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Research article

Transcriptome analysis and characteristics of drought resistance related genes in four varieties of foxtail millet [*Setaria italica*]

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ABSTRACT

Foxtail millet [*Setaria italica*] plays a crucial role as a multigrain crop in agricultural production. However, due to future extreme weather conditions, drought remains the main abiotic stress that limits foxtail millet yield, it is highly significant to screen for drought-tolerant varieties throughout the entire growth period and identify the regulatory genes associated with drought resistance in foxtail millet breeding. We identified 217 foxtail millet seed resources for drought resistance during the maturity stage in the field, and subsequently categorized them into different levels of drought resistance. Two cultivars with extremely strong drought resistance during the maturity stage in the field, JKH4 (Chi 5422) and JKH6 (Chigu 26), as well as two cultivars with extremely weak drought resistance during the maturity stage in the field, JRK3 (17M1309) and JRK6 (Canggu 9), were selected for physiological comparison and transcriptome sequencing before and after drought treatment. Transcriptome analysis at the seedling stage revealed that JRK3 and JRK6 cultivar primarily regulated phenylpropanoid biosynthesis, MAPK signaling pathogen-plant, and plant hormone signal transduction pathway in response to drought stress. On the other hand, the fatty acid elongation pathway of JKH4 and JKH6 variety was found to be more significant. Furthermore, 22 drought resistance related genes were screened through transcriptome analysis of four foxtail millet varieties. These findings could offer valuable theoretical guidance for breeding foxtail millet with enhanced drought resistance and potentially facilitate the development of genetically engineered drought-resistant foxtail millet varieties.

1. Introduction

Drought is the primary abiotic stressor that poses a threat to global food security and sustainability. It disrupts crucial physiological processes in plants due to the degradation of environmental conditions, thereby exacerbating the adverse effects of other abiotic stresses on plants [1–3]. Foxtail millet [*Setaria italica*], an ancient crop in China, may have a 10,000-year history of cultivation [4,5]. It is ranked as the world's second highest yielding millet after pearl millet and exhibits characteristics such as drought tolerance, barren tolerance, and saline-alkali soil tolerance [6]. The low water and fertilizer consumption of this crop holds great significance in

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mitigating drought in cereal-growing areas [7–9]. Additionally, foxtail millet is a C4 crop with a higher photosynthetic rate compared to C3 crops, as well as stronger drought tolerance and a smaller genome, making it more suitable for C4 model crop research. Yang et al. (2020) developed an ultra-early ripening mini foxtail millet called "Xiaomi." The growth period of "Xiaomi" is similar to that of the C3 model plant *Arabidopsis*, with a height of approximately 30 cm, making it suitable for cultivation in incubators and further advancing foxtail millet as a C4 model crop [10–12]. Studying the phenotypic variation of foxtail millet and its underlying regulatory mechanisms is crucial for molecular breeding of C4 crops. It also provides insights into the evolution of climate-adapted crops, which holds great significance in the current era of global climate change [13]. Cereals are the predominant crops in Central Asia, the Middle East, and North Africa, encompassing 48.5 % of the total agricultural land in Latin America and the Caribbean, and 87 % in Central Asia. The yields of foxtail millet, sorghum, and soybean crops are particularly susceptible to changes in weather patterns and other natural conditions in this region [14]. Nevertheless, the challenge of climate change's impact on global food security is one that must be confronted by the world in the 21st century [15]. In the future, drought will continue to be the primary abiotic stress factor constraining cereal yields due to extreme weather conditions. When 16 varieties of finger millet spikes are subjected to drought from emergence to filling, yield declines could reach as high as 36.6 % [16,17]. Consequently, it is imperative to prioritize the selection of foxtail millet varieties with strong drought resistance and explore regulatory genes related to drought resistance in foxtail millet due to the persistent influence of extreme weather conditions on foxtail millet production.

Regulation of gene expression plays a critical role in connecting genotype to phenotype [18]. High-throughput messenger RNA sequencing (RNA-seq) enables direct sequencing of cDNA libraries, offering a method for investigating gene expression and the potential to estimate isoform abundance and discover novel transcripts [19–21]. Sequencing is primarily carried out using next-generation platforms such as Illumina, with the sequence data ultimately aligned to annotated genes through sequence alignment [22,23]. Illumina sequencing technology, introduced in 2007, has been characterized by a consistent increase in read length and total number of reads per run, as well as a reduction in reaction cost and time [24,25]. In recent decades, RNA-seq has been hailed as a transformative tool for transcriptome analysis across animals, plants, and microorganisms. In addition, animal and plant models have been instrumental in uncovering the mechanisms that regulate transcriptional levels [26,27]. The primary application of transcriptome analysis is to identify differentially expressed (DE) genes - those showing differences in expression levels under different conditions associated with a given predictor or response - thus revealing gene regulation under varying conditions [28].

The identification of drought-related genes in foxtail millet holds significant importance. RNA-seq was employed to unveil the drought-resistant genes and regulatory pathways in foxtail millet under drought stress, contributing to a deeper understanding of the molecular mechanisms underlying drought tolerance in this crop species [29,30]. Previous transcriptomic studies on foxtail millet drought have identified several key genes associated with drought resistance, including LEA family genes, proline metabolism-related genes, and calcium-dependent protein kinase genes (CDPKs) [29,31,32]. The regulation of these genes can assist foxtail millet plants in maintaining water balance, reducing oxidative damage, and ultimately enhancing their resistance to drought conditions. However, the majority of previous drought-resistant genes have been identified through transient simulation of drought stress, potentially leading to an incomplete understanding of the mechanisms underlying foxtail millet's response to drought. Therefore, subjecting foxtail millet to long-term soil water deprivation and conducting transcriptome sequencing can provide a more comprehensive exploration of the regulatory network governing drought resistance in this crop. Through transcriptome and metabolic analysis, Yu et al. (2020) demonstrated that key genes and metabolites associated with the phenylpropanoid pathway may play a significant role in regulating drought resistance during the germination stage of foxtail millet, potentially mitigating drought by modulating endogenous chemosensory substance levels [33]; Qin et al. (2020) conducted transcriptome analysis of Jigu16 following drought treatment and rewatering, revealing SiP5CS2 as a potentially crucial gene for foxtail millet's drought tolerance [34]. Meanwhile, Tang et al. (2017) conducted transcriptomic analysis on the seedling stage of the drought-tolerant cultivar Yugu1 and the drought-sensitive cultivar An04. They integrated this analysis with drought-tolerant QTLs to identify 20 genes associated with drought tolerance, offering theoretical guidance for breeding and genetic improvement of drought tolerance in Gramineae [35]. Nevertheless, previous comprehensive transcriptional studies on foxtail millet drought tolerance have primarily focused on different developmental stages and tissues of one or two drought-tolerant foxtail millet cultivars, lacking the identification of drought tolerance throughout the entire growth period [33–36]. In this study, we identified the drought resistance of 217 millet samples in the field at maturity stage and categorized them into five grades based on their level of drought resistance. Additionally, two extremely drought-resistant varieties and two extremely weakly drought-resistant varieties were randomly selected for further evaluation of their resistance at seedling and germination stages. This approach aims to select foxtail millet varieties with consistent drought resistance across all growth stages and expand the scope of comparative transcriptome analysis, by conducting transcriptome analysis on four millet varieties during the seedling stage, we screened candidate genes associated with stress resistance, providing a theoretical foundation for exploring the mechanism underlying foxtail millet's response to drought stress as well as guiding future breeding efforts targeting enhanced drought resistance in gramineous crops.

2. Materials and methods

2.1. Experimental material

A total of 217 foxtail millet varieties (Supplementary Table S1) were tested in the Dunhuang area of China (48° 08′ N, 94° 43′ E) in 2020, characterized by an annual rainfall of less than 40 mm. During the period from foxtail millet flowering to maturing, the Dunhuang area received less than 6 mm of rainfall, which had minimal impact on soil water content. The average temperatures ranged from 34 °C ± 3 °C during the day to 17 °C ± 3 °C at night throughout the foxtail millet tasseling to maturity stage. Drought stress

treatment was implemented under natural conditions, with full irrigation and bottom fertilizer applied prior to sowing for seedling preservation (no water was provided throughout the entire growth period). The field was arranged in a randomized block design, consisting of three row areas with a row width of 25 cm and line length of 3.0 m. The foxtail millet seedling density per plot was set at 84 plants (equivalent to a seedling density of 25,000 plants). The control variety used was Longgu5, and the experimental design was based on interval contrast design method. Three replicates were established for each treatment combination. Phenological stages and biological characteristics were recorded during the experimental growth period. The 217 germplasms were systematically clustered based on spike weight, grain weight per spike, spike rate, grain emergence rate, yield, and yield increase range using Euclidean distance and sum of squared deviations methods. The germplasm samples were divided into five groups, including 7 extremely strong drought resistant germplasm samples, 76 drought resistant germplasm samples, 33 moderate drought resistant germplasm samples, 36 weak drought resistant germplasm samples and 65 extremely weak drought resistant germplasm samples (Supplementary Table S1). Two extremely drought resistant germplasm JKH4 (Chi 5422) and JKH6 (Chigu 26) and two extremely weak drought resistant germplasm JRK3 (17M1309) and JRK6 (Canggu 9) were selected for further experiments.

2.2. Growth conditions and drought stress in four millet cultivars

Four foxtail millet varieties (JKH4, JKH6, JRK3 and JRK6) were sterilized with 1 % NaClO and then placed in clean water, after soaking the seeds with uniform size and no obvious mechanical damage or disease overnight, the foxtail millet seeds were placed in nutrient soil (soil: vermiculite = 3: 1, v/v) and incubator (temperature 28 °C/20 °C, light 14 h/dark 10 h, humidity 60–70 %, light intensity 15000 lux), five seedlings per pot, four pots in one replicate, control (CK) and drought stress (DS) groups were set, and three replicates were set for each experiment. When the millet was grown to the five-leaf stage, the control group (CK) was watered normally, and the drought stress group (DS) stopped watering, until the foxtail millet appeared drought-resistant phenotype, so as to identify the drought resistance of the four foxtail millet varieties in the seedling stage. When the foxtail millet leaves in the drought treatment group appeared wilting, the material leaves of different foxtail millet varieties were taken and stored at −80 °C. For RNA-seq analysis and physiological parameter estimation.

2.3. Estimation of biochemical parameters

To assess various biochemical parameters under drought stress, four different cultivars of foxtail millet were cultivated until the five-leaf stage and then subjected to drought treatment. Drought tolerance indices, including chlorophyll content, proline content, and malondialdehyde content, were measured in both the control (CK) and drought-stressed (DS) groups. The chlorophyll content of foxtail millet leaves was measured using an SPAD-502 chlorophyll meter with three biological replicates and three technical replicates. Proline and malondialdehyde content were determined using the microplate method kit from Suzhou Geruisi Biological Company (http://www.geruisi-bio.com/), item numbers G0111W and G0109W respectively, with three biological replicates and two technical replicates. Foxtail millet germination under simulated drought conditions was assessed according to GB/T 5520-2011 Inspection of grain and oils-Germination test of seeds. Fifty seeds of each variety were subjected to 20 % PEG6000 osmotic stress for three repetitions after being sterilized with 1 % NaClO.

2.4. RNA extraction, cDNA library preparation, and transcriptome sequencing

Total RNA was extracted from leaf samples collected from four accessions of foxtail millet cultivars grown under CK and DS conditions using TRIzol (Tiangen, China) according to the method described by the manufacturer. Three biological replicates were performed for each sample. Total RNA was extracted and quality checked, first using 2.1 % agarose gel electrophoresis for RNA degradation and contamination, and then using Agilent 2100 Bioanaiyzer for RNA quality. In this study, mRNA with poly A structure in total RNA was enriched by Oligo (dT) magnetic beads, and ion interruption was used to interrupt the RNA to a fragment of about 300 bp in length. RNA was used as template to synthesize the first strand of cDNA using a 6-base random primer and reverse transcriptase, and the first strand cDNA was used as template for the second strand cDNA synthesis. After RNA extraction, purification and library construction, Next Generation Sequencing (NGS) technology was used to perform paired-end (PE) sequencing of these libraries based on Illumina sequencing platform.

2.5. Raw data processing and mapping

The samples were sequenced on the computer to obtain image files, which were transformed by the Illumina sequencing platform software to generate FASTQ Raw Data. The Raw Data of each sample were counted separately, including the sample name, N% (the percentage of ambiguous bases), Q20(%) (the percentage of bases identified with more than 99 % accuracy), and Q30(%) (the percentage of bases identified with more than 99.9 % accuracy). Sequencing data contain some Reads with connectors and low quality, and these sequences will cause great interference to the subsequent information analysis, so further filtering of sequencing data is needed. The criteria for data filtering mainly include: (1) using Cutadapt to remove sequences with junctions at the 3′ end; (2) removing Reads with average quality scores lower than Q20.

Using TopHat2 upgrade HISAT2 (http://ccb.jhu.edu/software/hisat2/index.shtml) software will be filtered reads than to the Ensemble plants on the foxtail millet reference genome Setaria_italica_v2.0 (https://plants.ensembl.org/Setaria_italica/Info/Index?db=core), HISAT2 uses a modified BWT algorith for faster speed and less resource consumption [37]. If the reference genome is

properly selected and there is no contamination in the relevant experiment, the Mapping ratio of the sequencing sequences generated by the experiment is generally higher than 70 %.

2.6. Identification of differentially expressed genes (DEG)

The HTSeq statistics were utilized for aligning the Read Count value of each gene as the original gene expression, while FPKM was employed to normalize the expression level. DESeq was used for conducting differential analysis of gene expression, with the criteria for screening differentially expressed genes being an expression differential fold $|\log_2\text{FoldChange}| > 1$ and a significance p-value <0.05. The ggplots2 software package in R language was utilized to generate volcano plots and MA plots of differentially expressed genes. Additionally, bidirectional cluster analysis of the union of differentially expressed genes and samples from all comparison groups was performed using the Pheatmap software package in R language. Clustering was based on both the expression level of the same gene across different samples and the expression pattern of different genes within the same sample, employing Euclidean distance calculation and hierarchical clustering method (Complete Linkage) with clustering.

2.7. Functional enrichment analysis

GO enrichment analyses were conducted using topGO, where both gene list and gene number per term were calculated based on GO annotations assigned to differential genes. Subsequently, hypergeometric distribution method was applied to calculate p-values (with significant enrichment defined as p-value <0.05). Significantly enriched GO terms among differentially expressed genes were determined to identify their main biological functions. The results of GO enrichment analysis for differentially expressed genes were

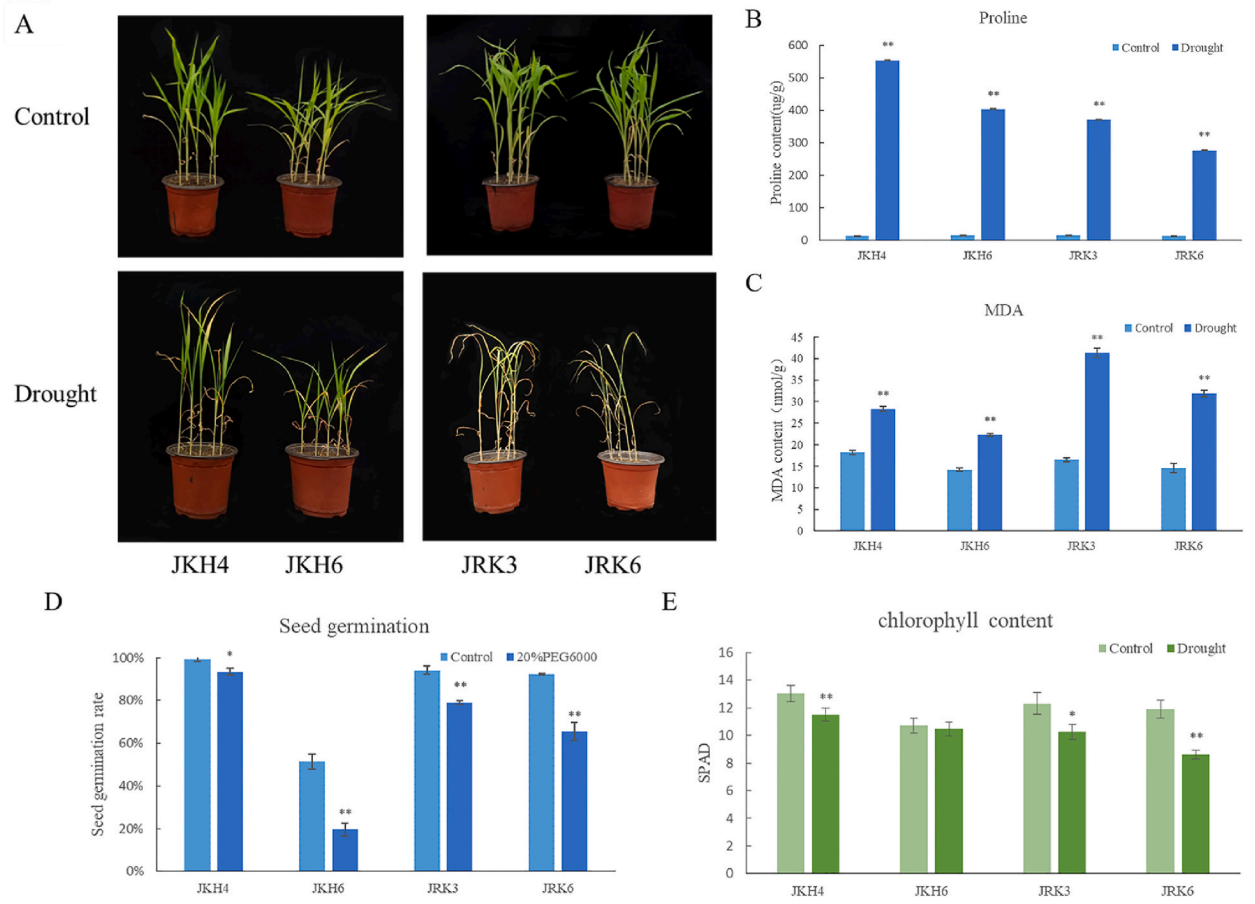

Fig. 1. Effects of drought stress on different foxtail millet genotypes. **(A)** Phenotypic changes in four millet cultivars during 14 days of drought stress at the five-leaf stage. **(B and C)** Determination of physiological indices in four foxtail millet varieties, **(B)** Determination of proline (Pro) content; **(C)** Determination of malondialdehyde (MDA) content. Standard deviations are indicated by error bars (mean ± SD and n = 3). **(D)** Determination of germination rates of the 20%PEG simulated drought treatment and normal control at the germination stage of four foxtail millet cultivars. **(E)** Determination of chlorophyll content in four foxtail millet varieties. Standard deviations are indicated by error bars (mean ± SD and n = 3). For each drought stress group was compared to the control group, and student's t-test was used to calculate significance: *indicates $p < 0.05$, and **indicates $p < 0.01$.

categorized into molecular function (MF), biological process (BP), and cell component (CC). The top 10 GO terms in each category with the smallest *p*-values (indicating the most significant enrichment) were selected for presentation, thereby identifying the primary biological function performed by the differential genes. Based on KEGG enrichment results, degree of enrichment was measured by Rich factor, FDR value, and number of enriched genes within each pathway; thus, identifying top 20 KEGG pathways with lowest FDR value were selected for display.

Conventional enrichment analysis based on hypergeometric distributions rely on significantly up-regulated or down-regulated genes, and tend to miss some genes that are not significantly differentially expressed but are biologically important. Gene Set Enrichment Analysis (GSEA) does not require to specify an explicit threshold of differentially expressed genes. All genes are ranked according to the degree of differential expression in the two groups of samples, and then a statistical method is used to test whether a prespecified gene set is enriched at the top or at the bottom of the ranking table. GSEA consists of three main steps: (1) calculating the Enrichment Score; (2) the significance level of enrichment scores was estimated.; (3) multiple hypothesis testing. In this study, we utilized the Gene Set Enrichment Analysis platform (https://www.gsea-msigdb.org/gsea/index.jsp) to analyze each gene's enrichment score. The normalized enrichment scores (NESs), nominal *p*-values, and false discovery rate (FDR) q-values were computed to derive the results. We considered $|NES| > 1$, a NOM *p*-value less than 0.05, and genomic pathways with an FDR q-value of less than 0.25 as significantly enriched. Transcription factors were predicted by comparing plants and animals with Plant Transcription Factor Database (Plant TFDB) and Animal Transcription Factor DataBase (Animal TFDB), respectively, so as to predict the transcription factors and the information about the families to which the transcription factors belong.

2.8. Reverse transcription quantitative PCR (RT-qPCR) validation

To validate the differential expression patterns of genes across different foxtail millet varieties and under drought stress conditions, Reverse Transcription quantitative PCR (RT-qPCR) was employed to confirm the expression levels of six randomly chosen genes. RNA samples were extracted from the leaves of four foxtail millet varieties subjected to both control (CK) and drought stress (DS) conditions. The EasyScript® One-step gDNA Removal and cDNA Synthesis Super Mix reverse transcription kit from TransGen Biotech (https://www.transgen.com/) was utilized for cDNA synthesis according to the manufacturer's protocol, followed by RT-qPCR using the 2 × TSINGKE® Master qPCR Mix (SYBR Green I) quantitative kit from Qingke Biological Company(https://www.tsingke.com.cn/) on a Quant Studio 7 Flex PCR system. The relative gene expression was calculated using $2^{-\Delta\Delta ct}$ and plotted accordingly, with SiActin-7 (NCBI sequence ID: XP_004970695) serving as an internal control. Details of the RT-qPCR primers can be found in Supplementary Table S2.

3. Results

3.1. Physiological morphological changes of foxtail millet under drought stress

Drought stress was imposed at the five-leaf stage of foxtail millet. After two weeks of drought stress, it was observed that the highly drought-resistant varieties JKH4 (Chi 5422) and JKH6 (Chigu 26) exhibited superior performance, while the extremely weak-resistant

Table 1
Illumina sequencing data filtering statistics and RNA-Seq Map statistics of four foxtail millet varieties.

Sample	Reads No.	Clean Reads No.	Clean Reads %	Total Mapped
JKH4-CK-1	52854130	48716406	92.17	46787647 (96.04 %)
JKH4-CK-2	51744096	46945064	90.72	45442413 (96.80 %)
JKH4-CK-3	59763958	53953376	90.27	51838264 (96.08 %)
JKH4-DS-1	42923804	39376160	91.73	38119689 (96.81 %)
JKH4-DS-2	55373528	51132270	92.34	49465518 (96.74 %)
JKH4-DS-3	67886832	60991280	89.84	58850045 (96.49 %)
JKH6-CK-1	50691472	47263030	93.23	44898226 (95.00 %)
JKH6-CK-2	62636974	58007100	92.6	55339170 (95.40 %)
JKH6-CK-3	51539822	45026652	87.36	42866516 (95.20 %)
JKH6-DS-1	40950256	38127198	93.1	36376334 (95.41 %)
JKH6-DS-2	40083586	37119138	92.6	35430160 (95.45 %)
JKH6-DS-3	54594524	49087652	89.91	46286966 (94.29 %)
JRK3-CK-1	42064510	38706540	92.01	37466705 (96.80 %)
JRK3-CK-2	52754544	48958738	92.8	47513641 (97.05 %)
JRK3-CK-3	46511074	41241218	88.66	39816602 (96.55 %)
JRK3-DS-1	42918516	40225890	93.72	38829706 (96.53 %)
JRK3-DS-2	46857988	42742496	91.21	41222300 (96.44 %)
JRK3-DS-3	90816500	79545266	87.58	77182501 (97.03 %)
JRK6-CK-1	45370562	41793844	92.11	40367120 (96.59 %)
JRK6-CK-2	47568990	44158394	92.83	42625395 (96.53 %)
JRK6-CK-3	69739744	64250084	92.12	62198411 (96.81 %)
JRK6-DS-1	50812314	46886888	92.27	45372565 (96.77 %)
JRK6-DS-2	82466082	73824236	89.52	71260810 (96.53 %)
JRK6-DS-3	73745636	63234764	85.74	61068813 (96.57 %)

varieties JRK3 (17M1309) and JRK6 (Canggu 9) displayed symptoms of yellowing leaves, weak stems, and poor performance (Fig. 1A). It was noted that under drought stress, proline levels increased in both drought-resistant and weak-resistant varieties (Fig. 1B). However, proline accumulation in the extremely drought-resistant varieties significantly surpassed that in the extremely weak-resistant varieties. Conversely, malondialdehyde levels showed an opposite trend (Fig. 1C). When subjected to drought stress, both drought-resistant and weak-resistant varieties showed a decrease in chlorophyll content. However, the decrease was less significant in the drought-resistant varieties JKH4 and JKH6, while weak-resistant varieties JRK3 and JRK6 exhibited a substantial decrease (Fig. 1E). Drought simulation experiments conducted at the germination stage revealed that the seed germination rate of JKH4 remained above 90 % when exposed to 20 % PEG6000. In contrast, JKH6, JRK3, and JRK6 all experienced a significant decrease in germination rate under the same conditions. Additionally, JKH6 demonstrated a lower germination rate (51.4 %) when exposed to distilled water but experienced a notable decline to 19.6 % when subjected to simulated drought with 20 % PEG6000 (Fig. 1D).

3.2. Data collation of transcriptome sequencing of samples under drought stress

The drought stress treatment group was divided into four groups: JKH4 drought stress (JKH4-DS) and JKH4 control group (JKH4-CK), JKH6 drought stress (JKH6-DS) and JKH6 control group (JKH6-CK); JRK3 drought stress (JRK3-DS) and JRK3 control group (JRK3-CK); JRK6 drought stress (JRK6-DS) and JRK6 control group (JRK6-CK). Each group consisted of three replicates, resulting in a total of 24 samples sequenced using the Illumina platform. The total number of reads obtained for each sample ranged from 40.08 million to 90.82 million. After removing the 3′ segment with splice sequences and filtering out low-quality sequences with an average quality score below Q20, clean reads ranging from 38.12 million to 79.55 million were obtained from the 24 samples. The mapping ratio of all 24 samples was above 95 % as shown in Table 1, indicating high sequencing quality for RNA-seq analysis.

3.3. Sample correlation analysis and screening of differential genes

To assess the disparity in transcription levels of millet under drought treatment and control treatment, we computed Pearson correlation coefficients (PCCs) for all expressed genes across all samples using FPKM values (Supplementary Fig. S1 and Table S3). The squared PCC (R2) between biological replicates for this sample ranged from 0.92 to 0.99, indicating a high degree of precision among the replicates. By using multiple expression differences with $|\log_2(\text{foldchange})| > 1$ and a significant p-value <0.05 as selection criteria, the foxtail millet varieties JKH4 and JKH6 exhibited a more than threefold increase in the number of up-regulated genes compared to down-regulated genes under drought stress, while the varieties JRK3 and JRK6 exhibited an equal number of up-regulated and down-regulated genes (Fig. 2; Supplementary Table S4). The volcano map displays the distribution of genes, the fold differences in gene expression, and the significance results of genes (Supplementary Fig. S2). Cluster analysis was utilized to identify expression patterns of differentially expressed genes under various experimental conditions, and potential biological connections between genes could be uncovered through expression clustering (Supplementary Fig. S3). The identification of additional up-regulated genes in foxtail millet varieties suggests their involvement in drought resistance mechanisms, contributing to the maintenance of higher levels of drought resistance. The roles of shared up-regulated and down-regulated differential genes in the response to drought stress in four foxtail millet varieties were further analyzed using Venn plots (Fig. 3A and B; Supplementary Table S5). The Venn plots revealed 16 shared up-regulated differential genes and 3 shared down-regulated genes after drought stress, indicating that these shared genes may play a key role in regulating the response to drought stress.

3.4. GO analysis of differentially expressed genes (DEGs)

To gain insights into the potential functions and roles of differentially expressed genes (DEGs) in the regulatory mechanism of drought resistance in foxtail millet, gene ontology (GO) analysis was conducted, the criterion for significant enrichment was set at a p-value <0.05. The leaves of JKH4 in were exposed to drought stress with a total of 239 GO terms (containing 2774 DEGs), including 20 CC (containing a total of 709 DEGs), 146 BP (containing a total of 1719 DEGs), and 73 MF (containing a total of 346 DEGs); JKH6 in

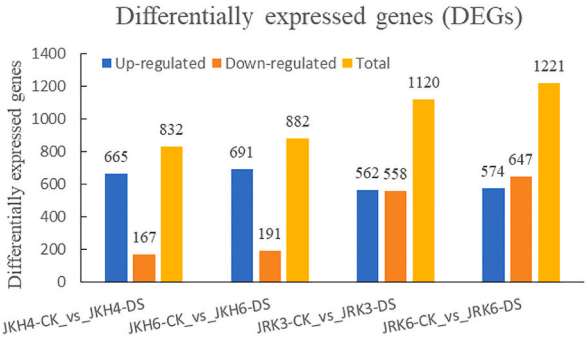

Fig. 2. Differentially expressed genes (DEGs) in different foxtail millet varieties after drought stress and control group, and using the expression differences multiples $|\log_2\text{FoldChange}| > 1$, the significant p-value <0.05 as selection criteria.

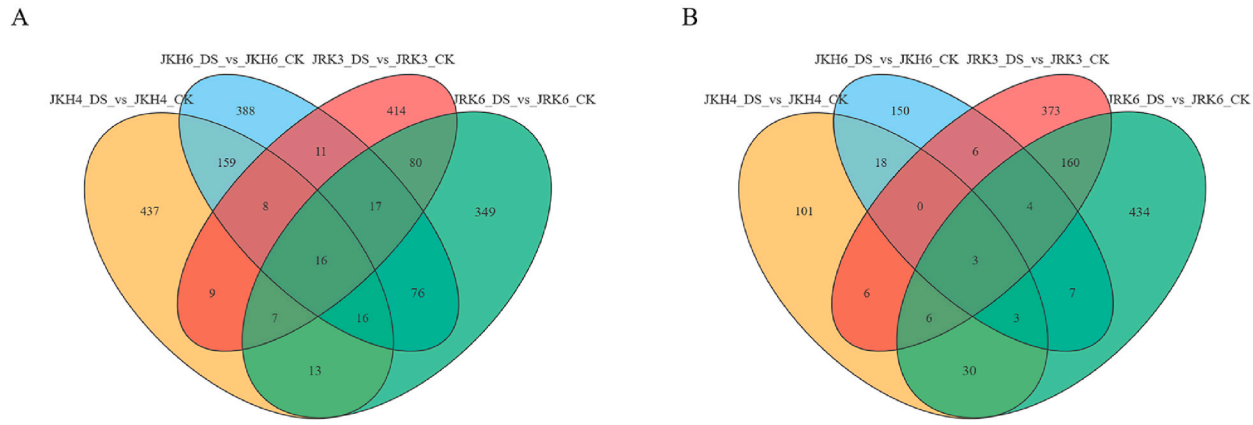

Fig. 3. Venn diagram shows the number of differentially expressed genes (DEGs) co-regulated in the leaves of the four foxtail millet varieties after drought stress treatment. **(A)** and **(B)** show the distribution of upregulated and downregulated DEGs in the four millet varieties, respectively.

leaves subjected to drought stress had a total of 286 GO terms (containing 4332 DEGs), including 16 CC (containing a total of 742 DEGs), 183 BP (containing a total of 2636 DEGs), and 87 MF (containing a total of 954 DEGs); there were 358 GO terms in JRK3 leaves under drought stress (containing 6539 DEGs), including 10 CC (containing 573 DEGs), 248 BP (containing 5039 DEGs), and 100 MF (containing 927 DEGs); JRK6 has 294 GO terms (containing 5615 DEGs), including 15 CC (containing 970 DEGs), 196 BP(containing 3645 DEGs), and 82 MF (containing 1000 DEGs) (Supplementary Table S6). After experiencing drought stress, a variety of differentially expressed genes (DGEs) in the four foxtail millet varieties exhibited diverse responses to chemical, response to hormone, response to endogenous stimulus, intrinsic component of membrane and response to acid chemical (Fig. 4A–D). This suggests that these responses may be associated with the leaves' reaction to water deprivation. In addition, foxtail millet cultivars with varying levels of drought tolerance under drought stress displayed significant alterations in biological processes, molecular functions, and cellular components.

3.5. KEGG pathway enrichment of differential genes

We conducted KEGG enrichment analysis to explore the primary pathways associated with DEGs. The results revealed that JKH4-DS identified 72 KEGG pathways compared to JKH4-CK, with 13 of them showing a p-value <0.05. Among these, pyruvate metabolism, fatty acid elongation, cutin, suberine and wax biosynthesis, phenylpropanoid biosynthesis, and plant-pathogen interaction were the most significantly enriched pathways (FDR<0.05) (Fig. 5A; Supplementary Table S7). JKH6-DS identified 70 KEGG pathways compared with JKH6-CK, there were 12 KEGG pathways with p-value <0.05, including fatty acid elongation, flavonoid biosynthesis, flavone and flavonol biosynthesis, phenylalanine metabolism, galactose metabolism and plant hormone signal transduction were the most significantly enriched pathways (FDR<0.05) (Fig. 5B; Supplementary Table S7); JRK3-DS identified 78 KEGG pathways compared with JRK3-CK, among them, there were 15 KEGG pathways with p-value <0.05, phenylpropanoid biosynthesis, MAPK signaling pathway-plant and plant hormone signal transduction was the most enriched pathway (FDR<0.05) (Fig. 5C; Supplementary Table S7); JRK6-DS identified 77 KEGG pathways compared with JRK6-CK, of which 10 KEGG pathways were p-value <0.05, MAPK signaling pathway - plant, phenylpropanoid biosynthesis and plant-pathogen interaction was the most enriched and significant pathway (FDR<0.05) (Fig. 5D; Supplementary Table S7).

3.6. Gene set enrichment analysis (GSEA)

In this study, FPKM was utilized for GSEA analysis on four foxtail millet varieties with varying levels of drought resistance under control and drought stress conditions, a total of 35,831 characteristic genes were enriched and analyzed (Supplementary Table S8). We considered gene set pathways with $|NES|>1$, NOM p-value<0.05, FDR q-value<0.25 as significantly enriched. GSEA results revealed that fatty acid elongation (SITA00062) was significantly enriched in both JKH4-DS and JKH6-DS datasets, while oxidative phosphorylation (SITA 00190) was significantly enriched in both JKH4-CK and JKH6-CK datasets (Supplementary Figs. S4 and S5). Additionally, plant hormone signal transduction (SITA04075) was co-enriched in JRK3-DS and JRK6-DS datasets, while sesquiterpenoid and triterpenoid biosynthesis (SITA 00909) along with plant-pathogen interaction (SITA04626) were co-enriched in JRK3-CK and JRK6-CK datasets (Supplementary Figs. S6 and S7). We identified the top 50 characteristic genes for each phenotype of every foxtail millet variety before and after exposure to drought stress, resulting in a total of 100 characteristic genes. Subsequently, we constructed a heat map (Fig. 6A–D). The genes exhibiting higher enrichment scores in each drought treatment group were further analyzed using gene set enrichment analysis (GSEA), potentially representing key genes involved in the response to drought.

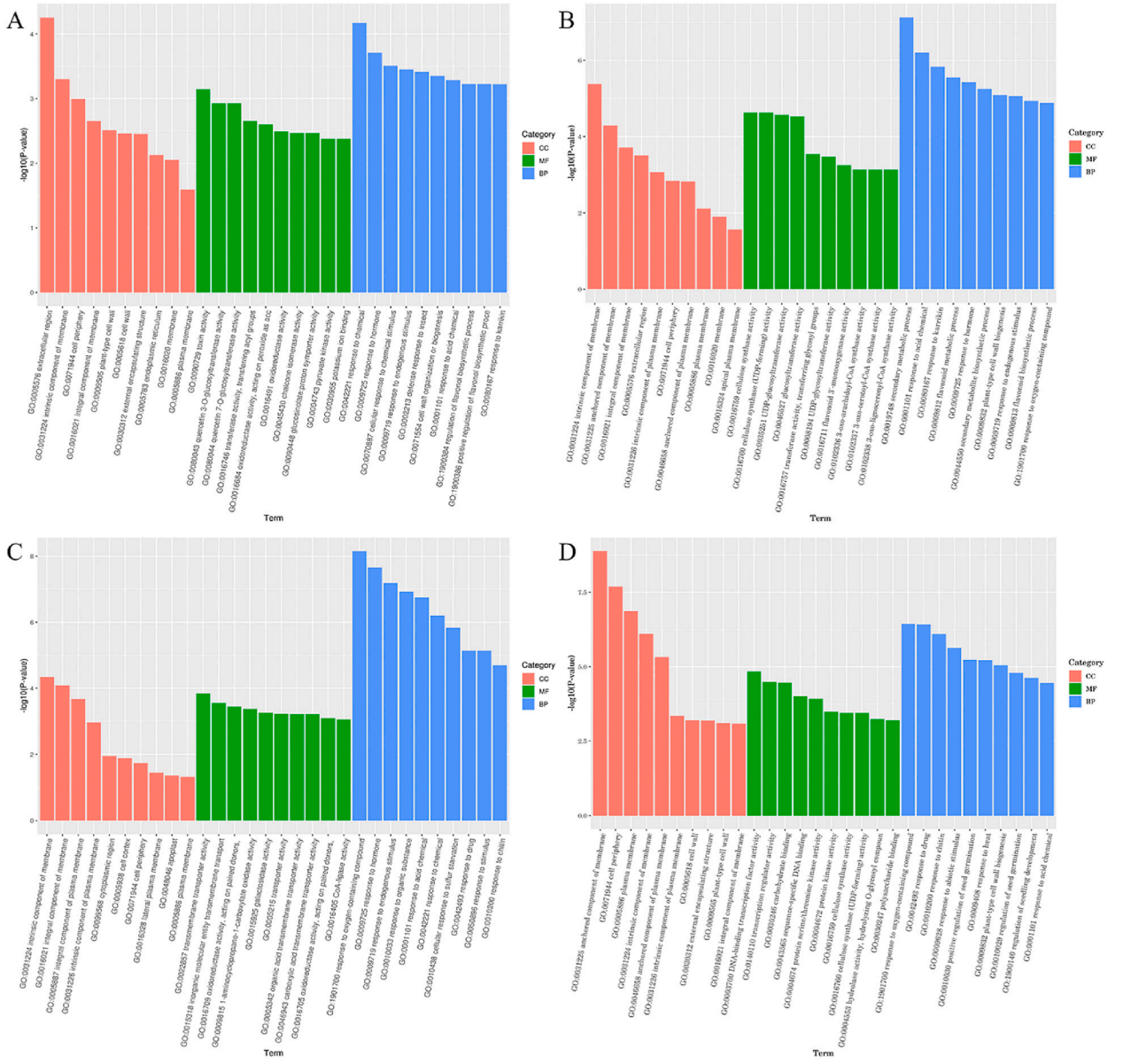

Fig. 4. Gene ontology (GO) enrichment of DEGs in response to drought in four different varieties of foxtail millet. The criterion for significant enrichment was p-value <0.05, and the top 10 GO terms with the smallest p-value (the most significant enrichment) in each GO category were selected for display. **(A)** GO analysis of DEGs in JKH4 foxtail millet varieties; **(B)** GO analysis of DEGs in JKH6 foxtail millet varieties;**(C)** GO analysis of DEGs in JRK3 foxtail millet varieties; **(D)** GO analysis of DEGs in JRK6 foxtail millet varieties.

3.7. Transcription factor (TF) analysis of DEGs

The role of transcription factors (TFs) in regulating cellular processes is crucial, and the number of predicted DEGs that are TFs was determined. A bar chart was used to illustrate the distribution of differentially expressed TFs across various transcription factor families within the comparison group (Fig. 7A–D; Supplementary Table S9). The WRKY, bHLH, HD-ZIP, and MYB families exhibited higher up-regulation in JKH4 and JKH6 foxtail millet varieties following drought stress (Fig. 7A and B). Conversely, the HD-ZIP and MYB transcription factor families showed greater up-regulation in JRK3 and JRK6 foxtail millet varieties after drought stress. Additionally, the WRKY and bHLH family members displayed more down-regulation (Fig. 7C and D). In this study, two HD-ZIP family members (SETIT_018237 mg and SETIT_010550 mg) were identified as being up-regulated after drought treatment across four foxtail millet varieties, indicating their potential role as key genes involved in regulating drought resistance.

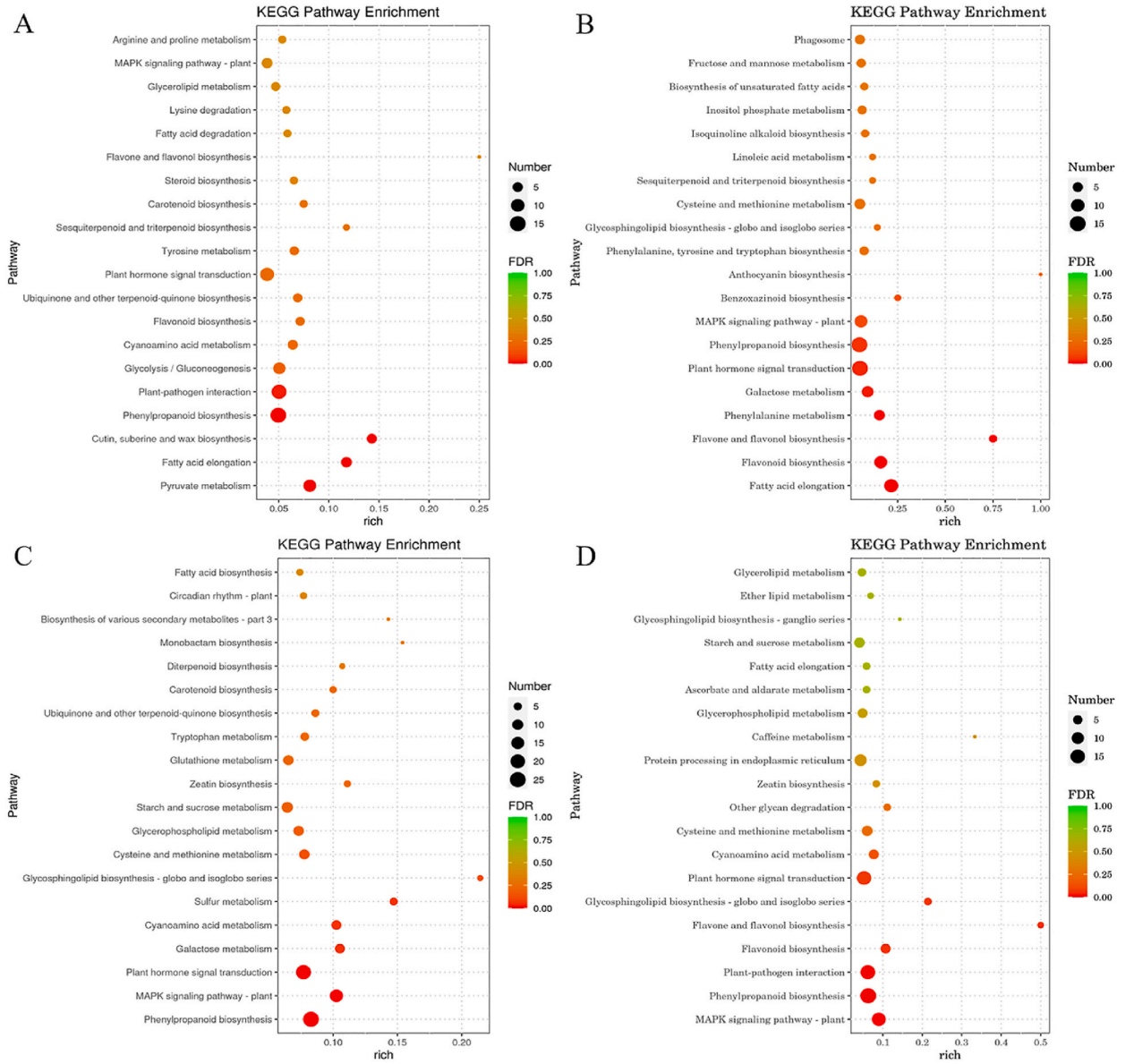

Fig. 5. KEGG analysis of DEGs identified in four different foxtail millet cultivars under drought conditions. Rich refers to the ratio of the number of differentially expressed genes enriched in the pathway to the number of differentially expressed genes annotated. FDR generally ranges from 0 to 1, red indicates close to zero and more significant enrichment, green indicates close to 1 and insignificant enrichment. **(A)** KEGG enriched pathways for DEGs of JKH4 foxtail millet variety. **(B)** KEGG enriched pathways for DEGs of JKH6 foxtail millet variety. **(C)** KEGG enriched pathways for DEGs of JRK3 foxtail millet variety. **(D)** KEGG enriched pathways for DEGs of JRK6 foxtail millet variety. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.8. Validation of transcriptome reliability by RT-qPCR

In order to validate the reliability of the transcriptome, a random selection of 3 up-regulated genes and 3 down-regulated genes that were consistently expressed across all 24 samples was performed for transcriptome verification. The results demonstrated concordance between RT-qPCR and transcriptome data (Fig. 8), thus confirming the high reliability of the transcriptome analysis.

3.9. Screening of key genes for drought resistance

Based on the analysis of differential gene expression and GESA enrichment scores in response to drought treatment, we identified 22 relevant genes and clarified the tissue-specificity of each gene by Setaria italica Functional Genomics Database (http://structuralbiology.cau.edu.cn/SIFGD/search.php) expression (Table 2). After analysis, we observed differential transcript abundance

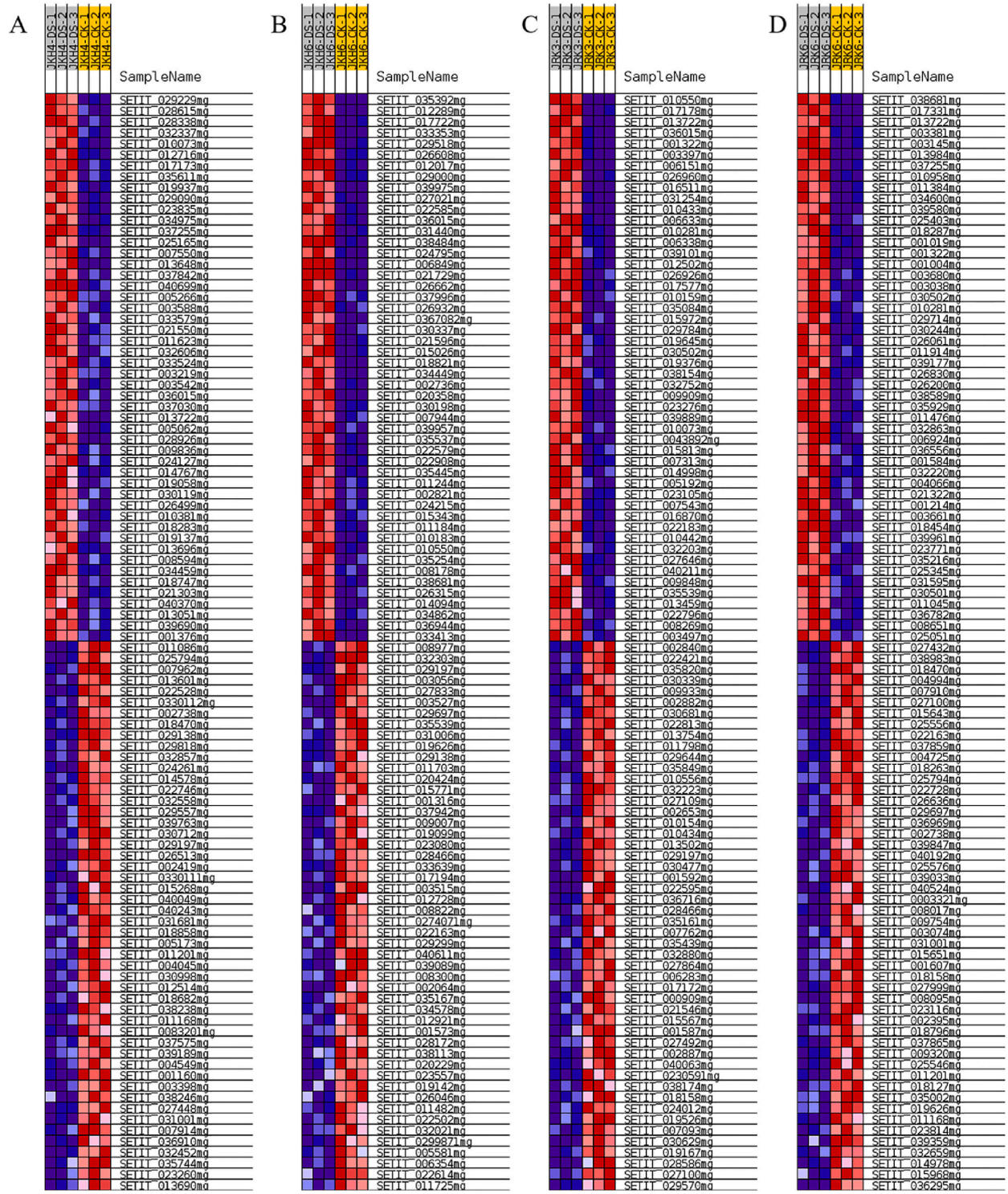

Fig. 6. Heat map of the top 50 features for each phenotype in fpkm regarding the drought stress group and control groups of the four foxtail millet cultivars. Up-regulated genes are indicated in red and downregulated in blue. **(A)** JKH4. **(B)** JKH6. **(C)** JRK3. **(D)** JRK6. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

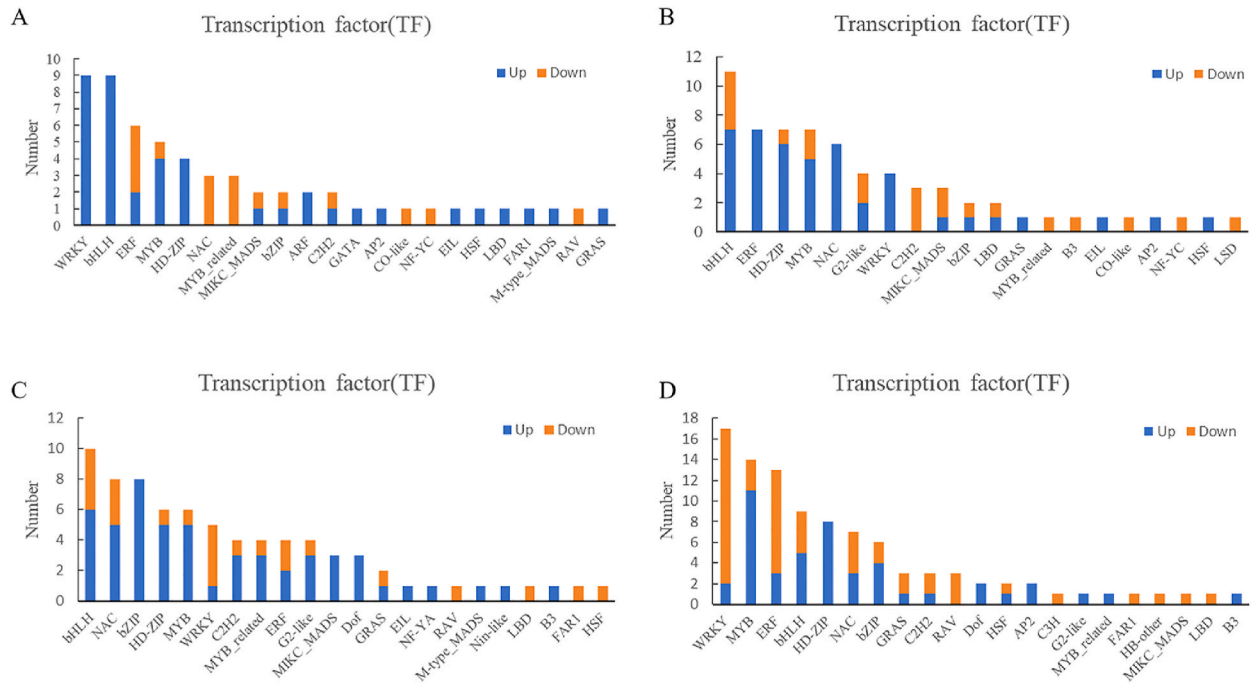

Fig. 7. Histogram of the distribution of differentially expressed transcription factor families in four foxtail millet cultivars under drought conditions, with upregulated transcription factors in blue and downregulated transcription factors in orange. **(A)** JKH4. **(B)** JKH6. **(C)** JRK3. **(D)** JRK6. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

of SETIT_035607 mg, SETIT_026926 mg, and SETIT_039957 mg between JKH4, JKH6 and JRK3, JRK6 varieties.

4. Discussion

Foxtail millet is known for its drought resistance; however, the main limiting factor in its production is still drought, especially the impact of extreme climate conditions in the future. Therefore, it is of great significance to select drought-resistant foxtail millet varieties throughout their life cycle and screen key drought-resistant genes for cereal breeding purposes. A total of 22 candidate genes related to drought resistance were identified in four foxtail millet varieties through a combination of shared differential gene analysis, GO analysis, KEGG pathway enrichment, and GESA enrichment scores in DEG analysis. These candidate genes include four PP2Cs genes (SETIT_013648 mg, SETIT_001708 mg, SETIT_024914 mg and SETIT_036015 mg) as well as a member of the protein kinase superfamily protein family designated as SETIT_036531 mg. The enriched pathways for these genes are plant hormone signal transduction and MAPK signaling pathway-plant pathways. When plants are exposed to external abiotic stresses, they respond to changes in their environment through a series of hormone signaling pathways and gene regulation. Among these, the ABA signaling pathway plays a crucial role in enabling plants to adapt to water-deficient environments during drought conditions [38,39]. The response of the ABA signaling pathway involves the activation of two enzymes, type 2C protein phosphatases (PP2Cs) and SNF-related kinase (SnRK2), which in turn activate down-stream response pathways, allowing plants to exhibit tolerance responses to drought [34,40]. Recently, the discovery of ZMPP84, encoding an active type 2c protein phosphatase (PP2C), has revealed its role in negatively regulating drought tolerance through stomatal closure in maize [36]. Similarly, the GhDRP1 gene in cotton encodes an active type 2c protein phosphatase (PP2C) and is implicated in the plant's response to drought stress by modulating the ABA signaling pathway and flavonoid biosynthesis pathway. This ultimately affects stomatal movement, leading to water loss and reactive oxygen species scavenging, thus influencing drought tolerance in cotton [41]. These findings suggest that members of the PP2C family play a significant regulatory role in the context of drought stress.

Transcription factors play a regulatory role in plants under abiotic stress. Through the prediction of transcription factors from DEGs, we identified two members of the HD-ZIP family, SETIT_010550 mg and SETIT_018237 mg, which show more than 60 % sequence similarity to the HB-7 gene in *Arabidopsis thaliana*. In *Arabidopsis thaliana*, AtHB7 is known to promote leaf development, chlorophyll levels, and photosynthesis in mature plants while decreasing stomatal conductance. It also mediates the induction of ATHB-7 strictly through ABA, suggesting a negative regulation of abscisic acid (ABA) signaling under drought stress [42–44]. In addition, we observed differential transcript abundance of SETIT_035607 mg, SETIT_026926 mg, and SETIT_039957 mg in the varieties JKH4, JKH6 and JRK3, JRK6. The gene SETIT_035607 mg encodes a UDP-Glycosyltransferase (UGT) superfamily protein responsible for glycosyl fragment transfer from UDP sugars to a wide range of receptor flavonoids. Glycosyltransferases play a role in plant responses to abiotic stresses [45,46]. The gene SETIT_026926 mg encodes a Dehydrin (DHN) family protein, which is highly

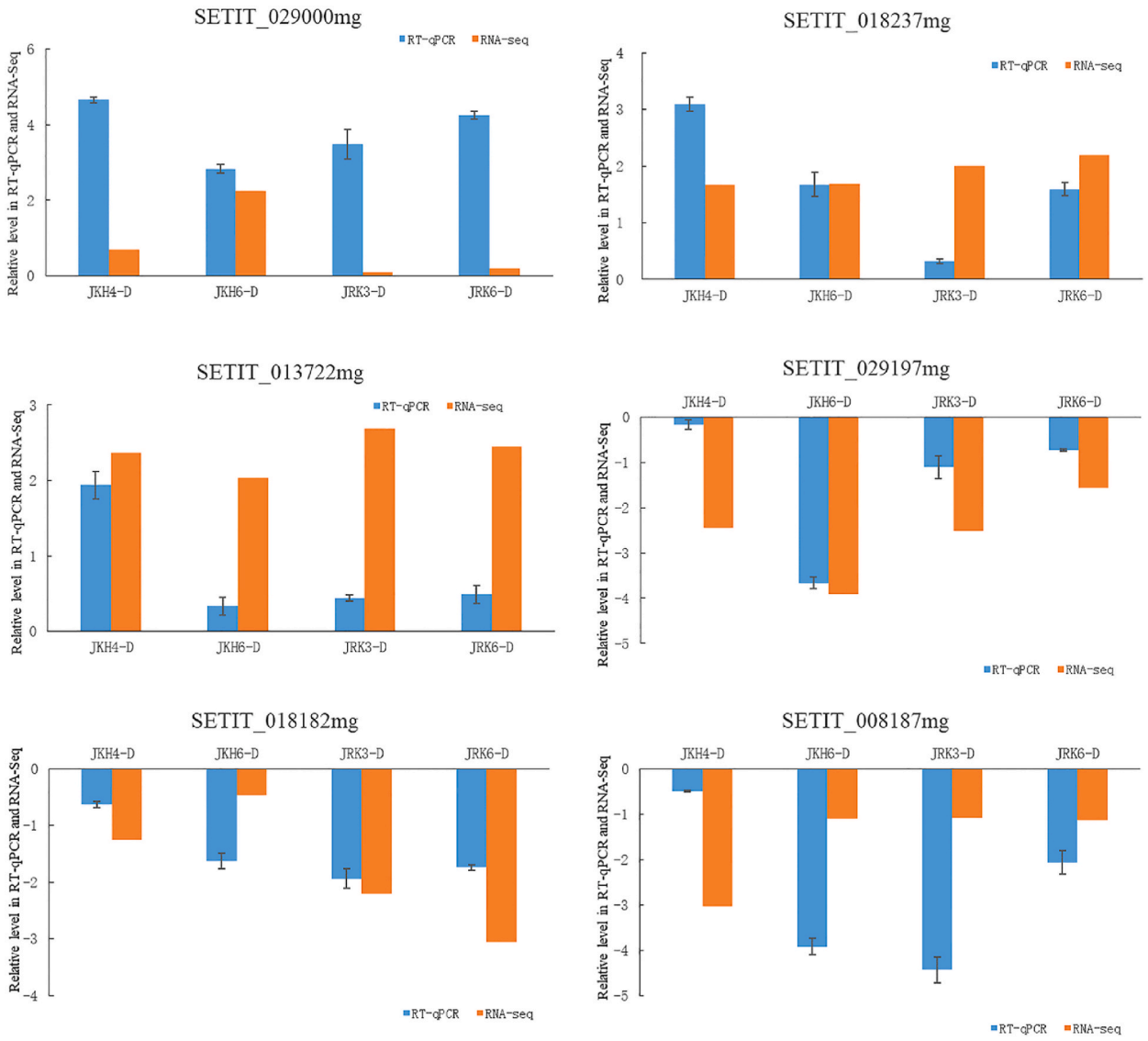

Fig. 8. The expression patterns of 6 differentially expressed genes (DEGs) in different foxtail millet varieties were analyzed, and the transcription levels were verified by RT-qPCR with SiActin-7 gene as an internal control. The X-axis shows the different foxtail millet varieties, and the Y-axis shows the relative expression of the selected genes. Blue indicates -ΔΔt values in RT-qPCR, orange indicates expression levels of genes evaluated by $\log_2$FPKM in RNA-seq data, and standard deviation is shown as error bars (mean ± SD and n = 3). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

hydrophilic and enhances drought stress tolerance by increasing water retention capacity. It can accumulate under conditions of drought and related stresses [47,48]. SETIT_039957 mg exhibited similarity to actin depolymerizing factor 11 (ADF11) in *Arabidopsis thaliana*. Actin-depolymerizing factors (ADFs) are crucial actin binding proteins (ABPs) in eukaryotes, playing a role in plant responses to biotic and abiotic stresses [49–51]. It is hypothesized that SETIT_035607 mg, SETIT_026926 mg, and SETIT_039957 mg may function differently, thereby contributing to a certain level of drought tolerance in various foxtail millet varieties. In conclusion, we identified 22 drought-related genes through transcriptome analysis of four foxtail millet varieties with varying levels of drought tolerance, providing theoretical guidance for breeding drought-tolerant cereal crops.

Previous transcriptomic studies on drought tolerance in foxtail millet have revealed the formation of a complex network involving various biological processes and path-ways, including photosynthesis, transcriptional regulation, signal transduction, osmo-regulation, and redox regulation, to regulate the drought resistance of foxtail millet after experiencing drought treatment [34,52]. Transcriptome analyses of two extremely drought-resistant varieties (JKH4 and JKH6) and two extremely weak drought-resistant varieties (JRK3 and JRK6) following seedling drought treatment revealed that the phenylpropanoid biosynthesis, MAPK signaling pathway, and plant hormone signaling pathway were the main regulatory mechanisms in JRK3 and JRK6 under water deficit stress. In contrast, the fatty acid elongation pathway was more prominent in JKH4 and JKH6, with JKH4 showing pathways related to pyruvate

Table 2
Identification of candidate genes associated with drought resistance in foxtail millet seedlings. R, L, S, and P represent root, leaf, stem, and spike, respectively, and color gradients indicate gene expression levels in different tissues. The expression levels of candidate genes in different foxtail millet varieties were expressed as Log_2FPKM values, and the *Arabidopsis* database was used for homology comparison and annotation.

Candidatee Gene ID	Phytozome Gene ID	tissue specific expression				JKH4	JKH6	JRK3	JRK6	Homology & Annotation
		R	L	S	P					
SETIT_013648mg	Seita.6G005300	2.53	0.92	0.92	1.92	2.36	2.26	1.45	2.93	HAI2; highly ABA-induced PP2C gene 2
SETIT_001708mg	Seita.5G379400	5.27	4.95	4.37	5.24	0.86	1.09	1.23	1.15	
SETIT_024914mg	Seita.3G218800	0.86	−3.17	1.90	0.08	1.14	1.37	1.34	1.01	
SETIT_036015mg	Seita.9G460200	5.36	1.94	1.94	4.39	2.00	2.66	2.79	1.37	
SETIT_013722mg	Seita.6G163200	3.49	4.49	3.49	6.13	2.37	2.03	2.69	2.45	ATCIPK6; CBL-interacting serine/threonine-protein kinase 6
SETIT_036531mg	Seita.9G318200	5.07	2.72	5.54	3.74	0.99	1.08	1.04	1.21	AT4G33950; protein kinase superfamily protein
SETIT_009836mg	Seita.7G023800	1.89	−3.12	2.16	2.42	2.05	1.53	1.27	1.03	FMO1; dimethylaniline monooxygenase 1
SETIT_035607mg	Seita.9G086400	−1.63	2.63	1.69	−1.64	3.55	1.63	0.81	0.86	AT3G02100; UDP-Glycosyltransferase superfamily protein
SETIT_038681mg	Seita.9G334800	2.90	0.36	0.36	−0.74	3.42	3.07	1.93	3.68	PRCE3; PSI-interacting root-cell enriched 3
SETIT_025269mg	Seita.3G193800	−3.62	NA	−2.24	−0.83	1.48	1.76	1.83	1.50	EDL3; EID1-like 3
SETIT_035904mg	Seita.9G208200	3.45	1.58	2.40	3.44	1.42	1.01	1.08	2.16	AGAL1; alpha-galactosidase 1
SETIT_036944mg	Seita.9G157500	0.78	−3.12	−3.12	−1.12	3.91	3.13	2.61	4.49	AT2G34610; highly expressed in root
SETIT_010550mg	Seita.7G184600	4.01	3.73	3.99	3.43	1.23	1.79	2.88	1.45	HB-7; homeobox 7
SETIT_018237mg	Seita.1G253700	5.34	3.18	4.47	2.94	1.67	1.68	2.00	2.19	HB-7; homeobox 7
SETIT_001322mg	Seita.5G346500	3.99	3.31	3.62	2.23	1.22	1.26	2.46	1.76	AtNRX1; nucleoredoxin 1
SETIT_023488mg	Seita.3G030100	−1.18	NA	0.02	0.42	0.86	1.27	2.05	1.44	KUOX1; KAR-UP oxidoreductase 1
SETIT_026926mg	Seita.8G115400	10.14	5.03	4.79	3.13	6.30	4.11	3.06	3.68	DHN1; Dehydrin family protein
SETIT_039957mg	Seita.9G034000	7.93	1.56	2.06	1.11	2.00	2.86	1.52	1.34	ADF11; actin depolymerizing factor 11
SETIT_009956mg	Seita.7G151700	3.13	0.24	5.20	4.74	4.84	1.08	1.24	2.38	AT3G07570; Cytochrome *b*561
SETIT_023276mg	Seita.3G209800	5.12	3.87	1.79	2.90	1.27	1.45	2.23	1.72	AT5G01300; PEBP family protein
SETIT_022163mg	Seita.3G330700	NA	6.31	2.20	−2.51	−2.13	−1.47	−1.01	−2.04	AT2G03200; Eukaryotic aspartyl protease family protein
SETIT_029197mg	Seita.2G342300	2.89	−2.13	2.71	−2.13	−2.45	−3.92	−2.51	−1.57	CRK25; cysteine-rich RLK 25

metabolism, cutin, suberine and wax biosynthesis, while JKH6 exhibited pathways related to fatty acid elongation, flavonoid biosynthesis and flavone/flavonol biosynthesis. This indicates that different drought-resistant varieties may possess distinct intrinsic mechanisms for coping with water deficit stress, highlighting the potential relevance of fatty acid elongation as a pathway for enhancing crop drought resistance.

The drought resistance of four different foxtail millet varieties in the germination, seedling and the maturity stage was identified, and found that the drought resistance of foxtail millet was complex. JKH4 variety was drought resistant at germination, seedling and maturity, while JKH6 was not drought resistant at germination, and was drought resistant at seedling and the maturity stage, JRK3 and JRK6 had more than 90 % of germination under normal treatment at germination, which was significantly reduced to 79 % and 65 % after treatment with 20%PEG6000 under simulated drought conditions, respectively, indicating that JRK did not have the ability to resist drought at germination, seedling and the maturity stage. The drought resistance performance of crops is associated with different developmental stages and indices for identifying drought resistance [53]. It is important to note that the results from the germination stage alone cannot fully represent the overall drought resistance throughout the seed-ling and entire growth period. Therefore, a combination of results from the entire growth period should be used to provide a more comprehensive material and theoretical basis for selecting drought-resistant varieties in foxtail millet and breeding new drought-resistant varieties.

5. Conclusion

In this study, 217 foxtail millet materials were evaluated for drought resistance during the maturity stage in the field and divided into five drought resistance grades, namely, extremely drought resistance, drought resistance, medium drought resistance, weak drought resistance and extremely weak drought resistance, from which we selected two extremely drought-resistant varieties (JKH4 and JKH6) and two extremely weakly drought-resistant varieties (JRK3 and JRK6) for seedling and germination drought resistance, thus screening out a full-life drought-resistant variety, JKH4 (Chi 5422). In addition, screening out 22 drought-resistant candidate genes to provide a theoretical basis for the drought-resistant breeding of cereal through the transcriptome analysis of four foxtail millet varieties.

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Data availability statement

The raw data from RNA-Seq is available in the NCBI Sequence Read Archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra) under the BioProject accession number: PRJNA1155684.

CRediT authorship contribution statement

Xiling Chang: Writing – original draft, Visualization, Validation, Software, Methodology, Data curation. **Shuangxing Zhang:** Writing – original draft, Validation, Methodology, Data curation. **Changyu Cao:** Validation, Software, Investigation, Data curation. **Jianfei Zhou:** Validation, Methodology, Data curation. **Xiaoxing Wang:** Software, Investigation. **Dingguo Zhang:** Software, Investigation. **Jishan Xiang:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38083.

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