https://doi.org/10.17221/27/2025-CJGPB

Mechanisms of overwintering frost tolerance in alfalfa roots based on 4D-Label-free quantitative proteomics

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Citation: Ma Y.J., Zhang Y., Zhang Q.X., Sun G., Yan Y.J., Wang C.A., Mian W.B., Yang T.H., Gao T. (2025): Mechanisms of overwintering frost tolerance in alfalfa roots based on 4D-Label-free quantitative proteomics. Czech J. Genet. Plant. Breed., 61: 181–192.

Abstract: The present study investigates the differential protein expression levels between the frost-tolerant variety Qingda No.1 and the frost-sensitive variety Gannong No.9. The analysis was conducted using 4D-Label-free quantitative proteomics technology, with the samples collected prior to and after overwintering. The results showed that the protein expression of Qingda No.1 changed more significantly during the overwintering process, with 451 differentially expressed proteins (DEPs) being identified, of which 224 were up-regulated and 227 down-regulated. In contrast, the protein expression of Gannong No.9 differed from that of alfalfa roots of the frost-sensitive variety, with 204 DEPs being identified, of which 93 were up-regulated and 111 down-regulated. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis showed that the phenylpropanoid biosynthesis pathway was significantly enriched in both groups. Furthermore, enzymes such as phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), and chalcone isomerase (CHI) were significantly up-regulated in the freezing-tolerant varieties. Protein interaction network analysis revealed the synergistic regulation mode of DEPs. The present study elucidated the metabolic adaptive mechanism of overwintering in alfalfa roots at the protein level, thus providing a theoretical basis for the selection and breeding of frost-tolerant varieties.

Keywords: differentially expressed protein; enrichment analysis; low-temperature stress; *Medicago sativa*; phenylpropanoid biosynthesis

Alfalfa (*Medicago sativa* L.), a globally important leguminous forage, occupies an extremely important position in animal husbandry due to its high nutritional value and wide adaptability. However, its yield and quality are often significantly affected by low temperature stress. The root system serves as a key organ for alfalfa to absorb water and nutrients, as well as a key part of its cold resistance. Under low temperatures, cell dehydration and membrane lipid peroxidation occur frequently, leading to root

Supported by the Ningxia Hui Autonomous Region Agricultural Science and Technology Independent Innovation Project, China, Project No. NKYJ-24-01.

https://doi.org/10.17221/27/2025-CJGPB

damage, which in turn affects plant growth and survival (Jaya & Suja 2022). Plants respond to low temperature stress by initiating a series of complex physiological and biochemical responses. Plants maintain intracellular osmotic balance by accumulating osmoregulators (e.g., proline) and activating antioxidant systems (e.g., superoxide dismutase (SOD), catalase (CAT) to scavenge reactive oxygen species (ROS) to mitigate oxidative damage (Wang et al. 2023). In addition, the regulation of secondary metabolic pathways (e.g., phenylpropanoid metabolism) also plays an important role in the low-temperature response of plants. Secondary metabolites such as lignin and flavonoids produced by the phenylpropanoid metabolic pathway not only enhance cell wall strength but also increase the antioxidant capacity of plants, thus helping them to withstand low-temperature stress (Kejík et al. 2021). Nevertheless, little is known about the proteomic mechanisms underlying freezing tolerance in alfalfa. As the main executors of various physiological and biochemical reactions in cells, changes in the expression levels of proteins can directly reflect the adaptive mechanisms of plants under low-temperature stress. Therefore, an in-depth study of protein expression differences in alfalfa roots during overwintering is of great significance in revealing the molecular basis of its freezing tolerance. 4D-Label-free quantitative proteomics technology, as an emerging proteomics method, has the advantages of label-free, high-throughput, and high-sensitivity, which enables large-scale identification and quantitative analysis of proteins in complex biological samples (Sa et al. 2023), and has become a powerful tool for analysing protein dynamics in response to plant adversity. Based on this technology, this study systematically revealed the protein regulatory network of alfalfa roots during the overwintering period for the first time. In this study, we analysed the protein expression profiles of alfalfa roots before and after the overwintering period by 4D-Label-free technology, and screened the key differentially expressed proteins (DEPs) and pathways by bioinformatics methods, aiming at revealing the key DEPs and pathways of alfalfa roots during the overwintering period. Combined with the bioinformatics method, key DEPs and pathways were screened, aiming to reveal the molecular basis of frost tolerance in alfalfa and to provide targets for cold-resistant breeding.

MATERIAL AND METHODS

Plant materials and culture conditions. The test varieties were the frost-tolerant variety Qingda No.1 (autumn dormancy score of 3.0) and the frost-sensitive variety Gannong No.9 (autumn dormancy score of 7.5). Seeds were provided by Qinghai University and Gansu Agricultural University, respectively. The experimental site was the Sanjiangyuan Ecological Environment and Grassland Animal Husbandry Innovation Base of Qinghai University, which is located in Xining City, Qinghai Province, at an altitude of 2 323.5 m above sea level, with a continental plateau semi-arid climate. The experiment was conducted in a one-way randomised block design, with a field plot area of 2 × 3 m and three replicates. The seeds were sown on April 20, 2022, and 750 kg/ha of phosphorus fertiliser and 340 kg/ha of nitrogen-phosphorus fertiliser were applied in combination with land preparation before sowing. Strip sowing was carried out at a spacing of 30 cm in rows, with a sowing rate of 15 kg/ha and a depth of 1.5–2.0 cm. Field management included seeding, mid-tillage, weeding, and irrigation at the right time.

Sample collection and processing. Sampling took place from September 1 to September 10 of the current year (before overwintering) and from March 1 to March 10 of the following year (after overwintering). Root samples were frozen in liquid nitrogen and stored at –80 °C until use. Since the test was carried out (2022-01-01 to 2023-06-15), the average high temperature is 14 °C, the average low temperature is –3 °C, the extreme high temperature is 35 °C, the extreme low temperature is –21 °C, the average wind speed is 6 km/h, the frost-free period is 100 days, the total rainfall is 943 mm, and the evaporation is 1 364 mm. 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.

Protein extraction and quantification. Proteins were extracted using SDT buffer (4% SDS, 100 mM Tris-HCl, pH 7.6) and quantified via the bicinchoninic acid (BCA) assay. Subsequently, 20 μg of protein per sample was mixed with 5× loading buffer, boiled for 5 min, separated on 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (180 V, 45 min), and visualised by Coomassie Blue R-250 staining. Tryptic digestion followed the filter-aided sample preparation (FASP) method (Matthias Mann): samples were first treated with dithiothreitol (DTT; 10 mM final concentration) at 37 °C with 600 rpm shaking for 1.5 h; after cooling to room temperature, indole-3-acetic acid (IAA) (20 mM final) was added for cysteine alkylation and incu-

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bated 30 min in darkness. Samples were then transferred to filters, washed sequentially with 100 µL uric acid (UA) buffer (three times) and 25 mM NH_4HCO_3 buffer (twice), followed by trypsin addition (1 : 50 enzyme : protein wt/wt ratio) and overnight incubation at 37 °C for 15–18 h. Resulting peptides were collected as filtrate, desalted using C18 cartridges (Empore™ SPE Cartridges C18, standard density, bed I.D. 7 mm, volume 3 mL, Sigma), concentrated via vacuum centrifugation, reconstituted in 40 µL of 0.1% (v/v) formic acid, and peptide content estimated by UV absorbance at 280 nm. Peptide separation was performed using a NanoElute liquid chromatography system (NanoElute® 2,Bruker, Germany), with mass spectrometry data acquired on a timsTOF Pro instrument in parallel accumulation–serial fragmentation (PASEF) mode (scan range 100–1 700 m/z; primary mass tolerance 6 ppm, secondary 20 ppm; Öztuğ 2024). Database searching against the UniProt alfalfa database (uniprotkb_Medicago_94538_2024_01_09.fasta) used MaxQuant v1.6.14 with peptide and protein false discovery rate (FDR) ≤ 0.01, fixed modification of cysteine carbamidomethylation, and variable modification of methionine oxidation (Arunachalam & Sasidharan 2021).

Bioinformatics analysis. Difference-in-difference analysis screening criteria: fold change > 2.0 (up-regulation) or < 0.5 (down-regulation) and $P < 0.05$ (Student's *t*-test). Gene ontology (GO) enrichment analysis was performed using Fisher's exact test ($P < 0.05$), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation was done by KEGG Orthology Based Annotation SystemKOBAS 3.0 (Yee et al. 2020). Protein interaction networks were constructed based on the Search Tool for the Retrieval of Interacting Genes/ProteinsSTRING database (confidence > 0.7) and visualised by Cytoscape 3.9.1.

RESULTS

Protein identification and differential analysis. The Venn diagram showed that there were 4 736 common proteins in the QD group before and after overwintering, while 4 752 in the LW group (Figure 1), which suggested that frost-tolerant varieties might have initiated a more complex protein regulatory network to enhance their cold tolerance under low temperature stress. In this study, a total of 193 521 spectra were matched, 28 378 peptides (including 24 065 unique peptides) were identified, and 4 990 proteins were quantified. 451 differential proteins (224 up-regulated and 227 down-regulated) were identified in the QD2_vs_QD1 group, which was obvious than that in the LW2_vs_LW1 group (204 proteins (93 up-regulated and 111 down-regulated)) (Figure 2). This indicates that the protein expression changes in QD1 were more significant during the overwintering process (Figure 3).

GO function enrichment. As shown below (Figures 4–6), in the QD2_vs_QD1 comparison group, drug catabolic process, cellular iron ion homeostasis, protein-chromophore linkage, deoxyribonucleic acid (DNA) metabolic process, phenylpropanoid biosynthetic process, ferric iron binding, tetrapyrrole binding, glucosidase activity, iron ion binding, serine-type endopeptidase activity, and other molecular functions. The DEPs were significantly enriched in the molecular functions such as ferrous iron binding, glucosidase activity, iron ion binding, serine-type endopeptidase activity, and the localised proteins such as photosystem II, photosystem, extracellular region, photosystem I, and anchored component of membrane. DEPs were mainly enriched in "phenylpropane metabolism" (biological process) (Figure 4A), "photosynthesis system" (cellular com-

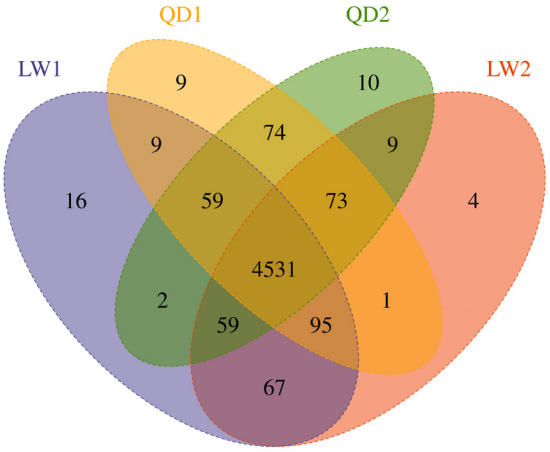

Figure 1. Venn diagram of proteins identified between all sample groups

In order to analyse the overlap of protein identifications between groups of samples, a Venn diagram was used to analyse the overlap of identifications between different groups; different colours show the overlap of identified proteins and protein sets between all sample groups, respectively; 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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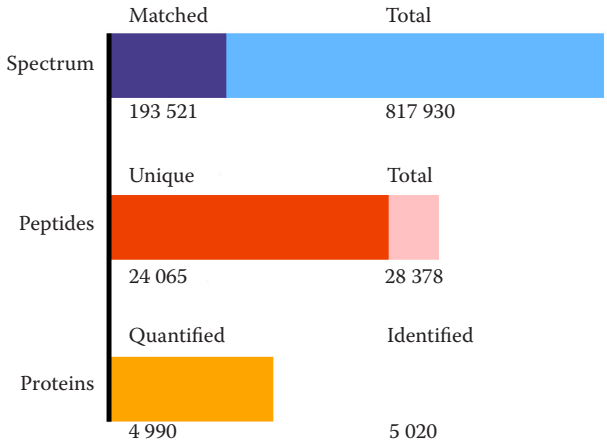

Figure 2. Histogram of identification and quantification results statistics

Total spectrum – total number of secondary spectra; matched spectrum – total number of matched spectra in the database; peptides – total number of peptides; unique peptides – total number of unique peptides; identified proteins – number of identified proteins; quantified proteins – number of quantifiable proteins; identified proteins – number of identified proteins; quantified proteins – number of quantifiable proteins, proteins with quantitative information in more than half of the biological replicates in at least one group

ponent) (Figure 5A), and "iron ion binding" (molecular function) (Figure 6A). This result suggests that protein expression changes in alfalfa roots during overwintering are closely related to int phenylpropanoidracellular iron ion metabolism, secondary metabolite synthesis, and photosynthesis-related processes. In particular, the significant enrichment of the phenylpropanoid metabolic pathway suggests that this pathway may play an important role in alfalfa frost tolerance. The biosynthesis-related entry (P = 3.2e-6) had an enrichment factor of 0.38, which was significantly higher than the other pathways. Thiamine (vitamin B1) plays a significant role in the cold acclimation of alfalfa, which can enhance antioxidant capacity and energy metabolism, help plants resist low-temperature stress, and maintain cell stability and physiological activity. Studies have shown that vitamin B1 and thiamine pyrophosphate (TPP) are important stress response molecules, which can alleviate the damage of oxidative stress under cold and other stress conditions.

During the overwintering adaptation process of *Medicago sativa* Gannong No.9, DEPs are significantly enriched in three major GO categories:

Biological process category (Figure 4B): photosynthetic dark reaction, reductive pentose phosphate cycle, and carbon fixation show an enrichment factor as high as 0.75 with extremely significant P-values ($\approx 10^{-15}$). These are accompanied by terms such as metal ion transport/homeostasis, regulation of stomatal movement, and defence response. This suggests that the plants ensure photosynthetic carbon supply and redox balance under low temperatures by enhancing the Calvin cycle, stably regulating divalent metal ions (Fe^{2+}/Mg^{2+}), and optimising stomatal behaviour.

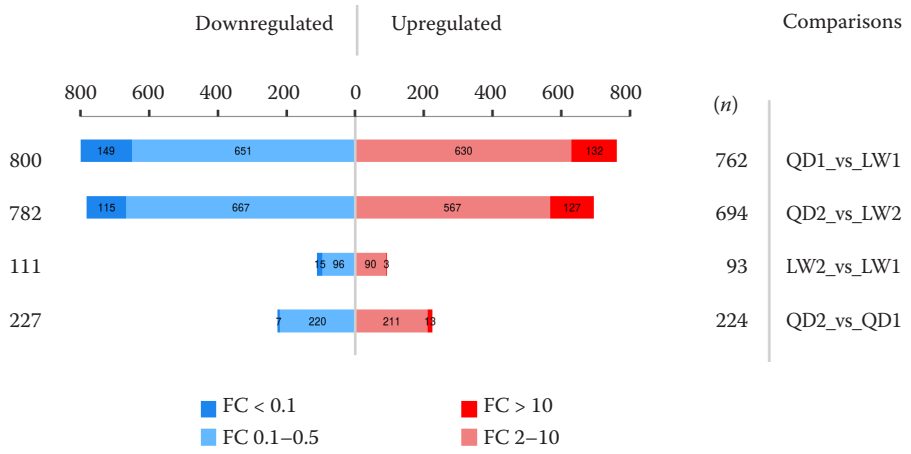

Figure 3. Histogram of quantitative protein difference results

Comparisons – differential comparison groups; upregulated – upregulated differentially expressed proteins; downregulated – downregulated expressed proteins; 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); FC – fold change

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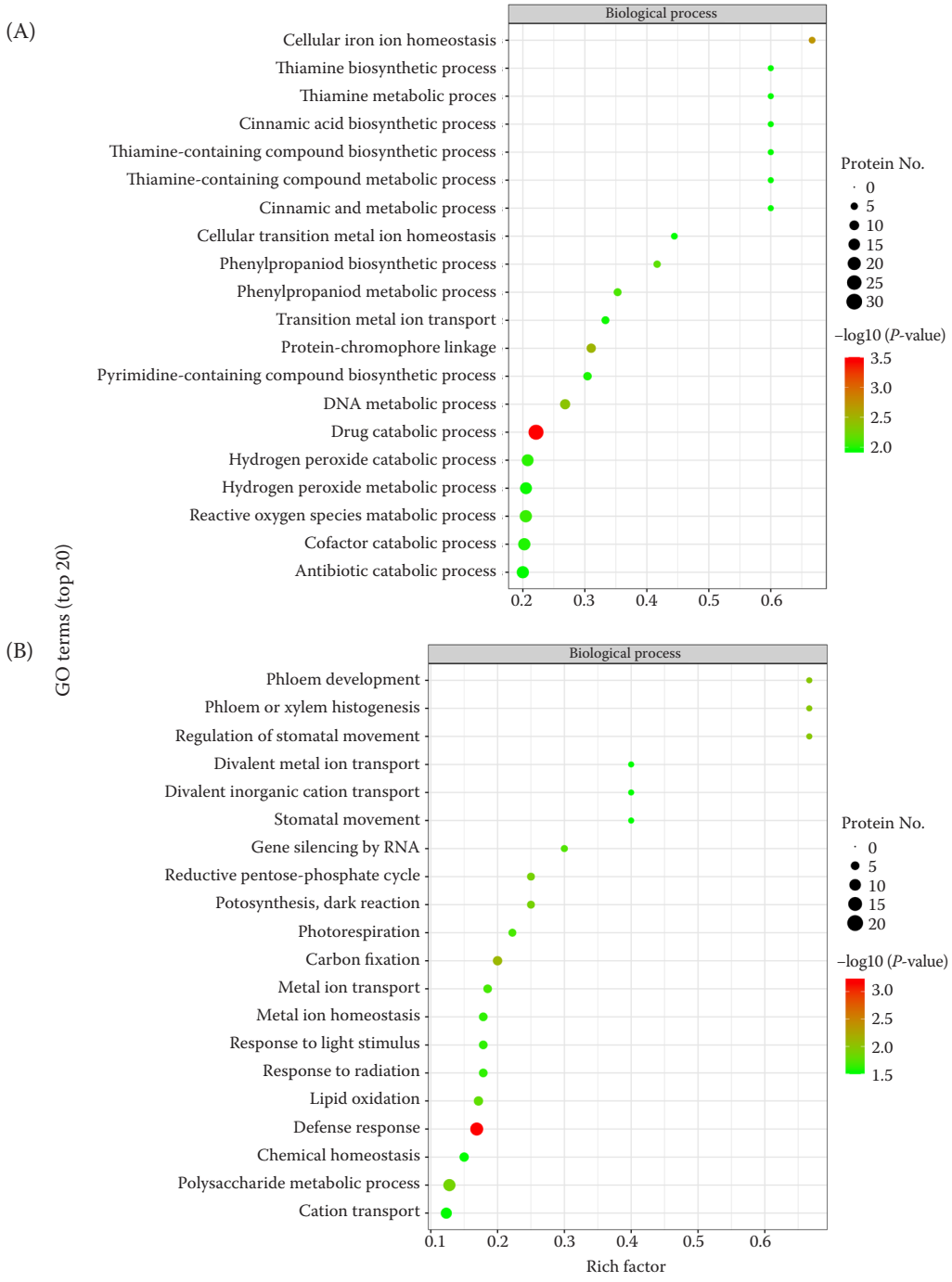

Figure 4. Bubble diagram of gene ontology (GO) functional enrichment under the classification of biological processes in the QD2_vs_QD1 group (A) and LW2_vs_LW1 group (B)

The horizontal coordinate in the figure is the enrichment factor (rich factor ≤ 1), which indicates the ratio of the number of differentially expressed proteins annotated to a GO functional category to the number of all identified proteins annotated to that GO functional category; the bubble size indicates the number of differentially expressed proteins under each GO functional category, and the bubble colour indicates the enrichment significance of a GO functional category, i.e., the *P*-value was calculated based on the Fisher's exact test, and the colour gradient represents the size of the *P*-value (taken as –log10), and the closer the colour is to the red colour represents the smaller the *P*-value corresponding to the higher the significance level of the GO functional category enrichment; LW1 – the Gannong No.9 samples before overwintering; LW2 – the Gannong No.9 samples after overwintering; QD1 – the Qingda No.1 samples before overwintering; QD2 – the Qingda No.1 samples after overwintering

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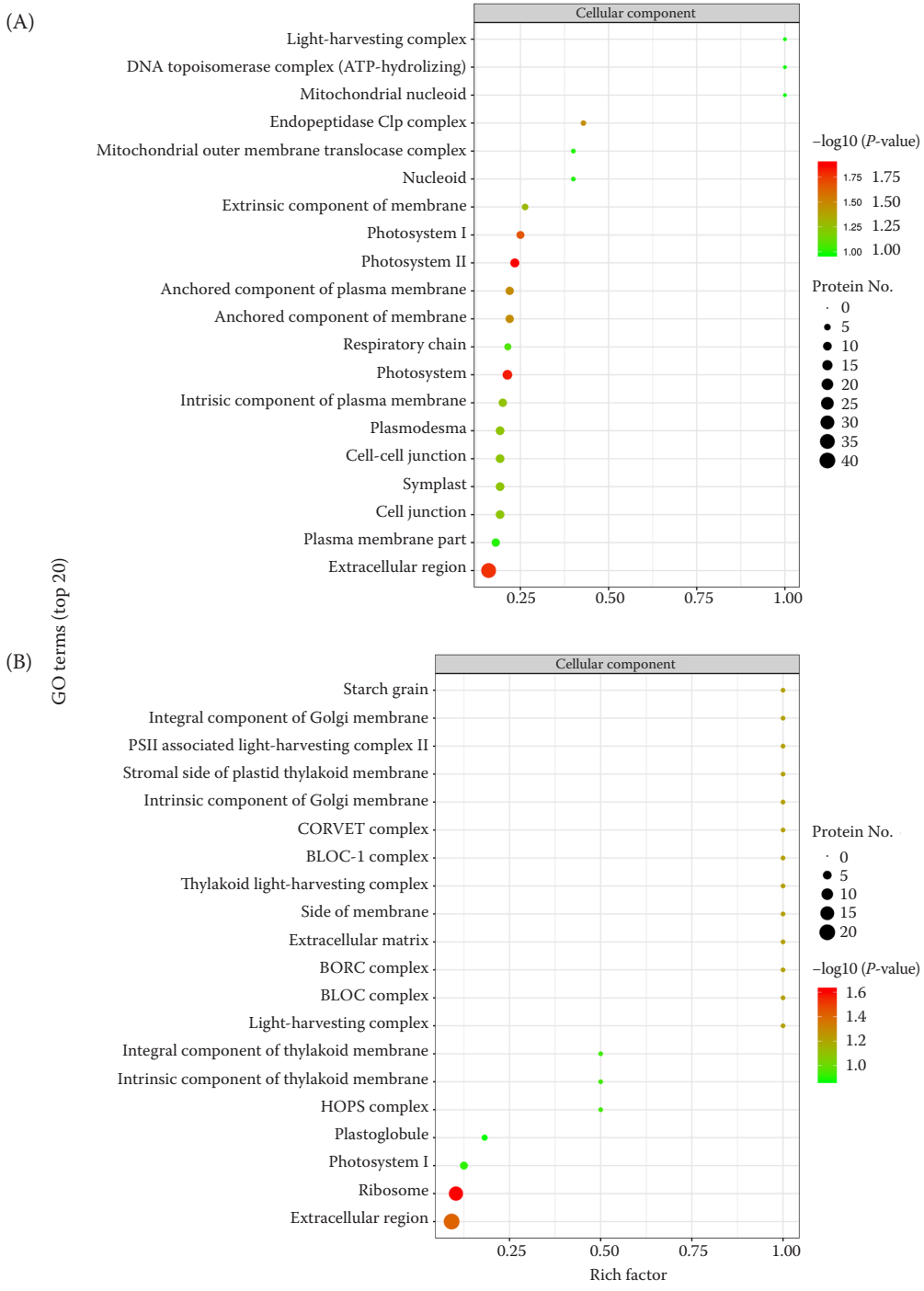

Figure 5. Bubble map of gene ontology (GO) functional enrichment under cellular component categorisation for QD2_vs_QD1group (A) and LW2_vs_LW1 group (B)

The horizontal coordinate in the figure is the enrichment factor (rich factor ≤ 1), which indicates the ratio of the number of differentially expressed proteins annotated to a GO functional category to the number of all identified proteins annotated to that GO functional category; the bubble size indicates the number of differentially expressed proteins under each GO functional category, and the bubble colour indicates the enrichment significance of a GO functional category, i.e., the *P*-value was calculated based on the Fisher's exact test, and the colour gradient represents the size of the *P*-value (taken as –log10), and the closer the colour is to the red colour represents the smaller the *P*-value corresponding to the higher the significance level of the GO functional category enrichment; LW1 – the Gannong No.9 samples before overwintering; LW2 – the Gannong No.9 samples after overwintering; QD1 – the Qingda No.1 samples before overwintering; QD2 – the Qingda No.1 samples after overwintering

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Figure 6. Bubble map of the gene ontology (GO) function enrichment under molecular function categorisation in QD2_vs_QD1 group (A) and LW2_vs_LW1 group (B)

The horizontal coordinate in the figure is the enrichment factor (rich factor ≤ 1), which indicates the ratio of the number of differentially expressed proteins annotated to a GO functional category to the number of all identified proteins annotated to that GO functional category; the bubble size indicates the number of differentially expressed proteins under each GO functional category, and the bubble colour indicates the enrichment significance of a GO functional category, i.e., the *P*-value was calculated based on the Fisher's exact test, and the colour gradient represents the size of the *P*-value (taken as –log10), and the closer the colour is to the red colour represents the smaller the *P*-value corresponding to the higher the significance level of the GO functional category enrichment; LW1 – the Gannong No.9 samples before overwintering; LW2 – the Gannong No.9 samples after overwintering; QD1 – the Qingda No.1 samples before overwintering; QD2 – the Qingda No.1 samples after overwintering

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Cellular component category (Figure 5B): enrichment is prominent in terms like PSII-associated light-harvesting complex II, thylakoid membrane integral component, and starch granule, indicating the coordinated remodelling of photosystem structure and temporary carbon storage. Meanwhile, extracellular region and extracellular matrix are also significantly recruited, implying that structural adjustments of the cell wall-membrane system are involved in low-temperature signal transduction and mechanical protection.

Molecular function category (Figure 6B): ribulose-1,5-bisphosphate carboxylase activity, tetrapyrrole binding, and β-amylase activity rank at the top ($P < 10^{-6}$), directly related to Rubisco catalytic efficiency, the stability of chlorophyll-heme prosthetic groups, and energy supply through starch degradation. In addition, defence-related functions such as chitinase activity and carbohydrate binding are synchronously upregulated, providing substrates and signals for osmotic adjustment, reactive oxygen species scavenging, and pathogen defence.

KEGG pathway analysis. The pathway map information annotated to each differential protein and its position in the pathway was demonstrated by KEGG pathway maps (Figure 7), showing the visualisation of one of the pathways, KeggEnrich-top1. KEGG pathway enrichment analysis revealed that the phenylpropanoid biosynthesis pathway (ko00940) was significantly enriched with a P value of 2.1×10^{-5}. Key enzymes in this pathway, such as phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and chalcone isomerase (CHI), were significantly up-regulated in the frost-tolerant variety

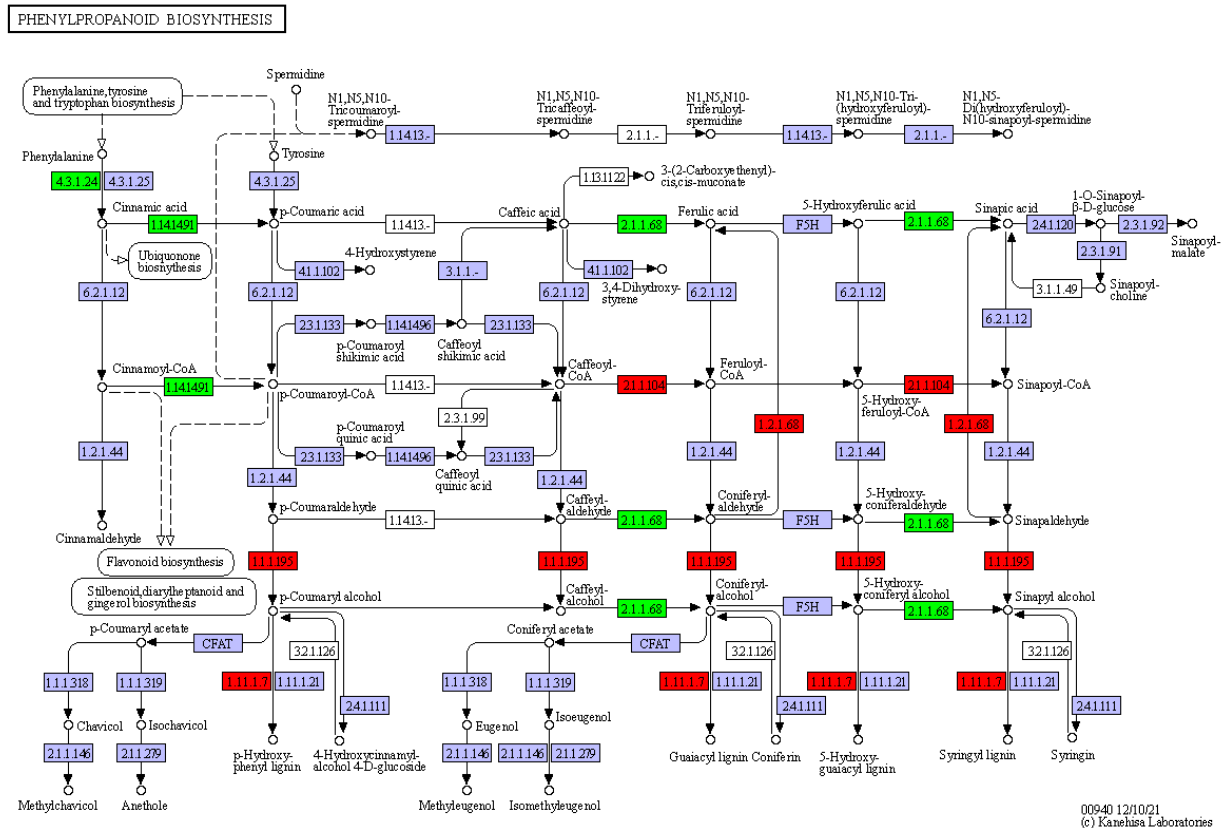

Figure 7. Example of KEGG-top1 pathway map for QD2_vs_QD1 group

Pathway names are shown in the upper left corner; boxes in red indicate that proteins are differentially up-regulated, boxes in green indicate that proteins that are differentially down-regulated, and boxes in blue indicate that multiple proteins are both up-regulated and down-regulated; small circles represent small metabolites, and large circles represent other pathways; light green boxes are species-specific proteins, light purple boxes are indistinguishable from species-specific proteins, and KEGG default disease-related proteins are in red; KEGG – Kyoto Encyclopedia of Genes and Genomes; QD1 – the Qingda No.1 samples before overwintering; QD2 – the Qingda No.1 samples after overwintering

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Qingda 1. These enzymes play a key role in the phenylpropanoid metabolic pathway by catalysing a series of reactions to generate secondary metabolites such as lignin and flavonoids. The accumulation of lignin can enhance the strength of the cell wall and improve the mechanical stability of the cell, thus resisting the cell damage caused by low temperature; flavonoids have an antioxidant effect, which can scavenge reactive oxygen species (ROS) and reduce the damage caused by oxidative stress on the cell. In addition, the "photosynthesis-antenna protein" and "oxidative phosphorylation" pathways were up-regulated in frost-tolerant varieties (Figure 8A), suggesting that these pathways may support low-temperature accli-

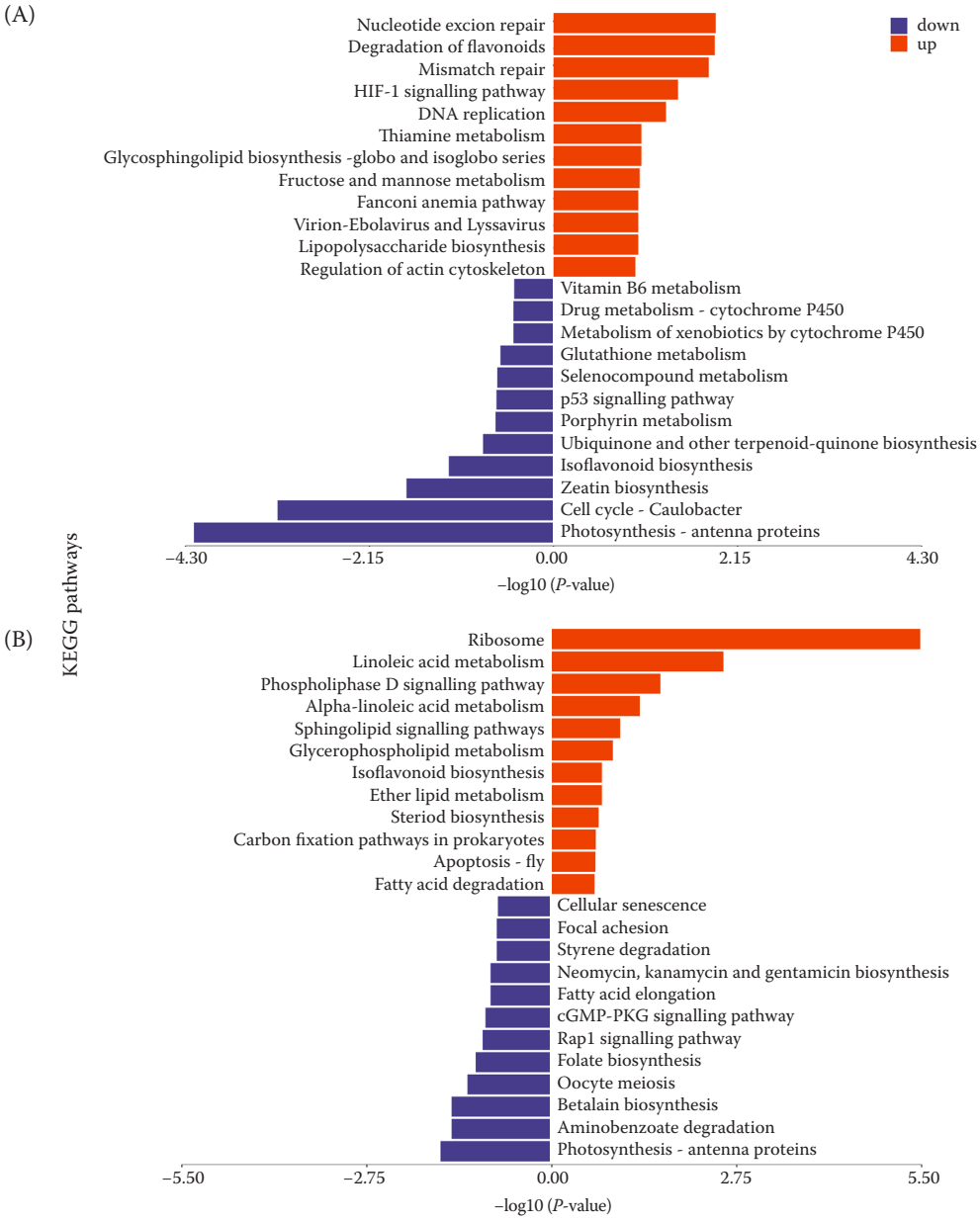

Figure 8. Pathway enrichment butterfly plots of up- and down-regulated differentially expressed proteins in the QD2_vs_QD1 group (A) and LW2_vs_LW1 group (B)

Horizontal coordinates are *P*-value values from Fisher's exact test (taken as logarithms with a base of 10), and vertical coordinates indicate pathway names; pathways involved in up- and down-regulated proteins are indicated by red (right) and blue (left) bars; KEGG – Kyoto Encyclopedia of Genes and Genomes; LW1 – the Gannong No.9 samples before overwintering; LW2 – the Gannong No.9 samples after overwintering; QD1 – the Qingda No.1 samples before overwintering; QD2 – the Qingda No.1 samples after overwintering

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matisation of plants during overwintering by maintaining light-energy utilisation efficiency and energy metabolism. During the overwintering adaptation of *Medicago sativa* Gannong No.9 (Figure 8B), differential proteins are significantly enriched in pathways such as photosynthesis-antenna proteins, carbon fixation pathway, fatty acid degradation/elongation, α-linolenic acid and linoleic acid metabolism, and glycerophospholipid/sphingolipid signalling (–log10*P* > 5.5). This indicates that it synergistically maintains membrane fluidity and energy homeostasis by enhancing photosynthetic light-harvesting and carbon assimilation efficiency, accelerating membrane lipid remodelling and reactive oxygen species scavenging, and regulating lipid signal transduction. Meanwhile, the upregulation of the flavonoid biosynthesis pathway suggests that the accumulation of secondary metabolites is involved in low-temperature defence, and the enrichment of the ribosome pathway ensures protein synthesis capacity under low temperatures, providing key metabolic support for alfalfa overwintering in cold and arid regions.

Protein interaction network analysis. One of the most important ways for proteins to fulfil their functions is to interact with other proteins and play biological regulatory roles through interprotein mediated pathways, or by forming complexes. Therefore, it is of great significance to study protein-protein interaction (PPI). In this study, the PPI network of DEPs was constructed based on the STRING database. The results showed (Figure S1 in Electronic Supplementary Material (ESM)) that DEPs formed multiple functional modules, among which phenylpropanoid metabolism-related proteins (e.g., PAL, C4H) had high connectivity, suggesting that these proteins may play a central regulatory role in freezing tolerance. Proteins with high connectivity usually have important regulatory functions in the cell, and they may form a complex regulatory network to coordinate multiple biological processes through interactions with other proteins. Based on the principle of topology identification, the proteins with high aggregation degree in the interaction network diagram were classified into different clusters, and each type of cluster was functionally categorised (Figure S2 in ESM). The results of this analysis not only revealed the interactions among DEPs but also provided important clues for further investigation of their co-regulatory mechanisms in freezing tolerance. Generally speaking, the greater the connectivity, the greater the perturbation of the whole system when the protein is changed, and it is more likely to be the key to maintaining the balance and stability of the system, making it a candidate protein for subsequent focused studies. In the PPI interaction network, highly aggregated proteins may have the same or similar functions and perform biological functions through synergistic interactions. Screening of key differentially expressed proteins: key differentially expressed proteins related to freezing tolerance were screened, including PAL, C4H and CHI. These proteins play important roles in the phenylpropanoid biosynthesis pathway and may enhance the freezing tolerance of alfalfa by regulating the synthesis of secondary metabolites.

DISSCUSION

Critical role of the phenylpropanoid biosynthetic pathway. The significant enrichment of the phenylpropanoid biosynthetic pathway in alfalfa freezing tolerance is one of the important findings of this study. This pathway occupies a central position in plant secondary metabolism, and its products include important secondary metabolites such as lignin and flavonoids (Shi et al. 2022). Accumulation of lignin enhances the strength and rigidity of the cell wall, thereby increasing the mechanical resistance of plants to low-temperature stress (Zhao et al. 2022). Flavonoids, on the other hand, play an important role in scavenging reactive oxygen species (ROS) due to their antioxidant properties and attenuate oxidative damage induced by low temperature stress (Feng et al. 2023). In this study, key enzymes in the phenylpropanoid biosynthesis pathway, such as PAL, C4H, and CHI, were significantly up-regulated in the frost-tolerant variety QD1, which further supports the critical role of phenylpropanoid metabolism in freezing tolerance of alfalfa. The up-regulation of these enzymes may promote the synthesis of lignin and flavonoids, which enhance the cell wall strength and antioxidant capacity, and help the plant to better adapt to the low-temperature environment.

Enrichment of photosynthesis-related proteins. Photosynthesis is a fundamental process in plant growth and development, and its efficiency directly affects the energy acquisition and material accumulation of plants. In this study, the photosynthesis-antenna protein pathway was significantly enriched in frost-tolerant varieties, suggesting that frost-tolerant varieties may regulate the expression of photosynthesis-related proteins during overwin-

https://doi.org/10.17221/27/2025-CJGPB

tering to maintain a high efficiency of light energy utilisation. Photosynthesis-antenna proteins play a key role in the capture and transfer of light energy (Levin & Schuster 2023), and their up-regulation may help plants to absorb and utilise light energy more efficiently under low temperatures, thus maintaining normal growth and metabolic activities. In addition, the efficient conduct of photosynthesis provides plants with sufficient energy to support their survival and recovery under low-temperature stress (Gusain et al. 2023). This sustained photosynthetic capacity is vital not only for basic carbon fixation but also for fuelling the high-energy demands of cold acclimation processes, such as the synthesis of cryoprotective compounds (e.g., osmolytes, antioxidants like flavonoids) and the repair of cold-induced cellular damage (Hüner et al. 2013; Gururani et al. 2015). This finding suggests that the regulation of the expression of photosynthesis-related proteins may be one of the important mechanisms of freezing tolerance in alfalfa, which deserves further in-depth study.

Synergistic regulation of protein-protein interaction networks. PPI network analysis revealed complex interactions among the DEPs identified in Qingda No.1 (Wimalagunasekara et al. 2022), offering a systems-level view of frost tolerance mechanisms. The network showed DEPs forming distinct functional modules. Notably, key phenylpropanoid enzymes (PAL, C4H) exhibited high connectivity (degree centrality), suggesting they act as central hubs within the network. Proteins with high connectivity often play critical regulatory roles, integrating signals and coordinating metabolic flux through interactions with numerous partners (Vranová et al. 2012; Vinayagam et al. 2013). In this context, PAL and C4H likely serve as pivotal nodes orchestrating the flux through the stress-responsive phenylpropanoid pathway. Furthermore, clustering analysis based on network topology grouped highly interconnected proteins into functional modules (e.g., phenylpropanoid biosynthesis, photosynthesis-related, stress response), highlighting the synergistic co-regulation of proteins within shared biological processes. This network architecture implies that frost tolerance in alfalfa is not governed by isolated proteins but emerges from the concerted action and functional integration of interconnected modules, facilitating coordinated responses across signalling and metabolic pathways.

Rationale for including Gannong No.9. While the frost-sensitive variety Gannong No.9 exhibited fewer protein expression changes (204 DEPs vs 451 in Qingda No.1) during overwintering, its inclusion served a critical comparative purpose. Firstly, it established a baseline of protein dynamics in a genotype lacking strong inherent frost tolerance. Secondly, and more importantly, contrasting the DEP profiles between the tolerant and sensitive varieties allowed us to pinpoint changes specifically associated with the enhanced cold acclimation capacity of Qingda No.1. The significant enrichment of pathways like phenylpropanoid biosynthesis and photosynthesis-antenna proteins predominantly or exclusively in the tolerant variety underscores their likely functional relevance to frost tolerance. This comparative proteomics approach strengthens the identification of key candidate mechanisms that distinguish the tolerant phenotype and would be less evident if studying only a single genotype.

CONCLUSION

In this study, the protein expression profiles of alfalfa roots of frost-tolerant variety Qingda No.1 and frost-sensitive variety Gannong No.9 were systematically analysed before and after overwintering by 4D-Label-free quantitative proteomics technology. A total of 5 020 proteins were identified, including 1 476 DEPs, indicating that significant protein expression changes occurred in alfalfa roots during overwintering. These changes reflected the adaptive response mechanism of plants under low temperature stress, revealing a unique mode of regulation at the molecular level in frost-tolerant varieties. Future studies can further validate the functions of these key proteins by transgenic or gene editing techniques and delve into their specific mechanisms of action in plant freezing tolerance. In addition, combined analysis with multi-omics techniques (e.g., metabolomics, transcriptomics) will help to more comprehensively analyse the molecular regulatory network of frost tolerance in alfalfa, and provide strong support for breeding more cold-tolerant alfalfa varieties.

Acknowledgements. We are grateful to Prof. Guozhu Yang for providing the experimental materials, to researcher Ting Gao for carefully revising the paper, and to the members of the group for helping each other in the joint research.

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Received: April 11, 2025
Accepted: August 4, 2025
Published online: September 17, 2025