

Differential expression analysis of genes in the root crown of alfalfa under low-temperature stress

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Abstract

Background: Alfalfa (*Medicago sativa*) is a perennial forage crop widely cultivated in northern China. The root crown is an important storage organ of alfalfa, especially in the wintering process, as it is closely related to winter hardiness. However, the molecular mechanism underlying the winter hardiness of the alfalfa root crown remains unclear. To investigate these gaps in knowledge, the RNA sequencing (RNA-Seq) technology was used to identify critical genes related to winter hardiness.

Results: In this study, the winter survival rate of the Lomgmu 806 variety was approximately 3.68-fold higher than that of the Sardi variety. We sequenced the transcriptomes of the root crown of the two alfalfa varieties. Among the 57,712 unigenes identified, 2,299 differentially expressed genes (DEGs) were upregulated, and 2,143 DEGs were downregulated. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotations showed that 1,159 unigenes were mainly annotated in 116 pathways. Seven DEGs belonging to the “plant hormone signalling transduction” pathway, the “peroxisome” pathway and transcription factor family (MYB, B3, AP2/ERF, and WRKY) and involved in alfalfa winter hardiness were identified. As a result, the expression patterns of seven DEGs were verified by real-time quantitative PCR (RT-qPCR) analyses, which verified the reliability of the RNA-Seq analyses.

Conclusions: The RNA-Seq data revealed the gene regulation response of alfalfa to low-temperature stress, which provides a valuable resource for the further identification and functional analysis of candidate genes related to winter hardiness in alfalfa. Furthermore, these data provide references for future in-depth studies of winter hardiness mechanisms in alfalfa.

Background

Alfalfa (*Medicago sativa* L.) is one of the most popular perennial legume forages and is known as the “king of the forages”, due to its high yield, rich nutritional value and palatability. This forage is not only the most widely cultivated in the world [1, 2] but also the most widely distributed in China [3, 4]. Alfalfa varieties introduced from abroad have the advantages of good quality and high yield, but their winter survival rates or spring returning green rates are low, which seriously limits their industrial development in northern China [2, 4]. In fact, there are also problems in the cultivation of alfalfa varieties that cannot safely survive under harsh winter conditions in the midwestern United States, Canada, Italy, Russia and other countries [5]. Due to the lack of winter-hardy varieties in some cold regions, understanding the molecular mechanism of low-temperature stress signal transduction pathways is very important for breeders aiming to improve the production of alfalfa.

Plants can survive safely in winter and enhance freezing resistance through a low-temperature adaptation process [6]. Previous studies on the mechanisms of plant responses to low temperature have mainly focused on plant physiology, phytohormones, and changes in low temperature-induced gene expression by regulating transcription factors (TFs) [7]. Plants undergo various changes under low-temperature stress, and the key response to low-temperature stress depends on the activation of

molecular networks involved in stress signal transduction pathways and the expression of specific sets of stress-related genes [8]. At present, the most widely studied C-repeat-binding factor (CBF) signalling pathway including *CBF1*, *CBF2*, and *CBF3*, and CBF TFs regulates the expression of COLD-RESPONSIVE (*COR*) functional genes, which are components of the low-temperature signal transduction pathway, thereby improving the cold tolerance of plants [9, 10]. A number of studies have shown that the CBF signalling pathway can improve the low-temperature tolerance of various plants, such as *Arabidopsis thaliana* [11, 12], *Triticum aestivum* [13], *Glycine max* [14], and *Malus domestica* [15]. The overexpression of *MtCBF3* cloned from *Medicago truncatula* induces *COR* gene expression and enhances the low-temperature tolerance of transgenic plants [16]. Recent studies have shown that CBFs may play an important role in regulating the low-temperature tolerance of *M. sativa* [17, 18]. This suggests that the regulation of the CBF pathway may have a potential role in improving the low-temperature tolerance of alfalfa, although it may not be the only method by which low-temperature tolerance is improved.

The plant hormone signal transduction pathway plays a central role in the plant low-temperature stress response and controls the CBF-dependent and CBF-independent pathways [19]. For example, abscisic acid (ABA) is an important abiotic stress-regulating hormone that plays an important role in abiotic stress signalling. Furthermore, the previous assumption suggested that ABA did not affect the expression of the *CBF* gene. Therefore, it has been suggested that the low-temperature response via ABA control is independent of CBF regulation [20]. However, a recent study suggested that ABA may affect the expression of the *COR* genes by regulating CBF transcription and may play an important role in plant low-temperature stress in plants [21]. Moreover, some functional genes also play an important role in protecting plants from cold and/or freezing stress. These genes are involved in signal transduction and transcriptional regulation. For example, TFs play a functional role by regulating the expression or status of stress-related genes. At present, some TFs have been identified, such as members of the MYB, B3, AP2/ERF, and WRKY families, and serve as important regulators of abiotic stress responses in plants [22–25]. In addition, in the plant response to abiotic stress, reactive oxygen species (ROS), which are harmful to cell membranes, proteins, and biological macromolecules, accumulate in plant cells destroying the homeostasis of the organism and causing serious damage to plants [26]. Therefore, the exact roles of the response factors of alfalfa in the wintering period, including transcriptional regulation, the plant hormone signal transduction pathway, and ROS scavenging, remain to be elucidated, and their functions should be studied in detail.

Recently, transcriptome sequencing has become essential for functional gene annotation, novel gene discovery, differential gene expression, and molecular marker research [27–29]. RNA sequencing (RNA-Seq) enables the study of gene expression at the transcriptome level and identifies genes involved in plant-specific biological processes [30–32]. For example, with the introduction of a new generation of RNA-Seq, transcriptome sequencing technology has been used to identify low-temperature stress response genes in *Ipomoea batatas* [33], *Populus tomentosa* [34], *Capsicum annuum* [35], and *Magnolia wufengensis* [36]. Moreover, some recent studies have reported that the use of transcriptome sequencing technology to analyze the expression of genes in the seedlings [7], taproots [37], and crown buds [38] of alfalfa, which provides valuable resources for functional genomics research on alfalfa cold tolerance.

However, the understanding of genetic regulation information regarding low-temperature stress remains limited. Therefore, in this study, alfalfa root crowns were collected during the wintering period for RNA-Seq to obtain genetic information on the response of alfalfa to low-temperature stress. Moreover, the specific molecular mechanisms underlying the low-temperature resistance of the alfalfa root crown during the wintering period remain unclear. Therefore, the systematic analysis of the low-temperature resistance of the Longmu 806 and Sardi varieties during winter not only aided in the understanding of the response mechanism of the alfalfa root crown during wintering but also provided a reference for functional genomics research on low-temperature stress in alfalfa.

Results

Winter survival

In the spring of 2019, Longmu 806 alfalfa exhibited the larger returning green plants than Sardi alfalfa in the experimental field (Fig. 1a). In September 2018, there was a significant difference in plant height (PH) between Longmu 806 and Sardi. The PH of Sardi alfalfa was significantly higher than that of Longmu 806 alfalfa ($P < 0.05$; Fig. 1b). The winter survival rate of Longmu 806 (98.09%) was approximately 3.68-fold higher than that of Sardi ($P < 0.05$, Fig. 1c).

De novo transcriptome assembly

Total RNA extracted from the root crown of the Longmu 806 and Sardi varieties during the wintering stage were constructed into six libraries for high-throughput sequencing. To ensure the quality of the information analysis, the original data were filtered. Among the raw reads, low-quality reads, reads containing adapter sequences, and reads containing low-quality bases were discarded, and 459,865,666 clean reads with a total of 68,515,729,607 nucleotides (nt) were generated from the six sequencing libraries. A total of 114,567 unigenes with an N50 value of 1,092 nt were assembled. The maximum length, mean length and minimum length were 15,687 nt, 740 nt and 201 nt, respectively (Fig. 2).

Functional annotation

A total of 57,712 unigenes (50.37% of the total 114,563 unigenes) were annotated against the non-redundant protein (Nr), A manually annotated and reviewed protein sequence database (SwissProt), Protein Family (Pfam), Clusters of Orthologous Groups of Proteins (COG), Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases using BLASTX (E-value $< 1 \times 10^{-5}$). Among the unigenes, 56,044 (48.92%), 34,764 (30.34%), 30,449 (26.58%), 6,670 (5.82%), 41,457 (36.19%), and 21,483 (18.75%) unigenes were annotated to the Nr, SwissProt, Pfam, COG, GO and KEGG databases, respectively (Fig. 3a). According to the Nr database, 5,933 (10.59%) of the unigenes ($1 \times 10^{-10} < \text{E-value} \leq 1 \times 10^{-5}$) showed homology, 14,978 (26.73%) of the unigenes ($1 \times 10^{-30} < \text{E-value} \leq 1 \times 10^{-10}$) showed high homology, and the remaining 35,133 (62.68%) of the unigenes ($\text{E-value} \leq 1 \times 10^{-30}$) had very high homology (Fig. 3b). Top ten BLASTX hits of the species distribution (Fig. 3c), 43,813 (78.28%), 3,482 (6.22%), 2,380 (4.25%), 1,140 (2.04%), 702 (1.25%), 548 (0.98%) and 388 (0.69%) unigenes were

homologous with *M. truncatula*, *Trifolium pratense*, *Trifolium subterraneum*, *Cicer arietinum*, *Quercus suber*, *Cajanus cajan* and *G. max*, respectively.

Identification and analysis of differentially expressed genes (DEGs)

With the Nr annotation results, BLAST2GO (2.5.0) software was used to perform GO functional annotations of unigenes. A total of 4,442 DEGs were assigned to one or more ontologies by the standard of $|\log_2 \text{fold change}| > 1$ and $P\text{-value} < 0.05$ (Additional file 1: Table S1; Fig. 4). There were 2,299 upregulated unigenes, and the other 2,143 unigenes were downregulated (Longmu 806 vs Sardi). These DEGs were used for the subsequent analyses.

To better understand the function of genes differentially expressed in alfalfa during the wintering period, GO functions were used to classify the function of DEGs (Additional file 2: Table S2). A total of 4,442 DEGs were summarized in three main functional categories: biological process, cellular component, and molecular function (Fig. 5). In the biological process category, “cellular process” (GO:0009987), “metabolic process” (GO:0008152) and “biological regulation” (GO:0065007) were the most common terms and contained 728, 702 and 260 unigenes, respectively. For “cellular process”, there were 326 upregulated unigenes and 366 downregulated unigenes. For “metabolic process”, there were 348 upregulated unigenes and 354 downregulated unigenes. For “biological regulation”, there were 148 upregulated unigenes and 112 downregulated unigenes. In the cellular component category, most of genes were classified into “cell” (207 upregulated unigenes and 306 downregulated unigenes), “cell part” (204 upregulated unigenes and 301 downregulated unigenes), and “organelle” (141 upregulated unigenes and 203 downregulated unigenes). In the molecular function category, genes were largely enriched in the subcategories “catalytic activity” (576 upregulated unigenes and 574 downregulated unigenes) and “binding” (593 upregulated unigenes and 516 downregulated unigenes).

KEGG pathway enrichment analysis

KEGG analysis of DEGs was performed using KOBAS (v2.1), and a total of 1,159 unigenes were assigned to 116 KEGG pathways (Additional file 3: Table S3). The top ten KEGG pathways with the highest representation of DEGs were “Starch and sucrose metabolism” (ko00500), “protein processing in endoplasmic reticulum” (ko04141), “plant-pathogen interaction” (ko04626), “phenylpropanoid biosynthesis” (ko00940), “plant hormone signal transduction” (ko04075), “ribosome” (ko03010), “MAPK signalling pathway-plant” (ko04016), “endocytosis” (ko04144), “glycolysis/gluconeogenesis” (ko00010), and “spliceosome” (ko03040). In the “plant hormone signal transduction” pathway (ko04075), DEGs associated with the ABA and ethylene biosynthetic pathways (including serine/threonine-protein kinase 2, SnRK2, and ethylene insensitive 3, EIN3) were upregulated. In the “peroxisome” pathway (ko04146), DEGs associated with superoxide dismutase X1 (SOD1) was upregulated. These annotations provided valuable resources for studying the specific functions and pathways of the alfalfa genes.

Stress response of TFs to low-temperature stress

A total of 34 TF families containing 1,364 unigenes were identified (Additional file 4, Table S4; Fig. 6). Based on the RNA-Seq data, the top six TF families with the highest representation were MYB (186), B3 (145), AP2/ERF (106), C2C2 (92), WRKY (88), and NAC (82). Many of these TF families have been reported to play an important role in plant responses to abiotic stresses, including low-temperature stress [39]. Among the MYB family members detected in our data, 86 were upregulated under low-temperature stress, and 67 were downregulated. Among the AP2/ERF family members detected in our data, 43 were upregulated. Moreover, the expression levels of most unigenes were upregulated in the WRKY family under low-temperature stress, and 45 unigenes in the B3 TF family were upregulated. In the C2C2, bHLH, bZIP, LOB, SRS, E2F/DP, GeBP and BBR-BPC TF families, the number of TFs with upregulated expression was less than the number of TFs with downregulated expression. Furthermore, the number of TFs with upregulated and downregulated expression was the same in the AP2/ERF, NAC, NF-Y, Nin-like, and NF-X1 families.

Validation of DEGs data by real-time quantitative PCR (RT-qPCR) analysis

To verify the reliability of the transcriptome sequencing data, differences in the expression of ten genes were detected using RT-qPCR, and these genes were well characterized by the NCBI Nr database (Fig. 7a). These genes included ethylene (*EIN3*), ABA (*SnRK2*), auxin (auxin response factors *ARF*), and jasmonic acid (JA; Jasmonate zim-domain protein, *JAZ*) responsive regulatory genes in the plant hormone signalling pathway; MYB (transcription factor JAMYB, *JAMYB*), B3 (AP2/ERF and B3 domain-containing transcription factor RAV1, *RAV1*), AP2/ERF (ethylene-responsive element binding factor, *AP2/EREBP*), and WRKY (WRKY family transcription factor 65, *WRKY 65*) TF families; and the ROS transcript *SOD1* associated with the low-temperature stress response (Additional file 5: Table S5). The expression levels of TF genes in the Longmu 806 variety were upregulated, and the expression levels of seven TF genes (*EIN3*, *SnRK2*, *SOD1*, *JAMYB*, *AP2/EREBP*, *WRKY 65*, and *RAV1*) were significantly higher than those in the Sardi variety. Moreover, RT-qPCR showed a high correlation ($R^2=0.8016$, $P<0.05$; Fig. 7b) of fold change between the RNA-Seq analysis and RT-qPCR. The expression patterns in the PCR assay were generally consistent with the results of RNA-Seq.

Discussion

Low-temperature stress is one of the main abiotic stresses affecting plant growth and development and alfalfa yield [2]. Generally, low-temperature freezing damage is likely to occur under severe winter conditions, which could result in reduced forage yields in the following year and seriously affect forage production. Therefore, the ability for regrowth in early spring following severe winter conditions reflects the degree of cold resistance in forage [40]. In this study, we also found that Longmu 806 alfalfa had more returning green plants than Sardi alfalfa in the early spring (Fig. 1a). In addition, a recent study has indicated that the winter survival rate of a fall dormant alfalfa (Maverick) was markedly higher than that of a non-dormant alfalfa (CUF101), suggesting that fall dormant alfalfa has a stronger cold tolerance

than non-dormant alfalfa [37]. Similar results were obtained in this study. The winter survival rate of Longmu 806 (fall dormant alfalfa) was significantly higher than that of Sardi (non-dormant alfalfa; Fig. 1c). In summary, the winter hardiness of Longmu 806 was stronger than that of Sardi. Because the winter hardiness of the alfalfa variety is a complex trait, it is not only affected by the growth environment but also related to the comprehensive genetic regulation of the variety [41]. To our knowledge, previous studies have reported the use of RNA-Seq technology to analyze DEGs related to alfalfa leaves, taproots, and crown buds [37, 38, 42]. However, unlike previous studies, to enhance our understanding of the comprehensive genetic regulation mechanism of alfalfa low-temperature resistance in alfalfa, we focused on the expression patterns of low-temperature responsive genes in the root crown of alfalfa.

Hormones in alfalfa in response to low-temperature stress

Hormones are the initiation factors of winter hardiness gene expression, and hormone-mediated abiotic stress responses involve multiple mechanisms. In perennial plants, the phytohormones ABA, ethylene, JA and auxin play important roles in regulating plant growth to adapt to low-temperature stress [43]. ABA is an important stress-regulating hormone in plants under low-temperature stress. A recent study found that the levels of ABA increased in plants treated with low temperature, and ABA-dependent and ABA-independent pathways can regulate low-temperature-responsive genes [44]. *SnRK2* is a positive regulator of the ABA signalling pathway, and the role of the ABA-activated *SnRK2* protein kinase in low-temperature stress signalling has been reported in *Kandelia obovata* [45]. In this study, the RNA-Seq data showed that the *SnRK2* gene was significantly upregulated in the Longmu 806 variety but downregulated in the Sardi variety. This result suggested that Longmu 806 was more likely to accumulate ABA than Sardi. The ABA-binding receptor inactivates *PP2C* and *PP2C*-mediated dephosphorylation of the *SnRK2* kinase in *Arabidopsis*, which phosphorylates the basic leucine zipper (bZIP) TFs (*ABFs/AREBs*). *ABFs* bind to ABA-responsive promoter elements (ABREs) to activate the transcription of downstream ABA-responsive genes [46, 47]. Therefore, the overexpression of the *SnRK2* gene in Longmu 806 regulated the low-temperature stress response by inducing the expression of ABA-responsive genes. Similar results have also been found with the overexpression of *TaSnRK2.3* in *Arabidopsis*, which results in an improved root-system structure and significantly enhanced the tolerance of this species to freezing stress [48]. In addition, low-temperature stress and ABA synergistically (in *Vitis vinifera* buds) increased the expression of *CBF/DREB1* TFs (*VvCBF2*, *VvCBF3*, *VvCBF4*, and *VvCBF6*) [49], and increased the cold resistance of the Longmu 806 variety, indicating that Longmu 806 is more likely to resist complex low-temperature environments than Sardi.

EIN3 is involved in ethylene signal transduction, and ethylene signalling promotes the transcription of multiple ethylene response factor (*ERF*) genes, ultimately guiding growth and physiological responses to abiotic stress [50]. The RNA-Seq data revealed that the *EIN3* gene was differentially expressed between the Longmu 806 and Sardi varieties. The RT-qPCR analysis showed that the expression level of *EIN3* in the Longmu 806 variety was approximately 2.2-fold higher than that of Sardi variety. This indicated that Longmu 806 was more likely to accumulate ethylene than Sardi. *EIN3* promotes the transcription of a

variety of *ERF* genes that ultimately regulate the expression of downstream stress-responsive genes. For example, overexpressing the *CBF/DREB* genes in tobacco and tomato enhanced freezing tolerance by regulating the expression of the *COR* genes [51]. In addition, a recent study revealed several mutants (*etr1-1*, *ein4-1*, *ein2-5*, *ein3-1*, and *ein3 eil1*) in the ethylene signalling pathway, in which the *ein3 eil1* mutant induced the expression of *CBF* genes (*CBF1*, *CBF2*, and *CBF3*) under low-temperature stress and then enhanced the freezing tolerance of *Arabidopsis* [52]. Interestingly, high concentrations of ABA inhibit root growth in *Arabidopsis* by increasing ethylene accumulation [53]. Therefore, although the mechanisms underlying the complex cross talk between ABA, ethylene, and low-temperature signalling remain unclear, it appears likely that the upregulation of ABA-responsive genes also helps the low-temperature tolerance of ethylene-insensitive mutants. This may be one of the reasons for the stronger winter hardiness in the Longmu 806 variety than in the Sardi variety.

JA is a lipid phytohormone that plays an important role in plant defenses. Many studies have reported that JA is involved in plant tolerance to low-temperature stress, and increasing the JA content positively regulates the INDUCER OF CBF EXPRESSION (ICE-CBF) transcriptional pathway and ultimately improves cold tolerance [54]. The RNA-Seq data showed that the *JAZ* gene in Longmu 806 alfalfa was upregulated but the downregulation of the gene in Sardi alfalfa was not significant. This indicated that Longmu 806 alfalfa may reduce *JAZ* expression through ICE-CBF regulatory pathways, releasing CBF TFs to induce the expression of the *COR* genes and ultimately improving alfalfa cold resistance [54]. Similarly, the expression of the *ARF* gene in Longmu 806 was upregulated compared with that in Sardi. Shu *et al.* [55] also found that the transcription of the *ARF* gene in *M.sativa* was upregulated under cold and freezing stresses, which indicated that the conserved miRNAs might regulate the alfalfa low-temperature stress response by controlling the alfalfa developmental process. Although the *ARF* gene is not directly involved in the alfalfa root overwintering, it may affect the elongation and dormancy of alfalfa roots under low-temperature stress by regulating auxin [38]. Therefore, although the *ARF* and *JAZ* genes of Longmu 806 were upregulated, their exact roles under low-temperature stress remain to be elucidated, and their functions require further study.

The response of DEGs to low-temperature stress revealed the relationship between the “plant hormone signal transduction” pathway (ko04075), “MAPK signalling pathway-plant” pathway (ko04016), and winter hardiness. The DEGs associated with ABA and ethylene biosynthetic pathways (including *SnRK2* and *EIN3*) in Longmu 806 alfalfa were upregulated. Hormones, as signalling molecules, play very important roles in regulating *CBF* gene expression in response to cold and freezing stresses in *M. sativa* (cv. Zhaodong) [18]. In summary, it is likely that Longmu 806 alfalfa has stronger winter hardiness than Sardi alfalfa due to the high levels of accumulated plant hormones. Increased expression of the *SnRK2* and *EIN3* genes caused an increase in the ABA and ethylene contents and then induced the expression of *CBF* genes, which may be a reason for increased cold resistance in the Longmu 806 variety.

TFs involved in the low-temperature stress response

TFs play important roles in the response to abiotic (low temperature, salt, and drought) stresses and directly control the expression of specific sets of stress-responsive genes [56, 57]. Most TF families identified play a vital role in plant responses to low-temperature stress, including the MYB, AP2/ERF, B3, and WRKY families [22–25]. A recent study has reported that many TF subfamilies belong to the AP2/ERF family, such as the AP2 subfamily, DREB subfamily, ERF subfamily, RAV subfamily and others, and a high percentage were involved in the low-temperature (cold and/or freezing) stress response [58]. In this study, we identified that the DEGs related to the GO terms “cellular process”, “metabolic process”, “cell part”, “cell”, “organelle”, “nucleic acid binding transcription factor activity”, and “binding” were enriched. The *AP2/EREBP* gene of the AP2/ERF family was significantly upregulated in low-temperature stress responses of the Longmu 806 variety, and similar results were obtained in *M. truncatula*. In particular, the AP2/ERF TF family includes a tandem array of *Mt-ERF* genes (also called *MtCBF* genes) on chromosome 6 that functions in the response to cold and freezing stresses in *M. truncatula* [22]. This result verifies previous reports in *Camellia sinensis* that five *CsAP2/ERF* genes from each AP2/ERF family (AP2, ERF, DREB, and RAV subfamilies) responded to temperature stresses [59]. In brief, this result confirmed that *AP2/EREBP* TFs have a positive response to low-temperature stress. The *AP2/EREBP* gene was upregulated in the Longmu 806 variety compared to the Sardi variety, suggesting that Longmu 806 has enhanced cold resistance.

The MYB TFs have been shown to play positive roles in the abiotic stress signalling process [60]. In the MYB family, the *JAmyb* genes induced by high salinity, high osmotic stresses and ROS, indicating that *JAmyb* is responsible for the abiotic stress response [61]. The RT-qPCR analysis showed that the expression level of the TF *JAMYB* in the Longmu 806 variety was approximately 2.5-fold higher than that in the Sardi variety. This indicated that *JAMYB* may be a response factor to low-temperature stress, but its specific function still needs further study. In addition, previous studies have reported that the WRKY family is involved in the low-temperature stress response. For example, the expression of TFs in most WRKY families (*WRKY 30, 33, 41, 46, 48, 51, 53, 65, 70*) is upregulated in *Hevea brasiliensis* under low-temperature stress (24 h cold treatment at 4 °C) [62]. In particular, the *WRKY 65* gene expression in *cassava* was upregulated under low-temperature stress [63], which was consistent with the results of our study. The RNA-Seq data showed that the expression of *WRKY 65* was upregulated in the Longmu 806 variety but downregulated in the Sardi variety. Increased expression of the *WRKY 65* gene can be quickly and significantly induced by ABA and cold treatments [63], indicating that the *WRKY 65* gene may positively regulate the cold signalling pathway in an ABA-dependent manner to improve the cold tolerance of Longmu 806 alfalfa. Similar results have also been confirmed in the B3 family. It has been reported that the AP2/ERF and B3 domain-containing TF gene *RAV1* was induced by low-temperature stress according to microarray analysis [24, 64], and thus may regulate plant growth under low-temperature stress. This indicated that *RAV1* may play an important role in restraining root growth under low-temperature stress in the Longmu 806 variety.

Antioxidant defense system-related genes in response to low-temperature stress

Under low-temperature stress, ROS destroy cellular components, disturbing the homeostasis of plants and causing programmed cell death [26]. To protect plants from ROS damage, plants have established a very complex physiological system to scavenge ROS [65]. For example, superoxide dismutase (SOD) is a plant-specific oxidation-reduction enzyme widely present in plants. SOD catalyzes the disproportionation of superoxide anions to oxygen, which protects cells from superoxide poisoning. Increased antioxidant enzyme activity can effectively protect plants against low temperature [66]. In this study, the DEGs associated with *SOD1* was upregulated in the “peroxisome” (ko04146) pathway, and the DEGs related to the GO terms “cellular process”, “response to stimulus”, “catalytic activity”, and “antioxidant activity” were enriched. The expression level of the *SOD1* gene in the Longmu 806 variety was approximately 2.8-times higher than that in the Sardi variety. The expression of *SOD1* in the Longmu 806 variety was significantly upregulated compared with that in the Sardi variety. A similar result was shown in *M. sativa* (cv. Zhaodong) under freezing stress, indicating that the *SOD1* gene has similar functions in response to low-temperature environments [38]. This study indicated that the key enzyme in the ROS-scavenging systems in the Longmu 806 variety was upregulated as a defense to resist low-temperature stress, and the upregulation of the *SOD1* gene may be a special protective mechanism in the Longmu 806 variety. The *SOD1* gene may reduce the root freezing damage directly by increasing the superoxide scavenging capacity or indirectly by increasing the flux of H₂O₂ [7], thereby maintaining a high survival rate of the alfalfa in severe winter conditions. The *SOD1* may be one of the reasons why the Longmu 806 variety has a higher winter survival rate than the Sardi variety.

Conclusion

In summary, our study identified 4,442 DEGs, and many potential low-temperature-responsive transcription genes. We found that the antioxidant defense system (*SOD1*) was involved in the “peroxisome” pathway, and the upregulation of the *SOD1* gene may play an important role in improving the low temperature resistance of alfalfa. Moreover, six candidate DEGs were involved in the “plant hormone signal transduction” pathway and in TF families (MYB, B3, AP2/ERF, and WRKY), directly protecting alfalfa from low-temperature stress and increasing the tolerance of alfalfa to a severe winter environment. The cold resistance of alfalfa led to the upregulation of a large number of genes, which may be a protective mechanism to ensure the survival of alfalfa in the winter. This study has improved our understanding of the mechanism underlying alfalfa winter hardiness. In addition, our RNA-Seq data greatly enriched the alfalfa gene resources, which provides a reference for the future in-depth studies of winter hardiness in alfalfa.

Methods

Plant material and sample collection

We selected two cultivars of tetraploid *M. sativa*: the “Longmu 806” variety (FD = 1.0), which exhibits strong winter hardiness, and the “Sardi” variety (FD = 7.0), which is a non-winter-hardy variety. Alfalfa seeds were obtained from the Branch of Animal Husbandry and Veterinary of Heilongjiang Academy of Agricultural Sciences. The two varieties were grown in experimental fields located in Hohhot City, Inner Mongolia, China (111°58'E, 40°39'N). Both varieties were initially planted in a greenhouse near the experimental fields to ensure uniformity and to minimize uncontrolled factors of stress. On 6 April 2018, alfalfa seeds were sown in cultivation pots (top diameter 8.0 cm, bottom diameter 5.5 cm, height 17.5 cm) that were filled with a nutrient soil matrix (provided by Worth Production Base, Shanghai, China). Each pot contained five to seven seeds with 300 pots (300 plants per replication, three replications) per variety. Seedlings were thinned by hand to three plants per pot two weeks after germinating. All plants were watered every three days. The temperature of the greenhouse was controlled from 18 °C to 27 °C and seedlings were grown under a natural photoperiod with approximately 11 h of daylight. Weeds were timely removed by hand. After six weeks of growth in the greenhouse, the two alfalfa varieties were transferred to the experimental field. The soil was mainly chestnut soil and contained (0–30 cm depth) 15.97 g/kg of organic matter (OM), 348.0 mg/kg of available nitrogen (AN), 54.0 mg/kg of available phosphorus (AP), and 328.0 mg/kg of available potassium (AK) with a pH value of 8.30 (1:2.5 in water). Each plot was 6.0 m long and 4.0 m wide with 0.4 m row spacing. Each row was planted with 30 plants and 0.2 m plant spacing. The test plots were designed as a randomized complete block with three replicates. The alfalfa cultivar Caoyuan No.3 was planted as a protective plant around the plot. The root crowns of the Longmu 806 and Sardi varieties were collected from one-year-old plants at the wintering stage in December. The root samples of alfalfa were immediately frozen in liquid nitrogen and then stored at –80 °C for future RNA extraction.

RNA extraction and RNA-Seq library construction

Total RNA was extracted from six samples (three biological replicates each *M. sativa* variety) using the RNA Prep Pure Plant Kit (Tiangen, Beijing, China) following the manufacturer's protocol. The quality of the RNA samples was measured by an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and then quantified using a NanoDrop–2000 (Thermo Scientific, Wilmington, DE, USA). The mRNA was isolated according to the poly (A) selection method using oligo (dT) beads and then fragmented in fragmentation buffer. Next, the enriched mRNA was constructed into a cDNA library with the SuperScript double-stranded cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) with random primers (Illumina) following the manufacturer's protocol. All cDNA libraries were created using the Tru-Seq RNA Sample Prep Kit (Illumina) according to the manufacturer's instructions (15 PCR cycles). Libraries were selected on 2% low-range ultra-agarose (Bio-Rad, Hercules, CA, USA). Target bands were size-selected on low-range ultra-agarose and quantified using the PicoGreen Assay (Life Technologies, Carlsbad, CA, USA) and a TBS380 fluorometer (Invitrogen).

Illumina deep sequencing

The samples of alfalfa were sent to Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. (Shanghai, China), and transcriptome sequencing was performed using an Illumina HiSeqTM 4000 system with 200 cycles (2 × 150 bp read length). The transcriptome sequencing data were saved in the National Center for Biotechnology Information Short Read Archive (NCBI/SRA) database.

***De novo* transcriptome assembly and unigene detection**

Since alfalfagenome information was not previously available, clean data from the samples of alfalfa were *de novo* assembled using the reference genome and Trinity (V.2.4.0) software (<http://trinityrnaseq.sf.net>) [67]. Trinity is a software package consisting of Inchworm, Chrysalis, and Butterfly. First, Inchworm fragments the reads, builds a k-mer (default k = 25) dictionary, selects the clean reads k-mer and extends both sides to form contigs. Second, Chrysalis fragments overlapping contig clustering to form components, and each component becomes a set of possible characterizations of variable shear isoforms or homologous genes. A corresponding de Bruijn graph is generated for each component. Finally, Butterfly simplifies the de Bruijn graph of each component, generates the full-length transcript of the variable splicing subtype, combines the transcripts corresponding to homologous genes, and finally obtains the splicing result file [68].

Unigene annotation

The unigenes were annotated using BLASTX alignment with an E-value threshold of 110^{-5} to the NCBI Nr database (<ftp://ftp.ncbi.nlm.nih.gov/blast/db/>), the SwissProt database (http://web.expasy.org/docs/swiss-prot_guideline.html), the Pfam database (<http://pfam.xfam.org/>), the GO database (<http://www.geneontology.org>), the KEGG database (<http://www.genome.jp/kegg>) and the COG database (<http://www.ncbi.nlm.nih.gov/COG/>). Protein functional annotations were obtained according to the best alignment results.

Analysis of DEGs

Gene expression analysis of unigenes were calculated and normalized by fragments per kilobase per million reads (FPKM). The DEGs were analyzed using DESeq2 software. After P-value threshold testing using the false discovery rate (FDR), genes with an $FDR \leq 0.05$ and a \log_2 fold change > 1 were taken as thresholds to select the DEGs. The DEGs were subjected to further GO enrichment analysis and KEGG pathway analysis.

RT-qPCR analysis

Total RNA was extracted from a *M. sativa* root crown sample by using the plant total RNAprep Kit (Tiangen Biotech, Beijing, China) and reverse transcribed using the High-Capacity cDNA Reverse

Transcription Kit (Applied Biosystems, Foster City, CA, USA). The experiments were performed using SYBR Premix ExTaq (Perfect real time; TaKaRa, Tokyo, Japan) and an ABI Prism 7500 Sequence Detector (Applied Biosystems). Primers for RT-qPCR were designed using Primer Premier 6.0 software (Premier, USA). All primer sequences are listed in the supplementary material, Table S5. A 20 µl reaction mix was set up containing 50 ng total RNA and 0.4 µM each primer. The RT-qPCR analysis was performed in three technical repeats, and the program was set at 95 °C for 3 minutes, followed by 35 cycles of 95 °C for 30 seconds, 52 °C for 30 seconds, and 72 °C for 40 seconds. The final melting curve analysis was set at 95 °C for 15 seconds, 60 °C for 1 minute and 95 °C for 15 seconds. The relative expression changes of the endogenous reference and tested genes were analyzed by the $2^{-\Delta\Delta CT}$ method [69]. Statistical analysis was performed using SAS 9.0 software (SAS Institute Inc., North Carolina State, USA) with an ANOVA test. Significant differences were confirmed using the Duncan multiple range test at the $P < 0.05$ significance level.

Abbreviations

ABA: Abscisic acid; AP2/ERF(*AP2/EREBP*): AP2 (APETALA2)/EREBP (Ethylene Responsive Element Binding Factor); *ARF*: Auxin response factors; ANOVA: One-way analysis of variance; BLAST: Basic Local Alignment Search Tool; CBF: C-repeat binding factor; COG: Clusters of Orthologous Groups of Proteins; DEGs: Differentially expressed genes; *EIN3*: Ethylene insensitive 3; ETR: Ethylene receptor protein; FD: fall dormancy; GO: Gene Ontology; JA: Jasmonic acid; *JAZ*: Jasmonate zim-domain protein; KEGG: Kyoto Encyclopedia of Genes and Genomes; *JAMYB*: Transcription factor JAMYB; NCBI/SRA: National Center for Biotechnology Information Short Read Archive; Nr: non-redundant protein; nt: Nucleotides; Pfam: Protein Family; *RAV1*: AP2/ERF and B3 domain-containing transcription factor RAV1; ROS: Reactive oxygen species; RNA-Seq: RNA sequencing; RT-qPCR: Real-Time quantitative PCR; SAS: Statistical Analysis System; *SOD1*: Superoxide dismutase X1; *SnRK2*: Serine/threonine-protein kinase 2; SwissProt: A manually annotated and reviewed protein sequence database; TFs: Transcription factors; *WRKY65*: WRKY family transcription factor 65.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Our transcriptome datasets are available at NCBI project PRJNA558269 with accession number SRP217137, and SRA with accession number SRR9888366, SRR9888367, SRR9888364, SRR9888365, SRR9888363, SRR9888362.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

FGM and HL designed the research and revised the manuscript. XLW and HQJ carried out the study and wrote the manuscript. XLW and SYY conducted the experimental work. KM and ZYJ carried out the data analysis. All authors discussed the results and reviewed the manuscript.

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Additional Material

Additional file 1: Table S1. Differential expression genes in the root crown of two alfalfa varieties.

Additional file 2: Table S2. GO functional annotations and the number of differentially expressed genes statistics.

Additional file 3: Table S3. KEGG pathway annotation of differential expressed genes in the root crown of two alfalfa varieties.

Additional file 4: Table S4. Transcription factors family statistical table.

Additional file 5: Table S5. Primers used for Real-Time quantitative PCR (RT-qPCR) analysis.

Figures

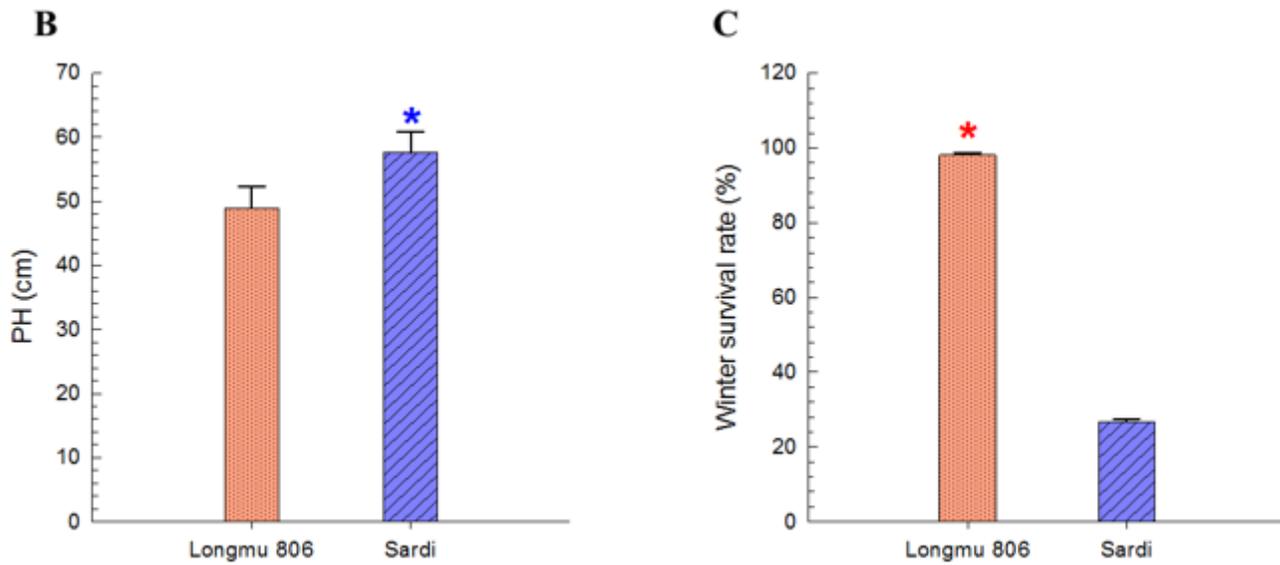


Figure 1

The returning green plants of the Longmu 806 and Sardi varieties in the field in April 2019 (a). The plant height (PH) in September 2018 (b) and winter survival rate (c) of the two alfalfa varieties were compared. Longmu 806 alfalfa (fall dormancy, FD=1) is a winter-hardy variety, while Sardi alfalfa (FD=7) is a non-winter-hardy variety. Data are presented as the mean \pm standard error. The star (*) represents significant differences at the 0.05 level of probability between the two alfalfa varieties.

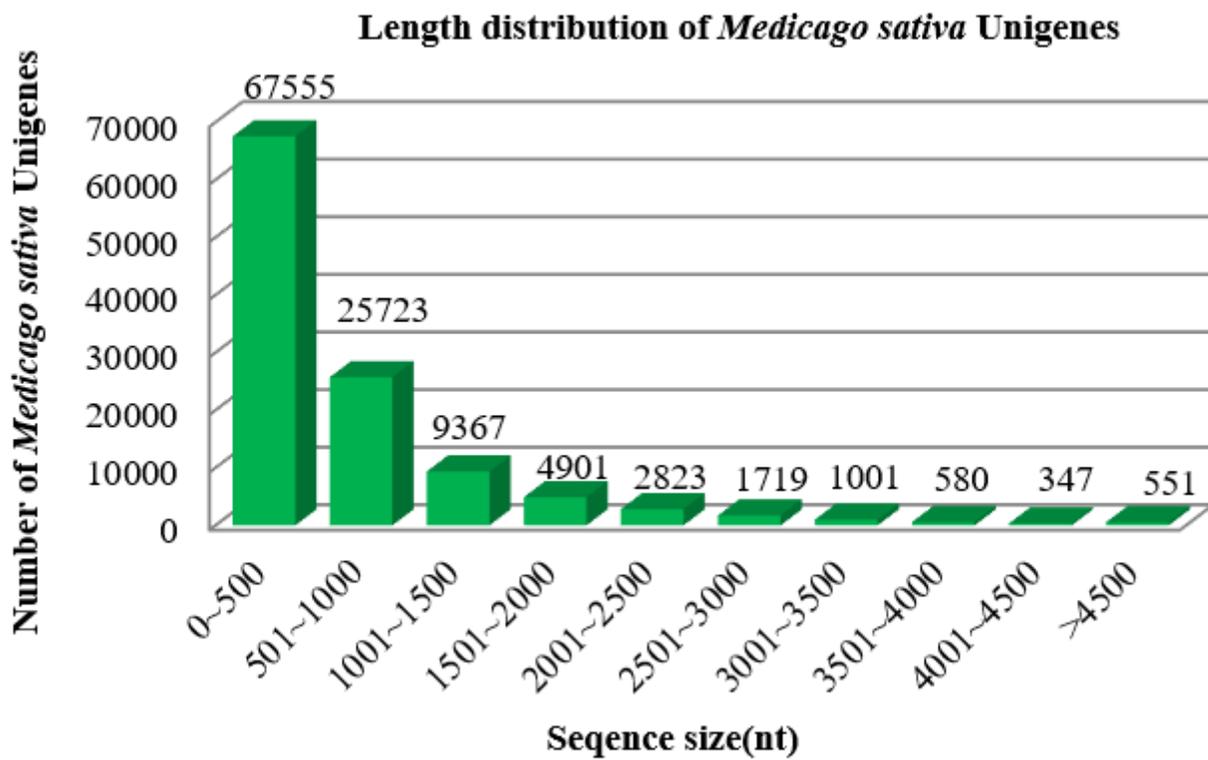


Figure 2

Sequence size distribution of unigenes *Medicago sativa* transcripts. The abscissa is the sequence size of the *M. sativa* unigenes from 0 nucleotides (nt) to > 4500 nt, and the ordinate is the number of *M. sativa* unigenes.

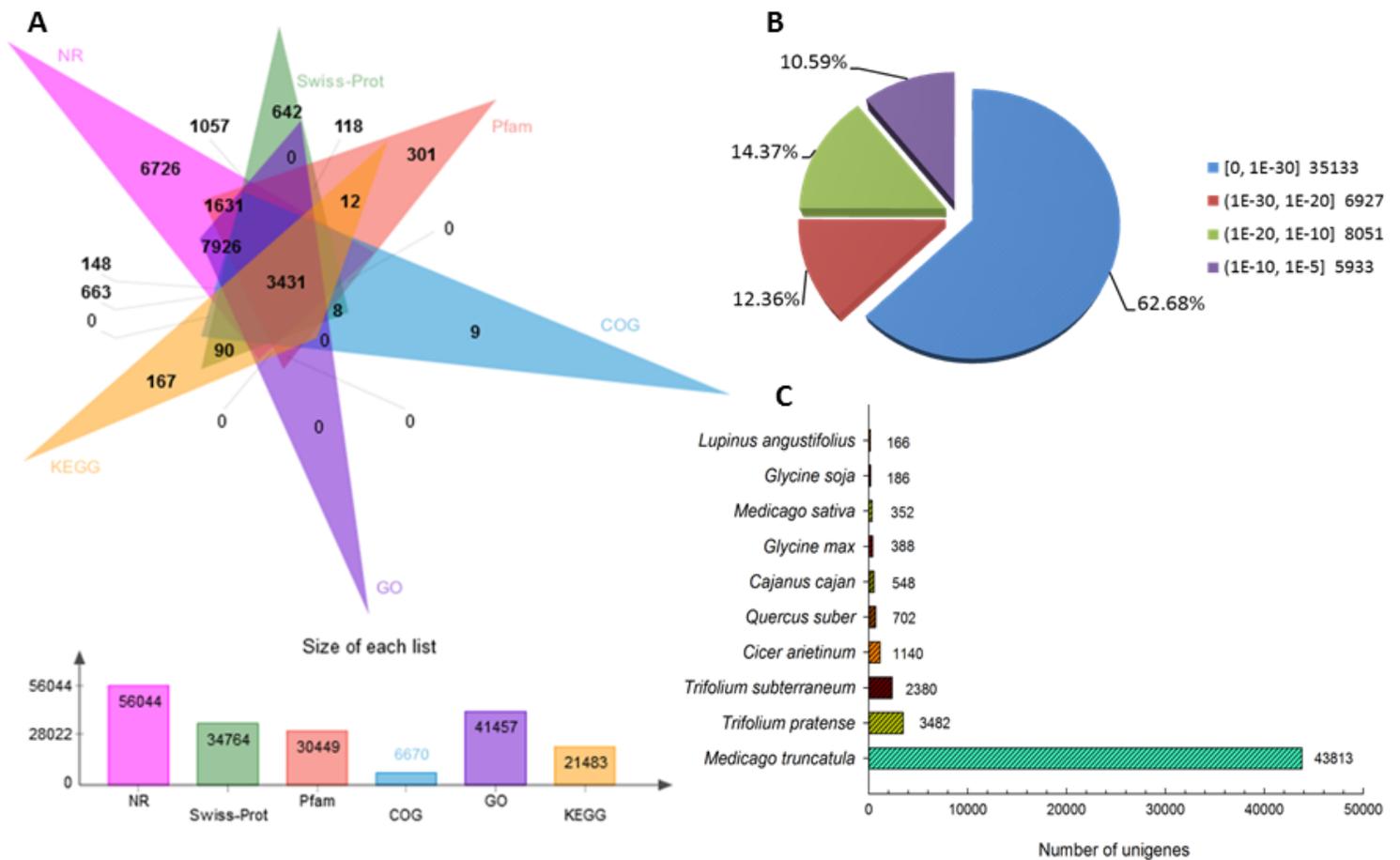


Figure 3

Homology search of *Medicago sativa* unigenes. The Venn diagram of the number of unigenes annotated by BLASTX with an E-value $< 1 \times 10^{-5}$ in the databases. The numbers in the triangles indicate the number of unigenes annotated by a single or multiple databases. The numbers in the bars indicate the number of total unigenes annotated in the databases (a). E-value distribution of *M. sativa* root crown transcriptome unigenes of the top BLASTX hits against the non-redundant protein (Nr) database (b). Top ten BLASTX hits of homologous sequences in the species distribution (c).

Sardi_VS_Longmu 806

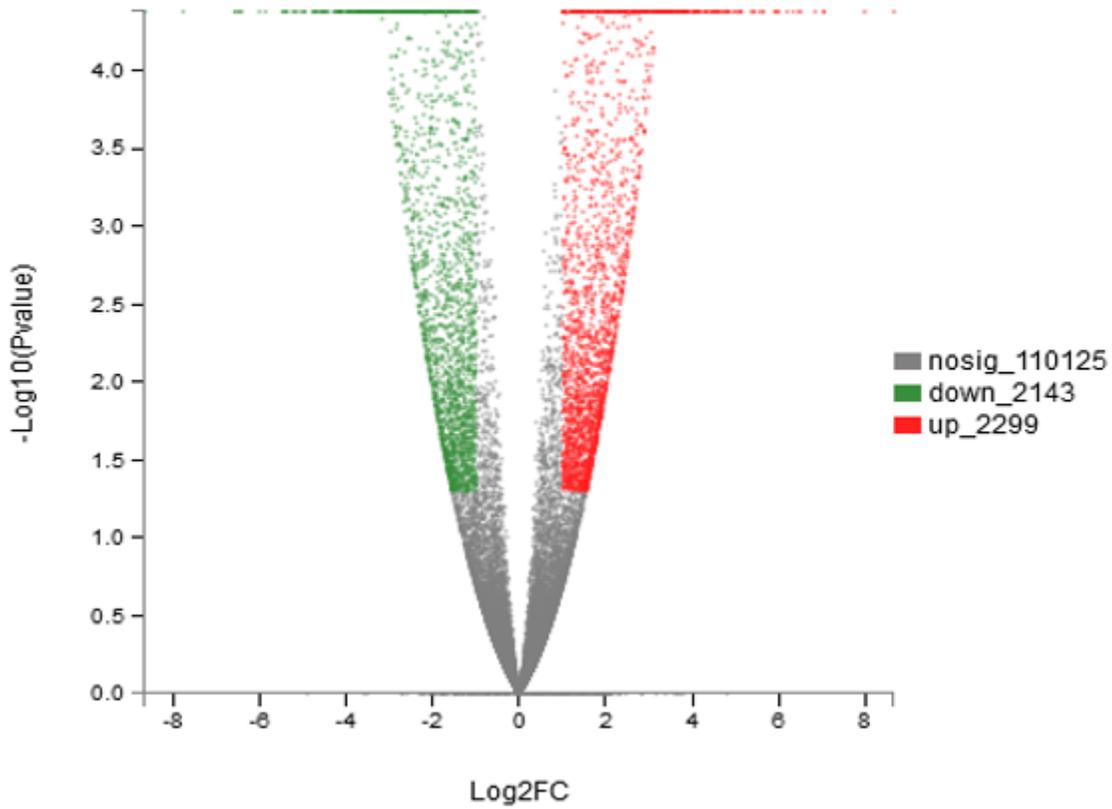


Figure 4

Sardi-vs-Longmu 806 differentially expressed genes (DEGs). DEGs were filtered using a false discovery rate (FDR) ≤ 0.05 and $|\text{Log}_2 \text{fold change}| > 1$ as thresholds. The volcano plot showed 4,442 DEGs, with 2,299 upregulated unigenes and 2,143 downregulated unigenes. Green spots indicate downregulated DEGs, and red spots represent upregulated DEGs. Those shown in black were unigenes that did not show significant expression.

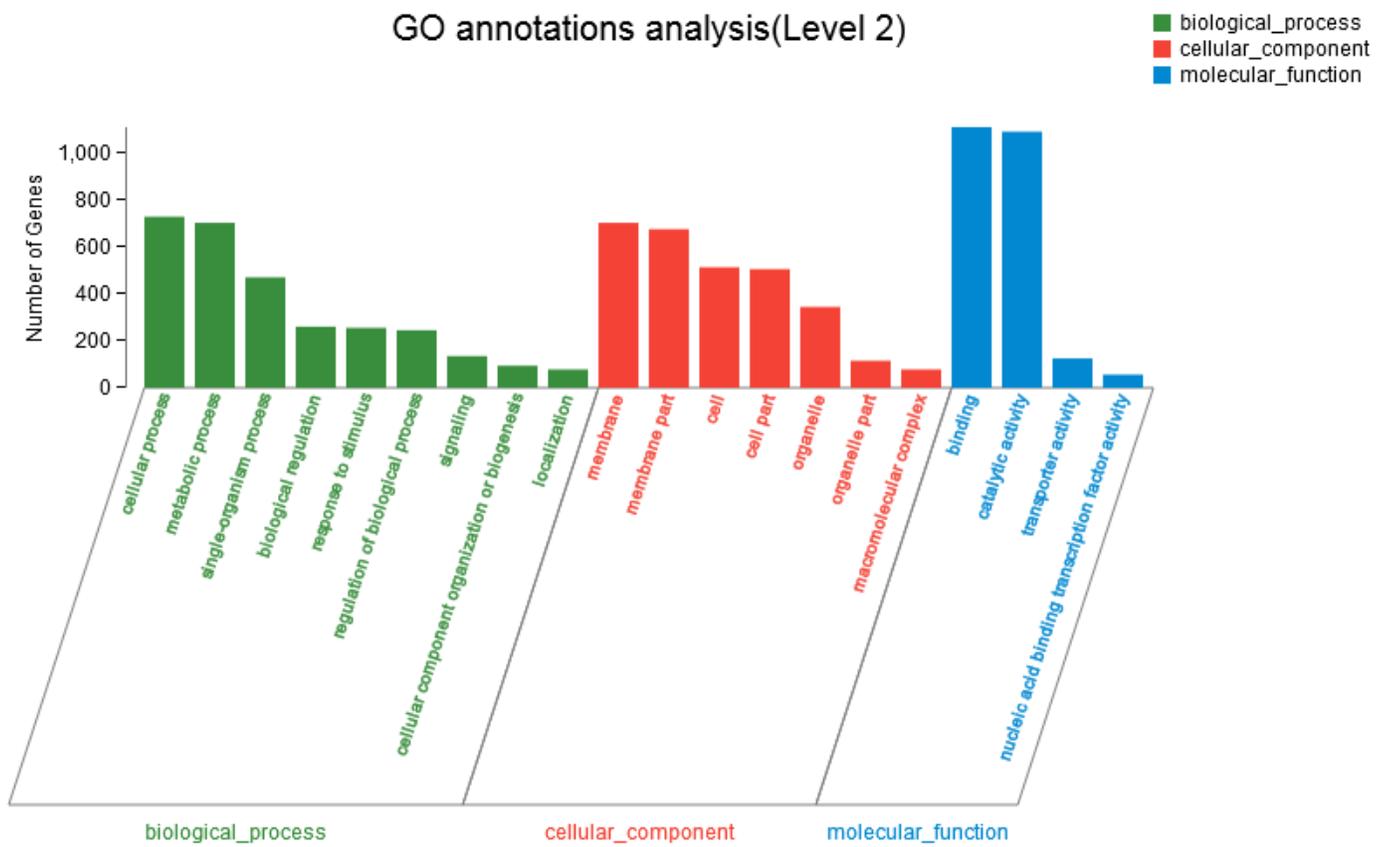


Figure 5

Gene Ontology (GO) annotation analysis of assembled unigenes. A total of 4,442 differentially expressed genes (DEGs) were categorized into three main categories: biological process, cellular component, and molecular function.

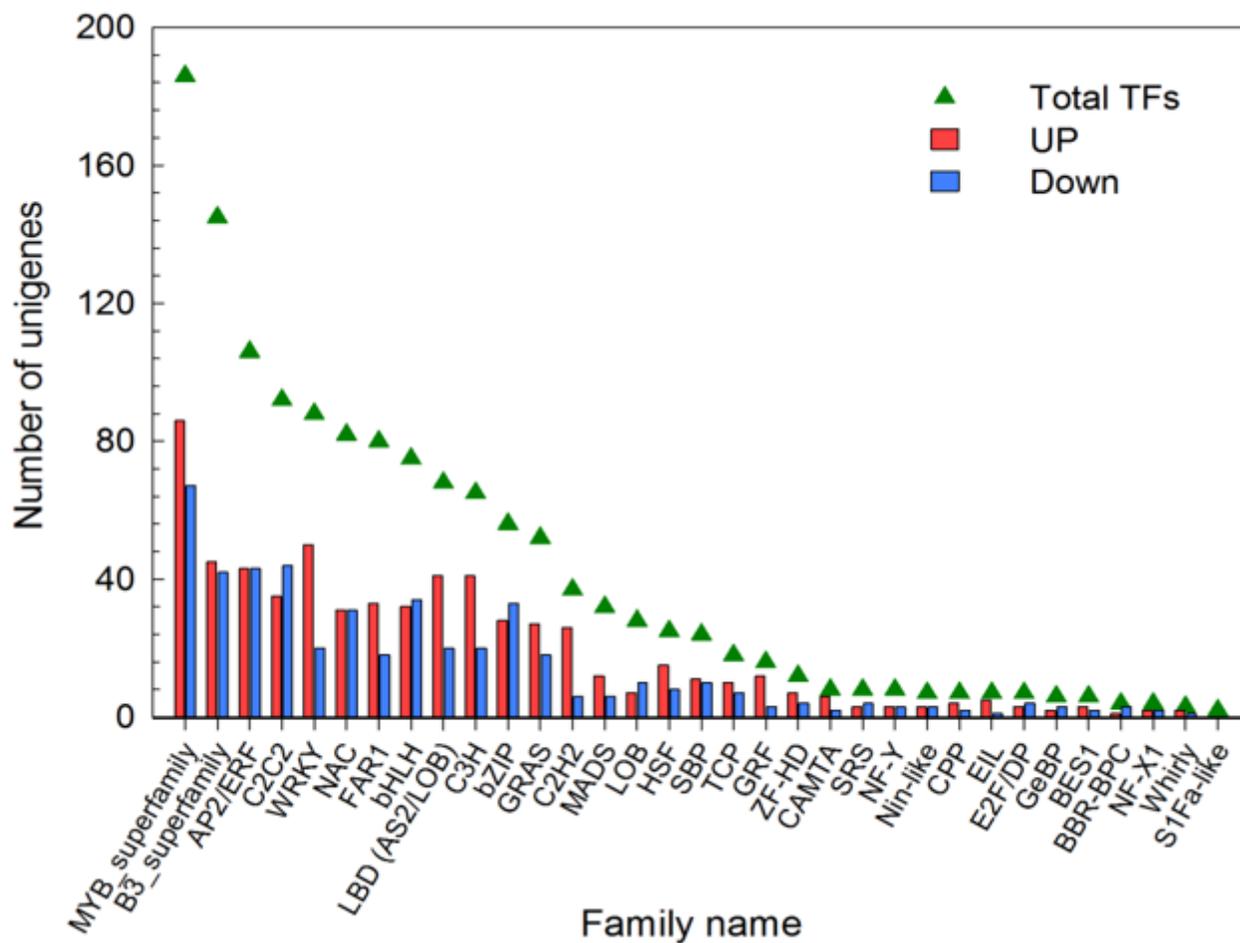


Figure 6

Distribution of differentially expressed transcription factor (TF) family. The abscissa is the TF family name, and the ordinate is the number of unigenes in the TF family. The triangles indicate the number of unigenes of among the total number of TFs. Blue bars indicate downregulated differentially expressed genes (DEGs), and red bars represent upregulated DEGs.

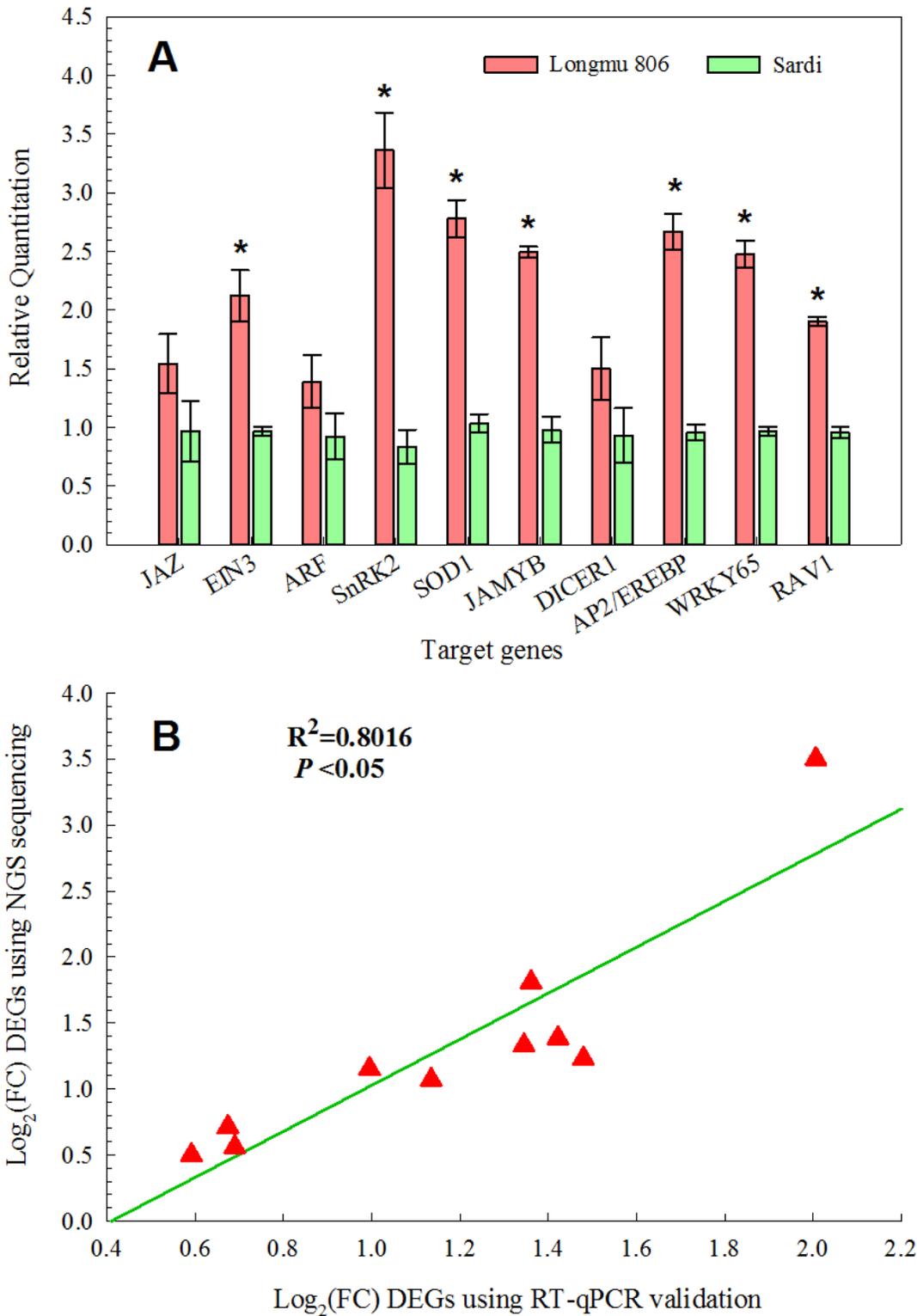


Figure 7

Expression analysis of the response of ten genes to low-temperature stress by real-time quantitative PCR (RT-qPCR). Data are presented as the mean \pm standard error. The star (*) represents significant differences at the 0.05 level of probability between the Longmu 806 and Sardi alfalfa varieties (a). Scatter plot shows expression changes (log₂ fold change) measured by RNA-Seq and RT-qPCR analyses of the selected genes under low-temperature stress (b).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS3.docx](#)
- [TableS5.docx](#)
- [TableS1.docx](#)
- [TableS2.docx](#)
- [TableS4.docx](#)