

Cloning of the GPCR-type G protein gene SikCOLD1 from *Saussurea involucrata* and functional validation and mechanistic analysis of its chilling tolerance in overexpressed processing tomato

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1 **Cloning of the GPCR-type G protein gene *SikCOLD1* from *Saussurea involucrata* and**
2 **functional validation and mechanistic analysis of its chilling tolerance in overexpressed**
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Abstract:

Chilling stress is a major abiotic constraint limiting the production of processing tomato, and the identification of superior chilling-tolerance genes and elucidation of their regulatory mechanisms are of great significance for genetic improvement of tomato stress resistance. *COLD1* (Chilling tolerance divergence 1) is a key cold sensor protein in plants that plays a central role in cold signal perception and response. However, the chilling-tolerance function and downstream regulatory network of *SikCOLD1*, derived from the alpine extreme plant *Saussurea involucrata*, remain largely unexplored in processing tomato. In this study, *SikCOLD1*-overexpressing processing tomato lines (OE) and the wild-type cultivar Riegel 87-5 (WT) were used as experimental materials and subjected to chilling stress at 4°C for 48 h. By integrating physiological and biochemical analyses, transcriptome sequencing, weighted gene co-expression network analysis (WGCNA), protein–protein interaction (PPI) network analysis, and qRT-PCR validation, we systematically investigated the physiological basis and molecular regulatory mechanisms underlying *SikCOLD1*-mediated chilling tolerance in tomato.

Keyword: *Saussurea involucrata*; *SikCOLD1*; transcriptome analysis; weighted gene co-expression network analysis (WGCNA);

Key Message: *SikCOLD1* improves antioxidant-osmotic defense and maintains photosynthesis under chilling stress.

Transcriptome reveals *SikCOLD1*-regulated chilling-response pathways.

WGCNA-PPI identifies key cold-tolerance modules and hub genes.

42 **Abbreviations :**

Abbreviation	Full name
ABA	Abscisic acid
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
AsA	Ascorbic acid
CAT	Catalase
cDNA	Complementary DNA
COLD1	Chilling tolerance divergence 1
CTAB	Cetyltrimethylammonium bromide
DEG	Differentially expressed gene
DEPC	Diethyl pyrocarbonate
FDR	False discovery rate
GO	Gene Ontology
GPCR	G protein-coupled receptor
GSH	Reduced glutathione
GS	Gene significance
JA	Jasmonic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MCM	Minichromosome maintenance
MM	Module membership
mRNA	Messenger RNA
NPQt	Non-photochemical quenching
OE	SikCOLD1-overexpressing line
PCA	Principal component analysis
Phi2	Actual photochemical quantum yield
PhiNPQ	Regulated non-photochemical energy dissipation quantum yield
POD	Peroxidase
PPI	Protein–protein interaction
PSII	Photosystem II
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
ROS	Reactive oxygen species
SOD	Superoxide dismutase
WGCNA	Weighted gene co-expression network analysis
WT	Wild type

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1. Introduction

Chilling stress is one of the major abiotic stresses limiting global crop production, significantly inhibiting plant growth and development and causing substantial yield losses (Ding & Yang, 2022). In recent years, extreme low-temperature events caused by global climate anomalies have occurred more frequently (Chaudhry & Sidhu, 2022). In addition, the increasing occurrence of agricultural meteorological disasters, such as spring cold spells and late autumn frost, has further intensified the threat of chilling stress to crop production (Jha et al., 2017). It has been estimated that more than half of the world's cultivated land is affected by chilling damage, particularly in high-latitude and high-altitude regions (Guo et al., 2024). Chilling stress can reduce crop yields by 10%–30% under normal production conditions and may even cause localized crop failure in severe disaster years, making it a critical environmental stress factor threatening global food and vegetable production security.

The damage caused by chilling stress in plants is characterized by multi-level and systemic effects. Chilling stress first induces phase transitions of membrane lipids, resulting in decreased membrane fluidity and increased membrane permeability (Chen et al., 2020). Meanwhile, it triggers excessive accumulation of reactive oxygen species (ROS) in cells (Chen et al., 2026), leading to lipid peroxidation (Rácz et al., 2008), protein denaturation, and nucleic acid damage (Janmohammadi et al., 2015), thereby disrupting cellular redox homeostasis (Carrillo et al., 2026). In addition, chilling stress inhibits photosynthetic activity (He et al., 2014), reduces carbon assimilation efficiency (Shi et al., 2025), and disturbs cellular osmotic balance and ion homeostasis, ultimately

causing growth inhibition or even plant death at the cellular, tissue, and whole-plant levels.

During long-term evolution, plants have developed multi-dimensional adaptation strategies to cope with low-temperature stress. The first strategy involves activation of the osmotic regulation system (Manacorda et al., 2026): plants accumulate osmoprotective compounds, such as proline (Avendaño et al., 2026), soluble proteins (Wang et al., 2023), and soluble sugars (Nie et al., 2026), to reduce cellular osmotic potential, maintain cell turgor and water uptake, and preserve the structural stability of biological macromolecules (Konrad et al., 2026). The second strategy is the enhancement of antioxidant defense systems (Xiao et al., 2026). Enzymatic antioxidants, including superoxide dismutase (SOD) (Zang et al., 2015), peroxidase (POD) (Kumar et al., 2022), and catalase (CAT), together with non-enzymatic antioxidants such as ascorbic acid (AsA) and reduced glutathione (GSH), function synergistically to eliminate excessive ROS and alleviate oxidative damage (H. Wang et al., 2025). The third strategy involves cascade regulation of signaling pathways (Ma et al., 2026). Calcium (Ca^{2+}) signaling, MAPK cascades (L. Wang et al., 2025), abscisic acid (ABA) signaling (Wei et al., 2025), jasmonic acid (JA) signaling, and other phytohormone-related pathways collectively form a complex regulatory network (Zhao et al., 2026), mediating cold signal transduction and the expression of downstream defense genes. At the level of cold signal perception, the cold sensor protein COLD1, first identified in rice, serves as a key regulatory factor (Ma et al., 2015). It is localized in the plasma membrane and endoplasmic reticulum and mediates cold-induced Ca^{2+}

influx through interaction with the G protein α subunit, thereby initiating downstream cold response programs (Luo et al., 2021). Subsequent studies have demonstrated that COLD1 homologs are widely conserved among terrestrial plants. For example, overexpression of COLD1 homologs in maize (Zhou et al., 2023) and *Populus euphratica* (Feng et al., 2025) significantly enhances chilling tolerance in recipient plants. Although plants possess these endogenous adaptive mechanisms, thermophilic crops such as tomato generally exhibit limited natural chilling tolerance. Therefore, it remains necessary to enhance cold resistance through germplasm innovation and genetic engineering approaches to ensure stable and high-yield crop production.

Tomato (*Solanum lycopersicum* L.) is the most widely cultivated and economically important solanaceous vegetable crop worldwide. It serves both fresh consumption and processing purposes and occupies a central position in the vegetable industry and human dietary nutrition (Morris & Taylor, 2017). Tomato fruits are rich in vitamins, lycopene, and various antioxidant compounds, making them an important source of dietary nutrients for humans (Abbamondi et al., 2026). Meanwhile, tomato is also a key raw material for the food processing industry and is widely processed into products such as tomato paste, juice, and canned foods, with its industrial chain spanning agricultural production and food processing sectors. With the rapid development of protected agriculture, tomato production has achieved year-round supply. However, originating from tropical and subtropical regions, tomato is a warm-season crop with poor cold tolerance and high sensitivity to chilling stress (Nie et al., 2024). In agricultural production, low temperatures during early spring transplanting, low-

temperature and low-light conditions in winter protected cultivation, and late autumn frost frequently result in delayed seedling growth, flower and fruit abscission, fruit deformity, and quality deterioration, representing major bottlenecks restricting stable and efficient year-round tomato production.

Current research on tomato chilling tolerance has made considerable progress, with several conserved cold-responsive pathways, including the ICE-CBF-COR cascade, MAPK signaling pathway, and phytohormone regulation, being identified (Guo et al., 2026). However, existing studies have mainly focused on the functional characterization of individual endogenous tomato genes, while the utilization of superior cold-tolerance genes from extreme cold-adapted plants for improving tomato chilling tolerance remains relatively limited. Meanwhile, chilling tolerance is a complex quantitative trait regulated by the coordinated action of multiple genes. Conventional differential expression analysis is insufficient to reveal synergistic regulatory relationships among genes and cannot comprehensively decipher the global regulatory networks underlying chilling tolerance formation. *Saussurea involucrata* Kar. et Kir. is a typical alpine plant with extreme chilling tolerance. It has evolved unique cold adaptation mechanisms through long-term natural selection under harsh environments characterized by high altitude, low temperature, and strong radiation, providing abundant genetic resources for stress resistance improvement. Previous studies have demonstrated that heterologous expression of multiple stress-resistance genes derived from *S. involucrata* can significantly enhance cold tolerance in recipient plants (Liu et al., 2023). As an upstream key regulator of cold signaling pathways, the COLD1 protein

from *S. involucrata* may possess enhanced cold signal perception and activation capabilities; however, its chilling-tolerance function and downstream regulatory network in tomato remain largely unexplored. Weighted gene co-expression network analysis (WGCNA) can construct co-expression modules based on genome-wide expression patterns (Wei et al., 2024). By integrating physiological traits, WGCNA enables the precise identification of key regulatory modules and core hub genes (Bhattarai et al., 2024), providing an effective approach for dissecting molecular regulatory networks underlying complex traits.

In this study, *SikCOLD1*-overexpressing tomato lines derived from *Saussurea involucrata* and the wild-type cultivar Riegel 87-5 were used as experimental materials and subjected to chilling stress at 4°C. By integrating physiological and biochemical measurements, transcriptome sequencing, differential expression analysis, WGCNA co-expression network construction, protein–protein interaction (PPI) network analysis, and qRT-PCR validation, we systematically investigated the physiological basis and molecular regulatory mechanisms underlying *SikCOLD1*-mediated enhancement of chilling tolerance in tomato. This study aims to elucidate the functional role of *SikCOLD1* in tomato responses to chilling stress and reveal the regulatory network and key targets mediated by *SikCOLD1* during chilling tolerance formation. The findings provide new theoretical insights and genetic resources for further understanding the cold-tolerance regulatory mechanisms of the plant COLD1 family and for molecular breeding of chilling-tolerant tomato varieties.

2. Materials and Methods

2.1 Plant Materials and Treatments

Tomato (*Solanum lycopersicum* L.) cultivar Riegel 87-5 (wild type, WT) and *SikCOLD1*-overexpressing lines (OE) previously generated by our research group were used as experimental materials in this study. The overexpression lines were constructed using the CaMV 35S promoter and the pCAMBIA1301 vector backbone, and homozygous T3 generation lines were obtained through multiple generations of screening.

WT and OE tomato seeds were sown in a mixed substrate containing vermiculite, perlite, and peat (1:1:1) and cultivated in seedling trays. The growth conditions were maintained at 28°C during the day and 25°C at night, with a photoperiod of 16 h light/8 h dark. After seedlings developed 2–3 true leaves, they were transplanted, and chilling stress treatment was applied when plants reached the 5–6 true-leaf stage. Four experimental groups were established, and chilling treatment was performed at 4°C under the same light conditions as normal cultivation for 48 h.

Mixed samples were collected from the fully expanded third to fourth functional leaves of each treatment group before chilling treatment (0 h) and after chilling treatment (48 h). Three biological replicates were collected for each treatment, and samples were rapidly frozen in liquid nitrogen and stored at –80°C for subsequent physiological measurements and molecular experiments.

The molecular identification procedure for transgenic lines was performed as follows: genomic DNA was extracted from tomato leaves using the CTAB method, and PCR amplification was conducted with *SikCOLD1*-specific primers. The amplification

products were detected by 1% agarose gel electrophoresis, with WT plants used as negative controls. Total RNA was further extracted from leaves and reverse-transcribed into cDNA. The expression level of *SikCOLD1* was analyzed by qRT-PCR, using tomato Actin as the reference gene. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, and homozygous lines with stable high *SikCOLD1* expression were selected for subsequent experiments.

2.2 Determination of Physiological and Biochemical Parameters and Photosynthetic Characteristics

Physiological and biochemical parameters were measured using samples collected from each group before and after chilling treatment. The concentrations of malondialdehyde (MDA), carotenoids, soluble proteins, soluble sugars, proline, reduced glutathione (GSH), ascorbic acid (AsA), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and superoxide dismutase (SOD) in tomato leaves were quantitatively determined using commercial assay kits (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions.

Photosynthetic pigment contents were determined using the ethanol extraction method. Leaf samples were collected using a 1 cm diameter puncher while avoiding major veins, and 0.1 g of samples was weighed and ground with a small amount of quartz sand and calcium carbonate powder. After thorough grinding, the extracts were washed with 95% ethanol, transferred into a 10 mL volumetric flask, and brought to volume. The samples were extracted in darkness for 24 h until the tissues became completely white. Absorbance values were measured at 665 nm, 649 nm, and 470 nm,

and chlorophyll and carotenoid contents were calculated according to the Lichtenthaler equations.

Chlorophyll fluorescence parameters were measured using a portable photosynthesis analyzer (MultispeQ v2.0, USA). Before measurement, leaves were dark-adapted for 30 min, followed by determination of chlorophyll fluorescence parameters, including regulated non-photochemical energy dissipation quantum yield (PhiNPQ), actual photochemical quantum yield (Phi2), and non-photochemical quenching (NPQt). All physiological measurements were performed with three biological replicates.

2.3 RNA Extraction, Library Construction, and Transcriptome Sequencing

Total RNA was extracted from leaves using the CTAB method, and the extracted RNA was dissolved in 50 µL of DEPC-treated water. The quality and quantity of total RNA were evaluated using a Qubit 2.0 fluorometer and a Qsep400 high-throughput fragment analyzer. mRNA was obtained using the poly(A) enrichment method, followed by mRNA fragmentation, first- and second-strand cDNA synthesis, end repair, adapter ligation, and PCR amplification to construct sequencing libraries. After passing quality control, the qualified libraries were subjected to 150 bp paired-end sequencing on an Illumina sequencing platform.

2.4 Bioinformatics Analysis of Transcriptome Data

Raw sequencing data were first subjected to quality control using the fastp software to remove low-quality reads and adapter sequences, generating clean reads. The clean reads were subsequently aligned to the tomato reference genome using

HISAT2, and transcript assembly was performed using StringTie. Gene expression levels were quantified using featureCounts. Differentially expressed gene (DEG) analysis was conducted using DESeq2, with $|\log_2\text{FoldChange}| \geq 1$ and $\text{FDR} < 0.05$ set as the screening criteria. The identified DEGs were further subjected to Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses, with an adjusted P-value < 0.05 considered statistically significant for enrichment.

2.5 Weighted Gene Co-expression Network Analysis (WGCNA)

Based on the gene expression matrix containing 27,932 genes from four groups with 12 samples, a weighted gene co-expression network was constructed using the Metware Cloud analysis platform. Genes were classified into different modules according to their expression patterns, and the module eigengenes were calculated for each module. Correlation analyses were further performed between module eigengenes and chilling stress-related physiological traits, including malondialdehyde (MDA), carotenoids, soluble proteins, soluble sugars, proline, reduced glutathione (GSH), ascorbic acid (AsA), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and superoxide dismutase (SOD). The parameters were set as follows: merge cut height = 0.25 and minimum module size = 50.

2.6 Protein–Protein Interaction (PPI) Network Analysis

Core hub genes were screened from the key co-expression modules using the criteria of module membership (MM) > 0.8 and gene significance (GS) > 0.6 . The identified hub genes were submitted to the STRING database (version 12.0) for

prediction of protein–protein interactions, with *Solanum lycopersicum* selected as the species and a medium confidence interaction threshold (≥ 0.7) applied. The predicted interaction data were imported into Cytoscape v3.9.1 for network visualization, and core network nodes were identified based on the degree value.

2.7 qRT-PCR Validation

Eight hub genes from the key co-expression module were selected for qRT-PCR validation. Tomato Actin was used as the reference gene, and primer sequences are provided in the supplementary table. Relative gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method. Each sample included three technical replicates and three biological replicates to verify the reliability of the transcriptome sequencing results.

2.8 Statistical Analysis

Physiological parameter data and qRT-PCR results were statistically analyzed and visualized using GraphPad Prism 8.0 (GraphPad Software, USA). Differences among groups were evaluated using one-way analysis of variance (One-way ANOVA), followed by Tukey's multiple comparison test. A P-value < 0.05 was considered statistically significant.

3. Results

3.1 Molecular Identification of *SikCOLDI*-Overexpressing Tomato Plants

PCR amplification was performed using genomic DNA as the template. The electrophoresis results showed that specific bands corresponding to the expected fragment size were detected in all OE plants, whereas no corresponding bands were observed in WT plants, indicating that the *SikCOLDI* gene had been successfully

integrated into the tomato genome (Figure S1).

qRT-PCR results showed that, compared with the OE-9 line exhibiting the lowest expression level, the relative expression levels of *SikCOLD1* in OE-3, OE-7, and OE-8 lines were increased by 11.7-, 16.2-, and 13.3-fold, respectively, with all differences reaching statistical significance. Therefore, these three high-expression lines were selected for subsequent chilling stress experiments (Figure S2).

3.2 Effects of Chilling Stress on Plant Phenotypes and Physiological and Biochemical Characteristics

Under normal growth conditions, WT and OE plants exhibited similar growth performance with no obvious phenotypic differences. After exposure to 4°C chilling stress for 48 h, WT plants showed obvious leaf wilting, yellowing, and growth inhibition, whereas OE plants maintained relatively healthy leaf morphology, indicating that *SikCOLD1* overexpression enhanced tomato tolerance to chilling stress (Figure 1A). Physiological analyses showed that under normal conditions, WT plants exhibited significantly lower proline and soluble protein contents but higher soluble sugar content compared with OE plants. After chilling stress, OE plants displayed lower malondialdehyde (MDA) accumulation than WT plants, while the increases in superoxide dismutase (SOD) activity, reduced glutathione (GSH), soluble protein, and proline contents were significantly greater than those in WT plants. These results indicated that transgenic plants possessed enhanced antioxidant defense and osmotic adjustment capacities. Chilling treatment significantly reduced chlorophyll content in WT plants, whereas OE plants maintained higher chlorophyll levels. Chlorophyll

fluorescence analysis further demonstrated that chilling stress markedly decreased NPQt, Phi2, and PhiNPQ values in WT plants, while OE plants maintained relatively higher levels of these parameters (Figure 1B–P)².

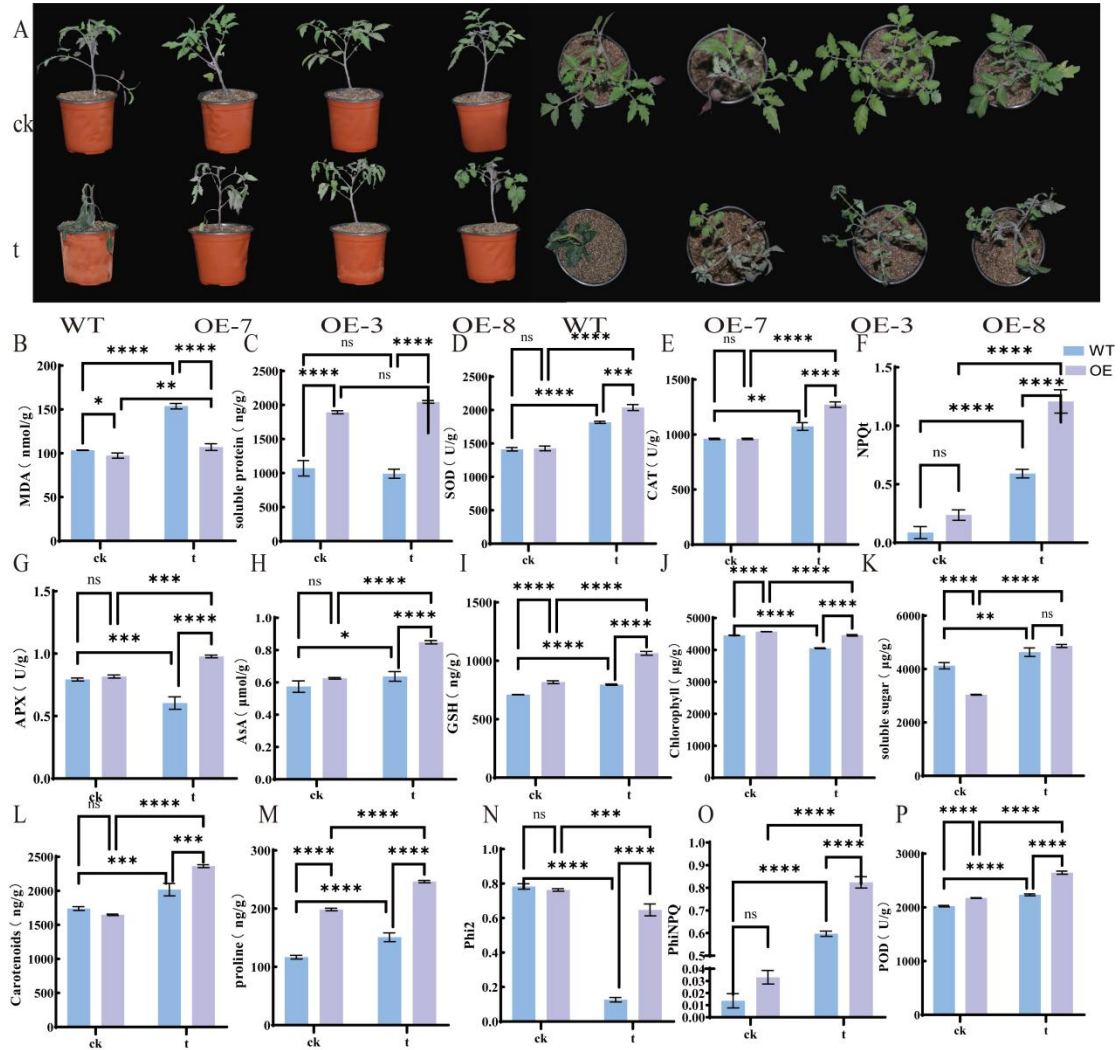


Figure 1. Phenotypic and physiological changes of WT and OE plants before and after chilling treatment. WT, wild-type plants under normal temperature conditions; T-WT, wild-type plants subjected to chilling stress; OE, SikCOLD1-overexpressing plants under normal temperature conditions; T-OE, SikCOLD1-overexpressing plants subjected to chilling stress. “ck” indicates normal growth conditions, and “t” indicates chilling stress treatment. (A) Phenotypic images: top and bottom views of WT and OE plants before and after chilling treatment. (B) Malondialdehyde (MDA). (C) Soluble protein. (D) Superoxide dismutase (SOD). (E) Catalase (CAT). (F) Non-photochemical quenching (NPQt). (G) Ascorbate peroxidase (APX). (H) Ascorbic acid (AsA). (I) Reduced glutathione (GSH). (J) Chlorophyll. (K) Soluble sugar. (L) Carotenoids. (M) Proline. (N) Effective quantum yield of photochemical energy conversion in photosystem II (Phi2). (O) Quantum yield of regulated energy dissipation (PhiNPQ). (P) Peroxidase (POD). Statistical significance was evaluated using one-way analysis of variance (ANOVA), and significant differences are indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Transcriptome sequencing was performed on a total of 12 samples from four groups: WT, T-WT, OE, and T-OE. After removing low-quality reads, the number of clean reads obtained from each sample ranged from 43,850,752 to 66,607,304. The Q30 base percentage of all samples exceeded 97.79%, the GC content ranged from 41.86% to 42.34%, and the alignment rates to the reference tomato genome ranged from 91.82% to 98.27% (Table S1). Principal component analysis (PCA) (Figure 2A) and sample correlation heatmap analysis showed clear separation among different treatment groups, while biological replicates within each group exhibited high reproducibility (Figure 2B).

3.4 Differentially Expressed Gene Analysis

3.4.1 WT vs T-WT: Basal Cold Response in Wild-Type Tomato

Differential expression analysis was performed between WT and T-WT plants, identifying a total of 11,872 differentially expressed genes (DEGs), including 5,644 upregulated and 6,228 downregulated genes (Table S2). PCA analysis showed a clear separation between WT and T-WT samples, indicating that low-temperature treatment significantly altered the overall transcriptional expression profile of tomato plants. The volcano plot further illustrated the overall distribution of differentially expressed genes, while the heatmap analysis demonstrated high consistency among biological replicates and clearly distinguished the expression patterns of cold-responsive genes among different treatment groups (Figure S3). GO and KEGG enrichment analyses revealed that upregulated DEGs were significantly enriched in pathways associated with ribosome biogenesis, RNA processing, proteasome activity, and vesicle transport (Figure 2C–F), suggesting that low-temperature stress induced the reconstruction of the

313 protein translation system and activated damaged protein degradation and cell
314 membrane repair processes in wild-type tomato plants to cope with cold stress. In
315 contrast, downregulated DEGs were mainly enriched in pathways related to
316 photosynthesis, photosynthetic antenna proteins, Calvin cycle carbon fixation, and
317 chlorophyll biosynthesis, indicating that low temperature suppressed the photosynthetic
318 system of tomato plants at the transcriptional level. Collectively, these results
319 demonstrate that low-temperature treatment induces the expression of numerous cold-
320 responsive genes while suppressing genes associated with photosynthesis and growth,
321 indicating that wild-type tomato plants can activate a typical cold stress response
322 program.

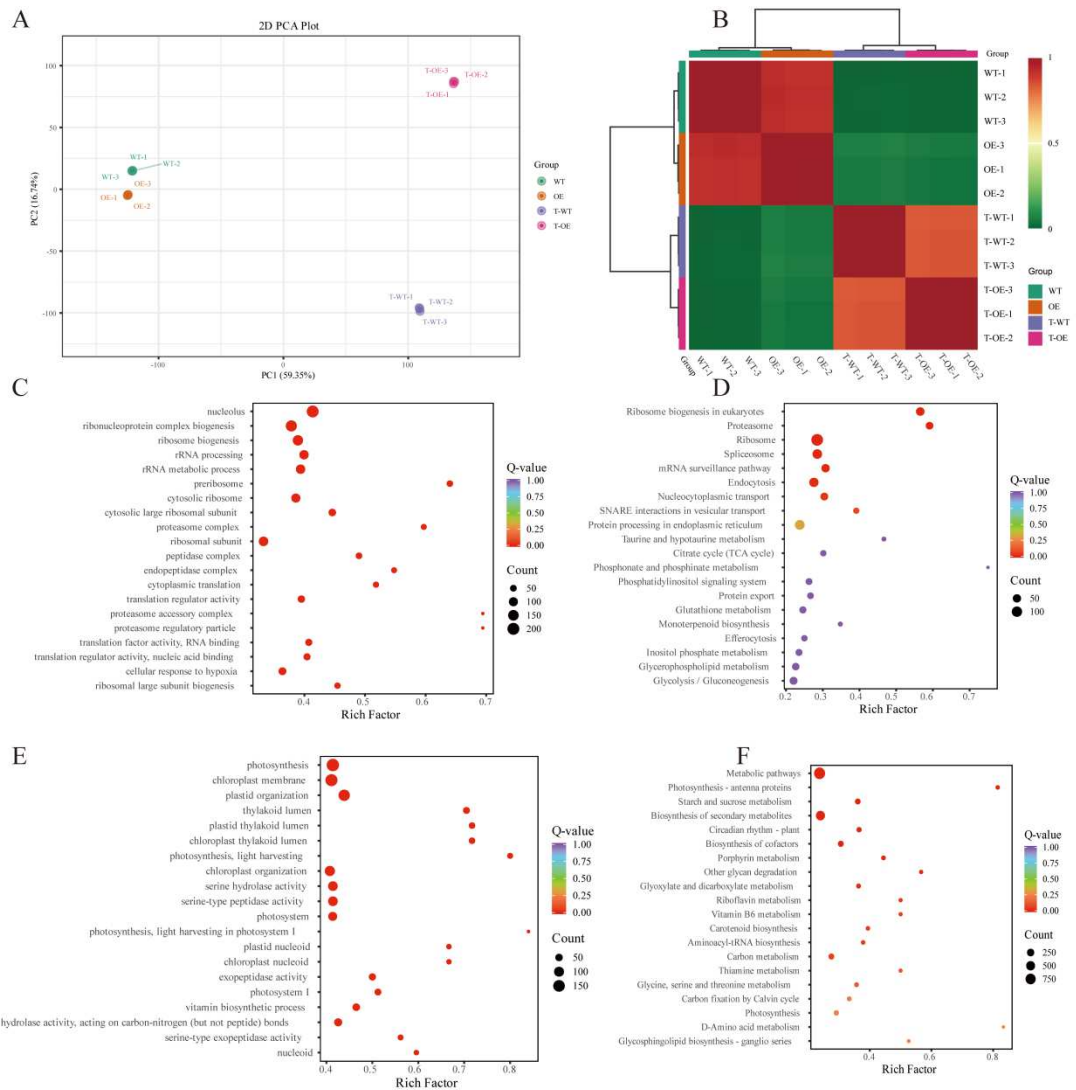


Figure 2. Quality assessment of transcriptome data and analysis of basal chilling responses in wild-type tomato. (A) Principal component analysis (PCA) of the 12 transcriptome samples. (B) Heatmap showing sample correlation among the 12 transcriptome datasets. (C) GO bubble plot analysis of upregulated differentially expressed genes (DEGs) involved in the basal chilling response of wild-type tomato. (D) KEGG bubble plot analysis of upregulated DEGs involved in the basal chilling response of wild-type tomato. (E) GO bubble plot analysis of downregulated DEGs involved in the basal chilling response of wild-type tomato. (F) KEGG bubble plot analysis of downregulated DEGs involved in the basal chilling response of wild-type tomato. The size of each bubble represents the number of enriched differentially expressed genes, and the color indicates the significance level of enrichment.

of differentially expressed genes after low-temperature treatment, indicating that overexpression enhanced the transcriptional responsiveness of tomato plants to cold stress. PCA analysis showed a clear separation between OE and T-OE samples, demonstrating that low-temperature treatment significantly altered the overall transcriptional expression patterns of tomato plants. The volcano plot further illustrated the global distribution of differentially expressed genes, while heatmap analysis showed high consistency among biological replicates and clearly distinguished the expression patterns of cold-responsive genes among different treatment groups (Figure S4). GO and KEGG enrichment analyses revealed that upregulated DEGs were significantly enriched in pathways associated with ribosome biogenesis and rRNA processing, and were additionally enriched in calcium ion-related signaling pathways and oxidative stress response-related processes. These results indicate that tomato plants with *SikCOLD1* overexpression can activate a broader and stronger cold-responsive regulatory network, providing a molecular basis for enhanced cold tolerance (Figure 3A–B).

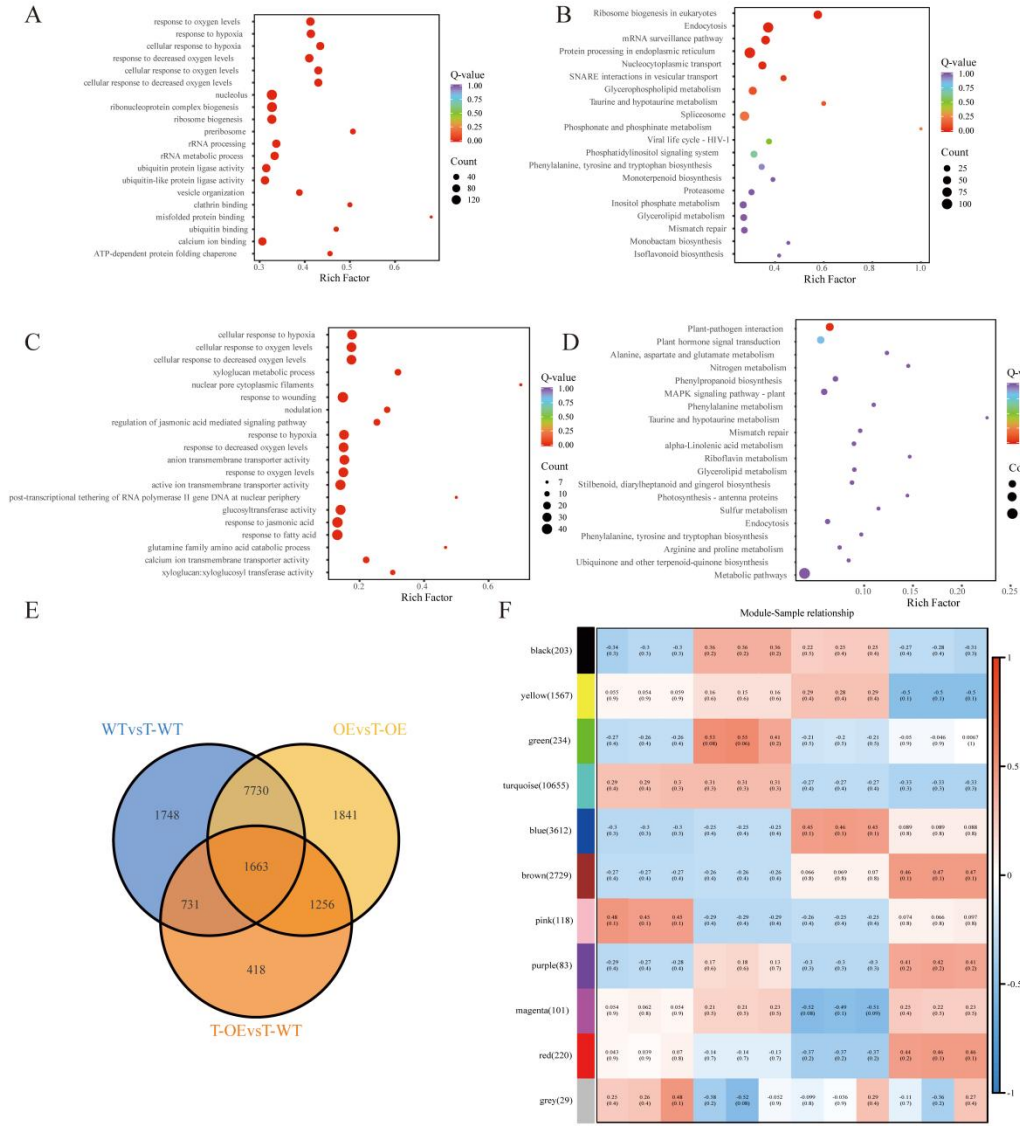


Figure 3. GO and KEGG enrichment analyses of transcriptomic differentially expressed genes, Venn diagram analysis, and co-expression module identification. (A) GO bubble plot analysis of upregulated differentially expressed genes (DEGs) under chilling stress in the *SikCOLD1*-overexpression background. (B) KEGG bubble plot analysis of upregulated DEGs under chilling stress in the *SikCOLD1*-overexpression background. (C) GO bubble plot analysis of genotype-dependent DEGs under chilling stress. (D) KEGG bubble plot analysis of genotype-dependent DEGs under chilling stress. (E) Venn diagram analysis of core chilling-responsive DEGs mediated by *SikCOLD1*. (F) Identification of gene co-expression modules using weighted gene co-expression network analysis (WGCNA). The bubble size represents the number of enriched differentially expressed genes, and the bubble color indicates the significance level of enrichment.

genes (DEGs) were identified, including 2,213 upregulated genes and 1,855 downregulated genes (Table S2). PCA analysis showed a clear separation between T-OE and T-WT samples, indicating that *SikCOLD1* overexpression significantly altered the global transcriptional expression pattern of tomato plants under cold stress conditions. The volcano plot illustrated the overall distribution of DEGs, while the heatmap analysis demonstrated high consistency among biological replicates and clearly distinguished the expression patterns of cold-responsive genes among different treatment groups (Figure S5). GO enrichment analysis showed that the identified DEGs were mainly associated with hypoxia response, jasmonic acid signaling pathways, ion transmembrane transport, and xyloglucan metabolism in the cell wall. KEGG enrichment analysis revealed significant enrichment in pathways related to plant hormone signal transduction, the plant MAPK signaling pathway, phenylpropanoid biosynthesis, and α -linolenic acid metabolism. Collectively, these results indicate that *SikCOLD1* significantly enhances the expression of cold defense-related genes and promotes the activation of antioxidant systems and membrane protection pathways, thereby maintaining cellular redox homeostasis and improving cold tolerance in tomato plants (Figure 3C–D).

3.5 Venn Analysis Identifies Core Cold-Responsive Genes Mediated by *SikCOLD1*

Venn analysis was performed on DEGs identified from three comparisons: WT vs T-WT, OE vs T-OE, and T-WT vs T-OE (Figure 3E). The results showed that the three comparison groups shared a subset of common differentially expressed genes, while also containing distinct groups of specifically responsive genes. Among them, the

11,872 DEGs identified from the T-WT vs WT comparison represented the basal cold-responsive genes involved in the endogenous response of tomato plants to cold stress, whereas the 12,490 DEGs identified from the T-OE vs OE comparison reflected the expanded cold-responsive transcriptional network under the *SikCOLD1* overexpression background. Further analysis revealed that 9,393 DEGs were simultaneously present in both the WT and OE cold-responsive gene sets, suggesting that these genes may participate in the basic cold adaptation process of tomato plants. Meanwhile, the 4,068 specific DEGs identified from the T-OE vs T-WT comparison mainly represented the transcriptional regulatory effects mediated by *SikCOLD1* under cold stress. Notably, intersection analysis between the T-WT vs WT and T-OE vs T-WT comparisons identified 2,394 candidate genes that simultaneously exhibited “cold-induced expression” and “enhanced expression by COLD1”. These genes were activated in WT plants upon cold stimulation and showed higher expression levels in *SikCOLD1*-overexpressing plants, suggesting that they may represent key responsive genes responsible for the enhanced cold tolerance mediated by *SikCOLD1*.

3.6 WGCNA Identifies Key Co-expression Modules Associated with Cold Tolerance

A weighted gene co-expression network was constructed based on the gene expression matrix, resulting in the identification of 11 gene co-expression modules. WGCNA module-trait correlation analysis revealed that the brown module, consisting of 2,729 genes, was significantly associated with multiple cold tolerance-related physiological traits (Figure 3F). The brown module showed highly significant positive correlations with antioxidant-related indicators, including SOD, CAT, POD,

393 AsA, and GSH, while osmotic adjustment-related compounds such as carotenoids,
394 soluble sugars, and proline were also significantly positively correlated with this
395 module. These results indicate that genes within the brown module participate in
396 antioxidant defense and osmotic regulation processes under cold stress and represent a
397 key regulatory module involved in *SikCOLDI*-mediated cold tolerance in
398 tomato. Expression pattern analysis showed that brown module genes exhibited
399 relatively low expression levels under normal conditions but were significantly
400 upregulated after cold treatment, with significantly higher expression levels in the T-
401 OE group than in the T-WT group, consistent with the enhanced cold tolerance
402 phenotype conferred by *SikCOLDI* overexpression (Figure 4A). Therefore, the brown
403 module was identified as a key regulatory module underlying *SikCOLDI*-mediated cold
404 tolerance in tomato. Functional enrichment analysis of genes within the brown module
405 showed that GO terms were significantly enriched in hypoxia response, response to
406 extracellular stimuli, ubiquitin protein ligase activity, calcium ion binding, vesicle
407 assembly, and regulation of jasmonic acid signaling pathways (Figure 4B). KEGG
408 pathway analysis revealed that these genes were mainly enriched in endoplasmic
409 reticulum protein processing, glycerophospholipid metabolism, vesicle transport, plant
410 hormone signal transduction, glutathione metabolism, and arginine and proline
411 metabolism pathways (Figure 4C).

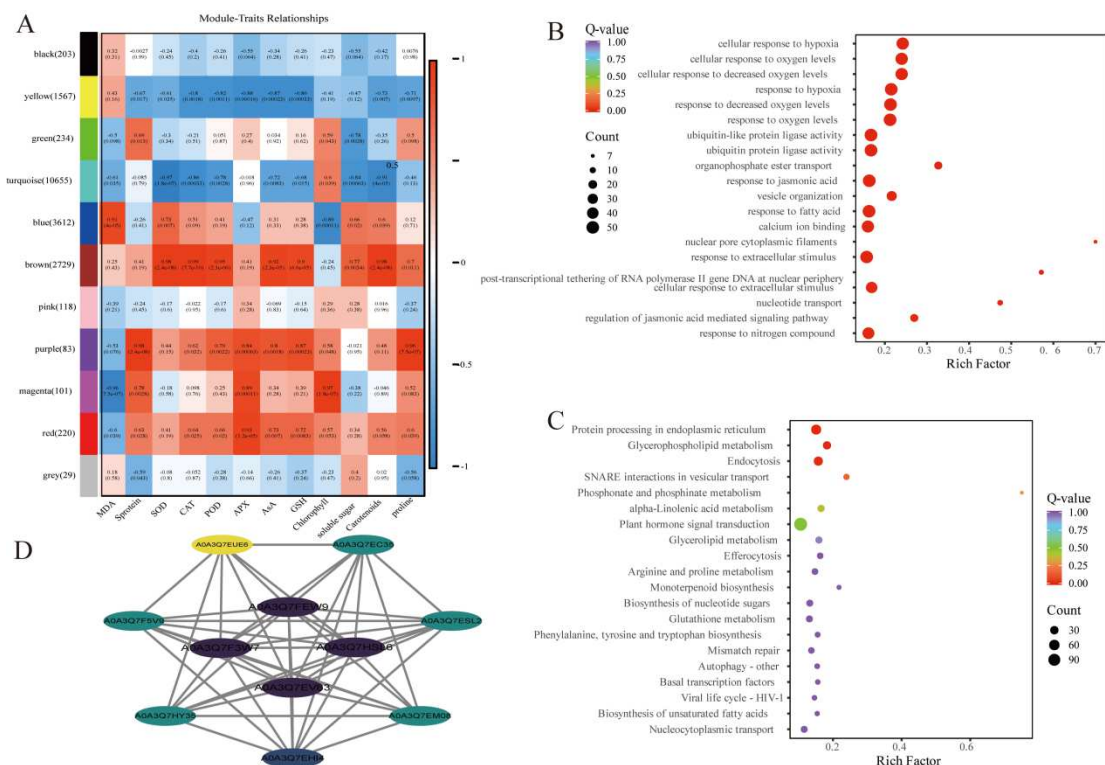


Figure 4. Identification of key co-expression modules associated with chilling tolerance by WGCNA and PPI network analysis of hub chilling-responsive genes. (A) Heatmap showing the correlations between WGCNA gene modules and physiological traits. (B) GO enrichment analysis of genes in the brown (2279) module. (C) KEGG enrichment analysis of genes in the brown (2279) module. (D) Protein-protein interaction (PPI) network analysis of hub genes in the brown (2279) module. The bubble size represents the number of enriched differentially expressed genes, and the bubble color indicates the significance level of enrichment.

licensing factor MCM3 (A0A3Q7F3W7), a non-specific serine/threonine protein kinase (A0A3Q7HSL6), and DNA replication licensing factor MCM4 (A0A3Q7EV63). These core Hub proteins were mainly involved in biological processes including cell cycle regulation, stress signal transduction, and maintenance of genome stability. These findings further support their potential roles as key regulatory nodes involved in *SikCOLDI*-mediated cold tolerance regulation (Figure 4D).

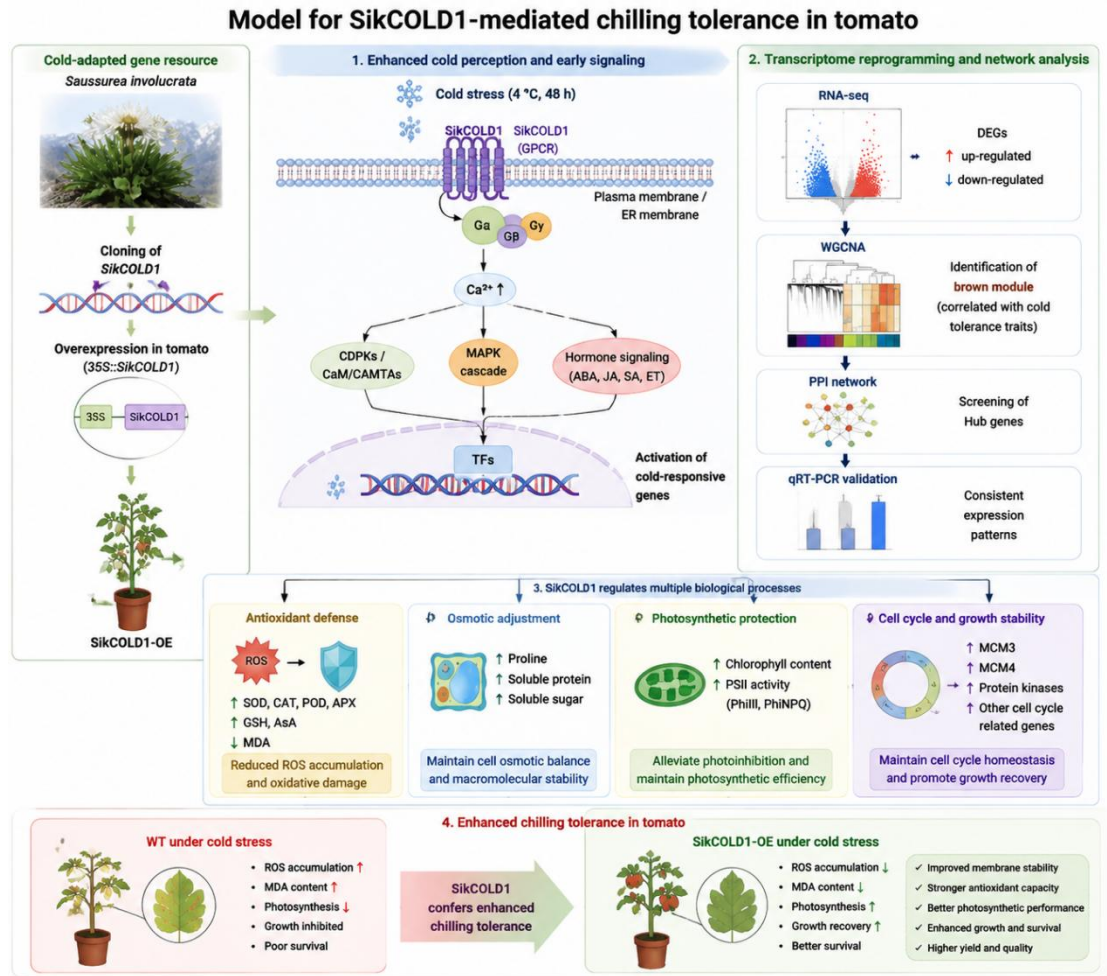
3.8 qRT-PCR Validation of the Reliability of Transcriptome Results

Eight representative genes from the Hub genes identified in the brown module were selected for qRT-PCR analysis, using WT, T-WT, OE, and T-OE plants as experimental materials to examine their expression patterns (Table S4). The results showed that the expression trends of these eight genes across the four treatment groups were fully consistent with the RNA-seq sequencing results (Table S5), confirming the accuracy and reliability of the transcriptome data (Figure S6).

3.9 Regulatory Model of *SikCOLDI*-Mediated Cold Tolerance in Tomato

Based on the integrated results of phenotypic analysis, physiological measurements, transcriptome profiling, WGCNA, PPI network analysis, and qRT-PCR validation, a molecular model was proposed to illustrate the regulatory mechanism of *SikCOLDI*-mediated cold tolerance in tomato (Figure 5). Under cold stress, *SikCOLDI* enhances cold signal perception, activates Ca²⁺ signaling, MAPK cascades, and plant hormone-related pathways, thereby promoting transcriptional reprogramming of cold-responsive genes. The brown module identified by WGCNA and the Hub genes screened through PPI network analysis further participate in the regulation of

antioxidant defense, osmotic adjustment, protein homeostasis maintenance, and photosynthetic system protection. Ultimately, *SikCOLD1* enhances cold tolerance in tomato plants by reducing oxidative damage, maintaining cellular homeostasis, and protecting photosynthetic functions.⁶



4 Discussion

4.1 *SikCOLD1* Overexpression Enhances Cold Stress Tolerance in Tomato at the Physiological Level

Cold stress is an important environmental factor affecting plant growth and development, with major detrimental effects including disruption of cell membrane

Figure 5. Regulatory model of SikCOLD1-mediated chilling tolerance in tomato.

integrity, excessive accumulation of reactive oxygen species (ROS), and impairment of photosynthetic system function. Under low-temperature conditions, alterations in membrane lipid phase transitions increase cell membrane permeability, and the accumulation of the lipid peroxidation product malondialdehyde (MDA) is widely considered an important indicator for evaluating the extent of plant cell membrane damage. In this study, after 48 h of treatment at 4°C, *SikCOLD1*-overexpressing tomato plants exhibited significantly lower MDA levels than wild-type plants, indicating that *SikCOLD1* effectively alleviates cold-induced membrane lipid peroxidation and enhances cell membrane stability (Díaz-Narváez et al., 2024).

Maintenance of ROS homeostasis is an important defense mechanism for plants to adapt to cold stress. Plants eliminate excessive ROS through the coordinated action of enzymatic antioxidant systems, including SOD, CAT, and POD, as well as non-enzymatic antioxidants such as GSH and AsA, thereby preventing oxidative damage. In this study, *SikCOLD1*-overexpressing plants exhibited higher activities of SOD, CAT, and POD, as well as increased levels of GSH and AsA after cold treatment, indicating that *SikCOLD1* enhances antioxidant defense capacity and reduces cold-induced oxidative stress in tomato plants (Ma et al., 2018). These findings are consistent with previous studies showing that COLD1 enhances antioxidant capacity and improves cold tolerance in plants such as *Populus euphratica* (Feng et al., 2025) and maize (Zhou et al., 2023), further suggesting that the stress regulatory function of COLD1 is evolutionarily conserved across different plant species.

Beyond oxidative defense, osmotic regulation is also an important physiological

strategy for plants to adapt to cold stress. Osmoprotectants, including proline, soluble sugars, and soluble proteins, contribute to maintaining cellular water balance and protecting the stability of proteins and membrane structures. In this study, *SikCOLD1*-overexpressing plants exhibited higher levels of proline and soluble protein accumulation even under normal conditions, and these levels were further enhanced after cold stress, suggesting that *SikCOLD1* may promote the establishment of a pre-adaptive defense state, thereby improving plant responsiveness to cold stimuli (Safhi et al., 2026). Cold stress also affects chlorophyll stability and photosystem II (PSII) activity, resulting in reduced light capture and electron transport efficiency (Ghosh et al., 2024). In the present study, *SikCOLD1*-overexpressing plants maintained higher chlorophyll content and exhibited increased levels of Phi2, PhiNPQ, and NPQt under cold conditions, indicating that *SikCOLD1* alleviates cold-induced damage to the photosynthetic apparatus and maintains PSII functional stability. Therefore, the enhanced cold tolerance mediated by *SikCOLD1* may be associated with the coordinated improvement of antioxidant capacity, osmotic adjustment, and photosynthetic system protection.

4.2 *SikCOLD1*-Mediated Global Transcriptional Reprogramming in Tomato Cold Response

Plant adaptation to cold stress involves extensive changes in gene expression and represents a complex process of transcriptional reprogramming. In this study, RNA-seq analysis revealed that cold treatment significantly altered the gene expression patterns of tomato plants. Under cold conditions, a large number of genes exhibited differential

expression in wild-type plants, with upregulated genes mainly involved in ribosome biogenesis, proteasome activity, and vesicle transport processes, whereas downregulated genes were primarily enriched in pathways associated with photosynthesis, photosynthetic antenna proteins, and chlorophyll biosynthesis. These findings indicate that cold stress promotes cellular protein homeostasis maintenance and damage repair on one hand, while suppressing photosynthetic activity on the other hand to reduce energy consumption and facilitate adaptation to adverse environmental conditions. Compared with wild-type plants, *SikCOLD1*-overexpressing plants exhibited a greater number of differentially expressed genes under cold stress, with further enrichment in processes related to calcium signaling, oxidative stress responses, and hormone signaling regulation, suggesting that *SikCOLD1* expands the transcriptional response capacity of tomato plants to cold stimuli (Zhao et al., 2026). As a previously identified plant cold sensor, COLD1 participates in cold signal perception and promotes Ca^{2+} signal transduction, thereby initiating downstream defense responses (Dong et al., 2025). In this study, the specific enrichment of calcium-related pathways in *SikCOLD1*-overexpressing plants was consistent with the known functions of COLD1, suggesting that *SikCOLD1* may activate downstream response networks by enhancing cold signal perception efficiency. Furthermore, under the same cold stress conditions, DEGs between *SikCOLD1*-overexpressing and wild-type plants were significantly enriched in pathways associated with plant hormone signal transduction, the MAPK signaling pathway, phenylpropanoid biosynthesis, and α -linolenic acid metabolism (Nguyen et al., 2009). Plant hormone signaling and MAPK cascades

coordinate the regulation of stress responses, while phenylpropanoid metabolism and lipid metabolism contribute to antioxidant compound synthesis and maintenance of membrane structural stability. Therefore, *SikCOLDI* may not function through a single cold-responsive pathway but rather enhance overall cold tolerance in tomato by integrating Ca^{2+} signaling, hormone signaling, MAPK cascades, and metabolic regulatory networks.

4.3 WGCNA Reveals Key Regulatory Modules Involved in *SikCOLDI*-Mediated Cold Tolerance

Differential expression analysis can identify cold-responsive genes but is insufficient to elucidate the coordinated regulatory relationships among genes during the formation of complex cold tolerance traits. In this study, WGCNA was further applied to construct a global gene co-expression network, resulting in the identification of 11 co-expression modules (Zhang et al., 2023). Among these modules, the brown module exhibited significant positive correlations with antioxidant-related indicators, including SOD, CAT, POD, GSH, and AsA, while showing a negative correlation with MDA. Its expression pattern was highly consistent with the enhanced cold tolerance phenotype observed in *SikCOLDI*-overexpressing plants, suggesting that it represents a key functional module involved in *SikCOLDI*-mediated cold tolerance. Functional enrichment analysis showed that genes within the brown module were mainly associated with calcium ion binding, ubiquitin protein ligase activity, vesicle assembly, plant hormone signaling regulation, glutathione metabolism, and protein processing. The ubiquitin–proteasome system maintains cellular protein homeostasis by

543 regulating the degradation of abnormal proteins and plays an important role in plant
544 stress responses. Endoplasmic reticulum protein processing and vesicle transport
545 contribute to protein quality control and membrane system repair, thereby helping
546 maintain normal cellular functions under cold conditions. Glutathione metabolism and
547 proline metabolism are closely associated with ROS scavenging and osmotic
548 protection. These results were consistent with the physiological changes observed in this
549 study, further suggesting that *SikCOLDI* may establish a comprehensive cold-
550 responsive network through the regulation of multiple biological processes. Hub genes
551 identified based on module membership (MM) and gene significance (GS) were further
552 analyzed through protein–protein interaction networks, revealing core regulatory nodes
553 within the brown module. These core nodes mainly included a non-specific
554 serine/threonine protein kinase, DNA replication licensing factors MCM3 and MCM4,
555 and one uncharacterized protein. The non-specific serine/threonine protein kinase is a
556 key component of eukaryotic signaling cascades and can regulate the activity of
557 downstream target proteins through phosphorylation modification. It is speculated that
558 this kinase may receive upstream calcium signaling inputs mediated by *SikCOLDI* and
559 participate in signal amplification and activation of downstream defense pathways
560 under cold stress. MCM3 and MCM4 belong to the minichromosome maintenance
561 protein family and are essential components of the MCM2–7 DNA helicase complex,
562 jointly regulating DNA replication initiation and genome stability. Cold stress
563 commonly induces DNA damage and cell cycle arrest; therefore, the upregulation of
564 these genes may alleviate cold-induced growth inhibition by maintaining normal cell

proliferation, which is consistent with the reduced damage phenotype observed in overexpression plants under cold stress. In addition, the uncharacterized protein located at the center of the network exhibited strong expression associations with multiple cold tolerance-related genes, suggesting that it may represent a novel regulatory factor involved in tomato cold response pathways with considerable potential for functional characterization. Integrating previous studies on *COLD1* function with the transcriptomic results obtained in this study, we propose that *SikCOLD1* may enhance cold adaptation in tomato by improving cold signal perception, promoting Ca^{2+} -related signal transduction, and further regulating transcription factors, antioxidant systems, and protein homeostasis-related processes. However, this proposed regulatory model requires further validation through genetic approaches and molecular interaction experiments.

4.4 Research Significance and Future Perspectives

Through physiological analysis, transcriptome sequencing, WGCNA co-expression network construction, and Hub gene validation, this study systematically elucidated the role of the *Saussurea involucrata*-derived *SikCOLD1* gene in regulating cold tolerance in tomato. The results demonstrated that *SikCOLD1* overexpression enhances antioxidant capacity, osmotic regulation ability, and photosynthetic system stability under cold stress, while promoting cold tolerance formation through the remodeling of cold-responsive transcriptional networks. This study further expands the functional understanding of the *COLD1* gene family across different plant species and provides a new strategy for improving crop stress tolerance by utilizing valuable stress-

resistance genes from plants adapted to extreme environments. Meanwhile, the key co-expression modules and Hub genes identified through WGCNA provide potential candidate targets for further elucidating the regulatory mechanisms underlying cold tolerance in tomato. However, several limitations remain in this study. First, the functional validation was mainly based on *SikCOLDI*-overexpressing materials; therefore, gene knockout or gene silencing approaches are still required to further confirm the necessity of *SikCOLDI* in tomato cold tolerance. Second, the regulatory relationships between Hub genes and *SikCOLDI* were primarily inferred from transcriptomic and network analyses and require further validation through experiments such as yeast two-hybrid assays, ChIP-qPCR, and dual-luciferase reporter assays. Furthermore, this study was mainly conducted under controlled environmental conditions, and future research should evaluate the effects of *SikCOLDI* on tomato growth, yield formation, and fruit quality under natural field cold stress conditions.

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Credit authorship contribution statement

M.W. conceived the study, designed the methodology, managed the project, performed investigation and formal analysis, curated data, collected resources, and wrote the original draft.

F.D. contributed to methodology development, investigation, formal analysis, data curation, and experimental coordination.

Z.Y., Q.L., Q.J., B.J., Q.L., B.C., Q.L., and X.S. contributed to data curation, investigation, experimental assistance, literature review, statistical analysis, and result validation.

L.Z. supervised the project, acquired funding, contributed to conceptualization, and revised the manuscript.

X.G. conceived and supervised the study, developed methodology, managed the project, acquired funding and resources, performed formal analysis, visualization, software development, experimental validation, and manuscript writing and revision.

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Ethical Compliance Statement: For studies involving endangered/protected species and wild genetic resources: A statement verifying compliance with the requirements of the *Convention on Biological Diversity* and the *Nagoya Protocol*.

Data Availability Statement : For publicly available data: Data will be made available on request.

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