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Identification Of *Arabidopsis* genes associated with cold tolerance based on integrated bioinformatics analysis

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ABSTRACT

Cold stress is a major environmental factor that limits plant growth and productivity. Plants have evolved various strategies to adapt to these environmental conditions. To better explain the mechanisms used to survive environmental challenges, we retrieved the cold-responsive genes of *Arabidopsis thaliana* from the Gene Expression Omnibus (GEO) database. The GEO raw data were normalized by the quantile method, and then the differentially expressed genes (DEGs) under cold stress were screened using the robust rank aggregation (RRA) algorithm, including 261 upregulated and 177 downregulated genes out of more than 20,000 genes. Further, the integrated bioinformatics analyses of PUBMED, PANTHER, DAVID, and STRING indicated that the upregulated DEGs were involved in cellular response to red light, negative regulation of circadian rhythm, photoprotection, monosaccharide transport, cold acclimation, and phosphate ion homeostasis, while the downregulated DEGs were associated with the indole glucosinolate biosynthetic process, regulation of RNA splicing, water transport, cell wall modification, cell wall loosening, cellular water homeostasis, and cell wall homeostasis. Furthermore, the up-regulated DEGs had about four times protein-protein-interactions (PPIs) than the down-regulated DEGs, and the cold-responsive genes were identified using Cytoscape software. Furthermore, qRT-PCR of low-temperature-responsive protein 78 (*LTI78*), transducin family protein (*SWA1*), and arginine methyltransferase 11 (*PRMT11*) were performed to validate the outcome of integrated bioinformatics analysis. Our work will improve our knowledge of cold-responsive mechanisms and these DEGs might be targets for plant cold stress-resistance research.

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KEYWORDS
Arabidopsis thaliana; cold stress; DEGs; integrated bioinformatics analysis; photoprotection; cell wall homeostasis

Introduction

Extreme low temperatures negatively affect crop productivity and threaten food security. Plants can perceive extreme temperatures and adjust their growth, reproduction, and development to adapt them. To better understand plant cold-resistance mechanisms and increase crop yield, *Arabidopsis thaliana* has been used as an ideal model plant to explore the response of differentially expressed genes (DEGs) to cold stress (Gong et al. 2020).

In previous studies, several important *Arabidopsis* genes and pathways, including calcium signaling, mitogen-activated protein kinase (MAPK) signaling, jasmonic acid (JA) signaling, and C-repeat/DREB binding factor (CBF) signaling, have been reported to be involved in the cold response (Shi et al. 2018; Wu et al. 2019; Yuan et al. 2018). For example, glutamate receptor family proteins (*ATGLR1.2* and *ATGLR1.3*) are glutamate-like receptors related to cold stress. Overexpression of *ATGLR1.2* and *ATGLR1.3* could enhance the gene expression of the CBF signaling pathway, which positively improves cold adaption in *Arabidopsis* (Zheng et al. 2018). The β-expansin gene (*TaEXPB7-B*) is another cold-responsive gene located in the *Arabidopsis* cell wall. Overexpression of *TaEXPB7-B* improved cellulose and lignin content, and increased antioxidant activity to survive at low-temperatures (Feng et al. 2019). The gene open stomata 1 (*OST1*) also plays an important role in enhancing low-temperature tolerance in *Arabidopsis*, and interacts with a plasma membrane-localized clade-E growth-regulating 2 (*EGR2*) phosphatase to facilitate better adaption of plants under cold stress (Ding et al. 2019). Abscisic acid (ABA) is associated with plant adaptation to cold stress. Overexpression of rice pyrabactin resistance-like gene 3 (*OsPYL3*) in *Arabidopsis* could increase the sensitivity of the ABA signaling pathway. As a result, the plant cold stress was enhanced (Lenka et al. 2018). *Arabidopsis thaliana* DEAD-box RNA helicase 7 (*AtRH7*) is a DEAD-box RNA helicase that interacts with cold shock domain protein 3 (*AtCSP3*), which plays a considerable role in cold tolerance. Mutants of *AtRH7* negatively affect pre-rRNA processing and cause delay in first leaf emergence in *Arabidopsis* (Liu et al. 2016). Ubiquitin-conjugating enzyme 13 (*UBC13*) is an important cold-responsive gene that participates in programmed cell death pathways in *Arabidopsis*. The UBC 13 mutant was involved in the lesion mimic phenotype and interacted with F-box and associated interaction domains-containing protein 1 (CPR1), which is an F-box protein that regulates TIR-NBS-LRR class disease resistance protein 1 (SNC1) degradation under cold stress (Wang et al. 2019). *Arabidopsis* cystatin A (*AtCYSa*) and cystatin B (*AtCYSb*) are cysteine

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proteinase inhibitors that can be induced by cold stress. Their promoter regions included a dehydration-responsive element (*DRE*) and abscisic acid-responsive element (*ABRE*). Therefore, *AtCYSa* and *AtCYSb* are target genes of the dehydration response element B1A (*DREB1A*) and AREB. Overexpression of *AtCYSa* and *AtCYSb* enhances plant tolerance to environmental stress (Zhang et al. 2008). By regulating the expression of cold-responsive salicylic acid and bZIP transcription factor family protein (TGA), the regulatory protein (NPR1) also plays a vital role in enhancing the cold acclimation of *Arabidopsis*. Interacting with *HSFA1* significantly promoted cold tolerance in plants, and *NPR1* might be an essential gene during plant cold acclimation (Olate et al. 2018).

Although cold-responsive genes have been reported by different laboratories, more comprehensive DEGs will better disclose the mechanisms of cold acclimation in plants. Recently, 5742 genes were differentially expressed to reveal the gene regulators and pathways involved in cold tolerance in *Brassica napus*. These DEGs were related to the inhibition of photosynthesis and the primary biological processes (Ke et al. 2020). To identify early responsive events in *Oryza sativa* under cold stress, a transcriptome profile was performed to identify 516 DEGs that were involved in Ca^{2+} and ROS-mediated signaling, and the DREB/CBF pathway (Dasgupta et al. 2020).

In the present study, to disclose the details of cold-responsive DEGs in *Arabidopsis*, the Gene Expression Omnibus database (GEO) was used to retrieve the DEGs of plant cold tolerance. Then, 261 upregulated and 177 downregulated genes from the GEO database were identified using a rank aggregation method. Integrated bioinformatics strategies have been applied to demonstrate the molecular functions, signaling pathways, and PPI interaction network of cold-responsive DEGs. These DEGs will greatly improve our knowledge of plant cold-response mechanisms and might become useful targets for crop plant growth, development, and crop output.

Materials and Methods

Retrieval of cold-responsive genes in Arabidopsis *from GEO database*

The eight responsive gene expression profiles of the microarray and RNA-seq data related to cold stress in *Arabidopsis* (Table 1) were downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/).

Microarray data normalization and robust rank aggregation (RRA) algorithm in Arabidopsis

Microarray data were downloaded from the GEO database in TXT format (https://www.ncbi.nlm.nih.gov/geo/). The R software package was used to process the matrix files and filter low-quality data. The resultant data were log2 transformed and processed with the limma package (http://www.bioconductor.org/) to retain the DEGs that had a p-value < 0.05, and a |log2fold change (FC)|> 1. Furthermore, the DEGs identified above were integrated into the robust rank aggregation (RRA) software package (https://cran.r-project.org/web/packages /RobustRankAggreg/index.html). Using a null hypothesis of uncorrelated inputs, the RRA algorithm defines genes ranked consistently better than expected, and then assigns a significance score to each gene. The hypothesis of the RRA method is that each gene is in a random order in each experiment. If a gene highly ranked in all experiments, its p-value is smaller (Niu et al. 2019).

Cluster analysis of cold-responsive DEGs in Arabidopsis

The resultant cold-responsive DEGs were clustered by Gene Cluster 3.0, using the C Clustering Library version 1.24 created by Michael de Hoon, Seiya Imoto and Satoru Miyano, and the results were viewed using Java TreeView software created by Alok (Version:1.0.4; http://jtreeview.sourceforge.net).

Gene ontology classification and KEGG Pathway in Arabidopsis

The bioinformatics tools of the PANTHER (Protein Analysis THrough Evolutionary Relationships) classification system (Version 15.0 released 2019_04) (http://pantherdb.org/) and DAVID (Database for annotation, visualization and integrated discovery, Version 6.8) (https://david.ncifcrf.gov/) were applied for gene ontology enrichment analysis of the DEGs. Each gene was classified into a single category.

Table 1. Detailed information about GEO data were showed in this study.

Dataset	Stress	Platform	Number of samples (Treatment/Control)	Experimental set-up	Organism	Data	Tissue
GSE3326	cold	GPL198	4(2/2)	0°C, 24h	Arabidopsis thaliana	RNA-seq	seedlings
GSE31837	cold	GPL198	6(3/3)	4°C, 3h	Arabidopsis thaliana	Microarray	T87 cells
GSE39090	cold	GPL198	4(2/2)	4°C, 24h	Arabidopsis thaliana	Microarray	seedlings
GSE43818	cold	GPL198	6(3/3)	4°C, 24h	Arabidopsis thaliana	RNA-seq	Leaves
GSE55907	cold	GPL198	4(2/2)	4°C, 24h	Arabidopsis thaliana	Microarray	seedlings
GSE106635	cold	GPL198	4(2/2)	4°C, 8h	Arabidopsis thaliana	Microarray	seedlings
GSE112389	cold	GPL198	4(2/2)	4°C, 8h	Arabidopsis thaliana	Microarray	Leaves
GSE113547	cold	GPL198	6(3/3)	4°C, 24h	Arabidopsis thaliana	Microarray	roots

Table 2. Primers Used in qRT-PCR.

gene names	forward primers (5′ to 3′)	reverse primers (5′ to 3′)
***Lti78* (At5g52310)**	CACCAGGCGTAACAGGTAAA	AACGTCGTCCTTACAGAATGAG
***Swa1* (At2g47990)**	GCACTGAGGCCTACGTATTT	CAACATTGGCCGGTTTCTTC
***Prmt11* (At4g29510)**	CAGTAACCACGACGAGAATGAG	GTAATCGGCACTGGTGGTATC
***Act2* (AT3G18780)**	ACCTTGCTGGACGTGACCTTACTGAT	GTTGTCTCGTGGATTCCAGCAGCTT

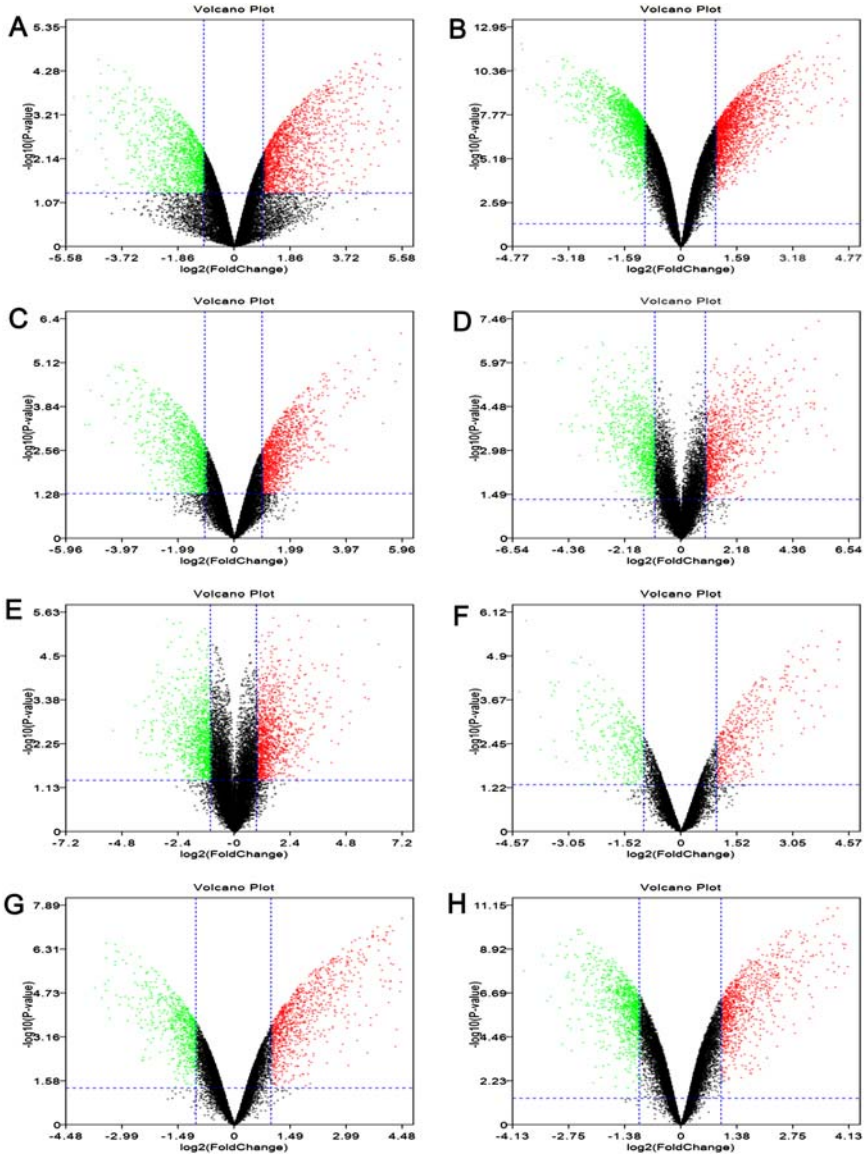

Figure 1. Differential expression genes of Arabidopsis responding to cold stress. A. GSE3326 (control: GSM74894, GSM74895, cold: GSM74900, GSM74901); B. GSE31837 (control: GSM789668, GSM789675, GSM789682, cold: GSM789671, GSM789678, GSM789685); C. GSE39090 (control: GSM955985,GSM955986, cold: GSM955989, GSM955990); D. GSE43818 (control: GSM1071668, GSM1071669, GSM1071670, cold: GSM1071671, GSM1071672, GSM1071673); E. GSE55907 (control: GSM1348266, GSM1348267, cold: GSM1348268, GSM1348269); F. GSE106635 (control: GSM2844128, GSM2844129, cold: GSM2844132, GSM2844133); G. GSE112389 (control: GSM3069395, GSM3069396, cold: GSM3069397, GSM3069398); H. GSE113547 (control: GSM3108856, GSM3108857, GSM3108858, cold: GSM3108862, GSM3108863, GSM3108864). The red points represent up-regulated genes (fold change > 2.0, P-value < 0.05. The green points represent down-regulated genes (fold change < 0.5, P-value < 0.05). The black points represent unchanged genes difference.

Protein-protein-interaction (PPI) networks in Arabidopsis

PPI networks were analyzed using the STRING (search tool for recurring instances of neighbouring genes) database (Version 11.0, released January 19, 2019) (http://string-db.org/). Subsequently, the maximal clique centrality (MCC) app in Cytoscape software was used to screen modules within the PPI network with the default parameters.

Growth conditions and harvest of Arabidopsis

According to a previous publication (Guo et al. 2020b), ecotype Col-0 *Arabidopsis* seeds were germinated on a normal medium plate at 22/20°C day/night, an 8/16 h light/dark cycle, and 60 μmol·m^{-2}s^{-1} of light intensity. After 7 days, the *A. thaliana* seedlings individually underwent cold stress by exposure to a temperature of 4°C for 12 h. Then, the *Arabidopsis* roots and leaves were harvested and stored at −80°C.

qRT-PCR analysis

According to the previous method of RNA isolation and qRT-PCR analysis (Guo et al. 2020b), the relative expression levels of *Arabidopsis* gene low-temperature-responsive protein 78 (*LTI78*), transducin family protein (*SWA1*), and arginine methyltransferase 11 (*PRMT11*) were determined and *Arabidopsis* gene actin 2 (AT3G18780) was used as an endogenous reference in *Arabidopsis* roots and leaves. Briefly, total RNA was extracted using the RNAiso Plus reagent (TaKaRa). RNA concentrations were determined by spectrophotometry (NanoDrop 2000/2000C, Thermo Scientific). Then, 2 μg of total RNA was reverse transcribed using ReverTra Ace (TOYOBO). Real-time RT–PCR analysis was performed in a Roter-Gene Q (QIAGEN) using the Platinum

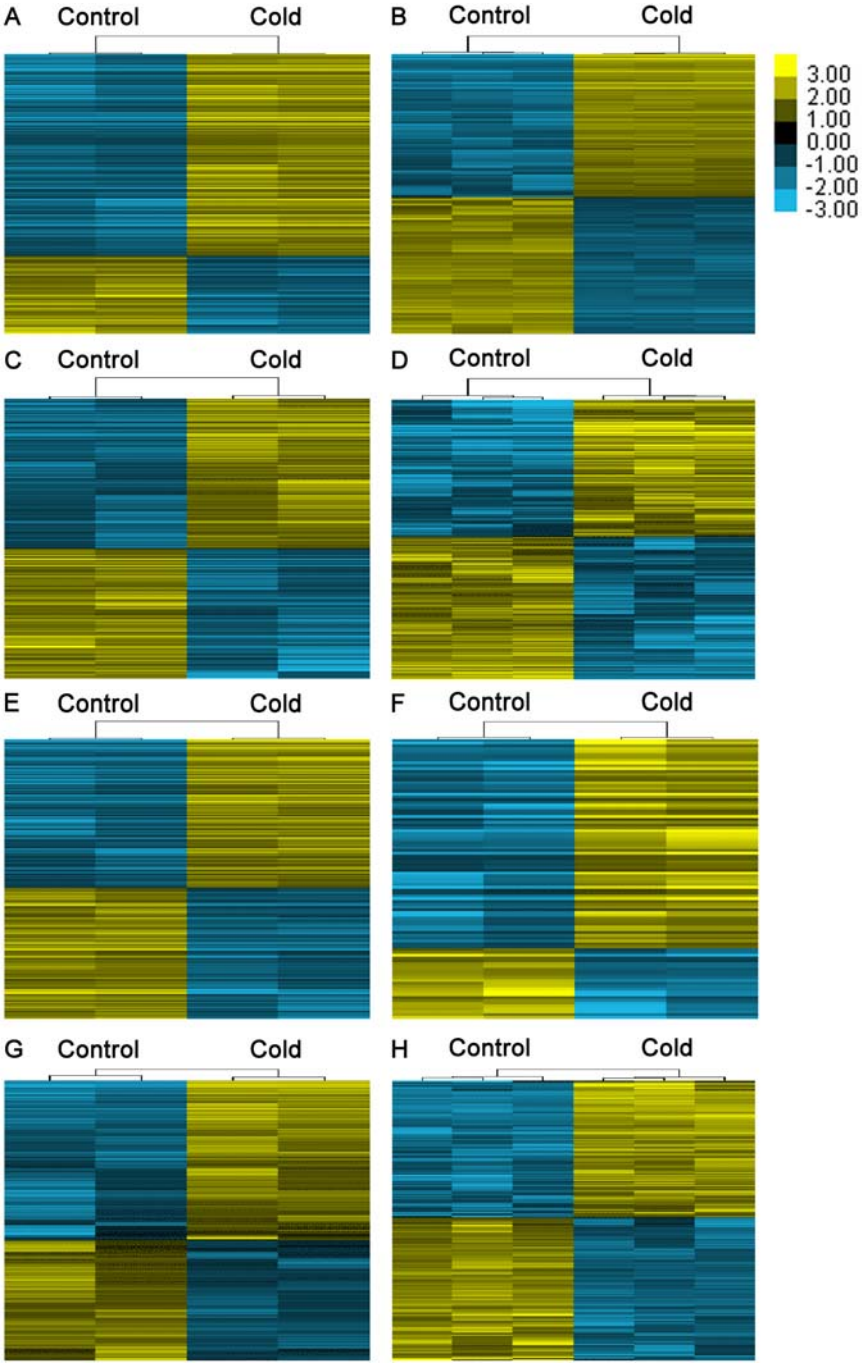

Figure 2. Hierarchial clustering of differential expression genes in *Arabidopsis* under cold stress. A. GSE3326 (control: GSM74894, GSM74895, cold: GSM74900, GSM74901); B. GSE31837 (control: GSM789668, GSM789675, GSM789682, cold: GSM789671, GSM789678, GSM789685); C. GSE39090 (control: GSM955985, GSM955986, cold: GSM955989, GSM955990); D. GSE43818 (control: GSM1071668, GSM1071669, GSM1071670, cold: GSM1071671, GSM1071672, GSM1071673); E. GSE55907 (control: GSM1348266, GSM1348267, cold: GSM1348268, GSM1348269); F. GSE106635 (control: GSM2844128, GSM2844129, cold: GSM2844132, GSM2844133); G. GSE112389 (control: GSM3069395, GSM3069396, cold: GSM3069397, GSM3069398); H. GSE113547 (control: GSM3108856, GSM3108857, GSM3108858, cold: GSM3108862, GSM3108863, GSM3108864).

SYBR Green qPCR SuperMix-UDG kit (Life Technologies Corporation). Cycling conditions were as follows: 50°C for 2 min, 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 45 s. The 2-ΔΔCt calculation was used to determine the differences in the cold-responsive genes. All experiments were performed in triplicate (Table 2).

Statistical analysis

The data of qRT-PCR analysis were statistically analyzed by means of an unpaired t-test using GraphPad Prism 7 (GraphPad Prism, La Jolla, CA, USA). Statistical significance was set at $p < 0.05$.

Results

Microarray data retrieving from GEO and raw data normalization in Arabidopsis

In the present study, eight retrieval cold-responsive microarray data (GSE3326 (control: GSM74894, GSM74895, cold: GSM74900, GSM74901); GSE31837 (control: GSM789668, GSM789675, GSM789682, cold: GSM789671, GSM789678, GSM789685); GSE39090 (control: GSM955985,GSM955986, cold: GSM955989, GSM955990); GSE43818 (control: GSM1071668, GSM1071669, GSM1071670, cold: GSM1071671, GSM1071672, GSM1071673); GSE55907 (control:

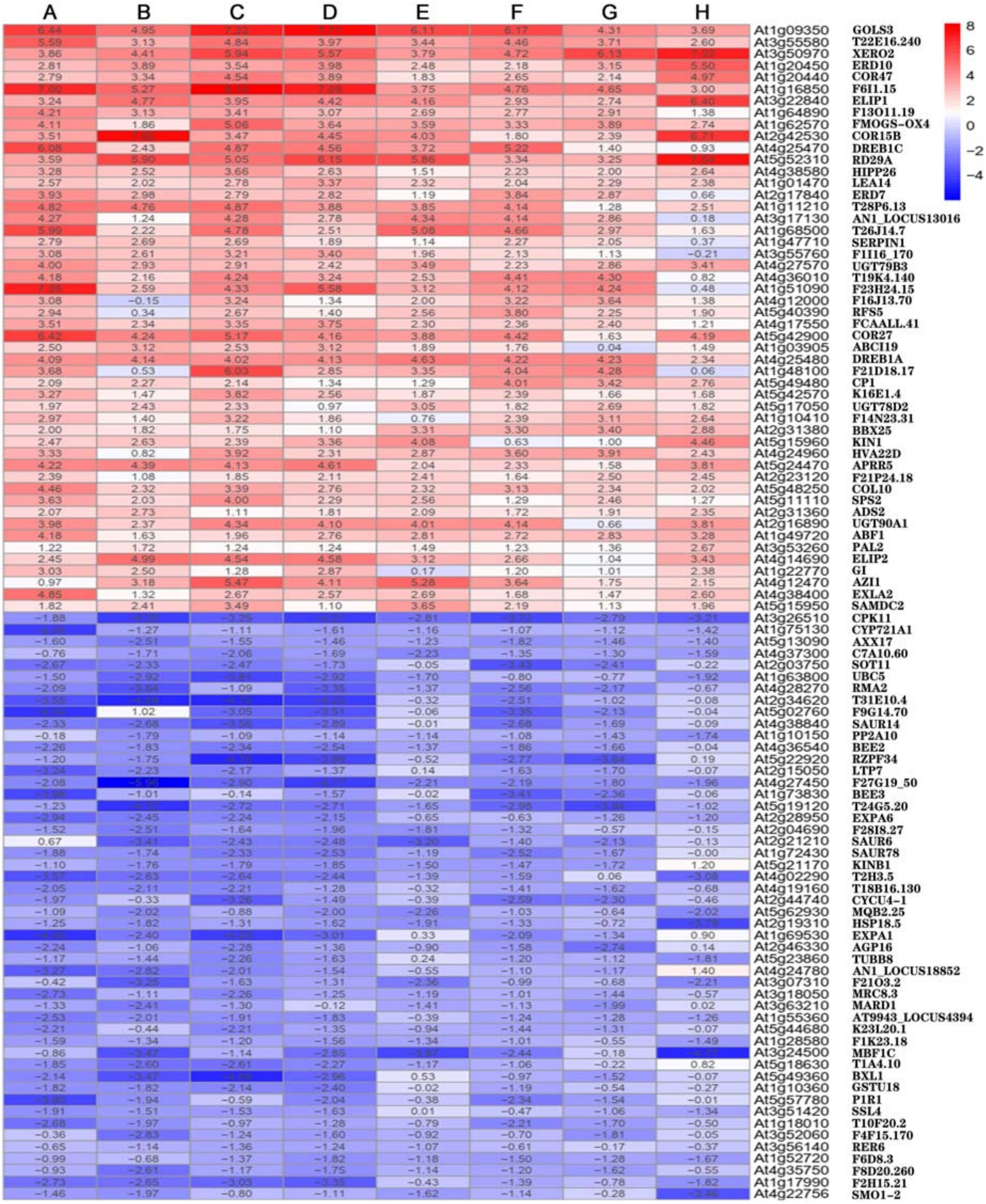
A
B
C
D
E
F
G
H
8
6
4
2
0
−2
−4
At1g09350 GOLS3
At3g55580 T22E16.240
At3g50970 XERO2
At1g20450 ERD10
At1g20440 COR47
At1g16850 F6I1.15
At3g22840 ELIP1
At1g64890 F13O11.19
At1g62570 FMOGS-OX4
At2g42530 COR15B
At4g25470 DREB1C
At5g52310 RD29A
At4g38580 HIPP26
At1g01470 LEA14
At2g17840 ERD7
At1g11210 T28P6.13
At3g17130 AN1_LOCUS13016
At1g68500 T26J14.7
At1g47710 SERPIN1
At3g55760 F1I16_170
At4g27570 UGT79B3
At4g36010 T19K4.140
At1g51090 F23H24.15
At4g12000 F16J13.70
At5g40390 RFS5
At4g17550 FCAALL.41
At5g42900 COR27
At1g03905 ABCI19
At4g25480 DREB1A
At1g48100 F21D18.17
At5g49480 CP1
At5g42570 K16E1.4
At5g17050 UGT78D2
At1g10410 F14N23.31
At2g31380 BBX25
At5g15960 KIN1
At4g24960 HVA22D
At5g24470 APRR5
At2g23120 F21P24.18
At5g48250 COL10
At5g11110 SPS2
At2g31360 ADS2
At2g16890 UGT90A1
At1g49720 ABF1
At3g53260 PAL2
At4g14690 ELIP2
At1g22770 GI
At4g12470 AZI1
At4g38400 EXLA2
At5g15950 SAMDC2
At3g26510 CPK11
At1g75130 CYP721A1
At5g13090 AXX17
At4g37300 C7A10.60
At2g03750 SOT11
At1g63800 UBC5
At4g28270 RMA2
At2g34620 T31E10.4
At5g02760 F9G14.70
At4g38840 SAUR14
At1g10150 PP2A10
At4g36540 BEE2
At5g22920 RZPF34
At2g15050 LTP7
At4g27450 F27G19_50
At1g73830 BEE3
At5g19120 T24G5.20
At2g28950 EXPA6
At2g04690 F28I8.27
At2g21210 SAUR6
At1g72430 SAUR78
At5g21170 KINB1
At4g02290 T2H3.5
At4g19160 T18B16.130
At2g44740 CYCU4-1
At5g62930 MQB2.25
At2g19310 HSP18.5
At1g69530 EXPA1
At2g46330 AGP16
At5g23860 TUBB8
At4g24780 AN1_LOCUS18852
At3g07310 F21O3.2
At3g18050 MRC8.3
At3g63210 MARD1
At1g55360 AT9943_LOCUS4394
At5g44680 K23L20.1
At1g28580 F1K23.18
At3g24500 MBF1C
At5g18630 T1A4.10
At5g49360 BXL1
At1g10360 GSTU18
At5g57780 P1R1
At3g51420 SSL4
At1g18010 T10F20.2
At3g52060 F4F15.170
At3g56140 RER6
At1g52720 F6D8.3
At4g35750 F8D20.260
At1g17990 F2H15.21
At4g22756 SMO1-2

Figure 3. The LogFC heatmap image of representative differential cold-responding genes in *Arabidopsis* by RRA. A. GSE3326 (control: GSM74894, GSM74895, cold: GSM74900, GSM74901); B. GSE113547 (control: GSM3108856, GSM3108857, GSM3108858, cold: GSM3108862, GSM3108863, GSM3108864); C. GSE31837 (control: GSM789668, GSM789675, GSM789682, cold: GSM789671, GSM789678, GSM789685); D. GSE39090 (control: GSM955985,GSM955986, cold: GSM955989, GSM955990); E. GSE43818 (control: GSM1071668, GSM1071669, GSM1071670, cold: GSM1071671, GSM1071672, GSM1071673); F. GSE112389 (control: GSM3069395, GSM3069396, cold: GSM3069397, GSM3069398); G. GSE106635 (control: GSM2844128, GSM2844129, cold: GSM2844132, GSM2844133); H. GSE55907 (control: GSM1348266, GSM1348267, cold: GSM1348268, GSM1348269).

GSM1348266, GSM1348267, cold: GSM1348268, GSM1348269); GSE106635 (control: GSM2844128, GSM2844129, cold: GSM2844132, GSM2844133); GSE112389 (control: GSM3069395, GSM3069396, cold: GSM3069397, GSM3069398); GSE113547 (control: GSM3108856, GSM3108857, GSM3108858, cold: GSM3108862, GSM3108863, GSM3108864)) were obtained from the GEO database (Table 1). To integrate the experimental data from different laboratories and platforms, the data were standardized using quantile normalization in R. Then, the eight GEO datasets were standardized as shown in Supplementary Figure 1.

Quantification of cold stress-responsive DEGs in Arabidopsis

These cold-responsive gene datasets were screened using the limma package in R with the following condition: p-value < 0.05 and $|\log_2 FC| > 1$ (Figure 1). In GSE3326, 2193 downregulated and 2452 upregulated DEGs were screened in

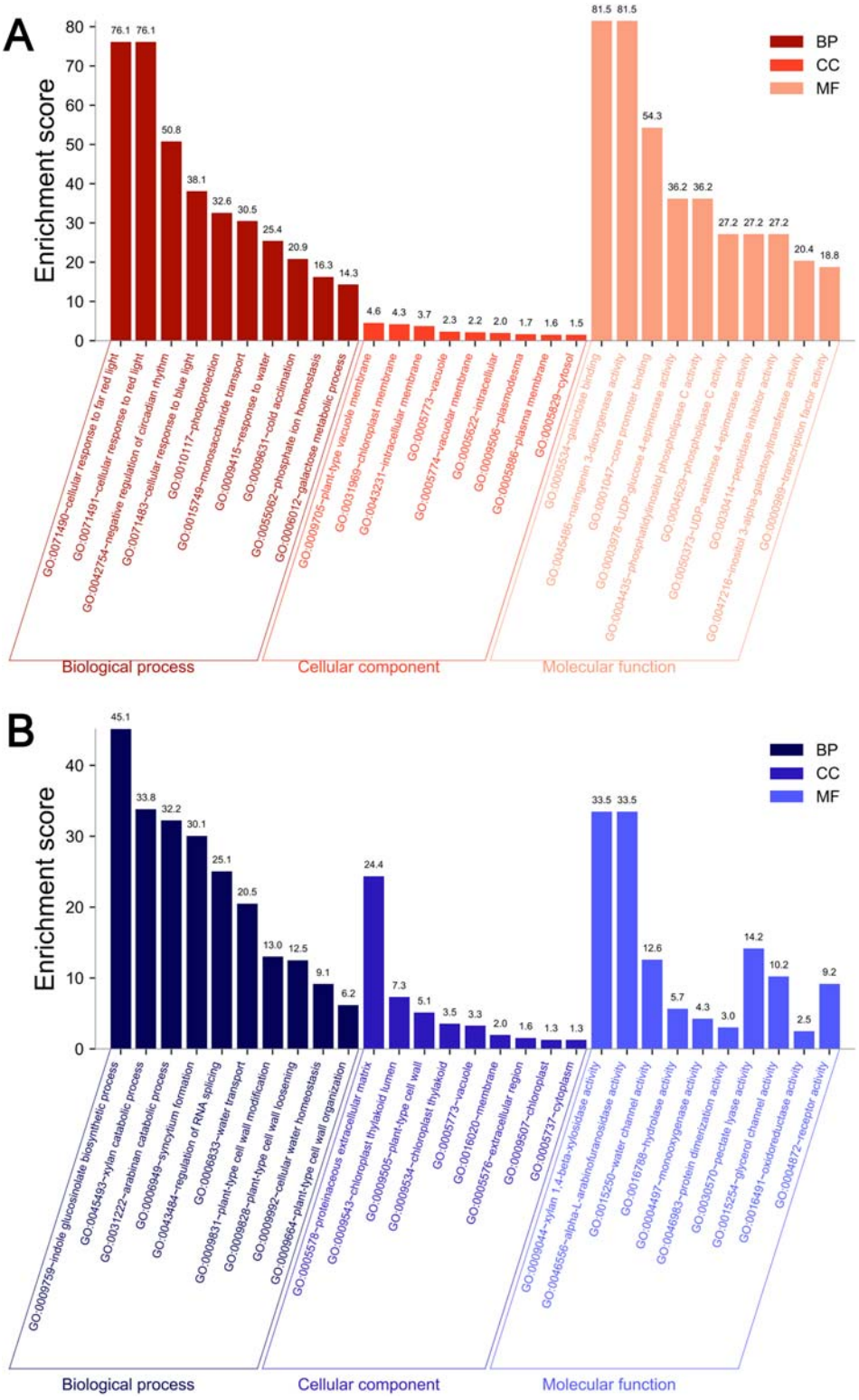

Figure 4. GO classification of differential cold stress-responding genes in *Arabidopsis*. (A) Up-regulated DEGs under cold stress (BP: biological process, CC: cell component, MF: molecular function, (B) Down-regulated DEGs under cold stress (BP: biological process, CC: cell component, MF: molecular function.

Arabidopsis. In GSE31837, 1872 downregulated and 1959 upregulated DEGs were screened in *Arabidopsis*. In GSE39090, 402 downregulated and 466 upregulated DEGs were screened in *Arabidopsis*. In GSE43818, 875 downregulated and 843 upregulated DEGs were identified in *Arabidopsis*. In GSE55907, 608 downregulated and 689 upregulated DEGs were screened in *Arabidopsis*. In GSE106635, 367 downregulated and 485 upregulated DEGs were identified in *Arabidopsis*. In GSE112389, 677 downregulated and 906 upregulated DEGs were identifie in *Arabidopsis*. In GSE113547, 1003 downregulated and 962 upregulated DEGs were screened in *Arabidopsis*. These cold-responsive DEGs were further clustered using Cluster 3.0 software, and viewed using Java TreeView software (Figure 2).

Cold-responsive DEGs screening by RRA algorithm in Arabidopsis

After the RRA method was used to integrate the above DEGs, 261 upregulated and 177 downregulated genes were

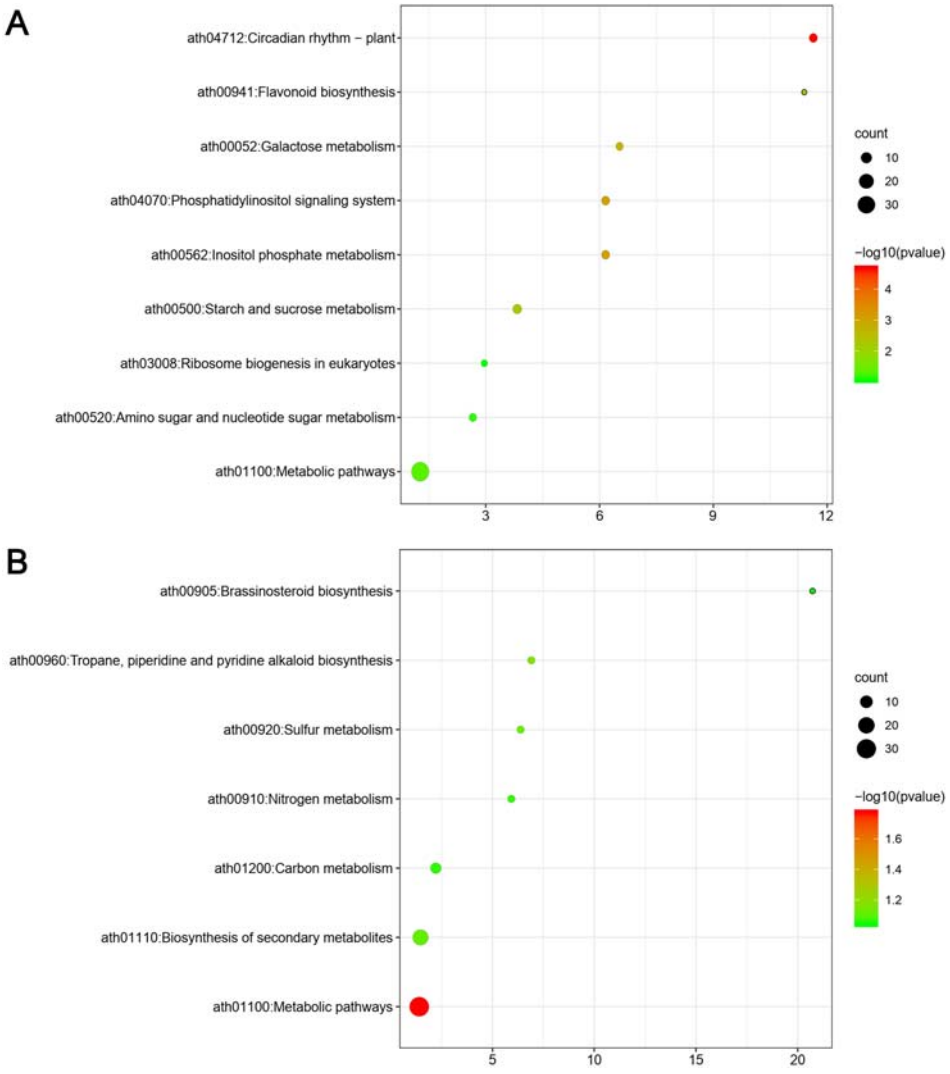

Figure 5. KEGG analysis of differential cold-responding genes in *Arabidopsis*. (A) KEGG enrichment analysis of up-regulated genes in *Arabidopsis*, (B) KEGG enrichment analysis of down-regulated genes in *Arabidopsis*. Circle size represents the number of genes, and circle color represents the p-value.

identified as the cold stress-responsive DEGs of *Arabidopsis* (Figure 3, Supplementary Table 1).

Bioinformatics enrichment of cold stress-responsive DEGs in Arabidopsis

Bioinformatics tools were further used to analyze 261 upregulated and 177 downregulated genes. For biological processes, the upregulated DEGs were mainly related to the regulation of transcription (20.3%), response to cold (14.2%), response to abscisic acid (11.5%), circadian rhythm (5.4%) and response to oxidative stress (4.2%) (Figure 4A), while the downregulated DEGs were involved in the oxidation-reduction process (12.4%) (Figure 4B). For molecular function, the upregulated DEGs were associated with transcription factor activity (17.6%), DNA binding (13.1%), and transferase activity (5.4%) (Figure 4A), and the downregulated DEGs were associated with transcription factor activity (11.9%), protein dimerization activity (4.5%) and oxidoreductase activity (4.5%) (Figure 4B). For the cellular component, the up-regulated DEGs were mainly located in the plasma membrane (22.6%), cytoplasm (22.6%), and vacuole (5.7%) (Figure 4A), while the upregulated DEGs were located in the cytoplasm (38%), chloroplast (19.2%), extracellular region (16.4%) and plant-type cell wall (6.8%) (Figure 4B).

KEGG pathway of cold-responsive DEGs in Arabidopsis

Based on the KEGG pathway, the upregulated DEGs were involved in circadian rhythm (p-value=2.18E-05), inositol phosphate metabolism (p-value=8.07E-04), phosphatidylinositol signaling system (p-value=8.07E-04), and galactose metabolism (p-value=0.0019), while the down-regulated DEGs were related to metabolic pathways (p-value=0.016) and tropane, piperidine and pyridine alkaloid biosynthesis (p-value=0.067) (Figure 5).

PPI network and genes of cold-responsive DEGs in Arabidopsis

Through the analysis of the online software STRING, 261 up-regulated cold stress-responsive DEGs were connected with 646 edges (PPI enrichment p-value=< 1.0e-16) (Figure 6A), and 177 downregulated cold stress-responsive DEGs were connected with 242 edges (PPI enrichment p-value=< 1.0e-16) (Figure 6B). Genes encoding *PRMT11*, fibrillarin 2 (*FIB2*), transducin (*YAO*), protein kinase

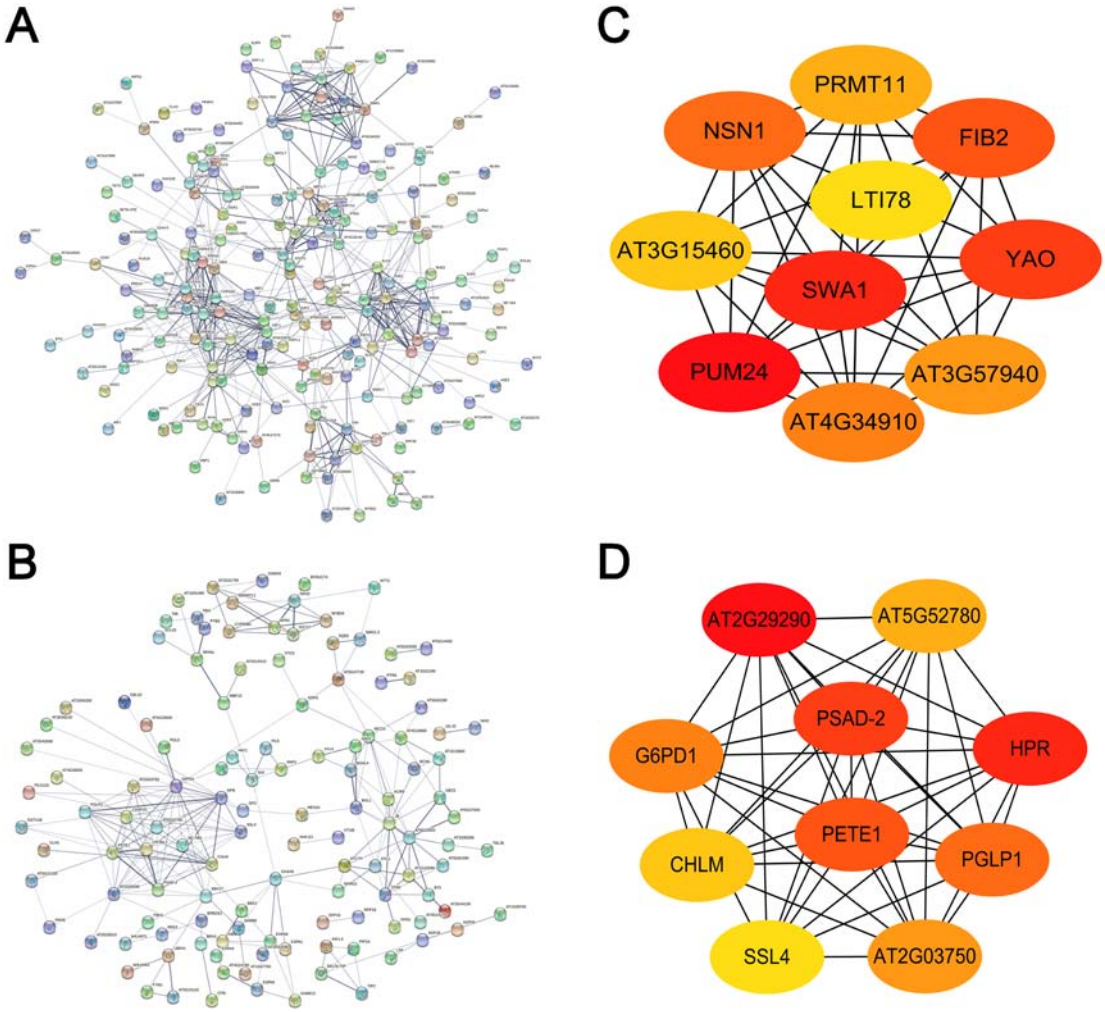

Figure 6. PPI networks of DEGs under cold stress and the interaction network of top 10 genes in *Arabidopsis*. (A) up-regulated DEGs, (B) down-regulated DEGs, (C) top 10 genes of up-regulated DEGs, (D) top 10 genes of down-regulated DEGs. DEG, differentially expressed genes; PPI, protein-protein interaction.

superfamily protein (*AT3G57640*), P-loop containing nucleoside triphosphate hydrolases superfamily protein (*AT4G34910*), pumilio 24 (*PUM24*), ribosomal RNA processing Brix domain protein (*AT3G15460*), GTP-binding family protein (*NSN1*), *LTI78*, and *SWA1* were upregulated. Meanwhile, NAD(P)-binding Rossmann-fold superfamily protein (*AT2G29290*), transmembrane protein (*AT5G52780*), hydroxypyruvate reductase (*HPR*), 2-phosphoglycolate phosphatase 1 (*PGLP1*), P-loop containing nucleoside triphosphate hydrolases superfamily protein (*AT2G03750*), strictosidine synthase-like 4 (*SSL4*), magnesium-protoporphyrin IX methyltransferase (*CHLM*), glucose-6-phosphate dehydrogenase 1 (*G6PD1*), photosystem I subunit D-2 (*PSAD-2*) and plastocyanin 1 (*PETE1*) were downregulated.

qRT-PCR analysis of cold-responsive genes in Arabidopsis

Our results showed that cold-responsive *Arabidopsis* genes *LTI78*, *SWA1*, and *PRMT11* were upregulated under cold stress. Therefore, the relative mRNA expression of Arabidopsis genes *LTI78*, *SWA1*, and *PRMT11* in both *Arabidopsis* roots and leaves were assayed under cold stress to validate the confidence of the bioinformatic outcome. The results indicated that the relative mRNA expression levels of *Arabidopsis* genes *LTI78*, *SWA1*, and *PRMT11* in *Arabidopsis* roots and leaves were all increased under cold stress (Figure 7).

Discussion

Cold stress negatively affects plant growth and crop yield. *Arabidopsis* has been used as a model plant for research on cold-responsive mechanisms. Our previous work indicated that there are complex signaling pathways and PPI interaction networks under salt stress in *Arabidopsis* (Guo et al. 2014; Guo et al. 2019a; Guo et al. 2020a; Guo et al. 2020b). However, it is still not clear how plants respond to other stresses, such as low temperatures, and further research is still needed.

Here, we retrieved the cold-responsive DEGs from the GEO database. To integrate the DEGs from the eight datasets, an RRA method was executed according to a previous publication (Niu et al. 2019). As a result, 261 upregulated and 177 downregulated genes were screened using the RRA algorithm. The integrated bioinformatics methods indicated that the DEGs were involved in biological processes, KEGG pathways, and PPI interaction networks. The upregulated DEGs were involved in the negative regulation of circadian rhythm, photoprotection, monosaccharide transport, cold acclimation, and phosphate ion homeostasis, while the downregulated DEGs were mainly involved in regulation of RNA splicing, water transport, cell wall modification,

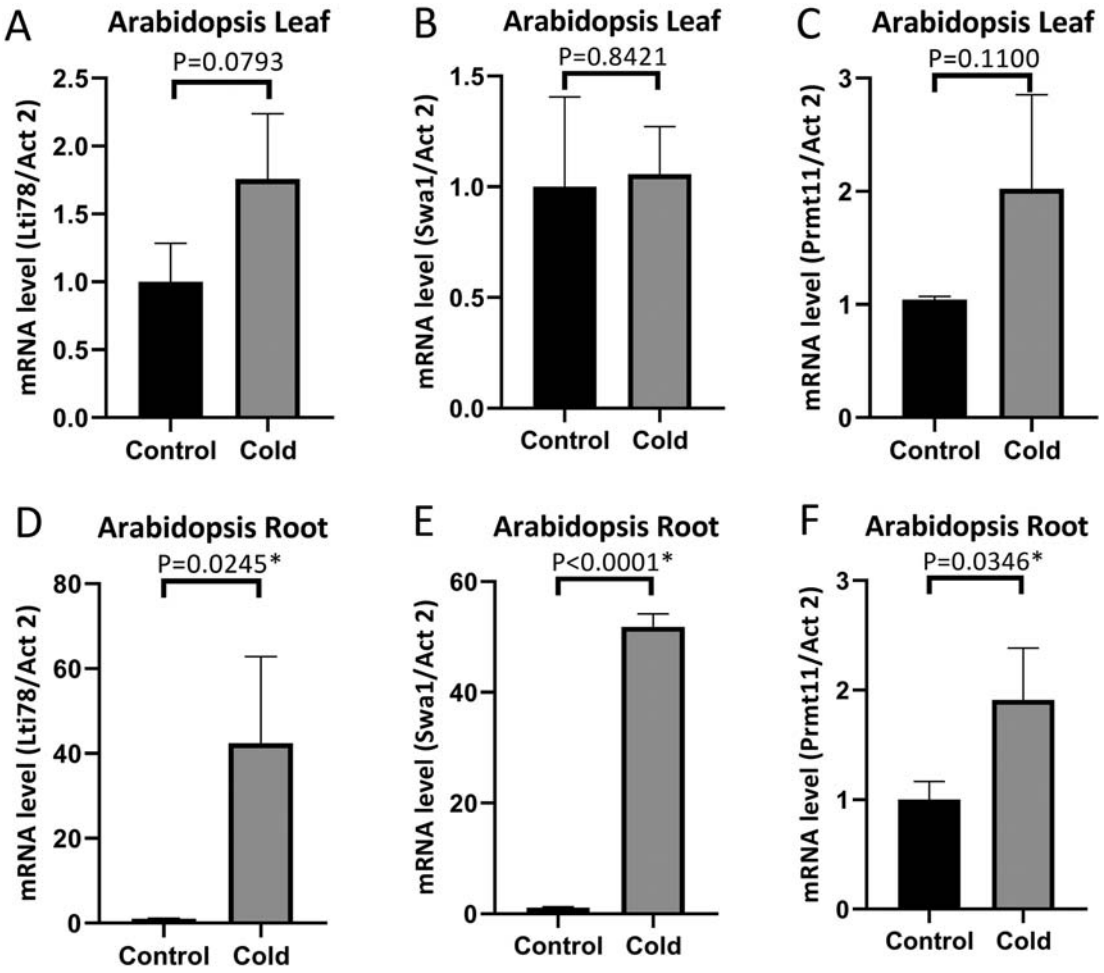

Figure 7. qRT-PCR analysis of Arabidopsis genes *Lti78 (At5g52310), Swa1 (At2g47990), and Prmt11 (At4g29510)* response to cold stress (Actin 2 (AT3G18780) was used as endogenous reference). (A) mRNA expression of *Lti78 (At5g52310)* in leaf, (B) mRNA expression of *Swa1 (At2g47990)* in leaf, (C) mRNA expression of *Prmt11 (At4g29510)* in leaf, (D) mRNA expression of *Lti78 (At5g52310)* in root, (E) mRNA expression of *Swa1 (At2g47990)* in root, (F) mRNA expression of *Prmt11 (At4g29510)* in root.

cell wall loosening, cellular water homeostasis, and cell wall homeostasis. Among the upregulated DEGs under cold stress, zinc finger protein ZAT12 (*ZAT12*) is a protein that is necessary for the expression of ascorbate peroxidases (*APXs*), maintaining the balance of ROS (Rizhsky et al. 2004). In the ABA signaling pathway, ABI3/VP1 1 (*RAV1*) is activated by serine/threonine-protein kinase (*SRK2*), which then downregulats the expression of ABI3, ABI4 and ABI5. In the present study, *RAV1* was upregulated, which was consistent with previous findings (Feng et al. 2014) and might play an important role in response to cold stress.

The ICE-CBF-COR pathway is an important signaling pathway associated with low-temperature stress (Guo et al. 2019b; Jin et al. 2018). In the present study, the *CBFs*, inducer of CBF expression (*ICEs*), and cold-responsive (*COR*) genes were differentially expressed under cold stress. Jasmonate zim-domain protein 1 (*JAZ1*) also increased in response to low temperature. It can inhibit the JA signaling pathway and regulate ICE1 expression (Hu et al. 2013). Furthermore, *JAZ1* interacted with ICE1 and regulated the expression of the CBF. CBF is regulated by the circadian clock late elongated hypocotyl (*LHY*) gene, related to plant circadian rhythm. *LYH* positively binds to the promoter of CBF. Furthermore, under cold stress, osmotically responsive gene 1 (*HOS1*) ubiquitinated ICE1, and SUMO E3 ligase (SIZ1) sumoylated ICE1. Then, the resultant ICE1 was degraded by the 26S proteasome pathway (Dong et al. 2006; Dong et al. 2011; Miura et al. 2007). Additionally, cold-regulated protein 28 (*COR28*) interacts with protein CCA1 (CCA1) and negatively regulates the expression of CBF. In the present study, JAZ1, ICE1, dehydration-responsive element-binding protein 1A/1B/1C (CBF1/2/3), LHY, CCA1, and COR27 were all overexpressed and interacted with each other, which played an important role in adapting to low temperature (Li et al. 2016). Furthermore, to compare with a previous publication, the DEGs involved in the CBF pathway under cold stress could also be found in rice through comparative transcriptome analysis (Dasgupta et al. 2020), which indicated the confidence and applicability of our results.

Conclusion

This work provides a new understanding of the details involved in tolerance of *Arabidopsis* under cold stress, which showed new signaling pathways, more cold-responsive DEGs, and more comprehensive interaction networks. This study has been helpful in demonstrating how plants survive under low temperature, and the mechanisms involved in cold tolerance might be potential targets for the research on cold-response in plants.

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MG conceived the study, and designed the experiments. MG, XL, YJ, JY, and TM performed the experiments. MG, XL, YJ, JY and TM analyzed the raw data, and drafted the manuscript. All the authors participated in the revision of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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