Different Genes Express Analysis of root crown of alfalfa under low temperature stress

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Abstract

Abstract Background: Alfalfa (Medicago sativa) is a perennial forage crop widely cultivated in northern China. The root crown of alfalfa is an important storage organ in the process of wintering, and it is closely related to the winter hardiness of alfalfa. At present, the specific molecular mechanism of response to winter hardiness in alfalfa root crown is unclear. The transcriptome database created by RNA sequencing (RNA-seq) is widely used to identify the critical genes related to winter hardiness. Results: The transcriptomes of alfalfa varieties, such as “Lomgmu 806” (with high winter survival rate) and “Sardi” (with low winter survival rate) have been sequenced in the study. Among the identified 57,712 unigenes, 2,299 differentially expressed genes (DEGs) were up-regulated, and 2,143 unigenes were down-regulated in the Lomgmu 806 vs Sardi root crown. The KEGG pathway annotations showed that 1,159 unigenes were mainly annotated to 116 pathways. Seven DEGs belonging to “plant hormone signaling transduction”, “peroxidase” pathway and transcription factors family (MYB, B3, AP2/ERF, WRKY) genes involved in alfalfa winter hardiness. Among them, the expression patterns of seven DEGs were verified by real-time quantitative PCR (RT-qPCR) analyses, which verified the reliable results of transcriptome sequencing analyses. Conclusions: RNA-Seq was used to discover genes associated with the wintering differences between alfalfa varieties. The transcriptome data showed that the gene regulation response of alfalfa to low temperature stress, which provides a valuable resource for further identification and functional analysis of candidate genes for winter hardiness of alfalfa. In addition, these data provide references for future study of genetic breeding and winter hardiness in alfalfa.

Background

Alfalfa (*Medicago sativa* L.) is one of the most popular perennial legume forages, and it is known as the “king of the forages”, beneficed to its high yield, rich nutritional value and good palatability. This forage is not only the most widely cultivated in the world [1-4], but also the most widely distributed and economically valuable in China [5, 6]. The alfalfa varieties introduced from abroad have the advantages of good quality and high yield, but winter survival rate or spring returning green rate is low, which seriously limits its industrial development in northern China [7]. 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 [8]. Due to the lack of winter hardiness varieties in some cold regions, understanding the winter hardiness mechanism of therefore alfalfa is important for improving the cold tolerance of alfalfa.

Plants can survive safely in winter and enhance freezing resistance through low temperature adaptation process [9]. The study of plant low temperature adaptation mechanism mainly focuses on physiological, phytohormones, and transcription factor regulation of low temperature induced gene expression changes [10]. Plants adapt to various changes in low temperature stress, and the critical response to low temperature stress conditions depends on the activation of molecular networks involved in the expression and signaling of specific stress-related genes [11]. At present, the most widely studied C-repeat-binding factor (CBF) signaling pathway including CBF1, CBF2, CBF3, and CBF transcription factor regulates the

expression of COLD-RESPONSIVE (COR) functional gene, which is a component of the low-temperature signal transduction pathway, thereby improving the cold tolerance of plants [12, 13]. So far, a number of studies have shown that CBF signaling pathway can improve the low temperature tolerance of various plants such as *Arabidopsis thaliana* [14, 15], *Medicago sativa* [16], *Triticum aestivum* [17], *Glycine max* [18], and *Malus domestica* [19]. Overexpression of cloned MtCBF3 from *Medicago truncatula* induces COR gene expression and enhances low temperature tolerance of transgenic plants [20]. A recent study showed that CBFs may play an important role in regulating the low temperature tolerance of alfalfa [21]. This suggests that 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 way.

Identification of low temperature responsive genes and their regulatory factors in alfalfa contributes to understand their function. In addition, the hormonal signal transduction pathway plays a central role in plant low temperature stress response and controls the CBF-dependence and independent pathway in plant [22]. For example, abscisic acid (ABA) is an important abiotic stress regulating hormone that plays an important role in abiotic stress signals. Furthermore, the previous assumption was that ABA did not affect the expression of CBF. Therefore, it has been suggested that the low temperature response of the ABA control is independent of the CBF regulation [23]. However, recent studies suggest that ABA may affect the expression of COR by regulating CBF transcription and may play a more important role in plant low temperature stress [24]. In addition, some functional genes play an important role in protecting plants from environmental stress. These genes are involved in signal transduction and transcriptional regulation. For example, transcription factors (TFs) play a crucial part in regulation the expression or status of other genes. At present, some TFs have been identified, such as AP2/ERF, MYB, B3, ICE and WRKY family members, and serve as important regulators of abiotic stress responses in plants [25-29]. Therefore, the exact role of the response factors of alfalfa in wintering period including transcription factors, hormone signals, and antioxidant system remains to be elucidated, and their function should be detailed study.

Recently, transcriptome sequencing is essential for functional gene annotation, novel gene discovery, differential gene expression, and molecular marker research [30-32]. RNA sequencing (RNA-seq) enables the study of gene expression at the transcriptome level and identifies genes involved in plant-specific biological processes [33-35]. So far, with the introduction of a new generation of RNA-seq, transcriptome sequencing technology has been used for identify low temperature stress response genes in *Ipomoea batatas* [36], *Brassica napus* [37], *Populus tomentosa* [38], *Capsicum annuum* [39], and *Magnolia wufengensis* [40]. In addition, studies on differentially expressed genes of low temperature resistance of alfalfa varieties have also been reported. A recent study reported that transcriptome high-throughput sequencing technology has been used to analysis the expression of the gene of the seedling [10], taproots [41], and crown buds [42] of alfalfa, which provides valuable resources for functional genomics research on plant cold tolerance in the future. However, the understanding of genetic response information for low temperature stress was limited, since some of the previous studies only RNA-seq the seedling, taproots, and crown buds of alfalfa. Therefore, this study collected the alfalfa root crown of wintering period for RNA-seq to obtain more genetic information on the response to low temperature

stress. Moreover, the molecular mechanism of specific low temperature resistance of the alfalfa root crown during the wintering period is still unclear. Therefore, through the systematic analysis of the low temperature resistance of Longmu 806 and Sardi varieties during winter, it not only helped to understand the response mechanism of alfalfa root crown during wintering, but also provided a reference for the functional genomics research on alfalfa low temperature stress.

Results

De novo transcriptome assembly

The total RNA extracted from the root crown of *Medicago sativa* during the wintering stage were constructed six libraries for high-throughput sequencing. In order to ensure the quality of information analysis, the original data was filtered. Among the raw reads, low quality reads, those containing adapter sequences, or low quality bases were discarded, and 459,865,666 clean reads with a total of 68,515,729,607 nucleotides (nt) were obtained from the six sequencing libraries. A total of 114,567 unigenes with an N50 of 1092 nt were assembled. The maximum length, minimum length and average length were 15,687 nt, 201 nt and 740 nt, respectively (Fig. 1).

Function annotation

A total of 57,712 unigenes (50.37% of total 114,563 unigenes) were annotated against Nr, SwissProt, Pfam, COG, GO and KEGG databases using BLASTX (E-value < 1×10^{-5}). Among them, 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. 2a). According to the Nr database, 10.59% (5,933) of unigenes showed homology ($1 \times E^{-10}$ < E-value ≤ $1 \times E^{-5}$), 14.37% (8,051) of unigenes showed strong homology ($1 \times E^{-20}$ < E-value ≤ $1E^{-10}$), and the remaining of 62.68% (35,133) unigenes had very strong homology (E-value ≤ $1E^{-30}$) (Fig. 2b). For the species distribution of the top BLASTX hits, 78.28% (43,813) unigenes matched to the homologous sequences of *Medicago truncatula*, while 3,482, 2380, 1,140, 548 and 388 unigenes matched to the homologous sequences of *Trifolium pretense*, *Trifolium subterraneum*, *Cicer arietinum*, *Cajanus cajan* and *Glycine max*, respectively (Fig. 2c).

Identification and analysis of differentially expressed genes (DEGs)

Using the Nr annotation results, BLAST2GO 2.5.0 software was used to perform GO functional annotations of unigenes. A total of 4,442 significant DEGs were assigned to one or more ontologies by the standard of $|\log_2$ fold change$|$ >1 and P-value < 0.05 (Additional file 1: Table S1; Fig. 3), there were 2,299 unigenes up-regulated, and the other 2,143 unigenes were down-regulated (Longmu 806 *vs* Sardi). Those DEGs were used for the next analysis.

In order to better understand the function of genes differentially expressed during the wintering period of alfalfa, GO functions were used to classify the function of DEGs (Additional file 2: Table S2). A total of

4,442 unigenes were summarized in three main functional categories “biological processes”, “cellular component”, and “molecular function” (Fig. 4). In the biological processes category, “cellular process” (GO:0009987), “metabolic process” (GO:0008152), “single-organism process” (GO:0044699) and “biological regulation” (GO:0065007) were the most frequent terms and contained 728, 702, 470 and 260 unigenes, respectively. In the molecular function category, genes were focused on subcategory including catalytic activity (GO:0003824) and binding (GO:0005488).

KEGG pathway enrichment analysis

KEGG analysis of differentially expressed genes using KOBAS (v2.1), a total of 1,159 unigenes were assigned to 116 KEGG pathways (Additional file 3: Table S3). The ten top KEGG pathways with the highest representation of the 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 signaling pathway-plant” (ko04016), “Endocytosis” (ko04144), “Glycolysis/Gluconeogenesis” (ko00010), “Spliceosome” (ko03040).

In the “Plant hormone signal transduction” pathway (ko04075), DEGs associated with ABA and ethylene biosynthetic pathways (including *SnRK2* and *EIN3*) were up-regulated, respectively. In the “peroxidase” pathway (ko04146), DEGs associated with superoxide dismutase X1 (*SOD1*) was up-regulated. These annotations provided valuable resources for studying the specific functions and pathways of the alfalfa gene.

Stress response of transcription factors (TFs) to low temperature stress

Transcription factors (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 [43, 44]. A total of 34 TFs families containing 1,364 unigenes were identified (Fig. 5). According to our data, these TFs families responding to low temperature stress included MYB, B3, AP2/ERF, C2C2, WRKY, NAC, FAR1, bHLH, LBD, C3H, bZIP, GRAS, C2H2, MADS, LOB, HSF, SBP, TCP, GRF, ZF-HD family (Additional file 4:Table S4). Many of these TFs families have been reported to play an important role in plant responses to abiotic stresses including low temperature stress [45], and have been utilized to improve plant abiotic stress tolerance by gene transfer technology [46].

Among these TFs, the top six TFs families with the highest representation were MYB (186), B3 (145), AP2-ERF (106), C2C2 (92), WRKY (88), and NAC (82). The MYB family members detected in our data 86 were up-regulated by low temperature stress. The AP2/ERF family was very important TFs family that regulates gene expression to cold and freezing stress in a variety of plants [47]. The AP2/ERF family members detected in our data 43 were up-regulated by low temperature stress. Moreover, most of the unigenes expression was up-regulated in the WRKY family under freezing stress, and 45 unigenes in the B3 TFs family were up-regulated under low temperature stress. In the C2C2, bHLH, bZIP, LOB, SRS, E2F/DP, GeBP and BBR-BPC TFs families, the number of TFs with up-regulated expression was less than

the number of TFs with down-regulated expression. In addition to the above TFs, five unigenes of the EIL family were also identified as up-regulated under low temperature stress. Furthermore, the number of TFs with up-regulated and down-regulated 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 transcriptome sequencing data, differences in expression of ten genes were detected using RT-qPCR, and these genes were well characterized by the NCBI Nr database (Fig. 6a). These genes included ethylene (*EIN3*), ABA (*SnRK2*), auxin (*ARF*), and jasmonic acid (*JAZ*) response regulatory genes in the plant hormone signaling pathway, and MYB, B3, AP2/ERF, WRKY TFs family, and detection of reactive oxygen species (ROS) transcript superoxide dismutase X1(*SOD1*) associated with low temperature stress response (Additional file 5: Table S5). The expression of transcription factor genes of Longmu 806 verity was up-regulated, and the expression of seven transcription factor genes was significantly higher than that of sardi verity under low temperature stress. By analyzing the expression profiles of selected genes during wintering, correlation analysis was performed with RT-qPCR analysis results and transcriptome sequencing results (Fig. 6b). Moreover, RT-qPCR showed a high correlation (R^2=0.8016, P<0.05) of fold change between RNA sequencing analysis and RT-qPCR. The expression patterns in PCR assay were generally in agreement with the results of the RNA-seq.

Discussion

Low temperature stress is one of the main abiotic stresses affecting plant growth and development and crop yield. Generally speaking, under severe winter conditions, low temperature freezing damage is likely to occur, resulting in lower yield of forage in the next year, which seriously affects the production benefit of forages. Therefore, the ability for regrowth in the spring reflects the cold resistance of the forages in the field [48]. A recent study have shown that different varieties of alfalfa have significant differences in winter cold resistance, mainly in dormancy and winter survival rate [41]. In this study, the winter survival rate of Longmu 806 variety (98.09%) was significantly higher than that of Sardi variety (26.69%, P<0.05, Fig. 7), indicating the higher winter hardiness of Longmu 806 variety than Sardi variety. The winter hardiness of alfalfa varieties was a complex trait, which was not only affected by the growth environment, but also closely related to the comprehensive genetic regulation of the varieties.

To our knowledge, some previous study have reported that transcriptome sequencing technology was used to analyze differentially expressed genes related to alfalfa leaves, taproots, and crown buds [41, 42, 49]. However, unlike previous studies, in order to understand the comprehensive genetic regulation mechanism of alfalfa low temperature resistance, this study focused on the expression patterns of low temperature response genes in the root crown of alfalfa, and explored the mechanism of winter hardiness response of different alfalfa roots during wintering.

Hormones in alfalfa response to low temperature stress

Hormones are the startup-factors of winter hardiness gene expression, and hormone-mediated abiotic stress responses involve multiple mechanisms. In perennial plants, phytohormone ABA, ethylene, auxin and jasmonic acid (JA) play important role in regulating plant growth to adapt to low temperature stress [50]. 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 independent pathways can regulate low temperature response genes [51]. *SnRK2* is the center of ABA signaling pathway, and the role of ABA-activated *SnRK2* protein kinase in low temperature stress signaling has also been reported in *Kandelia obovata* [52]. The ABA-binding receptor inactivates *PP2C*, leading to phosphorylation of *SnRK2* kinase, which then promoting downstream ABA response gene transcription [53, 54]. In our study, the expression level of the *SnRK2* gene in Longmu 806 variety was approximately 4.0 times higher than that of Sardi variety. Similar results have also been found that the overexpression of *TaSnRK2.3* in *Arabidopsis* results in improved root-system structure and significantly enhanced the tolerance of this species to freezing stress [55]. This indicated that the *SnRK2* gene regulated the low temperature stress response by controlling the expression of stress-responsive genes in a low temperature environment. Moreover, the low temperature stress and ABA synergistically increase the expression of *CBF/DREB1* transcription factors [56], which may help to the resistance of alfalfa to low temperature complex environments.

The *EIN3* is involved in ethylene signal transduction, and ethylene signaling promotes transcription of multiple ethylene response factor (ERF) genes, ultimately guiding growth and physiological responses abiotic stress [57]. In our study, the RT-qPCR result showed that the expression level of the *EIN3* gene in Longmu 806 variety was approximately 2.2 times higher than the Sardi variety. This study indicated that Longmu 806 variety was more likely to accumulate ethylene than that of Sardi variety. A recent found revealed the several ethylene-insensitive mutants (*etr1-1*, *ein4-1*, *ein2-5*, *ein3-1*, and *ein3 eil1*) are exhibited enhanced freezing tolerance in *Arabidopsis*. Genetic and biochemical analyses indicated that ethylene negatively regulates cold signaling, at least partially, through direct transcriptional control genes (cold-regulate *CBFs* and type-A *ARR*) via *EIN3* [58]. Interestingly, the high concentrations of ABA inhibit root growth in *Arabidopsis* by increasing ethylene accumulation [59]. Therefore, although the mechanisms underlying the complex cross talk between ABA, ethylene, and low temperature signaling still unclear, it appears likely that the up-regulation of ABA-responsive genes also help to the low temperature tolerance of ethylene-insensitive mutants. This may be one of the reasons for the stronger winter hardiness of Longmu 806 variety than that of Sardi variety.

Auxin reactive factors (*ARF*) are transcriptional activators and inhibitors of the early auxin responsive gene promoter. Compared with Sardi variety, the expression level of the *ARF* gene in Longmu 806 variety was up-regulated during the low temperature stress, but we did not find a significant difference between Longmu 806 and Sardi varieties. Shu *et al.* [60] also found that the expression of *ARF* in alfalfa was up-regulated under cold and freezing stresses, which indicated that the conserved miRNAs might regulate alfalfa low temperature stress response by controlling alfalfa developmental process. Therefore, these results indicate that *ARF* may affect the elongation and dormancy of alfalfa roots under low temperature stress by regulating auxin. Similarly, jasmonic acid is a lipid phytohormone that plays an important role in

plant defense. Many studies have reported that JA is involved in plant tolerance to low temperature stress, and increasing JA content positively regulates the ICE-CBF (INDUCER OF CBF EXPRESSION) transcriptional pathway and ultimately improve cold tolerance [61, 62]. Transcriptome analysis of root crown of alfalfa under low temperature stress showed that *JAZ* expression differed under low temperature stress ($|\log_2$ fold change$|$=0.712). The *JAZ* of Longmu 806 variety had higher expression, as compared to Sardi variety, but we did not find a significant difference between two alfalfa varieties. This study indicated that alfalfa may reduce *JAZ* expression through ICE-CBF regulatory pathways, release CBF TFs induce expression of COR genes, and ultimately improve alfalfa cold resistance. Therefore, the exact roles of *JAZ and ARF* under low temperature stress remains to be elucidated, and its function should be characterized in future.

The response of DEGs to low temperature stress revealed the relationship between plant hormone signaling pathway and winter hardiness. Transcriptome data and RT-qPCR results indicate that most of the hormonal responses were similar to the expression patterns of DEGs. The expression of hormones responsive DEGs increased during the low temperature stress period. As a signal molecule, hormones plays an important role in regulating gene expression which confirmed the results of the study on *Medicago sativa* (cv. *Zhaodong*) under cold and freezing stress response [16]. Therefore, this study was indicated that changing in the balance of alfalfa hormones in the field under low temperature might affect the winter hardiness of alfalfa.

Transcription factors (TFs) involved in low temperature stress response

Transcription factors (TFs) play an important role in plant responses to abiotic stresses (cold and freezing). In this study, most TFs families were identified that play a vital role in plant responses to low temperature stress, including MYB, AP2/ERF, B3, and WRKY family. The *MYB* TFs have been shown to play a positive role in abiotic stress signaling process [63]. In the MYB family, *MYB15* has been reported to be involved in cold-regulation of CBF genes. The *MYB15* gene transcript is induced up-regulated by cold stress [64]. Shu *et al*. [65] also reported that *MYB* transcription factor genes were induced up-regulated by cold and freezing stresses. In our study, was found that the expression level of the *MYB* transcription factor gene in Longmu 806 variety was approximately 2.5 times higher than that of Sardi variety. In the present study, we founded that many transcription factor subfamilies were belong to the AP2/ERF family, such as AP2 subfamily, DREB subfamily, ERF subfamily, RAV subfamily and others, and a high percentage of them were involved in the low temperature stress response [66]. The *AP2/EREBP* gene of AP2/ERF family was significantly up-regulated in low temperature stress responses for Longmu 806 variety, similar to our previous findings *Medicago truncatula*. In particular, AP2/ERF TFs family a tandem array of *Mt-ERF* genes on chromosome 6 was found to function in the response to cold and freezing stresses in *M. truncatula* [25]. These results verify previous reports in other plants, and confirmed that *MYB* and *AP2/EREBP* TFs have positive functions in the response to low temperature stress. In addition, many studies have reported that the WRKY family is involved in the low temperature stress response. For example, up-regulation of transcription factor expression in most WRKY gene families (*WRKY 30, 33, 41, 46, 48, 51, 53, 65, 70*) in *Hevea brasiliensis* under low temperature stress (24 h cold

treatment at 4 °C) [67]. In particular, the *WRKY65* gene expression in *cassava* was up-regulation under low temperature stress [68], consistent with the results of our study. Similar results have also been confirmed in other well-characterized low temperature stress response TFs families, such as the B3 family. It has been reported that microarray analysis identified the AP2/ERF and B3 domain containing transcription factor gene *RAV1* induced by low temperature stress [27, 69], which may regulate plant growth under low temperature stress. This indicated that the *RAV1* may play an important role in restraining root growth under low temperature stress in Longmu 806 variety.

Antioxidant defense system related genes in low temperature stress

As a signal molecule, reactive oxygen species (ROS) plays an important role in the low temperature adaptation of alfalfa. Under low temperature stress, reactive oxygen species (ROS), which are harmful to cell membranes, proteins, and biological macromolecules, accumulate in plant cells, ROS destroys cellular components, causing programmed cell death, destroying the homeostasis of plants and causing serious damage to plants [70]. Superoxide dismutase (SOD) is a plant specific oxidation-reduction enzyme widely present in plants. The SOD catalyzes the disproportionation of superoxide anion to oxygen, which protects cells from superoxide poisoning. Increased antioxidant enzyme activity can effectively protect plants against low temperature [71]. In this study, DEGs associated with superoxide dismutase X1 (*SOD1*) was up-regulated in the "peroxidase" pathway, consistent with the results of Song *et al.* [42]. Under the low temperature stress, the expression of DEGs in Longmu 806 variety *SOD1* was up-regulated, and the expression level of the *SOD1* gene in Longmu 806 variety was approximately 2.8 times higher than that of Sardi variety. In our study, the root crown of alfalfa was collected from December, during which alfalfa experienced low temperature acclimation. During low temperature acclimation, the plant's antioxidant enzyme system will increase with increasing stress [72]. Under low temperature stress in the field, compared with Sardi variety, the Longmu 806 variety *SOD1* was up-regulated during the wintering period. This study indicated that the Longmu 806 variety was up-regulated as a key enzyme to resist the low temperature stress, and the up-regulation of *SOD1* may be a special protective mechanism of alfalfa. The *SOD1* may reduce the root freezing damage by directly increasing the superoxide scavenging capacity or indirectly by increasing the flux of H_2O_2, thereby maintaining a high survival rate of the alfalfa in severe winter. The *SOD1* may be one of the reasons why the Longmu 806 variety has higher winter survival rate than that of Sardi variety.

Conclusion

With the Illumina platform for transcriptome sequencing of the root crown of cultivated alfalfa, we obtained 4,442 differentially expressed genes (DEGs), and many potential low temperature responsive transcription genes were identified and various key signal transduction components at the transcriptome level were found. This study involved the expression differences of a large number of genes with different biological functions under low temperature stress. Our results showed that the antioxidant defense system (*SOD1*) 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

transcription factors families (MYB, B3, AP2/ERF, and WRKY), directly protecting alfalfa from low temperature stress and increasing the tolerance of alfalfa to severe winter hardiness environment. The cold resistance of alfalfa led to the up-regulation of a large number of genes, which may be a protective mechanism to ensure the survival of alfalfa in the winter. This study had improved our understanding of the mechanism of alfalfa winter hardiness. In addition, our transcriptome data greatly enriched the alfalfa gene resources, which then provided a reference for the future wintering of alfalfa.

Methods

Plant material and sample collection

We selected two cultivars of tetraploid *M. sativa*: the “Longmu 806” variety that has strong winter hardiness and the “Sardi” variety that is not characterized by strong winter hardiness. 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. After six weeks of growth in the greenhouse, two alfalfa varieties were transferred to the experimental field. Each plot was 6.0 m long, 4.0 m wide with 0.4 m row spacing. Each row was planted with 20 plants and 0.3 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 crown of Longmu 806 and Sardi varieties were collected from first year old plant 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 RNA Prep Pure Plant Kit (Tiangen, Beijing, China) following the manufacturer’s protocol. The quality of the RNA samples was measured by 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 to a cDNA library by 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 the transcriptome sequencing was performed using an Illumina HiSeq™ 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 unigenes detection

Since alfalfa genome information was not previously available, clean data from the samples of alfalfa were *de novo* assembled using the reference genome Trinity (V.2.4.0) software (http://trinityrnaseq.sf.net) [73]. The Trinity is a software package consisting of Inchworm, Chrysalis, and Butterfly. Firstly, Inchworm breaks the reads, builds a k-mer (default k=25) dictionary, selects the clean reads k-mer and extends both sides to form contig. Secondly, Chrysalis will have overlapping contigs clustering to form components, each component becomes a set of possible characterizations of variable shear isoforms or homologous genes, each component will have a corresponding de Bruijn graph. Finally, Butterfly simplifies the de Bruijn graph of each component, outputs the full-length transcript of the variable splicing subtype, and combs the transcript corresponding to the homologous gene, and finally obtains the splicing result file [74].

Unigenes annotation

The unigenes were annotated using the BLASTX alignment with an E-value threshold of $1´10^{-5}$ to NCBI non-redundant protein (Nr) database (ftp://ftp.ncbi.nlm.nih.gov/ blast/db/), the Swiss-Prot protein 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 Kyoto Encyclopedia of Genes and Genomes (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 Differentially expressed genes (DEGs)

Gene expression analysis of unigenes were calculated and normalized by FPKM (Fragments per kilobases per millionreads). The differentially expressed genes (DEGs) were analyzed using DESeq2 software. After P-value threshold testing using false discovery rate (FDR), genes with a FDR≤0.05 and a ê$\log_2$ fold changeê> 1 were taken as thresholds to select the DEGs. The DEGs were carried out further GO enrichment analysis and KEGG pathway analysis.

Real-Time Quantitative PCR 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 the primers sequences are listed in supplementary material, Table S5. A total of 20 μl reaction

mix was set up containing 50 ng total RNA and 0.4 μM of each primer. The RT-qPCR analysis was performed in technical three 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, 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 $2^{-\triangle\triangle CT}$ method [75]. 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 Duncan multiple range test at the $P<0.05$ level of significance.

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; *DICER1*: Endoribonuclease dicer homolog 1; *EIN3*: Ethylene insensitive 3; ETR: Ethylene receptor protein; GO: Gene Ontology; JA: jasmonic acid; *JAZ*: Jasmonate zim-domain protein; KEGG: Kyoto Encyclopedia of Genes and Genomes; *MYB*: Transcription factor JAMYB; NCBI/SRA: National Center for Biotechnology Information Short Read Archive; Nr: NCBI nonredundant protein; nt: Nucleotides; *RAV1*: AP2/ERF and B3 domain containing transcription factor RAV1; ROS: Reactive oxygen species; RT-qPCR: Real-time quantitative PCR; SAS: Statistical analysis system; *SOD1*: Superoxide dismutase X1; *SnRK2*: Serine/threonine-protein kinase 2; Swiss-Prot: A manually annotated and reviewed protein sequence database; TFs: Transcription factors; *WRKY65*: WRKY family transcription factor 65.

Declarations

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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.

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.

Ethics approval and consent to participate

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Additional Material

Additional file 1: Table S1. Differentially expressed genes generated from two varieties of alfalfa roots.

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 different genes expressed from two varieties of alfalfa roots.

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

Additional file 5: Table S5. Primers used for RT-qPCR analysis.

Figures

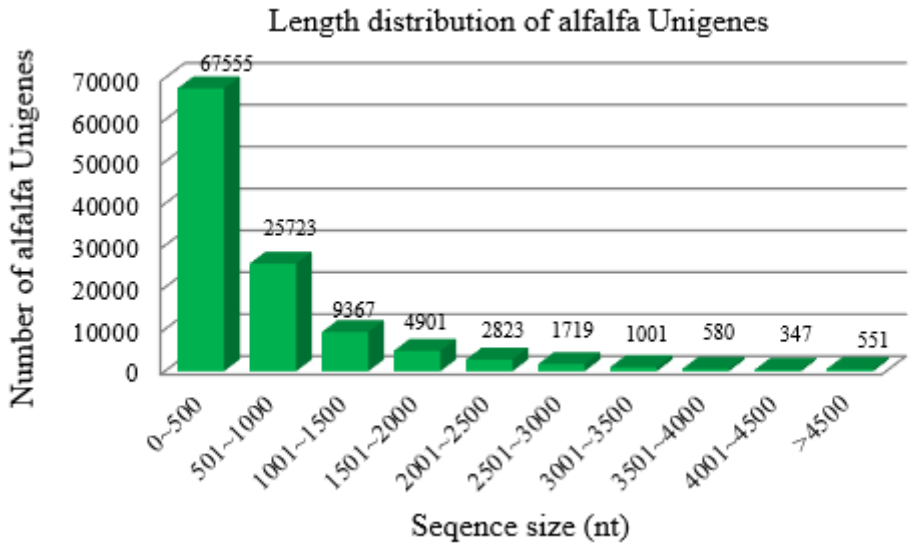
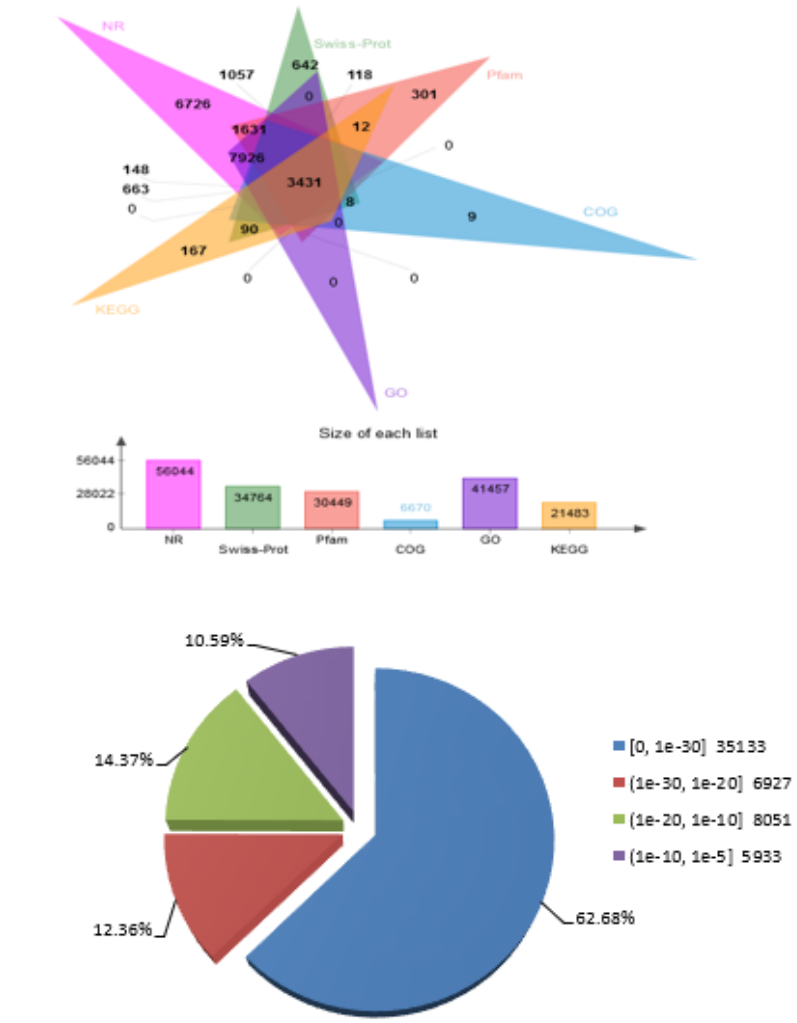


Figure 1

The length distribution of alfalfa unigenes. The abscissa is the length of the assembled unigenes, and the ordinate is the number of unigenes of the corresponding length



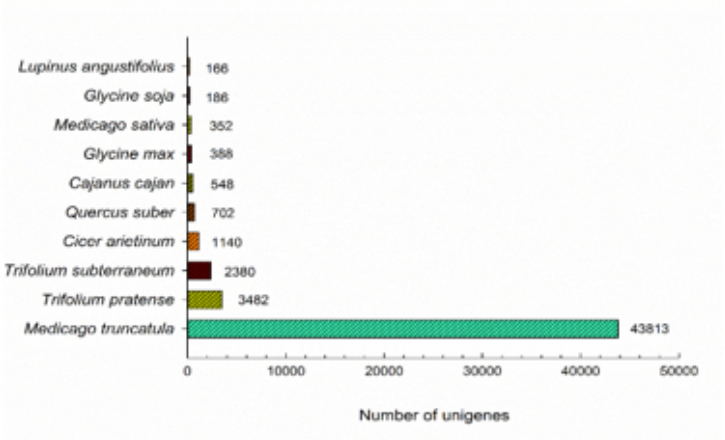


Figure 2

2a Function annotation of the Medicago sativa transcriptome. 2b E-value distribution of Medicago sativa root crown transcriptome unigenes of the top BLASTX hits against Nr database 2c Ten top BLASTX hits of homologous sequences for the species distribution

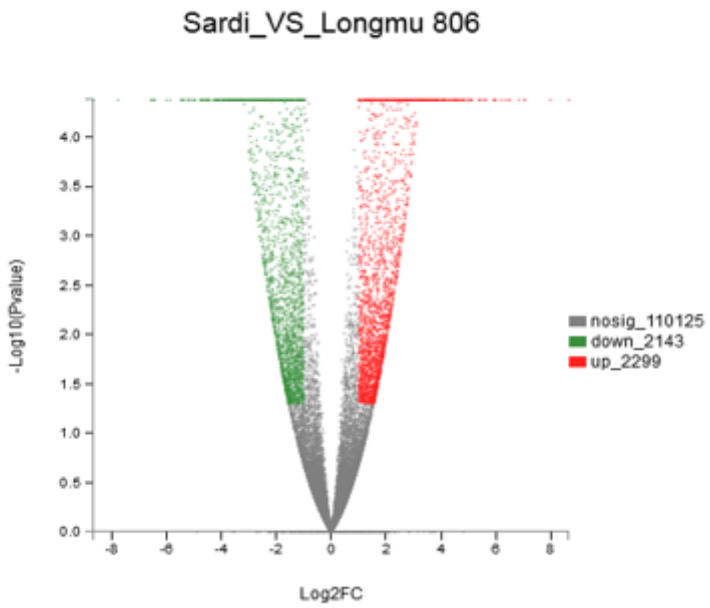


Figure 3

The volcano spots of DEGs variance analysis. The volcano spots showed 4,442 DEGs including 2,299 up-regulated and 2,143 down-regulated. Green spots indicate down-regulated DEGs and red spots represent up-regulated DEGs. Those shown in black were unigenes that did not significant expression. The screening condition for the differentially expressed genes was P-value <0.05

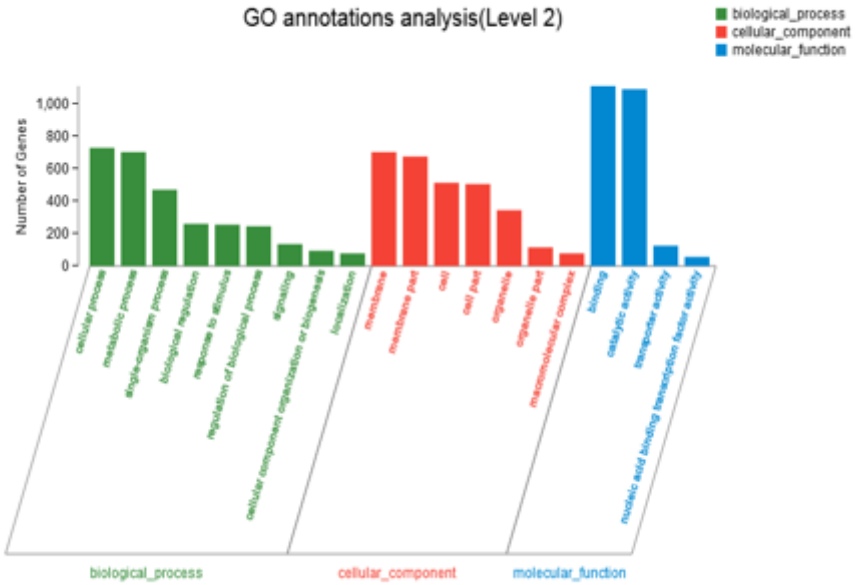


Figure 4

GO classification of assembled unigenes. A total of 4,442 unigenes were categorized into three main categories: biological process, cellular component, and molecular function

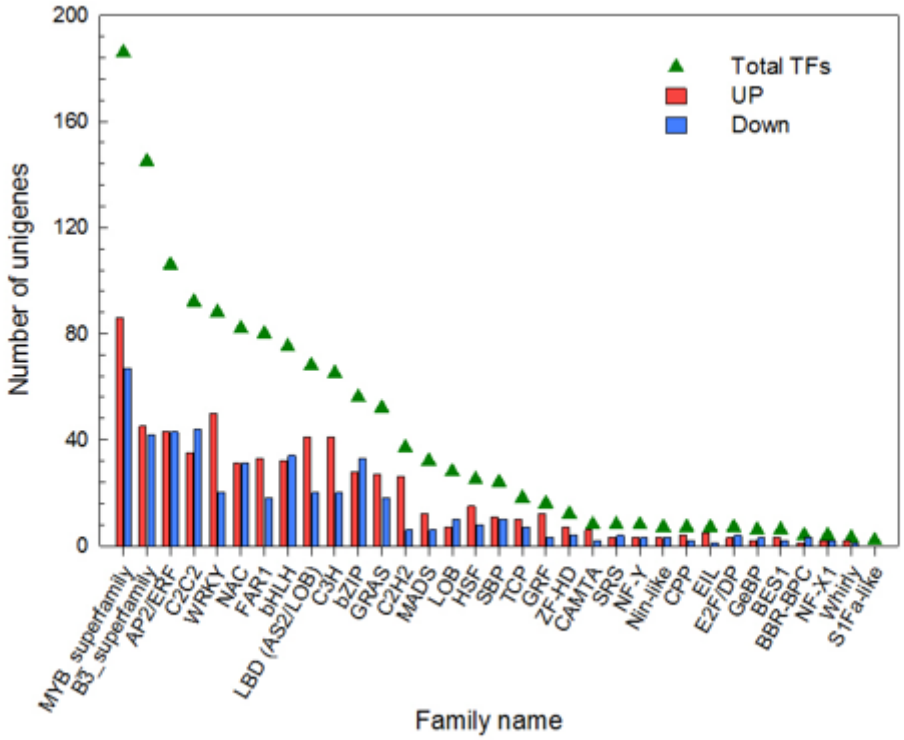


Figure 5

Distribution of differentially expressed transcription factors (TFs) families

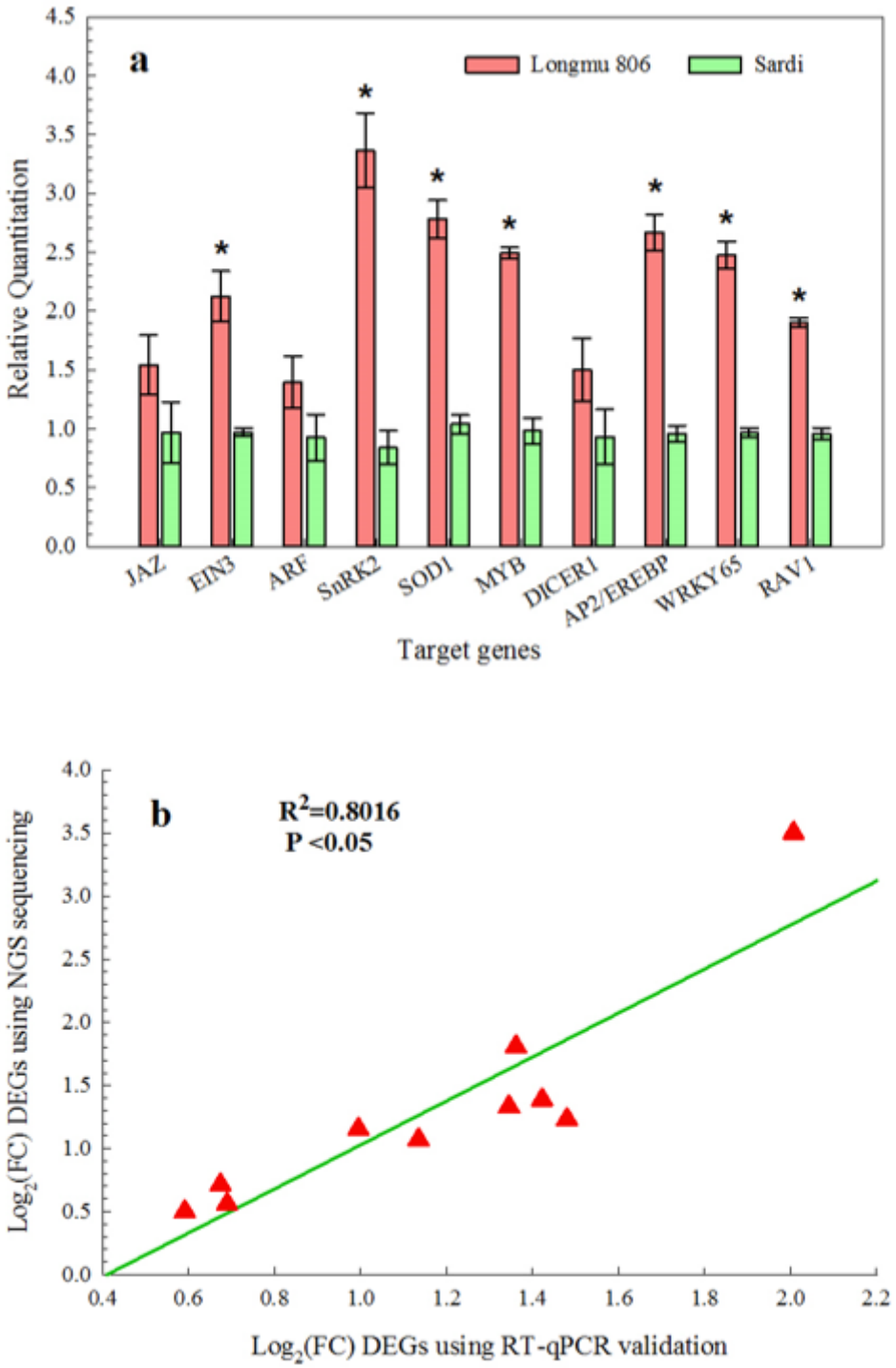


Figure 6

a Validation of transcriptome sequencing data by RT-qPCR analysis. Expression analysis of the response of 10 genes to low temperature stress by RT-qPCR. The star (□) within the figure show the significant difference (P<0.05) b Scatter plot shows expression changes (log2 Fold Change) measured by RNA-seq and by RT-qPCR analysis of the selected genes under low temperature stress

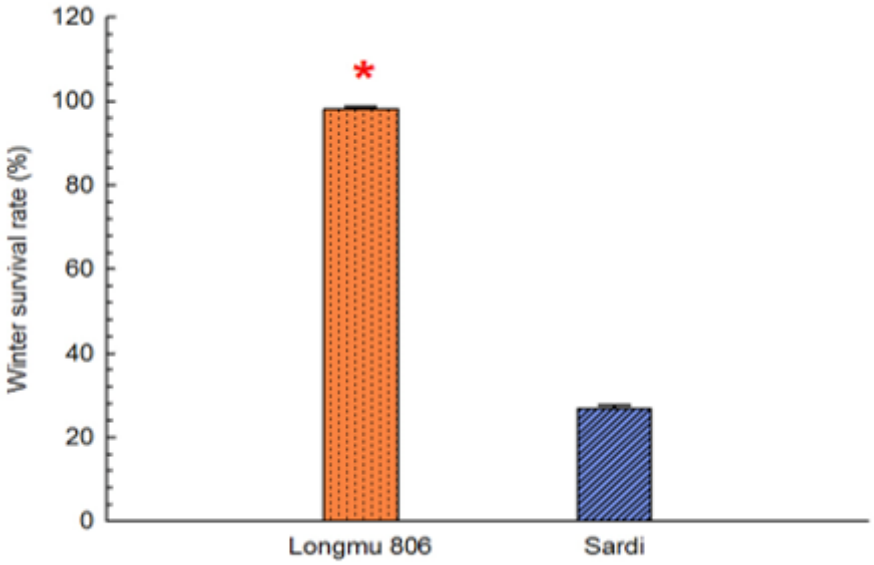


Figure 7

Comparison of winter survival rate of two alfalfa varieties. The star (□) within the figure show the significant difference ($P < 0.05$)

Supplementary Files

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

- TableS5.pdf
- TableS2.pdf
- TableS3.pdf
- TableS1.pdf
- TableS4.pdf