https://doi.org/10.17221/92/2023-CJGPB

Transcriptome analysis of alfalfa (*Medicago sativa* L.) roots reveals overwintering changes in different varieties

*Yajun Ma[1], Guozhu Yang[2], Ruijun Duan[2], Xiaoan Li[3], Siheng Zeng[3], Yajun Yan[1], Ce Zheng[4], Yueming Hu[1]**

[1]*College of Agriculture and Animal Husbandry, Qinghai University, Xining, Qinghai, P.R. China*
[2]*Ecological Environment Engineering Research Center, Qinghai University, Xining, Qinghai, P.R. China*
[3]*College of Ecological and Environmental Engineering, Qinghai University, Xining, Qinghai, P.R. China*
[4]*College of Finance and Economics, Qinghai University, Xining, Qinghai, P.R. China*

**Corresponding author: mayajun110@126.com*

Citation: Ma Y.J., Yang G.Z., Duan R.J., Li X.A., Zeng S.H., Yan Y.J., Zheng C., Hu Y.M. (2024): Transcriptome analysis of alfalfa (*Medicago sativa* L.) roots reveals overwintering changes in different varieties. Czech J. Genet. Plant Breed., 60: 97–104.

Abstract: Low temperatures are one of the major abiotic stresses that affect alfalfa's development and yield. Enhancing frost resistance through resistance-related genes is one of the most effective ways to address this issue in alfalfa. Therefore, exploring cold-resistant gene resources and the cultivation of cold-resistant alfalfa cultivars is inevitable in order to achieve high yield and quality. In this study, we conducted transcriptome profiling of roots obtained from two alfalfa genotypes, i.e., Qingda No.1 for freeze tolerance and Gannong No.9 for freeze sensitivity. We observed that Qingda No.1 had more lateral roots and a more developed root system after overwintering, while Gannong No.9 had fewer lateral roots and an underdeveloped root system. After overwintering, Qingda No.1 exhibited higher superoxide dismutase (SOD) activity compared to Gannong No.9, while Gannong No.9 showed higher perosuperoxide dismutasexidase (POD) activity than Qingda No.1. We identified 25,935 differentially expressed genes, with 12 979 and 12 956 differential genes found in the freeze-tolerant variety Qingda No.1 group and the freeze-sensitive Gannong No.9 group, respectively. The enrichment of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways also differed between the two groups. We also discovered several gene family members, and the most frequent transcription factors were *bHLH*, *B3*, *NAC*, *WRKY*, and *MYB_related*. These findings provide comprehensive information to further understand the molecular mechanisms of adaptation to freezing stress in alfalfa and offer potential functional candidate genes for adaptation to abiotic stress.

Keywords: expressed genes; frost resistance; low-temperature stress; lucerne; transcription factors

Alfalfa (*Medicago sativa* L.) is one of the most important cultivated crops in the world. It is a high-quality perennial legume forage with a high protein content and is known as the "king of forages" based on its high yield, rich nutritional value, good palatability, high nitrogen-fixing capacity, and strong ecological adaptability (Bacenetti et al. 2018). The yield of alfalfa is affected by the climate, mainly due to low temperatures and insufficient light, resulting in delayed flowering during the growing season (Wan

Supported by the Central Government Forestry and Grass Science and Technology Promotion Demonstration Project (Qing [2023] TG06) and (Qing [2023] TG03), and the Scientific and Technological Achievements Transformation Project (2022-NK-135) of the Key Research and Development and Transformation Program of the Department of Science and Technology of Qinghai Province.

https://doi.org/10.17221/92/2023-CJGPB

et al. 2023). As low winter temperatures approach, alfalfa can use dormancy to adjust the hormone levels in the body, which, in turn, can withstand adverse conditions and overwinter safely. Autumn dormancy is an adaptive response of alfalfa to changes in the growing environment such as shortened light and lower temperatures. Frost damage to alfalfa growth occurs when winter temperatures fall below the minimum temperature (–10 °C) for alfalfa survival, due to a large reduction in ambient temperature that completely exceeds the alfalfa's ability to tolerate that temperature (Rowland et al. 2004). Plant stems and leaves are the most susceptible to and reflect the effects of environmental factors. The thickness of the root neck, the number of branches, the plant height, and the weight of a single plant in the year (2016, at the University of Waikato, Hamilton, New Zealand) of sowing varied significantly among different sowing dates. The earlier the sowing date, the thicker the root neck the more branches, the higher the plant height and single plant weight, and the corresponding increase in alfalfa overwintering rate (Valverde-Barrantes et al. 2016). Cold tolerance of alfalfa is largely dependent on the root system, with branched root types tolerating freeze-pulling phenomena better than a single taproot. Alfalfa possesses four root types (i.e., taproot, branched root, rhizomatous root, and root-tiller type), where rhizomatous alfalfa is more resistant to severe cold due to a grape root neck and a root neck that penetrates deep into the soil (Nan et al. 2014). Low temperature induces the production of peroxide isoenzymes, and alfalfa enhances cold resistance by increasing the superoxide dismutase and catalase activities in the root neck, where the superoxide dismutase activity and hypoxidase can be used as physiological indicators of alfalfa's cold resistance strength (Chen et al. 2021). Through transcriptome sequencing, which mainly focuses on the processes of stress response, hormone regulation and antioxidant regulation, a further analysis revealed that the transcription factor family genes, such as *AP2/ERF*, *WRKY*, and *MYB*, play important regulatory roles in the cold stress process of alfalfa (Zhao et al. 2023). Alfalfa roots need to adapt to low temperatures and freeze-thaw cycles in winter to ensure the survival and growth of the plant. Transcriptome analysis is an important means to study the regulation of gene expression in organisms. Through an in-depth understanding of gene expression during root overwintering, the mechanism of cold tolerance and adaptive response of alfalfa can be revealed. In this experiment, RNA-seq was used to assess changes in the freezing stress response genes in the roots of freeze-tolerant and freeze-sensitive varieties with freezing stress based on the reference alfalfa genome, and several genes were identified that may strongly influence the freezing stress response in alfalfa. These results elucidated upon the mechanism of cold stress response and can be used to compare the difference between freeze-tolerant and freeze-sensitive alfalfa, and provide useful information for further research on the mechanism of cold tolerance and alfalfa breeding.

MATERIAL AND METHODS

Plant materials and culture conditions. The fall dormancy of alfalfa refers to the physiological dormancy phenomenon in northern latitudes caused by the reduction of light and temperature in autumn, which is a kind of growth characteristic of alfalfa, and this characteristic has a direct relationship on alfalfa's cold-resistance ability and production performance, which is an important theoretical basis for alfalfa seed introduction and ecological zoning. Planting varieties with a low level of fall dormancy can effectively improve the overwintering survival rate of alfalfa, and this correlation between alfalfa's fall dormancy and cold resistance has been fully confirmed in production practice. Therefore, two plant materials with different fall dormancy levels were selected for the experiment. The first was the frost-tolerant variety (autumn dormancy score 3.0), with seeds provided by Qinghai University (Qinghai, China). The second variety was the freeze-sensitive (autumn dormancy score 7.5), with seeds supplied by Gansu Agricultural University (Gansu, China). The experiment was conducted in a one-way randomised block design, with field plots of 2 × 3 m in size and three replications. A 1 m aisle was left around each plot, and a 1 m protection row was set up around the experimental area. Since the experiment was conducted (2022-01-01 to 2023-06-15), the maximum temperature (2022/07/07) was 35 °C, and the minimum temperature (2022/12/16) was –21 °C, and the relevant indices were measured in accordance with the contents of the experiment. The sampling time was from 1 September to 10 September of the current year, and from 1 March to 10 March of the following year. QD1 (QD1 stands for Qingda No.1 roots in September before overwintering), QD2 (QD2 stands for Qingda No.1 roots in March after

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overwintering), LW1 (LW1 stands for Gannong No.9 roots in September before overwintering), LW2 (LW2 stands for Gannong No.9 roots in March after overwintering); the roots were frozen with liquid nitrogen and stored at –80 °C.

Phenotype comparison and enzyme extraction and assay. The roots of the frost-tolerant variety Qingda No.1 and the frost-sensitive Gannong No.9 were dug up in September and March of the following year, cleaned with water, and observed for morphology using an HP scanner (HP LaserJet Pro M127, Hewlett-Packard, USA). We used 0.1 g of fresh roots, homogenised them in 1.5 mL of a potassium phosphate buffer (50 mM, pH 7.0) and centrifuged them at 12 000 rpm for 20 min at 4 °C to obtain the supernatants for the antioxidant activity. The superoxide dismutase (SOD) activity was determined by autoxidation with o-triol (Paoletti & Mocali 1990) and the perosuperoxide dismutasexidase (POD) activity was determined using guaiacol oxidation (Si et al. 2012). The experiment was repeated three times for each group. IBM SPSS Statistics 23 software was used to analyse the significance. The base quality value of the Illumina High-Throughput Sequencing Platform (Illumina Novaseq 6000) is a mapping of the probability of the error in the base identification. The higher the quality score, the more reliable the base recognition is and the less likely it is that a base will be measured incorrectly. When the sequencer recognises a base, it will give the probability *P* that each base is incorrectly recognised, and the Phred quality score of the base is –lg10P.

Differential expression analysis, GO enrichment analysis and KEGG pathway enrichment analysis. Differential expression analyses were performed for both conditions/groups using the DESeq R package (Ver. 1.10.1). DESeq provides statistical routines to determine the differential expression in numerical gene expression data using models based on negative binomial distributions. The GOseq R package based on the Wallenius non-central hypergeometric distribution (Young et al. 2010) performed the gene ontology (GO) enrichment analyses of the differentially expressed genes (DEGs), and we used the KOBAS (Mao et al. 2005) software to test for the statistical enrichment of the differentially expressed genes in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

RESULTS

Phenotypic changes and antioxidant enzymes in Gannong No.9 and Qingda No.1 in response to low temperature stress. We analysed the changes in the antioxidant enzymes and phenotypes in the alfalfa roots before and after overwintering to distinguish different frost-resistant types of alfalfa. The changes in the roots of the freeze-tolerance and the freeze-sensitivity were not obvious before overwintering (Figure 1A, B). To further demonstrate the phenotypic changes, we compared the roots of the two plants in March of the following year. Qingda No.1 had a high number of lateral roots and a well-developed root system, whereas Gannong No.9 had a low number of lateral roots and an undeveloped root system. The number of lateral roots of the freeze-tolerance variety was high and the root system was more developed, while the number of lateral roots of the freeze-sensitivity variety was low and the root system was undeveloped, which indicated that Qingda No.1 had higher tolerance compared with Gannong No.9. In order to test the effects of the low temperature stress on the SOD and POD activities in the alfalfa roots, we found that the SOD activities of Qingda No.1 and Gannong No.9 were similar before overwintering (September), and the SOD activities of Qingda No.1 and Gannong No.9 were similar after overwintering. After wintering, the SOD activities of Qingda No.1 and Gannong No.9 increased significantly, and importantly, Qingda No.1 had a greater degree of SOD activity than Gannong No.9. The change of POD activity was opposite to that of SOD, and the POD activity of Gannong No.9 was higher than that of Qingda No.1 after overwintering. The POD activity of both alfalfa varieties increased after overwintering (Figure 1D). From the phenotypes, it could be seen that the lignification of Gannong No.9 was higher than that of Qingda No.1. Taken together, the antioxidant activity of the frost-tolerant variety Qingda No.1 appeared later than that of the frost-sensitive variety Gannong No.9.

Analysis of the differentially expressed genes in the alfalfa roots of Gannong No.9 and Qingda No.1. We constructed comparative analyses of Qingda No.1 and Gannong No.9 in the treated roots after overwintering (March of the following year) and in the control roots before overwintering (September). Comparison and transcript splicing analyses were performed using star and Cufflinks software, respectively, and then all the genes were quantitatively analysed, and the differential genes were identified according to the differential combinations set in the experiments. 25 935 differential genes showed significant up-regulation/down-regulation,

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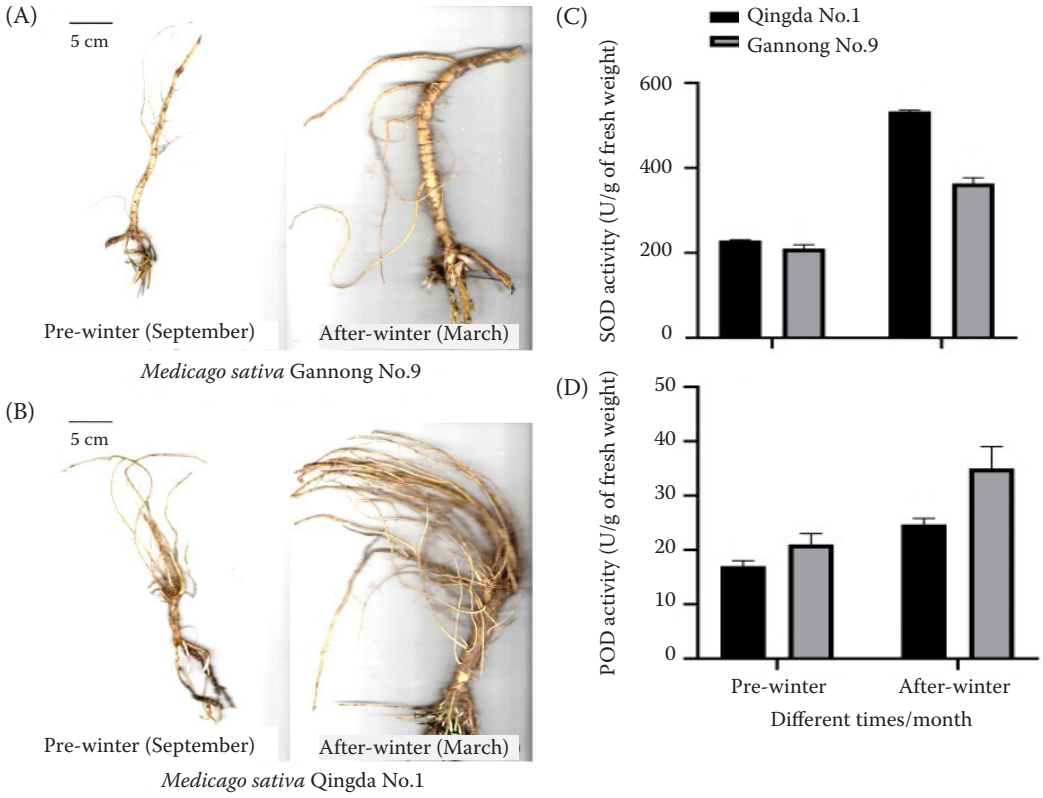

Figure 1. Phenotypic changes and antioxidant enzymes in Gannong No.9 and Qingda No.1 in response to low temperature stress: growth status of Gannong No.9 roots before (September) and after (March) overwintering (A), growth status of Qingda No.1 roots before (September) and after (March) overwintering (B), superoxide dismutase (SOD) activity (C) and perosuperoxide dismutasexidase (POD) activity (D) of the two alfalfa roots before and after overwintering

of which 12 956 differential genes and 12 979 differential genes were found in LW1vsLW2 and QD1 vsQD2, respectively (Figure 2).

GO enrichment and KEGG enrichment analyses of the differential genes in Gannong No.9 and Qingda No.1. In order to better understand the functions and roles of differentially expressed genes, the GO function annotation of differentially expressed genes was carried out, which was classified into three ontologies: molecular function, biological process and cellular composition. 9 495 and 9 321 differentially expressed genes were annotated into the 30 categories of the GO function in the freeze-tolerant variety Qingda No.1 and freeze-sensitive variety Gannong No.9, respectively. The frost-tolerant variety Qingda No.1 and the freeze-sensitive variety Gannong No.9 had 9 495 and 9 321 differentially expressed genes annotated into the 30 classifications of the GO function, respectively, and the proportions of biological process and molecular function were higher, and there were 9 subclasses in the biological process, among which the biological process,

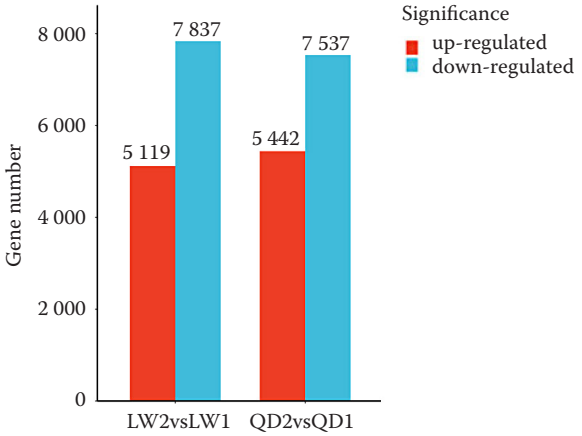

Figure 2. The number of differentially expressed genes in the alfalfa roots before and after overwintering

The genes with significant differentially expressed genes are indicated in red (up-regulated) and light blue (down-regulated); LW1 – the Gannong No.9 samples before overwintering (in September); LW2 – the Gannong No.9 samples after overwintering (in March of the following year); QD1 – the Qingda No.1 samples before overwintering (in September); QD2 – the Qingda No.1 samples after overwintering (in March of the following year)

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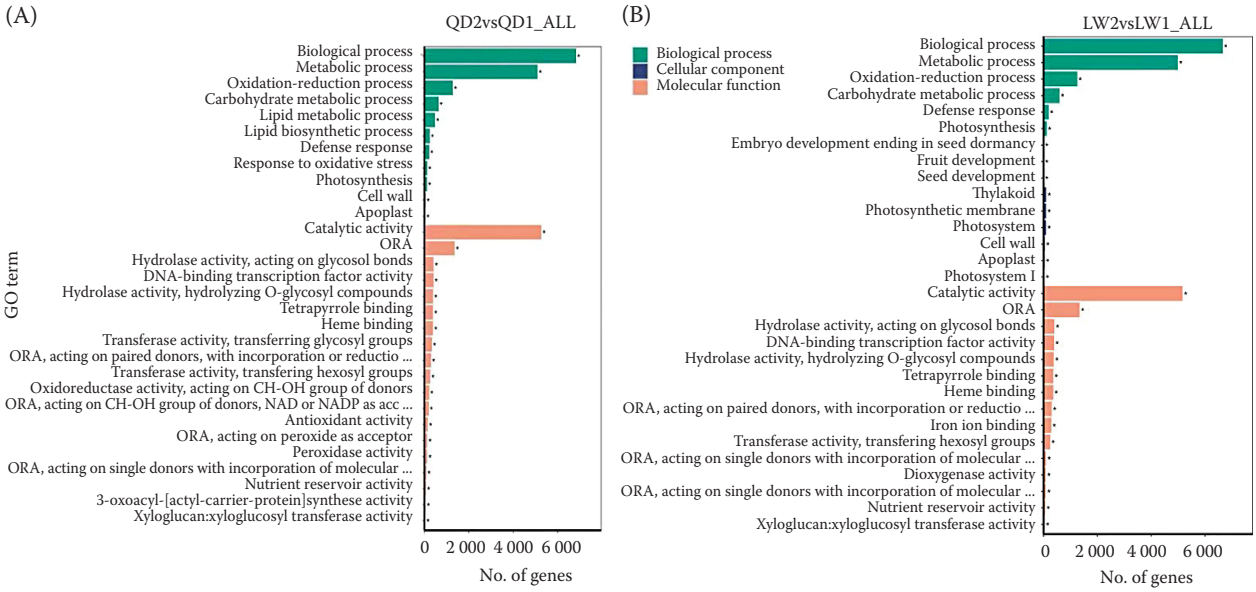

Figure 3. Histogram of the gene ontology (GO) enrichment: QD2vsQD1 (A), LW2vsLW1 (B)

QD1 – the Qingda No.1 samples before overwintering (in September); QD2 – the Qingda No.1 samples after overwintering (in March of the following year); LW1 – the Gannong No.9 samples before overwintering (in September); LW2 – the Gannong No.9 samples after overwintering (in March of the following year); ORA – oxidoreductase activity; the vertical coordinate is the enriched GO term, and the horizontal coordinate is the number of differentially expressed genes in the term; different colours are used to distinguish the biological processes, cellular components and molecular functions, and GO terms with "*" are significantly enriched

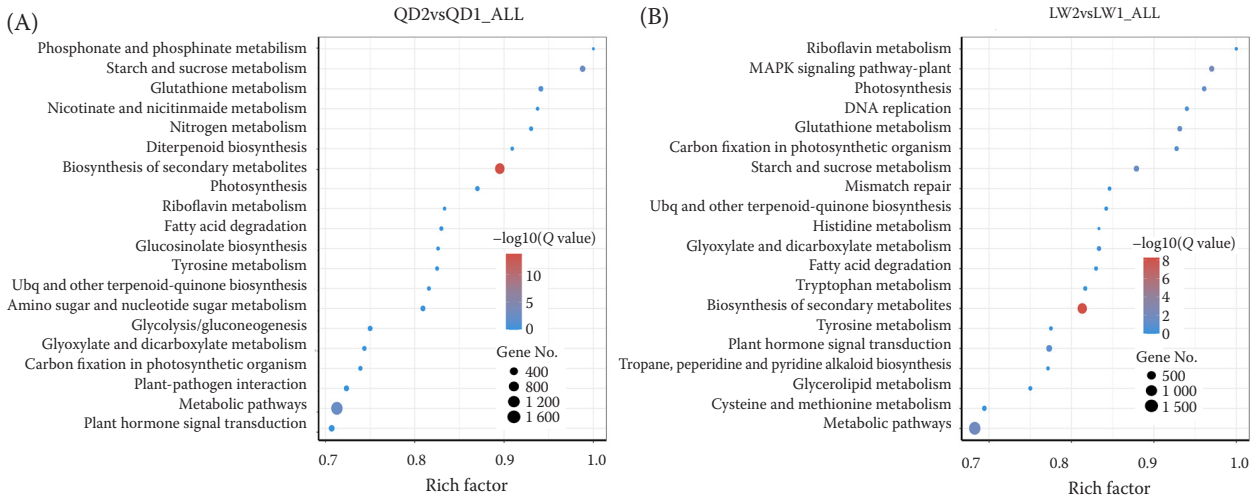

Figure 4. Scatter plot of the Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment of the differentially expressed genes: QD2vsQD1 (A) and LW2vsLW1 (B)

QD1 – the Qingda No.1 samples before overwintering (in September); QD2 – the Qingda No.1 samples after overwintering (in March of the following year); LW1 – the Gannong No.9 samples before overwintering (in September); LW2 – the Gannong No.9 samples after overwintering (in March of the following year); MAPK – mitogen activated protein kinase; Ubq – ubiquinone; the vertical axis indicates the pathway name, the horizontal axis indicates the rich factor; the size of the dots indicates the number of differentially expressed genes in the pathway, and the colour of the dots corresponds to the number of up- and down-regulated genes in the top 20 pathways that were significantly enriched in the KEGG enrichment analysis of the differentially expressed genes in the different *Q* value ranges in accordance with the order of *Q* value from smallest to largest

https://doi.org/10.17221/92/2023-CJGPB

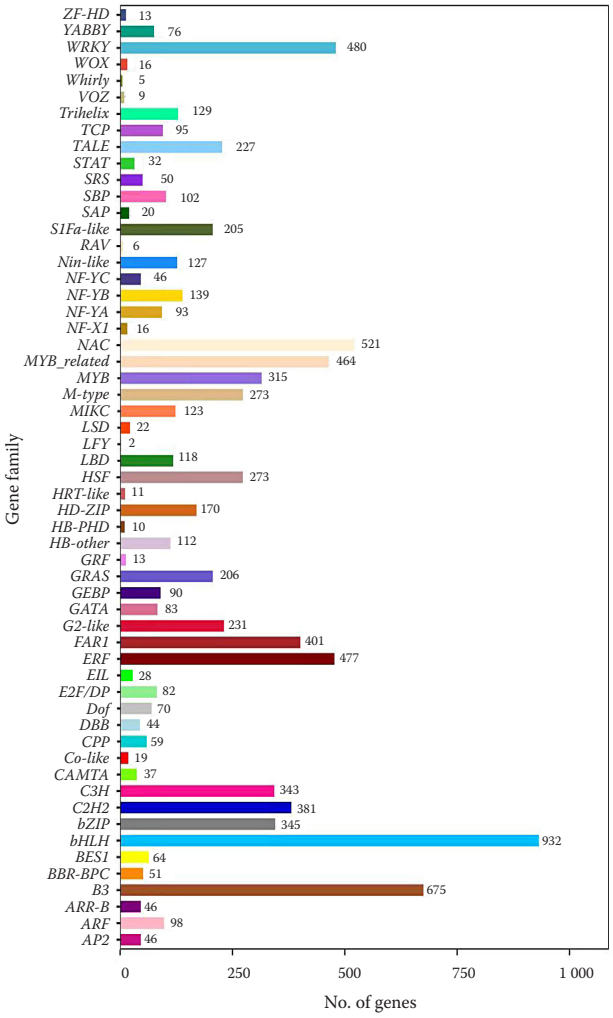

Figure 5. Map of the transcription factor family distribution
The left vertical axis indicates the gene family names and the horizontal axis the number of genes for the transcription factors

metabolic process, and redox process accounted for the largest proportion. The freeze-resistant variety Qingda No.1 had two subclasses in the cellular component, in which the cell wall and plasmodesmata dominated, and had 19 subclasses in the molecular function, in which the catalytic activity and oxidative reductase activity accounted for the largest proportion (Figure 3A). In the freeze-sensitive variety Gannong No.9, there were 6 subclasses in the cellular component, with cysts, photosynthetic membranes, and photosystems dominating, and 15 subclasses in the molecular function, with the catalytic activity and oxidoreductase activity accounting for the largest proportion (Figure 3B).

In the freeze-tolerant variety Qingda No.1 (QD-1vsQD2), the pathways with the strongest correlation between the KEGG enrichment of the differentially expressed genes were glycolysis/gluconeogenesis, glyoxylate and dicarboxylate metabolism, carbon sequestration by photosynthetic organisms, the plant-pathogen interaction metabolism pathway, and phytohormone signalling (Figure 4A). In the freeze-sensitive Gannong No.9 (LW1vsLW2) group, the pathways most relevant for the KEGG enrichment of the differentially expressed genes were the secondary metabolite biosynthesis, tyrosine metabolism, phytohormone signalling, tostones, piperidines and pyridine alkaloids biosynthesis, glycerolipid metabolism, cysteine and methionine metabolism, and metabolic pathways (Figure 4B).

Analysis of the TFs. The TF (transcription factor) is a class of proteins that bind DNA in a sequence-specific manner and play a key role in the regulation of gene transcription, ensuring that the target gene is expressed at a specific time and space with a specific intensity and playing a critical role in a variety of biological processes and disease development. Since TFs play a major role in the regulation of gene expression and plant stress response, we identified a number of gene family members (Figure 5). The five gene families with the most transcription factors are *bHLH*, *B3*, *NAC*, *WRKY*, and *MYB_related*.

DISSCUSION AND FUTURE PERSPECTIVES

According to statistics, low-temperature stress causes about a 40% annual crop yield reduction in temperate regions, while extreme cold weather causes a 51–82% crop yield loss globally (Zhang et al. 2022). The extent of crop damage caused by low-temperature stress is influenced by a variety of factors, including the plant's place of origin (temperate or tropical), species, stage of growth (nutritive or reproductive), damaged organs (shoots or roots), as well as the duration of the low-temperature stress and other environmental factors (Farooq et al. 2009).

Alfalfa with meristematic and rhizomatous roots can renew and produce new roots when the main root of the parent plant freezes to death, and therefore has greater cold tolerance than a taproot. Rhizomatous rooted alfalfa has many horizontal lateral roots, and thus has a stronger cold overwintering ability than taproot rooted alfalfa (Yang et al. 2023). The tolerance of alfalfa to low-temperature stress is closely related to the ability of its antioxidant system to scavenge reactive oxygen species (ROS). Under low-temperature stress, studies in rice (Wang

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et al. 2022a), maize (Xu et al. 2014), cucumber (Senadheera et al. 2023), and cotton (Egbuta et al. 2022) have shown that the antioxidant enzyme activities and the antioxidant content both showed an increase at first and then a decrease, and the antioxidant enzyme activity of cold-tolerant varieties was higher than that of cold-sensitive varieties under low-temperature stress. Importantly, our results are consistent with this conclusion, as the SOD activity in the roots of Qingda No.1 was higher than that of Gannong No.9, suggesting that the acquisition of freezing tolerance by alfalfa increases the activity of the antioxidant enzymes in the reactive oxygen species scavenging system. We conclude that alfalfa acquiring frost tolerance increases the activity of antioxidant enzymes in the reactive oxygen scavenging system. To explore the changes of the low-temperature stress-related gene expression in different alfalfa varieties, we constructed comparative analyses of Qingda No.1 and Gannong No.9 in the treated and pre-wintering (September) control roots after overwintering (March of the following year). It can be preliminarily speculated that the cold tolerance of alfalfa varieties is related to the number of DEGs and the number of genes with up-regulated expression after experiencing low temperatures. We reviewed other literature sources and found that in jasmine, the transcriptome differentially expressed genes are mainly involved in processes such as carbon fixation, photorespiration, peroxisomal fraction, messenger ribonucleoprotein complex, aspartyl esterase activity, and deoxyribonucleotide incorporation (Wang et al. 2022b).

Due to the major role of TFs in regulating the gene expression and plant stress response, the genes encoding TFs are major regulators in the stress response and also excellent candidates for crop improvement. The important role of transcription factors in enhancing crop stress tolerance has been revealed in recent studies through TF gene regulation and overexpression approaches. However, many mechanisms remain to be discovered regarding the regulatory strategies of TFs in different plants. Among more than 80 TF families, only a few (e.g., *NAC*, *MYB*, *WRKY*, *bZIP*, and *ERF/DREB*) have important roles in abiotic and biotic stress responses and have been intensively studied. (Nguyen & Lee H 2016). Future studies can further validate and functionally characterise these differential genes, as well as explore their roles in the regulation of cold tolerance in alfalfa.

CONCLUSION

In this study, we revealed the gene expression regulation mechanism of alfalfa roots during overwintering by transcriptome analysis. Through the functional annotation and enrichment analysis of the differential genes, we identified many genes and pathways related to the response to low-temperature adversity. These genes and pathways may be involved in the processes of cold adaptation, antioxidant response, and cell wall synthesis in plants. Future studies could further validate and functionally characterise these differential genes, as well as explore their role in the regulation of cold tolerance in alfalfa. These results provide useful information for a deeper understanding of the cold tolerance mechanism and adaptive response of alfalfa, as well as providing new ideas for alfalfa breeding and improvement. It provides comprehensive information to further understand the molecular mechanism of alfalfa adapting to freezing stress, and further provides functional candidate genes for adapting to abiotic stress, and also provides certain reference for alfalfa introduction in alpine regions such as the Qinghai-Tibetan Plateau.

Acknowledgements. We would like to thank Professor Guozhu Yang for providing the experimental materials, Professor Ruijun Duan for critically revising the paper, and thank the members of the research group for helping each other in the joint research.

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Received: November 16, 2023
Accepted: February 8, 2024
Published online: March 15, 2024