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Combined Physiological and Transcriptomic Analysis Reveals Key Regulatory Networks and Potential Hub Genes Controlling Chilling Tolerance During Soybean Germination

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Keywords: chilling tolerance | hub genes | physiological analysis | soybean germination | transcriptome analysis

ABSTRACT

Chilling is an important limiting factor for seed germination of soybean (*Glycine max* [L.] Merr.). To reveal the regulatory mechanism of chilling tolerance during the soybean germination stage, based on previous studies, the chilling tolerance line R48 and chilling sensitive line R89 in chromosome segment substitution lines were selected for physiological index determination and transcriptome sequencing. It was found that reactive oxygen species (ROS) scavenging system related enzymes, ROS, and osmotic regulators were significantly different between the two lines. Gene Ontology enrichment and Kyoto Encyclopedia of Genes and Genomes enrichment were performed on the differentially expressed genes obtained by transcriptome sequencing. It was found that terms or pathways related to flavonoids, unsaturated fatty acids, and abscisic acid were highly enriched. In addition, weighted gene coexpression network analysis (WGCNA) method was used to analyze the physiological index data and transcriptome sequencing data. Four main coexpression modules significantly related to physiological indicators were obtained, and the hub genes in each module were screened according to eigengene-based connectivity value. Haplotype analysis of important candidate genes using soybean germplasm resources showed that there were significant differences in germination indexes between different major haplotypes of *Glyma.17G163200*. Based on the results of enrichment analysis and WGCNA, the regulation model of low temperature tolerance during soybean germination was preliminarily drawn. This study will provide theoretical guidance for analyzing the molecular regulation mechanism of cold tolerance in soybean germination stage.

1 | Introduction

Temperature is an important factor affecting crop growth and development. Unsuitable temperature conditions have adverse effects on crops (YyGreaves 1996). Chilling injury refers to low-temperature stress on crops above the freezing point. The temperature causing chilling injury is generally 0°C–15°C (Somerville 1995). Soybean is rich in oil and protein, and it has

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become an important source of high-quality protein, edible oil, and animal husbandry feed (Clemente and Cahoon 2009; Chaudhary et al. 2015). Similar to many other crops, soybean is a thermophilic crop that is susceptible to chilling injury throughout the growth and development period and is extremely sensitive in the germination and seedling stages (Toda et al. 2011; Robison et al. 2017). The minimum temperatures required for germination of different varieties of soybean differ. Under low-temperature conditions, the germination of soybean is slow, and the germination rate is reduced, resulting in uneven yield (Tyagi and Tripathi 1983). It is very important to study the mechanism of chilling tolerance during soybean germination for soybean variety improvement and yield increase in soybean production (Dong et al. 2021).

Chilling injury mainly affects the structure, function, stability, and enzyme activity of the protoplast membrane, resulting in physiological drought and accumulation of toxic substances, thus adversely affecting plant growth and development (Steponkus 1984; Zhang et al. 2019; Athani 2022). Chilling tolerance refers to the adaptability of plants to low temperature (Somerville 1995). The ratio of saturated fatty acids to unsaturated fatty acids in the plasma membrane is the key to plant chilling tolerance. Physiological damage caused by low-temperature changes the membrane phase from a liquid crystal state to a gel state, which reduces membrane fluidity and leads to metabolic disorder. The higher the degree of unsaturated fatty acids in the membrane lipids is, the lower the phase transition temperature of the membrane lipids is (Welti et al. 2002; Ge et al. 2019; Liang et al. 2020). Plant chilling injury is not only closely related to plasma membrane components but also to functional proteins, the membrane lipid peroxidation defense system (Chi et al. 2021), osmotic adjustment substances (Siddique, Kandpal, and Kumar 2018; He et al. 2021), and plant hormones (Sadura and Janeczko 2018). Under low temperature during germination, the cell membrane structure can be damaged, and the membrane permeability increased, leading to leakage of ions and small molecular metabolites, which disrupts cellular metabolism and synthesis and finally reduces germination rate (Bewley 1997; Dubert 2021).

At present, the research on the molecular mechanism of plant cold tolerance has made a lot of progress. In a study of soybean cold tolerance genes, Gao et al. (2011) found low-temperature induced soybean *GmbZIP1* gene expression. The overexpressed exhibited an enhanced ABA response and caused stomatal closure under stress, which improved the tolerance of transgenic plants to low temperature (Gao et al. 2011). WRKY is a class of transcription factors that play a specific role in disease resistance and stress resistance in plants. According to the structural characteristics, WRKY transcription factors are divided into three types: The first type contains two WRKY domains, and the zinc finger structure is C2H2 type; the second type of zinc finger structure is the same as the first type but only contains a WRKY domain at the C-terminus; the third type contains a WRKY domain, and GmWRKY21 belongs to the second type. Zhou et al. transformed the soybean *GmWRKY21* gene into *Arabidopsis thaliana* and treated transgenic *A. thaliana* with drought, high salt, and low-temperature stress. The results showed that resistance to salt stress and drought stress was not found but there was good tolerance to low-temperature stress (Zhou et al. 2008). *AtTCF1*, a regulator of chromosome condensation 1 family protein, confers *A. thaliana* tolerance to chilling and freezing. *GmTCF1a* is the homolog of *AtTCF1* in soybean, the exogenous expression of which enhanced the cold tolerance of plants (Dong et al. 2021).

In the early stage of this study, the team constructed a chromosome segment substitution line (CSSL) population with the soybean cultivar suinong14 as the recurrent parent and the wild bean ZYD00006 as the recipient parent. In this study, based on phenotypic data, a chilling-sensitive line and a chilling-tolerant line were selected from the CSSL population for physiological performance determination and transcriptome sequencing to reveal the coexpression network of chilling tolerance genes as well as the key functional genes during soybean germination. This study will provide reference for the analysis of the chilling tolerance gene expression network and the utilization of appropriate genes in soybean breeding.

2 | Materials and Methods

2.1 | Field Planting and Management of Soybean

The soybean cultivar SN14 was used as the recurrent parent, and the wild soybean ZYD00006 was used as the recipient parent. Through hybridization, backcross, and selfing, a CSSL population containing 213 lines was constructed. The population was planted in an experimental field in Gongzhuling City, Jilin Province, China, in 2020. A randomized block design with plant spacing of 15-cm, 96-row spacing of 65 cm was used. The field management accord to local conventional soybean production.

2.2 | Identification of Chilling Tolerance During Seed Germination

The experiment was divided into two treatments: a stress group (6°C) and a control group (20°C). Each treatment was repeated three times. The seeds were sterilized with 1% sodium hypochlorite solution for 30–60 s and rinsed with distilled water three times. Each of the 40 seeds were placed in a sterilized 9-cm petri dish. A layer of sterile filter paper was laid on both sides of the culture dish, and the culture dish with seeds was placed in an incubator at 20°C for 12 h for imbibition. The stress group was transferred to a 6°C incubator, and the control group continued to be cultured in the 20°C incubator. The number of germinations was counted every other day. The germination standard of seeds is that the bud length is at least a quarter of the seed length. The germination potential (GP) was defined as the ratio of the number of germinated seeds in the first 4 days to the total number of seeds. The germination rate (GR) was defined as the ratio of the number of germinated seeds in the first 6 days to the total number of seeds. Relative germination potential (RGP) = number of seeds germinated at 6°C/20°C on Day 4; relative germination rate (RGR) = 6°C germination seed number/20°C germination seed number on the sixth day.

2.3 | Physiological Index Determination

The chilling-tolerant line R48 (BC_3F_6) and chilling-sensitive line R89 (BC3F7) in the CSSL population were used as sampling

materials for physiological index determination and transcriptome analysis. The test was divided into two treatments: the stress group (6°C) and the control group (20°C). For the germination test, refer to Section 2.1. The embryos of the stress group and the control group were taken as experimental materials at 0, 12, 24, 36, and 48 h after imbibition. Chilling stress samples at 0, 12, 24, 36, and 48 h were numbered 1–5, respectively; control treatment samples at 12, 24, 36, and 48 h were numbered 6–9, respectively. Five biological replicates were taken for each stage to determine physiological indexes.

All physiological indexes were determined using assay kits (Comin, www.cominbio.com, Suzhou, China). SPSS software was used to analyze the data. Paired sample *t* test was used to analyze the difference between the two varieties at the same time, and the significance level was set to 0.05. The difference in a single line in the same treatment at different time points was analyzed using one-way ANOVA. Duncan method was used for multiple comparison, and the significance level was set to 0.05. The Pearson correlation coefficient was used to analyze the correlation between physiological traits.

2.4 | RNA Extraction, Library Construction, and Sequence Analysis

Transcriptome analysis sampling method refers to the physiological index determination sampling method described in Section 2.3. Three biological replicates were taken for each stage to transcriptome analysis. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's protocol. The purity, concentration, and integrity of the RNA samples were identified using Nanodrop, Qubit 2.0, and Agilent 2100 instruments, respectively. After the samples were qualified, the poly(A) method was used to enrich the mRNA. First, oligo (dT) primers were used to reverse transcribe the mRNA, and the cDNA was fragmented. A QIAQuick PCR kit was used to purify the cDNA and repair the ends. After adding poly(A) and adaptors, agarose gel electrophoresis was used to select the fragment size, and PCR amplification was performed to construct the library. Finally, the Q-PCR method was used to accurately quantify the effective concentration of the library (the effective concentration of the library > 2 nM) to ensure the quality of the library. After the test was qualified, the sequencing platform Illumina HiSeq 4000 PE150 platform was used for sequencing. Because the sequence measured by RNA-Seq was a series of randomly interrupted cDNA fragments, in theory, the contents of G and C, A and T should be equal in each sequencing cycle, and the whole sequencing process is stable and horizontal. The RNA-Seq data have been deposited to the Sequence Read Archive (SRA) under accession number PRJNA999924.

2.5 | Transcriptome Assembly and Differential Expression Analysis

After obtaining the sequencing data, the following filtering methods were performed: reads containing connectors were removed; remove low-quality reads (including reads that remove more than 10% of N; and remove more than 50% of the reads with mass value $Q \leq 10$). The high-quality clean data obtained after a series of quality control are provided in FASTQ format. The high-quality clean data were compared with the reference genome using the HISAT system. The reference genome was *Glycine max* Wm82.a2.v1. Quantitative analysis of genes was performed using HTseq-count software. StringTie software was used to compare the assembled reads. The transcript reads were normalized using the DESeq2 package of the R software, and then, the fragments per kilobase of exon model per million mapped fragments (FPKM) value were calculated. The standardized FPKM value for each gene represented the expression level of that transcript. The DESeq2 software package was used to calculate the fold change (FC) of differentially expressed genes. Differentially expressed genes with $\log_2 FC \geq 2$ and p value ≤ 0.01 were screened as significantly differentially expressed genes.

2.6 | Construction of Gene Weighted Coepression Network

Coexpression network analysis was performed using the R package WGCNA. All genes were imported using the following parameter settings: FPKM ≥ 1 and variation of FPKM $= 0.5$. The hierarchical clustering of genes in the similarity-difference matrix was performed by the hclust function in the WGCNA package, and the generated clustering tree modules were defined by dynamic tree cutting. The module-trait association was estimated using the correlation between the module characteristic genes.

2.7 | Transcriptome Data Validation

The accuracy of the transcriptome results was verified by qRT-PCR. Refer to Section 2.3 for sampling of experimental materials, which was performed with three biological repeats. RNA was extracted by the TRIzol method. Primer5 software was used to design gene primers, which were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). cDNA was synthesized using TransGen reverse transcription kit (Beijing, China). Refer to previous research progress (Li et al. 2012); we selected UKN2, TUA5, Glyma05g27480, Glyma08g28550, and Glyma.20g141600 for stability verification. The validation materials were R48-2, R48-4, R48-6, R48-8, R89-2, R89-4, R89-6, and R89-8. Each material was biologically repeated three times. The NormFinder package was used to analyze the stability of the expression levels of the six internal reference genes in these materials, and the internal reference gene with the smallest coefficient of variation (CV) was selected as the internal reference gene for qRT-PCR in this study. Glyma08g28550 was selected as the internal reference gene for qRT-PCR (Table S1). The relative gene expression is given by

$$\text{Relative expression} = 2^{-\Delta CT}.$$

2.8 | Haplotype Analysis

The genotype data and phenotypic data of soybean germplasm resources used in haplotype analysis refer to the previous research results of the research group (Zheng et al. 2023).

DNASP5.0 software was used to analyze the haplotype distribution of candidate gene SNP sequences in the germplasm resources. If the number of germplasm resources contained in a haplotype ≥ 5, we use it as the main haplotype and analyze the significant difference in germination index between the main haplotypes.

3 | Results

3.1 | Determination of Physiological Indexes in Soybean Germination Period

According to the identification of the chilling tolerance of the CSSL population at the bud stage, the chilling tolerant line R48 (GP: 73.33%; GR: 92.50%) and chilling sensitive line R89 (GP: 6.67%; GR: 16.67%) were selected to determine the physiological indexes (Figure 1) that revealed the physiological responses of soybean to chilling stress at different stages within the bud stage (Figure 2). All physiological indexes include superoxide dismutase (SOD) activity, peroxidase (POD) activity, catalase (CAT) activity, malondialdehyde (MDA) content, soluble sugar (SS) content, soluble protein (SP) content, oxygen free radical (OFR) content, H_2O_2 content, and proline (PRO) content.

MDA is the final product of membrane lipid peroxidation, which can reflect the degree of damage to the cell membrane. The MDA content of the two lines peaked at 12 h of chilling stress, and that of R89 was significantly higher than that of R48. The MDA content of R89 was significantly higher than that of R48 at all time points, except at 36 h, and the MDA content of the same line under different treatments had different trends.

The metabolic balance of active oxygen is an important condition for the normal growth and development of plants. In this study, the content of reactive oxygen species (OFR and H_2O_2) and the activities of enzymes (CAT, SOD, and POD) related to the reactive oxygen scavenging system in R48 and R89 were determined. The OFR content of R48 did not change significantly under chilling stress. The OFR content of R89 increased significantly at 48 h and was significantly higher than that of R48. The H_2O_2 content in R48 and R89 increased first and then decreased under chilling stress. The H_2O_2 content of R89 was significantly higher than that of R48 at each time point of chilling stress. Compared with the control group, the content of H_2O_2 and OFR in most of the chilling stress periods was higher than that in the control group. The CAT activity of R48 was significantly higher than that of R89 under chilling stress for 48 h. The SOD activity of R48 increased first and then decreased from 0 to 36 h under chilling stress. The SOD activity of R48 was significantly higher than that of R89 at 24 and 48 h under chilling stress. The POD activity of R48 was significantly higher than that of R89 at 0, 36, and 48 h under chilling stress. The trends of the three enzyme activities in the stress group and control group differed. When R48 and R89 were subjected to chilling stress, H_2O_2 and OFR increased, indicating that the balance of active oxygen metabolism in R48 and R89 was destroyed. The activity of enzymes related to the active oxygen scavenging system in R48 was greater than that in R89, and the corresponding ability to clean up reactive oxygen species was stronger. Therefore, R48 line showed more chilling tolerance.

SS, SP, and PRO are important regulators of plant osmotic pressure. The content of these regulators in R48 and R89 was determined in this study. Under chilling stress, the PRO content of R48 first increased and then decreased. At 24 and 48 h, the PRO content of R89 was significantly lower than that of R48. The SS content of R48 first decreased and then increased under chilling stress. At 36 and 48 h, the SS content of R48 was significantly higher than that of R89. The SP content of R89 was stable at chilling. R48 fluctuated greatly, increased significantly at 48 h, and was significantly higher than that of R89. Compared with the control, the content and trends of PRO, SS, and SP under chilling stress changed. Under chilling stress, the content of osmotic potential regulators of SS, SP, and PRO in R48 was higher than that in R89, so that the ability to maintain osmotic pressure steady state was stronger and the performance was more resistant to chilling.

The results showed that CAT activity was positively correlated with SP content and the correlation coefficient was 0.59. In addition, CAT activity was significantly positively correlated with POD activity, with a correlation coefficient of 0.45. MDA was significantly negatively correlated with SS content. SOD activity was significantly positively correlated with POD activity, and the correlation coefficient was 0.49. SOD activity was also significantly negatively correlated with H_2O_2 content, and the correlation coefficient was −0.47. There was a correlation between osmoregulatory substances and ROS and their scavenging systems.

In summary, under chilling stress, the change trend of the above physiological indexes was different from that of the control, and the difference between R48 and R89 was significant. These physiological indexes have different responses to chilling stress, which may be the reason for the difference in

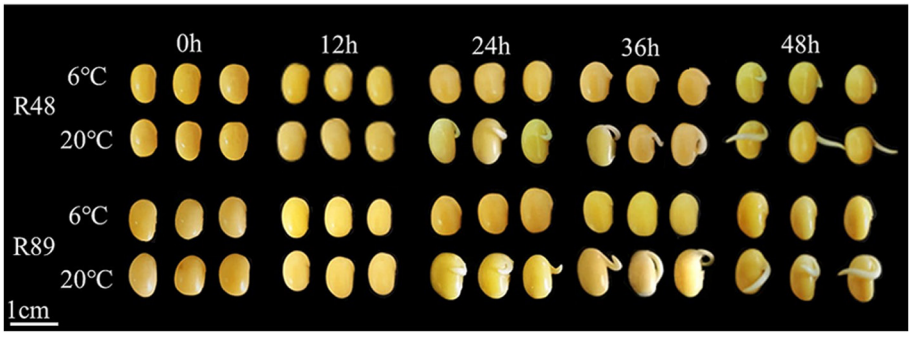


FIGURE 1 | Comparison of germination of R48 and R89 at 6°C and 20°C.

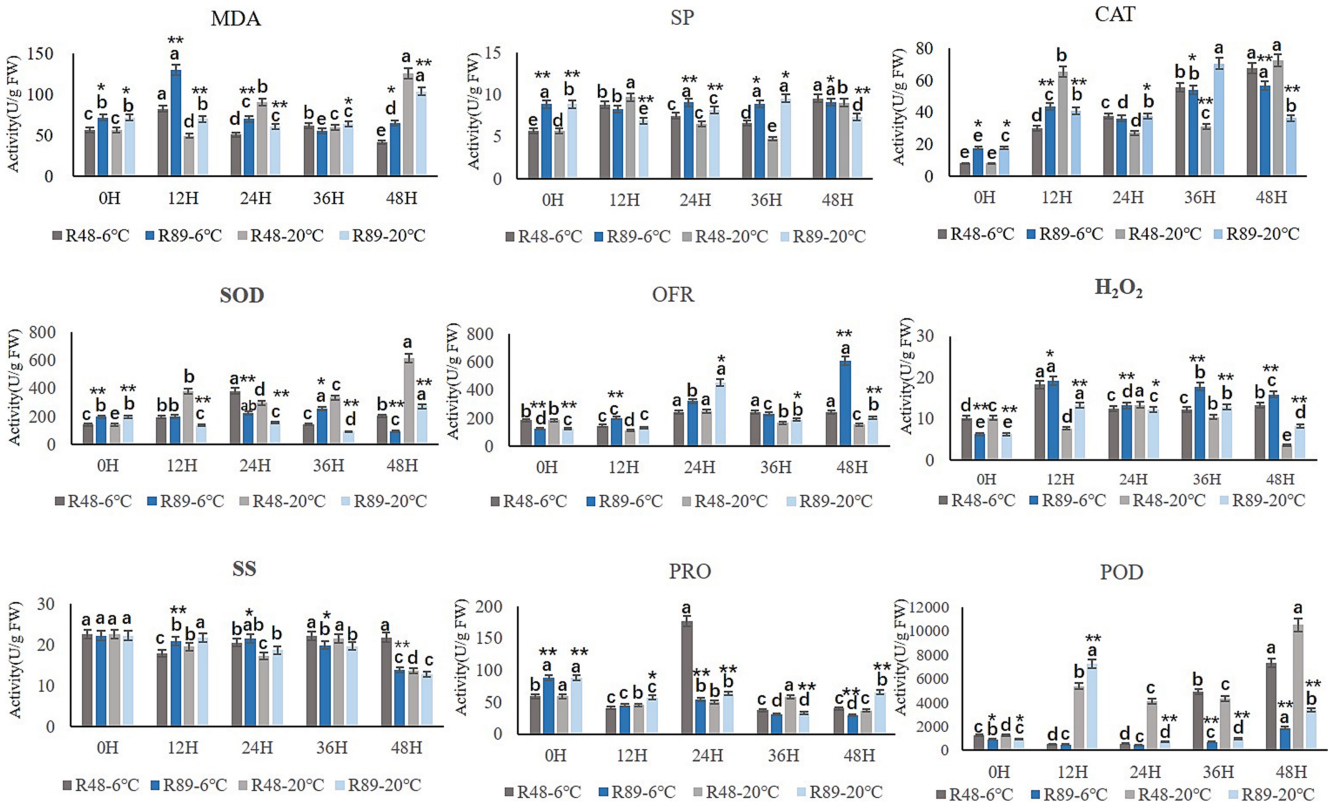


FIGURE 2 | Determination of the physiological indexes of the chilling treatment group and the control group of R48 and R89 in each test period. An asterisk indicates a significant difference between R48 and R89 at the same treatment time point (*$p < 0.05$, **$p < 0.01$). Letters indicate whether the same strain in the same treatment group changes significantly over time (different letters indicate $p < 0.05$).

chilling tolerance between the two lines. On the whole, under chilling stress, the activities of CAT, POD, and SOD in R89 were significantly lower than those in R48, resulting in a significant increase in the content of OFR and H_2O_2, resulting in membrane lipid peroxidation and a large accumulation of MDA. The content of SS, SP, and PRO in R89 was also significantly lower than that in R48, and the osmotic homeostasis was difficult to maintain, which was more vulnerable to chilling damage (Figure 3).

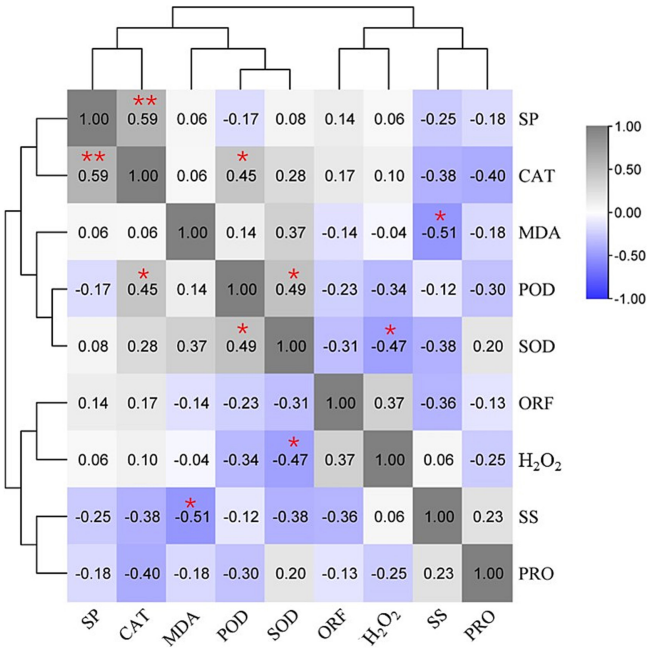


FIGURE 3 | Correlation analysis of physiological indexes (*correlation at the 0.05 level; **correlation at the 0.01 level).

3.2 | RNA-Seq Analysis

To explore the molecular mechanism of these physiological responses during the adaptation of soybean germination to chilling stress, the transcriptional levels of R89 and R48 at 0, 12, 24, and 48 h under 6°C chilling stress with a corresponding control group (20°C) at each time point were compared and analyzed. Between 19.1125 and 24.2508 million clean reads were obtained from each sample, with an average of 20.723 million sequences. The GC content (G + C bases) was 43.79%–45.76%, the average was 44.50%, and the Q30 was 93.33%–95.23% with an average of 94.50%. In each sample, 91.51%–96.56% of clean reads were mapped to the reference genome, of which 85.26%–94.32% were uniquely mapped (Table S2). DESeq2 software was used to analyze the differentially expressed genes between the two lines at the same testing time point under the same treatment, with a cut-off of $p \leq 0.01$ and fold change ≥ 2. After chilling stress, the number of differentially expressed genes at each time point decreased compared with the control, and the number of upregulated genes was greater than the number of downregulated genes (Figure 4). These results indicated that at the molecular level, the two lines each had a response mechanism to resist chilling stress and the different expression levels

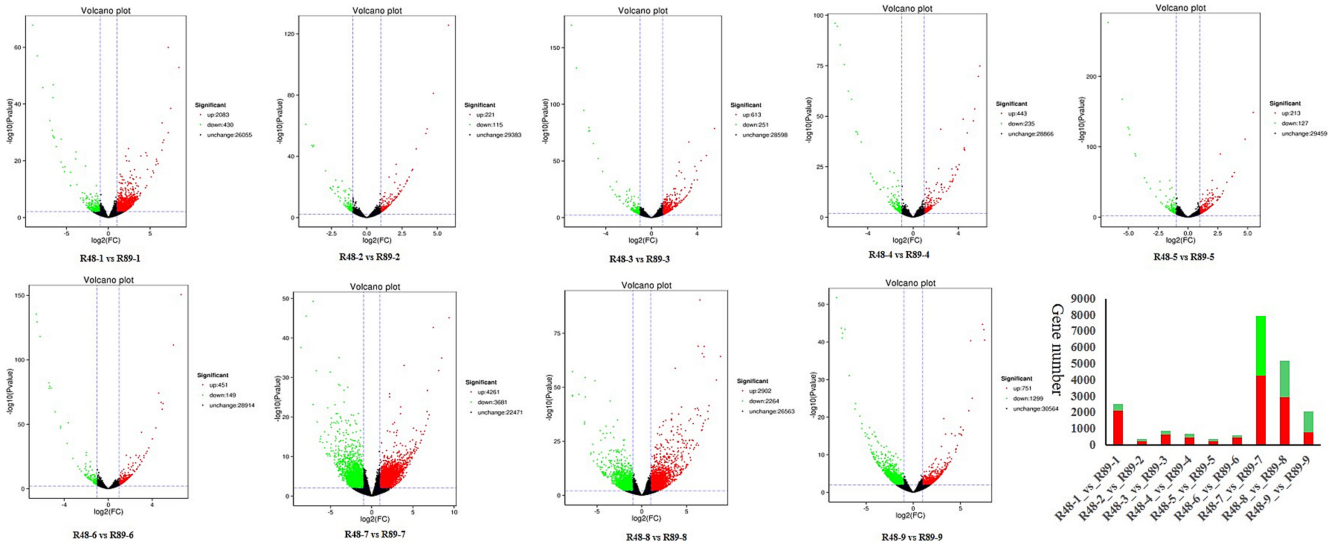


FIGURE 4 | Volcano map of differentially expressed genes and quantitative statistics of differentially expressed genes at each time period for R48 and R89. Red represents upregulated gene expression, and green represents downregulated gene expression.

of chilling response genes led to the difference in chilling tolerance between the two lines.

Gene Ontology (GO) enrichment analysis was performed on the differentially expressed genes, and REVIGO software was used to remove redundancies from the enriched GO terms (Table S3). In the biological process category, the frequency of the GO term related to ABA, "response to abscisic acid-activated signaling pathway," was high. In addition, the frequency of the GO terms "response to hydrogen peroxide" and "lipid storage" was also high, and the frequency of the GO term "plasma membrane" was high in the cell components category. In the molecular function category, "oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen" appeared frequently. In addition, we also performed Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis on these differentially expressed genes (Table S4), which identified that the differentially expressed genes were related to the "flavonoid biosynthesis" pathway, "starch and sucrose metabolism" pathway, and the metabolism pathways associated with unsaturated fatty acids such as "α-linoleic acid." ABA is an important hormone regulating plant stress tolerance. Flavonoids, unsaturated fatty acids, hydrogen peroxide, and other substances are related to membrane lipid peroxidation. Starch and sugar are important regulators to maintain cell homeostasis. Therefore, the differentially expressed genes enriched above may be related to the chilling tolerance of soybean during germination.

3.3 | WGCNA

To understand more comprehensively the molecular regulatory network under cold stress in the soybean germination stage, the gene expression data obtained by RNA-seq analysis were used for weighted gene coexpression network analysis. Four main modules were identified: the blue module, the brown module, the green module, and the turquoise module (Figure 5A,B). The corresponding numbers of genes in each module were 1607, 1874, 150, and 6005, respectively. In addition, to explore the biological significance of each module, the relationship between each module and each physiological index was also analyzed (Figure 5C). The turquoise module was significantly positively correlated with POD activity and SOD activity, and the correlation coefficients were 0.52 and 0.6, respectively. This module was also significantly negatively correlated with H_2O_2 and SS content, and the correlation coefficients were −0.55 and −0.53, respectively. The blue module was significantly negatively correlated with CAT activity, with a correlation coefficient of −0.54. The brown module was significantly negatively correlated with POD activity, with a correlation coefficient of 0.03. The green module was positively correlated with CAT activity, and the correlation coefficient was 0.47.

The hub gene is a gene that plays a vital role in biological processes. In related pathways, the regulation of other genes is often affected by the hub gene. It has a high correlation with other genes in the coexpression network (Figure 6). The eigengene-based connectivity (KME) value can be used to evaluate the connectivity between genes. The first five genes with the highest KME value in each module were selected as hub genes, and a total of 20 hub genes were obtained (Tables S5 and S6). The hub genes in the blue module were *Glyma.07G120900*, *Glyma.08G253000*, *Glyma.13G296400*, *Glyma.13G261200*, and *Glyma.09G066700*; the hub genes in the brown module were *Glyma.13G236600*, *Glycine_max_newGene_8571*, *Glyma.02G257600*, *Glyma.01G009400*, and *Glyma.08G095600*; the hub genes in the green module were *Glyma.20G179600*, *Glyma.09G187200*, *Glyma.05G070400*, *Glyma.06G100100*, and *Glyma.06G309000*; and the hub genes in the turquoise module were *Glyma.12G183100*, *Glyma.01G213400*, *Glyma.11G215500*, *Glyma.17G163200*, and *Glyma.12G192200*.

According to Table S5, the functions of these hub genes are ERF domain protein 12, proline-rich family protein, Aldo/keto reductase family, Peroxidase, Glutamine synthetase, and so on. Previous studies have shown that these functions are involved in the regulation of plant stress tolerance (Jung et al. 2021; Sun et al. 2018); therefore, these genes may be the key genes affecting the chilling tolerance of soybean at germination stage.

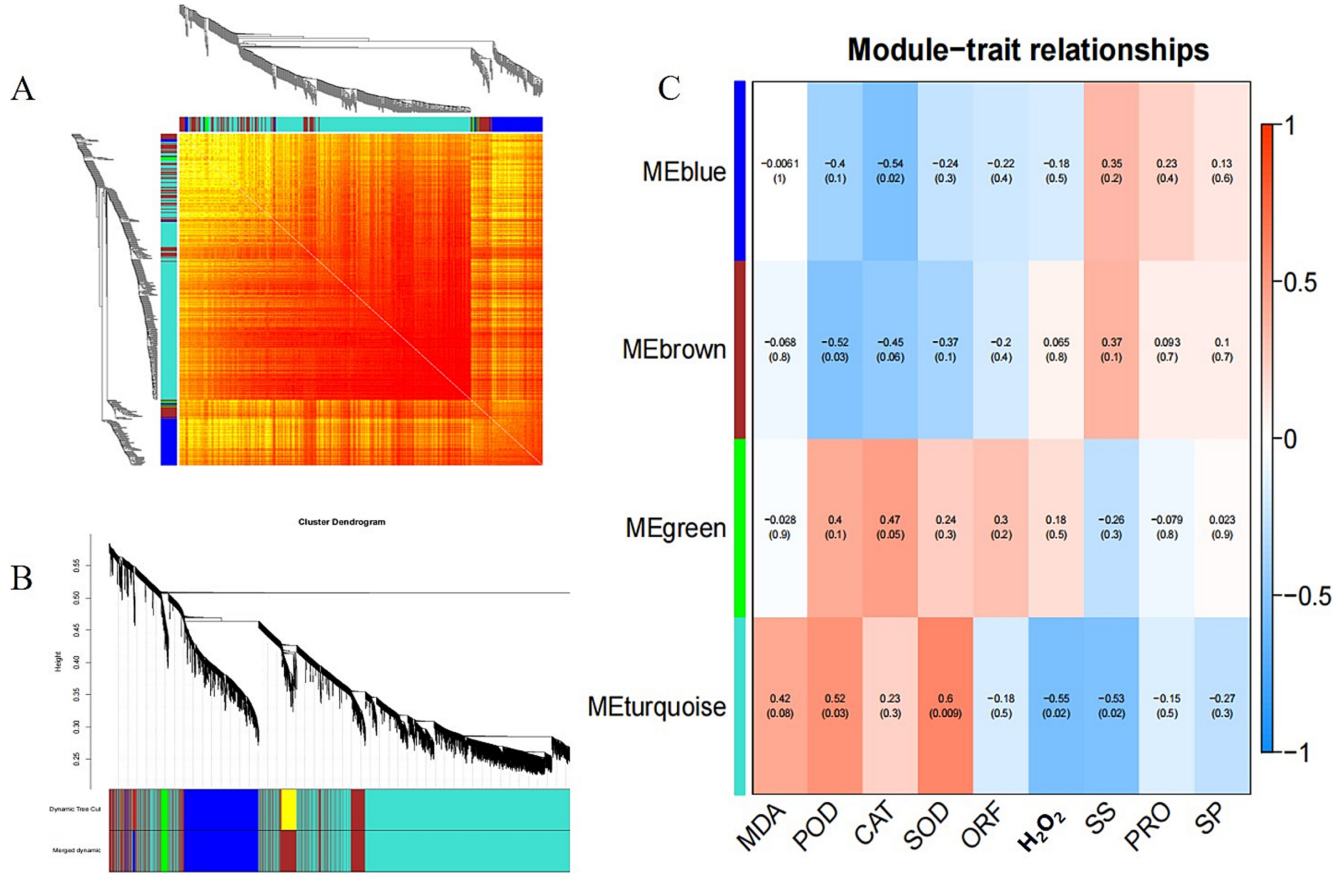


FIGURE 5 | Coexpression network analysis of all genes in the chilling tolerant transcriptome sequencing of R48 and R89 at the germination stage. (A) Gene coexpression network correlation heat map. (B) Hierarchical clustering tree of the coexpression module. Each leaf on the tree represents a gene, and different colors represent different modules. (C) Correlation analysis between the gene coexpression network modules and physiological indicators. The transverse axis represents different physiological characteristics, and the vertical axis represents the module characteristics in each module.

3.4 | Transcription Factor Analysis

We analyzed the transcription factors of the four main modules (Figure 7). The top five transcription factors in the blue module were AP2/ERF-ERF, MYB, WRKY, C2H2, and GRAS, with frequencies 31, 18, 17, 13, and 10, respectively. The top five transcription factors in the brown module were AP2/ERF-ERF, MYB, BHLH, C2H2, and NAC, and the frequencies were 15, 15, 11, 11, and 11, respectively; the top five transcription factors in the green module were AP2/ERF-ERF, Trihelix, AP2/ERF-AP2, C3H, and GARP-ARR-B, and the frequencies were 3, 2, 1, 1, and 1, respectively; the top five transcription factors in the turquoise module were BHLH, MYB, AP2/ERF-ERF, bZIP, and GARP-G2-like, and the frequencies were 56, 41, 40, 32, and 15, respectively. The frequencies of AP2/ERF-ERF, MYB, and BHLH were the highest in the four modules, suggesting that they may be related to the cold tolerance of soybean sprouts. A number of studies have shown that AP2/ERF-ERF, MYB, and BHLH are involved in the regulation of plant stress tolerance, so the genes encoding these transcription factors may be functional genes that affect the chilling tolerance of soybean during germination (Lin et al. 2014; Liu et al. 2014).

3.5 | Verification of RNA-Seq Accuracy With qRT-PCR

To test the accuracy of the RNA-seq results, eight genes were randomly selected from the important genes identified in the functional enrichment analyses and WGCNA for qRT-PCR verification. Genes and primers are shown in Table S7. The relative expression data obtained by qRT-PCR and per million fragments per kilogram of bases (FPKM) data obtained by RNA-seq were normalized by Tbtools v1.1043 software, and a heatmap was constructed. It can be seen from Figure S1 that the trend of the gene expression data obtained by the two methods was consistent. The correlation coefficients calculated by SPSS 17.0 software ranged from 0.584 to 0.945, and the significance was less than 0.05 (Table S8). These results indicate that the RNA-seq results were accurate.

3.6 | Haplotype Analysis

Haplotype analysis of the above verified important genes was performed using 261 soybean germplasm resources, and the RGP and RGR were compared among the main haplotypes. It was found that there were significant differences in RGE

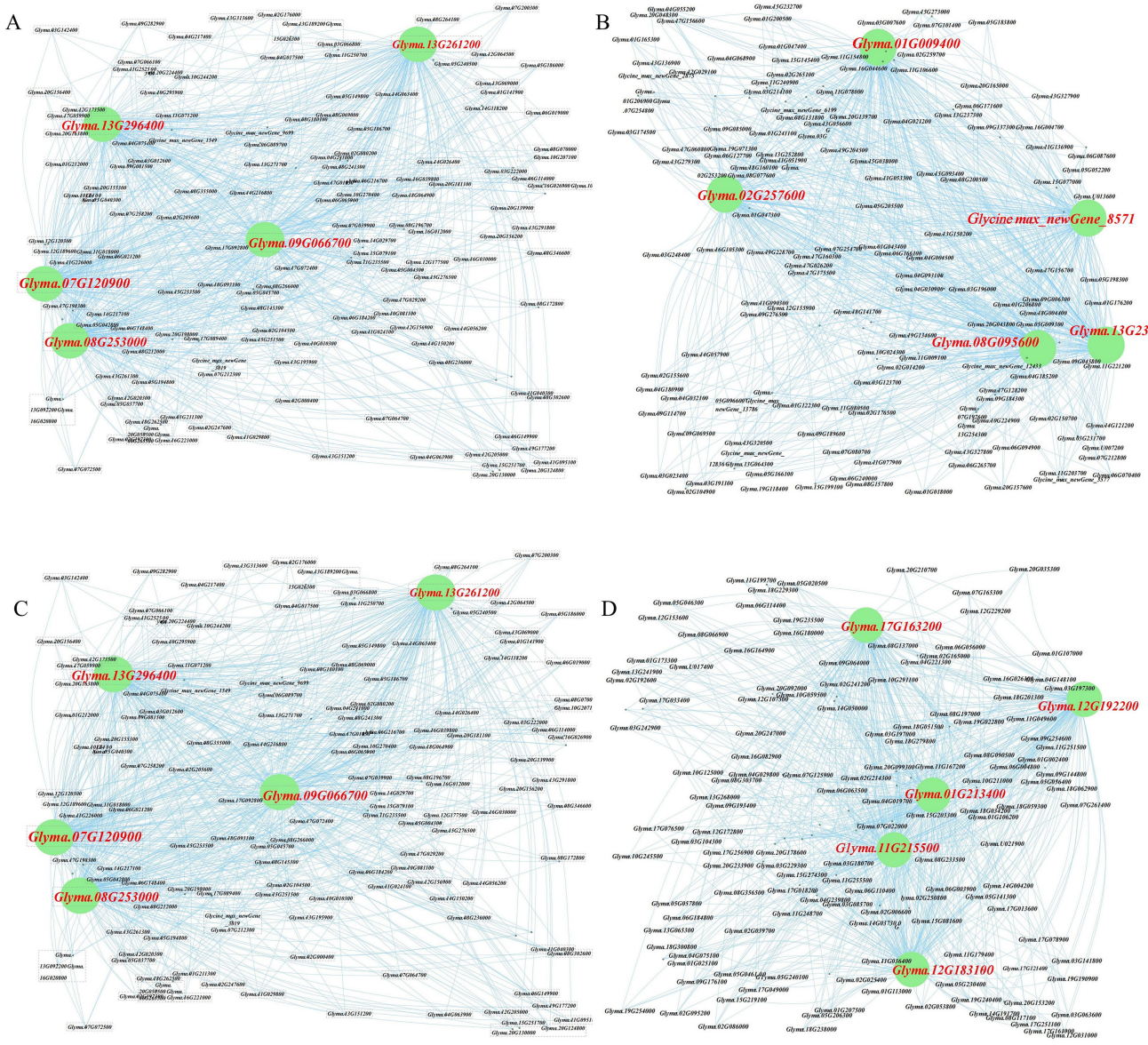


FIGURE 6 | Analysis of the hub gene coexpression network of the main modules. (A) Blue module, (B) brown module, (C) green module, and (D) turquoise module. Genes with high connectivity are represented by large green nodes.

and RGR among the main haplotypes of *Glyma.17G163200* (Figure 8A). The RGR of Hap2 was significantly higher than that of Hap4, and the RGP of Hap1 and Hap2 was significantly higher than that of Hap4. *Glyma.17G163200* major haplotypes SNP variation is shown in Figure 8B,C.

4 | Discussion

Chilling is one of the main abiotic stresses encountered in the process of crop growth and development. If chilling stress is encountered in the bud stage, it will seriously affect the rate and quality of germination. Studying the response mechanism of crop germination to chilling stress is of great significance for reducing and alleviating chilling damage during crop germination. At present, there are few studies on the regulatory mechanism by which soybean adapts to chilling stress. In this study, we screened two soybean lines, R48 and R89, with different sensitivities to chilling stress at the germination stage, and studied the physiological changes and gene expression changes under chilling stress at the germination stage by physiological index determination and RNA-seq. The changes in physiological indexes, such as osmotic adjustment substances, active oxygen, and related enzymes, in soybean sprouts under chilling stress were expounded. Several pathways and key genes related to chilling stress in soybean sprouts were identified.

4.1 | Physiological Response of Soybean to Chilling Stress at the Germination Stage

Chilling stress adversely affects plant growth and development, limiting plant species distribution and crop yield (Pearce and RS 2001). In the long process of evolution, plants

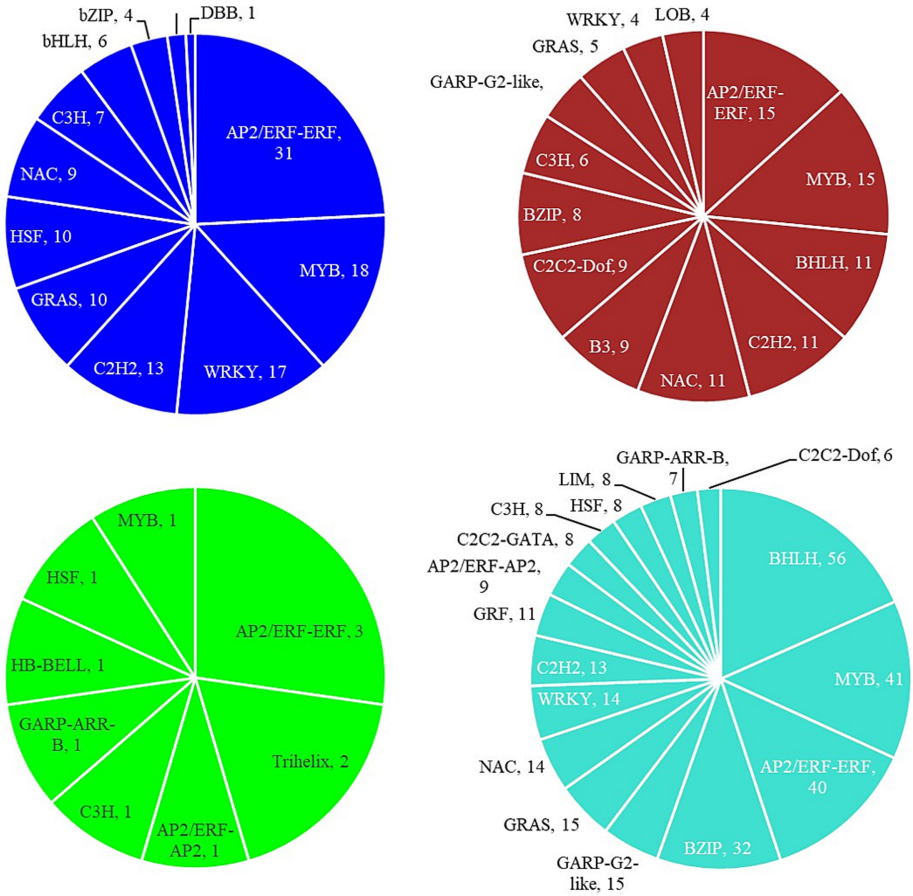


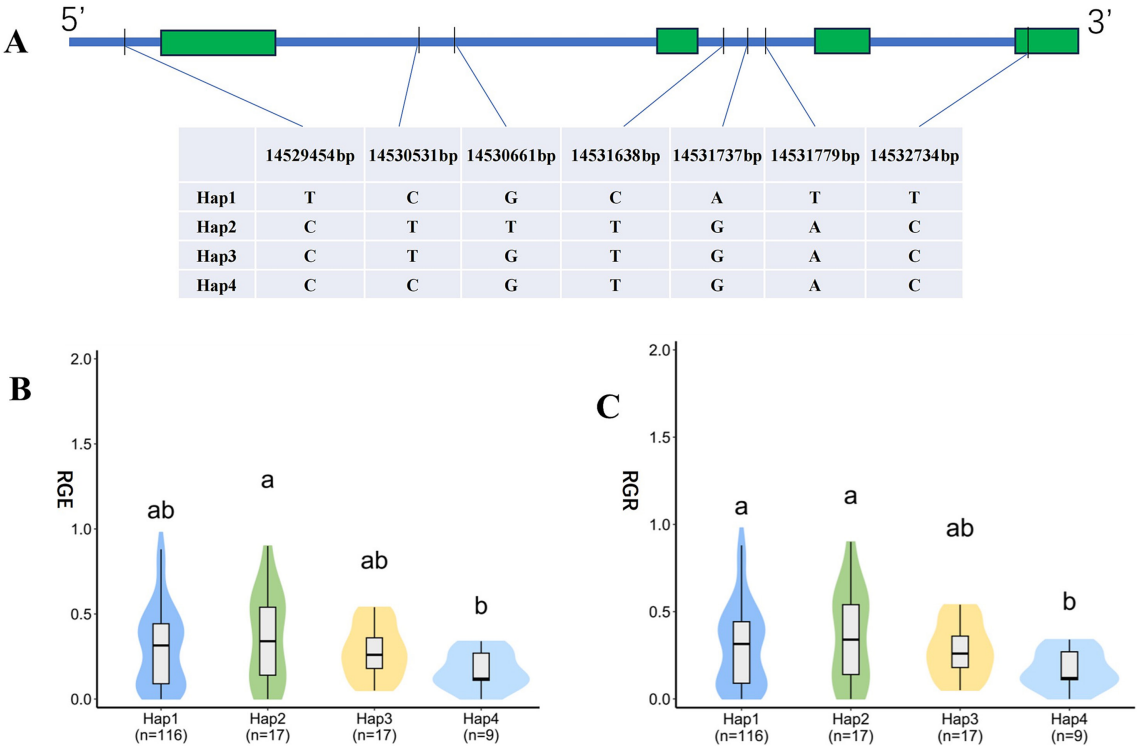
FIGURE 7 | Main module transcription factor analysis. The pie color corresponds to the module color.

have acquired a certain ability to adapt to chilling stress, and chilling can produce a series of physiological responses to resist stress (Ding, Shi, and Yang 2019). Under chilling stress, an imbalance in active oxygen metabolism and aggravation of membrane lipid peroxidation affects the structure and function of biomembranes and macromolecules (Gill and Tuteja 2010; Banerjee, Tripathi, and Roychoudhury 2018). This study found that the accumulation of H_2O_2 and OFR was higher in R89 than in R48, which was consistent with their tolerances to cold stress. The accumulation peaks of H_2O_2 and OFR appeared at 12 and 48 h under chilling stress, respectively, which may be related to the transmission mechanism of the ROS signal. Puntarulo, Sánchez, and Boveris (1988) found that soybean seeds produce H_2O_2 and OFR during imbibition and germination and that H_2O_2 was produced faster than OFR, which was consistent with the changes in H_2O_2 and OFR in the control group in this study.

MDA is one of the important products of membrane lipid peroxidation, and it is also a marker to identify whether the cell membrane is destroyed (Yasar, Ellialtioglu, and Yildiz 2008). Therefore, MDA content is often used to characterize the extent to which plants are subjected to stress in resistance studies (Foyer and Noctor 2016). In this study, the MDA content of R89 was significantly increased at 12 h under chilling stress and was significantly higher than that of R48, and the MDA content gradually decreased at the later stages of stress. This result shows that R89 is more sensitive to cold stress, and the decrease in MDA content in the later stage may be due to the initiation of a cold tolerance mechanism to reduce the degree of membrane lipid peroxidation.

POD, SOD, and CAT are important components of the active oxygen scavenging system. Numerous studies have shown that under chilling stress, the stronger the enzyme activity of the ROS scavenging system, the better the resistance of plant varieties (Cheng et al. 2020; Zhang et al. 2022a). In this study, compared with the control, chilling stress seriously affected the POD enzyme activity at 12 h of germination. At 24 h, R48 was still severely inhibited. At 36 and 48 h, the POD enzyme activity of R48 was greatly improved and significantly higher than that of R89, which was consistent with the resistance of R48 and R89 to low temperature. Correlation analysis showed that POD activity was positively correlated with CAT and SOD activity, which was consistent with the results of Zhang et al. (2022a).

Under chilling stress, genes related to osmotic regulation in plants are expressed, and the content of various osmotic regulators increases, giving plants a line of osmotic regulation capabilities. Studies have shown that osmotic adjustment substances, such as soluble proteins, soluble sugar, and prolines, are important osmotic adjustment factors when plants are subjected to abiotic stresses, such as low temperature (Airaki et al. 2012; Zheng, Li, and Sun 2015). In this study, under chilling stress, the SS content of R48 was significantly higher than that of R89 at 36 h, whereas the SP content of R48 was significantly higher than that of R89 at 48 h.



	14529454bp	14530531bp	14530661bp	14531638bp	14531737bp	14531779bp	14532734bp
Hap1	T	C	G	C	A	T	T
Hap2	C	T	T	T	G	A	C
Hap3	C	T	G	T	G	A	C
Hap4	C	C	G	T	G	A	C

FIGURE 8 | Haplotype analysis of *Glyma.17G163200*. (A) The main haplotype SNP variation of *Glyma.17G163200*, (B) The significance analysis of RGP difference between the main haplotypes. (C) The significance analysis of RGR difference between the main haplotypes (different letters indicate $p < 0.05$).

This may be because plants accumulate soluble sugar under chilling stress, promoting the accumulation of ABA and indirectly inducing protein synthesis (Monroy, Sangwan, and Dhindsa 1998). In general, the higher the proline content, the better the cold resistance of plants (Nejadsadeghi et al. 2014). In this study, the proline content of R48 increased significantly at 24 h of chilling stress, which was significantly higher than that of R89, and it was higher than that of R89 at 36 and 48 h, which was consistent with previous studies. It is worth noting that the proline content of R48 and R89 decreased at 36 and 48 h under chilling stress. This may be because after a long period of chilling stress, the supply of carbohydrates in plants was seriously hindered, which affected the formation of glutamic acid and the synthesis of proline.

4.2 | Analysis of Differentially Expressed Genes Between R49 and R89

In this study, GO enrichment and KEGG enrichment analyses were performed on the differentially expressed genes obtained from transcriptome sequencing. GO terms or KEGG metabolic pathways related to flavonoids, unsaturated fatty acids, ABA, hydrogen peroxide, starch, and sucrose were highly enriched. Flavonoids include flavonoids, flavonols, isoflavones, and anthocyanins. Flavonoids have strong free radical scavenging activity and antioxidant activity. In plants, flavonoids are often involved in the oxidative stress response (Ariga, Koshiyama, and Fukushima 1988; Landrault et al. 2001; Chapman et al. 2019). Previously, a near-isogenic line was used to map the gene locus T/t associated with cold tolerance in soybean. Subsequent studies showed that the T allele encoded flavonoid-3′-hydroxylase, the antioxidant activity of which contributes to cold tolerance in plants (Takahashi and Asanuma 1996; Toda et al. 2002). In this study, the gene encoding shikimate O-hydroxycinnamoyl transferase was found to be downregulated at 12, 24, and 36 h under chilling stress, but it was also downregulated at 24 and 46 h at 20°C, indicating that the gene may be related to the response to chilling stress at the soybean germination stage.

The increase in membrane lipid desaturation and unsaturated fatty acid concentration at low temperature can improve membrane fluidity and enhance the cold resistance of plants (Scott et al. 1997; Routaboul, Fischer, and Browse 2000). Unsaturated fatty acids mainly include linoleic acid, linolenic acid, and arachidonic acid. Lanna et al. (2005) studied the effect of temperature on the accumulation of unsaturated fatty acids in soybean and found that the activities of CDP-choline:1,2-diacylglycerolcholine phosphotransferase (CTP) and acyl-CoA: Lysophosphatidylcholine acyltransferase (LPCAT) and the accumulation of linolenic acid were all higher at low temperature. The intrinsic relationship between unsaturated fatty acids and low temperature was explained. In this study, the linoleic acid and linolenic acid pathways were enriched several folds. The gene *Glyma.13G347700* encoding linoleate 9S-lipoxygenase in the linoleic acid pathway was subjected to chilling stress for 36 and 48 h. The expression level of R48 was greater than that of R89, indicating that R48 may produce more linoleic acid to resist stress.

ABA is a key regulator of plant tolerance to abiotic stresses and an endogenous inducer of these stresses (Xiong and Yang 2003; Wei et al. 2015). In general, low temperature, drought, and salt stress can cause osmotic stress, which leads to the rapid accumulation

of ABA. ABA regulates the expression of downstream stress response genes by integrating multiple stress signals (Kim et al. 2011). In the process of differential gene analysis in this study, GO terms related to ABA were highly enriched. The expression levels of repeated genes (*Glyma.11G252800*, *Glyma.16G192000*, and *Glyma.18G300500*) in these differential groups were significantly increased under chilling stress. This indicates that these genes may reduce the damage caused by chilling stress on soybean embryos by increasing the response to ABA. It should also be noted that the expression of these genes in the stressed R89 group was greater than that in the R48 group, which was not completely consistent with the difference in resistance to chilling stress. This result indicated that the response mechanism of soybean sprouts to chilling stress was complex and not dependent on only a certain pathway. Previous studies have shown that plants respond not only through ABA-dependent signaling pathways but also through ABA-independent signaling pathways under cold stress. The main regulator of the ABA-dependent signaling pathway is the bZIP transcription factor, and the main regulator of the ABA-independent signaling pathway is the CBF transcription factor (Rihan, Al-Issawi, and Fuller 2017).

Glyma.06G017900 is a CAT biosynthetic gene, which was upregulated at 0 h under chilling stress and 24 h at 20°C, and there was no differential expression in other stages, indicating that the gene responded to chilling stress and changed its expression pattern. Similar to *Glyma.06G017900*, the SOD biosynthetic gene *Glyma.16G153900* was upregulated at 24 and 36 h at 20°C, but there was no differential expression at other stages, indicating that the gene also changed its expression pattern due to chilling stress. A total of 37 POD biosynthetic genes were differentially expressed, which had significant advantages over the number of differentially expressed genes related to SOD biosynthesis and the number of differentially expressed genes related to CAT biosynthesis. Therefore, it is speculated that POD plays a more important role in soybean resistance to chilling stress during germination.

4.3 | WGCNA

In this study, we identified four gene coexpression modules that were significantly correlated with physiological indicators through the joint analysis of WGCNA and physiological studies. The turquoise module hub gene *Glyma.17G163200* encoded a peroxidase, which is related to hydrogen peroxide catabolism. In this study, the gene expression of R48 was higher than that of R89 at 24, 36, and 48 h under chilling stress. *Glyma.11G215500* was also a turquoise module hub gene encoding glutamate synthase. Glutamate plays a central role in amino acid metabolism, plant defense, and productivity in higher plants (Michard et al. 2011; Jia et al. 2015). Zheng et al. (2018) found that *Arabidopsis* mutants of the glutamate synthase genes *AtGLR1.2* and *AtGLR1.3* were sensitive to cold stress and overexpression of *AtGLR1.2* and *AtGLR1.3* improved the adaptability of *Arabidopsis* to cold stress. In this study, the expression of *Glyma.11G215500* in R48 was higher than that in R89 at 24, 36, and 48 h under chilling stress.

The hub gene *Glyma.07G120900* in the blue module belongs to the dynamin-related protein (DRP). Dynamin and DRP comprise a family of structurally similar but functionally diverse GTP-binding proteins (van der Bliek 1999). DRP can form a ring structure around the membrane and force membrane rupture to promote biofilm fusion and fission (Koch et al. 2004; Praefcke and Mcmahon 2004) and are involved in peroxisome biosynthesis (Hu et al. 2009). Peroxisomes are small single-membrane organelles found in almost all eukaryotic cells. Plant peroxisomes mediate multiple biochemical processes, including fatty acid oxidation, photorespiration, hydrogen peroxide metabolism, and phytohormone synthesis (Olsen and Harada 1995; Hayashi and Nishimura 2003).

The hub gene *Glyma.13G236600* of the brown module encodes an ethylene response factor (ERF). ERF is a subfamily of the apetala2/ethylene response factor (AP2/ERF) transcription factor family and contains an AP2 domain. ERF plays an important role in the plant response to abiotic stresses (Jung et al. 2021). Previous studies have found that PtrERF9 acts downstream of the ethylene signal in trifoliate orange under chilling stress. This protein plays an active role in plant cold tolerance by regulating the transcription of *PtrGSTU17* to maintain the ROS balance in plants (Zhang et al. 2022b). Sun et al. (2018) transformed the *Vitis amurensis VaERF080/087* gene into *A. thaliana*. The transgenic plants had higher activity of active oxygen scavenging enzymes and showed better chilling resistance than the wild-type plants under chilling stress. This study found that *Glyma.13G236600* was upregulated in R48 at 12 h and upregulated in R89 at 36 h compared with the control under chilling stress. The expression level of *Glyma.13G236600* in R48 was higher than that in R89 at 12 h under chilling stress, indicating that R48 may respond to chilling stress by upregulating *Glyma.13G236600* in the early stage of stress.

The green module hub gene *Glyma.06G309000* encodes a proline-rich family protein (PRP). PRPs are rich in proline residues and are combined with other amino acids to form repetitive sequences (Stein et al. 2011). Previous studies showed that the expression of the PRP gene seems to be regulated under stress according to the needs of plants. Gothandam et al. (2010) found that overexpression of *OsPRP3* enhanced cold resistance in rice. There are many reports suggesting that overexpression of the PRP gene improved plant tolerance to abiotic stress (Qin et al. 2013; Mellacheruvu et al. 2016). However, some researchers believe that downregulation of the PRP gene under drought stress is helpful for increasing cell proline content to resist adversity (Ting and Mao-Mao 2015, Gujjar et al. 2018). Thus, PRP appears to be a beneficial protein under all types of environmental stresses (Gujjar et al. 2019).

Transcription factors play an important role in response to gene expression regulation caused by environmental factors. Among the four modules, AP2/ERF-ERF, BHLH, and MYB transcription factors had the highest frequency of occurrence. The function and structure of ERF transcription factors have been described previously. The bHLH transcription factor is named so because it contains a highly conserved basic/helix–loop–helix region. bHLH transcription factors are involved in plant responses to adversity, including low-temperature, drought, salinity, and other stresses (Lin et al. 2014; Liu et al. 2014). Similar to ERF transcription factor, ethylene can induce the expression of the bHLH transcription factor gene. Huang et al. (2013) cloned

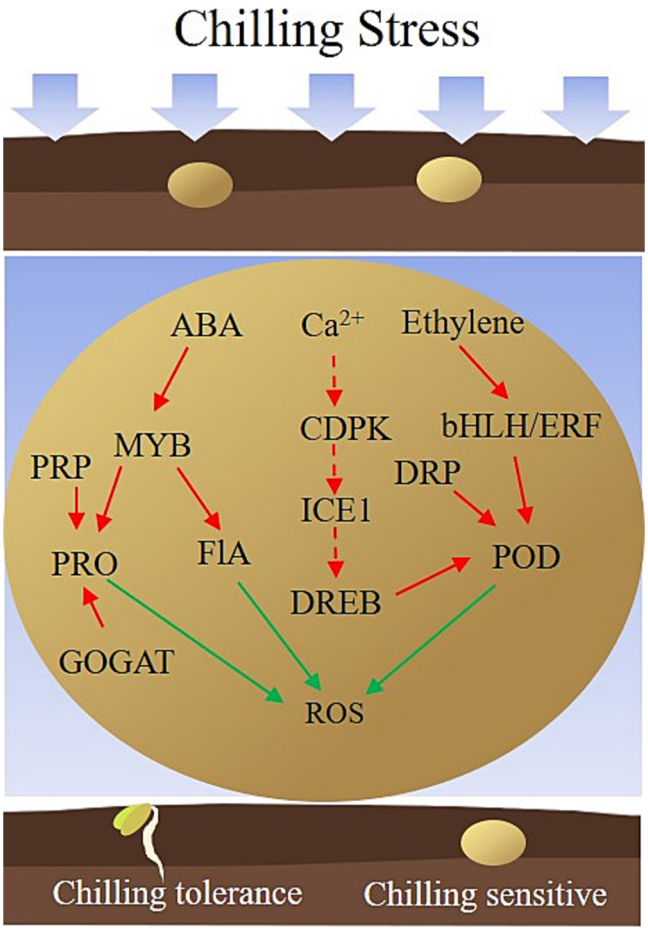


FIGURE 9 | Proposed model of chilling tolerance pathways during soybean germination. ABA, abscisic acid; CDPK, calcium-dependent protein kinase; FLA, flavonoid; GOGAT, glutamate synthase; ICE1, CBF expression inducing factor 1; PRP, proline-rich protein. The red arrow represents promotion, and the green arrow represents inhibition. The dotted arrow is the interaction obtained in other studies.

PtrbHLH induced by low temperature from *Poncirus trifoliata* and found that it participated in the plant response to cold stress signaling by regulating POD-mediated H_2O_2 scavenging. As the largest transcription factor in plant metabolic regulation, MYB transcription factors are named by the N-terminal conserved MYB domain (Ng, Abeysinghe, and Kamali 2018). Studies have shown that in *Arabidopsis*, AtMYB12 promoted flavonoid synthesis, scavenged reactive oxygen species, and upregulated the expression of ABA and proline synthesis-related genes (Wang et al. 2016). *GmMYB176* regulates isoflavone biosynthesis by activating chalcone synthase gene expression in soybean (Anguraj Vadivel et al. 2019). In response to chilling stress, previous studies have found that overexpression of the maize *ZmMYB-IF35* gene enhanced the resistance of transgenic *Arabidopsis* to chilling and oxidative stress (Meng and Sui 2019). Li et al. (2019) found that *ZmMYB31* enhanced cold resistance in maize by positively regulating CBF gene expression. These results show that MYB, ERF, and bHLH transcription factors play an important role in the response mechanism of chilling stress, and there is an interaction between the three, which provides an additional approach for the analysis of chilling tolerance mechanisms of soybean sprouts.

4.4 | A Possible Regulation Model of Chilling Tolerance During Soybean Germination

Based on the above results and previous research progress, this study proposed a possible molecular regulation model of chilling tolerance during soybean germination. The cell membrane first senses cold signals and triggers Ca^{2+}, ABA, ethylene, and so on to respond. Then, mediated by signal molecules such as CDPK, MYB, and ERF, the activities of proline, flavonoids, and POD enzymes increase, and the content of H_2O_2 decreases, which enhances the chilling tolerance of soybean during germination (Figure 9).

5 | Conclusion

In general, our study showed that soybean would accumulate ROS and lead to membrane lipid peroxidation under chilling stress during germination. At the same time, the activity of active oxygen scavenging system related enzymes and the content of osmotic adjustment substances will increase, as far as possible to maintain the internal physiological balance of soybean under chilling stress. Transcriptome sequencing analysis revealed that genes related to flavonoid synthesis, linoleic acid, and catalase were enriched. According to WGCNA, four gene coexpression modules were obtained, and 20 hub genes were screened. These results will provide theoretical guidance for the study of molecular regulation mechanism of chilling tolerance during soybean germination.

Author Contributions

Formal analysis: Xuhong Fan. *Funding acquisition*: Shuming Wang and Hongwei Jiang. *Investigation*: Fanfan Meng and Xingmiao Sun. *Methodology*: Wei Zhang. *Resources*: Yunfeng Zhang and Mingliang Wang. *Software*: Guang Li. *Supervision*: Qingshan Chen. *Validation*: Yuhong Zheng. *Writing – original draft*: Jianguo Xie.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.