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RESEARCH

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Transcriptome analysis reveals candidate genes for different root types of alfalfa (*Medicago sativa*) after water stress induced by PEG-6000

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Abstract

Background We aimed to gain insight into the response mechanism of alfalfa (*Medicago sativa*) to drought stress by recognizing and analyzing drought-responsive genes in the roots of different root types of alfalfa. The rhizomatous-rooted *M. sativa* cv.'Qingshui' (QS), tap-rooted *M. sativa* cv.'Longdong' (LD), and creeping-rooted *M. varia* cv. 'Gongnong No. 4' (GN) were used to analyze the transcriptome information and physiological characteristics of the root systems of the cultivars under simulated drought stress using PEG-6000.

Results It was found that aridity caused a significant increase in the content of osmotic stress substances and antioxidant enzyme activity. The content of malondialdehyde (MDA) in QS was lower than that in LD and GN under moisture stress, indicating a stronger accumulation capacity of osmotic regulatory substances. Based on sequencing results, 14,475, 9336, and 9243 upregulated DEGs from QS, LD, and GN were annotated into 26, 29, and 28 transcription factor families, respectively. QS showed more DEGs than LD and GN. KEGG enrichment analysis identified that DEGs were significantly enriched in metabolic pathways such as amino acid biosynthesis, phenylpropanoid biosynthesis, plant hormone signaling transduction, and MAPK pathways. This suggests a strong correlation between these pathways and drought stress. The results also show that genes associated with ABA hormone signaling (*MS*. gene93372, *MS*. gene072046, and *MS*. gene012975) are crucial for plant's adaptation to drought stress.

Conclusions These genes, such as serine/threonine protein kinases and abscisic acid receptors, play a crucial role in plant hormone signaling and MAPK pathways. They could serve as potential candidate genes for drought resistance research in alfalfa, providing a molecular foundation for studying drought resistance.

Keywords Alfalfa, Drought stress, Transcriptome, Physiological characteristics

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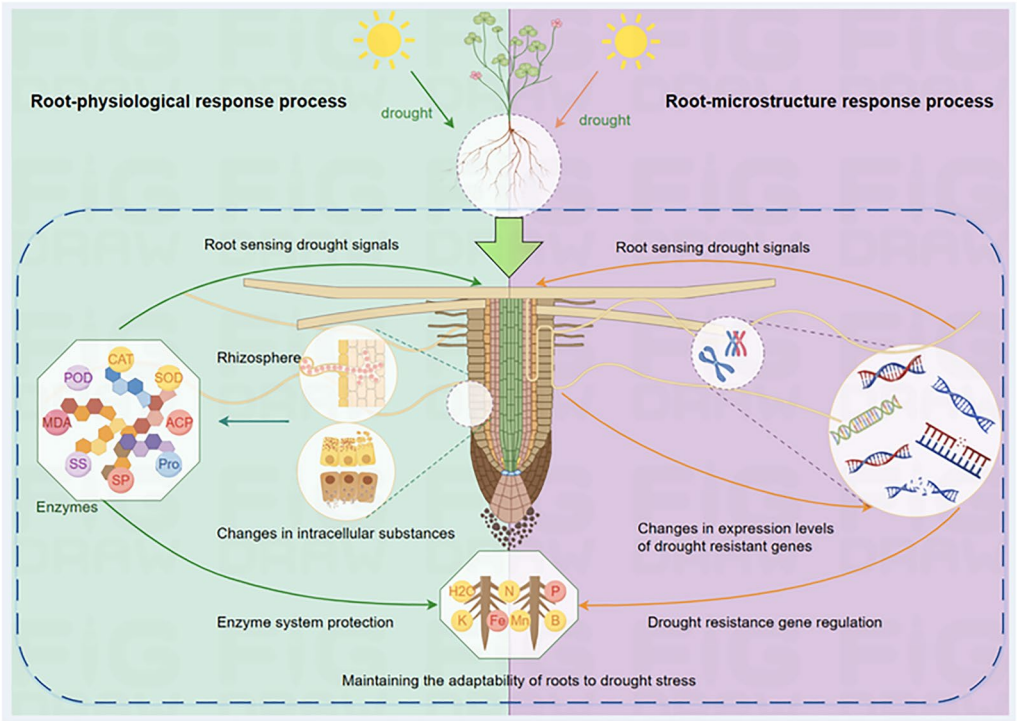
Graphical Abstract



Introduction

Medicago sativa or alfalfa, a high-quality legume forage, could be used as a protein source for livestock and improve soil fertility by fixing nitrogen in the air [1]. Over 4×10^7 hectares of land worldwide were planted with alfalfa [2, 3]. Alfalfa was capable of great drought resistance due to its more developed root system compared to other forage species [4]. Nevertheless, the primary factor impeding alfalfa output continues to be drought [5]. The northern regions of China, such as Gansu, Inner Mongolia, Ningxia, Shaanxi, and Xinjiang have emerged as the primary production areas for alfalfa, accounting for 70% of the nation's total planting area [6]. Meanwhile, these regions also experienced climate conditions of drought and low rainfall, which significantly affected stable production and economic benefits. Due to the incapacity of domestic cultivation and production to meet demand, alfalfa hay imports currently account for a substantial portion of the domestic market's needs [7]. Therefore, it was crucial to investigate how alfalfa responds to water deficits to improve its drought resistance and dry matter production.

Plants have developed a unique set of mechanisms to withstand and adapt to aridity, enabling them to cope with the complex and continuously changing natural environment over a prolonged evolutionary process [8]. Plants respond to water deficits through a complex network of genes, including regulatory genes and functional genes, that work together to regulate their growth. These regulatory mechanisms include stomatal movement, the clearance of active oxygen, stress hormones, the synthesis of osmoregulatory substances, energy metabolism, and phenotypic changes [4, 9–11]. For instance, drought disrupts the balance between the production and elimination of active oxygen. Excessive accumulation can lead to cell membrane damage, nucleic acid destruction, protein oxidation, and seriously affect normal metabolism [12, 13]. As a result, plants could significantly increase the activity of related enzymes like catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) within their systems to mitigate oxidative harm triggered by arid conditions [14].

Transcriptome sequencing technology emerged in 2008, which makes it possible to investigate the mechanisms behind plants' responses to stress and the genes associated with stress alleviation by examining the expression of differentially expressed genes (DEGs) in plants under specific time and condition sets [15–17].

The application of this research method has screened many genes and transcription factors (TFs) related to drought resistance in alfalfa. TFs involved in regulating drought response in alfalfa mainly include families such as NAC, WRKY, bHLH, MYB, and B3 [18]. When Fang et al. originally constructed a full-length transcriptome of drought-stressed alfalfa roots, they found that the majority of DEGs were primarily concentrated in the families of AP2/ERF-ERF, C2H2, bHLH, and bZIP, followed by enrichment in the C3H, MYB, WRKY, GRAS, and NAC families [19]. Consequently, there has been plenty of enthusiasm for investigating gene families, such as the PLD, PYL/RCAR, and ZF-HD gene families [20–23]. Many DEGs involved in alfalfa's drought response are linked to numerous stress processes, such as sugar metabolism, lignin, and wax biosynthesis [4]. Genes associated with abscisic acid metabolism, root growth, antioxidant enzyme activity, cell membrane stability, ubiquitination, and genetic processing all respond to drought stress [24–26], and expression patterns of DEGs involved in the abscisic acid and auxin hormone signaling pathways have been found to change under stress in numerous studies [27, 28]. Alfalfa circular RNA (circRNA) and long noncoding RNA (lncRNA) exhibit diversity under drought and high salt stress, which may be key node genes regulating stress [29]. Various genotypes or varieties of alfalfa may respond to drought in multiple ways, and some have been demonstrated to perform well under such circumstances [30]. Previously, research on alfalfa's drought-resistant adaptation mechanism focused mainly on the aboveground parts, with studies on the root system only recently gaining prominence [5, 31].

Alfalfa's root systems are classified into four types: rhizomatous-rooted, tap-rooted, creeping-rooted, and branch-rooted [32]. The tap-rooted variety of alfalfa has notably developed main roots that are immediately distinguishable from lateral roots due to their thickness and length. Rhizomatous-rooted alfalfa can grow horizontally from transverse stem nodes originating from the central axis of the roots and sprout new shoots to grow upwards, forming new branches. Unlike rhizomatous-rooted alfalfa, creeping-rooted alfalfa can sprout adventitious buds from the shallow root neck and extend to the surface to form new branches. Nevertheless, rhizomatous alfalfa only has a 60% tiller plant rate due to the influence of gene sources and the ecological environment (Fig. 1). The development of the root system in branch-rooted alfalfa is affected by the growing environment, planting density, growth time, and soil texture [33, 34]. Currently, no branch-rooted alfalfa varieties have been cultivated in China.

The various drought resistance mechanisms of alfalfa are determined by its genes. Therefore, it is crucial to explore the relationship between its specific gene expression and drought resistance. There has been a general consensus that drought severely restricts the development of the alfalfa industry. Improving the drought resistance traits of alfalfa through molecular biology methods and techniques to develop new varieties with high yield, high resistance, and high quality is a more direct and effective approach to carrying out alfalfa breeding programs. For this reason, this work examined the DEGs of three root types of alfalfa using high-throughput sequencing techniques under drought stress. The analysis focused on signal transduction, regulation, metabolic mechanisms, and exploring drought resistance-related

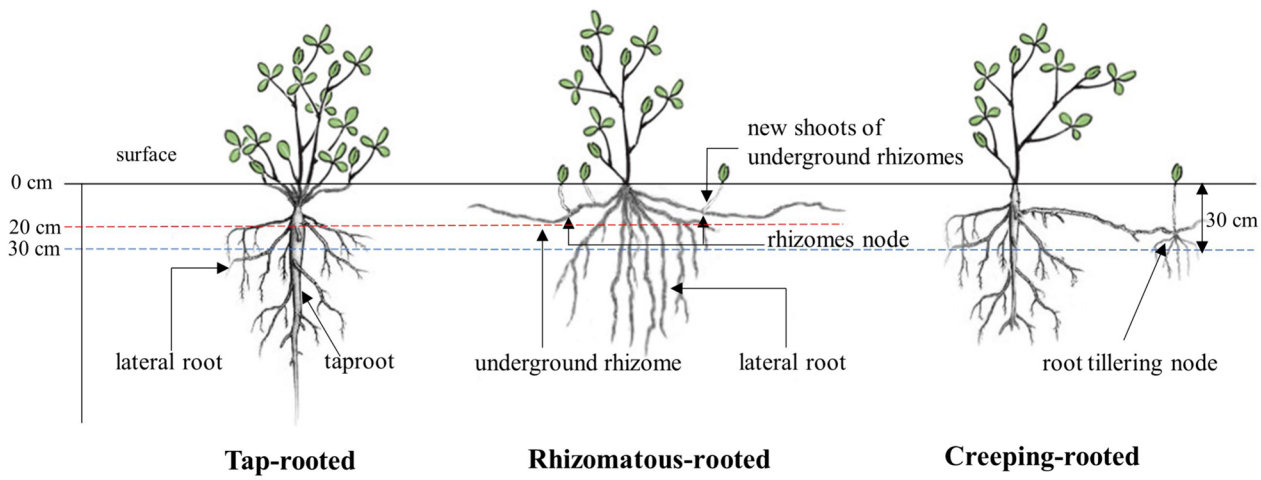


Fig. 1 Root structure diagram of alfalfa showing different types of roots

genes to lay the foundation for understanding the molecular mechanisms of alfalfa in response to drought stress.

Materials and methods

Plant materials and experimental conditions

The rhizomatous-rooted *M. sativa* 'Qingshui' (QS), taproot *M. sativa* 'Longdong' (LD), and creeping-rooted *M. varia* 'Gongnong' No. 4 (GN) of alfalfa were used in the experiment. After disinfecting the seeds with 5% NaClO, they were sown in pots to ensure that the number of seeds sown in each pot is consistent. After sprouting, thinning was carried out to have 20 evenly distributed seedlings in each pot. The pot were then placed in the growth chamber (YSTH-B8-20, EshengTaiHe Ctrl Tech, China). The growth conditions included a16 h: 8 h of light: dark roation, relative humidity of 60%, and a light flux density of 450 mol m^{-2} s^{-1}. The necessary water and nutrients for seedling growth were supplied using Hoagland's nutrient solution [35].

Treatment of stress

After more than 60 days of growth, the average height of alfalfa seedlings reached about 40 cm, and PEG-6000 stress was applied on July 10, 2021. The PEG-6000 was dissolved in the Hoagland nutrient solution in proportion and watered into the corresponding pots to induce osmotic stress on alfalfa. The dissolution of PEG-6000 had three levels of osmotic stress on alfalfa: 0 MPa (control, CK), − 1.0 MPa (moderate drought, M), and -2.0 MPa (severe drought, S) [36]. The experiment started on May 10, 2021, and ended on July 17.

Sample collection

After 7 days of stress treatment, significant changes were observed in the aboveground morphological characteristics of alfalfa, and samples were collected on June 17, 2021. The root system was separated from the aboveground part, dug up and then thoroughly washed. Subsequently, multiple repeated samples of the same treatment were promptly frozen in liquid nitrogen (stored at − 80 °C) for subsequent indicator determination [7].

Determining the concentration of physiological and total flavonoids

The malondialdehyde (MDA) was determined using the method of Christou et al. [37]. Proline (Pro) was determined following Bates et al.'s method [38]. Soluble sugar (SS) and soluble protein (SP) were analyzed using the methods of Bradford and Buysse et al. [39, 40]. Superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activities were measured following the methods of Meloni et al. and Wassie et al. [41, 42]. The colorimetric technique with aluminum chloride was used to determine the total flavonoid content [43, 44]. All the above indicators were measured three times with biological replicates.

RNA extraction and sequencing

RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The construction and sequencing of cDNA libraries were conducted by the Beijing Nuohe Zhiyuan Technology platform. Total RNA was enriched with polyA-tail mRNA using Oligo (dT) magnetic beads and then randomly interrupted with divalent cations in a fragmentation buffer. The first cDNA strand was synthesized using the M-MuLV reverse transcriptase system with fragmented mRNA as a template, followed by the synthesis of the second cDNA strand using dNTPs in the DNA polymerase I system. The cDNA was screened at approximately 370–420 bp, subjected to PCR amplification, and purified using AMPureXP beads to obtain the library. The insert size of the library was assessed using an Agilent 2100 Bioanalyzer after library construction, and qRT-PCR was used to accurately quantify the effective concentration of the library (the effective concentration should be higher than 2 nM) to ensure library quality. Three biological replicates were processed of each sample transcriptome during execution. The sequencing data have been uploaded to the NCBI website (BioProject ID: PRJNA1098431).

Differential expression gene screening

Gene's relative expression levels were estimated using the RPKM (Reads Per Kilobase Per Million Reads) technique. DEGs were screened using the DESeq technique, with a threshold padj < 0.05 and $\log_2 X$ [X = (Fold Change, FC)] > 2.

qRT-PCR analysis

qRT-PCR analysis was conducted using the Roche LightCycler® 96 real-time fluorescence quantitative PCR system (Roche LightCycler® 96, Switzerland). Primers were designed using Primer 5.0 (Table 1). Reaction procedure: 94 °C for 30 s, 98 °C for 10 s, 55 °C for 30 s, 72 °C for 1 min, 35 cycles, 72 °C for 2 min. The $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels [45].

Analysis of the WGCNA co-expression module

WGCNA is a systematic biological method commonly used to describe gene association patterns between different samples as well as causal relationships between module features and phenotypes [46, 47]. It involves selecting appropriate thresholds in the construction of the co-expression network through WGCNA analysis, and identifying the module with the highest weight and

Table 1 Primers used in qRT-PCR

Gene ID	Primer 5′–3′	
MS.gene012975	F:ATGAATTTCAAGGGTTTTGG	R:TTAGCATAACTTCTGACCAAATG
MS.gene26446	F:ATGAACTTCGCTTGGGATG	R:CTACCATGGACCTGTAAGTGT
MS.gene29514	F:CTGGAAGTCAACGATGGAGAAGAG	R:ACGGCAACAACAGCAGTAGAAC
MS.gene38710	F:ATGAATTTCAAGGGTTTTGG	R:TTAGCATAACTTCTGACCAAATGTT
MS.gene040004	F:ATGCCATCAAGTTTTTCTCTTCAGC	R:TTATTGCTCGGAGTTGTTTCTG
MS.gene072046	F:ATGGAGAAGTACGAGGTGGTTAAGGAT	R:TTAGTTGACATGGATTTCTCCGCTTTC
MS.gene95282	F:ATGAACAACGGTTGTGAACAACAACAG	R:TTATGGGTTAATATTGATAGGATCGGT
MS.gene93372	F:ATCACCAACCACCACGACCTC	R:GACGACCGCCGAGCATAATTG
Actin 2	F:AAAAGGATGCCTATGTTGGTG	R: TAAGTGGAGCCTCAGTTAGAAGTA

the core genes selected from the optimal module ($P < 0.05$ and $\log_2 X > 2$). Finally, significantly enriched functions and signaling pathways were selected [48]. This paper utilizes an online website (https://cloud.majorbio.com/page/tools/) to conduct WGCNA analysis of DEGs and phenotypes.

Statistical analysis

Excel 2019 was used to statistically analyze and screen genetic data. Significant difference in physiological indicators between leaves and roots under different stress gradients were determined using one-way analysis of variance. Origin 2021 and R (Version 3.5.0) software was used for the above statistical analysis and mapping.

Results

Quality assessment of transcriptome data

Illumina sequencing was used to analyze the alfalfa roots after PEG-6000 stress. QS, LD, and GN generated raw read numbers of QS, LD and GN ranged from 42295080 ~ 49738736, 41205642 ~ 46098132, and 41824044 ~ 50649804, respectively (Table 2). The number of clean reads ranged from 40954086 ~ 48104590, 38388564 ~ 44808706, and 39350418 ~ 49168332. The error rate remains below 3%, with Q30 above 92.90%, 93.17%, and 93.22%. The GC content ranged from 42.01% ~ 43.48%, 41.79% ~ 44.55%, and 42.14% ~ 43.76%. Meeting the requirements for database construction.

Analysis of DEGs in different root types of alfalfa

To identify PEG-6000 stress response genes in three root types of alfalfa, we analyzed DEGs in the root transcriptome dates after stress treatment (Fig. 2 and Fig. 3). Groups M and S obtained 16,260 and 14,475, 6920 and 9336, and 9295 and 9243 upregulated DEGs in QS, LD, and GN, accounting for 52.85% and 49.82%, 53.19% and 46.56%, and 46.65% and 43.96% of the total DEGs, respectively. The number of DEGs showed more downregulation than upregulation in QS, LD, and GN under S stress. The number of unique DEGs 6419, 5319, and 6247 in QS, LD, and GN caused by PEG-6000 stress, respectively (Fig. 4). QS contained the most unique genes, which were 20.68% and 2.75% higher than LD and GN, respectively, while LD has the least unique genes.

GO enrichment of DEGs in different root types of alfalfa

GO functional enrichment analysis was performed on significantly enriched DEGs (Fig. 5), which were enriched in biological processes, cellular components, and molecular functions. The DEGs of QS were significantly enriched in 13 subcategories of 3 major categories (accounting for 36.30%, 10.25%, and 53.45%). Annotate biological processes such as response to acid chemical (17) and oxygen-containing compounds (17), cellular components such as extracellular region (65) and apoplast (48), and additionally, molecular functions include antioxidant (181) and peroxidase (171) activity. The DEGs of LD were enriched in 9 subclasses of biological processes and molecular functions (accounting for 65.20% and 34.80%). The former included the nicotianamine metabolic process (11) and the tricarboxylic acid biosynthetic process (74). The latter included nicotianamine synthase activity (30) and carboxylic acid binding (30). The DEGs of GN were significantly enriched in 24 subcategories of 3 major categories (accounting for 42.88%, 21.61%, and 36.50%). They were biological processes such as polysaccharide metabolism (113) and cellular glucan metabolism (99), as well as cellular components such as apoplast (57) and extracellular region (72). Molecular functions included xyloglucosyl transferase activity (57) and glucosyltransferase activity (105). GO enrichment analysis revealed that QS, LD, and GN were mostly associated with antioxidant enzyme activity, organic acid and amino acid metabolism, and carbohydrate metabolism in response to PEG-6000 stress.

Table 2 Sequencing data quality evaluation

Sample	Raw reads	Clean reads	Clean bases(G)	Error rate/%	Q30/%	GC/%
QS-CK1	49738736	48104590	7.22G	0.03	92.90	42.09
QS-CK2	42295080	40954086	6.14G	0.03	93.22	42.01
QS-CK3	44404126	43142002	6.47G	0.03	93.29	42.15
QS-M1	43571776	42363038	6.35G	0.03	93.34	42.35
QS-M2	48023774	46383080	6.96G	0.03	93.29	42.08
QS-M3	46717846	45298850	6.79G	0.03	93.47	42.38
QS-S1	48443752	47345,924	7.10G	0.03	93.31	43.82
QS-S2	49417132	47965114	7.19G	0.03	93.41	42.19
QS-S3	49044460	48,064126	7.21G	0.03	93.49	43.48
LD-CK1	46098132	44808706	6.72G	0.03	93.69	43.11
LD-CK2	44746788	43350280	6.50G	0.03	93.17	43.40
LD-CK3	42874662	40676240	6.10G	0.03	93.46	44.55
LD-M1	41791026	39298696	5.89G	0.03	93.67	42.43
LD-M2	42831566	39893498	5.98G	0.03	93.63	41.79
LD-M3	43979520	41926712	6.29G	0.03	93.58	43.41
LD-S1	41205642	38388564	5.76G	0.03	93.60	42.12
LD-S2	41625256	40142472	6.02G	0.03	93.93	42.61
LD-S3	43372678	42,490936	6.37G	0.03	93.64	43.43
GN-CK1	42542450	41329922	6.20G	0.03	93.81	42.81
GN-CK2	41824044	39350418	5.90G	0.03	93.94	42.14
GN-CK3	50649804	49168332	7.38G	0.03	93.62	43.76
GN-M1	44582194	43224728	6.48G	0.03	93.22	42.80
GN-M2	48293874	46770928	7.02G	0.03	93.42	42.37
GN-M3	47298264	45573462	6.84G	0.03	93.30	42.81
GN-S1	43212272	41764264	6.26G	0.03	93.70	42.69
GN-S2	43972,484	41743872	6.26G	0.03	93.62	42.62
GN-S3	42283948	40422082	6.06G	0.03	93.76	42.42

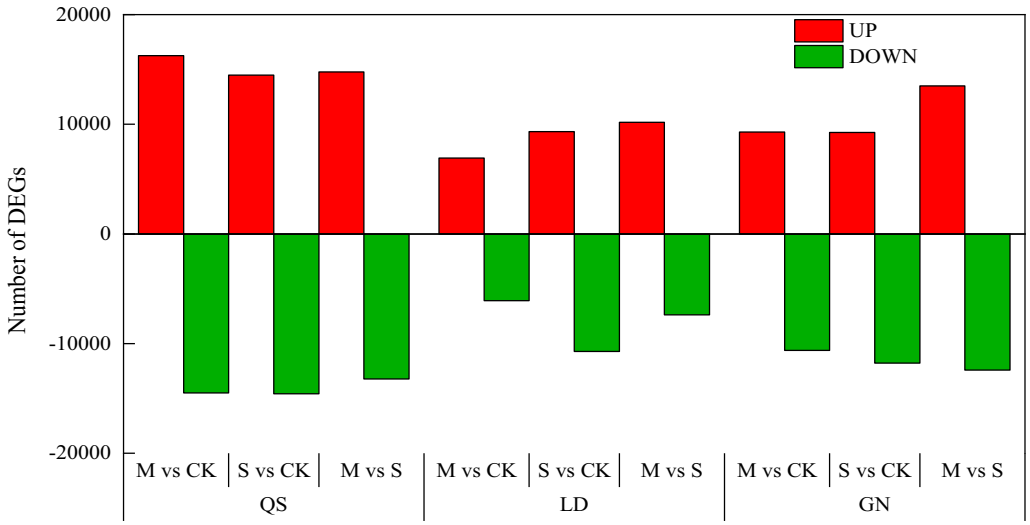


Fig. 2 Expression analysis of DEGs of different root types of alfalfa under drought. The horizontal axis in the figure represents each treatment, and the vertical axis represents the number of DEGs. Red and green represent upregulated and downregulated genes, respectively. QS: rhizomatous-rooted *Medicago sativa* 'Qingshui'; LD: *M. sativa* tap-rooted 'Longdong'; GN: creeping-rooted *M. varia* Martin 'Gongnong No.4'. CK: control; M: medium stress; S: severe stress

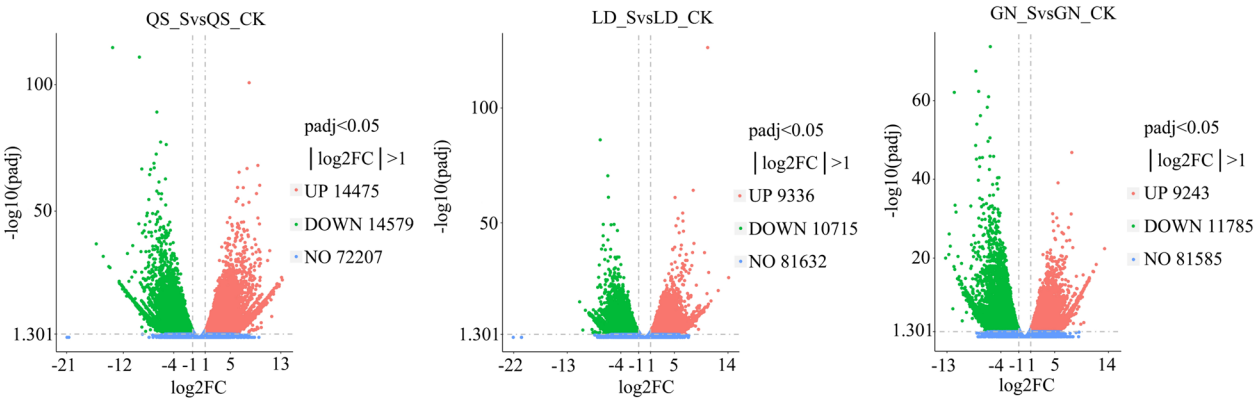


Fig. 3 Volcano plot of DEGs. The horizontal axis in the figure represents the log_2FoldChange value, and the vertical axis represents -log_{10}padj or -log_{10}pvalue. The dashed line represents the threshold line for screening differential genes. Red and green represent upregulated and downregulated genes, respectively. QS: rhizomatous-rooted *Medicago sativa* 'Qingshui'; LD: *M. sativa* tap-rooted 'Longdong'; GN: creeping-rooted *M. varia* Martin 'Gongnong No.4'. CK: control; M: medium stress; S: severe stress

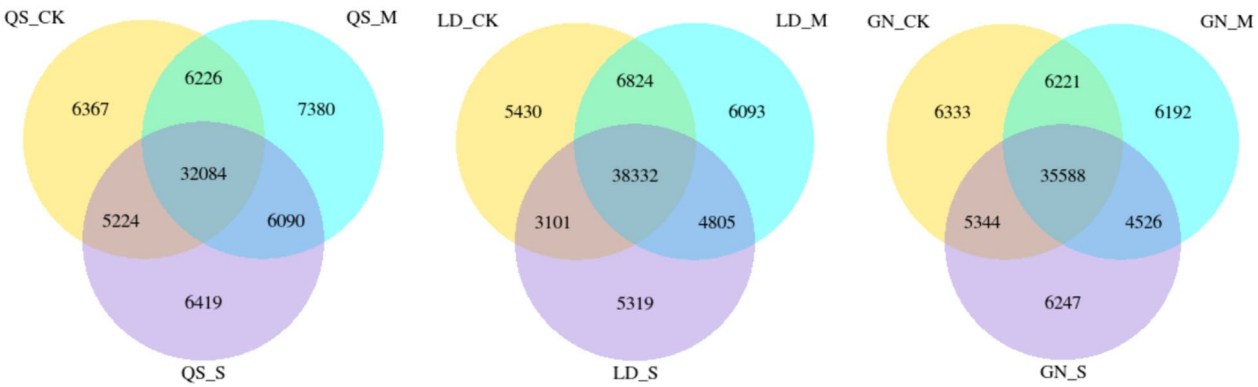


Fig. 4 DEGs Venn diagram. QS: rhizomatous-rooted *Medicago sativa* 'Qingshui'; LD: *M. sativa* tap-rooted 'Longdong'; GN: creeping-rooted *M. varia* Martin 'Gongnong No.4'. CK: control; M: medium stress; S: severe stress

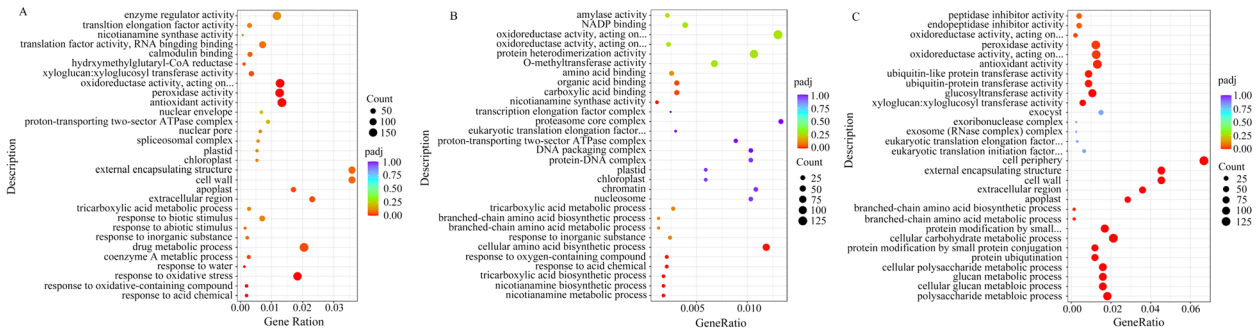


Fig. 5 GO enrichment analysis. The horizontal axis in the figure represents the ratio of the number of DEGs annotated on GO term to the total number of DEGs, while the vertical axis represents GO term. QS: rhizomatous-rooted *Medicago sativa* 'Qingshui' (4A); LD: *M. sativa* tap-rooted 'Longdong' (4B); GN: creeping-rooted *M. varia* Martin 'Gongnong No.4' (4D)

KEGG pathway enrichment of DEGs in different root types of alfalfa

KEGG enrichment analysis revealed that QS DEGs were enriched in pathways (Fig. 6) such as starch and sucrose metabolism (61), arginine and proline metabolism (32), linoleic acid metabolism (14), phenylpropanoid biosynthesis (60), carbon fixation (23), and similar alpha linolenic acid metabolism (23). The metabolic pathways that were markedly enriched in LD mainly included oxidative phosphorylation (34), amino acid biosynthesis (61), spliceosomes (58), plant hormone signaling transduction (58), and the MAPK pathway-plants (37). DEGs were significantly enriched in metabolic pathways such as brassinosteroid biosynthesis (5), RNA transport (52), plant–pathogen interaction (54), and the MAPK pathway (46) in GN. The mentioned metabolic pathways could have crucial to how alfalfa responded to PEG-6000 stress.

Analysis of differentially expressed TFs in different root types of alfalfa

Different transcription factor (TF) families' gene expression levels fluctuated in response to PEG-6000 stress. 772 DEGs from 26 TF families were identified in QS. Among them, 374 exhibited upregulation, while the remainder showed downregulation. A total of 736 DEGs from 29 TF families were found in LD, with 364 upregulated and 372 downregulated. There were 794 DEGs from 28 TF families in GN, with 326 upregulated and 468 downregulated. The top 10 TF families with the most DEGs were AP2/ERF, MYB, C2, bZIP, WRKY, GRAS, SNF2, FAR1, SET, and B3 (Fig. 7). The TF families with the highest number of upregulated DEGs expressions were C2H2, MYB, AP2, and WRKY in QS, LD, and GN.

Screening of TFs involved in the PEG-6000 stress response of different root types of alfalfa

In order to further explore the key regulatory factors of different root types of alfalfa in response to PEG-6000 stress, we conducted a detailed screening of the transcriptome data (Table 3). By analyzing the significant metabolic pathways and annotation results by DEGs, we identified 8 TFs from 5 families, all of which showed upregulation during stress. The fold changes belonging to the MYB (*MS.* gene020647) and WRKY (*MS.* gene058475) families were above 9.51. Both of them exhibited high expression under drought stress. Therefore, we hypothesized that they might be key players in controlling alfalfa's adaptability to water-deficient conditions, which warrants further validation.

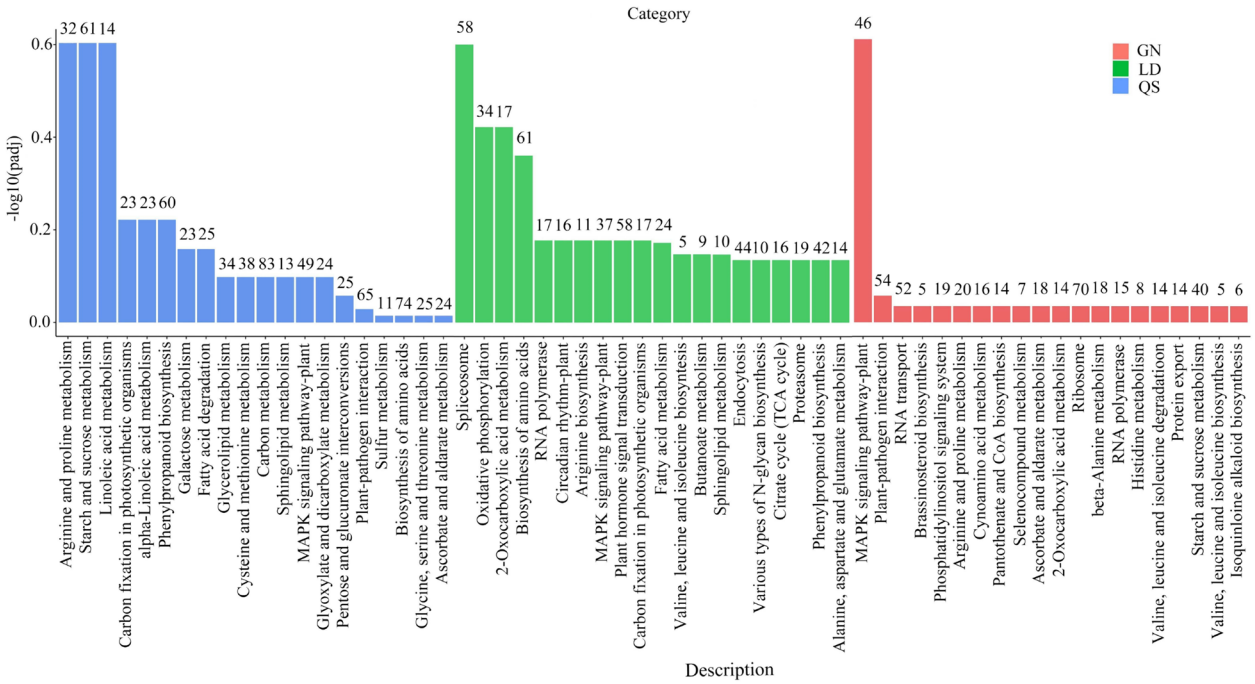


Fig. 6 KEGG enrichment analysis. The horizontal axis in the figure represents the KEGG pathway, and the vertical axis represents the significance level of pathway enrichment. QS: rhizomatous-rooted *Medicago sativa* 'Qingshui'; LD: *M. sativa* tap-rooted 'Longdong'; GN: creeping-rooted *M. varia* Martin 'Gongnong No.4'. CK: control; M: medium stress; S: severe stress

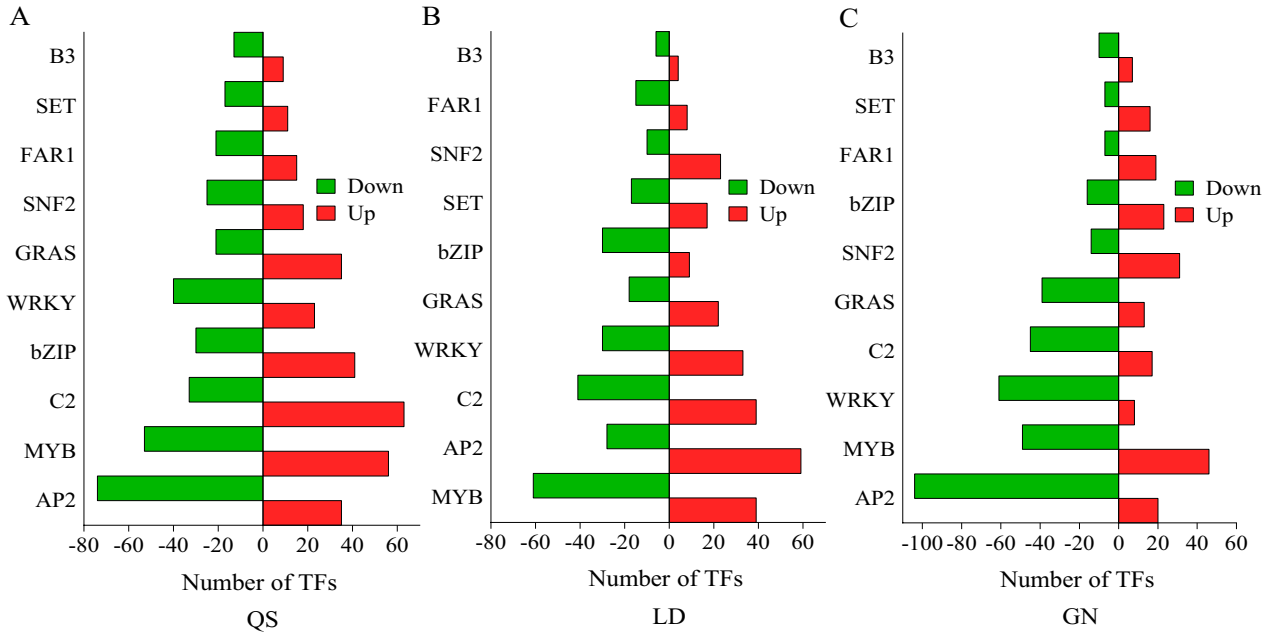


Fig. 7 Statistics of differential expressed TFs. The horizontal axis in the figure represents the number of genes, while the vertical axis represents the transcription factor family. Red and green represent upregulated and downregulated genes, respectively. QS: rhizomatous-rooted *Medicago sativa* 'Qingshui'; LD: *M. sativa* tap-rooted 'Longdong'; GN: creeping-rooted *M. varia* Martin 'Gongnong No.4'. CK: control; M: medium stress; S: severe stress

Table 3 Differentially expressed TFs of different root types of alfalfa under drought stress

Gene ID	Log_2FC	Family	Gene description
MS.gene020647	10.06	MYB	Transcription factor MYB36
MS.gene57543	9.86	C2	C2 domain-containing protein At1g63220
MS.gene36782	9.41	AP2	Ethylene-responsive transcription factor ERF039
MS.gene026023	8.35	bZIP	Light-inducible protein CPRF2
MS.gene042854	7.84	C2	Protein C2-DOMAIN ABA-RELATED 4
MS.gene87956	7.72	AP2	Ethylene-responsive transcription factor RAP2-11
MS.gene058475	9.51	WRKY	WRKY transcription factor 40
MS.gene051664	6.75	AP2	AP2-like ethylene-responsive transcription factor ANT

DEGs screening and qRT-PCR validation of different root types of alfalfa

The DEGs with high expression after PEG-6000 stress were analyzed in the KEGG database. It was discovered that they were enriched in metabolic pathways such as carbon, galactose, and linoleic acid metabolism; plant hormone signaling; N-glycan biosynthesis; oxidative phosphorylation; and phenylpropanoid biosynthesis in three root types of alfalfa (Table 4). We focused on DEGs with high fold changes involved in the plant hormone signaling pathways within these metabolic pathways. We specifically selected genes that were significantly upregulated in this pathway, such as ABA hormone signaling-related genes (*MS.* gene93372, *MS.* gene012975, *and MS.* gene072046), and hypothesized that these genes might play a role in water utilization and osmotic regulation during water deficiency (Table 5).

The expression of 8 genes was randomly detected using the qRT-PCR method. The findings demonstrated that these genes corresponded with the expression trend of DEGs in transcriptome sequencing (Fig. 8), indicating that the information obtained from transcriptome sequencing in this study was highly reliable. However, only a few genes had different expression trends. For example, the expression levels of ABA-responsive element binding proteins (*MS.* gene012975 and *MS.* gene38710) and protein

Table 4 DEGs of different root types of alfalfa under drought stress

Gene ID	log_2FC	Gene description
MS.gene89315	6.37	Aldo-keto reductase family 4 member C9
MS.gene037735	8.51	NAD-dependent malic enzyme 62 kDa isoform, mitochondrial
MS.gene69076	8.32	ATP-dependent 6-phosphofructokinase 3
MS.gene93372	6.04	Abscisic acid receptor PYL4
MS.gene93870	2.12	Alpha-1,3 1,6-mannosyltransferase ALG2
MS.gene052700	3.70	Linoleate 9S-lipoxygenase
MS.gene024661	7.36	V-type proton ATPase subunit E
MS.gene072046	3.47	Serine/threonine-protein kinase SRK2A
MS.gene88971	7.44	Peroxidase 64
MS.gene012975	4.21	ABA-responsive element-binding protein

Table 5 Statistics of target DEGs and TFs under drought stress

Gene ID	log_2FC	Gene description
MS.gene020647	10.06	Transcription factor MYB36
MS.gene058475	9.51	WRKY transcription factor 40
MS.gene93372	6.04	Abscisic acid receptor PYL4
MS.gene072046	3.47	Serine/threonine-protein kinase SRK2A
MS.gene012975	4.21	ABA-responsive element-binding protein

phosphatase (*MS*. gene29514) in GN were higher than those in QS and LD after stress, while the expression levels of ubiquitin ligase (*MS*. gene26466), abscisic acid receptor PYL9 (*MS*. gene95282), serine/threonine protein kinase (*MS*. gene072046), and abscisic acid receptor PYL4 (*MS*. gene040004) were similar in QS, LD, and GN after stress.

Physiological and total flavonoids responses to PEG-6000 stress

The levels of SP, SS, Pro, MDA, SOD, POD, CAT and total flavonoids in the roots increased as the degree of stress increased (Fig. 9). The content of these indexes increased in QS, LD, and GN after severe stress (by 13.54%, 4.26%, 11.77%; 246.84%, 269.51%, 81.16%; 165.06%, 182.63%, 200.68%; 9.68%, 175.94%, 63.49%; 73.71%, 23.71%, 32.24%; 129.94%, 85.15%, 30.41%; and 20.41%, 29.61%, 23.79%; and 117.46%, 148.94%, 150.79%, respectively). Different root types of alfalfa exhibited varying tendencies; for example, QS exhibited much higher POD activity than LD and GN ($P<0.05$), but MDA displayed the reverse pattern. The CAT activity and SS content of LD were significantly higher than those of QS and GN ($P<0.05$). SOD activity was substantially lower in GN than in QS ($P<0.05$), and the Pro content of GN was significantly higher than in QS and LD ($P<0.05$). In contrast to QS and LD, GN's total flavonoid content increased significantly under − 1.0 MPa and is much higher than that of the other two. The tendency to consist of greater root content than aboveground was observed in all osmotic stress substances and antioxidant enzymes, except for POD.

WGCNA module clustering tree diagram and correlation between each module and traits

WGCNA analysis based on the phenotypes and DEGs expression levels revealed that DEGs were divided into 16 modules (Fig. 10). The modules that were positively correlated with SS, SP, and MDA were MEpurple and MEsalmon, while the modules negatively correlated with MDA were MEgreenyellow, MEturquoise, and MEgrey. SOD, POD, and CAT were positively correlated with the modules MEpurple, MEcyan, MEmidnightblue, MEbrown, MEblack, and MEmagenta. KEGG enrichment analysis revealed that genes in the MEpurple, MEsalmon, MEgreenyellow, MEturquoise, and MEgrey modules were enriched in amino acid biosynthesis, carbon metabolism, N-sugar biosynthesis, and arginine and proline metabolism pathways. The gene modules positively correlated with antioxidant enzymes were enriched in flavonoid and sugar biosynthesis, carbon, linoleic acid, arginine, and proline metabolism; proteasomes, citric acid cycle, and peroxisomes. It was found that the total flavonoid and soluble sugar content in alfalfa roots significantly increased with stress. This suggests that genes involved in regulating flavonoid and sugar synthesis may have a potential regulatory relationship with antioxidant enzyme synthesis.

Discussion

Plant productivity is mostly restricted by drought in low rainfall regions [49]. Plants alter the expression of genes associated with stress responses when encountering water scarcity, thereby regulating cell metabolism and physiological procedures that aid the plant's survival in water-deprived environments [50, 51]. Therefore, studying alterations in the expression of relevant genes in plant tissues offers crucial insights into their molecular response mechanisms and valuable information for developing possible drought resistance strategies.

(See figure on next page.)

Fig. 8 qRT-PCR validation of gene after drought stress. The horizontal axis in the figure represents each treatment, and the vertical axis represents the relative expression level. QS: rhizomatous-rooted *Medicago sativa* 'Qingshui'; LD: *M. sativa* tap-rooted 'Longdong'; GN: creeping-rooted *M. varia* Martin 'Gongnong No.4'. CK: control; M: medium stress; S: severe stress

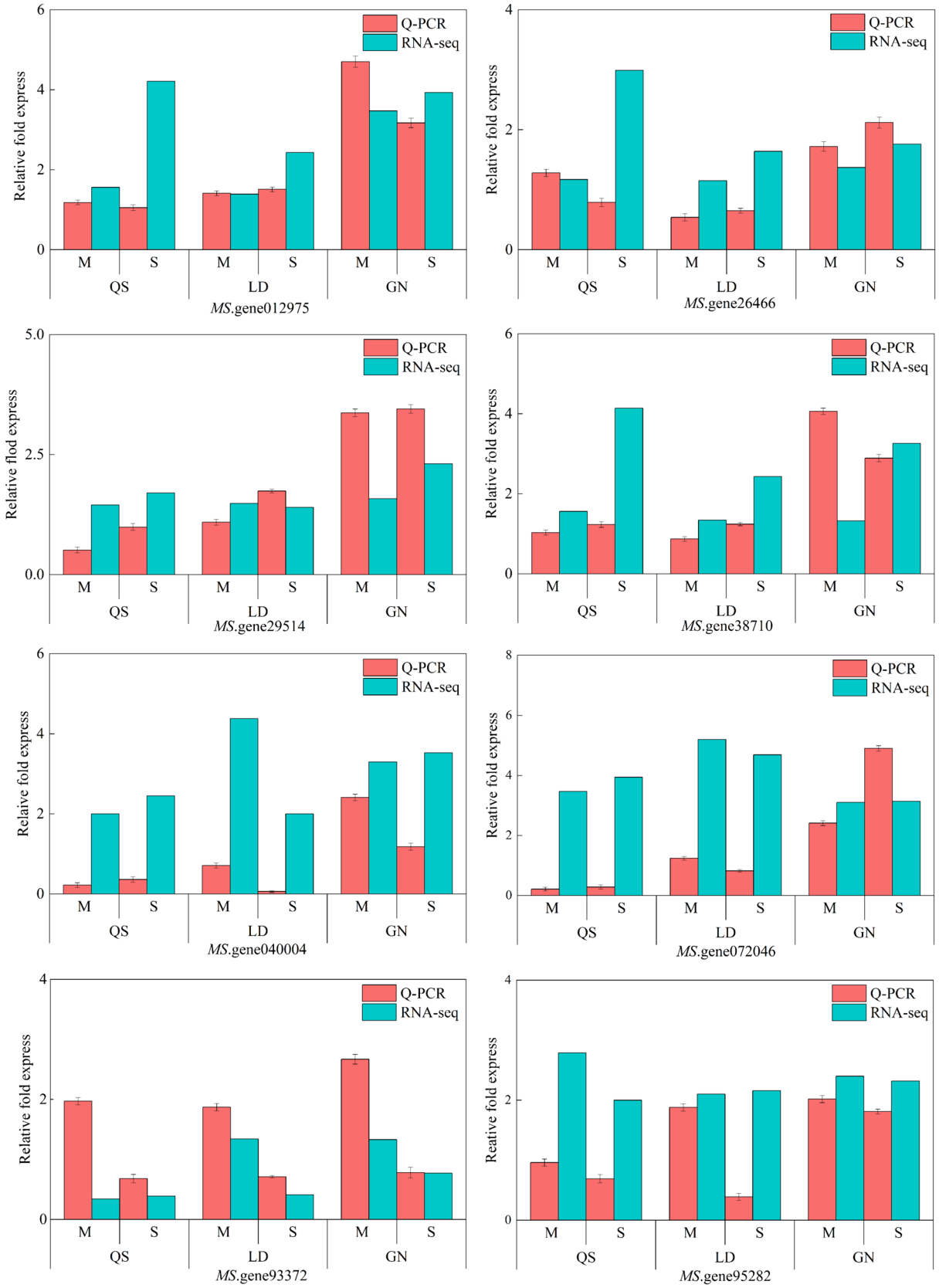


Fig. 8 (See legend on previous page.)

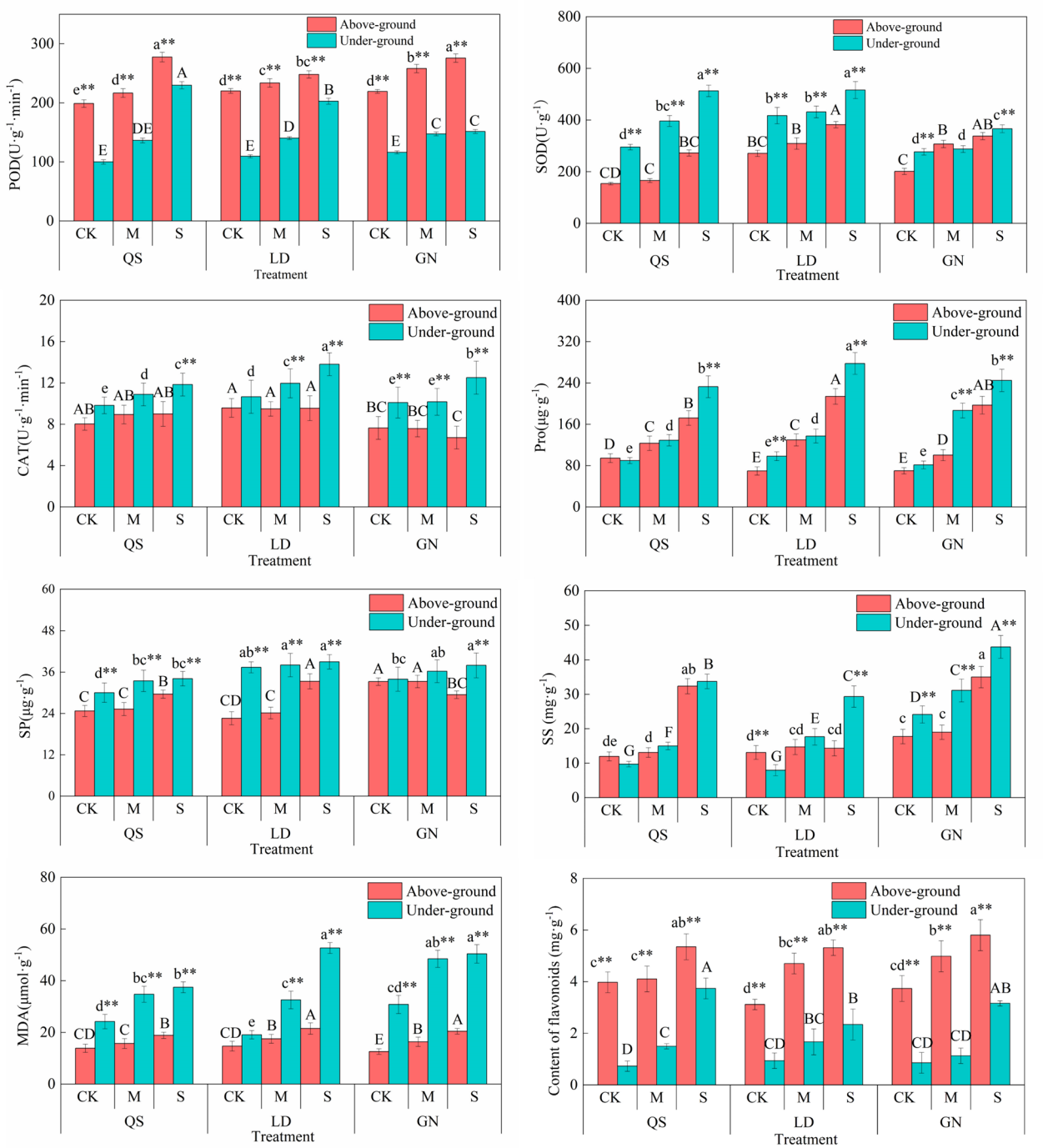


Fig. 9 Effect of physiological characteristics of alfalfa under drought stress. Capital letters and lowercase letters represent the significance of aboveground and underground parts among different treatments, while * represents the significance between aboveground and underground parts under one treatment. Red and blue represent the above ground and underground parts, respectively. QS: rhizomatous-rooted *Medicago sativa* 'Qingshui'; LD: *M. sativa* tap-rooted 'Longdong'; GN: creeping-rooted *M. varia* Martin 'Gongnong No.4'. *CK* control; *M* medium stress; *S* severe stress

According to GO functional enrichment analysis, the DEGs of QS, LD, and GN were markedly enriched in response to oxidative stress, acidic chemicals, and polysaccharide metabolism. This suggests that these pathways are the primary molecular response strategies to cope with oxidative damage induced by reactive oxygen species under PEG-6000 stress. The POD and SOD could eliminate the damage of ROS to cells, while the

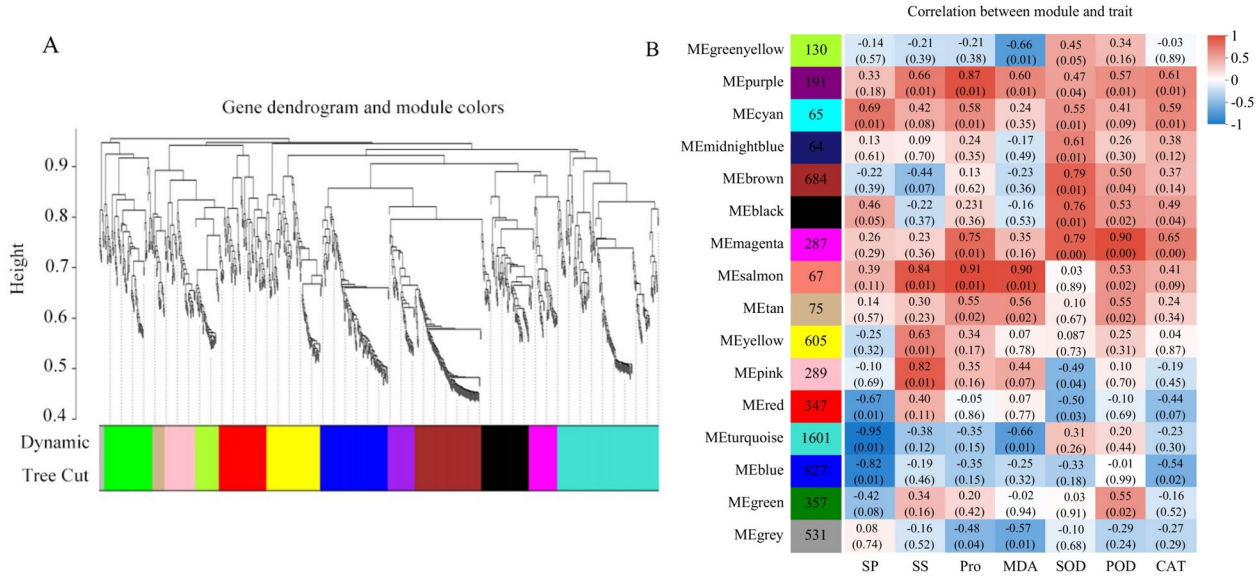


Fig. 10 Cluster dendrogram and module-trait relationships of WGCNA

enhancement of enzyme activity is closely related to gene expression levels. Studies have shown that P5CS (delta-1-pyrroline-5-carboxylate synthesis) is related to the synthesis of POD and SOD, and is highly expressed after alfalfa suffered PEG-6000 stress [3], which aligns with the results of this research (*MS*. gene89313). The main pathways by which plants cope with a lack of moisture include the phenylpropanoid biosynthesis pathway, plant hormone signal transduction, amino acid biosynthesis, and plant–pathogen interaction [18, 52–54]. The present investigation revealed that the DEGs exhibited notable enrichment in starch and sucrose metabolism, phenylpropanoid and amino acid biosynthesis, phytohormone signal transduction, plant–pathogen interaction, and the MAPK pathway.

This demonstrates that the metabolic pathways mentioned above play a crucial regulatory role in defending against PEG-6000 stress in three root types of alfalfa. When plants detect drought signals, they transmit these signals to relevant organelles to trigger gene expression associated with carbohydrate and amino acid metabolism pathways, promoting the activation of metabolism in a manner that supports osmotic regulation [55]. Primarily, small molecules are created through the combination of sugars and amino acids to regulate osmotic pressure[56] and prevent further water loss from plant cells [57]. The present research observed that genes related to sucrose synthase and sucrose transferase were upregulated. This suggests that alfalfa can tolerate drought stress by increasing the concentration of small molecules (glucose) and so enhancing osmotic potential [4]. Previous studies have revealed that drought markedly enriches the phenylalanine biosynthesis pathway, mainly attributed to the metabolism of flavonoids relying on the activities of phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) in this pathway [58]. Similarly, in this study, QS was found to be highly enriched in some DEGs in the phenylpropanoid biosynthesis pathway after exposure to PEG-6000 stress. This suggests that genes related to this pathway undergo expression changes during stress and regulate plant adaptation to water-deficient conditions. Phytohormone signals are significant signal transmitters in plants [59], and these DEGs of the metabolic pathways are markedly enriched in LD alfalfa. This means that hormone signals are particularly important in LD alfalfa's response to water scarcity. Studies indicate that plants detect stress signals and accumulate ABA as one of their initial defense mechanisms [60]. *MS*.gen38710, as a binding factor for ABA response elements and documented in multiple studies, can be phosphorylated by upstream genes to regulate the gene expression in response to arid stress [61, 62]. Its enrichment post-stress makes it a promising candidate for studying drought resistance further. The MAPK cascade pathway is a key signal transduction system in plants, and numerous cis-acting elements associated with responses to biotic and abiotic stress have been identified in many plant species [63]. The relationship between phytohormones and MAPK signaling is intricate, and specific MAPK members can function as upstream regulators to regulate the transport or synthesis of hormones [64]. In this research, the MAPK pathway was markedly enriched in all three root

types of alfalfa, and the annotated DEGs accounted for 6.75%, 7.13%, and 10.11% of the total number of genes in the top 20 metabolic pathways, respectively. This indicates a strong association between the MAPK pathway and plant growth, development, and stress response. TFs are crucial regulatory proteins in plants that respond to various hormones and environmental factors, transmit intracellular signals, and control gene expression. When plants are under drought stress, TFs play an indispensable role [65]. It could also interact with other regulatory factors or bind to cis-elements to control the expression of genes associated with downstream defense mechanisms [66]. When PEG stress treatment was applied to the full-length transcriptome of alfalfa roots, research revealed that TFs were primarily enriched in the FAR1, NAC, bZIP, bHLH, AP2/ERF, WRKY, Myb-related, and MYB families [18, 19, 67]. ERF, AP2/ERF, WRKY, bHLH, MYB, and other families are common TFs that regulate plant growth, development, and environmental adaptation [68–71]. *Arabidopsis* plants overexpressing *TaWRKY1-2D* and *MfERF053* promote root growth and increase the fresh weight of lateral roots under drought stress [72, 73]. The majority of the differential genes in the present research originate from families like AP2/ERF, MYB, C2, bZIP, WRKY, GRAS, SNF2, FAR1, SET, and B3, and there is a great expression of TFs associated with drought resistance. The TFs identified in the aforementioned families enhance alfalfa's tolerance by acting as regulators in the plant's response to arid stress.

Plants possess an intricate signal network that allows them to quickly detect changes in their surroundings rapidly, control specific gene expression, and establish a series of morphological and physiological defense mechanisms. A study found that subjecting *Arabidopsis* to drought stress and overexpressing *IbWRKY2* results in increased SOD activity and decreased MDA and H_2O_2 levels. Simultaneously, there was a great expression of genes linked to the proline biosynthesis pathway, antioxidant enzymes, and the ABA signal transduction system, indicating that the WRKY TF enhances drought tolerance in transgenic *Arabidopsis* [74]. Consequently, the greatly enriched WRKY-TFs may also have the potential to improve the drought resistance of alfalfa in this research. Alfalfa also changes the activity of antioxidant enzymes by regulating the expression of genes associated with them. Increased activity of SOD, POD, and CAT enzymes has the potential to reverse drought stress-related damage [18]. This work revealed that the manufacture of flavonoids is primarily regulated by genes that exhibit a positive correlation with antioxidant enzymes. To enhance alfalfa's resistance to drought, further research could focus on the genes related to antioxidant enzymes and flavonoids.

Conclusion

PEG-6000 stress induced changes in the root transcriptome information of QS, LD, and GN. 14,475, 9336, and 9243 upregulated DEGs were identified and annotated into 26 (QS), 29 (LD), and 28 (GN) TF families, respectively. KEGG annotation of DEGs indicated their involvement in pathways such as phenylalanine biosynthesis, arginine and proline metabolism, amino acid biosynthesis, plant hormone signal transduction, and the MAPK pathway. The TFs *MsMYB36* (*MS*. gene020647) and *MsWRKY40* (*MS*. gene058475), as well as ABA hormone signaling-related genes (*MS*. gene93372 and *MS*. gene072046), identified through further screening of differentially expressed genes, play crucial roles in responding to drought stress. Through WGCNA analysis, it was discovered that the biosynthesis of flavonoids was mostly mediated by genes that positively connect with antioxidant enzymes. These research results provide new directions and ideas for further exploration of drought resistance in alfalfa and can help establish a molecular foundation for investigating drought resistance in alfalfa.

Author contributions
L-LN planned and designed the research. KW analyzed the data, wrote, and revised the manuscript. JX, Y-HY, JC, and J-RC made plentiful valuable comments for the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
This research has been confirmed for publication in the journal.

Competing interests
The authors declare no competing interests.

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