and 35%, respectively, all lower than the rate of 64% for NIP (**Supplementary Figure 9D**). However, no significant difference was observed between single and triple mutants (**Supplementary Figure 9D**). These results indicate that the *OsDREB1C* and *OsDREB1G* genes, but not the *OsDREB1E* gene, are positive regulators of salt tolerance in rice.

For drought stress, seedlings at three-leaf stage were withheld of water for 10 days and then re-watered for 7 days. No significant difference in survival rate was observed among *dreb1c-2*, *dreb1e-1,* and NIP (**Supplementary Figures 10A,B**). The survival rate for the *dreb1g-1* mutant was 17%, lower than 53% for NIP (**Supplementary Figure 10C**). The survival rate for the *dreb1ceg* mutant was 29%, lower than 52% for NIP (**Figure 6D**). Compared drought tolerance between the *dreb1g* and *dreb1ceg* mutant, no significant difference was observed (**Supplementary Figure 10D**). These data indicate that the *OsDREB1G* gene but not *OsDREB1C* or *OsDREB1E* positively regulates drought tolerance in rice.

Accumulation of ROS and cell death was examined in the *dreb1ceg* mutant after each of the stress treatments: 24 h of 48°C, 13 days of 200 mM NaCl, and 6 days of water withheld. DAB staining revealed a higher H_2O_2 accumulation in leaves of the *dreb1ceg* mutant compared to that in NIP after all three stress treatments: heat, salt, and drought (**Figure 6E**). Similarly, leaves of the *dreb1ceg* mutant had more cell death than NIP after these stress treatments as indicated by trypan blue staining (**Figure 6F**). Therefore, *OsDREB1C/E/G* genes function to repress

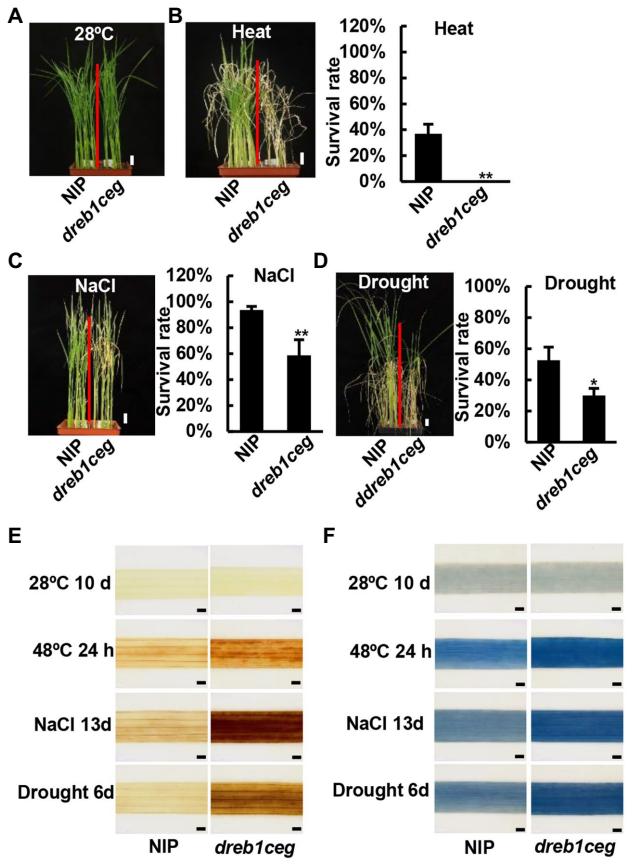

FIGURE 6 | The *dreb1ceg* mutant is more susceptible to heat, salt and drought stress at seedling stage. **(A)** Growth phenotype of NIP and *dreb1ceg* under control condition of 28°C. **(B)** Growth phenotype and survival rates of NIP and *dreb1ceg* after heat treatment (48°C for 2 days) and 7-day recovery growth at 28°C. **(C)** Growth phenotype and survival rates of NIP and *dreb1ceg* after salt treatment (200 mM NaCl) for 23 days. **(D)** Growth phenotype and survival rates of NIP and *dreb1ceg* after drought treatment for 10 days followed by a 7-day recovery. **(E,F)** DAB **(E)** and trypan blue **(F)** staining of the second leaves of NIP and *dreb1ceg* under control condition (28°C), heat (48°C for 24 h), salt (200 mM for 13 days), and drought stress (6 days of water withheld). Bars in plant images represent 2 cm, and bars in staining images represent 1 mm. Survival rates in **(B–D)** are expressed as mean ± SD from three biological replicates. * and ** indicate significant differences at $p < 0.05$, and $p < 0.01$, respectively, by Student's *t*-test.

ROS overaccumulation and cell death under heat, salt, and drought stresses.

DISCUSSION

DREB1 transcription factors are implicated in stress tolerance, especially cold tolerance. Three members in Arabidopsis, *AtDREB1A*, *AtDREB1B,* and *AtDREB1C*, are key regulators of cold acclimation for freezing tolerance, while *AtDREB1D* is a positive regulator of drought tolerance. The function of DREB1 proteins in other plant species are largely unexplored except for some overexpression studies in heterologous systems. Here, we report the function of three of the 10 *DREB1* genes in rice, *OsDREB1C/E/G*, through mutant characterizations. These three genes are found to be critical for basal chilling tolerance and low temperature growth in rice. They are also important for stress tolerance to heat, salt and drought. These indicate that *DREB1* genes have conserved function in stress tolerance in higher plants. These findings offer an explanation on previous observations that stress tolerance can be improved by overexpressing *DREB1* genes in heterologous systems.

The rice *OsDREB1C/E/G* genes, similar to the Arabidopsis *CBF1/2/3* genes, are positive regulators of cold tolerance. However, the Arabidopsis *AtDREB1A/B/C* genes are required for cold acclimation but not significantly for basal freezing tolerance (Jia et al., 2016). In contrast, the rice *OsDREB1C/E/G* genes are required for basal chilling tolerance but not for cold acclimation (**Figure 4**). This may suggest a species-specific function of *DREB1* genes in basal cold tolerance and acclimation-induced cold tolerance. It could also result from an expansion of the *DREB1* gene family in rice, and other *OsDREB1* genes may function in cold acclimation or have overlapping function with *OsDREB1C/E/G* in cold acclimation. Further investigation on other *OsDREB1* genes, especially *OsDREB1F*, could resolve this question. Alternatively, this differential function in cold acclimation may reflect a difference of cold tolerance between Arabidopsis and rice. Arabidopsis is chilling tolerant (surviving at 4°C), while rice is chilling susceptible (not surviving at 4°C). Cold acclimation for Arabidopsis happens at chilling temperatures such as 4°C to 10°C, while basal chilling tolerance in rice happens at chilling temperatures in the same range. Therefore, the *DREB1* genes in Arabidopsis and rice genes may function under the same low temperature range.

Strikingly, we found that *OsDREB1C/E/G* genes are required for stress tolerance to chilling, salt, drought, and heat stress in rice. Although salt and drought tolerances have been reported for some *OsDREB1* genes from overexpression studies, this is the first study reporting a regulation of heat tolerance by any *OsDREB1* genes in rice. Not all three genes have a detectable function in all stress responses. While all three genes promote chilling tolerance, *OsDREB1C* and *OsDREB1G* promote salt tolerance, while *OsDREB1G* promotes drought tolerance. More detailed analysis is needed to reveal if these three genes have overlapping functions in each of the stress tolerance. It will also be interesting to determine the function of other seven *OsDREB1* genes in stress tolerance.

Transcriptome analysis of the rice *dreb1* mutants suggests that chilling tolerance promoted by the *OsDREB1C/E/G* genes is at least partially through scavenging ROS and reducing cell death. Genes with reduced expression in the *dreb1ceg* mutant compared to the WT were enriched in the GO terms "plant-type hypersensitive response" and "hydrogen peroxide catabolic process" (**Supplementary Figures 5**, **6**). The *dreb1* single mutants, and more so the triple mutant, have more H_2O_2 accumulation and cell death than the WT (**Figures 2G,H**). In addition, the *dreb1ceg* mutant has more H_2O_2 accumulation and cell death than the WT after treatment of other abiotic stresses (**Figures 6E,F**). Together, these results indicate that the *OsDREB1C/E/G* genes may regulate various abiotic stress responses through shared mechanisms such as ROS scavenging

and cell death control. Early studies found a higher ROS accumulation and a more robust ROS response in the more chilling tolerant Japonica rice varieties compared to the less tolerant Indica rice varieties (Zhang et al., 2016). Here, a higher ROS in the *dreb1* mutants compared to the WT (all in Japonica NIP background) is associated with more chilling susceptibility, suggesting that a controlled ROS accumulation is critical for chilling tolerance. This supports the dual role of ROS as signaling molecules and damaging molecules.

This study identified potential target genes that could mediate the function of OsDREB1C/E/G in chilling tolerance or general stress tolerance. A total of 53 potential OsDREB1C/E/G target genes based on the features that they contain the DRE/CRT element in the promoter region and that they have a lower expression in the *dreb1ceg* mutant compared to the WT at 0, 4, or 24 h of chilling treatment. This is likely a stringent criterion because some of the direct target genes of Arabidopsis DREB1 proteins do not have a DRE/CRT element in their promoters (Song et al., 2021). Therefore, more genes could be direct regulatory targets of OsDREB1C/E/G proteins. Nevertheless, some of the 53 candidate target genes could mediate the function of *OsDREB1C/E/G* in chilling tolerance. Among the 10 genes with functional information, some have been shown to promote chilling tolerance or growth. These genes include *OsDREB1F*, *OsNAP,* and *OsGNA1*, all of which have been shown to be positive regulators of chilling tolerance in rice (Jiang et al., 2005; Wang et al., 2008; Chen et al., 2014). Their lower expression in the *dreb1ceg* mutant after chilling compared to the WT may lead to reduced chilling tolerance. The nitrate reductase coding gene *OsNIA2* could also mediate the function of *OsDREB1C/E/G* in chilling tolerance because nitrate reductase-regulated NO level is positively associated with freezing tolerance in Arabidopsis (Zhao et al., 2009). *OsHAN1* is a negative regulator of chilling tolerance (Mao et al., 2019). Its lower expression at 0 h but higher expression in *dreb1ceg* at 24 h may contribute to chilling susceptibility. In addition, OsDREB1C/E/G proteins could regulate expression of genes involved in ROS and cell death control. One potential target gene *OsGRX2* encodes glutaredoxin, and its lower expression in *dreb1ceg* could be one of the causes of high accumulation of ROS and cell death associated with more chilling susceptibility. Four other candidate target genes had GO terms of "plant-type hypersensitive response" and "hydrogen peroxide catabolic process," suggesting that regulation on ROS and cell death could be one major mechanism for DREB1C/E/G to promote chilling tolerance. As regulation of ROS and cell death is also key to tolerance to other abiotic stresses, this could also be one mechanism for these proteins to promote tolerance to heat, drought and salt.

CONCLUSION

We revealed an important role of three out of 10 rice *DREB1* genes (*OsDREB1C/E/G*) in stress tolerance to chilling, heat, salt, and drought in rice. In contrast to the Arabidopsis *CBF1/2/3* genes, these rice genes are critical for basal chilling tolerance but not cold acclimation. This study also suggests that ROS scavenging and cell death control might be shared mechanisms for these *DREB1* genes to promote tolerance to multiple stresses. This study enhances mechanistic understanding of general stress tolerance in plants and provides genetic materials for enhancing abiotic stress tolerance in crop 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 at: https://www.ncbi.nlm.nih.gov; PRJNA797855.

AUTHOR CONTRIBUTIONS

JH, BZ, HW, and SL designed the experiments and wrote the manuscript. HW and XG performed the experimental work. HW, YJ, and BW processed and analyzed RNA-Seq data. All authors contributed to the article and approved the submitted version.

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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.2022.851731/full#supplementary-material

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Low Temperature Stress Tolerance: An Insight Into the Omics Approaches for Legume Crops

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The change in climatic conditions is the major cause for decline in crop production worldwide. Decreasing crop productivity will further lead to increase in global hunger rate. Climate change results in environmental stress which has negative impact on plant-like deficiencies in growth, crop yield, permanent damage, or death if the plant remains in the stress conditions for prolonged period. Cold stress is one of the main abiotic stresses which have already affected the global crop production. Cold stress adversely affects the plants leading to necrosis, chlorosis, and growth retardation. Various physiological, biochemical, and molecular responses under cold stress have revealed that the cold resistance is more complex than perceived which involves multiple pathways. Like other crops, legumes are also affected by cold stress and therefore, an effective technique to mitigate cold-mediated damage is critical for long-term legume production. Earlier, crop improvement for any stress was challenging for scientific community as conventional breeding approaches like inter-specific or inter-generic hybridization had limited success in crop improvement. The availability of genome sequence, transcriptome, and proteome data provides in-depth sight into different complex mechanisms under cold stress. Identification of QTLs, genes, and proteins responsible for cold stress tolerance will help in improving or developing stress-tolerant legume crop. Cold stress can alter gene expression which further leads to increases in stress protecting metabolites to cope up the plant against the temperature fluctuations. Moreover, genetic engineering can help in development of new cold stress-tolerant varieties of legume crop. This paper provides a general insight into the "omics" approaches for cold stress in legume crops.

Keywords: cold stress, omics approaches, legumes, tolerance, transcriptional factors, proteomics

INTRODUCTION

In addition to the expanding population rate, increased biotic and abiotic pressures and limited agricultural land availability are significant restraints for farming and food production. Today, the most important concern for mankind is ensuring food security for an expanding population. It is well-known fact that the world population is increasing exponentially and believed to reach ten billion by 2050, needing a 60–100% increase in global food production (FAOSTAT, 2021). In one or other way, both these issues have adversely impacted agriculture sector. Crop yield and production of a particular crop is affected by numerous abiotic and biotic factors. Legumes are one of the essential staple foods after cereal crop. More than 1,300 legume species are grown worldwide out of which only 20 legume species are consumed by humans, because of its high protein, fiber, carbohydrate, and low-fat content (Câmara et al., 2013). Legumes like common bean, pea, and soybean are great source of proteins, micronutrients, dietary fibers, and carbohydrates for humans as well as animals (Zargar et al., 2017). Furthermore, legumes can serve as a great source of animal feed and can help in improvement of soil as it fixes atmospheric nitrogen in the soil (Meena and Lal, 2018). Since late last century, legume production has declined due to various environmental, socio-economic, and genetic factors. Various types of abiotic stresses *viz* drought, extreme temperature, salinity, and heavy metal stress have declined the legume production by approximately 50% worldwide (Jan et al., 2022). Cold stress alone has resulted in a decline of the overall productivity of legume crops, such as Chickpea and soybean by ~60% and mungbean by ~70% in recent years (Jan et al., 2022). Extreme temperature (either cold or heat) is one of the most important factors affecting overall developmental stages of plant and crop phenology that leads to loss in productivity and yield (Repo et al., 2008). Cold stress is differentiated on the basis of degree of temperature and is categorized into chilling (0–15°C) or freezing (<0°C) stress. Cold stress primarily affects the plasma membrane of the cell resulting in leakage of different types of ions, proteins, and lipids (Cheng et al., 2010). Physiological as well as cellular perturbations occur in a variety of legume species after encountering cold stress. Plants must retain cell behavior and activity under cold stress, especially the stability of the cell membrane and the structure of the protein with biological activity, in order to survive harsh settings. Earlier developing stress-tolerant varieties *via* conventional breeding was laborious and time-consuming process which was eased down by the new modern breeding approaches as well as by multi-omic techniques that simplify the improvement and development of new cold stress-tolerant varieties in various crops. Thus, to survive the cold stress, plants adapt different approaches which include gene expression, reprogramming, and alteration in different metabolic processes which help in modulation of various proteins of stress induction (Jan et al., 2019). In-depth knowledge of how plants react to cold stress could provide critical information and biological resources for improving crop cold-stress tolerance. Because of multigenicity, understanding abiotic stress tolerance, without breeding consideration had proven difficult which resulted in non-existence of traditional crop line that has cumulative tolerance for complex trait of drought and high salinity stress. Many plant biologists are of the opinion that to decipher the tolerance pathway mechanisms by understanding correlative evidence from different plant species rely on biophysical and biochemical mechanisms that regulate stress tolerance (Levitt, 1980). These discoveries offered guiding concepts for progressing from phenotype to protein and enzyme analysis, gene structure, and gene expression investigations, leading to the production as well as analysis of transgenic and mutant species. Here, in this review, we have summarized different available multi-omic techniques used for identifying the cold tolerance genes/protein and to study the role of different cold-responsive proteins in legume plants.

IMPACT OF COLD STRESS IN LEGUMES

For appropriate growth and development, each plant requires a specific set of temperatures. For example, a set of temperature ideal for one plant may be stressful for another. Plants native to warm settings have been seen to show damage signs when exposed to low temperatures. Plants like soybean (*Glycine max*), show indications of harm when exposed to temperatures below 10–15°C (Maqbool et al., 2010). The emergence of damage signs, on the other hand, is dependent on a plant's sensitivity to cold stress and differs from plant to plant. Plants are affected by stressful conditions at all stages of development. Unfavorable temperatures can have a direct impact on seed germination and emergence, as well as early survival and growth of seedlings (Maqbool et al., 2010). For example, cold stress during germination in chickpea increased susceptibility to soil-borne diseases, as well as poor crop establishment and even seedling death. Cold stress causes phenotypic changes in plants, such as reduced leaf expansion, wilting, and chlorosis (leaf yellowing), which can lead to necrosis (death of tissue). Plants reproductive development is also harmed by cold stress (Shafiq et al., 2012). Poor germination, stunted seedlings, yellowing of leaves, wilting, and diminished tillering are all symptoms of cold stress. The effects of cold stress on plants at the reproductive stage delay heading and result in pollen sterility, which is regarded to be one of the main causes of crop production loss (Suzuki et al., 2008). The most serious side effect of cold stress in plant has been plasma membrane damage. This has been documented as a result of dehydration caused by cold stress. Plasma membranes become more static at low temperatures, limiting fluidity. As a result, the membrane becomes more rigid and may lose its function (Sanghera et al., 2011; Hatfield and Prueger, 2015). Rupturing of plasma membrane and tonoplast resulted in solute leakage. Cold stress, when combined, causes a loss of membrane integrity, which leads to solute leakage (Maqbool et al., 2010). The research of phase transitions in mungbean cell membranes was hailed as a breakthrough in the field of legumes. Increased electrolyte leakage in five-day-old seedlings exposed to cold temperature (4°C) caused irreversible chilling harm. Furthermore, cold stress compromises the integrity of intracellular organelles, resulting in compartmentalization loss. Plants that are subjected to cold stress have a reduction in photosynthesis, protein synthesis, and general metabolic functions. Different physiological, morphological, biochemical and molecular

changes take place when a particular plant faces stress condition (**Figure 1**). Multi-omic approaches for understanding cold stress tolerance involves analysis of stress perception to downstream signaling and data processing for improving traits to develop legumes with better cold tolerance (**Figure 2**).

GENOMICS: A KEY TO UNDERSTAND QTLs/MTA ASSOCIATED WITH COLD STRESS TOLERANCE

Genomics deals with study of genetic variation as well as identification of novel QTLs/trait-specific genes *via* different genetic techniques to generate superior cultivars. The availability of genome sequence of a particular crop helps in identification of quantitative trait genes which ultimately leads to crop improvement. Till date, genome sequence of 15 legume crops are available in database. **Table 1** summarizes different legume species with respective genome sizes. The discovery of candidate genes associated with many quantitative traits particular to crops pave a way for identification and selection of superior lines that can further be used in marker-assisted selection (MAS), Genome Assisted Breeding (GAB), and other breeding programs in agricultural crops (Jaganathan et al., 2020). Development of mapping population (RILs, NILs, MAGIC) and diverse germplasm of cold stress tolerance in legume crops could be important for improving the legume breeding. PCR and hybridization-based molecular markers are utilized for identification of QTLs and candidate gene in various biotic and abiotic stress in crops. In case of cold tolerance in legumes, not much work has been done so far. Thus to understand the impact of cold stress on different parameters at genetic level there is need to identify QTLs/genes associated with these parameters.

QTLs CONTRIBUTING TO COLD STRESS TOLERANCE

Advances in phenomics and genome sequencing molecular breeding techniques are potential tools for crop development but cost-effectiveness of such approaches restricts their use. SSR markers were frequently employed in rice to identify QTLs in stress and gene flow programs (Kumar et al., 2014). Applying SNP markers in a layered aided linkage map, many climate QTLs in maize were discovered (Li et al., 2016). For legume crops, various markers have been used to study genetic diversity and other genetic level research for crop improvement program or for development of smart climate resilience crops. Various QTLs/SNPs identified in different legumes under cold stress are tabulated in **Table 2**. In chickpea, very less genomic resources are studied on cold stress (Jha et al., 2020). SSR markers have been combined with DArT and CAPS markers to identify a favorable QTL for abiotic stress and effectively introgressed, leading to the release of superior chickpea varieties (Varshney et al., 2014; Jaganathan et al., 2020). Other legumes, such as peanuts and pigeonpea, have also benefited from genomic-assisted breeding for abiotic and biotic stress tolerance (Varshney et al., 2019). While completing QTL research in the population derived from ICC 4958 × PI 489777, QTLs for chilling resistance in six different environmental conditions were identified

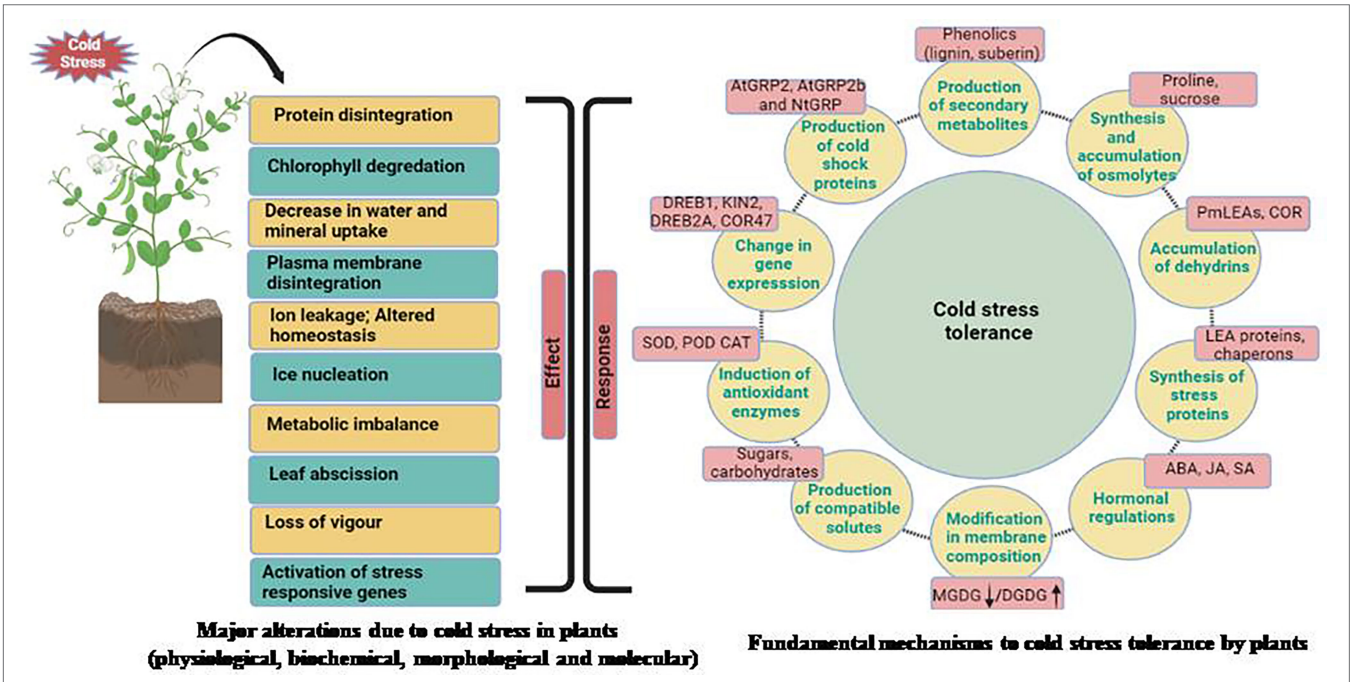

FIGURE 1 | Impact of cold stress at morphological, physiological, biochemical, and molecular levels and different mechanisms adapted by plants for combating cold stress.

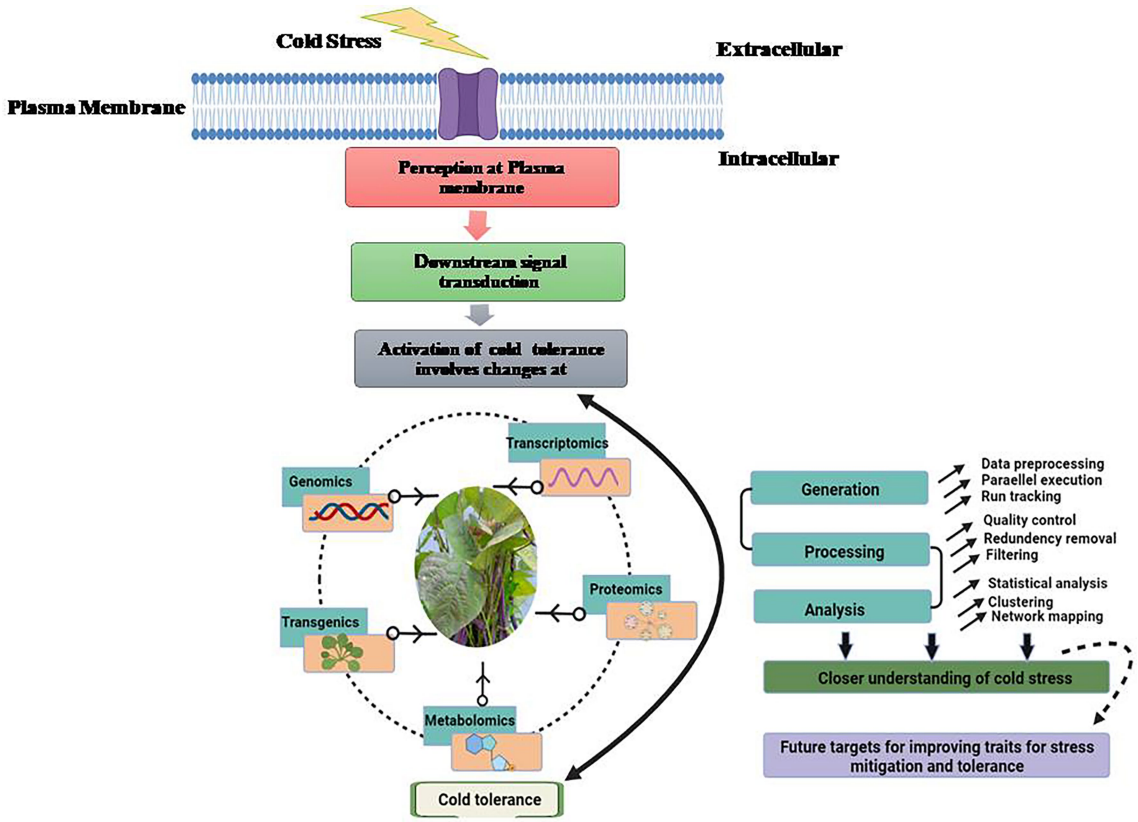

FIGURE 2 | Multi-omic approaches for cold stress tolerance involving analysis of cold stress perception to downstream signaling and data processing with analysis and future targets for improving traits for cold stress.

TABLE 1 | Available genome size of various legumes along with scientific name.

S. No.	Common name	Scientific name	Genome size (GB)	Reference
1	Senna	Alexandrian senna	1.76	Macas et al., 2015
2	Gum arabic	Senegalia senegal	1.47	Macas et al., 2015
3	Lupinus	Lupinus polyphyllus	0.90	Macas et al., 2015
4	Ground nut	Arachis hypogaea	1.74	Chang et al., 2018
5	Common bean	Phaseolus vulgaris	0.59	Macas et al., 2015
6	Mungbean	Vigna radiata	0.52	Macas et al., 2015
7	Soybean	Glycine max	1.10	Schmutz et al., 2010
8	Pigeon pea	Cajanus cajan	0.86	Singh et al., 2012
9	Sweet clover	Melilotus officinalis	1.10	Macas et al., 2015
10	Clover	Trifolium	0.96	Macas et al., 2015
11	Barrel medic	Medicago truncatula	0.47	Macas et al., 2015
12	Garden pea	Pisum sativum	4.36	Macas et al., 2015
13	Broad bean	Vicia faba	13.06	Macas et al., 2015
14	Black locust	Robinia pseudoacacia	0.64	Macas et al., 2015
15	Birdsfoottrefoil	Lotus japonicus	0.47	Sato et al., 2008

(Mugabe et al., 2019). Three QTLs were identified on LG1B, LG3, and LG8, as well as CT Ca-3.1 (on LG3) and CT Ca-8.1 (on LG8) of which one QTL was detected in single environment while other two QTLs were important for all six environments by using CIM QTL mapping (Mugabe et al., 2019). Regarding plant height and seed content, two QTLs were discovered (Ht Ca-4 was discovered on LG 4 with a LOD value of 6.5, and Ht Ca-8 on LG 8 with a LOD value of 6.5). Ht Ca-4 accounted for 20.21% of the variance, while Ht Ca-8 accounted for 19.97% of the polymorphism. However, two QTLs were discovered for seed ellipsoid volume, the first QTL, SEV Ca-1(A), with a LOD value of 7.4, was discovered on LG 1(A), representing 15.93% of phenotypic variance and SEV Ca-4, the second QTL, was discovered on LG 4 and has a LOD score of 11.8, explaining 29.41% of

TABLE 2 | Various QTLs/SNPs identified in different legumes under cold stress that can be used in MAS/MAB for cold tolerance in legumes.

S.No.	Legume	Bi parental cross/diverse germplasm	Approach	QTL/ SNP	Linked trait	Reference
1	Chickpea (*Cicer arietinum*)	ICC 4958 × PI 489777	QTL mapping	7 QTLs	Plant height and seed content	Mugabe et al., 2019
2	Pea (*Pisum sativum*)	China (JI1491) × Caméor	QTL mapping	161 QTLs	Internode length, branching type, hilum color, seed weight, harvest index and seed protein content.	Klein et al., 2014
		Champagne × Terese	QTL mapping	25 QTLs	Leakage of electrolytes,sugar concentration, osmotic pressure, and RuBisCO activity	Dumont et al., 2009
3	White clover (*Trifolium repens*)	192 diverse germplasm	GWAS	17 SNPs	Stolon dry weight, diameter, length, water soluble carbohydrate degradation rate, Petiole length, Leaf area, dry matter Annual dry matter	Inostroza et al., 2018
4	Pea (*Pisum sativum*)	365 diverse pea accessions	GWAS	62 SNPs	Frost damage (FD) loci	Beji et al., 2020
5	Sorghum [*Sorghum bicolor* (L.) Moench]	Chinese landrace 'Shan Qui Red,' (SQR, cold-tolerant) and SRN39 (cold-sensitive)	QTL mapping	2 QTLs	Germination	Knoll, 2008
		242 accesions from ICRISAT	GWAS	1 SNP	Low-temperature germination	Upadhyaya et al., 2016
6	*Medicago sativa* L.	3,010 x CW 1010 (F_1 mapping population)	QTL mapping	20 QTLs	Visual rating-based FT, percentage survival (PS), control regrowth ratio (RR), and control biomass ratio (BR)	Adhikari et al., 2021
7	Winter faba bean	Côte d'Or 1 (French landrace), and BeanPureLine 4,628 (BPL4628, Chinese inbred line)	QTL mapping	17 QTLs	11 frost tolerant and physiological traits.	Sallam et al., 2016
		Gottingen Winter Bean population (GWBP)	GWAS	25 SNPs	Three traits AUSPC (after hardening), LTAF, and LCAF	Sallam et al., 2016

variability (Mugabe et al., 2019). Enhancing cold tolerance for production of winter period pea variety is a challenging task. When breeding cold-season varieties, chilling tolerance, as well as seed output and purity, along with genetic factors of cold/frost tolerance, genetic connections with developmental and yield characteristics must be taken into account (Klein et al., 2014). Groups of hybrid inbred lines were examined in six distinct climatic situations using a newly discovered basis of cold stress. A genomic map with 679 microsatellite tools spread over seven clusters and a total length of 947.1 cM was created. For all assessed variables, 186 QTLs were discovered, accounting for 9 to 71% of variations. Specific frost tolerance may be developed to increase seed growth and yield in winter pea crop (Klein et al., 2014). Cold stress has affected sugar concentration, leakage of electrolytes, osmotic pressure, and activity of ribulose 1,5 bisphosphate in pea plant. RILs developed from the cross of Champagne (frost tolerant) and Terese (frost sensitive) were used for the identification of chromosomal regions linked to frost tolerance (QTLs) associated with sugar content under frost condition (Dumont et al., 2009). Effect of Raffinose metabolism and RuBisCO activity have an essential role in acclimatizing cold stress in pea (Dumont et al., 2009).

MARKER TRAIT ASSOCIATION (MTA): FOR COLD TOLERANCE

Marker–trait association (MTA) or Genome-wide association (GWAS) studies deal with association of desired trait and molecular markers across the genome of a particular crop. This technique has advantage over biparental mapping as it involves study of highly diverse germplasm for identification of associated markers or candidate genes. Since last 20 years, GWAS/MTA approach has been used in various plant species to understand the mechanism of acclimatization to different stresses and to look about the genetic basis of desired traits during stress induction. In case of legumes, GWAS/MTA studies related to cold stress tolerance are limited. GWAS was conducted in white clover for identification of cold tolerance-related traits. 53 loci associated with cold tolerance traits were identified from 192 diverse germplasm collected from Patagonia region of South America. 17 of the 53 SNP trait relationships regulated several traits and were stable across many sites, according to the study carried by Inostroza and co-workers. This work was the first one to establish a QTL for cold tolerance-related phenotypes, and it revealed its genetic basis as well as prospective genomic areas for future functional validation investigations (Inostroza et al., 2018). In pea plant, 62 SNPs significantly associated with frost tolerance at six different linkage groups were identified by GWAS in set of 365 pea accessions (Beji et al., 2020). A total of 50 candidate genes representing significant SNPs annotation linked to frost damage (FD) loci were detected by GWAS (Beji et al., 2020).

TRANSCRIPTOMICS: A LINK TO HAVE INSIGHT INTO GENES REGULATING COLD STRESS

Transcriptional response of the genome varies under multiple biotic and abiotic stresses in different tissues. Transcriptomic analysis initially relied on the identification of differentially

expressed genes. Gene microarray, expressed sequence tag-based method (EST, SAGE), and Next-Generation Sequencing (NGS) based RNA-sequencing technology were developed to obtain the transcriptome as well as identifying differentially expressed genes (Mortazavi et al., 2008; Nagalakshmi et al., 2008; Wilhelm et al., 2008). The rise of NGS technology and availability of genome sequence has made it possible to further study species or previously unidentified species in resequencing and de-novo sequencing aspects (Miao et al., 2015). The technology (RNA-Seq), is making important in-roads to genome annotation by allowing the sequencing of the entire transcriptome and provides expression profiles of either coding or non-coding RNAs (Miao et al., 2015). Many important genes/transcriptional factors (TFs) expressed during developmental, physiological, or pathological mechanisms, biotic and abiotic stresses were identified by these means. Gene expression analysis under cold stress in different plant model systems was reported by many researchers. Many transcriptomic studies in *A. thaliana* have been undertaken in order to decipher cold responses (Bahrman et al., 2019). Some high throughput transcriptomic analyses have been performed in some legumes (Fabaceae), such as *Vicia faba* (Lyu et al., 2021), *Pisum sativum* (Bahrman et al., 2019), *Arachis hypogaea* (Jiang et al., 2020), *Ammopiptanthus mongolicus* (Pang et al., 2013), *Glycine max* (Kidokoro et al., 2015), *Lotus japonicus* (Calzadilla et al., 2016), *Vigna unguiculata*, subspecies sesquipedalis (Tan et al., 2016), *Vigna subterranean* (Bonthala et al., 2016), *Medicago falcate* (Miao et al., 2015), *Medicago sativa* (Song et al., 2016), and *Cicer arietinum* (Sharma and Nayyar, 2014). Transcriptomic studies in these legume species under the exposure of cold stress have led to altered transcript of genes (Buti et al., 2018; Guan et al., 2019). These changes in the transcriptome are regulated by a large number of different TFs and various other key regulatory genes. TFs act as transcriptional inducers or repressors in the regulation of development, metabolic processes, biotic and abiotic stresses (Ng et al., 2018) including cold stress (Jiang et al., 2020). TF family members like APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF) (Nie et al., 2018), WRKY (Birkenbihl et al., 2017), and basic helix–loop–helix protein (bHLH) are differentially expressed and play a significant role in providing low temperature stress tolerance in plants (Sun et al., 2018). Identification of cold-responsive TFs helped to demonstrate cold resistance and tolerance in legume plants. For instance, one of the family member of AP2/ERF TF, such as C-repeat binding factors/dehydration responsive element binding factor1 proteins (CBFs/DREB1s), are proved to play an important role in plant cold tolerance (Jiang et al., 2020). CBFs in response to cold stress are rapidly over expressed in a short period of time (15 min) in plants (Kidokoro et al., 2015). CBFs are reported to directly regulate the expression levels of cold-responsive (COR) genes, thus can help in enhancing cold tolerance in many legume species (An et al., 2016; Zhang et al., 2018). Although, there is a need to recognize different cis elements present in CBF promoters and combine them with different TFs to regulate the expression of CBFs under cold stress in legume plants. ICE1 (inducer of CBF expression 1) is an MYC-like bHLH transcriptional activator, which induces the expression of CBF genes by binding to the CBFs' promoters under cold stress in plants (Kim et al., 2015). Detail of some important genes/transporters/transcription factors identified by various scientific groups on expression studies under cold stress in legumes are tabulated in **Table 3**.

PROTEOMICS: TO STUDY THE CHANGE IN PROTEOME CAUSED BY COLD STRESS

Proteomics deals with the in-depth study of proteome (gene product present in different tissue or organelle at distinct cell developmental stages), protein–protein interactions, protein accumulation, post-translational modifications (PTMs), and analysis of gene product (Chakraborty et al., 2015; Larance and Lamond, 2015; Minton, 2016). The post-transcriptional modifications like RNA splicing leading to different protein isoforms and the PTMs as processes resulting in multiple functional proteoforms encoded by a single gene make the proteome analysis complex indicating modulation of final protein product of a single gene in response to environmental cue (Kosová et al., 2021) Proteins can act as molecular chaperones, enzymes, and TFs that might play an important role in regulating stress signaling and protecting plants from stress (Rodziewicz et al., 2014). Numerous cold stress-related studies conducted on various plant species revealed that cold stress led to change in proteins response. Different protein families that play a vital role in cold stress response have been identified while studying differential proteomics in different plant species (Xiaoqin et al., 2009; Weckwerth et al., 2015). Several proteomic studies have been carried out in different legumes under various biotic and abiotic stresses (**Table 4**). However, a few studies related to cold stress have been conducted in legumes like soybean, chickpea, common bean, mung bean, grass pea, and forage legumes (Jan et al., 2022). Some of the cold tolerance proteomic studies in important legume crops are discussed under this section:

Cold stress has affected soybean production by 47–63% (Vara Prasad et al., 2000). Different gel-based and gel-free proteomics methods like 2DE, mass spectrometry, LC–MS, MALDI, iTRAQ have been utilized for analyzing protein change in tissue or organelle proteome under cold stress (Swigonska and Weidner, 2013). The proteome analysis of roots of soybean cv. Aldana under prolonged cold and osmotic stress revealed 59 differentially expressed proteins in control as well as treated samples (Swigonska and Weidner, 2013). LC/nanoESI-MS method was employed for identification of differentially expressed proteins that were involved in different functional categories like plant defense, translocation and storage, various metabolic pathways, secondary metabolism, protein synthesis, growth and development, cellular and electron transport (Swigonska and Weidner, 2013). It was also revealed that 24% of identified proteins were responsible for growth and development followed by 22% for translocation and storage (Swigonska and Weidner, 2013). Another study on seed

TABLE 3 | Genes/transcription factors identified having role in cold tolerance in different legumes by using RNA seq technology.

S.No.	Gene/transcription factors	Regulation	Tissue	Plant	Reference
1	WCOR413-15785	Down	Leaf	*Vicia faba*	Lyu et al., 2021
2	DHN2-12403	Up			
3	DHN2-14197	Up			
4	DHN2-14797	Down			
5	HVA22-15951	Up			
6	COR15-14478	Down			
7	DREBs	Up	Leaf	*Arachis hypogaea*	Wang et al., 2021
8	Phytochrome interacting factors	Up			
9	Raffinose synthases	Up			
10	Galactinol synthase	Up			
11	CBF1	Up	Shoot	*Lotus japonicus*	Calzadilla et al., 2016
12	CBF3	Up			
13	ICE1	Down			
14	RD29A	Up			
15	COR47	Up			
16	NAC	Up	Leaf	*Arachis hypogaea.*	Jiang et al., 2020
17	WRKY	Up			
18	ERF	Up			
19	MYB	Up			
20	C2H2	Up			
21	AP2-EREBP	Up	Leaf	*Pisum sativum*	Bahrman et al., 2019
22	bHLH	Up			
23	AP2-EREBP	Up			
24	Cold, circadian rhythm, RNA-binding 2, GRP7	Up			
25	Cation efflux system protein	Up	Leaf	*Cicer arietinum*	Sharma and Nayyar, 2014
26	L-ascorbate oxidase like protein	Up	Gynoecium		
27	Beta-galactosidase	Up			
28	Sucrose phosphorylase	Up	Anther		
29	Translation initiation factor EIF-2B epsilon	Up			
30	Peroxisomal ABC transporter	Up			
31	Wound responsive protein	Up	Root		
32	zinc finger family (including C2C2, C2H2, and C3H)	Up	Leaf	*Vigna unguiculata*	Tan et al., 2016
33	JUMONJI	Up			
33	Psudo ARR	Up			
34	PHD	Up			
35	ELF3	Up			
36	AtSR	Up			
37	Auxin responsive factor	Up			
38	WHIRLY2	Up	Leaflet	*Vigna subterranea*	Bonthala et al., 2016
39	GATA9	Down			
40	GRAS	Up	Leaf	*Medicago sativa*	Zhuo et al., 2018
41	HSF	Up			
42	FAR1	Up	Leaf	*Medicago falcata*	Miao et al., 2015
43	Orphans	Up			
44	MADS	Up			

proteomics of soybean under cold stress suggested that there is an upregulation in expression of the different proteins like glutathione S transferase, sucrose binding protein, and dehydrins while downregulation in expression of proteins responsible for cell division and growth, transcription, protein synthesis and storage metabolism (Cheng et al., 2010). Further analysis of leaf proteome of cv. Guliqing (cold-tolerant) and cv. Nannong 513 (cold-sensitive) soybean cultivar., 57 differentially abundant proteins were identified by MALDI-TOF/MS method (Tian et al., 2015). The identified proteins play an important role in various metabolic pathways and cellular processes, such as transcription and translation regulation, photosynthesis, protein folding and assembly, defense, storage proteins, signal transduction, metabolic pathways (carbohydrate, lipid, energy, amino acid, and nitrogen) (Tian et al., 2015). This study suggested that the presence of more proteins related to lipid and polyamine biosynthesis, photosynthesis, and metabolic recycling whereas less ROS production, low protein proteolysis, and energy depletion is responsible for cold stress tolerance in soybean cv. Guliqing under cold stress. However, these findings could provide an in-depth knowledge on cold stress responses and cold tolerance mechanisms in spring soybean (Tian et al., 2015).

Like other legume crops, pea and common bean crop yield is also affected by cold stress. Proteomics analysis of different pea cultivar revealed the role of various proteins, such as cyclophilin, caffeoyl-CoA O-methyltransferase, plastoglobulin, glycine decarboxylase-H subunits protein disulfide isomerase,

TABLE 4 | Proteomic studies related to cold stress conducted in various legumes.

S.No.	Plant	Source	Approach used	No. of protein identified	Functions	Reference
1	Soybean (*Glycine Max*)	Root	LC/nanoESI-MS	59	Plant defense, translocation and storage, various metabolic pathways, secondary metabolism, protein synthesis, growth and development, cellular and electron transport	Swigonska and Weidner, 2013
		Seed	2-DE and MALDI-TOF/MS	40	Cell defense, energy, protein synthesis, cell growth/division, storage, transcription and transport.	Cheng et al., 2010
		Leaf	2-DE and MALDI-TOF/TOF MS	57	Transcription and translation regulation; photosynthesis; protein folding and assembly; defense; storage proteins; signal transduction; metabolic pathways (carbohydrate, lipid, energy, amino acid, nitrogen)	Tian et al., 2015
2	Pea (*Pisum sativum*)	Leaf, stem, root	2DE, ElectroSpraylonisation (ESI)	68	Photosynthesis and defense, energy metabolism	Dumont et al., 2011
		Leaf (stromal and luminal chloroplasts proteome)	2DE, 2D-DIGE, MALDI TOF-TOF	620 spots in the stromal pea proteome and 400 spots in the lumenal pea proteome	Soluble sugar synthesis, antioxidant potential, regulation of mRNA transcription and translation	Grimaud et al., 2013
		Leaf(mitochondrial proteome)	2DE, Q-TOF MS	33	Photosynthetic and respiratory rates	Taylor et al., 2005
		Root	2DE, MALDI TOF/TOF	74	Ca^{2+} dependent signal transduction pathways associated proteins and cell division and expansion	Badowiec et al., 2013
3	Common bean (*Phaseolus vulgaris*)	Root	2DE, MALDI TOF/TOF	64	Protection against stress, cell cycle regulation and hormone synthesis, regulating metabolic pathways	Badowiec and Weidner, 2014
4	Chick pea (*Cicer arietinum*)	Seedling	MALDI-TOF-TOF and LC–MS/MS	70	cellular organelles (mitochondria, chloroplast), protein involved in defense system, metabolic pathways	Heidarvand and Maali-Amiri, 2013
5	Mung bean (*Vigna radiata*)	plumule or epicotyl	2DE, MALDI-quadrupole (Q)-TOF MS/MS and Western blotting	17	cell growth, wall formation, ATP production, the stress response, and methionine assimilation.	Huang et al., 2006
6	Red clover (*Trifolium pratense*)	Roots	2D-DIGE, MALDI TOF-TOF/MS	408	carbohydrate and energy metabolism, amino acid metabolism,signal transduction, molecular chaperones and protein folding, transcription and translation and metabolite transport	Bertrand et al., 2016

and disease resistance protein for providing tolerance in pea under cold stress (Sharma et al., 2005; Dumont et al., 2011). In a study on pea crop, proteomic technique 2DE followed by Electrosprayionization (ESI) was used to have better insight into cold tolerance in two contrasting pea lines, that is, Champagne (resistant) and Terese (sensitive). Three different tissues (leaf, stem, and root) were studied and 68 differentially expressed proteins were identified (Dumont et al., 2011). An increase in the protein expressions responsible for photosynthesis and defense are responsible for more adaptations to cold stress (Dumont et al., 2011). Frost resistance in pea crop might be due to re-orientation of proteins involved in energy metabolism (Dumont et al., 2011). Effect of cold stress on chloroplast of two contrasting pea lines (Champagne and Térèse) was assessed by 2D-DIGE technique suggested that chilling tolerance might be due to change in proteins associated with soluble sugar synthesis, antioxidant potential, regulation of mRNA transcription and translation in chloroplast (Grimaud et al., 2013). An increase in proteins related to carbohydrate, protein synthesis, and photosynthesis was observed in pea plant chloroplast under cold stress (Grimaud et al., 2013). Cold stress leads to alteration in photosynthetic and respiratory rates of pea leaves (Taylor et al., 2005). To understand the impact of environmental stresses like cold, drought, and herbicide on mitochondrial proteome, gel electrophoresis, and MS approach was utilized in pea crop (Taylor et al., 2005). Cold stress significantly affected the leaf metabolism and caused oxidation of mitochondrial proteins without leading to accumulation of lipid peroxidation products inside mitochondria (Taylor et al., 2005). This also suggested that the oxidative stress due to cold and drought conditions is not severe like oxidative stress caused by application of herbicides to pea plant (Taylor et al., 2005). Root growth and

development in pea and common bean plant is also affected by both long and short-term cold stress. Root proteome analysis of pea as well as common bean plant under long and short-term cold stress by using 2DE, MALDI-TOF approaches identified proteins involved in regulating metabolic pathways, protection against stress, cell cycle regulation, and hormone synthesis that might have effect on root growth and development in the early stages of plant life (Badowiec et al., 2013; Badowiec and Weidner, 2014). An increase in the Ca^{2+}-dependent signal transduction pathways associated proteins and cell division and expansion regulating proteins can be seen in roots of pea and common bean plant affected by cold stress (Badowiec et al., 2013; Badowiec and Weidner, 2014). Response of legume like pea and common bean to cold stress is directly proportional to the length and manner of cold stress exposure, which ultimately alter the root proteome of plants (Badowiec and Weidner, 2014).

In case of chickpea, cold stress declines the crop production by 45–61% (Sharma et al., 2016). MALDI-TOF and LC–MS/MS were used to analyze change in proteome of chickpea seedlings of cv. Sel 96Th11439 at early developmental stage under cold stress (Heidarvand and Maali-Amiri, 2013). Increase in expression of various proteins localized in different cellular organelles (mitochondria, chloroplast), protein involved in defense system, metabolic pathways were found in providing cold stress tolerance to chickpea under prolonged stress (Heidarvand and Maali-Amiri, 2013). A total of 70 differential expressed proteins were identified out of which 4 major proteins involved in providing cold stress tolerance in chickpea are globulin protein, FK506-binding protein (FKBP), $NADP^+$ binding Rossmann domain-containing protein, and a protein containing cyclophilin ABH-like region (Heidarvand and Maali-Amiri, 2013). Brassinosteroid (BR) synthetic gene plays an important role in cell elongation, plant growth, development, and responses to several stresses (cold, heat, and drought). The function of BR synthetic gene is suppressed by cold stress in various plants. Applying exogenous BR to epicotyls of mung bean plant helps in regulating epicotyl growth and to overcome inhibitory growth effect by cold stress (Huang et al., 2006).

METABOLOMICS: KNOWLEDGE OF METABOLITES REGULATING COLD STRESS RESPONSE

Metabolomics is the scientific study of the metabolites and processes that comprise cellular metabolism. In specific, it is concerned with distinguishing the characteristics of individual cells, and laying down their consequences (Daviss, 2005; Gong et al., 2013). Molecular metabolomics has made it possible to gain greater insights into multiple tolerance mechanisms at metabolic levels under abiotic stress (Bokszczanin and Fragkostefanakis, 2013). Physiological, biochemical, and molecular mechanisms are implemented by plants against a wide variety of stresses, such as biosynthesis of various metabolites, activation of antioxidant enzymes, transport of ions, accumulation of osmoprotectants, and release of different plant hormones (Pandey et al., 2015; Singhal et al., 2021). Furthermore, plants undergo different metabolic changes against stress conditions by synthesizing compatible solutes, antioxidants, and stress-responsive proteins, which act as anti-stress factors (Ramalingam et al., 2015). The ability of plants to withstand low temperature leads to an increase in freezing resistance and tolerance by induction of different mechanisms, such as cold acclimation (Dale and Fortin, 2014). When metabolite profiles are studied at large scale, it is possible to acquire observations of precursors, intermediates, and consequences of metabolic processes. Using it, one can identify and observe undiscovered mass spectral tags (MSTs) as well as establish metabolites, which play significant roles in metabolism, physiology, and stress tolerance (Zhou et al., 2019). Researchers used metabolomics in legumes to study reaction of *Medicago truncatula* to diverse stressors (Bell et al., 2001). In terms of metabolites, such as amino acids, carbohydrates, organic acids, and free fatty acids, both technologies provide complementary perspectives. However, majority of metabolomic platforms still require quality assurance and method validation. In order for metabolomics to improve in the future, we need databases, experimental standards, and data exchangeability between laboratories to be fulfilled (Sansone et al., 2007; Weckwerth, 2011). Even in the midst of the difficulty of conducting comprehensive metabolomic studies, it has been possible to conduct a variety of specific analyses to investigate the role of subsets of metabolites in various conditions, such as cold stress. The potential significance of genome research in crop development is further emphasized by preliminary findings from integrating metabolic techniques with transgenics, which suggest a possibility to boost intrinsic stress resistance in legume crops (He and Dixon, 2000; Wu and VanEtten, 2004). Some of the metabolites studied under cold stress in legumes are enlisted in **Table 5**. Further, the role of metabolomics during cold stress conditions in different legumes is discussed in following sections:

Alfalfa *(Medicago sativa L.)* is a high feed crop commonly grown in different countries where winters are bitterly cold which pose a barrier to regeneration and growth of alfalfa, resulting in significant economic deficits. Enhancing alfalfa's capacity to withstand the winter has emerged as a critical production concern. A study was performed by employing biochemical and metabolomic assays to compare the resistance against freezing in alfalfa grown under two different soil water regimes to understand how water shortage impacts tolerance against freezing stress. Water-stressed alfalfa had lower semi-lethal temperatures than the well-watered alfalfa. Under water scarce and low-temperature circumstances, the pool proportions of total soluble amino acids, sugars, and proline altered significantly. Metabolomic findings indicated 72 distinct subclasses of differential metabolites *viz* lipid and lipid-like molecules (e.g., glycerophospholipids, fatty acids, and unsaturated fatty acids) and peptides, amino acids, and analogues (e.g., proline betaine), were highly expressed in water scarce conditions. Certain flavonoids and carbohydrates, like raffinose and D-maltose, were elevated at low temperatures. Investigations of the Kyoto Encyclopedia of Genes and Genomes indicated the existence of 18 highly enriched pathways implicated in

TABLE 5 | Overview of metabolic studies related to cold stress effects on some legumes.

Legume	Tissue	Method	Metabolites studied	Reference(s)
Peanut (*Arachis hypogaea*)	Leaf	GC–MS	Amino acids, sugars, sugar alchols, fatty acids	Patel et al., 2022
Lotus (*Lotus japonicus*)	Shoot	Expression analysis, Illumina	Sucrose, terpenoids, anthrocyanin	Calzadilla et al., 2016
Chickpea (*Cicer arietinum*)	Leaf	GC–MS, qRTPCR	Oxalic acid, polyamines, putrescine, CAT, LOX, SOD	Kazemi Shahandashti et al., 2013; Amini et al., 2021
Common bean (*Phaseolus vulgaris*)	Seed	MS, Hybrid Orbitrap	Flavoinoids, phenol lipids, isoflavoinoids	Mecha et al., 2022
Alfalfa (*Medicago sativa*)	Leaf	Biochemical assays	Raffinose and D-maltose, total soluble amino acids, sugars, and proline	Zhuo et al., 2018; Shi et al., 2019
Soybean (*Glycine max*)	Seedling	GC–MS/ HPLC	Genistein, genistin, daidzein, succinate, pyruvate	Janas et al., 2002; Ramalingam et al., 2015; Yeshi et al., 2021

the production and metabolism of amino acids, carbohydrates, glycerophospholipids, and unsaturated fatty acids (Shi et al., 2019). Another study carried by Zhou and co-workers where alfalfa was subjected to cold stress at 4°C for different time lines. The high-throughput sequencing identified 50,809 annotated unigenes and 5,283 genes that were differentially expressed (DEGs). According to metabolic pathway enrichment analysis, DEGs were shown to be implicated in plant hormone signal transduction, glucose metabolism, photosynthetic signal transduction, and synthesis of amino acids. The peroxidase and catalase activity were also found to fluctuate in a manner that was in line with changes in the gene transcript profiles that were being studied (Zhuo et al., 2018).

Chickpea is the second most essential edible crop in the world (FAOSTAT, 2021). Chickpea is one of the important sources of easily digestible protein and minerals. As a key method to reduce yield loss during typical spring sowing in regions with hot and dry weather, early planting of chickpea in autumn or spring is preferred in Mediterranean regions. A key limiting factor for early chickpea seeding is, however cold stress sensitivity (Kazemi-Shahandashti et al., 2014). When a stress signal is sensed by receptors, a variety of components of the signaling pathway get involved in signal transduction, including cell membrane lipids, reactive oxygen species (ROS), calcium, hormones, protein kinases, and phosphatases (Kazemi-Shahandashti and Maali-Amiri, 2018). Plants adapt to cold stress by changing transcription factors, reprogramming their gene expression in response to stress, and altering their metabolism (Novák et al., 2016) as cold stress is perceived at the plasma membrane with activation of downstream signaling cascade (**Figure 3**). Chickpeas grow poorly at low temperatures since sporogenesis, pollen germination, pod abortions, and blooming is impaired (Kumar et al., 2010). The decline in enzyme activity and reaction rates associated with temperature is not only due to a decrease in enzyme activity and reaction rates (Levitt, 1980), but also because of active metabolic re-organization (Cook et al., 2004; Kaplan et al., 2004). When cold stress is applied to immature microspores, transcription of a particular gene OCINV4 (tapetum specific invertase gene) is inhibited thus preventing sugars from reaching the tapetum and pollen grains and allowing another type of sucrose to accumulate (Oliver et al., 2005). Affected by sterility of the pollen, the entire reproductive process is affected and polyamine levels fluctuate in response to cold stress and then fall over the course of prolonged exposure (Nayyar et al., 2005). This polyamine putrescine increases the weight of viable pods and seeds which in turn enhances tolerance of plants to cold stress. Defensive and mitochondrial responses among the sensitive (ILC533) and tolerant (Sel96Th11439) genotypes of chickpea which are responsible for cold stress tolerance were identified in a study (Karami-Moalem et al., 2018). Cold stress responses are translated into different physiological changes as a consequence of direct or indirect gene expression and are regulated by factors, such as DNA methylation. During cold stress tolerance, there is significant increase in the antioxidant enzymatic activities, such as catalases (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX), along with the increase in non-enzymatic molecules, such as proline and ascorbate, to eliminate the effects of oxidative stress in chickpea thus, validating the active scavenging system against ROS (Karami-Moalem et al., 2018). Chickpea tolerant cells in response to cold stress modify their genome, such as the number of bands, was found to be decreased in tolerant genotypes. During Cold stress, the methylation levels are found higher in comparison to de-methylation (27.92 *vs* 22.09%) in susceptible ones and (29.05 vs. 19.79%) in tolerant genotypes (Rakei et al., 2016). The approaches for cold stress analysis, such as physiological and biochemical analysis, established the involvement of lipoxygenases (LOX), antioxidants, fatty acid content as cell responses during cold stress. The alterations in membrane fatty acid compositions, defense machinery (such as anti-oxidative enzymes), and damage indicators, including the malondialdehyde (MDA) and electrolyte leakage index (ELI) in chickpeas were found throughout the cold acclimation (CA), cold stress and recovery (R) phases. According to these findings, the proportion of unsaturated to saturated fatty acids increased, which is a mark of cold resistance, notably after the CA phase. Antioxidant enzymes were influential throughout the CA and R stages, but cold stress decreased their activity, suggesting that other metabolites or enzymes are involved in plant cold tolerance development. The expression pattern of certain enzymes like CAT, LOX, and SOD, was investigated during experimental treatments employing quantitative real-time PCR. The LOX activity exhibited a bidirectional association with membrane damage index in CA and an exciting link

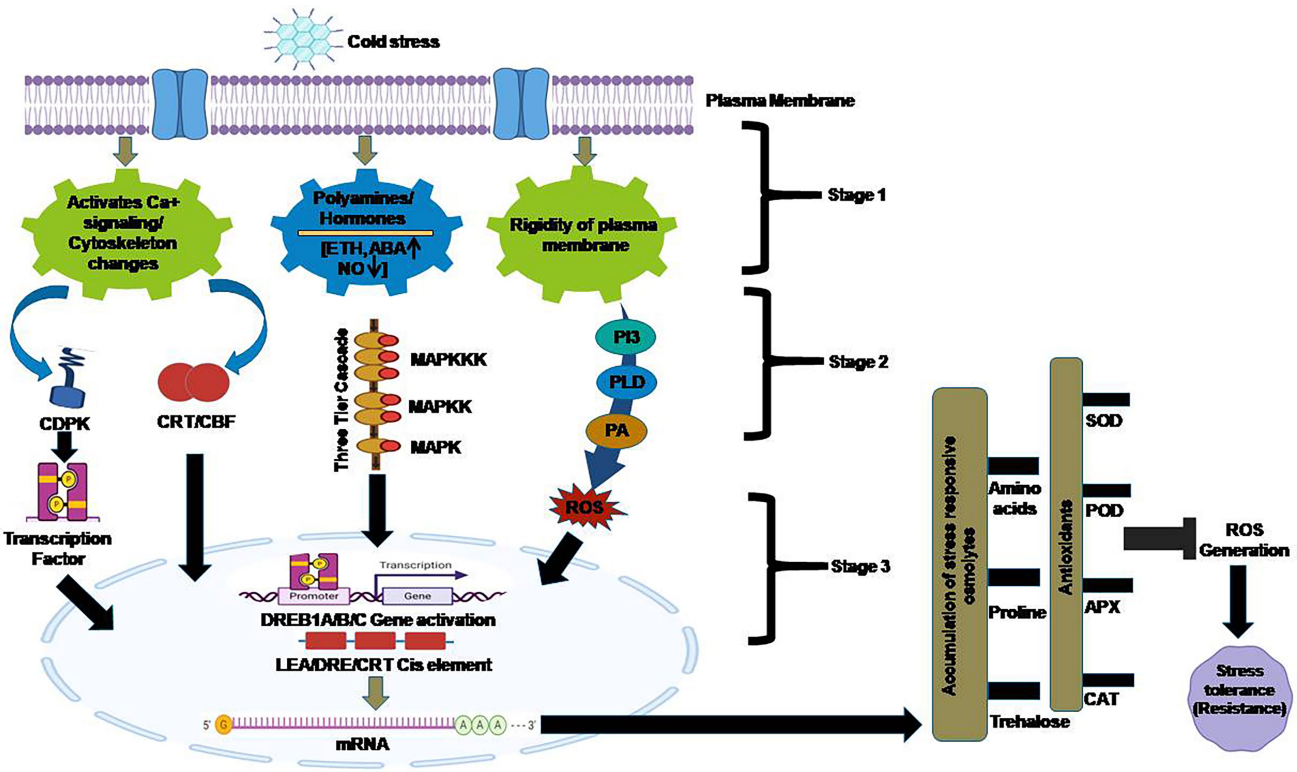

FIGURE 3 | Cold stress-induced signal transduction and response: Cold stress is perceived at the plasma membrane with activation of downstream signaling cascade *viz* activation of calcium signaling, polyamines and hormone signaling and rigidity of plasma membrane which in turn activates multiple cytoplasmic proteins and expression of different genes which ultimately leads to cold tolerance. CDPK: Calcium-dependent Kinases; CBF: C repeat binding factors; MAPK: Mitogen-activated protein kinase; ROS: Reactive oxygen species.

with double bond index in cold stress, implying a role in secondary metabolite signaling pathways, such as jasmonic acid signaling (Kazemi Shahandashti et al., 2013).

Soybean, an important food crop worldwide is a valuable source of proteins consumed globally, (Islam et al., 2019). As an additional method of improving soil and its fertility, soybeans utilize natural nitrogen fixation in the roots. However, a number of abiotic factors, such as cold, heat, drought, and high salt concentration hinder soybean growth and productivity (Hasanuzzaman et al., 2016). There are a number of defense mechanisms activated by complex transcriptional regulatory networks at the physiological and biochemical level, including activation of chaperones, detoxifying enzymes, transporters, and enzymes for metabolite production (Pandey et al., 2021). A study was carried out using 5 concentrations of 5-aminolevulinic acid (ALA) added to the Hoagland solution for 12 h for the purpose of increasing soybean plant cold stress resistance. The plants treated with ALA were exposed to cold stress at 4°C for 48 h after treatment. On average, ALA at low concentrations (5–10 uM) increased relative water content (RWC) and chlorophyll content compared to non-ALA-treated plants. The amount of reactive species generated by thiobarbituric acid (TBARS) in plants pre-treated with ALA (15–40 uM) increased dose dependently with about 117% in plants treated with 40 uM. Furthermore, 5 uM ALA pre-treatment resulted in maximum cold tolerance (Balestrasse et al., 2010). This suggests that heme oxygenase (HO-1) is an antioxidant as well as a rate-limiting enzyme in the heme catabolism. By increasing the activity of heme proteins, such as CAT and promoting heme catabolism, ALA, an endogenous plant growth regulator, is an effective treatment for combating cold stress in soybean. As a consequence, the highly antioxidant substance biliverdin is produced, along with carbon monoxide without affecting growth (Balestrasse et al., 2010). A study carried out by Xu and co-workers on different temperature conditions of soybean was carried out where a controlled environment with low (22/18°C), optimal (28/24°C), and high (36/32°C) temperatures was used to assess the cumulative influence of temperature and CO_2 on leaf metabolites, photosynthetic activities, and growth. Through the range of CO_2 levels studied, the photosynthesis rate, mesophyll, and stomatal conductance, photosystem II quantum yield, and electron transport increased. The photosynthetic restriction was temperature-dependent and affected by photo-biochemical and metabolic processes. Under well-watered and nutrient-sufficient circumstances, CO_2 tended to fully or partially compensate for the respective low temperature and high temperature stressors (Xu et al., 2016).

In semi-arid tropical and subtropical regions, peanut forms an important grain legume that is farmed for its edible oil content and proteins (Zhang et al., 2019). Temperature is an

important factor in the development and growth of this crop, as it demands a high range of temperature throughout the entire process (Wang et al., 2003). It is known that peanuts germinate at 12–15°C, and reach their optimum growth at 28°C, but these plants suffer drastic metabolic disturbances below 12°C, a kind of stress condition (Bell et al., 1994). All of these factors influence chilling tolerance that includes their structure, composition, and metabolism. One of the primary ways in which plants adapt to temperature changes is through the distribution ratio of lipids on their membranes and un-saturation of the glycerol lipid group. Peanut plasma membranes are less elastic, and membrane lipids are reduced to a gel-like structure under cold stress (Murata et al., 1982), which results in poor protoplast flow and increased membrane permeability, causing electrolyte leakage and an imbalance of intracellular ions (Huang et al., 2015). A group of cold-responsive metabolites was discovered during the metabolomic study of two peanut cultivars exposed to chilling stress, including various sugars and polyamines. Under cold stress, these compounds accumulated more in the cold-resistant variety (SLH) than in the cold-susceptible variety (ZH12), demonstrating their role in protecting peanuts against chilling damage (Wang et al., 2021). Cold-tolerant plants exhibit greater growth amplitude of these osmotic regulatory molecules than cold-sensitive plants due to their increased levels of proline, soluble carbohydrates, and soluble proteins in the cytosol. During extremely cold temperatures, these compounds are drastically reduced in the cytosol of the plant (Bai et al., 2018; Kazemi-Shahandashti and Maali-Amiri, 2018). Considering that most metabolic pathways are connected in intricate systems, modifying one metabolic pathway may have adverse consequences on another, which may lead to undermining the manipulated crop. A large-scale metabolic experiment is required in order to observe the metabolic networks that have a significant role in the development and growth of legumes.

TRANS-GENOMICS: USE OF GENE-BASED APPROACH TO UNDERSTAND COLD TOLERANCE

Traditionally, crop development for cold tolerance by a standard breeding approach has been limited due to stress-tolerant trait complexity, absence of precise phenotyping methods, selection criteria, and little genetic diversity in the respective breeding populations (Sanghera et al., 2011). Advances in recombinant DNA technology and development of effective genetic engineering methods can aid in the development of precise methodology and strategies for producing cold-tolerant cultivars in a variety of agricultural species (Wani et al., 2016). Transgenic technology or simply transgenomics by the use of gene-based approaches may prove valuable for gaining deep insight into stress tolerance mechanisms and, as a complementary means, for genetically improving crops, which in turn can help to alleviate some of the major pitfalls to crop improvement (Sharma and Ortiz, 2000). A deep study has been carried out for identifying different genes which play an important role in enhancing cold or freezing tolerance in plants. The central principle for transformation experiments revolves around enzymes important for biosynthesis of various osmoprotectants, detoxifying enzymes, late embryogenesis abundant (LEA) proteins and those which encode for membrane lipids (Eapen, 2008). Many aspects of cold adaptation are under transcriptional control which helps in choosing regulatory factors for introgression of these genes that are believed to be responsible during putative tolerance and stress response, thus paving a way for developing varieties with improved cold tolerance. There have been reports of genetic transformation of all important legume crops, including *Vigna species, Chrysanthemum arietinum, Chrysanthemum cajan, Phaseolus* species, *Lupinus* species, *Vicia* species, and soybeans. With the exception of soybean, transgenic grain legumes, unlike their cereal counterparts, are yet to migrate out of laboratories to major farm fields, regardless of their importance to tropical agriculture (Eapen, 2008). Transgenic alfalfa's increased tolerance to aluminum toxicity (Tesfaye et al., 2001) and soybean's increased tolerance to cyanamide toxicity (Zhang et al., 2005) show this method can be applicable to legumes also. At International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), training on efficient transformation techniques has been provided for legume crops, such as peanuts, pigeon peas, and chick peas. Transgenes are being used to overcome abiotic stressors in various leguminous crops and thus different transgenic methods are developed to improve stress resistance, including those that encode enzymes responsible for biosynthesis of osmoprotectants (Sharma and Lavanya, 2002) or that modify membrane lipids (Sinha et al., 2015), or LEA proteins (Wang et al., 2019), and detoxification enzymes (Kazemi-Shahandashti and Maali-Amiri, 2018). Both Agrobacterium-mediated transformations (Joshi et al., 2018) and particle bombardment methods were utilized in transformation experiments in soybean (Pareddy et al., 2020). Commercially, it is the only legume-based transgenic crop which is currently been grown. Several great reviews on gene technology uses in soybean have been published from time to time to understand the stress biology of the crop (Debbarma et al., 2019). The transformation experiment for abiotic stress tolerance on transgenic soybean where harmful cyanamide was converted to urea, from the soil fungus *Myrothecium* using cyanamide hydratase (Cah) coding sequence has been reported in recent. Cah expression reduced cyanamide levels in soybean leaf callus and embryogenic cultures as evidenced by cyanamide resistance (Zhang et al., 2005). Another study found that transgenic soybeans with constitutive expression of the nectarin 1 (ntr1) gene from *Brassica campestris* accumulated more methyl jasmonate (MeJA). The NTR1 gene codes a plant regulator called jasmonic acid carboxyl methyl transferase that regulates the expression of various plant defense genes against diverse challenges, such as wounding, dehydration, and infections. The transgenic soybean plants' greater levels of MeJA provided dehydration tolerance throughout seed germination and seedling growth, as measured by the percentage of fresh weight of seedlings.

Significant efforts have been made in soybean to improve and optimize the plant's cold tolerance. Soybean plants reprogram

the expression of many cold-responsive genes against cold stress. Nevertheless, the inherent mechanism underpinning soybean's cold-stress tolerance is unknown. Research revealed a soybean homolog of the AtTCF1 gene (identified as GmTCF1a), which is involved in tolerance of plant to low temperatures. Cold stress induces GmTCF1a robustly and precisely. Remarkably, higher expression of GmTCF1a ectopically in *Arabidopsis* significantly boosted plant survival. GmTCF1a was mainly induced in response to cold stressors, and ectopic expression of GmTCF1a increased cold resistance and levels of COR15a. These findings suggest that GmTCF1a positively influences cold resistance in soybean, which may bring unique insights into cold-tolerant genetic improvements in crops (Dong et al., 2021). A DREB orthologue, GmDREB3, was identified from soybean employing the RACE technique. Northern blot study revealed that GmDREB3 expression was elevated in soybean seedlings post 0.5 h of cold stress but was not found after 3 h. However, it was not generated by excessive salt or drought stress, nor was it triggered by abscisic acid (ABA) therapy. This reaction was comparable to members of the A-1 subgroup but distinct from other members of the A-5 subgroup, indicating that GmDREB3 gene was engaged in a cold stress-responsive signaling pathway that is independent of ABA (Chen et al., 2009).

MtCTLK1, a cold-tolerant LRR-RLK gene, was discovered in *Medicago truncatula* using transgenic lines overexpressing MtCTLK1 (MtCTLK1-OE). MtCTLK1-OE lines had greater cold resistance than the wild type, but MtCTLK1 lines exhibited lower cold resistance. The decreased tolerance against cold in MtCTLK1 might be compensated for the transgenic expression of MtCTLK1 or its *Medicago falcata* homolog MfCTLK1. Proline buildup, antioxidant enzyme activity, and transcript levels of related genes were enhanced in response to cold, with greater levels in MtCTLK1-OE lines or reduced levels in mtctlk1 lines relative to wild type (Geng et al., 2021). Another investigation found MfAIR12 in *Medicago falcata,* legume germplasm with exceptional cold resistance. MfAIR12 and its *Medicago truncatula* homolog MtAIR12 transcript levels were triggered at low temperature. MfAIR12 overexpression resulted in water buildup in the apoplast with enhancement in cold resistance, which was prevented by water scavengers, demonstrating that the improved tolerance to cold was reliant on the accumulated water. Additionally, a decrease in cold tolerance in *Arabidopsis* mutant air12 was detected, which was recovered by overexpression of MfAIR12. In comparison to wild type, MfAIR12 transgenic lines had elevated levels of ascorbate redox state, and ascorbic acid and transcripts of the CBF transcription factors and their downstream cold-responsive genes, but decreased levels in air12 mutant lines (Wang et al., 2021). Also, MfERF1, a cold-responsive ERF, was identified from *Medicago falcata,* with exceptional cold tolerance. MfERF1 overexpression boosted the tolerance of transgenic tobacco plants to chilling and freezing, but downregulation of the ortholog of MfERF1 in *Medicago truncatula* led to decreased freezing resistance in RNAi plants (Zhuo et al., 2018). Another cold-responsive PIP2 was derived from *Medicago falcate,* with remarkable cold resistance, and the tolerance of transgenic tobacco plants overexpressing MfPIP2-7 to different stressors was evaluated, including nitrate reduction, chilling, and freezing. Four to twelve hours of cold therapy and two hours of ABA treatment activated the MfPIP2-7 transcript. Pretreatment with ABA inhibitor production suppressed cold-induced transcription of MfPIP2-7 in *M falcata,* demonstrating that ABA was engaged in cold-induced transcription of MfPIP2-7 whose overexpression increased the tolerance of transgenic tobacco plants to chilling, freezing, and NO_3 deficiency compared to the wild type. Additionally, it was found that MfPIP2-7 facilitates water transport in yeast (Zhuo et al., 2016).

Chickpea photosynthetic machinery, for example, can survive oxidative stress by expressing a prokaryotic homolog of choline oxidase (codA) which was proved by an experiment on transgenic chickpea chloroplasts to determine the survival rate during photo inhibitory damage, and it was found that wild-type plants exposed to high light intensity lost a greater level of PS II activity than transgenic chickpea chloroplasts under the same conditions (Sharmila et al., 2009). Researchers found H_2O_2 produced during glycine betaine synthesis in the chloroplasts of transgenic chickpea plants is responsible for the development of a stronger antioxidant system (Sharmila et al., 2009). It is important to mention that the P5CSF129A gene, encoding a mutant D1-pyrroline-5-carboxylate synthetase (P5CS) promoting proline overproduction in chickpeas, was introduced in field trials at ICRISAT (Bhatnagar-Mathur et al., 2009). Reduction in MDA levels, as measured by the formation of proline under drought and cold stress, was associated with enhanced levels of proline synthesis and formation in the leaves (Ejaz et al., 2020). During the progressive drying phase, only very few occurrences showed a noticeable increase in biomass, suggesting that over-expression of proline had no useful effect on accumulation of biomass. Over-expression of the P5CSF129A gene only slightly increased transpiration efficiency (TE), indicating that proline was not able to affect the components of yield architecture which are important in reducing the detrimental effects of stress in chickpea (Bhatnagar-Mathur et al., 2009). The effects of cold stress on growth indices and antioxidant responses in seedlings subjected to cold (4°C) stress for 5 days were studied by Oktem and co-workers. They found that the length and fresh weight of shoots fell dramatically, in contrast to an increase in both growth parameters for roots under the same conditions. Under cold stress, the increase in proline levels in shoots and roots was more pronounced (Öktem et al., 2008). These results, like those in other crops, were in line with prior findings. Genes that affect a single protein tend to be less effective when it comes to handling cold stress. Targeting transcription factors that govern the expression of many genes linked with abiotic stress is one way to address the multigenicity of the agricultural plant response to stress. Researchers previously showed that transcription factors that regulate the coordinated expression of multiple stress-related genes in heterologous transgenic plants under laboratory conditions improved stress tolerance in these plants. Hence, a significant number of transgenic chickpeas containing *Arabidopsis thaliana* DREB1A transcription factors, driven by *A. thaliana* RD29A gene are being generated (Khan et al., 2018). To date, individual genes had a limited impact on stress tolerance in plants. However, the simultaneous activation of a subset of those genes by transcription factors is a promising

technique (Srivastava and Sahoo, 2021). It has been shown that transgenic plants containing AP2/EREBP genes (DREB1A) can enhance abiotic stress tolerance in crop plants (Singh et al., 2012). Under a constitutive promoter, over-expression of DREB1A proved to be harmful under normal conditions, whereas it was beneficial when a stress was applied. DREB1A expression was reduced in peanuts by using the stress inducible promoter from rd29A instead of CaMV35S (Puli et al., 2021). Peanut genotypes with high transpiration efficiency (TE) and stomatal conductance have largely failed to develop due to the difficulty of solving the stress tolerance problem. Under greenhouse conditions, plants containing *A. thaliana* DREB1A transcription factor, driven by the rd29A promoter, have been found to confer improved cold stress resistance (Gantait et al., 2020). Several transgenic events with diverse responses were chosen for more comprehensive studies on leaves for gas exchange characteristics. To better understand the mechanism behind transgenic environment stress tolerance, the biochemical reactions have been studied under identical cold stress conditions (Khan et al., 2018).

CONCLUSION AND FUTURE PERSPECTIVE

Global climate change has resulted in the emergence of complex stress combinations and their effects on crop yield and overall growth in modern day agriculture. Climate change is a multifaceted issue with long-term consequences in the form of many abiotic pressures. Cold tolerance is among one of those abiotic stresses with a complicated feature that results from multiple molecular interactions in an organism's genome, transcriptome, proteome, and metabolome. Cold tolerance is stage-specific and appears as a response to cell stability toward external stimuli. Despite the fact that genomic loci influencing cold stress tolerance in plants share a degree of homology across species, the relative expression and location of protein/gene products varies with systems between species, and the context defining cold response may differ entirely. Moreover, the function of "omics" technologies in understanding cold stress tolerance in plant species is of enormous importance and immediate solicitation in elucidating new pathways behind such processes. Plants mainly undergo morphological, biochemical, and molecular changes as a result of cold stress. Although most legumes can withstand these fluctuations at low temperature but extended exposure could cause partial or complete failure of the final product. Multiple changes occur throughout the crop life cycle, from germination to harvesting, as a result of such an environment/climate. Crop plants also show a variety of biochemical and molecular manifestations under cold stress. Cold acclimation in response to the freezing stress has been studied extensively in various plants to understand the molecular and physiological status. Contrary to this a little is known about the responses in legumes for the process of deacclimation and reacclimation. Low temperature alters the gene expression of some important gene products which include the dehydrin, Cor, and CBF genes. Legumes are nutritionally as well as economically important crop species and to understand the process of acclimation during freezing or cold stress a lot of research needs to be carried out. Environmental and genotypic impacts, as well as their interactions, end up making elucidating generic responses difficult. When designing studies and evaluating acclimation, deacclimation and reacclimation reactions affecting cold stress tolerance, plant developmental stages, tissue type, and other characteristics, such as light conditions, must be taken into account. Finally, the function of "omics" technologies in understanding cold stress resistance in plant species is of enormous importance with direct solicitation in drawing out new pathways behind such a mechanism and deciphering the genes providing cold tolerance will help to solve the problem of growing seasons of the legumes and help in growing legumes in different seasons of the year. There is a greater need to investigate and concentrate on the genetic features of legumes that enables to resist cold stress effects and continue to grow and develop normally. It was improved even more by finding a wide range of genetic features and mapping them using various gene mapping approaches like QTL mapping and genome-wide association studies. Apart from that, several other osmolytes (such as glycine betaine) and plant hormones (such as SA, ABA, brassinosteroids, and strigolactone) are currently overlooked and can be more promising at regulating plant responses to cold stress using multi-omic approaches. As a result, adopting integrated multidisciplinary methodologies to investigate these gaps and open up new research vistas is urgently required.

AUTHOR CONTRIBUTIONS

KAB, RM, MMP, and UU collected the literature and prepared the first draft. KAB and ZB helped in preparing figures. AAS, AA, and PAS helped in editing the manuscript. BB helped in revising the manuscript. AM finalized figures, tables and helped in revising the manuscript. SMZ conceive the idea, prepared the outline, and helped in finalizing manuscript and its revision. All authors contributed to the article and approved the submitted version.

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Light Quality Modulates Plant Cold Response and Freezing Tolerance

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The cold acclimation process is regulated by many factors like ambient temperature, day length, light intensity, or hormonal status. Experiments with plants grown under different light quality conditions indicate that the plant response to cold is also a light-quality-dependent process. Here, the role of light quality in the cold response was studied in 1-month-old *Arabidopsis thaliana* (Col-0) plants exposed for 1 week to 4°C at short-day conditions under white (100 and 20 μmol $m^{-2}s^{-1}$), blue, or red (20 μmol $m^{-2}s^{-1}$) light conditions. An upregulated expression of *CBF1*, inhibition of photosynthesis, and an increase in membrane damage showed that blue light enhanced the effect of low temperature. Interestingly, cold-treated plants under blue and red light showed only limited freezing tolerance compared to white light cold-treated plants. Next, the specificity of the light quality signal in cold response was evaluated in *Arabidopsis* accessions originating from different and contrasting latitudes. In all but one *Arabidopsis* accession, blue light increased the effect of cold on photosynthetic parameters and electrolyte leakage. This effect was not found for Ws-0, which lacks functional CRY2 protein, indicating its role in the cold response. Proteomics data confirmed significant differences between red and blue light-treated plants at low temperatures and showed that the cold response is highly accession-specific. In general, blue light increased mainly the cold-stress-related proteins and red light-induced higher expression of chloroplast-related proteins, which correlated with higher photosynthetic parameters in red light cold-treated plants. Altogether, our data suggest that light modulates two distinct mechanisms during the cold treatment - red light-driven cell function maintaining program and blue light-activated specific cold response. The importance of mutual complementarity of these mechanisms was demonstrated by significantly higher freezing tolerance of cold-treated plants under white light.

Keywords: *Arabidopsis thaliana* (*Arabidopsis*), accession, cold, freezing tolerance, light intensity, light quality, photosynthesis, proteome

INTRODUCTION

Low temperature is one of the main factors limiting plant growth and overall crop production. However, plants that originate from various geographical areas have developed different levels of cold tolerance (Chen et al., 2014). Temperate climate plants can optimize their metabolism and growth in response to low temperatures in a process known as cold acclimation (Thomashow, 1999).

The primary targets of cold stress damage are cell membranes (reviewed in Mukhopadhyay and Roychoudhury, 2018). Low temperature induces changes in the composition of membranes and has a direct impact on their biophysical properties (Örvar et al., 2000; Palma et al., 2008; reviewed in Raju et al., 2018, and Yu et al., 2021). Tissue damages induced by freezing are characterized by loss of membrane integrity and higher permeability for molecules that can be followed and quantified by electrolyte leakage assay (Thalhammer et al., 2020). The first and most severely affected organelles in plants by cold stress are chloroplasts (reviewed in Liu et al., 2018a). Low temperature has an inhibitory effect on photosynthesis and promotes reactive oxygen species (ROS) generation (Liu et al., 2012; Zhang et al., 2020). ROS are toxic byproducts of inevitable processes like photosynthesis or respiration, and plants modulate their levels during cold stress (Luo et al., 2020; Lv et al., 2020). However, ROS also play an important role in mediating response to cold stress. Plant pre-treatment with scavenger of hydrogen peroxide or inhibitors of NADPH oxidases could alleviate cold tolerance (Zhou et al., 2012; Si et al., 2018).

Molecular and biochemical mechanisms supporting plant chilling and freezing tolerance are very complex and involve the activation of various genes in cell membrane metabolism, production of cryoprotective proteins and solutes like sucrose or proline, and production of ROS scavengers (Hannah et al., 2005; John et al., 2016). In *Arabidopsis*, low-temperature treatment modulates the expression of up to four thousand genes, and approximately half of these genes are upregulated and half down-regulated (Park et al., 2015; Zhao et al., 2016).

Expression analysis of mutant lines revealed that 10 - 20% of cold-modulated genes are regulated by C-repeat (CRT)-binding factors (CBFs), also known as dehydration responsive element (DRE)-binding factor1 (DREB1) proteins (Jia et al., 2016; Zhao et al., 2016). CBF/DREB1 is a subfamily of AP2/ERF DNA-binding transcription factors, and its members are involved in abiotic stress responses. Three members of this family (CBF1, CBF2, and CBF3) are well-known for their response to cold stress (reviewed in Shi et al., 2018). *CBF* expression is upstream regulated by the ICE1 transcription factor (Chinnusamy et al., 2003), and an increase in their expression is detectable already within minutes after cold exposure (Gilmour et al., 1998). Besides the transcriptional control, the CBF activity is regulated by post-translation modification *via* thioredoxin H2 in a temperature-dependent manner (Lee et al., 2021). The molecular analyses of *CBF* genes confirmed their positive effect on plant freezing tolerance accompanied by induction of downstream COLD-REGULATED (*COR*) genes (Gilmour et al., 2004; Jeknić et al., 2014).

A growing number of studies provide evidence that temperature and light signaling are closely connected, and light quantity and quality play a significant role in cold stress tolerance (Catalá et al., 2011; Jiang et al., 2017; Ahres et al., 2020; Prerostová et al., 2021a). Low light intensity was shown to decrease freezing tolerance in diverse plant species (Gray et al., 1997; Janda et al., 2007). However, the overall freezing tolerance depends probably also on the specific conditions for the cold acclimation, way of cultivation, and freezing treatment because plants can significantly increase freezing tolerance also under low light conditions, as we have recently reported (Novák et al., 2021). In addition to low temperatures, *CBF* genes are regulated by light quality, period, and circadian clocks (Franklin and Whitelam, 2007; Soitamo et al., 2008; Dong et al., 2011; Lee and Thomashow, 2012). At dusk and dawn, the ratio of red and far-red light (R/FR) decreases and positively regulates the expression of *CBFs* in *Arabidopsis* even at temperatures higher than those required for cold acclimation (Franklin and Whitelam, 2007). In tomato plants, a low R/FR light ratio increased the level of CBF transcript and overall cold tolerance of plants. The same effect of light was observed in *phyB* mutants but not in *phyA* mutants suggesting their diverse roles in response to cold (Wang et al., 2016). Phytochrome B was also shown to have a negative impact on cold tolerance in *Arabidopsis* (Franklin and Whitelam, 2007) and rice (He et al., 2016). On the contrary, a recent study shows PHYB to be a positive regulator of cold tolerance in tomato plants (Jiang et al., 2020). Recently, the blue light and photoreceptors cryptochromes were shown to play important role in cold tolerance and cold acclimation (Imai et al., 2021; Li et al., 2021a; Ma et al., 2021). These works confirmed the role of blue light in cold tolerance and suggested the presence of blue light-modulated cold tolerance mechanisms, including the activity of CRY2.

Besides the light, plant hormones also contribute to the cold stress response, and this is not limited only to well-known stress-related hormones like abscisic, salicylic, or jasmonic acid, but also cytokinin (reviewed in Jeon et al., 2010; Eremina et al., 2016; Prerostová et al., 2021b). Low temperature modulates cytokinin metabolism (Novák et al., 2021) and multiple signaling elements (Jeon et al., 2010; Zwack et al., 2016) to adjust freezing tolerance. Recently, the role of strigolactones in cold response was also discovered (Zhang et al., 2020).

The model species *Arabidopsis thaliana* is an annual plant with a wide geographical distribution. *Arabidopsis* originates in Eurasia, and its occurrence extends from North Africa to northern Europe through eastern and central Asia and North America. Its natural habitats thus include areas that have very different local climates and different winter temperatures (Hoffmann, 2002; Koornneef et al., 2004). The natural variation of *Arabidopsis* accessions provides an excellent tool for exploring the molecular basis of adaptation. It was reported that ecotypes differ in their tolerance against abiotic stresses, including cold, with those coming from colder northern latitudes being more tolerant to frost than those from southern areas (Hannah et al., 2006; Gery et al., 2011; Gehan et al., 2015).

Previously, we have shown that *Arabidopsis* plants could be hardened even at low white light intensity (Novák et al.,

2021). However, there is limited knowledge about the interaction between low temperature and light quality. In particular, the comparison of the effect of red and blue light was not studied in detail. We have assessed the quantity of blue and red light in our previous cultivation setup and treated plants with low temperature and specific light quality and intensity. We show that blue light promotes the effect of the low temperature on the level of *Arabidopsis* physiology parameters, gene expression, and proteome. Our experiments with selected contrasting *Arabidopsis* accessions revealed a correlation of accession latitude with cold-induced ion leakage and accession-specific cold response proteins. Furthermore, our data indicate that light quality played a regulatory role in response to low temperatures. We showed that cold treatment under red light attenuated oxidative stress and promoted photosynthesis and that blue light cold-treated plants upregulated stress marker genes, the key cold-response regulatory genes CBFs, and accumulated a higher level of cold response proteins. Finally, the plant freezing tolerance assay suggested the importance of complementarity of both light-quality modulated responses.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana accessions Colombia (Col-0), RRS (RRS-7 and RRS-10), Wassilewskija (Ws-0 and Ws-2), Tossa de Mar (Ts-1), and Tammisari (Tamm-2) were grown in a hydroponic system (Araponics, Liege, Belgium). Seeds were surface-sterilized with 70% EtOH and 50% bleach with 0.05% Triton X-100. Then, the seeds were stratified for 2 days at 4°C in the dark and sown on 0.7% (w/v) agar in seed holders immersed in regularly refreshed ½ Murashige and Skoog (MS) medium. The germinating plants were cultivated under short-day conditions (8 h light) in an AR-36L growth chamber (Percival Scientific Inc, Perry, IA, United States) with 65% relative humidity and 21/19°C day/night temperatures with 100 μmol m^{-2} s^{-1} PPFD provided by Philips TL-D fluorescent tubes. Plants aged 5 weeks were divided into five groups ($n > 30$) and were cold-treated at 4°C for 7 days in short-day conditions under (i) standard light intensity and white light (W100), (ii) low light intensity and white light (W20), (iii) low light intensity of blue light (430 nm; B20), or (iv) low light intensity of red light (660 nm; R20). The control group of plants continued to grow at 21 /19°C and standard white light intensity (100 μmol m^{-2} s^{-1}); 20 and 100 μmol m^{-2} s^{-1} PPFD were chosen as the 'low' and 'standard' light intensities for reasons outlined in the Discussion. The photoperiod and humidity were the same in all treatments. After this period, young leaves from at least six plants per sample and experimental replicate were collected, flash-frozen in liquid nitrogen, homogenized, and aliquoted for the molecular analyses. The whole experimental design is visualized in **Supplementary Material S1**.

For freezing experiments, *Arabidopsis thaliana* plants (Col-0) were cultivated *in vitro* on Petri dishes on 0.8% ½ MS medium at short-day conditions (8/16 day/night) and 21°C/19°C day/night temperatures with 100 μmol m^{-2} s^{-1} PPFD during the day. After 2 weeks of horizontal cultivation, the plants were transferred to low temperature (4°C) for 1 week at different light conditions: W100, W20, B20, and R20. At this point, plants reached the growth stage 1.04 (Boyes et al., 2001). The freezing treatment was performed in dark conditions. The cold-treated plants and 2-weeks-old control plants without cold treatment were exposed to a decreasing temperature at a rate of 2°C per hour until it reached−6°C and at this point, ice nucleation was induced. Then, the temperature was reduced to a final−7°C. After 2 h of exposure to−7°C, the plates with plants were removed and allowed to thaw at 4°C. The freezing tolerance was evaluated after 14 days of recovery at standard conditions (21°C/19°C day/night temperatures, 100 μmol m^{-2} s^{-1} PPFD). The freezing tolerance rating was determined by counting the number of plants which continued growth, reaching at least the growth stage 1.06. Plants which did not match this criterion (dead or growth-arrested) were considered freezing intolerant.

Evaluation of Plant Physiology

The plants' growth at both the 1.14 growth stage (start of the experiment) and 7 days later (the end of the cold treatment at 4 and 21°C control treatments) was evaluated by quantifying rosette dimensions using ImageJ software (Schneider et al., 2012). The average shoot fresh weight and dry weight of plants subjected to each treatment were evaluated at the end of the cold period. Chlorophyll fluorescence measurements were performed using a kinetic imaging fluorometer (Handy FluorCam, model FC 1000-H, Photon Systems Instruments; www.psi.cz) as previously described (Novák et al., 2021). Anthocyanins were determined according to Zhang et al. (2011) with the following modifications: 0.08 g of leaf tissue was extracted overnight in 0.45 ml of 1% (v/v) hydrochloric acid in methanol, then chlorophyll was removed by partitioning into a chloroform fraction formed by adding 0.15 ml chloroform and 0.30 ml water. Spectrophotometrically determined levels of pigments were normalized to dry weight. The extent of lipid peroxidation was determined by estimating their malondialdehyde content (MDA) according to Novák et al. (2013). The membrane damage was determined by electrolyte leakage measurement. Whole-leaf rosettes (5-6 for each variant) were incubated in 0.01% Silwet solution (Crompton) for 1.5 h at RT with gentle shaking. The initial electrolyte leakage of samples (EL_{in}) and blank (EL_{B1}) was measured by electrical conductivity. To release all electrolytes, the samples were boiled for 0.5 h at 100°C and after cooling down to RT, the conductivity of the solution was measured again for samples (EL_{fin}) and blank (EL_{B2}). The electrolyte leakage was calculated using the formula: $EL\ (\%) = [(EL_{in} - EL_{B1})/ (EL_{fin} - EL_{B2}) \times 100]$.

RT-qPCR Analysis

The total mRNA was extracted from portions of the samples according to Valledor et al. (2014) with some modifications (**Supplementary Material S2**). RT-qPCR was performed as described by Novák et al. (2015), using primers listed in **Supplementary Material S3**. Three biological replicates of each variant were analyzed. The relative levels of gene expression were normalized by the delta-delta Ct method (Pfaffl, 2001), using *ACT2*, and *EF1-α* as the reference genes.

Proteomic Analysis

Total protein extracts were prepared as described by Valledor et al. (2014), then portions of samples corresponding to 5 μg peptide were analyzed by nanoflow reverse-phase liquid chromatography-mass spectrometry using a 15 cm C18 Zorbax column (Agilent), a Dionex Ultimate 3000 RSLC nano-UPLC system and Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher). Acquired spectra were recalibrated and searched against the Araport 11 protein database following Berková et al. (2020). Only proteins represented by at least two unique peptides were considered for the quantitative analysis. The quantitative differences were determined by Minora, employing precursor ion quantification followed by normalization and calculation of relative protein abundances. The mass spectrometry proteomics dataset has been deposited in the ProteomeXchange Consortium database (Vizcaíno et al., 2016) *via* the PRIDE partner repository with the identifier PXD033102.

Statistical Analysis

Statistical tests were implemented using Instant Clue (Nolte et al., 2018), Rapid Miner (www.rapidminer.com; Mierswa et al., 2006), R (R Development Core Team, www.r-project.org), MetaboAnalyst 5.0 (Chong et al., 2019), SIMCA 14.1, Proteome Discoverer 2.4, Compound Discoverer 3.2, and Statistica 12 software packages. The detected differences between samples were deemed significant if $p < 0.05$.

RESULTS

Light Intensity and Spectral Quality Affect Response to the Low Temperature of *Arabidopsis thaliana* (Col-0)

The 5-week-old *Arabidopsis thaliana* (Col-0) plants grown in a hydroponic system were used to elucidate the effects of light quality in low-temperature treatment (**Figure 1A**). The plants were cold-treated at 4°C for 7 days in short-day conditions at (i) standard light intensity and white light (W100), (ii) decreased light intensity and white light (W20), (iii) decreased light intensity of blue light (B20), or (iv) decreased light intensity of red light (R20). The leaves of cold-treated plants showed a similar pattern at the end of the cold treatment but were significantly inhibited in growth compared to non-treated plants grown at short-day conditions with standard white light intensity and 21°C (control; **Figure 1B**). The cold-treated W100 plants had darker leaf coloration reflected in higher anthocyanin accumulation compared to cold-treated plants grown at low light intensity (under white, blue, or red light; **Figure 1C**).

The analysis of chlorophyll fluorescence did not confirm a significant change in maximum efficiency of photosystem II (Fv/Fm ratio) in the cold-treated plants (**Figure 1D**), but cold stress was followed by a significant decrease in efficiency of photosystem II in a light steady state (Qy_Lss; **Figure 1E**). The most significant inhibition was observed in plants grown under blue light with a more than 50% decrease in this parameter compared to non-treated control plants. Similarly, non-photochemical quenching (NPQ, which reflects protective mechanisms of energy dissipation by heat) and vitality index (Rfd) were reduced in cold-treated plants with the most significant change in blue light-grown plants (**Figures 1F,G**).

Physiological experiments have shown that the cold response of *Arabidopsis* is affected by both light intensity and spectral properties of light, and photosynthetic parameters suggested a significant role of blue light in response to low temperature.

Cold-Treated Plants Under Standard White Light and Low Blue Light Show Increased Cold Stress Markers

The role of light quantity and quality in cold response was evaluated by ion leakage measurement. Results did not show any difference among W20, R20, and control plants but a significant ($p<0.05$) increase in ion leakage for W100 and B20 cold-treated plants (**Figure 2A**). The relative content of malondialdehyde, the product of lipid peroxidation, confirmed that only W100 and B20 plants showed a response to oxidative stress higher than the control plants (**Figure 2B**). Interestingly, the comparison of B20 and R20 plants showed that B20 plants had a more than 45% higher ion leakage and 130% higher MDA level than R20 plants.

Cold-Treated Plants Under Blue and Red Light Showed Reduced Tolerance to Freezing

The role of light conditions in the acquired freezing tolerance of plants was tested. For this validation experiment, a modified experimental setup was utilized, employing 2-week-old plants grown on Petri dishes and then exposed to 4°C under W100, W20, B20, or R20 light conditions for a week. The cold treatment period was followed by a freezing stress experiment confirming the positive role of low-temperature treatment on plant freezing tolerance. In contrast to cold-treated plants, control plants (3-week-old plants without cold treatment) were not able to survive freezing treatment. However, there were significant differences among the cold-treated light variants. Cold-treated plants under standard light conditions W100 showed the highest freezing tolerance (**Figures 3A,B**). Cold treatment under low light intensity was very sensitive to the light spectrum. W20 cold-treated plants acquired similar tolerance to freezing as W100 plants, but B20 and R20 cold-treated plants showed very limited tolerance to freezing stress.

Blue Light Amplifies the Effect of Low Temperature in Diverse *Arabidopsis* Accessions

Arabidopsis is a widespread plant species, and its accessions had to adapt to their location-specific temperature profiles and light conditions. Previous studies have shown a correlation between the cold response of *Arabidopsis* accessions and latitude or temperature in the original growth habitat (Zuther et al., 2012). The light intensity and spectral properties of sunlight vary across latitudes over the year, therefore, we have tested the specific

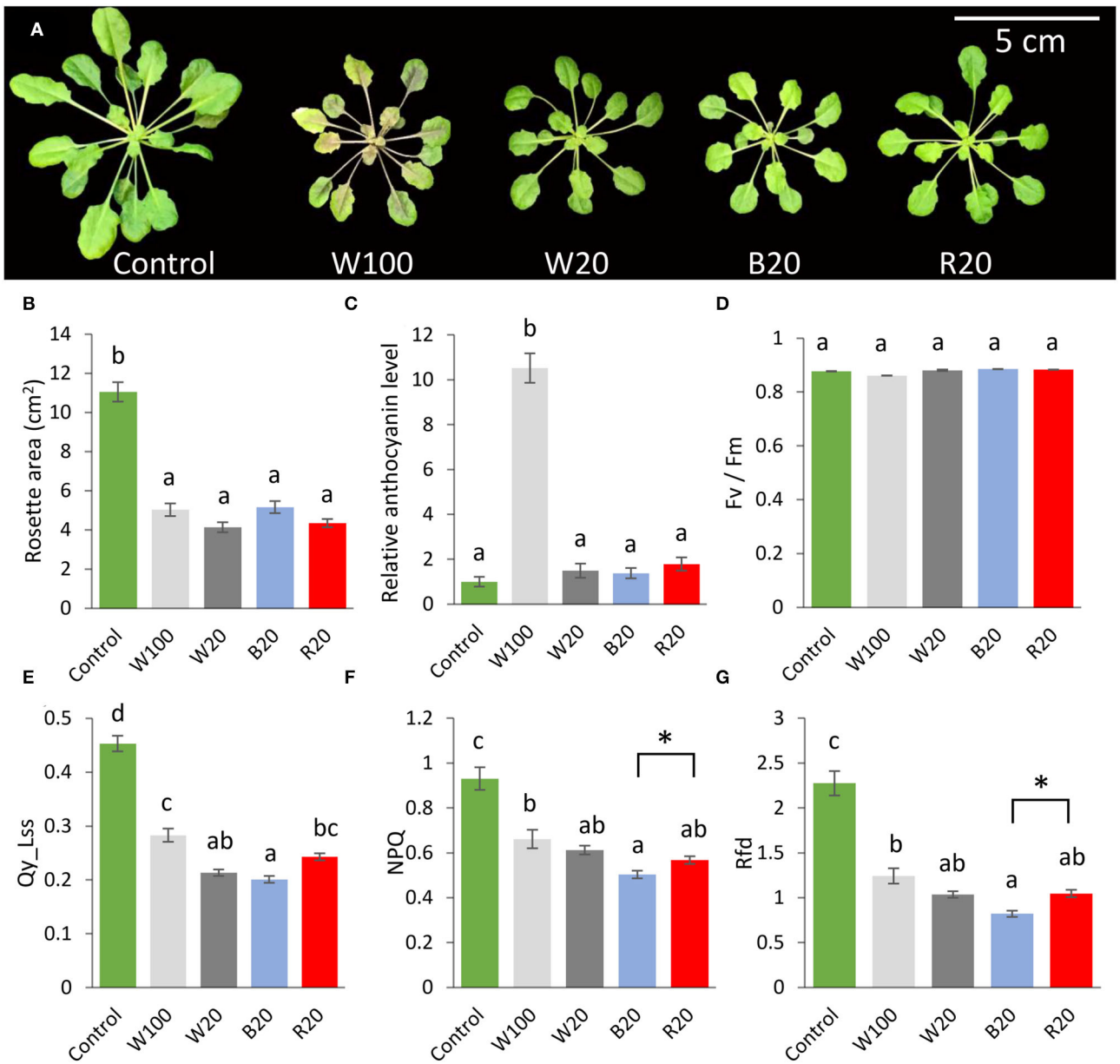

FIGURE 1 | Light intensity and quality modulate *Arabidopsis* response to low temperature. **(A)** Representative images of *Arabidopsis* Col-0 plants grown for 7 days in indicated conditions. **(B)** Rosette area. **(C)** Relative anthocyanin contents normalized to dry weight (relative to non-treated control plants). **(D)** The maximum quantum efficiency of photosystem II in the dark-adapted state. **(E)** Quantum efficiency of photosystem II in light steady state. **(F)** Non-photochemical quenching in light steady state. **(G)** Photosynthetic parameter Rfd (fluorescence decline ratio in light steady state). Results are means of three biological replicates, error bars indicate standard errors, and letters in all panels indicate significant differences according to one-way ANOVA with Tukey's HSD *post hoc* test ($p < 0.05$). Asterisks indicate significant differences according to Student's *t*-test between marked variants ($p < 0.05$). W100, cold treatment under 100 μmol m^{-2} s^{-1} white light; W20, cold treatment under 20 μmol m^{-2} s^{-1} white light; B20, cold treatment under 20 μmol m^{-2} s^{-1} blue light; R20, cold treatment under 20 μmol m^{-2} s^{-1} red light. For details, see Materials and methods.

role of light quality in cold response in another six accessions ranging between 40 and 60°, and adapted to different average temperatures (**Supplementary Material S4**).

All accessions were grown for 5 weeks in a hydroponic system in short-day conditions with a standard light photon flux density of 100 μmol m^{-2} s^{-1} before cold hardening. At this growth stage, different accessions showed diverse rosette and leaf shapes (**Figure 4A**). Most significant was a reduction of rosette compactness of Ts-1 and Tamm-2.

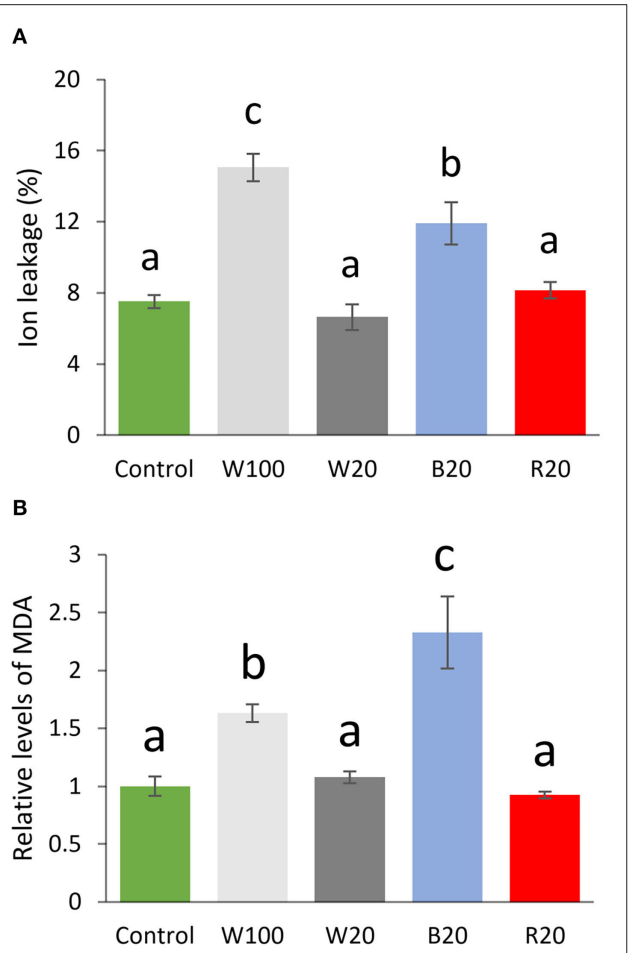

FIGURE 2 | Membrane damage and lipid peroxidation of plants treated by low temperature under different light regimes. **(A)** Ion leakage and **(B)** relative level of malondialdehyde (MDA) normalized to control and compared to control. Results represent means of three biological replicates with standard errors, letters indicate significant differences (ANOVA, Tukey's HSD *post hoc* test, $p < 0.05$). W100, cold treatment under 100 μmol m^{-2} s^{-1} white light; W20, cold treatment under 20 μmol m^{-2} s^{-1} white light; B20, cold treatment under 20 μmol m^{-2} s^{-1} blue light; R20, cold treatment under 20 μmol m^{-2} s^{-1} red light. For details, see Materials and methods.

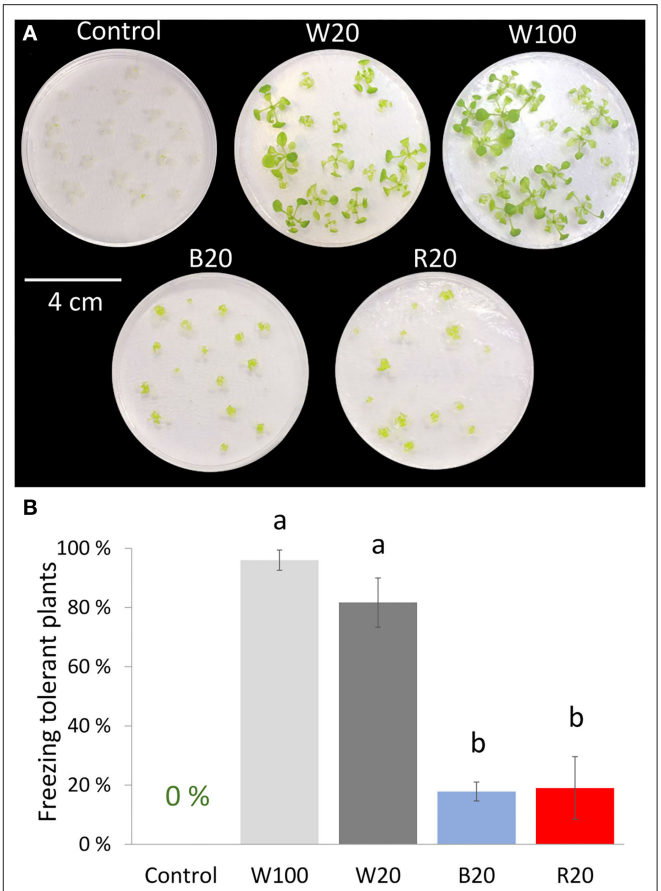

FIGURE 3 | Freezing tolerance of cold-treated plants under different light regimes. **(A)** Representative images of *Arabidopsis* Col-0 plants 2 weeks after freezing stress; 2-week-old plants cultivated on Petri dishes in standard conditions were treated at low temperature (4°C) under different light conditions for a week (W100, W20, B20, R20) and then exposed to freezing stress. Control plants represent 2-week-old plants exposed to freezing stress without the cold treatment. Plants were transferred to control conditions after the freezing stress and recovered for another 2 weeks. **(B)** Freezing tolerant plants. Plants that produced new leaves were evaluated as freezing tolerant. Results represent means of three biological replicates with standard errors, letters indicate significant differences (ANOVA, Tukey's HSD *post hoc* test, $p < 0.05$). For details, see Materials and methods.

Next, plants were cold-treated under low temperature (4°C) and blue or red light with a photon flux density of 20 μmol m^{-2} s^{-1}. At the end of the cold treatment period, ion leakage was determined (**Figure 4B**). All accession except Ws-0 showed higher ion leakage if cold-treated under blue light. Ion leakage of blue light cold-treated plants correlated statistically significantly ($p<0,05$) to the average (January to March) temperature of the original location of accession (**Supplementary Material S4**) and was on average almost 50% higher compared to red light (**Figure 4B**). The ion leakage of B20 plants also negatively correlated with the number of ground frost days and latitude of the original location (**Supplementary Material S5**). The ion leakage of R20 plants correlated with the temperature of the original location to a lower extent, and this correlation was not statistically significant. The main factor reducing correlation was probably a high decrease in ion leakage of red light cold-treated Ts-1 accession.

The analysis of photosynthetic parameters confirmed the blue light as an amplifier of the low-temperature treatment. The determination of Qy_Lss showed more than 40 and 60% decrease in cold-treated plants under red and blue light respectively (**Figure 4C**). The most significant change between blue and red light-treated accession was found in Ts-1. Interestingly, Qy_Lss in cold-treated plants under blue and red light showed an inverse correlation with the temperature of the original location of accession (**Supplementary Material S5**). NPQ was also more significantly affected by the blue light during cold treatment

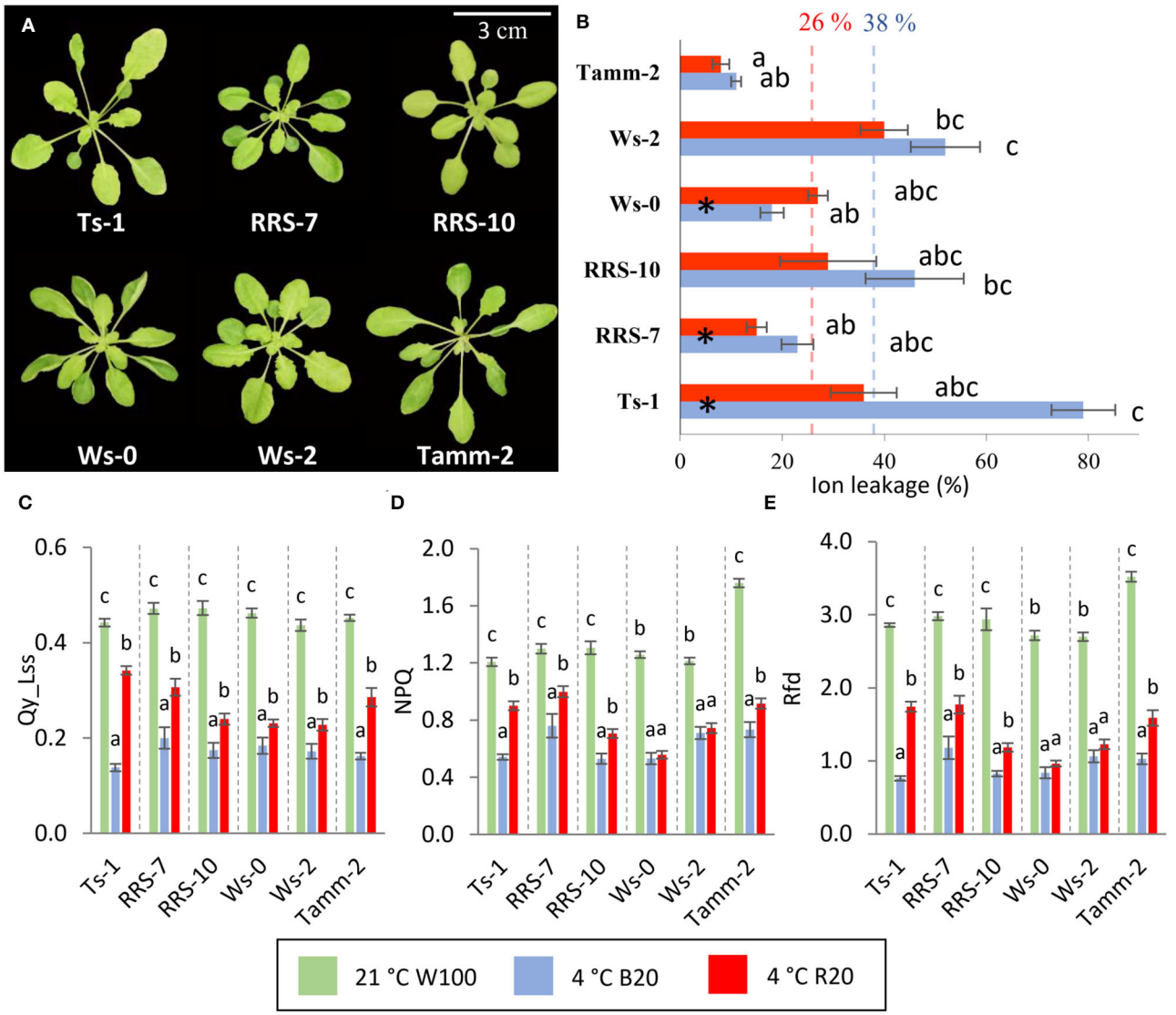

FIGURE 4 | Role of blue and red light in the cold-treated *Arabidopsis* accessions. **(A)** Representative images of 5 weeks old hydroponically grown *Arabidopsis* accessions grown in control conditions and used for cold treatment experiments. Plants were treated for 7 days at 4°C under 20 μmol m^{-2} s^{-1} blue or red light. **(B)** Ion leakage of cold-treated plants in respective conditions. Results are means of three biological replicates, error bars indicate standard errors, and letters indicate significant differences according to the Kruskal–Walli's test with *post hoc* analysis ($p < 0.05$). Black asterisks in columns indicate significant differences between red and blue light cold-treated accession according to Student's *t*-test ($p < 0.05$). Colored dashed lines represent the average value of ion leakage for blue and red light cold-treated plants. **(C)** Quantum efficiency of photosystem II in light steady state, **(D)** non-photochemical quenching in light steady state, **(E)** photosynthetic parameter RFD. Results of photosynthetic parameters are means of three biological replicates, error bars indicate standard errors. The statistical significance of the results was evaluated separately for each accession. Letters indicate significant differences according to one-way ANOVA with Tukey's HSD *post hoc* test ($p < 0.05$). Accessions Ts-1, RRS-7, RRS-10, Ws-0, Ws-2, and Tamm-2 are described in **Supplementary Material S4**.

but not in all accessions (**Figure 4D**). Ws-0 and Ws-2 did not show a significant difference between blue and red light. The highest NPQ was measured in northern accession Tamm-2 and accession RRS-7. Vitality parameter Rfd showed a similar pattern to Qy_Lss, a higher reduction in plants treated by low temperature under blue light, and the highest impact of light in accession Ts-1 (**Figure 4E**).

Physiological experiments employing diverse *Arabidopsis* accessions supported the amplifying role of blue light in low-temperature response and confirmed its general activity across accessions. Statistical analysis showed the correlation of cold-induced membrane damage with physical parameters of accession habitats.

Different Light Quality During Cold Treatment Does Not Change the Expression Profile of Cytokinin-Related Genes But Plays a Significant Role in the Expression of CBF Genes

The CBF signaling pathway regulates essential aspects of cold response and cold tolerance. CBF genes are activated within minutes following cold treatment and stimulate downstream targets known as CBF regulon. The role of light quality in the expression of CBF genes and cold-related genes was analyzed by RT-qPCR (**Figure 5**). The analysis confirmed the importance of light quality in the regulation of CBF expression. Plants treated by low temperature under blue light have significantly increased expression of CBF genes in comparison to red light cold-treated plants. The exception was northern accession Tamm-2 and southern accession Ts-1 with the expression of CBF independent of light quality. Interestingly, light-dependent expression was not significant for two analyzed COR genes *RD29A* and *COR47*.

The recently published works highlighted the potential role of cytokinin in cold response. The analysis of expression of cytokinin-related genes at the end of the cold treatment period did not confirm differences in response to cold under blue or red light (**Figure 5**). The analysis confirmed that the expression of cytokinin transcription factors CRF and ARR-B was predominantly down-regulated. Similarly, the cytokinin response genes ARR-A were down-regulated (**Figure 5**). Out of seven different accessions, only Ts-1 showed statistically significant differences in the expression of cytokinin biosynthetic enzyme, namely *IPT3*.

Light Quality Modulated Cold-Stress Responsive Proteome and the Response Was Predominantly Accession-Specific

The analysis of shoot proteome of *Arabidopsis* accessions provided identification and quantitative data for 3,124 and 2,290 *Arabidopsis* proteins, respectively. The effect of cold stress was found to be both light-quality- and accession-dependent (**Figures 6A–D**). The lowest response to cold treatment was found in the accession Ws-2 with only 105 (R20) and 118 (B20) differentially abundant proteins compared to the plants grown under control conditions (**Figure 6A**). In contrast, the abundances of over 600 proteins were significantly altered in B20 Ws-0 plants. In most accessions, B20 plants showed a higher response to cold stress, with more cold-responsive proteins with an increase in abundance and fewer proteins with a decrease in abundance than the corresponding R20 plants (**Figure 6B**). The comparison of B20 and R20 plants revealed only limited overlap, but the majority of R20 and B20 responsive proteins showed a similar response to cold stress (**Figure 6C**). Interestingly, Col-0 plants showed the second weakest response to cold, and the similarity between R20 and B20 plants was the highest for this accession with more than 50% of shared cold-responsive proteins. As illustrated in the B20 cold response, most cold-responsive proteins were accession-specific (**Figure 6D**). The accession-specific differentially abundant proteins represented 60 and 66% of all differentially abundant proteins in B20 and R20 plants, respectively.

Identification of Proteins With a Putative Role in Contrasting Cold Responses in R20 and B20 Plants

Proteomics data supported significant differences between R20 and B20 plants under cold stress. To identify putative protein markers contributing to the observed differences in ion leakage and photosynthetic parameters, ratios between R20 and B20 protein abundances were analyzed by partial least squares regression analysis (PLS; **Figures 7A–D**). The analysis separated accessions Ts-1 and Ws-0 (**Figure 7A**), and the consecutive VIP (variables of importance in projection) highlighted 108 and 35 proteins that showed positive and negative correlations with the observed separation, respectively (**Figure 7B**). Proteins that were found to be more abundant in R20 plants (**Figure 7C**) were predominantly localized in chloroplasts (58%), indicating that this organelle is critical for the observed differences between R20 and B20 response to cold stress. The functional characterization of these proteins highlighted an increase in the components of photosystems, protein import into chloroplasts, and mechanisms involved in plastidic protein repair and quality control. A higher abundance was also found for carbohydrate-active enzymes (CAZymes), proteins of ROS metabolism and signaling, components of proteasome, translation machinery, cytoskeleton, and phenylpropanoid pathway. Proteins correlating with the observed B20-promoted cold stress response were enriched only in two categories, namely vesicular transport and fatty acid metabolism (**Figure 7D**).

DISCUSSION

Role of Light Quality and Quantity in Response to Low Temperature

Our previous study showed that *Arabidopsis* plants exposed to low temperature under a reduced white light intensity of 20 μmol m^{-2} s^{-1} could increase their resistance to freezing (e.g., Novák et al., 2021; Prerostová et al., 2021a). However, the role of light quality was not analyzed in detail. Other groups have reported and highlighted the impact of the blue and red light spectrum, or R/FR light ratio in plant response to low temperature (Franklin and Whitelam, 2007; Imai et al., 2021). But the overall effect of red and blue light on plants treated with low temperature was not compared. Here, to further deepen our knowledge about the role of the light spectrum in cold response, month-old plants grown under 100 μmol m^{-2} s^{-1} white light were exposed for 7 days to a low non-freezing temperature under 20 μmol m^{-2} s^{-1} of red or blue light. The selected photon flux density of 20 μmol m^{-2} s^{-1} was matching the corresponding photon flux density of blue and red components in 100 μmol m^{-2} s^{-1} white light fluorescent tube sources. This experimental design resolved the specific effect of individual light regions and separated the integrative net effect of remaining white light components. Moreover, these light intensities correspond to natural conditions that plants encounter in winter (Robson

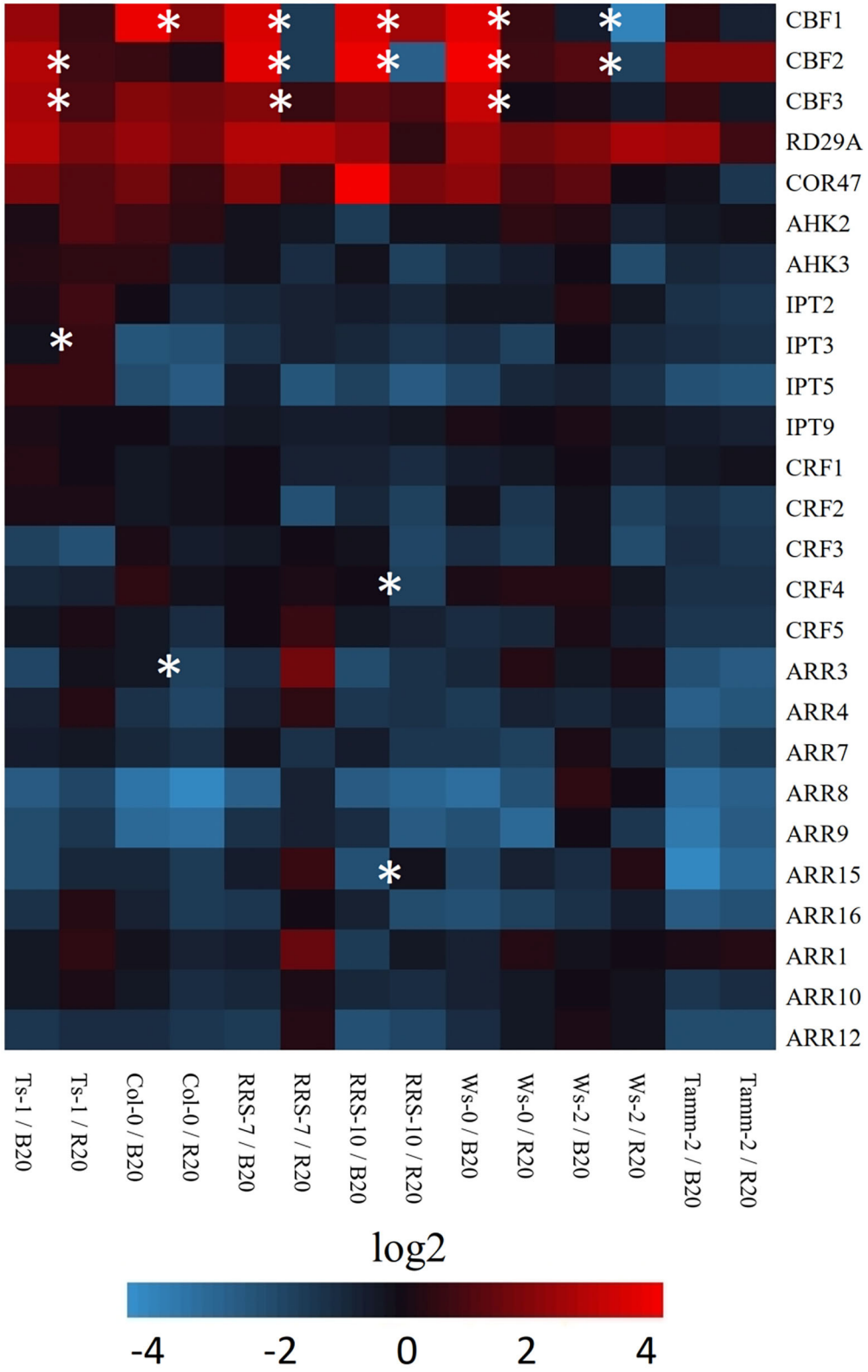

FIGURE 5 | Heat map visualization of expression of selected cold-related and cytokinin-related genes. The expression was measured in leaves of *Arabidopsis* accessions treated 7 days by low temperature under red (R20, 20 μmol m^{-2} s^{-1}) or blue light (B20, 20 μmol m^{-2} s^{-1}) conditions by RT-qPCR. Data were normalized as specified in Material and methods. Results are means of three biological replicates, and asterisks indicate significant differences between red and blue light-grown accession according to Student's *t*-test ($p < 0.05$). Accessions Ts-1, RRS-7, RRS-10, Ws-0, Ws-2, and Tamm-2 are described in **Supplementary Material S4**.

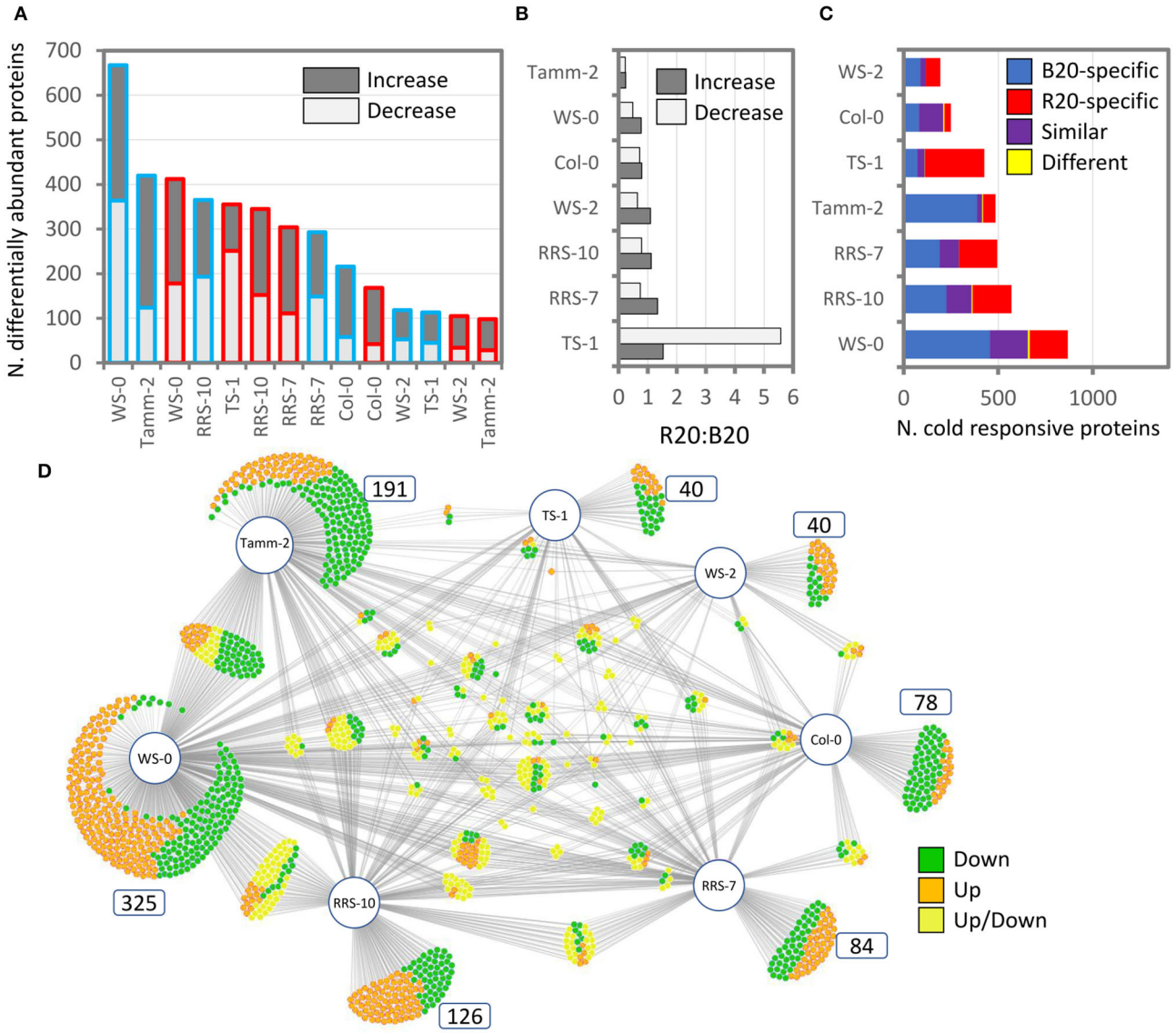

FIGURE 6 | Comparison of cold stress response in *Arabidopsis* accessions. **(A)** Differentially abundant proteins in leaf proteomes of plants grown under red (R20; red outline) or blue (B20; blue outline) light at 4°C for 7 days compared to respective accessions grown under standard conditions (21°C, W100). **(B)** Comparison of ratios between the number of cold-responsive proteins in R20 vs. B20 plants. **(C)** Cold stress in R20 and B20 plants elicits unique sets of proteins. **(D)** The cold response is accession-specific. Comparison of response to cold stress in B20 plants visualized with DiVenn 2.0 (Sun et al., 2019). For details, see **Supplementary Material S6**.

and Aphalo, 2019). Furthermore, photoreceptors of blue and red light are sufficiently activated at this intensity (Park et al., 2012; Liu et al., 2020b), and reduced temperature prolongs their activated state (Legris et al., 2016; Pooam et al., 2021). The action spectrum for photosynthesis is comparable for the blue and red light components, and approximately the same amount of energy was available for photosynthesis in our experiment. Thus, it is expected that the observed changes in physiology and molecular processes are predominantly proportional to the differences in light perception and signaling.

One of the well-known effects of cold stress is the inhibition of plant growth. In accordance, there was no statistically significant difference in the rosette size R20, B20, W20, and W100 plants after 7 days of cold stress (**Figure 1B**). It has been reported that the inhibitory effect of suboptimal temperature can be compensated by red light, and plants grown under red light at 16°C had a much larger leaf rosette compared to the variant under blue light (Liu et al., 2020a). Results reported here indicate that the effect of temperature (4°C) on growth overcomes the impact of light quality. Growth inhibition could be the result of metabolic activity attenuation and seems to be critical for cold resilience. Indeed, activation of growth signals under low temperature has a negative impact on plants chilling tolerance (Lange et al., 2020; Li et al., 2021b).

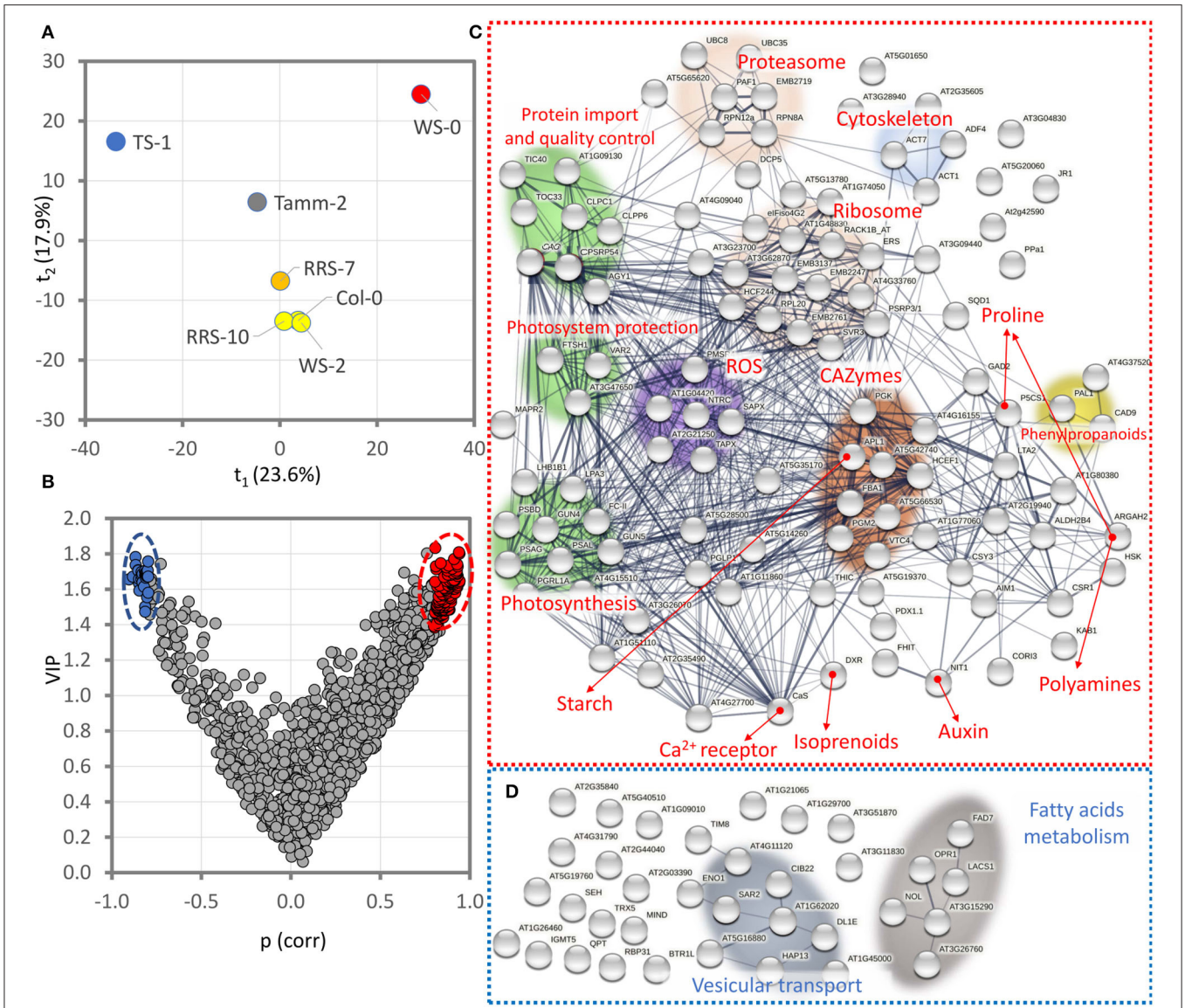

FIGURE 7 | Identification of putative protein markers of the contrasting response in R20 and B20 plants under cold stress. The ratios in protein abundance, ion leakage, Qy_lss, NPQ, and RFD were analyzed by partial least squares regression analysis **(A)** and VIP variables of importance in projection. **(B)** Projected ratios represent the means of at least four biological replicates. **(C,D)** Functional characterization of identified proteins showing positive correlations [red, p(corr)>0.8, VIP>1.4] and negative correlations [blue, p(corr)<-0.8, VIP>1.4] with the OPLS distribution. Visualized by STRING (Szklarczyk et al., 2021). For details, see **Supplementary Material S7**.

Cold-Induced Anthocyanin Accumulation Is Lost in R20 and B20 Plants

The effect of reduced temperature is associated with reprogramming of metabolism and activation of pathways for the synthesis of protective compounds, including anthocyanins (Novák et al., 2021). These cold-responsive compounds may contribute to plant resistance to low temperatures (Catalá et al., 2011). A significant accumulation of anthocyanins was detectable only in plants grown under white light (at optimal light intensity; **Figure 1C**). At normal temperatures, the accumulation of anthocyanins is more sensitive to blue light than red light (Liu et al., 2018b), and significant differences can be detected at low light intensities (Vandenbussche et al., 2007). That indicates that the light-induced anthocyanin biosynthesis regulation is temperature-dependent, requiring sufficient light intensity.

Blue and Red Light Cold-Treated Plants Showed Similar Freezing Tolerance But Different Level of Cold-Induced Stress Markers

The comparison of B20 and R20 plants showed significant differences in ion leakage and photosynthetic parameters

(**Figures 1D–G**, **2A**). The observed significant increase in ion leakage and lower value of RFD indicated higher oxidative stress in B20 plants, and that was confirmed by measuring the product of membrane oxidation MDA (**Figure 2B**). Reactive oxygen species (ROS) play a significant role in cold acclimation and their levels are increased in response to cold stress (Li et al., 2014; Zhang et al., 2020). It is well-known that ROS accumulation is harmful to the plant (e.g., Novák et al., 2013; Saxena et al., 2016), but recent reports indicate that ROS participate in the ICE-CBF pathway regulation and may (at a certain level) serve as signaling molecules and increase freezing tolerance (Wang et al., 2018; Devireddy et al., 2021; Fang et al., 2021). In addition, it has been shown that the activity of the CBF transcription factors is regulated by redox-mediated switching by TRX-H2, the thioredoxin translocated from cytoplasm to nucleus in response to low temperature (Lee et al., 2021). Our proteomics data indicated that two proteins of the TRX-H family were significantly less abundant in some B20 and R20 accessions (TRX-H3, TRX-H5). However, these changes did not seem to correlate with the freezing tolerance (**Table S5**). Interestingly, thioredoxin reductases were implicated in the contrasting cold responses in R20 and B20, namely NTR3 and FTRC (**Figure 7C**, **Supplementary Material S6**). That is well in line with the previously reported role of cellular redox status in an optimal cold response (Lee et al., 2021).

A higher ROS accumulation in B20 plants may coincide with the cold-regulated cryptochrome activity (Pooam et al., 2021). Cryptochromes positively affect ROS in the cell nuclei as a part of the signaling cascade (Consentino et al., 2015). This production does not represent a significant part of the ROS production at normal temperature (El-Esawi et al., 2017), but it is possible that its importance increases with the cold-induced inhibition of photosynthetic light reactions (**Figures 1E–G**). This may be another factor in amplifying the cold response in the blue light cold-treated plants.

Increased stress markers did not correlate with plant freezing tolerance (**Figure 3**). Similar to Li et al. (2021a), plants exposed to low temperatures under white light showed the highest freezing tolerance. High freezing tolerance in W20 cold-treated plants and reduced tolerance in B20 and R20 plants suggest a higher impact of light quality than light quantity in the cold response and cold acclimation process.

Accession-Specific Responses May Reflect *Arabidopsis* Adaptation or Development-Dependent Cold Response

Cold stress-responsive proteins were predominantly accession-specific (**Figure 6**), and the observed differences supported the contrasting results of the stress response evaluation *via* determination of photosynthetic parameters and conductivity (**Figures 4B–E**). It is tempting to speculate that the results reflect adaptation of these accessions and documented high-impact mutations in the genes of the light signaling pathway (**Supplementary Material S8**). However, it should be noted that all accessions were cultivated for 4 weeks under conditions that were optimal for Col-0 growth, and differences in plants' phenotypes were visible (**Figure 4A**). A similar experimental setup has been previously employed in the evaluation of *Arabidopsis* accessions response to cold stress (e.g., Rasmussen et al., 2013; Cvetkovic et al., 2017; Leuendorf et al., 2020), and it cannot be excluded that at least part of the accession-specific response could be development-dependent.

B20 Plants Accumulated More Cold-Stress Responsive Proteins

The comparison of B20 and R20 plants revealed 35 proteins that were more abundant in B20 plants, and the B20:R20 protein abundance ratio showed a positive correlation with the stress response documented by ion leakage and selected photosynthetic parameters (**Figures 7B,D**). It does not seem that these proteins would be negative markers of stress tolerance, and it is likely that the increased abundance only reflects a higher cold stress response. These proteins include chloroplast RNA-binding protein CP31A (AT4G24770) that stabilizes transcripts under cold stress conditions and may confer cold stress tolerance (Kupsch et al., 2012). Cold-induced modulation of membrane lipid composition is likely reflected in increased abundances of enzymes associated with fatty acid metabolism, including sn-2 acyl-lipid omega-3 desaturase (biosynthesis of 16:3 and 18:3 fatty acids; AT3G11170), long-chain acyl-CoA synthetase 1 (AT2G47240), 12-oxophytodienoate reductase 1 (AT1G76680), and 3-hydroxyacyl-CoA dehydrogenase (AT3G15290). Similarly, sucrose accumulation is a well-documented cold stress response (e.g., Novák et al., 2021) and correlates with the observed accumulation of sucrose biosynthetic enzyme (sucrose-phosphatase 2; AT2G35840).

The list of proteins with a higher abundance in B20 plants indicated a putative stimulatory effect on protein trafficking, including the coatomer subunit (AT1G62020) that mediates protein transport from the endoplasmic reticulum, protein AT5G16880 ESCRT (endosomal sorting complex required for transport), a subunit of clathrin-associated adapter protein complex HAP13 (AT1G60780), dynamin-related protein 13 (AT3G60190), secretion-associated protein SAR2 (AT4G0208), and a protein with a reported role in actin and tubulin folding (AT3G11830). Interestingly, two proteins indicated an increase in biotic stress defense mechanisms, namely, glucosinolate biosynthetic enzyme (indole glucosinolate O-methyltransferase 5; AT1G76790) and RNA-binding protein BTR1 that has a role in plant defense against virus infection (Fujisaki and Ishikawa, 2008).

R20 Plants Invest More Resources Into the Chloroplast Development and Maintenance of Photosynthetic Apparatus

Protein analyses indicated that R20 plants possess an enhanced production of isoprenoids (1-Deoxy-d-xylulose 5-phosphate reductoisomerase, AT5G62790) without significant effect on cytokinin signaling (**Figure 5**), phytohormone auxin (Nitrilase 1, AT3G44310), and an osmoprotectant proline (rate-limiting enzyme Δ^1-pyrroline-5-carboxylate synthase A, AT2G39800).

The functional analysis also showed that proteins with a higher abundance in R20 plants mediate chloroplast development and photosynthesis (**Figure 7C**). The R20 plants had a lower abundance of an inhibitor of plastid division (MIND1, AT5G24020; Kanamaru et al., 2000), and accumulated multiple proteins essential for chloroplast biogenesis, development, and integrity, including CLPP6 protease (AT1G11750; Sjögren et al., 2006) and its protein interactor CLPC1 (AT5G50920), proteases FTSH1 and FTSH2 (AT1G50250, AT2G30950; Chen et al., 2006), calcium-sensing receptor CaS (AT5G23060; Huang et al., 2012), and protein translocase subunit SECA1 (AT4G01800; Skalitzky et al., 2011). An increase in abundance was found also for a protein required for chlorophyll accumulation under normal growth conditions (GUN4, AT3G59400; Larkin et al., 2003), a chloroplastic elongation factor required for proper chloroplast rRNA processing and/or translation at low temperature (AT5G13650, Liu et al., 2010), ferrochelatase regulating heme biosynthesis (AT2G30390), and multiple subunits of photosynthetic apparatus (**Figure 7C**). Taken together, the comparison of B20 and R20 plants showed that R20 plants invest more resources into the maintenance of efficient photosynthesis, and that is well in line with the measured photosynthetic parameters (**Figures 4C–E**). It is likely that the higher photosynthetic activity stimulates carbohydrate metabolism and starch production (a higher abundance of glucose-1-phosphate adenylyltransferase, AT5G19220) and could be responsible for an increase in ROS metabolism enzymes (ascorbate peroxidases AT1G7749, AT4G08390; peroxidase AT4G37520).

Prolonged Cold Treatment Inhibits Cytokinin Signaling in a Light Quality-Independent Manner

Plant hormone cytokinin is well-known for its multifaceted function in growth and development (e.g., Skalák et al., 2019). Similarly, its role in abiotic stress is also well-documented (Pavlů et al., 2018). Previously, it was shown that a brief period of cold treatment upregulated cytokinin responsive genes but did not change the cytokinin content (Jeon et al., 2010). However, prolonged cold stress has a negative impact on the active cytokinin pool (Novák et al., 2021; Prerostová et al., 2021b). That is like other abiotic stressors (Zwack and Rashotte, 2015) and may reflect the growth arrest. The exact role of cytokinin in cold response and cold tolerance is far from understood. For instance, cytokinin receptors were shown to be negative regulators of freezing tolerance, but an increase in cytokinin pool by an exogenous application or promoted biosynthesis has a positive effect (Jeon et al., 2010; Prerostová et al., 2021b). Similarly, previous studies have reported cytokinin responsive genes ARR-A to be both positive and negative regulators of freezing tolerance (Jeon et al., 2010; Shi et al., 2012). Our previous results showed that cytokinin signaling in cold-treated plants is modulated by ambient light and that higher light intensity has a negative impact on its output (Novák et al., 2021). Surprisingly, B20- and R20-treated plants showed a somewhat similar gene expression profile (**Figure 5**) to that of plants treated with higher white light intensity (Novák et al., 2021). This profile was observed in all tested accessions suggesting a light quality-independent general attenuation of cytokinin signaling. The inhibition of cytokinin signaling is likely a growth regulatory mechanism restricting excess growth under suboptimal environmental conditions, and that is consistent with cytokinin/abscisic acid antagonism (Huang et al., 2018).

Do Different Parts of the Light Spectrum Activate Specific Cold Tolerance Mechanisms?

The presented data demonstrate that *Arabidopsis* response to low temperature is strongly light-quality specific. The blue light treatment results in low temperature in higher expression of key cold-responsive transcription factors CBFs, higher accumulation of cold-stress responsive proteins, but more attenuated photosynthetic processes and increased oxidation status indicating a promoted ROS signaling. In contrast, red light inhibits CBF expression, but promotes the expression of chloroplast and ROS scavenging proteins, maintains photosynthetic processes, and decreases level of oxidation and membrane damage. Altogether, it seems that red light activates the program for maintaining cell function, and blue light induces a specific cold defense program. Neither of these mechanisms on their own can successfully protect plants from subsequent freezing damage, as seen by freezing stress tolerance. The W20 plants showed that the intensity of light is not the key limiting factor in this process, indicating the importance of complementarity in maintaining growth and specific cold protection mechanisms. Experiments with *Arabidopsis* accessions showed that the blue light perception *via* CRY2 is an integral part of cold response under blue light and its absence in Ws-0 (**Supplementary Material S8**) significantly altered plant physiology and proteome. Interestingly, it has been demonstrated that plants have dynamic life also in cold under the snowpack (reviewed in Körner, 2003). Robson and Aphalo (2019) found that the blue: red (B:R) ratio positively correlates with the depth of the snow surface. Taken together with the observations reported here, it seems that a low B:R ratio acts as a plant signal for promoting active photosynthesis and restoration of the vital processes (i.e., plant growing close to snowpack surface or in the melting snow), and an increase in B:R ratio signals less suitable conditions that require a stronger cold-specific response.

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: http://proteomecentral.proteomexchange.org/; PXD033102.

AUTHOR CONTRIBUTIONS

BB, RV, MC, and JN: conceptualization. MK: methodology and evaluation of the data, freezing assay, RT-qPCR, and physiology

experiments. VO: physiology experiments. MC: proteomic analysis and evaluation of the data. LH: MDA analysis. MB: proteomic analysis. JN: physiology experiments and evaluation of the data. BB: evaluation of the data. MC, JN, MK, and BB: manuscript preparation. BB and RV: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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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.2022.887103/full#supplementary-material

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