

Revealing the Response Mechanism of Winter Rapeseed (*Brassica rapa* L. subsp. *oleifera*) to Freezing Stress Based on Transcriptome Analysis

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Research Article

Keywords: winter rapeseed, freezing stress, root, transcriptome, differentially expressed genes

Posted Date: November 17th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-7920671/v1>

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Additional Declarations: No competing interests reported.

1 **Revealing the Response Mechanism of Winter Rapeseed (*Brassica rapa* L. subsp.**
2 ***oleifera*) to Freezing Stress Based on Transcriptome Analysis**

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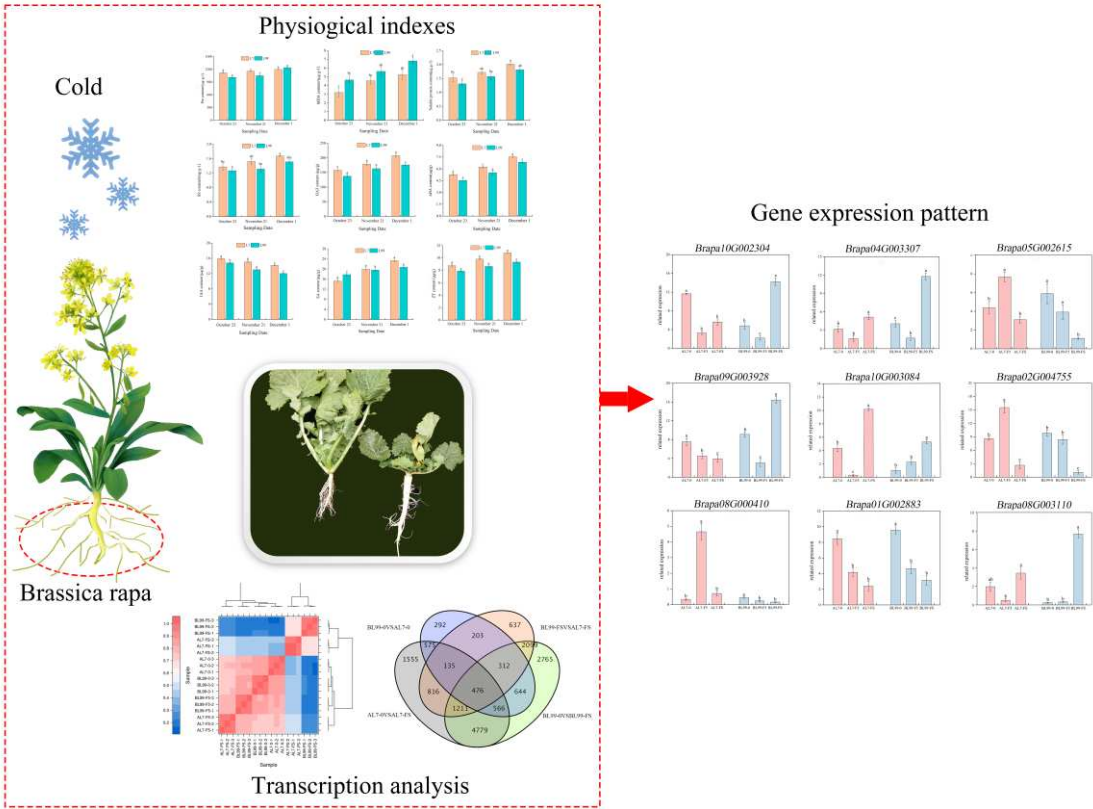
11

12 **Abstract**

13 Winter rapeseed (*Brassica rapa* L.*subsp.oleifera*) is an important oilseed crop in
14 northern China, where low-temperature stress imposes severe constraints on its yield.
15 Its leaves dry up and wither during the overwintering period, but its roots stay deeply
16 embedded in the soil. Following March of the next year, new leaves grow from the
17 growing point and it enters reproductive growth. Therefore, we carried out this research
18 for exploring the regulatory mechanisms and physiological-biochemical responses of
19 roots in different rapeseed varieties as the temperature decrease before wintering. This
20 study used a strong cold-tolerant variety "Longyou 7 (L7)" and a moderate cold-tolerant
21 variety "Longyou 99 (L99)" as experimental materials. The materials were planted in
22 field to analyze the effects of natural low-temperature stress on rapeseed growth,
23 physiological characteristics, hormone levels, and transcriptional levels before
24 wintering. Additionally, transcriptome sequencing (RNA-seq) was combined to
25 decipher the molecular regulatory mechanisms. Results showed that as temperature
26 decrease, contents of proline (Pro), soluble sugar (SS), soluble protein (SP), and
27 salicylic acid (SA) in roots of the strong cold-tolerant variety L7 were all significantly
28 higher than those in L99, while contents of malondialdehyde (MDA) and gibberellin
29 (GA₃) in L7 were significantly lower than those in L99. RNA-seq results revealed that
30 differentially expressed genes (DEGs) were significantly involved in phenylpropane
31 biosynthesis, carbon metabolism, starch and sucrose metabolism, MAPK signaling
32 pathway, ribosome, proteasome, and protein processing. Among these, the differences
33 in the expression of genes related to cell signal transduction (MAPK signaling pathway)
34 and metabolism were particularly prominent. Through weighted gene co-expression
35 network analysis (WGCNA), 9 candidate genes related to protein kinases, plant
36 hormones, transcription factors, and signal transduction were identified in the MAPK
37 and 2 other modules.

38 **Keywords:** winter rapeseed; freezing stress; root; transcriptome; differentially
39 expressed genes.

40 **Graphical Abstract**



42 **1. Introduction**

43 Winter rapeseed (*Brassica rapa* L. subsp. *oleifera*) is a crucial oilseed crop in northern
44 China, widely cultivated for its strong cold tolerance and water conservation
45 functions^[1]. However, the extreme cold climate in this region poses a severe threat to
46 its growth, overwintering, and reproduction processes. Furthermore, low-temperature
47 stress is one of the primary factors affecting plant growth and development, which
48 restricts the geographical distribution of crops and the development of the crop
49 industry^[2]. Therefore, exploring the key regulatory mechanisms underlying cold
50 tolerance in winter rapeseed can alleviate the constraints imposed by the extreme low-
51 temperature climate in northern China on its growth, overwintering, and reproduction,
52 and improve the yield and quality of rapeseed in this region.

53 Winter rapeseed is typically sown in the end of August in northern China. When it
54 reaches to 8 or 9 true-leaf stage, its aboveground tissues begin to wither, in contrast, the
55 underground root system—including the shoot apical meristem—maintains viability to

persist through the winter. With the arrival of warm spring conditions, the aboveground parts of winter rapeseed initiate regreening^[3]. Therefore, identifying hub genes associated with strong cold tolerance in winter rapeseed and enhancing its cold tolerance is of great significance for the sustainable development of the rapeseed industry.

To date, numerous studies have focused on the morphological structure, physiological and biochemical levels, and molecular biological levels of cold tolerance in *Brassica rapa* L. *subsp. oleifera*^[4]. At the morphological and physiological levels, low temperature significantly alters root morphology^{[5]-[6]} and the content of osmotic regulatory substances^{[7]-[8]}, thereby reducing the root system's efficiency in absorbing water and nutrients and affecting the overall development of the plant. Low temperature increases membrane permeability and causes intracellular ion imbalance^[9], while inhibiting enzyme activity and disrupting metabolic pathways^[10]; furthermore, severe freezing damage can disrupt the biological membrane system and even cause plant death. Furthermore, extreme freezing injury can cause disruption of the cellular biomembrane system, and in severe cases, it may lead to plant death^{[11]-[12]}.

To cope with low-temperature stress, catalase (CAT) in plants maintains intracellular reactive oxygen species homeostasis, while osmotic regulatory substances and malondialdehyde (MDA) alleviate damage to the plasma membrane by balancing cellular osmotic pressure^{[13]-[15]}. Furthermore, low temperature can trigger calcium ion (Ca^{2+}) signal transduction, activating the expression of defense genes and the synthesis of secondary metabolites to help plants adapt to the environment^[16].

Recent analyses of transcriptomics, proteomics, miRNA sequencing, and exogenous factor treatments have shown that under low-temperature stress, the magnitude of changes in metabolism-related proteins in cold-tolerant varieties of winter-type *Brassica rapa* L. is greater than that in moderately cold-tolerant varieties^{[17]-[20]}. Low temperature can rapidly induce the expression of stress-responsive genes, such as those belonging to transcription factor (TF) families; these genes enhance cold tolerance by regulating downstream pathways, in which the calcium ion (Ca^{2+})/ROS cascade and the MAPK pathway play a key role. Additionally, studies have clarified the variation patterns of endogenous hormone contents in different winter rapeseed varieties under low-temperature conditions, as well as the mechanism by which endogenous hormones regulate cold tolerance in winter rapeseed^{[21]-[24]}.

Results showed that, compared with moderate cold-tolerant variety grown in the field, cold-tolerant varieties exhibited lower contents of malondialdehyde (MDA) and indole-3-acetic acid (IAA), along with higher contents of soluble sugar (SS) and soluble protein (SP). However, what are the hub genes affecting winter rapeseed strong cold resistance, and what are the mechanisms. These warrant in-depth investigation. This study systematically analyzed the transcriptomic changes in roots of two winter

rapeseed accessions (L7 and L99) under cold stress at the transcriptional level, aiming to screen for key differentially expressed genes (DEGs), enriched metabolic pathways, and core regulatory factors that mediate the phenotypic differences in cold tolerance between the two accessions. Specifically, this study compared the gene expression profiles of L7 and L99 at different low-temperature treatment stages, and further verified the function of candidate genes via quantitative real-time PCR (qRT-PCR). It is expected that the results of this study will elucidate the molecular mechanism underlying the differences in cold tolerance between L7 and L99, and provide a theoretical basis and genetic resources for the breeding of cold-tolerant rapeseed varieties.

2. Materials and Methods

2.1 Plant material and treatment

We used a strong cold-tolerant variety "Longyou 7 (L7)" and a moderate cold-tolerant variety "Longyou 99 (L99)" as experimental materials. Samples were collected during the autumn-to-winter growth period of winter rapeseed, with simultaneous determination of plant phenotypic traits. Three sampling time points were established for root samples, October 23rd (0°C), November 21st (-5°C), and December 1st (-10°C). Among these, October 23rd, 2024, was the first sampling day, with a minimum air temperature of 0°C; November 21, 2024, had a minimum air temperature of -5°C, and December 1, 2024, had a minimum air temperature of -10°C. To eliminate the interference of circadian rhythm on experimental results, all field samples were uniformly collected between 10:30 a.m. and 11:30 a.m. on their respective sampling days, ensuring temporal consistency in sample collection. All root samples obtained in this study were used for subsequent determination of physiological indices, hormone level detection, and transcriptomic analysis, providing basic materials for the subsequent exploration of the response mechanism of winter rapeseed roots under low-temperature stress. The sample processing procedure was as follows: rapeseed roots were washed with deionised water, tightly wrapped in aluminium foil, rapidly frozen in liquid nitrogen, and then stored at -80°C in an ultra-low temperature freezer for later use.

2.2 Measurement of root phenotype indicators

In the field trial, 10 plants were first selected from each of the two materials and their chlorophyll content was measured using a SPAD520 portable chlorophyll meter. Subsequently, 6 plants were selected from each material, their fresh weight and dry weight were measured, and their roots were washed with distilled water and scanned

using a GXY-A root system phenotyping analysis system to obtain root system phenotyping data.

2.3 Determination of root physiological indices

When rapeseed plants grew to the 3-5 leaf stage, root samples of Longyou 7 (L7) and Longyou 99 (L99) were collected in field on October 23rd, November 21st, and December 1st. A 0.5 g portion of fresh root sample was weighed and rapidly ground in liquid nitrogen. Proline (Pro) content was determined using acidic ninhydrin mixing method^[25]. Malondialdehyde (MDA) content was measured by thiobarbituric acid method^[26]. Soluble protein (SP) content was assayed via Coomassie brilliant blue method^[27]; and soluble sugar (SS) content was determined using anthrone method^[28]. Three biological replicates were set for each treatment.

2.4 Determination of endogenous hormone content in root

Root samples of two cultivars were collected at the same time as that in 2.3. Endogenous hormones contents were determined by high-performance liquid chromatography (HPLC). The reagents used in the experiment were acetonitrile and methanol. The extraction and preparation of samples were carried out with reference to the method by Fan Junqiang^[29] with slight modifications. The chromatographic conditions were as follows: a C18W column was used; mobile phase was prepared with methanol and 1% phosphoric acid; detection wavelength was 280 nm; flow rate was 1 ml/min; column temperature was 30 °C; injection volume was 10 µl; and detection time for a single sample was 30 min. The calculation of endogenous hormone contents was performed with reference to the method by Niu Yajie et al.^[30].

2.5 Determination of calcium ion flux in root

Roots with consistent status were selected and fixed in a Petri dish. They were immersed in a room-temperature test solution (0.2 mM CaCl₂, 0.2 mM MES, pH 5.8) and allowed to stand for 0.5 h. Then, using non-invasive micro-test technology (NMT) and a Ca²⁺ flux microsensor, calcium ion flux was measured at root surface site 350 µm from apex of root tip^[31]; inductively coupled plasma optical emission spectrometry was used to determine calcium ion content^[32].

2.6 Transcriptomics analysis

The frozen rapeseed roots were sent to Beijing Bemaik Biotechnology Co., Ltd. for transcriptomic sequencing and identification (<https://www.biocloud.net/>). The purity and concentration of RNA were determined using a NanoDrop 2000 spectrophotometer

and Agilent 2100/LabChip GX. Subsequently, library construction and quality control were performed, and effective concentration of the library was accurately quantified using Q-PCR method. HISAT2^[33] software was used to rapidly and accurately align Clean Reads with the reference genome, obtaining their positioning information on the reference genome; gene expression levels were measured using fragments per million mapped fragments per thousand base pairs (FPKM). For differential analysis, groups with biological replicates used DESeq2^[34] software, while groups without biological replicates used edgeR^[35] software. For ClusterProfiler, hypergeometric tests were used to perform GO annotation analysis on enriched genes, including enrichment analysis of biological processes, molecular functions, and cellular components. KEGG (Kyoto Encyclopedia of Genes and Genomes) is a database for systematic analysis of gene function and genomic information, and it was used to analyse the enrichment of differentially expressed genes in KEGG pathways (<https://www.genome.jp/kegg>).

2.7 Weighted gene co-expression network analysis (WGCNA)

WGCNA (Weighted Gene Co-Expression Network Analysis) of differentially expressed genes (DEGs) was performed using the Biomarker Cloud Service Platform (<https://www.biocloud.net/>) to construct a gene co-expression network, and Cytoscape (version 3.10.1) was used for network visualization. A co-expression network with a power value of 30 was constructed to ensure high gene connectivity. The module merging height threshold was set to merge CutHeight = 0.25 (corresponding to 75% similarity), and genes with similar expression patterns were clustered into the same module. Important modules were screened by calculating kME (module eigengene connectivity) ($kME > 0.7$), which were then subjected to correlation analysis with physiological traits. Finally, hub genes with the highest connectivity among genes in each module were screened based on the module eigengene values and the number of gene-gene connections (edge counts). Cytoscape (version 3.10.1) was used to visualize the top 20 hub genes in the selected modules and their interaction networks.

2.8 qRT-PCR validation

Real-time PCR amplification was carried out on a QuantStudio 5 real-time PCR system (ABI, United States) using SYBR Green I (Takara, RR820A, Dalian, China) to validate the transcriptome sequencing results. Based on the transcriptome data, we selected 6 differentially expressed genes (DEGs) and 9 candidate genes from two materials, L7 and L99, to validate their relative expression levels. The detection was conducted using TaKaRa fluorescent quantitative reagent TB Green kit in accordance with the instruction manual. With reference to the rapeseed genome sequence, specific primers were designed using the Primer-BLAST tool of National Center for Biotechnology

Information (NCBI) (Tables 1、Table2). The $2^{-\Delta\Delta C_t}$ method was used to calculate gene expression levels, and Origin 2024 software was employed for graphing and analysis.

Table 1: qRT-PCR primer sequences of differentially expressed genes

Gene ID	Upstream primer(5'-3')	Downstream primer(5'-3')
<i>Brapa09G006484</i>	GGTCCCATTCAACTTCTTGGAAT	TGCTTTGGTCGGTCTGTACC
<i>Brapa09G004404</i>	GCCTATCCATCGCCACTCAA	CTCCTCTATTACCTGCTTGTGAAC
<i>Brapa04G000176</i>	CCTGGTAATGCTCTCCGCAT	CGCATATTCATCCATCCTTCCA
<i>Brapa07G002667</i>	ACGGTTCTGTTTTCGCTTCG	AACCTAACATGCGCCAGACA
<i>Brapa09G003893</i>	GAGGTTCAGCAACGGAAGGA	GAAACCCGCTGGCCTAATTC
<i>Brapa01G003538</i>	GAAGACTTCAGAGGGCATTCTG	CCGATCACTTCATCAAGCTACTG

Table 2: qRT-PCR primer sequences of candidate genes

Gene ID	Upstream primer(5'-3')	Downstream primer(5'-3')
<i>Brapa08G000410</i>	AAGGTTCCAGCTAAAAAGGTTGAG	GGCTACCACAGGTTTCATCATCT
<i>Brapa01G002883</i>	CGAGGCTGATGAGGGTCAAA	TGGGATGGCGAAGTGATCTG
<i>Brapa05G002615</i>	CAGGGCGAAGCAAAGCC	ACCCATGAGCTAAGACGAAGA
<i>Brapa08G003110</i>	TGAAGGGTCCGGTTTCAGTC	CCTCAACTGCTTTACGCTTGG
<i>Brapa02G004755</i>	GTATGGAAACCCGATTGGCG	AAGCATTCTCCGCCGTAT
<i>Brapa10G003084</i>	GGGTTTAATATGTCGGTGGGG	TCCGTCCATATCCTCTTCCGA
<i>Brapa04G003370</i>	ATGAAGCTCCCTTCCCTTCG	TACTCCTCAGTCCGGGAGAG
<i>Brapa10G002304</i>	AGAAACGAGCAGGGAGGAAG	GGTCGGAAAAGTCCCTAACCA
<i>Brapa09G003928</i>	TCGCGTTTCTCTTACCTCCT	TTTCTTCCACCCTTCCCCG

3. Results

3.1 The effects of freezing temperature stress on the phenotypic characteristics, physiological properties, and endogenous hormones of rapeseed

The results showed that, on October 23rd, neither variety exhibited obvious symptoms of low-temperature stress; on November 21st, leaves of L7 showed mild wilting and yellowing, while L99 had no wilting or yellowing; on December 1st, most leaves of L7 were withered and obviously damaged with frost, whereas L99 had milder leaf wilting accompanied by frost (**Figure 1**). It can be concluded that freezing temperature had significant effects on both varieties, leading to gradual leaf wilting and withering, but the wilting degree of L99 was lower than that of L7. This indicates that with the decrease in temperature, the aboveground parts of L7 exhibited phenotypes of growth stagnation, leaf chlorosis, wilting, and even withering within a short period; in contrast, the aboveground parts of L99 only showed the wilting phenotype when the temperature dropped to -10°C.

A correlation analysis was performed on root phenotypic indices of two cultivars. Results of correlation analysis demonstrated that there was an extremely significant correlation between dry and fresh weights of above-ground and underground parts, as well as root-shoot ratio. Specifically, root-shoot ratio was negatively correlated with fresh weight (-0.529) and dry weight (-0.536) of above-ground parts. Leaf water content showed a significant positive correlation with shoot fresh weight (0.158) and was positively correlated with all other indices. Moreover, main root length was extremely significantly correlated with number of root tips (0.867), and number of root tips was significantly correlated with most indices (**Figure 2**). The above analysis results suggest that freezing injury of plants under freezing temperature stress can be reflected through the corresponding indices of leaves and growth status of roots. That is, under freezing temperature stress, although the leaves of rapeseed cultivars with strong cold resistance are severely damaged, their roots can maintain relatively intact cellular structures and physiological functions. As a cultivar with strong cold resistance, well-developed root tissues of L7 may serve as an important basis for maintaining a good physiological state under low-temperature environments, which further confirms the close relationship between root growth status and plant cold resistance.

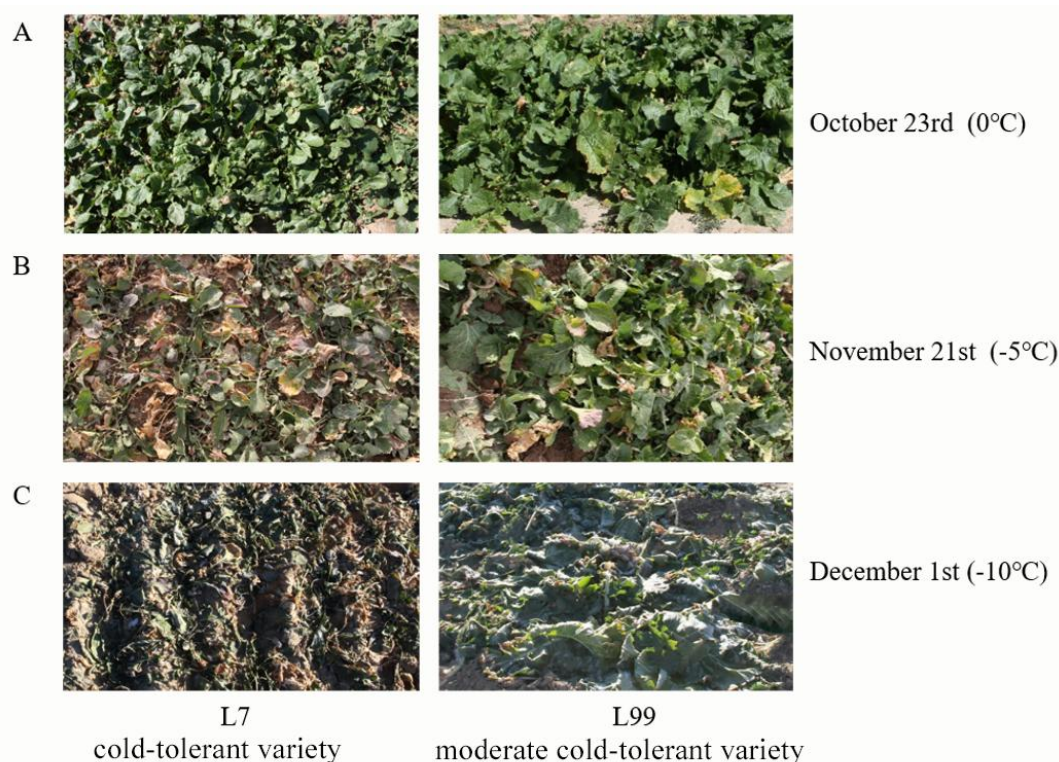


Figure 1: Phenotypic characteristics of rapeseed under low-temperature stress in the field.
Note: (A) October 23rd (0°C), (B) November 21st (-5°C), (C) December 1st (-10°C). L7(left)
L99 (right)

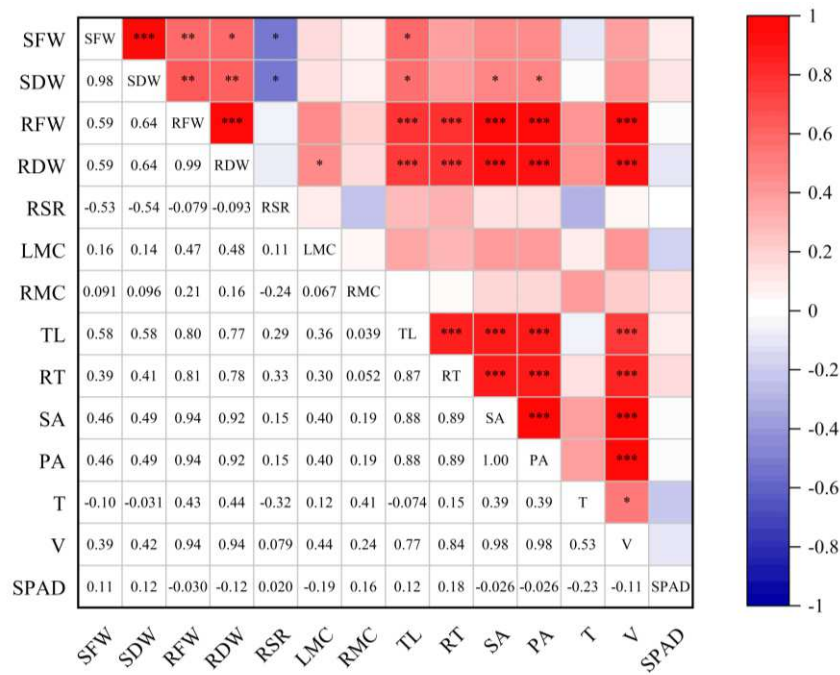


Figure 2: Correlation analysis of phenotypic indicators

Note: *indicates a significant difference at $p < 0.05$, **indicates an extremely significant difference at $p < 0.01$, ***indicates an extremely significant difference at $p < 0.001$, the same below.

SFW: Shoot fresh weight; SDW: Shoot dry weight; RFW: Root fresh weight; RDW: Root dry weight; RSR: Root shoot ratio; LMC: Leaf mositure content; RMC: Root mositure content; TL: Taproot length; RT: Number of root tips; SA: Root surface area; PA: Root projection area; T: Root tip Diameter; V: Root volume.

Studies on roots and physiological indicators of L7 and L99 under freezing temperature stress revealed that as temperature decreased, content of pPro and a osmotic regulatory substance in roots of L7 were significantly higher than that in L99 (**Figure 3A**). The content of MDA in L7 showed a significant decreasing trend (**Figure 3B**); simultaneously, SS and SP contents in L7 were significantly higher than those in L99 (**Figures 3Cand D**). From a temporal perspective, GA₃ and ABA contents in both L7 and L99 continued to rise over time, with L7 maintaining a consistently higher level (**Figures 3E and 3F**). IAA content exhibited a pattern of first increasing and then decreasing (**Figure 3G**); SA content continued to rise (**Figure 3H**); and ZT content first decreased and then recovered (**Figure 3I**). Overall, under freezing stress, osmotic regulatory substances and protective chemicals play a key role in regulating plant growth and development and stress responses. L7 demonstrates superior cold tolerance compared to L99 through more efficient osmotic regulation, substance accumulation, and hormone regulation, providing a physiological basis for its adaptation to low-temperature environments.

The correlation analysis of root physiological indices and hormone levels between the two materials (**Figure 4A**) showed that most indices were significantly or extremely

significantly correlated. MDA was significantly positively correlated with SS and SP (correlation coefficients 0.586 and 0.475, respectively). SP was significantly positively correlated with SPAD. Pro was significantly correlated with SS, SP and MDA, indicating that different physiological indices have an impact on root cold resistance. IAA was significantly negatively correlated with Pro, SP and SS, suggesting that it plays a protective role in root tissues under low temperature stresses. Calcium ion flux is involved in the low-temperature response of rapeseed roots, maintenance of osmotic balance, and defense signal transduction. Before low-temperature treatment (approximately 0-288s), the efflux and uptake of Ca^{2+} in roots of L7 and L99 were in a balanced state, with the lines fluctuating around zero value. After treatment, Ca^{2+} flux rapidly turned negative and decreased significantly, activating physiological process of active Ca^{2+} uptake by roots. Moreover, L7 showed a greater variation amplitude and a higher uptake rate. Since the increase in calcium ion uptake and efflux is associated with cold resistance to a certain extent, this leads L7 to exhibit stronger cold resistance (Figures 4B and C).

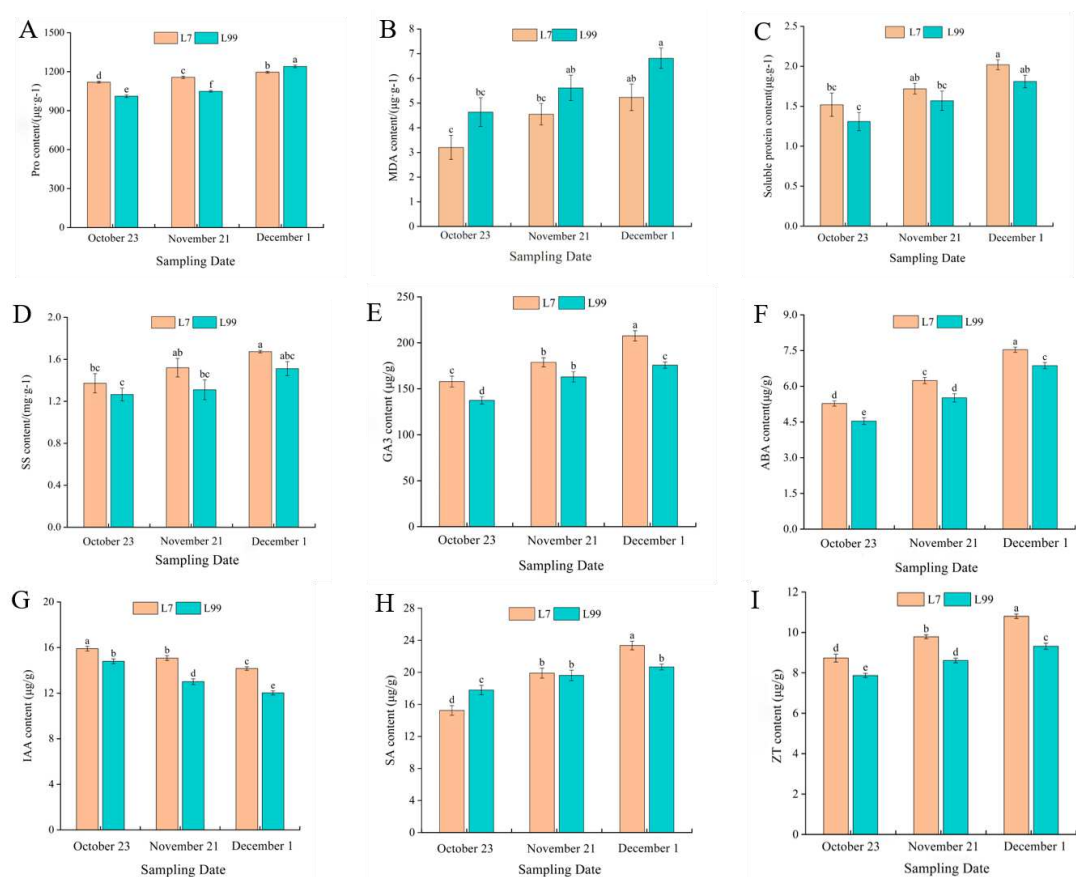


Figure 3: Physiological characteristics and hormone levels in rapeseed roots under low-temperature stress.

Note: (A) Proline (B) MDA (C) Soluble protein (D) Soluble sugar (E) GA₃ (F) ABA (G) IAA (H) SA (I) ZT. The results were shown as mean ± standard deviation values (n=3). Different lowercase letters above the bars indicated significant differences at the level of P < 0.05, the

289 same below.

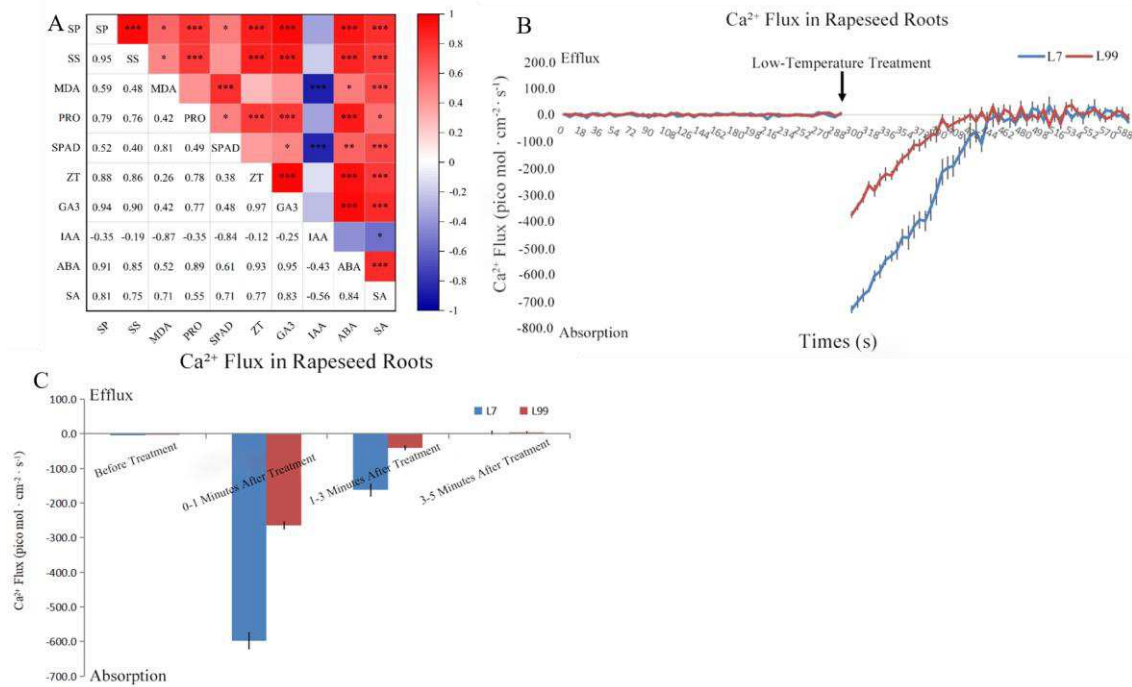


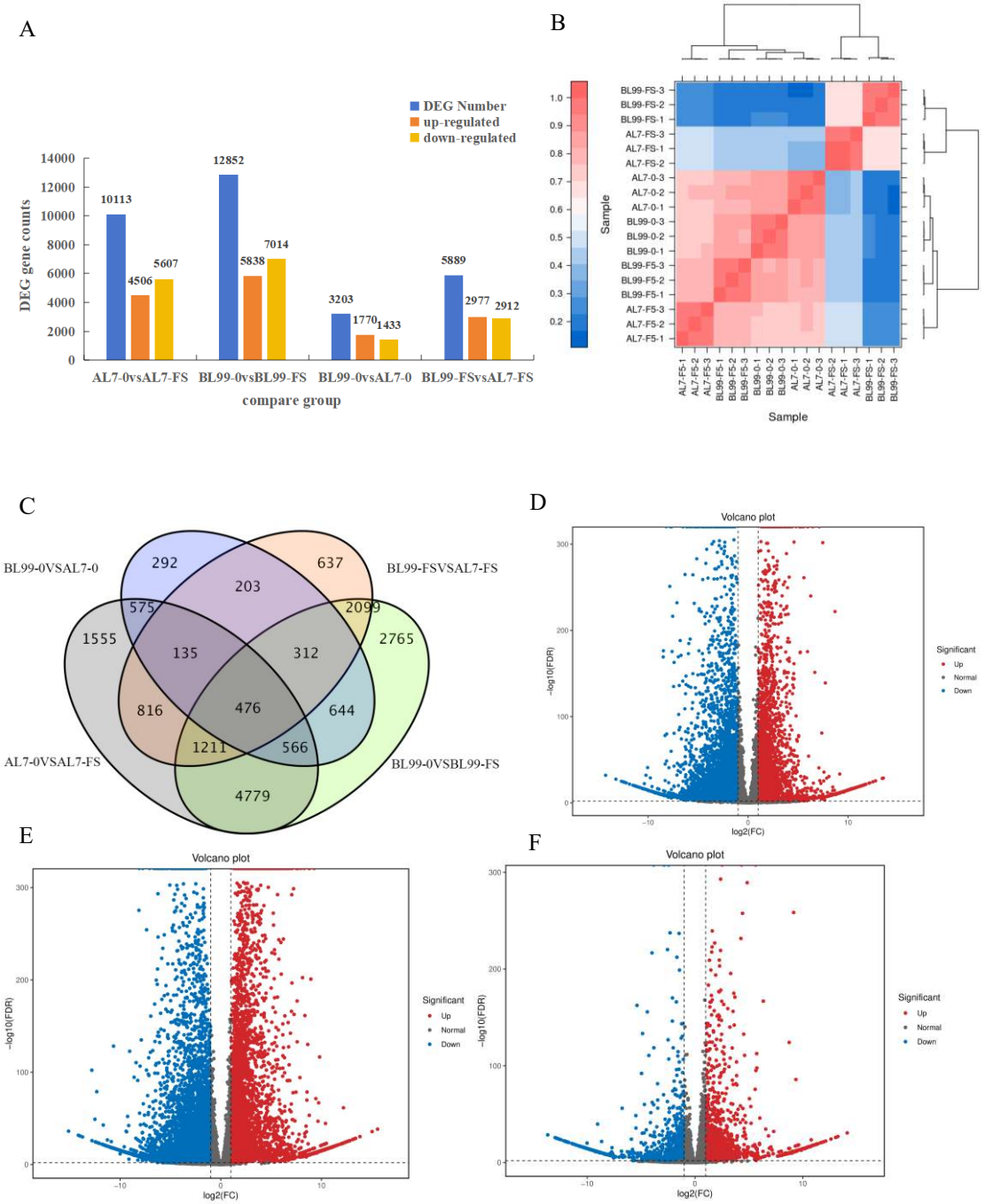
Figure 4: (A) Correlation analysis of physiological characteristics. (B) Line chart comparing Ca²⁺ flux in rapeseed roots before and after low-temperature treatment. (C) Bar graph showing Ca²⁺ flux in rapeseed roots before and after low-temperature treatment.

3.2 Transcriptome analyses

Transcriptome sequencing and data quality control were performed on rapeseed roots under freezing temperature stress. After filtering low-quality data, a total of 108.79 Gb of Clean Data was obtained, with the percentage of Q30 bases being $\geq 94.60\%$, indicating that the sequencing quality was qualified (**Supplementary Table 1**). The alignment efficiency of the Clean Data from the 18 samples obtained in this study ranged from 80.02% to 87.49%, which suggested that data utilization rate was high and could be used for subsequent analyses (**Supplementary Table 2**).

To analyze the transcriptional level differences of winter rapeseed under different low-temperature stresses, we set $\log_2FC \geq 2$ and $p < 0.05$ to screen for differentially expressed genes (DEGs) to identify key genes and further analyse their expression results. The comparison group AL7-0 vs AL7-FS identified 10113 differentially expressed genes (DEGs), of which 4506 were upregulated and 5607 were downregulated. BL99-0 vs BL99-FS had 12852 DEGs, with 5838 up-regulated and 7014 down-regulated; BL99-0 vs AL7-0 had 3203 DEGs, with 1433 up-regulated and 1770 down-regulated; BL99-FS vs AL7-FS had 5889 DEGs, with 2912 up-regulated and 2977 down-regulated (**Figure 5A**). This indicates that the two materials exhibit significant differences in transcriptional levels under low-temperature stress. Cluster

heatmaps show that samples under different treatment conditions were grouped into distinct clusters, indicating significant differences in their gene expression patterns (Figure 5B). The Venn diagram shows that the two materials share 476 DEGs across the four comparison groups (Figure 5C). The volcano plot indicates that there are a large number of significantly differentially expressed genes between the treatment and control groups, which may play important roles in the biological effects induced by the treatment (Figures 5D-G).



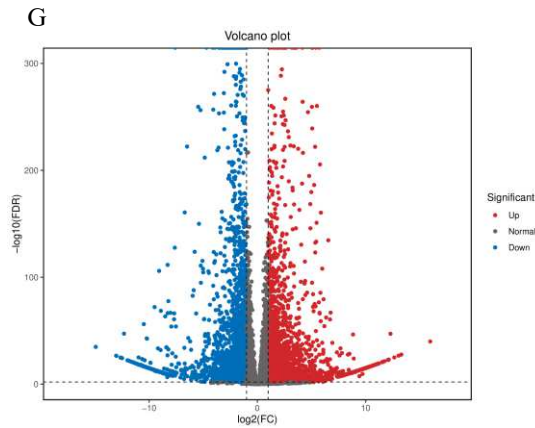


Figure 5: Transcriptome analysis of rapeseed under low-temperature stress.

Note: (A) The number of DEGs in AL7-0vsAL7-FS, BL99-0vsBL99-FS, BL99-0vsAL7-0 and BL99-FSvsAL7-FS. (B) The Pearson correlation analysis between samples. (C) Venn diagram showing the number of differentially expressed genes (DEGs) in the comparison groups: AL7-0 vs AL7-FS, BL99-0 vs BL99-FS, BL99-0 vs AL7-0, and BL99-FS vs AL7-FS. The found DEGs in comparison groups (D) AL7-0 vs AL7-FS (E) BL99-0 vs BL99-FS (F) BL99-0 vs AL7-0 (G) BL99-FS vs AL7-FS, In the volcano plot, statistical significance $-\log_{10}$ of q value, Y-axis, have been plotted against $\log_2$ fold change (X-axis). Significantly upregulated genes are represented by red dots, while downregulated genes are represented by blue dots, and non-significant genes are represented by gray dots.

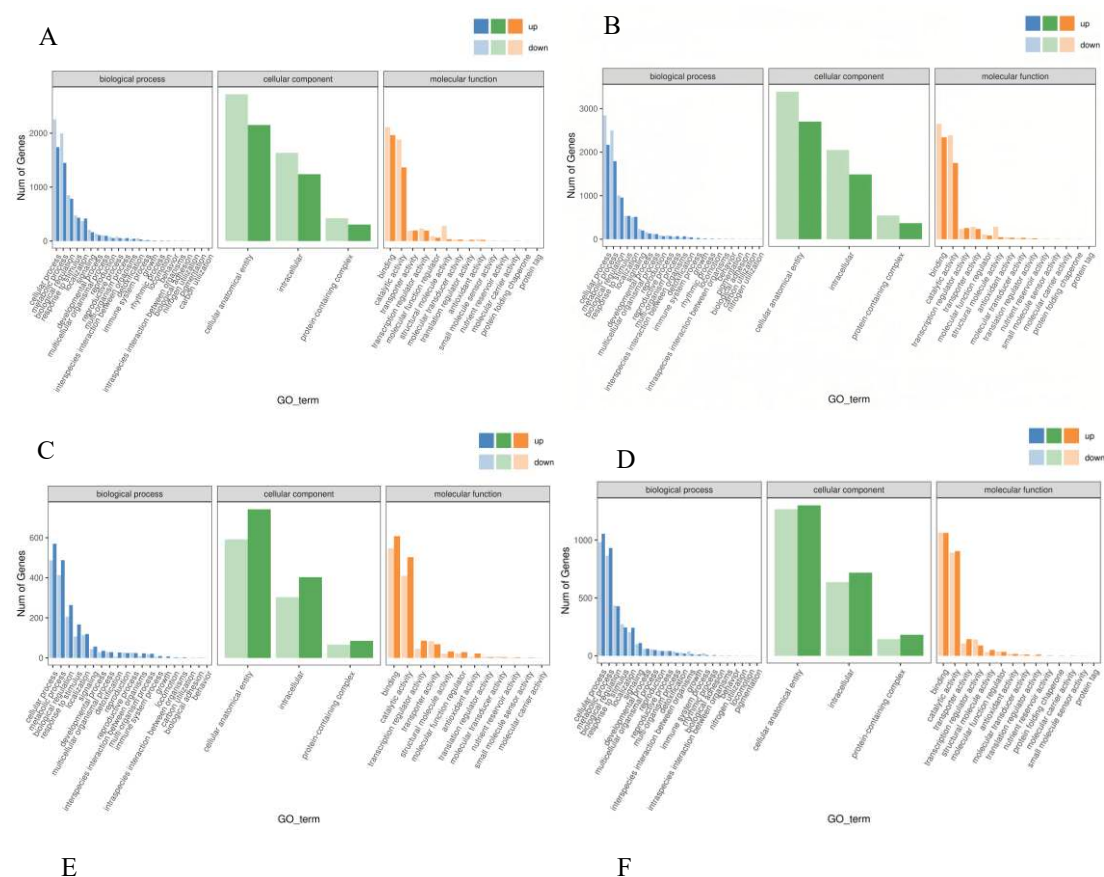
3.3 GO and KEGG enrichment analysis of DEGs

To clarify the functional distribution of differentially expressed genes (DEGs) in roots of the two varieties under low-temperature stress, GO annotation analysis was conducted on DEGs from two groups of comparisons: the same variety under different treatments (AL7-0 vs AL7-FS; BL99-0 vs BL99-FS) and different varieties under the same treatments (BL99-0 vs AL7-0; BL99-FS vs AL7-FS) (**Figures 6A-D**). The DEGs were mainly enriched in biological processes (BP), cellular components (CC), and molecular functions (MF). In the comparison groups of different varieties, most DEGs were enriched in BP, while fewer were in CC, with the enriched GO terms mostly including "regulation of biosynthetic process", "developmental process", "catalytic activity", etc.

To investigate changes in the growth and metabolic pathways of rapeseed roots, KEGG classification statistics were performed on differentially expressed genes (DEGs) (**Figure 6E-H**). The results showed that DEGs in the comparisons of AL7-0 vs AL7-FS and BL99-0 vs BL99-FS were involved in 50 KEGG pathways. Most of these pathways were categorized into metabolic pathways (such as amino acid biosynthesis and carbon metabolism), while the remaining were related to cellular processes,

environmental information processing, and genetic information processing. Among them, the most enriched pathways included plant hormone signal transduction and amino acid biosynthesis. In L7, the KEGG-specific enriched pathways include plant circadian rhythm, plant hormone signal transduction, and brassinosteroid biosynthesis; whereas the pathways uniquely enriched in L99 are ubiquitin-mediated proteolysis, photosynthesis-antenna proteins, and amino sugar and nucleotide sugar metabolism. In the comparisons of BL99-0 vs AL7-0 and BL99-FS vs AL7-FS, the significantly enriched pathways included plant hormone signal transduction and MAPK signaling pathway. These results indicate that starch and sucrose metabolism, amino acid biosynthesis, and MAPK signaling pathway play important roles in the growth and development of rapeseed roots.

Transcription factors (TFs), as important upstream regulators, can directly regulate the expression of stress-responsive genes and play a key role in plant responses to abiotic stresses. We conducted statistical analysis on differentially expressed transcription factors (**Figure 6I**). The top five transcription factor families with the largest number of differentially expressed genes in the two varieties were AP2/ERF-ERF, MYB, bHLH, C2H2 and NAC, among which the first three families had 245, 240 and 231 differentially expressed TF genes, respectively.



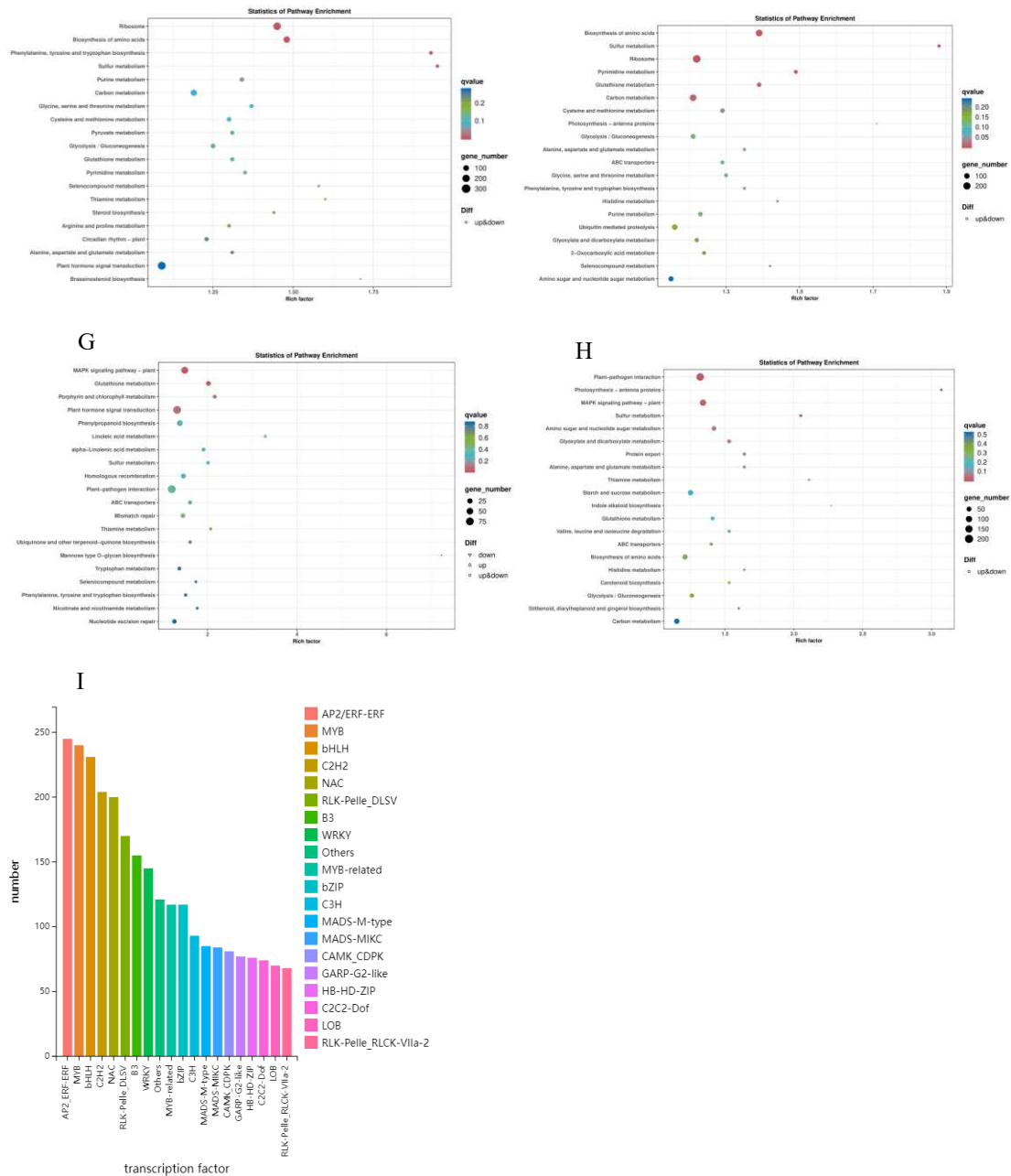


Figure 6: Analysis of GO and KEGG enrichment results of differentially expressed genes. Note: GO functional enrichment analysis of differentially expressed genes (DEGs) in rapeseed under low-temperature stress. (A)AL7-0 vs AL7-FS (B) BL99-0 vs BL99-FS (C) BL99-0 vs AL7-0 and (D) BL99-FS vs AL7-FS. KEGG pathway enrichment analysis of differentially expressed genes (DEGs) in rapeseed under low-temperature stress. (E)AL7-0 vs AL7-FS (F) BL99-0 vs BL99-FS (G) BL99-0 vs AL7-0 and (H) BL99-FS vs AL7-FS. (I) Prediction results of transcription factor families.

3.4 qRT-PCR verification of differential gene expression levels

To verify the accuracy of the transcriptome results, six cold stress-related genes were selected from the above modules for qRT-PCR validation. The results showed that the qRT-PCR results were highly correlated with the RNA-seq data, confirming the

reliability of the transcriptome sequencing data (Figure 7).

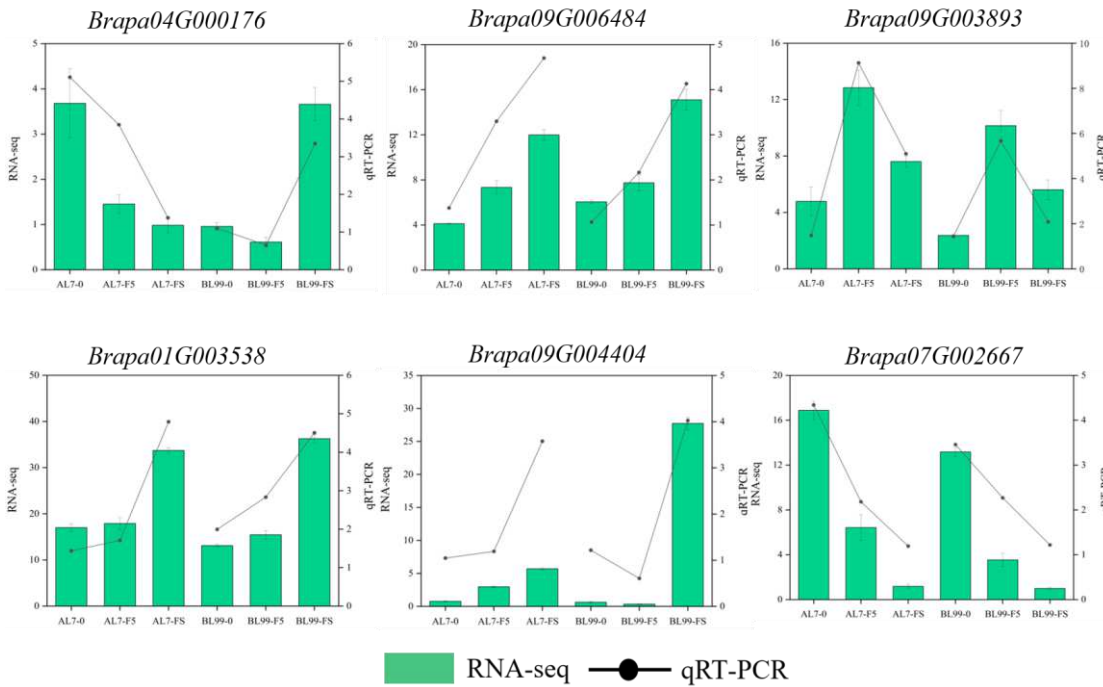


Figure 7: qRT-PCR analysis of relative gene expression levels.

Note: The bar charts and black lines represent the results of RNA-seq and qRT-PCR, respectively.

3.5 Weighted gene co-expression network analysis and identification of Hub genes

Based on RNA-seq data, key modules were identified, a network was constructed, and core genes were screened through weighted gene co-expression network analysis^[36]. To fully understand the molecular mechanism of rapeseed under freezing stress in the field, 287 genes identified from 18 samples of roots transcriptome were processed by filtering out those with low expression variation (standard deviation ≤ 0.5), and the remaining 1943 genes were used for WGCNA network construction. Meanwhile, the transcriptome data were combined with physiological indicators including MDA, SP, SS, and Pro to identify co-expression modules, thereby screening out core genes.

3.6 Module screening and trait association analysis

In the results analysis of 18 samples, a total of 10416 genes were identified and clustered into 20 modules (Figure 8E), among which blue module contained the largest number of genes (2203), while darkslateblue module had the smallest number (41). The obtained modules were then analysed for their association with physiological traits (Figure 9A-D). It was found that MEgreenyellow module had the strongest association with SP and SS; MEbrown module showed the strongest association with MDA and

Pro, and these modules were positively correlated with the traits. This correlation can be attributed to the characteristic genes associated with these modules, which are related to the response of rapeseed roots to low-temperature stress (**Figure 8D**). Therefore, the DEGs from the MEgreenyellow and MEbrown modules were selected for GO and KEGG enrichment analysis.

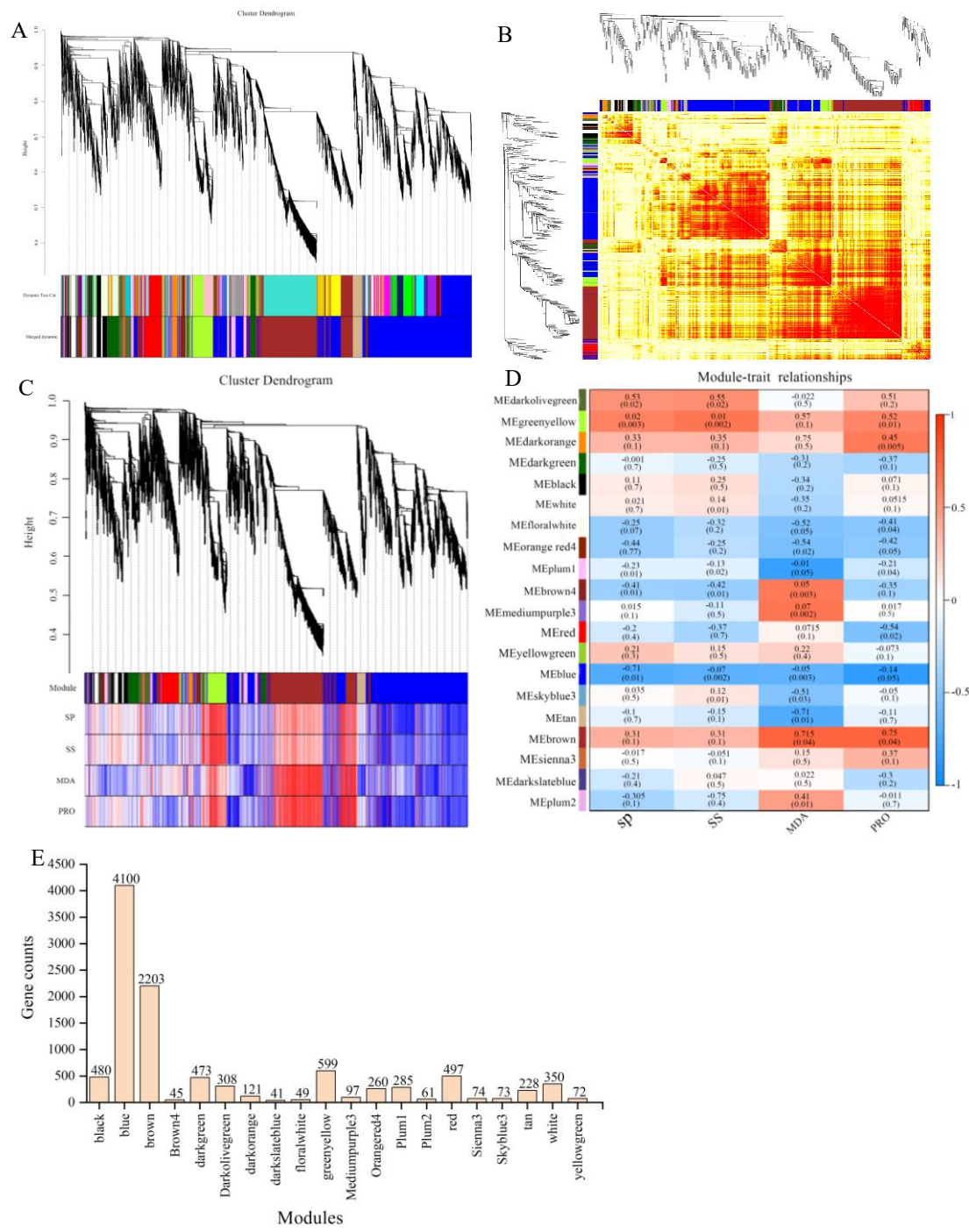


Figure 8: Weighted gene co-expression network analysis of differentially expressed genes (DEGs)

Note: (A) Gene clustering tree and distribution of modules. The higher the merging position of branches in the dendrogram, the greater the difference between the two merged groups of genes.

Different colors represent different gene modules. (B) Trait-module association heatmap. (C) Module-clustering dendrogram. Similar samples or genes are classified into the same module, with different colors corresponding to different modules. (D) Heat map of trait module associations. Each row represents a module, and each column corresponds to a specific sample. The values in each cell indicate the correlation coefficient (r) and the p-value of module-trait associations. Red indicates a positive correlation between the module and the sample, while blue indicates a negative correlation. (E) Number of genes in each module.

GO and KEGG enrichment analyses were performed on the differentially expressed genes (DEGs) screened from the two modules. The GO terms in the two modules include metabolic processes, transcription factor activity, and structural molecule activity. The main KEGG-enriched pathways involve plant hormone signal transduction, starch and sucrose metabolism, carbon metabolism, and plant circadian rhythm. Based on the edge weights and node scores from WGCNA, the top 20 core genes were screened out from the Greenyellow and Brown modules, and their expression patterns are shown in the figure (Figure 10). These genes encode proteins with high connectivity in the network and play important roles in biological processes (Figure 11).

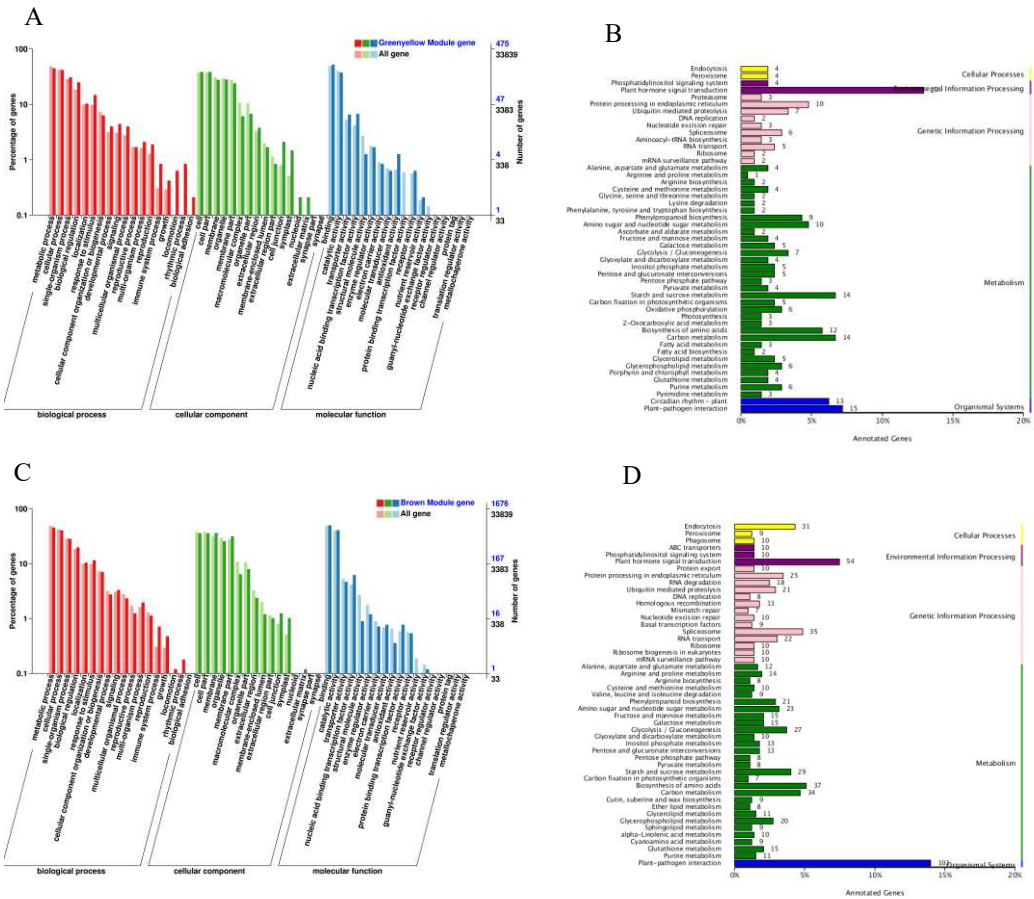


Figure 9: (A) represents the GO function enrichment analysis in the MEgreenyellow module; (B) represents the KEGG pathway enrichment analysis in the MEgreenyellow module; (C) represents the GO function enrichment analysis in the MEBrown module; (D) represents the KEGG pathway enrichment analysis in the MEBrown module.

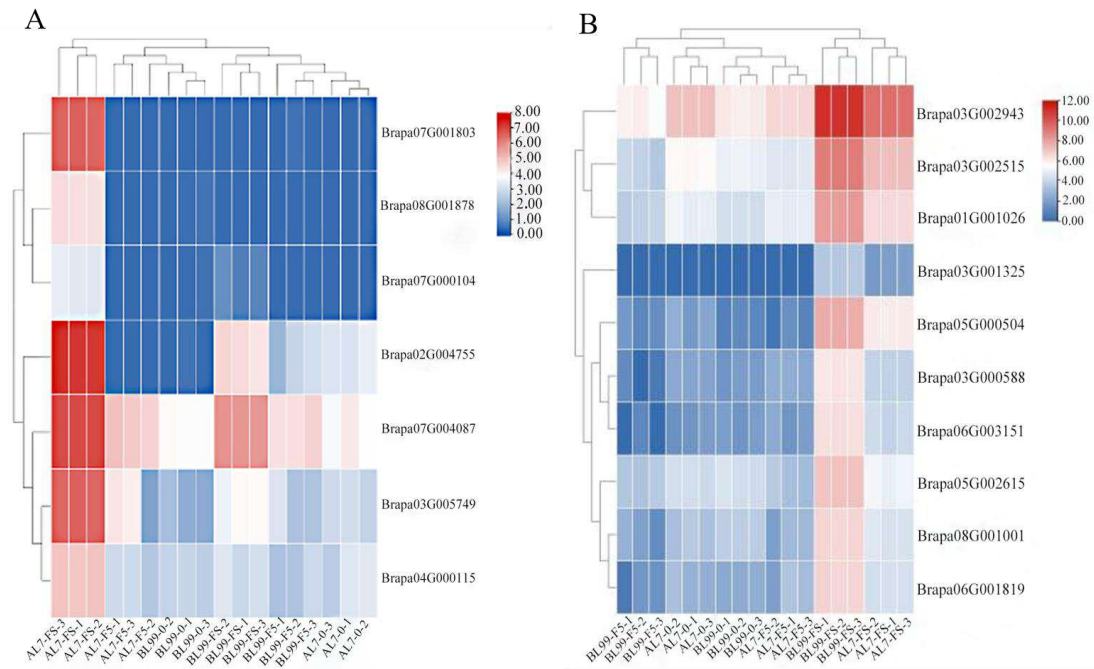


Figure 10: (A) Heatmap of the expression levels of core genes in the greenyellow module. (B) Heatmap of the expression levels of core genes in the brown module.

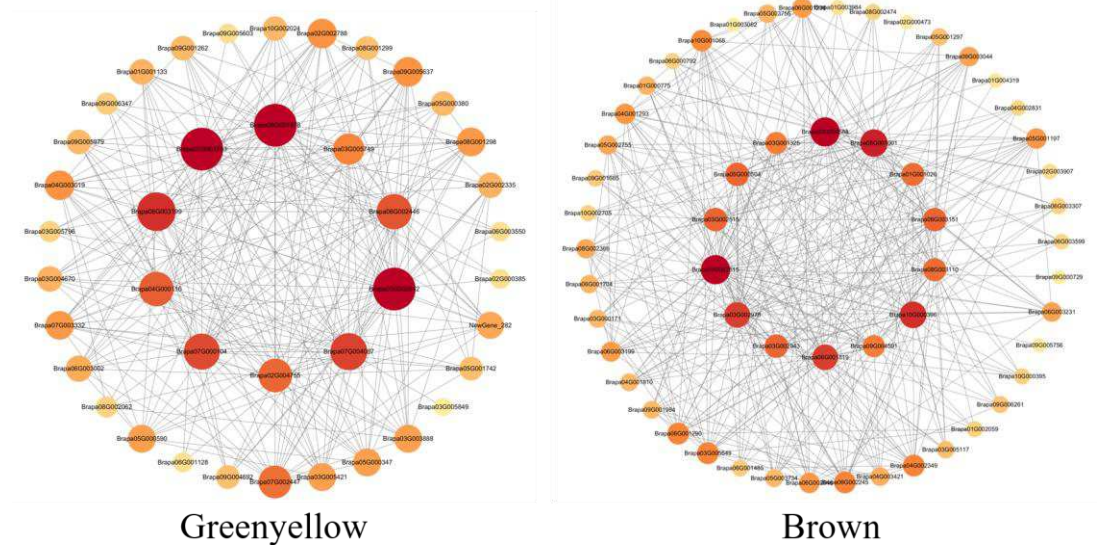
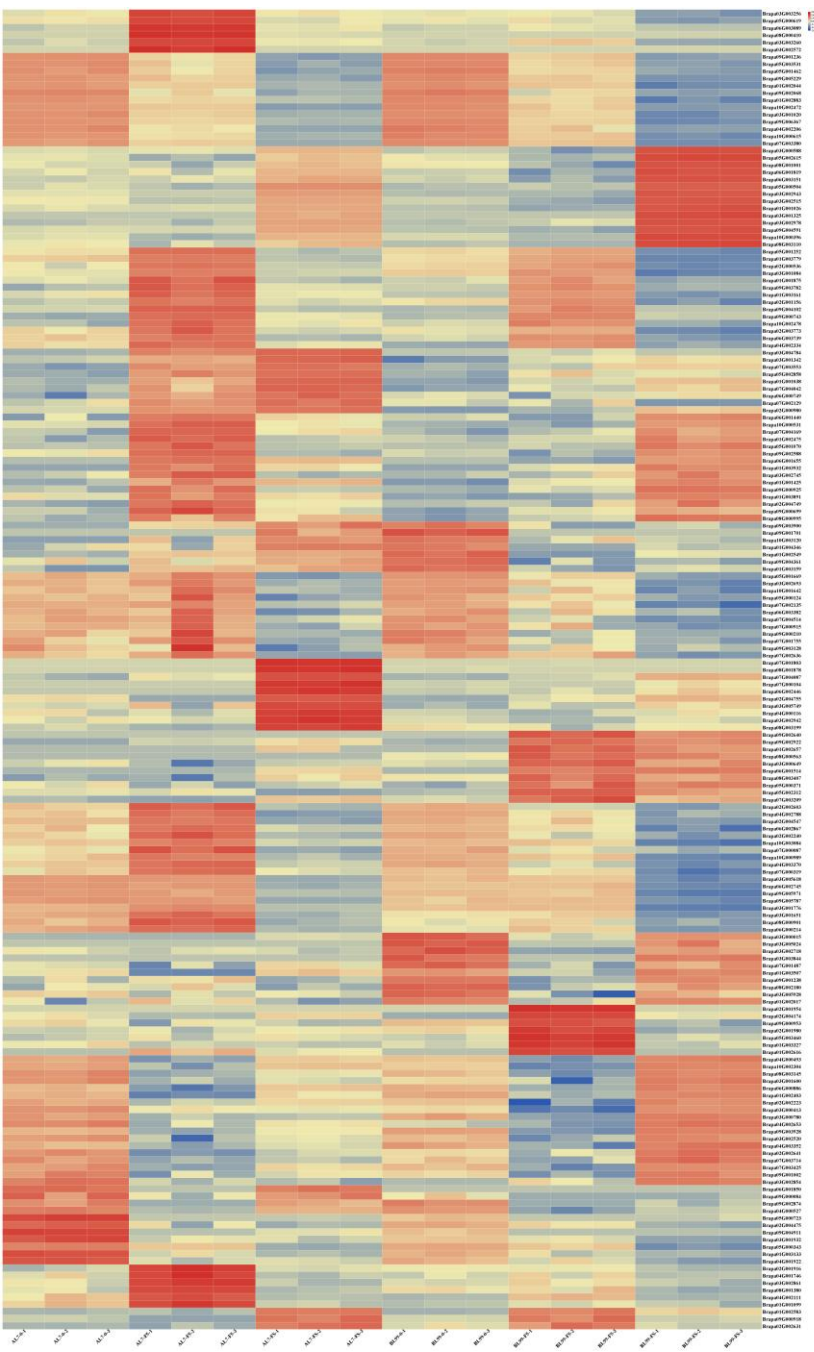


Figure 11: Visualization of WGCNA key genes in greenyellow module and brown module. Note: The inner circle represents the screened core genes.

To further explore freezing resistance genes in roots, WGCNA was performed on 183 core genes from 20 modules. Their expression levels are shown in Figure 11A, with most genes being up-regulated under low-temperature stress. Based on WGCNA edge weights and degree scores, 9 cold resistance candidate genes were finally screened out, and their expression levels are presented in Figure 11B. Among them, the genes involved in the MAPK signaling pathway include *Brapa01G002883*, *Brapa10G003084*, and *Brapa09G003928*.

Functional annotation of these candidate genes was conducted using the TAIR (<https://www.arabidopsis.org/>) and NCBI (<https://www.ncbi.nlm.nih.gov/>) databases (Table 3).

A



B

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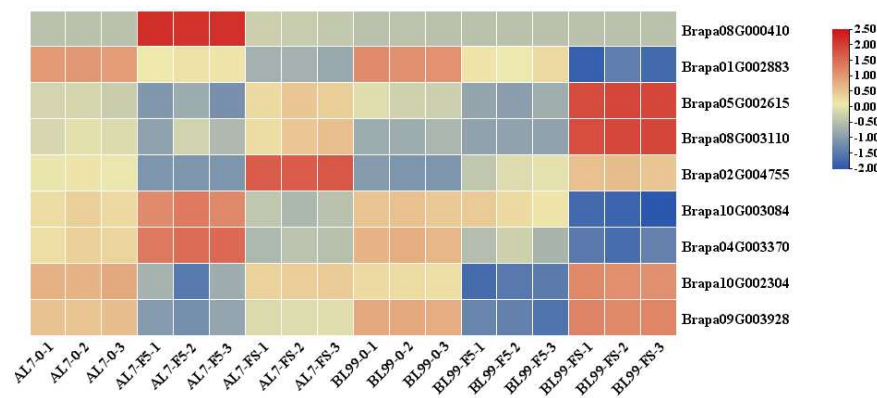


Figure 12: (A) Expression levels of all core genes in 20 modules. (B) Expression levels of candidate genes.

Note: Red represents the expression level of up-regulated genes, and blue represents the expression level of down-regulated genes.

Table 3: Functional annotation of candidate genes

Gene ID	Hub gene ID	Gene abbreviation	Functional annotation
Brapa08G000410	LOC103833240	AT1G48920 (NUC-L1)	RCI2B (Rare-cold-inducible 2B), LTI6B (Lowtemperature induced protein 6B)
Brapa01G002883	LOC103830167	AT1G60940 (SNRK2.10)	SNRK2.10 (Snf1-relatedprotein kinase 2.10), SRK2B (Snf1- related kinase 2B), SNRK2-10 (Sucrose nonfermenting 1-related protein kinase 2-10)
Brapa05G002615	LOC103869481	AT3G19240 (DEM2)	DEM2 (Defective embryo and meristems 2)
Brapa08G003110	LOC103836110	AT1G13340 (ISTL6)	ISTL6 (Ist1-like 6)
Brapa02G004755	LOC103855350	AT5G66400 (RAB18)	RAB18 (responsive to ABA 18), ATDI8 (Arabidopsis thaliana drought-induced 8)
Brapa10G003084	LOC103844644	AT1G01360 (RCAR1)	RCAR1 (Regulatory component of ABA receptor 1), PYL9 (Pyrabactin resistance 1-like 9)
Brapa04G003370	LOC103866268	AT2G46140 (LEA27)	LEA27 (Late embryogenesis ab- undant 27)
Brapa10G002304	LOC106436839	AT5G51990 (CBF4)	CBF4 (C-repeat-binding factor 4), DREB1D (Element-binding prot- ein 1D)
Brapa09G003928	LOC103833884	AT4G11280 (ACS6)	ACS6 (1-aminocyclopropane-1- carboxylic acid (ACC) synthase 6), ATACS6

3.7 Response of MAPK pathway to low-temperature stress

Transcriptome analysis revealed that differentially expressed genes (DEGs) are mostly enriched in MAPK pathway as well as starch and sucrose metabolism pathways. When plants are subjected to adverse conditions such as pathogen infection, cold stress, and drought stress at low temperatures, the corresponding cascade pathways are activated

to induce the expression of downstream genes.

When plants are infected by pathogens, the pathogen molecule (*flg22*) is recognized by the receptor *FLS2/BAK1*. A total of 45 DEGs are involved in the cascade pathway of this process. Under freezing stress, most of these genes are down-regulated, with similar expression trends in the two materials.

When plants are exposed to adverse stresses (salt, drought, osmotic stress), abscisic acid (ABA) accumulation is induced. ABA binds to the receptor *PYR/PYL* to relieve the inhibition of *SnRK2*. A total of 11 genes are enriched in this pathway: the PP2C-encoding genes *Brapa05G000590*, *Brapa06G003883*, and *Brapa06G003781* are up-regulated in cold-tolerant varieties but down-regulated in moderate cold-tolerant variety; the SnRK2-encoding gene *Brapa01G002883* shows similar expression trends in the two materials.

When plants suffer external wounding, calcium signaling (Ca^{2+}) is triggered to activate *CaM4*, and ROS homeostasis is maintained through *OXII*. A total of 23 genes are enriched in this pathway, among which 9 genes are associated with *CaM4*, and *Brapa02G000975* is up-regulated in both materials; among the MPK8-encoding genes, all are down-regulated except *Brapa09G000896*.

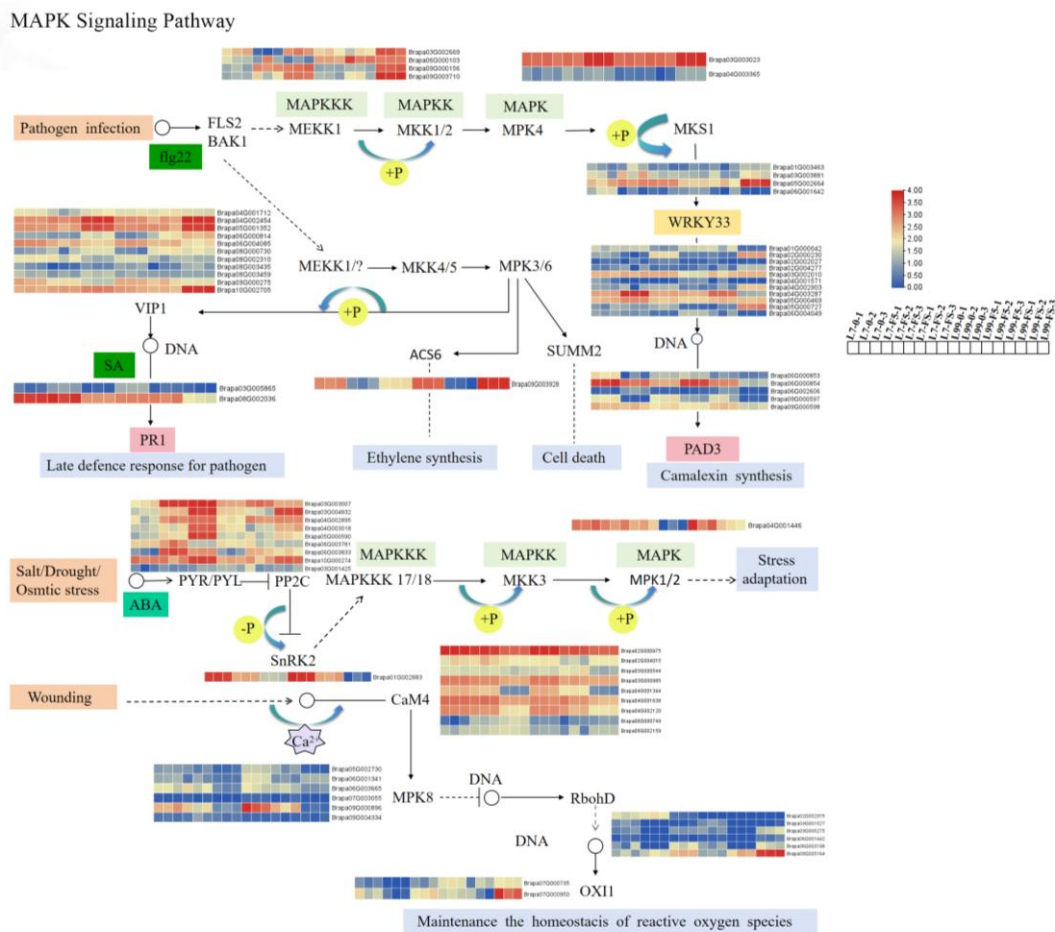


Figure 13: Expression profiles of differentially expressed genes (DEGs) in MAPK signaling pathway.

Note: The expression levels of differentially expressed genes are indicated by different colors.

3.8 qRT-PCR validation of candidate genes

Nine candidate genes screened from the modules were validated using qRT-PCR. *Brapa04G003370* and *Brapa10G003084* exhibited a sharp increase in expression in L7-F5, suggesting they may play key roles in the early response to low-temperature stress. *Brapa10G002304*, *Brapa05G002615*, and *Brapa09G003928* were highly expressed in L99-FS; they might be involved in stress responses but with weak effects, synergistically regulating moderate cold-tolerant phenotypes. *Brapa02G004755* and *Brapa08G000410* showed high expression in L7, indicating they could be freezing stress regulatory genes specific to high cold-tolerant varieties. *Brapa01G002883* decreased gradually in L7, possibly due to cells entering a state of protective metabolic suppression. *Brapa08G003110* was significantly up-regulated in L7, while it showed a burst of high expression in L99-FS, which might serve as a marker of extreme low-temperature stress overload.

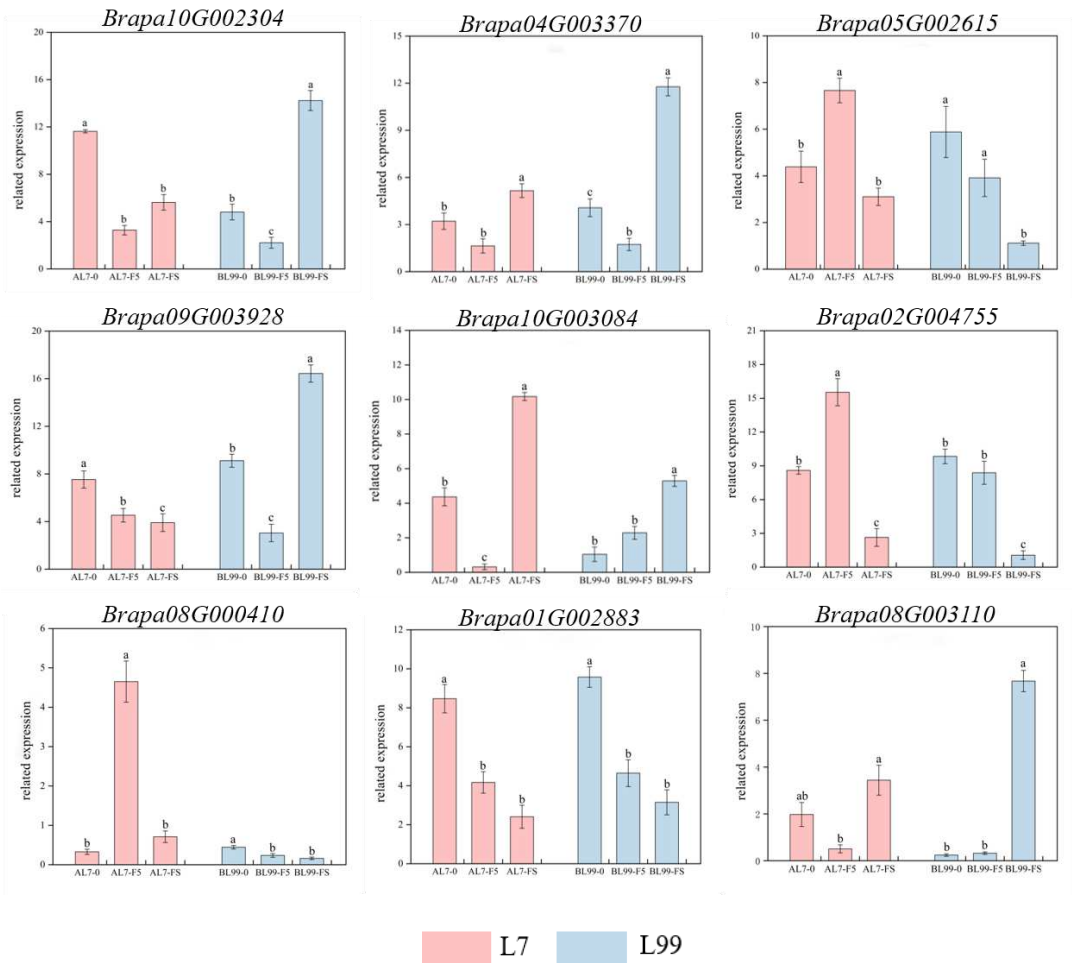


Figure 14: Relative expression of 9 candidate genes.

Note: Different lowercase letters indicate significant differences between the same variety at

different times at the $p<0.05$ level.

4. Discussion

4.1 Physicochemical changes in rapeseed under low-temperature stress

Cold stress not only affects plant growth and development but also reduces crop yields^[37]. Cold stress in plants is a complex process involving a series of physiological and molecular changes^[38]. Previous studies have extensively investigated the adverse effects of cold stress on plant growth and development^[39]. Clarifying the cold stress response mechanisms of two varieties can provide valuable information and genetic resources for enhancing crop cold tolerance. This study observed physiological and biochemical changes in rapeseed seedlings under freezing stress conditions in the field, and found that they exhibited wilting, drying, and leaf curling (**Figure 1**). These findings are consistent with previous studies^[40]. However, our results indicate that Longyou 7 enhances root development by reducing nutrient supply to the aboveground parts, resulting in a higher root-to-shoot ratio compared to Longyou 99 (**Figure 2**), which is consistent with previous findings^[41]. These results indicate that there are differences in root development among different winter rapeseed varieties: cold-tolerant varieties can prioritise the allocation of more photosynthetic products to the underground parts, store more organic matter to promote root development^[42], and restrict aboveground leaf growth during the early stages of overwintering, thereby enhancing overwintering capacity. As field temperatures decrease, freezing stress damages root cell membranes, affecting the accumulation of osmotic regulatory substances (**Figures 3A-D**), which maintain cellular structural stability and enhance plant stress resistance. Plant hormones (IAA, ABA, GA, ZT, SA) are central regulators of root growth and development and interact with one another^{[43]-[45]}. In this study, the cold-tolerant varieties (L7) enhances cold tolerance through hormone regulation to adapt to environmental changes (**Figure 3E-I**).

4.2 Mechanism of response to low-temperature stress at the transcriptome level

Studying gene expression changes at the transcriptional level can elucidate the mechanisms underlying plant responses to cold stress. Transcriptome and WGCNA analyses of rice H5 and H9 identified 161 candidate genes (79 cold-responsive genes and 82 cold-tolerant genes) involved in various metabolic processes, providing new strategies for enhancing rice yield and cold tolerance^[46]. In this study, transcriptomic sequencing yielded 108.79 GB of clean data, with an effective read ratio of approximately 94.6%. Transcriptomic analysis of freezing stress in roots of L7 and L99 (with different cold tolerance levels) showed that the number of DEGs in L7 was higher

than that in L99 (**Figure 5A**), consistent with findings in tomato^[47], potato^[48], rice^[49], and creeping bentgrass^[50], indicating that L7 and L99 exhibit significant differences in gene expression induced by freezing stress. L7 may adapt to freezing temperature stress by inducing more cold-response genes, thereby enhancing the plant's cold tolerance, while cold-response genes in L99 are suppressed by low-temperature stress, leading to reduced cold resistance in the plant. As temperature decreases (below freezing), the number of DEGs increases, suggesting that plants adapt to low temperatures by activating the high expression of a large number of genes. GO and KEGG analyses indicate that sucrose metabolism, amino acid biosynthesis, and the MAPK signalling pathway play important roles in the low-temperature response of rapeseed.

4.3 Identification of key modules and hub genes by WGCNA

To identify key genes involved in rapeseed root responses to freezing stress, this study constructed a gene regulatory network using weighted gene co-expression network analysis (WGCNA). The results showed that greenyellow module was most significantly correlated with the contents of SP and SS, while the brown module was most significantly correlated with MDA and Pro. The upregulated genes in these modules may respond to cold stress by maintaining osmotic balance, protecting cell membrane stability, and alleviating oxidative damage (**Figure 8D**). Transcriptome data combined with WGCNA for modular classification of genes is an effective method to explore key genes related to environmental stress and their interactions^[51]. In L7 and L99, 10416 differentially expressed genes (DEGs) were classified as trait-module genes, and 183 candidate DEGs were predicted, involving processes such as signal transduction, MAPK pathway, and starch and sucrose metabolism.

In addition, WGCNA analysis of DEGs identified hub genes in key modules, which exhibit high connectivity in gene expression networks and significant biological relevance. This study identified 9 candidate genes from 20 modules, belonging to transcription factor families such as PMP3, SNRK2 and CBF. The Pmp3 gene encodes a plasma membrane protein that senses external stimuli and induces gene expression through the MAPK cascade pathway, thereby enhancing cold tolerance. In this study, the expression level of CBF (*Brapa10G002304*) increased with decreasing temperature, possibly because CBF4 enhances the tolerance of rapeseed. Research has found that increased CBF expression promotes the production of soluble sugars, including glucose, raffinose, sucrose, and fructose. This, in turn, enhances the cold tolerance of pomegranate plants^{[52][52]}. Li^[53] reported that three CBF genes in peach branches were significantly upregulated under cold stress. Additionally, transcription factors such as DREB/CBF, bZIP, and MYB also participate in the transcriptional regulation of cold-tolerant genes^[54].

5. Conclusion

This study utilised RNA-seq technology to analyse the cold tolerance mechanisms of the root systems of Longyou 7 (L7) and Longyou 99 (L99), identifying differentially expressed genes primarily involved in growth metabolism, stress responses, transcriptional regulation, and various biosynthetic pathways. By combining transcriptomics sequencing with weighted co-expression networks, we screened for hub genes that play a central regulatory role, as these hub genes often perform key functions in specific biological processes. In summary, this study focuses on the low-temperature response mechanisms of rapeseed, aiming to reveal the molecular regulatory patterns and physiological metabolic characteristics of its response to low-temperature stress, thereby laying a solid foundation for future research aimed at enhancing the cold tolerance of Chinese cabbage-type rapeseed.

Ethics approval and consent to participate

The authors declared that experimental research works on the plants described in this paper comply with institutional, national and international guidelines.

Consent for publication

Not applicable.

Availability of data and materials

Data and materials will be made available on request.

Competing Interests

The authors declare no competing interests.

Funding

This research was funded by Gansu Province college industry support plan project (2023CYZC-51), Major Science and Technology Project of Gansu Province (24ZDNA007) and China Agriculture Research System of MOF and MARA (CARS-12-09). Project supported by the Key Program of International Cooperation of Gansu Province (No:25YFWA019).

Authors' contributions

J. W., W. S., L. M. conceived and designed the study. X.L. authored the research methodology. X. L., J. Z. did the qRT-PCR analysis. G. Y., Y. P., W. W. Conducted formal analysis of the data. P. Y., L. L., T. F. Conducted data curation. G. Y., T. F., W. W. and L. L. contributed materials and analysis tools. J. W., Y. P. wrote the paper. J. W., X. L., L. M. reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgements

We thank Yahong Zhang and Feng Li for identification of the cold tolerance of materials. Reviewers are acknowledged for their contribution to the improvement of the manuscript in the revision process.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

634 **References**

- 635 [1] Sun, W.C., Wu, J.Y., Fang, Y., Liu, Q., Yang, R.Y., Ma, W.G., Li, X.C., Zhang, J.J., Zhang, P.F.,
636 Lei, J.M. (2020). Growth and development characteristics of winter rapeseed northern-
637 extended from the cold and arid regions in China. *Acta Agron. Sin.* 36(12): 2124-2134. DOI:
638 10.3724/SP.J.1006.2010.02124
- 639 [2] Chinnusamy V., Zhu J. K., Sunkar R. (2010). Gene regulation during cold stress acclimation
640 in plants. *Meth. Mol. Biol.* 639, 39–55. DOI: 10.1007/978-1-60761-702-0_3
- 641 [3] Liu Z. G., Sun W. C., Yang N. N., Wang Y., He L., Zhao C. X., et al. (2013). Morphology and
642 physiological characteristics of cultivars with different levels of cold-resistance in winter
643 rapeseed (*Brassica campestris* L.) during cold acclimation. *Sci. Agric. Sin.* 46(22): 4679–4687.
644 DOI: 10.3864/j.issn.0578-1752.2013.22.005
- 645 [4] Yang N. N., Sun W. C., Liu Z. G., Shi P. H., Fang Y., Wu J. Y., et al. (2014). Morphological
646 characters and physiological mechanisms of cold resistance of winter rapeseed in northern
647 China. *Sci. Agric. Sin.* 47(3): 452–461. DOI: 10.3864/j.issn.0578-1752.2014.03.005
- 648 [5] Ma H, Liu C, Li Z, Ran Q, Xie G, Wang B, Fang S, Chu J, Zhang J. (2018). ZmbZIP4
649 Contributes to Stress Resistance in Maize by Regulating ABA Synthesis and Root
650 Development. *Plant Physiol.* 178(2):753-770. DOI: 10.1104/pp.18.00436.
- 651 [6] Bian L, Fahim AM, Wu J, Liu L, Pu Y, Ma L, Fang Y, Zhang D, Yang G, Wang W, Fan T, Yang
652 X, Wang J, Shi Y, Sun W. (2024). Systematic Analysis of the BrHAT Gene Family and
653 Physiological Characteristics of *Brassica rapa* L. Treated with Histone Acetylase and
654 Deacetylase Inhibitors under Low Temperature. *Int J Mol Sci.* DOI: 10.3390/ijms25179200.
- 655 [7] Qi W, Wang F, Ma L, Qi Z, Liu S, Chen C, Wu J, Wang P, Yang C, Wu Y, Sun W. (2020).
656 Physiological and Biochemical Mechanisms and Cytology of Cold Tolerance in *Brassica napus*.
657 *Front Plant Sci.* 11:1241. DOI: 10.3389/fpls.2020.01241.
- 658 [8] Lei P, Xu Z, Ding Y, Tang B, Zhang Y, Li H, Feng X, Xu H. (2015). Effect of Poly(γ -glutamic
659 acid) on the Physiological Responses and Calcium Signaling of Rape Seedlings (*Brassica*
660 *napus* L.) under Cold Stress. *J Agric Food Chem.* 63(48):10399-406. DOI: 10.1021/acs. jafc.
661 5b04523.
- 662 [9] Bai, C, Fang, M, Zhai, B, Ma, L, Fu, A, Gao, L, et al. (2021). Regulations of m⁶A methylation
663 on tomato fruit chilling injury. *Hortic. Plant J.* 7(5), 434-442. DOI: 10.1016/j.hpj. 2021.05.005
- 664 [10] Zhao, Y , Song, C, Brummell, D. A, Qi, S, Lin, Q, Bi, J, et al. (2021). Salicylic acid treatment
665 mitigates chilling injury in peach fruit by regulation of sucrose metabolism and soluble sugar
666 content. *Food Chem.* 358:129867. DOI: 10.1016/j.foodchem.2021.129867.
- 667 [11] Sun S, Yang Y, Hao S, Liu Y, Zhang X, Yang P, Zhang X, Luo Y. (2024). Comparison of
668 transcriptome and metabolome analysis revealed cold-resistant metabolic pathways in
669 cucumber roots under low-temperature stress in root zone. *Front Plant Sci.* 15:1413716. DOI:
670 10.3389/fpls.2024.1413716.
- 671 [12] Takahashi D, Uemura M, Kawamura Y. (2018). Freezing Tolerance of Plant Cells: From the
672 Aspect of Plasma Membrane and Microdomain. *Adv Exp Med Biol.* 1081:61-79. DOI:
673 10.1007/978-981-13-1244-1_4
- 674 [13] Furlong, M. J., Wright, D. J., and Dosdall, L. M. (2013). Diamondback moth ecology and
675 management: problems, progress, and prospects. *Annu. Rev. Entomol.* 58:517-41. DOI:
676 10.1146/annurev-ento-120811-153605.

- 677 [14] Kovinich, N., Kayanja, G., Chanoca, A., Otegui, M. S., and Grotewold, E. (2015). Abiotic
678 stresses induce different localizations of anthocyanins in arabidopsis. *Plant Signal Behav.* 10(7):
679 e1027850. DOI: 10.1080/15592324.2015.1027850.
- 680 [15] Li Y.B, Kang M, Song W. (2015). Effects of low temperature on physiological indexes of cold
681 tolerance in progeny of cultivated rice transferred the total genomic DNA of chilling wild rice.
682 *Crop Research* 29(01): 1-4. DOI: 10. 3969 /j. issn. 1001-5280. 2015. 01. 01
- 683 [16] Zhu, J. K. (2016). Abiotic stress signaling and responses in plants. *Cell* 167(2):313-324. DOI:
684 10.1016/j.cell.2016.08.029
- 685 [17] Liu L, Pu Y, Niu Z, Wu J, Fang Y, Xu J, Xu F, Yue J, Ma L, Li X, Sun W. (2022). Transcriptomic
686 Insights Into Root Development and Overwintering Transcriptional Memory of *Brassica rapa*
687 L. Grown in the Field. *Front Plant Sci.* 13:900708. DOI: 10.3389/fpls.2022.900708.
- 688 [18] Niu Z., Liu L., Pu Y., Ma L., Wu J., Hu F., et al. (2021). iTRAQ-based quantitative proteome
689 analysis insights into cold stress of Winter Rapeseed (*Brassica rapa* L.) grown in the field. *Sci.*
690 *Rep.* 11(1):23434. DOI:10.1038/s41598-021-02707-z.
- 691 [19] Ma L, Tao X, Wang W, Jiao J, Pu Y, Yang G, Liu L, Fang Y, Wu J, Sun W. (2023). Genome-
692 wide identification of RNA recognition motif (*RRM1*) in *Brassica rapa* and functional analysis
693 of RNA-binding protein (BrRBP) under low-temperature stress. *BMC Plant Biol.* 23(1):621.
694 DOI:10.1186/s12870-023-04639-4.
- 695 [20] Junyan W, Qiaowen P, Fahim AM, Lulu Z, Hui G, Lijun L, Gang Y, Wangtian W, Yuanyuan P,
696 Yan F, Li M, Wancang S. (2024). Effects of exogenous calcium and calcium inhibitor on
697 physiological characteristics of winter turnip rape (*Brassica rapa*) under low temperature stress.
698 *BMC Plant Biol.* 24(1):937. DOI: 10.1186/s12870-024-05556-w.
- 699 [21] Zhang, Y., Wu, L., Liu, L., Jia, B., Ye, Z., Tang, X., et al. (2024). Functional analysis of
700 PbbZIP11 transcription factor in response to cold stress in *Arabidopsis* and pear. *Plants(Basel).*
701 13(1):24. DOI: 10.3390/plants13010024.
- 702 [22] Dasgupta P, Das A, Datta S, Banerjee I, Tripathy S, Chaudhuri S. (2020). Understanding the
703 early cold response mechanism in IR64 indica rice variety through comparative transcriptome
704 analysis. *BMC Genomics.* 21(1): 425. DOI: 10.1186/s12864-020-06841-2
- 705 [23] Liu Y, Wu C, Hu X, Gao H, Wang Y, Luo H, Cai S, Li G, Zheng Y, Lin C, Zhu Q. (2020)
706 Transcriptome profiling reveals the crucial biological pathways involved in cold response in
707 Moso bamboo (*Phyllostachys edulis*). *Tree Physiol.* 40(4):538-556. DOI:
708 10.1093/treephys/tpz133.
- 709 [24] Xu Y. Z., Sun W. C., Fang Y., Sun B.L. (2020). Changes of endogenous hormones and total
710 polyphenols for winter turnip rape (*Brassica rapa* L.) subjected to low temperature. *Nucl.*
711 *Agric. Sci.* 34(7):1551-1560. DOI: 10.11869/j.issn.100-8551.2020.07.1551
- 712 [25] Bates, L., Waldren, R., and Teare, I. (1973). Rapid determination of free proline for wa-ter-
713 stress studies. *Plant and Soil*, 39, 205-207.
- 714 [26] Dhindsa, R., Plumb-Dhindsa, P., and Thorpe, T. (1981). Leaf senescence: correlated with
715 increased levels of membrane permeability and lipid peroxidation, and decreased levels of
716 superoxide dismutase and catalase. *Journal of Experimental Botany*, 32, 93-101.
- 717 [27] Dacheng, J., Shan, G., Hailun, G., and Nianwei, Q. (2018). The details of protein content
718 determination by coomassie brilliant blue staining. *Experiment Science and Technology*,
719 16(04), 143-147. DOI: 10.3969 /j.issn.1672-4550.2018.04.035
- 720 [28] Liu H X, Zeng S X, Wang Y R, Li P, Chen D F, Guo J Y. Effects of low temperature on

- superoxide dismutase (SOD) in various organelles of cotyledons of cucumber (*Cucumis sativus*) seedlings with different cold hardiness[J]. *Plant Physiology Communications*, 1985 (01): 48-57.
- [29] Fan J Q, Lu X M, Wang H W, et al. (2023). Correlation between cold hardiness and leaf hormone contents of winter *Brassica napus* under low temperature stress[J]. *Jiangsu Journal of Agricultural Sciences*, 39(01): 15-21. DOI:10.3969/j.issn.1000-4440.2023.01.002.
- [30] Niu Y J, Yuan J F, Chen H X, et al. Germination characteristics of *Corethroedendron fruticosum* seeds after dehulling and cold stratification, and changes in endogenous hormone ABA and IAA contents determined by HPLC[J]. *Chinese Agricultural Science Bulletin*, 2015, 31(04): 34-39.
- [31] Ren, Q, Xu, Z, Xue, Y, Yang, R, Ma, X, Sun, J, Wang, J, Lin, S, Wang, W, Yang, L, and Sun, Z. (2022). Mechanism of calcium signal response to cadmium stress in duckweed. *Plant Signaling & Behavior*, 17(1):2119340. DOI: 10.1080/15592324.2022.2119340.
- [32] Fassel, V., and Kniseley, R. (1974). Inductively coupled plasma. Optical emission spectroscopy. *Analytical Chemistry*, 46, 1110A-1120a.
- [33] Kim D, Langmead B, Salzberg S L.(2015). HISAT: a fast spliced aligner with low memory requirements. *Nature methods*,12(4): 357-360. DOI: 10.1038/nmeth.3317.
- [34] Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, 15(12):550. DOI: 10.1186/s13059-014-0550-8.
- [35] Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26(1), 139-40. DOI: 10.1093/bioinformatics/btp616.
- [36] Llanes, A, Arbona, V, Go'mez-Cadenas A, Luna, V.(2016). Metabolomic profiling of the halophyte *Prosopis strombulifera* shows sodium salt-specific response. *Plant Physiol Biochem*. 108:145-157. DOI: 10.1016/j.plaphy.2016.07.010.
- [37] Eremina M, Rozhon W, Poppenberger B. (2015). Hormonal control of cold stress responses in plants. *Cell Mol Life Sci*. 73(4):797-810. DOI: 10.1007/s00018-015-2089-6.
- [38] Ding Y, Shi Y, Yang S. (2019). Advances and challenges in uncovering cold tolerance regulatory mechanisms in plants. *New Phytol*. 222(4): 1690-1704. DOI: 10.1111/nph.15696.
- [39] Niu Z, Liu L, Pu Y, Ma L, Wu J, Hu F, Fang Y, Li X, Sun W, Wang W, Bai C. (2021). iTRAQ-based quantitative proteome analysis insights into cold stress of Winter Rapeseed (*Brassica rapa* L.) grown in the field.Sci Rep. 11(1):23434. DOI: 10.1038/s41598-021-02707-z.
- [40] Gu H, Yang Y, Xing M, Yue C, Wei F, Zhang Y, Zhao W, Huang J.(2019). Physiological and transcriptome analyses of *Opisthopappus taihangensis* in response to drought stress. *Cell Biosci*. 9:56. DOI: 10.1186/s13578-019-0318-7.
- [41] Karlova R, Boer D, Hayes S, Testerink C. (2021). Root plasticity under abiotic stress. *Plant Physiol*. 187(3):1057-1070. DOI: 10.1093/plphys/kiab392.
- [42] Li T, Zhu T, Liu Z, Yang N, Wang Z, Yang T, Gao K. (2024). Evaluation of Cold Resistance in Alfalfa Varieties Based on Root Traits and Winter Survival in Horqin Sandy Land. *Biology (Basel)*. 13(12):1042. DOI: 10.3390/biology13121042.
- [43] Lewis DR, Negi S, Sukumar P, Muday GK. (2011). Ethylene inhibits lateral root development, increases IAA transport and expression of PIN3 and PIN7 auxin efflux carriers. *Development*. 138(16):3485-95. DOI: 10.1242/dev.065102.
- [44] Liu J, Moore S, Chen C, Lindsey K. (2017). Crosstalk Complexities between Auxin, Cytokinin,

- and Ethylene in Arabidopsis Root Development: From Experiments to Systems Modeling, and Back Again. *Mol Plant*. 10(12):1480-1496. DOI: 10.1016/j.molp.2017.11.002.
- [45] Qin H, He L, Huang R. (2019). The Coordination of Ethylene and Other Hormones in Primary Root Development. *Front Plant Sci*. 10:874. DOI: 10.3389/fpls.2019.00874.
- [46] Niu Y, Fan S, Cheng B, Li H, Wu J, Zhao H, Huang Z, Yan F, Qi B, Zhang L, Zhang G. (2023). Comparative transcriptomics and co-expression networks reveal cultivar-specific molecular signatures associated with reproductive-stage cold stress in rice. *Plant Cell Rep*. 42(4): 707-722. DOI: 10.1007/s00299-023-02984-0.
- [47] Zhou R, Yu X, Zhao T, Ottosen CO, Rosenqvist E, Wu Z. (2019). Physiological analysis and transcriptome sequencing reveal the effects of combined cold and drought on tomato leaf. *BMC Plant Biol*. 19(1):377. DOI: 10.1186/s12870-019-1982-9.
- [48] Chen Y, Chu Y, Wang J, Liu S, Zuo Y, Yao C, Dong J, Wang Q, Liu T, Tu W, Qin J, Chen L, Song B. (2025). Natural allelic variation in the CBF2 transcription factor is a pivotal factor controlling cold resistance in potato. *Plant Physiol*. DOI: 10.1093/plphys/kiaf428.
- [49] Luo F, Yin M, Zhou J, Zhou X, Wang C, Zhang W, Chen L, Lee D. (2024). Integrative Transcriptomic and Small RNA Analysis Uncovers Key Genes for Cold Resistance in Rice. *Genes (Basel)*. 16(1):38. DOI: 10.3390/genes16010038.
- [50] Fu J, Miao Y, Shao L, Hu T, Yang P. (2016). De novo transcriptome sequencing and gene expression profiling of *Elymus nutans* under cold stress. *17(1):870*. DOI: 10.1186/s12864-016-3222-0.
- [51] Tarun JA, Mauleon R, Arbelaez JD, Catausan S, Dixit S, Kumar A, Brown P, Kohli A, Kretschmar T. (2020). Comparative transcriptomics and co-expression networks reveal tissue-and Genotype-Specific Responses of *qDTYs* to Reproductive-Stage Drought stress in Rice (*Oryza sativa* L.). *11(10):1124*. DOI: 10.3390/genes11101124.
- [52] Wang L, Wang S, Tong R, Wang S, Yao J, Jiao J, Wan R, Wang M, Shi J, Zheng X. (2022). Overexpression of PgCBF3 and PgCBF7 Transcription Factors from Pomegranate Enhances Freezing Tolerance in Arabidopsis under the Promoter Activity Positively Regulated by PgICE1. *23 (16):9439*. DOI: 10.3390/ijms23169439.
- [53] Li Y, Tian Q, Wang Z, Li J, Liu S, Chang R, Chen H, Liu G. Integrated analysis of transcriptomics and metabolomics of peach under cold stress. *Front. Plant Sci*. 14:1153902. DOI: 10.3389/fpls.2023.1153902.
- [54] Huo C, Zhang B, Wang R. (2022). Research progress on plant noncoding RNAs in response to low-temperature stress. *Plant Signal Behav*. 17(1):2004035. DOI:10.1080/ 15592324. 2021. 2004035.

806 **Appendix Table**

807 **Appendix Table 1:** Statistics of sequencing quality

Samples	Clean reads	Clean bases	GC Content (%)	Q30 (%)
AL7-0-1	19,082,198	5,702,035,863	47.38%	97.92%
AL7-0-2	19,382,514	5,794,861,756	46.77%	97.64%
AL7-0-3	19,507,603	5,832,358,771	46.63%	98.46%
AL7-F5-1	19,739,444	5,900,555,907	46.24%	96.99%
AL7-F5-2	19,599,098	5,857,635,710	46.24%	96.37%
AL7-F5-3	21,075,339	6,298,072,397	46.37%	94.60%
AL7-FS-1	20,126,763	6,019,493,637	46.91%	98.27%
AL7-FS-2	20,047,268	5,996,569,195	46.81%	98.38%
AL7-FS-3	20,648,143	6,176,693,408	46.81%	98.06%
BL99-0-1	20,681,947	6,184,911,865	46.65%	98.18%
BL99-0-2	19,983,956	5,974,704,291	46.66%	97.89%
BL99-0-3	21,124,131	6,313,539,212	46.71%	98.90%
BL99-F5-1	20,142,528	6,016,819,532	46.54%	96.87%
BL99-F5-2	19,941,887	5,957,290,841	46.44%	97.48%
BL99-F5-3	19,787,086	5,915,627,473	46.56%	97.41%
BL99-FS-1	24,283,344	7,261,051,482	46.98%	96.49%
BL99-FS-2	19,138,934	5,718,376,050	46.89%	97.93%
BL99-FS-3	19,626,314	5,868,689,739	46.83%	98.32%

808 Note: AL7 and BL99 represent two different sample names; 0, F5, and FS represent three different low-
809 temperature stresses at 0°C, -5°C, and -10°C respectively; the numbers 1, 2, and 3 after the sample names
810 indicate three biological replicates under the same temperature treatment.

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813 **Appendix Table 2:** Statistics of transcriptome sample sequencing data and comparison efficiency with
814 rapeseed reference genome

Sample	Total Reads	Mapped Reads	Uniq Mapped Reads	Multiple Map Reads	Reads Map to '+'	Reads Map to '-'
AL7-0-1	38,164,396	30,540,409 (80.02%)	29,794,144 (78.07%)	746,265 (1.96%)	15,740,604 (41.24%)	15,731,550 (41.22%)
AL7-0-2	38,765,028	33,366,878 (86.07%)	32,545,787 (83.96%)	821,091 (2.12%)	17,197,840 (44.36%)	17,183,717 (44.33%)
AL7-0-3	39,015,206	33,969,847 (87.07%)	17,183,717 (44.33%)	805,943 (2.07%)	17,490,434 (44.83%)	17,482,884 (44.81%)
AL7-F5-1	39,478,888	33,364,417 (84.51%)	33,163,904 (85.00%)	805,943 (2.07%)	17,193,752 (43.55%)	17,196,507 (43.56%)
AL7-F5-2	39,198,196	31,937,543 (81.48%)	17,482,884 (44.81%)	787,684 (2.00%)	16,452,491 (41.97%)	16,461,009 (41.99%)
AL7-F5-3	42,150,678	34,418,307 (81.66%)	32,576,733 (82.52%)	737,165 (1.88%)	17,748,749 (42.11%)	17,750,951 (42.11%)

AL7-FS-1	40,253,526	34,799,900 (86.45%)	33,987,342 (84.43%)	812,558 (2.02%)	17,941,474 (44.57%)	17,895,369 (44.46%)
AL7-FS-2	40,094,536	35,049,780 (87.42%)	34,238,302 (85.39%)	811,478 (2.02%)	18,065,872 (45.06%)	18,022,306 (44.95%)
AL7-FS-3	41,296,286	35,918,300 (86.98%)	35,080,264 (84.95%)	838,036 (2.03%)	18,512,521 (44.83%)	18,477,909 (44.74%)
BL99-0-1	41,363,894	35,755,676 (86.44%)	34,860,790 (84.28%)	894,886 (2.16%)	18,442,456 (44.59%)	18,437,684 (44.57%)
BL99-0-2	39,967,912	34,649,614 (86.69%)	33,800,190 (84.57%)	849,424 (2.13%)	17,857,914 (44.68%)	17,848,352 (44.66%)
BL99-0-3	42,248,262	36,962,478 (87.49%)	36,041,211 (85.31%)	921,267 (2.18%)	19,061,501 (45.12%)	19,058,643 (45.11%)
BL99-F5-1	40,285,056	33,811,300 (83.93%)	32,979,397 (81.87%)	831,903 (2.07%)	17,449,080 (43.31%)	17,442,890 (43.30%)
BL99-F5-2	39,883,774	33,466,616 (83.91%)	32,632,536 (81.82%)	834,080 (2.09%)	17,272,500 (43.31%)	17,271,848 (43.31%)
BL99-F5-3	39,574,172	32,793,600 (82.87%)	31,957,937 (80.75%)	835,663 (2.11%)	16,949,069 (42.83%)	16,933,387 (42.79%)
BL99-FS-1	48,566,688	40,700,872 (83.80%)	39,776,799 (81.90%)	924,073 (1.90%)	20,988,505 (43.22%)	20,920,562 (43.08%)
BL99-FS-2	38,277,868	32,428,338 (84.72%)	31,701,710 (82.82%)	726,628 (1.90%)	16,706,206 (43.64%)	16,659,868 (43.52%)
BL99-FS-3	39,252,628	33,086,739 (84.29%)	32,319,996 (82.34%)	766,743 (1.95%)	17,073,317 (43.50%)	17,015,139 (43.35%)

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