

Comparative transcriptomics reveals key genes contributing to the differences in drought tolerance among three cultivars of Foxtail millet (*Setaria italica*)

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1 Comparative transcriptomics reveals key genes contributing to the differences in drought
2 tolerance among three cultivars of Foxtail millet (*Setaria italica*)

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8
9 **Abstract**

10 Foxtail millet (*Setaria italica* L.) is an important food crop with strong drought stress
11 tolerance. The response of foxtail millet to drought stress is a complex regulatory network,
12 and the physiological mechanism of foxtail millet's adaptation to drought stress is still unclear.
13 This study investigated three different cultivars of foxtail millet with different drought
14 resistance. Drought stress reduced the water content and chlorophyll content of foxtail millet
15 leaves and increased the content of MDA and soluble protein. From strong to weak drought
16 resistance order was Jigu39, Jingu21, and Longgu16. The transcriptome analysis of these
17 three cultivars was carried out. 2954, 1531 and 2344 differentially expressed genes under
18 drought stress were identified in Jigu39, Jingu21 and Longgu16. The GO and KEGG pathway
19 analysis identified DEGs significantly enriched in photosynthesis, chlorophyll metabolism,
20 amino acid metabolism, and carbohydrate metabolism in all three cultivars. In addition, we
21 identified 46 genes whose trends of transcription change were consistent with the drought
22 resistance trends among three cultivars of foxtail millet. Among them, 32 genes were first
23 identified related to drought response in foxtail millet, which was worth for further research.

24
25 **Keywords:** *Setaria italica*, drought stress, transcriptome, drought response genes

26
27 **Introduction**

28
29 Global climate conditions are deteriorating due to increased human activities such as
30 urbanization, rapid industrialization, and overuse of non-biodegradable commodities, thereby
31 exposing plants to harsh and extreme climatic conditions, suffering abiotic stresses such as
32 drought, salinity, and cold, adversely affecting plant morphology, growth and development,
33 and molecular processes (Chaudhry and Sidhu, 2022). Drought is considered to be one of
34 the major abiotic stresses limiting crop production worldwide because it reduces the supply of
35 water in the crop and seriously affects the growth rate and biomass of plants, thereby
36 affecting global food security (Delauney and Verma, 1993; Mishra and Cherkauer, 2010;
37 Anjum et al., 2011).

38 Foxtail millet (*Setaria italica* L.) is an important food crop in arid and semi-arid regions
39 (He et al., 2015). As a C4 plant, foxtail millet can maintain a high photosynthetic rate under
40 drought conditions than other traditional C3 crops such as wheat and rice (Pant et al., 2016).
41 Foxtail millet has a small genome (515 Mb), less repeated DNA, and a short life cycle,
42 making it an ideal model system for the C4 plant study (Li and Brutnell, 2011; Bennetzen et

al., 2012; Zhang et al., 2012; Qin et al., 2020). Foxtail millet also requires less soil moisture, and its transpiration efficiency and water use efficiency (WUE) are much higher than other species, such as sorghum, maize, and wheat (Tang et al., 2017a). Research focusing on foxtail millet will help understand the mechanism of plant stress tolerance.

The adaptation of foxtail millet to drought stress includes biochemical and physiological changes. Foxtail millet weakens or offsets the adverse effects of drought by reducing its net photosynthetic rate, regulating stomatal closure, synthesizing osmotic regulators to balance cell osmotic pressure, and synthesizing various antioxidants (Lawlor and Cornic, 2002). However, the response of foxtail millet to drought stress is a complex regulatory network, and the physiological mechanism is still unclear.

At present, the deep transcriptional studies on the drought tolerance in foxtail millet mainly focus on the different development stages and tissues of one or two foxtail millet cultivars with different drought tolerance (Tang et al., 2017b; Xu et al., 2019; Qin et al., 2020; Zhang et al., 2022). In our previous study, we detected the transcription levels of 52 *SiSNARE* genes in Jingu21 and Longgu16 under drought stress (Wang et al., 2021) and found that *SiNPSN11* (*Seita. 4G280200*) was only down-regulated in Jingu21, which was different from the report of Qi et al. (2013). The above results indicated that transcriptional regulation of some drought-induced genes was different in different cultivars of foxtail millet. Therefore, In this study, we further expanded the scope of the comparative transcriptome and compared the drought resistance of three foxtail millet cultivars (Jigu39, Jingu21, and Longgu16). Jigu39, Jingu21, and Longgu16 were selected and bred by the Chinese Academy of Agricultural Sciences in Hebei, Shanxi, and Gansu provinces. The results showed that they had different drought tolerance. Jigu39 had the strongest drought resistance, followed by Jingu21 and Longgu16. Then, we used these three cultivars as materials and performed transcriptome analysis under drought stress by stopping watering. Differentially expressed genes (DEGs) related to drought resistance were isolated and identified. Through gene clustering and related metabolic pathway analysis, the mechanisms of foxtail millet response to drought were systematically expounded from the physiological, biochemical, and transcriptome levels. In total, 46 genes were identified, whose trends of transcription change were consistent with the drought resistance trends among three cultivars. Furthermore, 32 genes of them were firstly reported as drought-related in foxtail millet. Our research provided useful information and a theoretical basis for further exploring the mechanism of foxtail millet drought resistance.

Materials and methods

Plant materials, growth conditions and stress treatment

In this study, three different cultivars of foxtail millet (Longgu16, Jingu21, and Jigu39) were used as experimental materials (referred to as Jigu, Jingu and Longgu, respectively). All three seeds were collected from Prof. Lizhen Zhang (School of Life Science of Shanxi University) for free and used for research only. Three cultivars of foxtail millet were planted in a mixture (nutrient soil: vermiculite = 1:1) to grow in a greenhouse for four days, and growth-consistent seedlings were moved to the plastic pot. Seedlings were grown for 14 days

in a greenhouse at 16 h light/8 h darkness, 23-26 °C, 50000 Lux light, and 60-70 % relative humidity, and then treated with drought stress by stopping watering for another 14 days. The leaves were collected from the plant with normal watering or drought stress, immediately frozen in liquid nitrogen and stored at -80 °C for RNA sequencing and qRT-PCR analysis. There were three biological replicates and three technical replicates per experiment to ensure the reliability of experimental data.

Physiology assay

The measurement of the water loss rate was performed as a previous report (Aguilar et al., 2000). The detached fresh leaves of 14-day-old seedlings were dried at room temperature for 48 h (15-25% relative humidity), and the weight of the leaves was measured. The weight of detached leaves calculated the water loss rate at each time point marked. The weight of 0 h was fresh weight (FW), and the weight of leaves at the corresponding time was recorded as dry weight (DW). According to the following formula, the leaf water loss rate at the corresponding time was calculated.

$$\text{Leaf water loss rate (\%)} = (\text{FW}-\text{DW})/\text{FW} \times 100.$$

The contents of MDA, chlorophyll, and soluble protein were determined by Comin biochemical test kit (MDA-1-Y, CPL-1-G, KMSP-1-W, Suzhou, Jiangsu, China) according to the manufacturer's instructions.

RNA Isolation and Library Preparation

The transcriptome sequencing and preliminary analysis were carried out by OE Biotech (Shanghai, China). Total RNA was extracted by the TRIzol method (Ambion, Thermo Scientific Inc., USA). After removing the DNA in total RNA using DNase, the mRNA was enriched by magnetic beads and interrupted by the reagent. The double-stranded cDNA was gradually synthesized as a template, and the end of the double-stranded cDNA was filled. A tail was added, and the adapter was added. Finally, the library was constructed by PCR amplification, and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was used for quality inspection.

RNA Sequencing and Differentially Expressed Genes Analysis

The constructed libraries were sequenced using the Illumina HiSeq X Ten platform, processed to obtain clean readings and mapped via HISAT2 (Kim et al., 2015) to the *Setaria italica* v2.2 transcripts in the JGI transcriptome reference database (<https://phytozome-next.jgi.doe.gov/>).

After the genes were obtained, their FPKM values were calculated, readings were taken using HTSeq count, and differential expression analysis was performed using the DESeq R software package to obtain DEGs. P-value < 0.05 and foldchange > 2 or foldchange < 0.5 were used as the thresholds for significant difference expression. Hierarchical clustering analysis, GO enrichment and KEGG pathway enrichment analysis were performed on DEGs.

qPCR validation in the Illumina data

The accuracy of the RNA-seq data needs to be verified by quantitative real-time PCR (qRT-PCR). The total RNA of foxtail millet leaves was extracted using TransZol™ UP Plus RNA Kit (TransGen Biotech, Beijing, China) and reversely transcribed using EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech, Beijing, China). QRT-PCR was performed using the PerfectStart Green qPCR SuperMix kit (TransGen Biotech, Beijing, China), and the results were analyzed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). *SiAct2* (*Seita. 8G043100*) is used as an internal reference. All primers used in the experiment were synthesized by Sangon Biotech, Shanghai (Supplement Table 1). Three biological and technical replicates were performed for each sample in the qRT-PCR to reduce the impact of biological and technical differences.

Statistical analysis

Each experiment has at least three completely randomized block designs with biological repetitions to ensure the accuracy of the experimental results. Student's t-tests or ANOVA were performed in SPSS Statistics 22.0 to evaluate the data of physiological analysis (SPSS Inc., Chicago, IL, USA). Each data value is expressed as mean $\pm$ standard error (SE). $P < 0.05$ indicated that there was a statistically significant difference.

Results

Longgu shows better drought stress tolerance than the other two cultivars

After germination, all cultivars showed similar growth and development pattern during the time range of the experiment, suggesting there was no significant development difference among the three cultivars (Supplement Figure 1). The phenotype response of Jigu, Jingu and Longgu to drought stress are shown in Figure 1A. After drought treatment, the leaves of all three cultivars became yellow and wrinkled to a certain extent. Longgu showed the most serious of wrinkles. After plants are subjected to drought stress, they will reduce the water loss by closing stomata. The water loss in detached leaves can reflect the drought resistance of plants (Carmody et al., 2016). Here, the water loss rate of detached leaves within 48 h was detected, and the water loss rate was calculated. The results showed that the leaf water loss rate of Longgu was significantly higher than that of Jigu and Jingu at the same time, and that of Jigu was the slowest (Figure 1B), supporting their phenotypes shown in Figure 1A.

MDA is an indicator of membrane lipid peroxidation under stress. The change of MDA content can reflect the damage degree of membrane lipid under drought stress and indirectly reflect the drought resistance of crops (Hu et al., 2013). As shown in Figure 1C, the MDA contents of the three cultivars of seedlings increased, indicating that their cell membrane was damaged under drought stress (Figure 1C). Chlorophyll content can indicate the utilization rate of light energy in crops. When crops are under drought stress, the enzyme synthesis of chlorophyll is hindered (Impa et al., 2012), resulting in a decrease of chlorophyll content (Meher et al., 2018). This study found that the chlorophyll contents of Jigu, Jingu and Longgu

leaves decreased significantly after drought stress (Figure 1D). Again, Jigu showed a higher level of chlorophyll content, even after drought stress, which might be related to its better drought tolerance.

Soluble proteins are important osmoregulation substances. The accumulation of soluble proteins improves the water retention ability of cells and protects the vital substances and biofilm of cells, so they are often used as plant stress resistance indicators (Smirnoff, 1993; Sharma and Dubey, 2005). As shown in Figure 1E, it was found that after drought stress, the soluble protein contents in Jigu leaves were significantly increased, while the soluble protein contents in Longgu leaves had no significant change (Figure 1E), which also supported the results above.

In conclusion, Jigu showed the highest drought stress tolerance, while Longgu was the weakest among the three cultivars.

RNA-seq analysis

To identify the key genes of foxtail millet in response to drought stress, leaves from three cultivars of foxtail millet were taken for RNA-seq analysis with or without stop watering treatment. Each treatment had three biological replicates, and 18 cDNA libraries were constructed (marker as Jigu and JiguX, Jingu, and JinguX, Longgu and LongguX, with X meaning sample under drought stress). A total of 120.99 G of clean data was acquired from the referenced transcriptome sequencing of 18 samples. The effective data volume of each sample was distributed in 6.45-7.09 G, the Q30 base was distributed in 94.92-95.33%, and the average GC content was 53.87%. Use HISAT2 (Kim et al., 2015) to align clean reads with the specified reference genome. The sequencing and alignment results are summarized in Supplement Table 2. The reads were mapped to the reference genome and showed high alignment rates. Alignment rates ranged from 96.85 to 98.05 %, and unique mapped reads ranged from 94.33 % to 95.81 %.

Differential expression analysis of transcripts

Htseq-count software (Anders et al., 2015) was used to obtain the number of reads on protein-coding genes. The cufflinks software (Trapnell et al., 2010) was used to calculate the FPKM value (Roberts et al., 2011) of these genes. According to the gene expression of all samples, principal component analysis (PCA) and cluster analysis were performed by R software to evaluate the repeatability and correlation of samples. It can be seen from Supplement Figure 2 that the repeatability of three biological repeats in each treatment is satisfactory, and the difference between treatments is obvious, indicating that the current RNA-Seq data is reliable for subsequent analysis.

The three repeated data of each treatment were summarized and compared as JiguX with Jigu (JiguX/Jigu), JinguX with Jingu (JinguX/Jingu), and Longgux with Longgu (LongguX/Longgu) to further identify differentially expressed genes (DEGs) in response to drought stress, P value < 0.05 and $|\log_2FC| > 2$ was set as the threshold for significantly differential expression. The number of DEGs in different cultivars was summarized in Figure 2A. Before and after drought stress, 24966 DEGs were identified in three cultivars of foxtail

millet. After drought stress, there were 4581 up-regulated DEGs and 4896 down-regulated DEGs in Jigu, 3442 up-regulated DEGs and 3160 down-regulated DEGs in Jingu, and 4068 up-regulated DEGs and 4819 down-regulated DEGs in Longgu. The overall distribution of differentially expressed genes is shown in the volcano map (Figure 3).

In order to further understand the regulatory mechanism of response to drought stress in foxtail millet, we compared DEGs among JiguX/Jigu, JinguX/Jingu, and LongguX/Longgu. A total of 2717 (18.69%) overlapping genes were found in the three cultivars. 94.81% of overlapping genes were up-regulated and down-regulated in the same trend among the three cultivars, and these overlapping DEGs were more likely to participate in the metabolic pathways that affected the growth and development of foxtail millet under drought stress. In addition, there were 2954, 1531 and 2344 cultivar specific DEGs in Jigu, Jingu, and Longgu, respectively. Among these cultivar specific DEGs, there were 1655, 809, 1310 up-regulated genes and 1299, 722, 1034 down-regulated genes in three cultivars (Figure 2A). The expression specificity of many differential genes in the three cultivars of foxtail millet showed different regulation modes of drought stress, which may be one of the reasons why the tested cultivars were sensitive or tolerant to drought stress.

Gene ontology (GO) analysis of DEGs

After obtaining DEGs, GO enrichment analysis of DEGs was performed, and their functions were described combined with GO annotation results. Based on GO annotation, in JiguX/Jigu comparison group, drought stress mainly affects two biological processes: cellular process and metabolic process. The cellular component mainly affects the cell, cell part, membrane, complex, membrane part, organelle, and organelle part. For molecular function, drought stress mainly affects binding and catalytic activity, structural molecule activity, and transporter activity (Supplement Figure 3). The main changes in biological process, cellular component, and molecular function of JinguX/Jingu and LongguX/Longgu are similar to that of JiguX/Jigu (Supplement Figure 3). In addition, the results for significantly enriched Top30 GO terms ($P_{adj}\text{-value}<0.05$) are presented in Supplement Table 3. In addition, to further understand the regulatory network of drought stress response of three foxtail millet cultivars, we also performed GO enrichment analysis on the overlapping and specific DEGs in this study (Supplement Figure 4).

Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway analysis of DEGs

Based on KEGG pathway enrichment analysis, 2052 (JiguX/Jigu), 1227 (JinguX/Jingu) and 1838 (LongguX/Longgu) DEGs were assigned to 372, 355 and 377 pathways (Supplement Table 4). These three comparisons were different in the metabolic pathways of DEGs. Jigu, with strong drought resistance, was significantly enriched in photosynthesis antenna proteins, biosynthesis of ansamycins, ribosome, photosynthesis, ribosome biogenesis in eukaryotes, glyoxylate and dicarboxylate metabolism, and carbon fixation in photosynthetic organism pathways (Figure 4A). DEGs in Jingu are mainly enriched in plant hormone signal transduction, MAPK signalling pathway-plant, type I diabetes mellitus and

other pathways (Figure 4B). DEGs' main enriched metabolic pathways in Longgu included biosynthesis of ansamycins, photosynthesis-antenna proteins, glyoxylate and dicarboxylate metabolism, photosynthesis, porphyrin and chlorophyll metabolism, and carbon fixation in the photosynthetic organisms (Figure 4C).

Drought stress-responsive transcriptional factors

Transcription factors (TFs) play a central role in regulating biological and abiotic stress responses and various biological processes by regulating functional genes in plants (Mickelbart et al., 2015; Baillo et al., 2019). This study identified 122 TFs from 26 TF families in the detected DEGs (Supplement Table 5, Supplement Table 6). These TFs identified in the leaves of three cultivars of foxtail millet included many *WRKY*, *ERF*, *MYB*, *NAC*, *bHLH*, *bZIP*, and *HSF* TFs that have been confirmed to be involved in the transcriptional regulation of multiple stress responses in plants (Lata et al., 2013; Muthamilarasan et al., 2015; Todaka et al., 2015; Wang et al., 2018). The number of target genes regulated by the *DOF* family was the largest. In addition, in this study, the number of DEGs identified by *DOF* and *C2H2* families in Jigu with the strongest drought resistance was higher than that of the other two cultivars, while the number of DEGs identified by the *MIKC-type MADS* family was the largest in Longgu with the weakest drought resistance (Supplement Table 5). We also found that some TFs had different expression patterns among the three foxtail millet cultivars in response to drought stress (Supplement Table 6). Notably, three TFs, including *Seita.1G043800* (belonging to the *bZIP* family) and *Seita.9G432200* (belonging to the *MYB* family), and *Seita.2G206000* (belonging to the *WRKY* family), expressed opposite patterns in JiguX/Jigu and JinguX/Jingu. The first two were down-regulated in JiguX/Jigu but up-regulated in JinguX/Jingu, whereas *Seita.2G206000* was the opposite. Of interest, none of the three TFs was expressed significantly in LongguX/Longgu (Supplement Table 6). In summary, the expression changes of the three cultivars under drought stress indicated that the TF encoding gene played an important role in the drought tolerance of foxtail millet.

DEGs involved in chlorophyll metabolism and photosynthesis

A total of 57 DEGs involved in the Photosynthesis (ko00195) pathway were identified in transcriptome analysis (Figure 5). In Jigu and Longgu, genes related to photosystem II, photosystem I, cytochrome b6/f complex, and F-type ATPase were significantly down-regulated. The gene encoding plastocyanin (PC) was significantly down-regulated in photosynthetic electron transport, while the genes encoding ferredoxin (Fd) and ferredoxin-NADP⁺ reductase (FNR) was partially up-regulated and partially down-regulated (Figure 6). The number of DEGs responding to drought stress was the least in the photosynthetic pathway in Jingu. Under drought stress, the transcription levels of the genes encoding Fd and cytochrome c6 (cyt c6) in Jingu were up-regulated (Figure 6). In transcriptome analysis, 35 DEGs involved in porphyrin and chlorophyll metabolic pathways were found, of which 11 were up-regulated and 24 were down-regulated (Figure 7). The transcription level changes of 8 DEGs in the two pathways were consistent with the drought resistance trend of the three foxtail millet genotypes. *FDX6* (*Seita.5G390200*), *psaB* (*Seita.9G143100*), *psbA*

(*Seita.J017900*) and *psbC* (*Seita.J018300*) participated in the photosynthesis pathway. *COX15* (*Seita.6G195500*), *POX2* (*Seita.7G147400*), *HO1* (*Seita.4G223200*) and *Seita.3G298600* were involved in the chlorophyll metabolic pathway (Figure 5, Figure 7).

DEGs involved in amino acid metabolism and carbohydrate metabolism

There were 170 DEGs (86 up-regulated and 84 down-regulated) in JiguX/Jigu, 112 DEGs (51 up-regulated and 61 down-regulated) in JinguX/Jingu, and 202 DEGs (103 up-regulated and 99 down-regulated) in LongguX/Longgu were involved in amino acid metabolism (Supplement Table 7). There were only 21 overlapping DEGs in all three groups. They were all down-regulated (Figure 8, Supplement Table 8). In addition, DEGs of the three groups were enriched in some pathways related to carbohydrate metabolisms, such as glyoxylate and dicarboxylate metabolism (ko00630), pentose phosphate pathway (ko00030), glycolysis/gluconeogenesis (ko00010), pantothenate and CoA synthetase (ko00770), fructose and mannose metabolism (ko00051), pyruvate metabolism (ko00620), ascorbate and aldarate metabolism (ko00053), starch and sucrose metabolism (ko00500), amino and nucleotide sugar metabolism (ko00520), galactose metabolism (ko00052), and citrate cycle (TCA cycle) (ko00020) (Table 1).

Table 1. Carbohydrate metabolic pathways significantly enriched by DEGs in three cultivars of foxtail millet.

ID	Term	JiguX/Jigu		JinguX/Jingu		LongguX/Longgu	
		Up	Down	Up	Down	Up	Down
ko00630	Glyoxylate and dicarboxylate metabolism	12	31	7	8	13	32
ko00030	Pentose phosphate pathway	15	15	5	11	14	17
ko00010	Glycolysis / Gluconeogenesis	25	39	19	18	28	35
ko00770	Pantothenate and CoA biosynthesis	9	11	7	8	8	11
ko00051	Fructose and mannose metabolism	10	20	9	12	14	15
ko00620	Pyruvate metabolism	17	18	15	9	18	19
ko00053	Ascorbate and aldarate metabolism	8	12	11	4	13	11
ko00500	Starch and sucrose metabolism	25	33	23	16	24	32
ko00520	Amino sugar and nucleotide sugar metabolism	26	30	20	16	32	30
ko00052	Galactose metabolism	11	13	12	7	18	11
ko00020	Citrate cycle (TCA cycle)	13	5	9	3	11	4

Prediction of drought resistance genes in differentially expressed genes

According to the expression levels and differential expression multiples of the differential genes, we identified 46 genes, and the upward or downward trend of their expression levels under drought stress was consistent with the resistance trend of three cultivars (Table 2). These genes may play an important role in the response of foxtail millet to drought stress.

In order to verify the results of DEGs in RNA-seq data, we randomly selected nine genes from the above 46 genes whose transcription levels are consistent with the drought resistance trend among three genotypes of foxtail millet and performed the qRT-PCR analysis (Supplement Table 1). The qRT-PCR results of the candidate genes are shown in

Supplement Figure 5. The expression patterns of these genes obtained by qRT-PCR were consistent with the results of RNA-seq (Figure 9). Pearson correlation coefficient showed a significantly positive correlation between RNA seq and qRT-PCR data. In JiguX/Jigu, r value is 0.814, in JinguX/Jingu, r value is 0.907, and in LongguX/Longgu, r value is 0.912 (Figure 9).

Table 2. Prediction table of drought stress response genes

Gene ID	Description	log ₂ FoldChange			References
		JiguX/Jigu	JinguX/Jingu	LongguX/Longgu	
Seita.1G239600	phosphoenolpyruvate carboxylase kinase 2 (PPCK2)	0.747	1.220	3.774	(Shi et al., 2018)
Seita.1G241800	Unknown	1.483	3.138	3.454	
Seita.1G326200	protein EXORDIUM (PHI-1)	3.873	3.360	-3.189	(Qin et al., 2020)
Seita.1G335200*	1-aminocyclopropane-1-carboxylate oxidase 1 (ACO1)	0.860	1.931	3.255	(Puranik et al., 2011)
Seita.1G342400	18.6 kDa class III heat shock protein (HSP18.6)	0.811	4.277	5.306	
Seita.1G349800*	serine carboxypeptidase 24 (SCPL24)	0.730	2.349	4.056	(Zhao et al., 2021)
Seita.2G141500	Unknown	4.630	5.747	8.776	(Zhang et al., 2022)
Seita.3G281700	CASP-like protein 4B4	0.860	1.653	1.968	
Seita.3G300900	Unknown	-1.701	0.824	1.340	
Seita.4G020900	protein DECREASED SIZE EXCLUSION LIMIT 1 (DSE1)	0.414	1.926	2.819	
Seita.4G099200	ATP-dependent zinc metalloprotease (FTSH 6)	1.533	4.574	6.013	
Seita.4G108300	pyruvate dehydrogenase E1 component subunit alpha-2, mitochondrial	2.383	2.554	5.330	
Seita.4G155500	Unknown	-2.978	-2.410	-2.316	
Seita.4G235500*	ent-kaurene oxidase 2 (CYP701A6)	-2.027	-2.754	-3.533	
Seita.4G256300	Unknown	4.652	1.881	1.596	
Seita.4G266100*	auxin-responsive protein (SAUR40)	-1.560	-1.937	-3.081	
Seita.4G273900	mitogen-activated protein kinase 12 (MPK12)	3.325	1.106	-0.170	(Puranik et al., 2011)
Seita.5G013800	membrane-associated kinase regulator 2 (MAKR2)	-1.582	-1.749	-2.901	

Seita.5G022000	ethylene-responsive transcription factor (ERF071)	4.406	1.296	-0.625	(Shi et al., 2018)
Seita.5G069300	ricin B-like lectin (R40C1)	2.063	2.181	4.256	
Seita.5G294000	probable protein phosphatase 2C 50 (PP2C50)	-1.031	1.106	1.465	(Puranik et al., 2011; Qin et al., 2020)
Seita.5G326900	protein NRT1/PTR FAMILY 1.2 (NPF1.2)	4.598	3.589	3.053	
Seita.5G393800	bZIP transcription factor 12 (BZIP12)	1.924	2.145	4.247	(Liu et al., 2016)
Seita.5G427100*	E3 ubiquitin ligase PQT3-like	1.716	2.442	3.658	(Song et al., 2016)
Seita.6G096800	zinc finger protein CONSTANS-LIKE 1	-4.815	-2.614	-1.468	
Seita.7G045200	transcription factor bHLH35	5.714	2.687	2.480	(Qin et al., 2020)
Seita.7G113700*	putative lipoxygenase 5	3.453	2.919	0.775	(Zhang et al., 2021)
Seita.7G115200	non-functional NADPH-dependent codeinone reductase 2 isoform X2 (DMAS1-D)	1.990	1.921	1.490	
Seita.7G117700	esterase OVCA2-like isoform X1 (STR6)	0.920	1.378	1.533	
Seita.7G120900	Unknown	2.261	2.100	1.024	
Seita.7G126700*	vacuolar iron transporter 1.1 (VIT1)	-0.233	1.790	1.896	
Seita.7G184300	Trihelix transcription factor GT-2-like 1 (GTL1)	0.540	1.465	1.638	
Seita.7G199600	probable serine/threonine-protei n kinase (PIX13)	3.863	1.776	1.579	
Seita.7G203700	RHOMBOID-like protein (RBL1)	1.794	1.749	0.242	
Seita.7G226600*	E3 ubiquitin-protein ligase MBR2 (MBR2)	-3.737	-2.518	-0.983	
Seita.7G230500	Unknown	0.630	1.617	1.896	
Seita.7G312500	protein ZINC INDUCED FACILITATOR-LIKE 1 isoform X2 (ZIFL1)	3.978	1.960	-1.530	

Seita.8G025700	calcium-binding protein CML50 (CBP)	0.855	1.544	1.726	
Seita.9G149500	E3 ubiquitin ligase PQT3-like isoform X1	1.265	1.592	1.903	(Song et al., 2016)
Seita.9G166700	B3 domain-containing protein	-0.256	-2.377	-7.031	
Seita.9G196800	Unknown	-3.606	-7.259	-7.489	
Seita.9G271000*	mitochondrial import inner membrane translocase subunit (TIM22-3)	1.916	1.575	1.269	
Seita.9G288400	thaumatin-like protein 1b (TLP1)	4.720	1.781	-1.746	
Seita.9G379000	serine/threonine-protein kinase (SAPK1)	0.027	0.374	3.010	(Puranik et al., 2011; Shi et al., 2018)
Seita.9G544600	beta-amylase 2, chloroplastic (BAMY2)	1.314	2.024	3.803	
Seita.J013800	RIBOSOMAL PROTEIN L20 (rpl20)	0.485	0.941	1.865	

344 *the gene was verified in qRT-PCR.

Discussion

Plants are challenged by various biotic and abiotic stresses in the whole life process (Boyer, 1982). Crops respond to these stresses through biochemical and physiological processes (Shinozaki et al., 2003). Drought is one of the major abiotic stresses affecting agricultural. In recent years, in order to improve crop productivity in regions with limited water resources, the transcriptional regulation mechanism of drought response in many grain crops such as rice (Lenka et al., 2011) and sorghum (Dugas et al., 2011), wheat (Ergen et al., 2009) and maize (Zheng et al., 2010) has been extensively studied. Foxtail millet, as a member of Gramineae plants, has outstanding tolerance to drought and has the potential to become a new C4 model plant (Peng and Zhang, 2021). Although the research on foxtail millet is increasing day by day, we still know little about the complex network of drought response of foxtail millet.

Moreover, most transcriptional research is concentrated on one or two cultivars, and there is a lack of comparison data among multiple cultivars. Therefore, it is still quite challenging to deeply explore the drought-resistant mechanism of foxtail millet. This study performed transcriptome sequencing of three foxtail millet cultivars with different drought resistance to identify the transcriptome characteristics common to the three cultivars, thereby defining the core genes for drought resistance in foxtail millet, eliminating the specificity caused by differences in individual genetic backgrounds.

Three cultivars of foxtail millet had different drought tolerance mechanism

This study measured leaf physiological status changes and transcriptome changes of three foxtail millet cultivars after drought stress. From the physiological data, the drought resistance of Jigu was better than that of Jingu and Longgu. Its water loss rate and chlorophyll content decreased mildly, and its soluble protein content increased significantly (Figure 1B, figure 1D and figure 1E). Under drought stress, the MDA content of three cultivars of seedling leaves increased to varying degrees (Figure 1C). The above results supported previous reports that O_2 produced in chloroplasts could receive electrons from the photosynthetic electron transport chain and transform into $O_2^{\cdot -}$ under drought stress, causing oxidative damage to the photosynthetic pigment and plasma membrane (Gill and Tuteja, 2010; Exposito-Rodriguez et al., 2017). In addition, to eliminate the difference in growth trend among millet cultivars, we studied the growth of three cultivars at different development stages during the experimental time. The results showed that the development states of the three cultivars were similar (Supplement Figure 1). In summary, the study showed that among the three cultivars of foxtail millet, Jigu had the strongest drought resistance, and Longgu had the weakest drought resistance (Figure 1A), which laid the foundation for our further research on drought tolerance mechanism by RNA-Seq analysis.

RNA-seq analyzed the transcriptome of these three cultivars under drought stress, and many DEGs were detected in the three cultivars, which revealed the transcriptome reconstruction of foxtail millet under drought stress. In addition, according to GO and KEGG analysis of DEGs in JiguX/Jigu, JinguX/Jingu, and LongguX/Longgu, there were differences in molecular functions and pathways of DEGs. In order to further understand the differences

in the regulatory mechanisms of three cultivars of foxtail millet in response to drought stress, we also conducted GO enrichment analysis on the overlapping and specific DEGs (Supplement Figure 4). The study found that the DEGs with overlapping of the three genotypes were significantly enriched in the biological process, such as "recognition of pollen", "protein phosphorylation", "protein", and "response to stress" (Supplement Figure 4A). Interestingly, we found that these DEGs significantly enriched GO terms related to photosynthesis and energy metabolism in cellular and molecular functions, respectively. The overlapping DEGs of the three cultivars might play a conservative role in the drought stress response of foxtail millet. In addition, it should be noted that the specific DEGs of the JiguX/Jigu were significantly enriched in the GO term of "translation", and the specific DEGs of the JinguX/Jingu were significantly enriched in the GO terms of "carbohydrate metabolic process" and "metabolic process". The specific DEGs of the LongguX/Longgu were significantly enriched in the GO term of "embryo development" (Supplement Figure 4). These specific genes were enriched into different GO terms, indicating that different cultivars of foxtail millet had different regulatory mechanisms in response to drought stress.

Based on KEGG pathway analysis, we found that the up-regulated DEGs in JiguX/Jigu were significantly enriched in "ribosome" (ko03010), "ribosome biogenesis in eukaryotes" (ko03008). The ribosome is the molecular factory of protein synthesis, and ribosome biosynthesis is the basis of cell growth ability (Lempiainen and Shore, 2009). This result indicated that Jigu could ensure the correct assembly of ribosomal proteins under drought stress. Among three cultivars, fewer DEGs were identified in JinguX/Jingu. The DEGs of JinguX/Jingu involved in chlorophyll metabolism, amino acid metabolism, and carbohydrate metabolism pathways were fewer than those in JinguX/Jingu. There were a large number of DEGs enriched in some amino acid metabolic pathways in LongguX/Longgu, such as "glycine, serine and threonine metabolism" (ko00260), "cysteine and methionine metabolism" (ko00270), "valine, leucine and isoleucine degradation" (ko00280), "arginine and proline metabolism" (ko00330), "alanine, aspartate and glutamate metabolism" (ko00250).

In conclusion, different physiological and transcriptomic changes of the three cultivars suggested that they have different mechanisms of drought stress response.

Drought response pathways and related genes in foxtail millet

Photosynthesis is an important way for plants to accumulate organic matter (Xiong et al., 2019). Based on the GO enrichment results, it was found that many down-regulated DEGs were enriched in the GO term of "photosynthesis" (GO:0015979) in the three cultivars of foxtail millet, indicating that drought stress would affect the photosynthesis (Supplement Table 3), consistent with previous reports (Galmes et al., 2007; Chaves et al., 2009; Zhang et al., 2016; Shi et al., 2018). We have sorted out the DEGs that are significantly enriched in the pathway of photosynthesis (Figure 5). Cluster analysis showed that the transcriptional regulation of the photosynthesis pathway in Jigu and Longgu was more similar (Figure 5). The studies found that the transcription levels of most genes related to photosystem II (PSII), cytochrome b6/f (Cytb6f), photosystem I (PSI) and carbon fixation were down-regulated in Jigu and Longgu (Figure 6). However, compared with Longgu, the regulation mechanism of photosynthesis is more complex in Jigu. The transcription levels of *psbA* (*Seita.J017900*),

psbB (Seita.J014000), *psbK* (Seita.1G126100), *psbT* (Seita.J014100), *psbZ* (Seita.J018400), *psaA* (Seita.J011100), *petG* (Seita.J013400), and *atpB* (Seita.J020700) were only down-regulated in Jigu (Figure 6). Further analysis showed that under drought stress, the transcription level of the *PsaO* subunit responsible for binding to light-harvesting complex II in the PSI reaction centre was significantly down-regulated in three cultivars of foxtail millet (Gorski et al., 2022). The transcription levels of *PNSL3*, a subunit of the chloroplast NADH dehydrogenase-like (NDH) complex involved in photosystem I (PSI) cycle electron flow, as well as *FdC1* and *FdC2*, two FD-like protein members encoded by the plant genome with an extension at the C- terminal of the 2Fe-2S cluster, were significantly down-regulated in the drought-sensitive Longgu. Therefore, we speculated that the down-regulation of transcription levels of *PsaO*, *PNSL3*, *FdC1* and *FdC2* in drought-sensitive Longgu was higher than that in Jigu, which might be one of the reasons why the drought resistance of Longgu was weaker than Jigu (Figure 5). It indicated that chloroplast photosynthesis was involved in plant response to drought stress.

Photosynthesis is related to photosynthetic pigments, photosynthetic gene expression, and redox regulation (Li and Yi, 2020). Chlorophyll is the main photosynthetic pigment in plants and plays a key role in the light absorption of photosynthesis. A stable supply of chlorophyll is the key factor for the plant development of photosynthesis. (Xiong et al., 2019). In our study, the down-regulated DEGs in JiguX/Jigu and LongguX/Longgu were significantly enriched in the 'Porphyrin and chlorophyll metabolism' (ko00860) pathway and the number of DEGs involved in this pathway in Jingu was the least (Figure 8, Supplement Table 4). Under drought stress, the expression levels of protein-coding genes involved in chlorophyll metabolism in JiguX/Jigu and LongguX/Longgu showed significant synchronous changes (Figure 8). Except for the up-regulation of some enzymes, such as heme A synthase (COX15) and chlorophyll b reductase (NOL, NYC1), the expression levels of most protein-coding genes were down-regulated in all three cultivars. The pheophorbide a oxygenase (PAO) gene was significantly up-regulated in LongguX/Longgu, which played an important role in chlorophyll degradation. The genes of protochlorophyllide reductase (PORA) and magnesium protoporphyrin IX methyltransferase, chloroplastic (CHLM), which were related to chlorophyll biosynthesis, were significantly down-regulated. This result may be one of the reasons for the relatively weak drought resistance of Longgu (Figure 8).

Carbohydrates are substrates for energy production, osmoregulation, and osmoprotection in response to drought and are associated with growth, development, and carbon status. Among the main enriched carbohydrate metabolic pathways, the numbers of DEGs in Jigu and Longgu were similar in each pathway, while Jingu was relatively small (Table 1).

Amino acids are the synthetic units of proteins and play a role in compound biosynthesis, signalling, and stress responses (Jiang et al., 2021; Ming et al., 2021). In plants, the levels of free amino acids are tightly controlled by metabolic and developmental factors. Their degradation not only contributes to osmoregulation to maintain cellular homeostasis but also contributes to the energy state of plants, providing a link between carbon and nitrogen metabolism. Under drought stress, amino acid and carbohydrate metabolism are important for stress adaptation (Diniz et al., 2020). This study also sorted out the DEGs involved in amino acid metabolism under drought stress (Supplement Table 7). The number of DEGs in

Longgu was the largest, while that in Jingu was the smallest. After comparison, only 21 overlapping genes (5.98%) were identified in three foxtail millet cultivars, and all DEGs were down-regulated. This situation shows that three cultivars have different regulatory mechanisms in amino acid metabolism under drought stress, which may be one of the reasons they show different tolerance to drought stress.

Transcription factors play an important role in drought tolerance

Transcription factors control the expression of many target genes in plants under drought stress (Qin et al., 2020). In this study, we found that the number of target genes regulated by the *DOF* family was the most among three cultivars of foxtail millet, followed by the *ERF* family (Supplement Table 5), indicating that the transcription factors of these two families played an important role in the drought response of foxtail millet. The *DOF* family is involved in many basic processes in higher plants, from response to light and plant hormones to flowering time and seed maturation (Yu et al., 2020). Studies have shown that the *DOF* and *MYB* families play important regulatory roles in barley seed germination (Seven and Akdemir, 2020). However, the relationship between *DOF*-type TFs and abiotic stress tolerance remains largely unknown (Corrales et al., 2017). The *ERF* family is a large gene family of transcription factors (Nakano et al., 2006). In contrast to the *DOF* family, many proteins of the *ERF* family were identified in our study. They are involved in many different functions in plant growth and development, such as hormone signal transduction and disease resistance pathways (Gutterson and Reuber, 2004), responses to biotic and abiotic stresses (Gu et al., 2000; Gutterson and Reuber, 2004; Mizoi et al., 2012), and regulation of metabolism (van der Fits and Memelink, 2000; Aharoni et al., 2004; Broun et al., 2004; Zhang et al., 2005). We identified 5 *DOF* and 12 *ERF* differentially expressed in the leaves of three cultivars. The number of differentially expressed transcription factors (DETFs) encoded by *DOF* and *ERF* in the tolerant cultivar (Jigu) was higher than that in the sensitive cultivars (Jingu and Longgu) (Supplement Table 5). In Jigu, DETFs of four *DOF* families were all down-regulated. The only existed *DOF* (*Seita.1G290700*) in the other two cultivars was up-regulated. Its homology was presumed to be a key regulator involved in carbohydrate metabolism and drought tolerance in *Betula platyphylla* (Tarelkina et al., 2020). In addition, *ERF* family proteins are considered to be key regulators in response to environmental stimuli in Arabidopsis and rice (Nakano et al., 2006). It is worth noting that two up-regulated DETFs (*Seita.1G342700* and *Seita.2G230500*) and three down-regulated DETFs (*Seita.1G055900*, *Seita.8G081600* and *Seita.9G320700*) of this family only exist in drought tolerance cultivar (Jigu). They were likely to play an important role in the drought tolerance of Jigu. Previous research has shown that the expression level of *EREBP1* (homology of *Seita.1G342700*) in drought-resistant cultivars of *Lemon Balm* was significantly higher than that in drought-sensitive cultivars (Rahimi et al., 2016).

Prediction of drought stress response DEGs

This study identified 46 genes whose expression levels changed regularly in JiguX/Jigu, JinguX/Jingu, and LongguX/Longgu (Table 2). In previous studies, *Seita.2G141500* (Zhang et

al., 2022), *Seita.1G239600*, *Seita.5G022000*, *Seita.9G379000* (Shi et al., 2018), *Seita.1G326200*, *Seita.5G294000*, *Seita.7G045200* (Qin et al., 2020), *Seita.1G349800* (Zhao et al., 2021), *Seita.5G393800* (Liu et al., 2016), and *Seita.7G113700* (Zhang et al., 2021) have been identified to play an important role in the drought process of foxtail millet. In addition, *SiBZIP12* (*Seita.5G393800*), *SiPP2C50* (*Seita.5G294000*), *SiSAPK1* (*Seita.9G379000*), *Seita.7G113700*, *SiACO1* (*Seita.1G335200*) and *SiMPK12* (*Seita.4G273900*) were also detected to be involved in the salt stress response of foxtail millet in previous studies (Puranik et al., 2011; Liu et al., 2016; Zhang et al., 2021). In addition to foxtail millet, these genes were also identified in plant abiotic stress responses in other species. For example, in *Populus euphratica*, *BHLH35* (homology of *Seita.7G045200*) has been identified as a positive regulator of the drought stress response by regulating stomatal density, stomatal opening, photosynthesis, and growth (Dong et al., 2014). The expression of *SAPK1* in rice was increased by drought, NaCl, and PEG treatment, and it also affected the ROS detoxification in salt stress response (Lou et al., 2018).

In addition to the above genes, in previous studies, some genes were identified as important regulatory factors involved in plant abiotic stress responses in some plant species, but they have not been reported in foxtail millet. For Example, *ACO1* (homology of *Seita.1G335200*) is related to drought resistance in *Arabidopsis* and tomato. Plants can regulate the synthesis of ethylene in plants by increasing its expression levels, thereby improving the drought resistance of plants (Eglous et al., 2013). *FTSH6* (homology of *Seita.4G099200*), identified as a potential drought tolerance marker in *Quercus ilex* (Guerrero-Sanchez et al., 2021), was highly induced when the tomato was subjected to water stress (Tapia et al., 2021). The expression of *R40C1* (homology of *Seita.5G069300*) and *rpl20* (homology of *Seita.J013800*) were related to the drought tolerance potential of rice and *Caragana cultivars*, respectively (Jiang et al., 2018; Sahid et al., 2020). In cotton, *GT-2* (homology of *Seita.7G184300*) was highly up-regulated under drought and salt stress. Overexpression of *GT-2* enhanced drought tolerance, decreased MDA and H₂O₂ contents, and increased reactive oxygen species scavenging enzyme activity in cotton (Magwanga et al., 2019). In *Arabidopsis*, plants expressing *CBP* (homology of *Seita.8G025700*) have a better survival rate and root growth under intermittent drought or high salt stress (Tsou et al., 2012).

Lipoxygenase catalyzes the production of oxytocin and plays a key role in the whole growth period of plants (such as seed germination, plant growth, and stress resistance). A previous study by Zhang et al. (2021) showed that the transcription level of *Seita.7G113700* was decreased in foxtail millet under drought stress, which was different from our results. In addition, in our study, *HSP18.6* (*Seita.1G342400*) was involved in the response process of foxtail millet to drought stress, which was also different from the result reported by Coca et al. (1996) in sunflowers. These results indicated that the transcriptional regulation of the same gene was different in different species or even among different cultivars under drought stress. As the drought resistance mechanism of foxtail millet is relatively complex and involves various factors, these problems need to be further studied in the future.

We also found some drought-related genes in foxtail millet (Table 2) that previous studies have not reported as stress-related. Among these identified new genes, the functions of some genes' homologous in other species have been characterized, such as *DMAS1-D* (homology

of *Seita.7G115200*) and *ZIFL1* (homology of *Seita.7G312500*), they maintain iron homeostasis in gramineous plants such as rice and barley, and *CYP701A6* (homology of *Seita.4G235500*) necessary for GA biosynthesis in rice (Wang et al., 2012; Beasley et al., 2017; Lee et al., 2021). However, there are still some genes, such as *Seita.7G230500*, *Seita.4G155500* and *Seita.6G096800*, whose functions in plant growth and development need further study. In conclusion, these genes were probably related to the drought resistance mechanism of foxtail millet.

Conclusion

The plant drought stress response is complex, which involves the survival optimization at multiple levels (Diniz et al., 2020). To understand the drought stress response mechanism, the physiological and transcriptional changes of three foxtail millet cultivars were studied. We found that these three cultivars had different drought tolerance. Jigu has the strongest drought tolerance, followed by Jingu, and Longgu was the weakest. This study performed transcriptome sequencing on these three cultivars to identify genes whose transcriptional level change trends were consistent with drought resistance trends. From the deep transcriptome investigation of these three cultivars of foxtail millet, we found that foxtail millet initiated a system composed of multiple genes and multiple steps, which regulated various pathways to resist drought stress. GO enrichment showed that differentially expressed genes were mainly enriched in the GO terms related to photosynthesis, amino acid metabolism, and carbohydrate metabolism, indicating that some key DEGs involved in these pathways played an important role in drought resistance foxtail millet. Based on the analysis of the KEGG pathway, we found that there were differences in the pathways of apparent enrichment in the three cultivars of foxtail millet. In addition, we found that two transcription factor families, *DOF* and *ERF*, played important roles in the drought response of foxtail millet. We identified 46 genes whose expression levels shared the same trends with the drought resistance of the three cultivars of foxtail millet. Among them, 32 genes were firstly reported as drought stress response-related genes in foxtail millet. Our results provide a theoretical basis for further research of drought-resistant mechanism.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its

supplementary information files.

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Chen et al., 2021) in National Genomics Data Center (Members and Partners, 2022), China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (PRJCA009384; CRA006802) that are publicly accessible at <https://ngdc.cncb.ac.cn/gsa/>.

Competing interests

All authors declare that they have no conflict of interest.

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Authors' contributions

Experiments were designed by BZ, YG, and PY. Experiments were performed by YG, DDH, HW, and XXW. BZ and YG, HW, and PY analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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Figure 1. Effects of drought stress on different Foxtail millet cultivars.
A. Phenotypic alterations of Jigu, Jingu and Longgu in response to drought. B. Water loss rate. C. Malondialdehyde (MDA). D. Chlorophyll. E. Soluble protein. Asterisk indicates that the gene expression after drought stress treatment has a significant difference compared with the control (** $p < 0.01$).

Figure 2. Common and unique differentially expressed genes between different comparison groups.

Figure 3. Differentially expressed volcano map.
Gray is the gene with a non-significant difference, red and green are the genes with a significant difference, the horizontal axis is $\log_2\text{FoldChange}$, and the vertical axis is $-\log_{10} p\text{Value}$.

Figure 4. KEGG pathway enrichment analysis based on the differentially expressed genes in three comparisons.

A. JiguX/Jigu, B. JinguX/Jingu and C. LongguX/Longgu.

Figure 5. DEGs involved in the photosynthesis pathway.
The gene ID, gene symbol and description are shown on the right side of the figure. The values in the figure are $\log_2\text{FC}$ values of each gene.

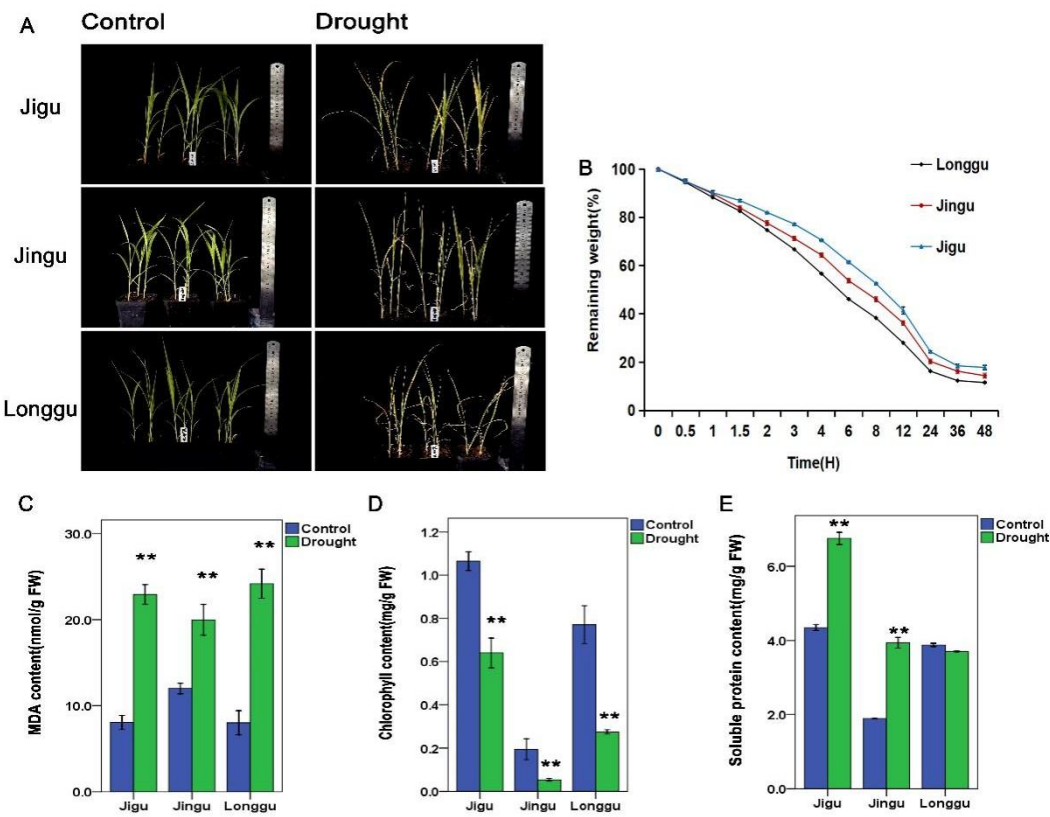
Figure 6. KEGG pathway diagram of photosynthesis.
Green indicates down-regulated genes, and yellow indicates that there are both up-regulated and down-regulated genes.

Figure 7. DEGs involved in the porphyrin and chlorophyll metabolic pathways.
The gene ID, gene symbol and description are shown on the right side of the figure. The values in the figure are $\log_2\text{FC}$ values of each gene.

Figure 8. Statistical histogram of differentially expressed genes in amino acid metabolic pathways.
The horizontal axis is the name of the three comparison groups. The longitudinal axis is the number of differential genes in the comparison group. Up is the number of up-regulated genes with significant differences, and down is the number of down-regulated genes with significant differences.

Figure 9. Linear correlation analysis of fold change data between qRT-PCR and RNA-seq using Pearson correlation coefficient (r).

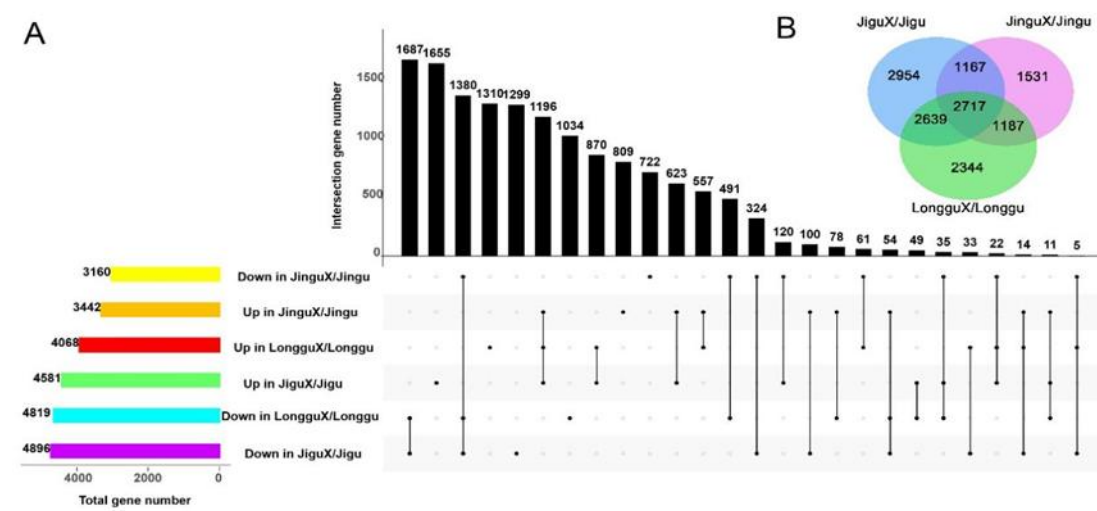
969 Figure 1 Effects of drought stress on different Foxtail millet cultivars.



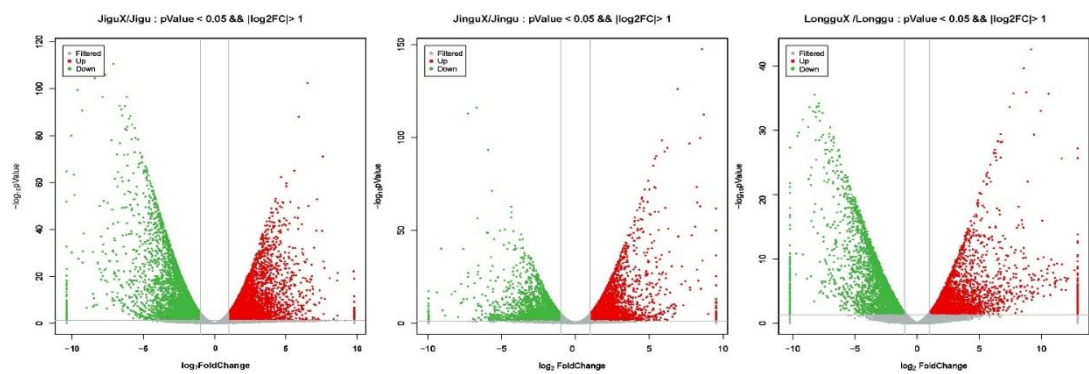
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Figure 2. Common and unique differentially expressed genes between different comparison groups.

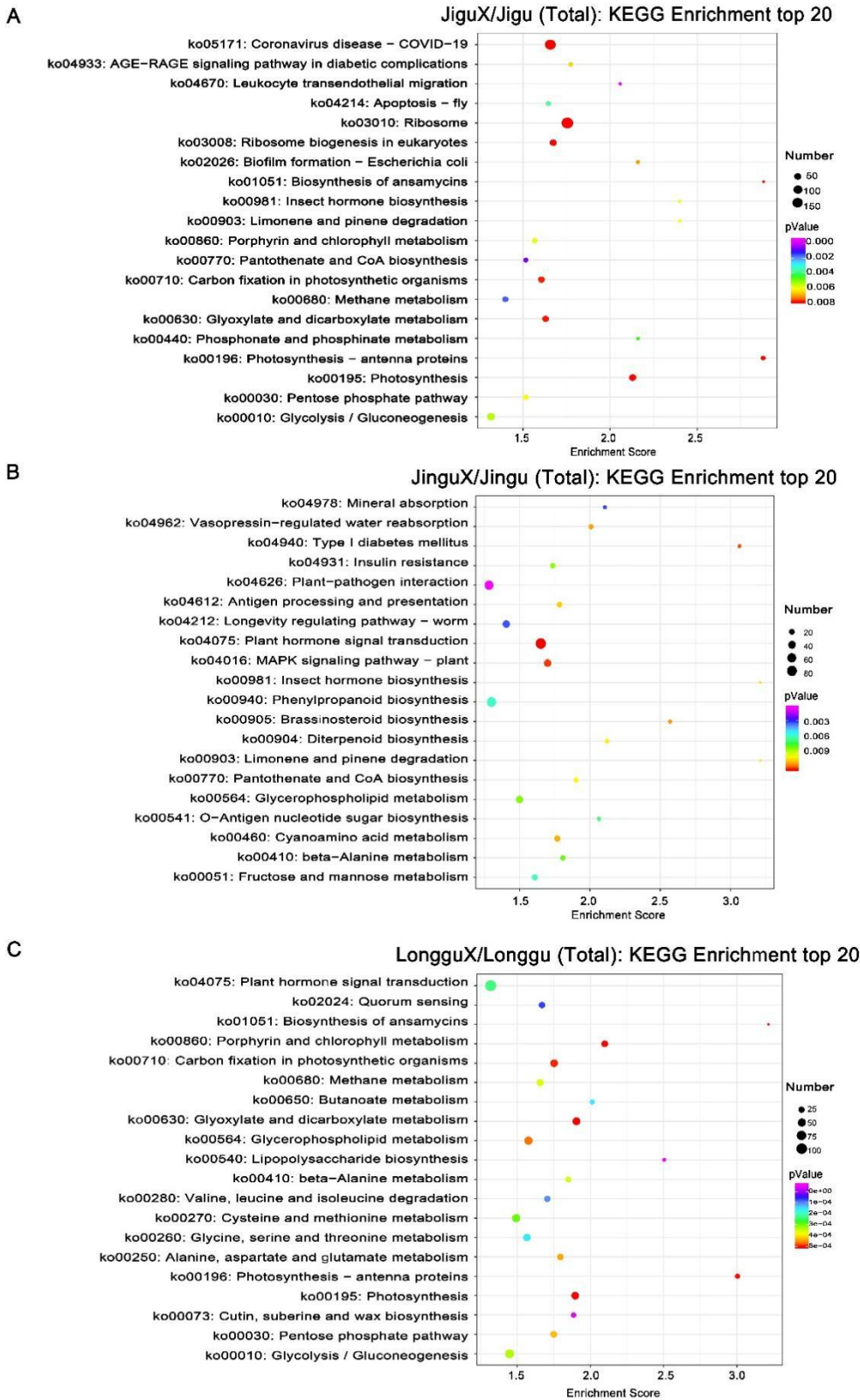


976 Figure 3. Differentially expressed volcano map.

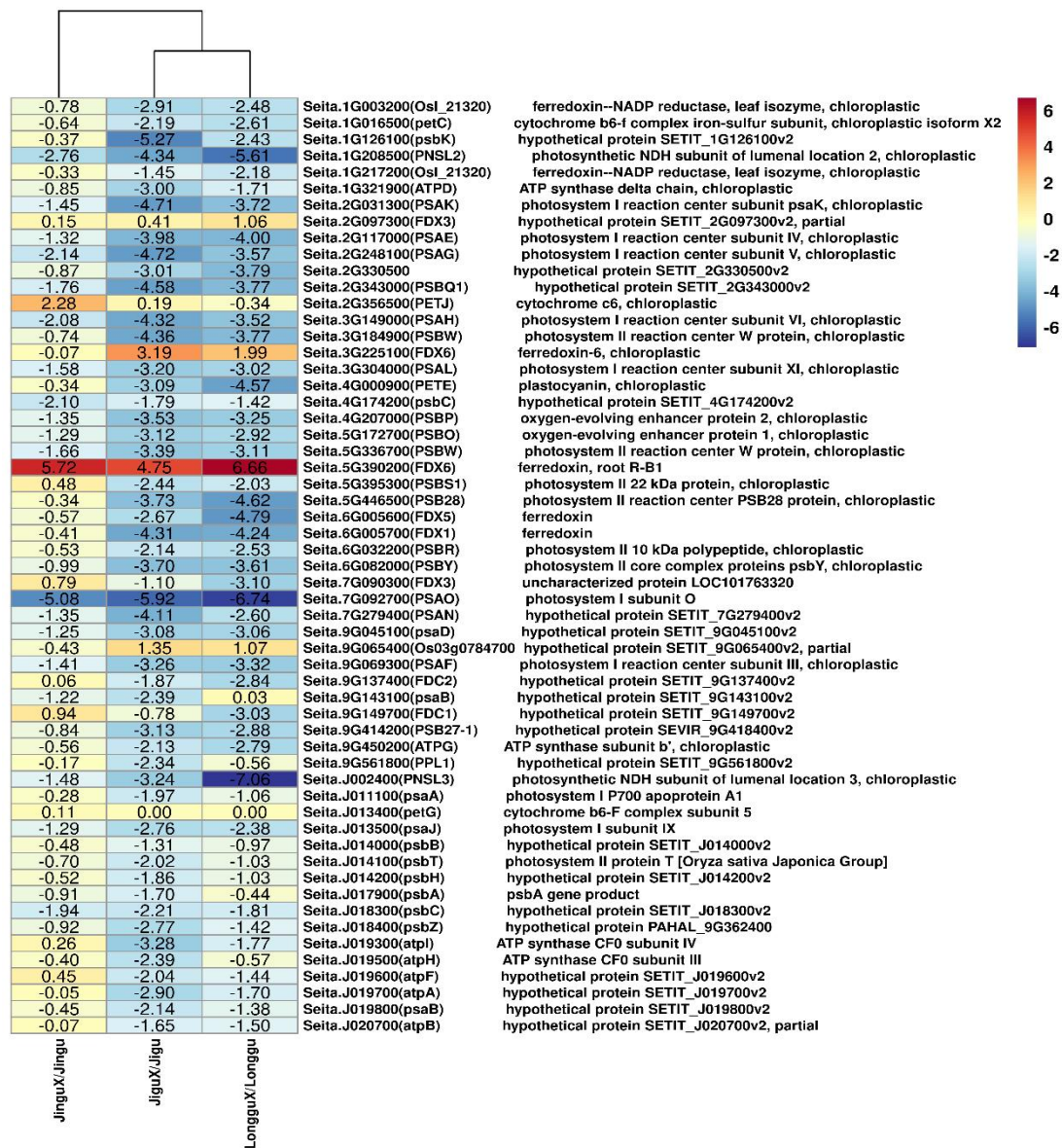


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978 Figure 4. KEGG pathway enrichment analysis based on the differentially expressed genes in
 979 three comparisons.



981 Figure 5. DEGs involved in photosynthesis pathway.





986 Figure 7. DEGs involved in the porphyrin and chlorophyll metabolic pathways.

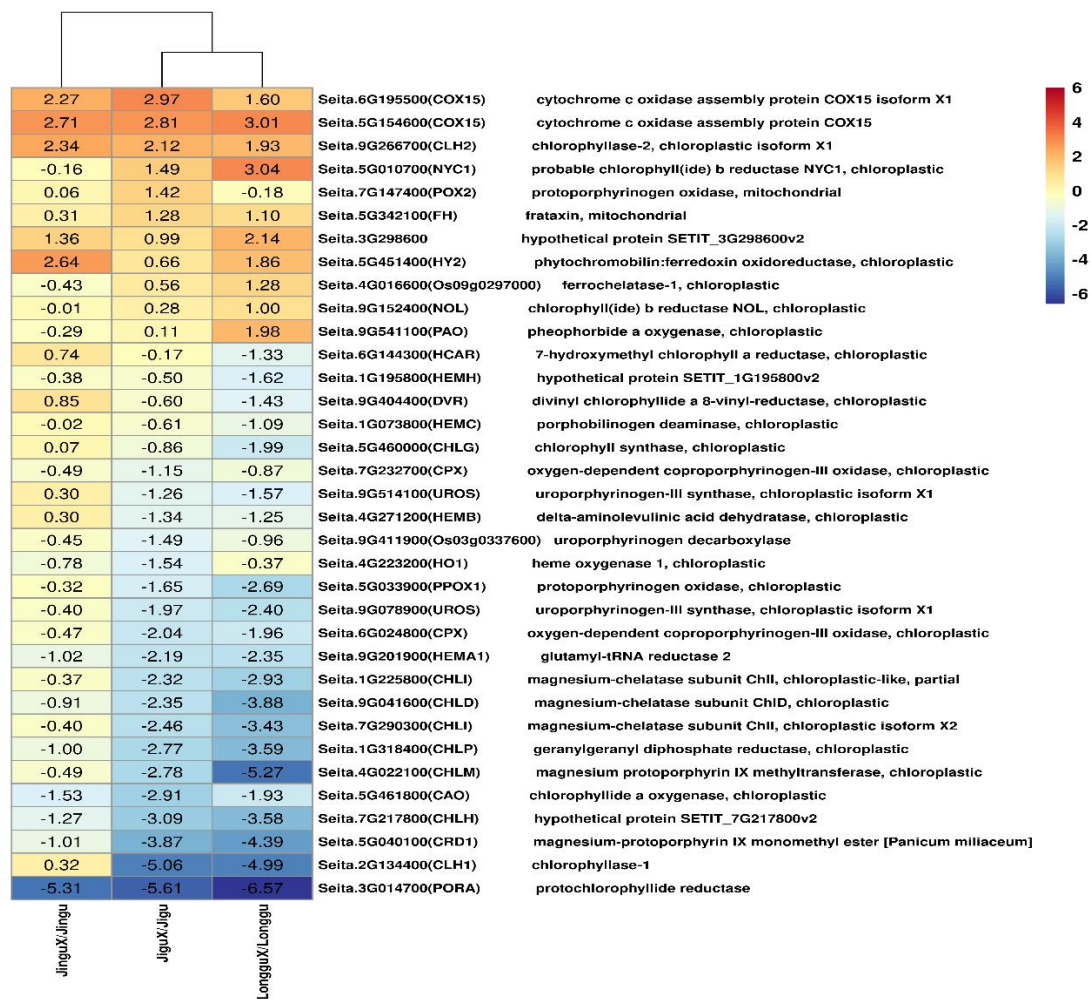


Figure 8. Statistical histogram of differentially expressed genes in amino acid metabolic pathways.

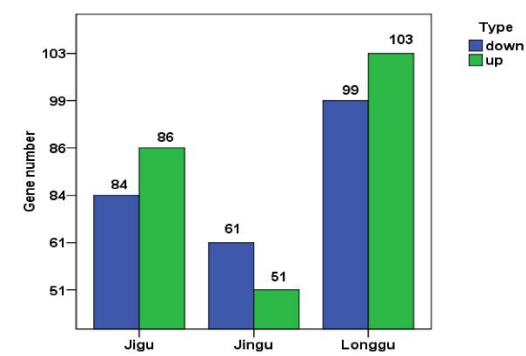
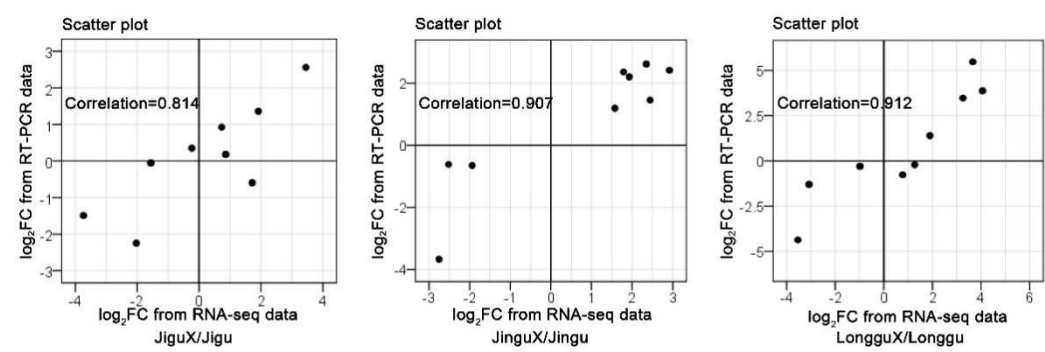


Figure 9. Linear correlation analysis of fold change data between qRT-PCR and RNA-seq using Pearson correlation coefficient (r).



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