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DROUGHT-INDUCED TRANSCRIPTION OF RESISTANT AND SENSITIVE COMMON MILLET VARIETIES

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ABSTRACT

As a well-adapted plant to dry soil and short growing seasons, common millet holds great potential as a drought-resistant crop. Here, we performed a comparative RNA-Seq analysis of two common millet genotypes, Huangmizi (tolerant) and Zhenyuandamizi (sensitive) grown under stress conditions (20% Polyethylene Glycol 6000 with an average molecular weight of ~5,400-7,000 solution). Leaves of seedling plants were harvested from a control and also after 1 and 3 h stress treatment and pooled for RNA isolation. Using Illumina paired-end sequencing, approximately 46.5 million clean reads were generated. Following a *de novo* assembly, a total of 42,240 Unigenes were obtained. A total of 2,301 SSRs and 1,447,148 SNPs were also identified and 75% of Unigenes were annotated. A total of 21,556 and 8,304 Unigenes aligned to the GO and COG databases, respectively. The GO classification showed that the Unigenes were distributed into three categories with 74.30% having biological functions, 76.51% having molecular functions and 79.30% having cellular-level functions. By cross-referencing against KEGGs, 5,535 Unigenes were assigned to 63 metabolic pathways. Additionally, 701 DEGs including 187 up-regulated and 514 down-regulated genes were detected. Transcripts identified in this study will accelerate our understanding of the molecular mechanisms of drought tolerance in common millet.

Key word: Common millet (*Panicum miliaceum* L.), Drought, Transcription.

INTRODUCTION

Common millet (*Panicum miliaceum* L.) belongs to the Gramineae family and is one of the oldest cultivated crops. It is distributed throughout semiarid regions in Asia, Europe, America and Oceania (Chai, 1999; Lágler *et al*., 2005; Fuller, 2006; Crawford *et al*., 2006; Lu *et al*., 2009; Hunt *et al*., 2008). In the US, it is utilized for wild bird seed (Graybosch and Baltensperger, 2009). In China, it has been cultivated for more than 10,000 years between latitudes of 19°15′N (Qionghai, Hainan) to 48°N (Habahe, Xinjiang) and 49°18′N (Haila'er, Inner Mongolia), and from longitudes of 76°E (Atushi and Kashi in Xinjiang) to 143°E (Tongjiang and Hulin in Heilongjiang). In China, it has been cultivated at variable altitudes from 200 m (Rizhao, Shandong) to 3000 m above sea level (Zhada and Pulan Tibet) (Chai, 1999; Chai *et al*., 2012). Due to its relatively low productivity and the introduction of new crops with better yields (e.g. corn, potatoes, sweet potatoes), common millet's production decreased from 2×10^4 km^2 in the 1950s to $1 - 1.2 \times 10^4$ km^2 in the 1990s to approximately $7 - 10 \times 10^3$ km^2 in 2012 (Chai, 1999; Chai *et al*., 2012). Nevertheless, common millet plays an important role in Chinese food and culture because it is richer in nutrients than most other cereal crops therefore it has great potential for further development. Research has shown that supplementing a diet with common millet can help to decrease the incidence rate of chronic diseases such as cardiovascular diseases, cancers, chronic respiratory diseases and diabetes (Nishizawa and Fudamo, 1995; Denery-Papini *et al*., 1999; Nishizawa *et al*., 2002; Park *et al*., 2011; Zhang *et al*., 2014).

Global temperatures are rising and drier conditions are increasing, so cultivating drought-tolerant plants is essential to overcome food and water shortages. Common millet is well-adapted to saline soil, is tolerant to droughts and high temperatures and efficiently uses water, with the lowest water consumption recorded among cereal crops (Chai, 1999). It also has a short growing season. Often it is used as a substitute crop when other crops that require a longer growing season cannot be grown due to a lack of expected seasonal rain. For example, in 1962 China experienced the worst year of natural disasters in recorded history with severe droughts across a vast area. During this crisis, common millet was cultivated in many provinces to cope with the drought and alleviate famine. In that year, common millet covered the largest planted area in China, with a planting area of 6.88×10^3 km^2 in Inner Mongolia and 2.91×10^3 km^2 in Shaanxi (Chai, 1999).

Most drought-tolerance research on common millet has focused on its morphology, physiology and anatomy (Wang *et al*., 2007; Mu *et al*., 2010; Zhang *et al*., 2010; Feng and Zhang, 2012; Zhang *et al*., 2012; Feng *et al*., 2013; Wang *et al*., 2014). There have also been several studies on the molecular mechanisms of drought resistance (Lin *et al*., 2006; Lin *et al*., 2007), but only two genes related to drought tolerance have been characterized (Lin *et al*., 2008; Hu *et al*., 2008). By contrast, many drought resistance genes have been functionally characterized in monocotyledons (rice, maize, oat, barley) and dicotyledons (*Arabidopsis*, tomato, soybean and canola). Most of these genes were first identified in rice and *Arabidopsis* (Yang *et al*., 2010). Since drought-resistance is an extremely desirable trait, we sought to identify additional genes involved in drought-tolerance of common millet that are not present in other plants. Until now the genome of common millet has not been well-characterized due to its allotetraploid nature. Sequencing large genomes is expensive, even when using next-generation sequencing (NGS) technology (Liu *et al*., 2012). An optimal alternative methodology to whole-genome sequencing is RNA-seq, due to its high-throughput nature, reproducibility and accuracy. Over the past several years, RNA-seq has been used in many plant species to explore the genes responsible for abiotic stress, growth, development and terpenoid biosynthesis (Qiu *et al*., 2011; Liu *et al*., 2012; Han *et al*., 2013; Yu *et al*., 2013). In this study, we investigated the transcriptome of common millet using Illumina sequencing and *de novo* assembly. A total of 92,523 transcripts and 42,240 Unigenes were identified. Additionally, a total of 2,301 simple sequence repeats (SSRs) and 1,447,148 single nucleotide polymorphisms (SNPs) were identified. The genomic dataset generated here will facilitate future genetic and breeding studies on this species and other crops that are drought-tolerant.

MATERIALS AND METHODS

Plant materials and growing conditions: This study was carried out at the experimental station of the Agronomy College, Shanxi Agricultural University (37°25′ N, 112°35′ E), Taigu, Shanxi Province, China. Two common millet genotypes, Huangmizi-tolerant (HM) and Zhenyuandamizi-sensitive (ZY), were used as plant materials. HM and ZY originated from Dingbian, Shaanxi province of China (accession number 00005272) and Zhenyuan, Gansu province of China, respectively. These two accessions are grown widely in local areas. The difference in drought resistance between these two varieties was determined by physiological/biochemical indicators and the leaf character by paraffin sections (data not shown). They were grown in a phytotron with day/night temperatures of 24℃ and 18℃, respectively, and a 14 h day light length. The relative humidity was kept at 40% during day and night. Twenty days later, the seedlings were removed and the vermiculite was washed away carefully. Leaves from three seedlings were harvested directly as a control, while the roots of the other seedlings were put into a 20% PEG 6000 solution. Leaves were collected from three seedlings at two time points t = 1 hour and t = 3 hours, frozen in liquid nitrogen and stored at -80℃ until RNA extraction.

RNA extraction and quality determination: Total RNA was extracted using an RNAiso PLUS kit (Takara Biotechnology) and dissolved in 10 mm Tris (pH 7.6). The A260/A280 ratios of the RNA ranged from 1.9 to 2.1. Using an Agilent 2100 Bioanalyzer, the integrity of the RNA samples ranged from 8.6 to 10.0, with no signs of degradation. RNA samples of each genotype were used for cDNA preparation and subsequent RNA-Seq analysis.

Illumina cDNA library preparation and sequencing: Approximately 30 µg of total RNA with a concentration of ≥ 250 ng/ µL was used for cDNA synthesis and Solexa sequencing. Initially mRNA was enriched excluding ribosomal RNA using a Next Poly (A) mRNA Magnetic Isolation Module kit [New England BioLabs (NEB) E7490]. Next, sequencing libraries were constructed from cleaved mRNA using a Next mRNA Library Prep Master Mix Set for Illumina (NEB, E6110) and a Next Multiplex Oligos for Illumina (NEB, E7500). The fragment size of mRNA libraries was detected using electrophoresis on a 1.8% agarose gel and also using quantitative PCR prepared utilising the Library Quantification Kit-Illumina GA Universal (Kapa, KK4824). Using Illumina cBot (San Diego, CA, USA), clusters were generated based on the qualified libraries. Finally, we constructed two paired-end cDNA libraries with insert sizes of 200 bp. Libraries were sequenced using an Illumina HiSeq^{TM}2500 (San Diego, CA, USA) Genome Analyzer according to the manufacturer's protocols, with a cDNA read length of 100 bp.

Molecular marker development / polymorphism detection: In order to explore the common millet-specific molecular markers, SSR and SNP detection were performed with MISA (http://pgrc.ipk-gatersleben.de/misa) and SOAPsnp software (Li *et al.*, 2009), respectively. Putative SSRs were analyzed for identified Unigenes over 1 kb in length. Putative SNPs were tagged if the depth of sequencing for the minor allele was between 10 and 100 times and their scores of soapsnp were above 30.

***De novo* transcriptome assembly and assessment:** We assembled reads from each library separately using in-house developed Perl scripts. Adapter sequences and reads containing many unknown bases (> 8) or low quality bases (> 50% of the bases with a quality score ≤ 5) were removed. First, contigs were constructed using Trinity software (Grabherr *et al.*, 2011). Next, the contigs

were classified according to the similarity between paired-end information of the reads and the contigs. Then, the collected reads were locally assembled into transcripts. Finally, the major transcripts were screened from the locally assembled ones and identified as Unigene.

In order to get high-quality sequences for further annotation and analysis, the following steps were carried out. First, the sequences with non-coding RNAs were excluded after comparing them with known non-coding RNAs deposited in the Rfam database (http://www.sanger.ac.uk/resources/databases/rfam.html, release 10.0). Next, the sequences assigned to microbial (http://mbgd.genome.ad.jp), fungal and virus sources (according to data downloaded from the NCBI) were also filtered out. Then, sequences for which greater than 50% of the bases aligned with those in the UTRdb (http://utrdb.ba.itb.cnr.it/) were removed. In addition, sequences containing less than 200 non-UTR bases were excluded.

In order to evaluate the content of the transcriptome assemblies, we searched our Unigene sequences by BLAST (Altschul *et al.*, 1997) against the NR (non-redundant) (Deng *et al.*, 2006), SwissProt (Apweiler *et al.*, 2004), GO (Ashburner *et al.*, 2000), COG (Tatusouv *et al.*, 2000), KEGG (Kanehisa *et al.*, 2004) and NT (http://www.ncbi.nlm.nih.gov/BLAST/blastcgihelp.shtml) databases.

Functional annotation: The sequences were annotated based on a set of sequential BLAST searches and the most descriptive annotation for each sequence was found. Using the BLASTN algorithm, the assembled unique transcripts were compared with sequences in GenBank's NR database. According to their molecular, biological and cellular functional ontologies, the GO accessions were mapped to GO terms. Applying a typical *E*-value cutoff of $<10^{-5}$, those remaining sequences that putatively encoded proteins were searched against the COG, SwissProt protein and the KEGG pathway databases.

Analysis of gene expression levels: For the RNA-Seq analyses, gene expression levels were measured as numbers of reads per kb of exon in a given gene per million mapped reads (RPKM) (Mortazavi *et al.*, 2008). To identify genes regulated by drought tolerance, we determined the number of reads for each coding region in the control and drought-stress leaf libraries. We then calculated the ratio of reads between the two libraries. Statistically significant differences in gene expression were determined using the method described by Leng *et al*. (2013). Results of the statistical tests were corrected for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) (Benjamini and Hochberg, 1995). Two sequences were recognized to have significantly different expression levels if their adjusted *p*-values were < 0.001 and there was at least a two fold change (> 1 or < -1 in the log 2 ratio value) in the sequence count between the two libraries.

To investigate the biological significance of the differences in gene expression, Unigenes with significant expression differences under drought stress were assigned to functional classifications.

Quantitative real time PCR (qRT-PCR) analysis: Total RNA was extracted from the seedling leaves of common millet using TRIzol reagent (Invitrogen), and 1 mg of total RNA from each sample was used for the reverse transcription reaction. The expression level of 9 differential expression genes (DEGs) and tubulin (an internal standard) was analyzed using qRT-PCR and the primers are listed in Table 1.

Data Availability: The *Panicum miliaceum* transcriptome sequences and assembly data are available through NCBI (Accessions: SAMN03164242, SAMN03252933 and SAMN03252967; PID: PRJNA266434) see: http://www.ncbi.nlm.nih.gov/bioproject/266434.

RESULTS AND DISCUSSION

ORF prediction: An open reading frame (ORF) is a gene's sequence that is uninterrupted by stop codons that could potentially encode for a protein. ORF identification can provide the first evidence of a new sequence of DNA. Using Getorf software (http://emboss.sourceforge.net/apps/cvs/emboss/apps/getorf.html), the ORFs of the Unigenes were predicted as the longest ORF identified. The coding and protein sequences were then obtained from the Unigenes. The length distribution of the ORFs is shown in Figure 1 and Table 2. The total number and total length of ORFs was 41,952 and 20,057,046 bp, respectively. ORFs with 0 - 300 bp length were the most abundant, accounting for 59.1% of the total sequence length identified. ORFs with length greater than 2,000 bp were the least abundant, accounting for just 3.19% of the total length.

Polymorphism (SSR, SNP) Detection: Sequence polymorphisms are important aspects of genomic resources (Bansal *et al.*, 2014) that are valuable in marker assisted breeding programs.

Using the MISA program, a total of 2,301 SSRs were identified from 11,187 Unigenes, with an average of 4.86 SSRs / Unigene (Table 3). Among all of the SSRs, the numbers of mono-, di-, tri-, tetra- and penta-nucleotide repeats were 649, 386, 1,204, 52 and 10, respectively. These genetic resources should facilitate the development of common millet-specific SSRs related to drought-tolerance. Several common millet-specific SSRs have been utilised in the assessment of genetic diversity and phylogeography (Hunt *et al.*, 2011), however, no

common millet-specific SSRs involved in drought-tolerance have been developed. Using the sequence information obtained here, a set of common millet-specific SSRs molecular markers can be designed in drought-resistance research.

Using SOAPsnp software, a total of 1,447,148 SNPs (Table 4) were detected. The SNPs were identified as variations within each common millet genotype, variations between two common millet genotypes and reference assembly, and variations between two common millet genotypes. Among them, 99.94% were heterozygous and only 831 were homozygous. The majority of these polymorphisms represent allelic SNPs (intra-genotype SNPs) that are not useful as molecular markers for common millet breeding. Only those putative SNPs identified as variable between tolerant and sensitive genotypes can be used as molecular markers for crop improvement. There were also several common millet-specific SNPs (2 SNP loci were identified from ORFs for the waxy endosperm GBSS1-L gene) that were related to the waxy endosperm starch phenotype (Hunt *et al.*, 2010; Hunt *et al.*, 2013). The SNP information generated here will provide a platform to develop common millet-specific SNPs that will be helpful to drought-tolerance breeding programs.

***De novo* assembly and quantitative assessment of the Illumina ESTs:** Using RNA-Seq analysis, we generated a total of 55 million raw reads and 11.6 gigabases (Gb), respectively. Results showed that the mean quality score, Q20 (indicating a 1% chance of error) was 90.02%. After removing the low quality sequences and trimming adapter sequences, 22 and 23 million clean reads were produced from two cDNA libraries (Table 5). The total length of the reads was 9.40 Gb and Q30 was over 80%, indicating the sequencing quality was reliable enough for further analysis. Using Trinity software, *de novo* assembly was carried out (Table 6). A total of 2,907,357 contigs with an average length of 55 bp were identified. After using paired-end information to join the contigs, we generated 92,523 transcripts with an average length of 1,181 bp. Among the transcripts, 42,240 Unigenes with an average length of 834 bp were obtained. Transcripts with a length between 1,000 - 2,000 bp were the most abundant, accounting for 27.59% of the total. The total lengths of the contigs, transcripts and Unigenes were 158,915,393, 109,298,129 and 35,221,928 bp, respectively; and the N50 lengths of contigs, transcripts and Unigenes were 49, 1,825 and 1,450 bp, respectively. Generally speaking, transcripts with over 800 bp of N50 lengths indicated that the sequence integrity was good enough for further research.

Annotation of Unigenes and DEGs: We annotated Unigenes and DEGs by comparing sequences with the NR, SwissProt, TreEMBL, GO, COG, KEGG and NT databases using BLAST software (Table 7). We identified 701 DEGs between the two libraries, including 187 up-regulated and 514 down-regulated genes. Most sequences could be annotated using the NR, TreEMBL and NT databases.

Among the 187 up-regulated genes of 701 DEGs, gene comp31450_c0 (Table S1) with large expression (log2FC = 3.25), had significant homology with the dehydration-responsive element-binding protein 1C in rice as indicated by the Swissprot annotation results. This gene had significant homology with dehydration-responsive element-binding protein 1C-like in *Setaria italica* as indicated by the Nr annotation results. Nt annotation suggested that this gene showed similarity to the dehydration-responsive element-binding protein 1C-like (LOC101777280) in *Setaria italica*. GO annotation showed that gene comp31450_c0 was predicted with the molecular function of sequence-specific DNA binding transcription factor activity and biological process of regulation of transcription and response to stress. Previous reports have shown that genes in this family including *OsNAC6*, *OsDREB*, *AtABF3* were responsive to drought stress (Oh *et al.*, 2005; Nakashima *et al.*, 2007).

Among the 701 DEGs, 629 were annotated and 72 were not. Similarly, among the 187 up-regulated DEGs, 161 were annotated and 26 were not. Among the 514 down-regulated DEGs, 468 were annotated and 46 were not. The above annotated genes can be cloned, expressed and transformed into other crops to improve their drought-resistance. The non-annotated genes may relate to a novel gene, a gene located in the 3′-terminal or 5′-UTR gene regions with abundant variations, or they did not have protein homology with the species in the databases used (Li *et al.*, 2007). This may provide an opportunity to discover novel genes that are specific to common millet.

Functional annotation and characterization of the transcripts: The DEG sets between the sensitive and tolerant varieties were annotated based on their similarities to known or putative sequences in public databases. An inferred putative function including biological, cellular and molecular functions are shown in Figure 2. A total of 21,545 Unigenes were distributed into three categories including biological functions, molecular functions and cellular functions. Each GO term was further clustered with its parent term. The three largest biological functions were involved in metabolism, cellular processes and responses to stimuli. Most of the genes classified in the molecular functions category were involved in binding, catalytic activity and structural molecular activity. In the cellular function category, the major classifications were for cell parts, cells and organelles. Thus, most of the sequenced genes were responsible for fundamental biological regulation and metabolism.

To better understand the functional significance of the differentially regulated ESTs, those genes that played key roles in well-characterized metabolic pathways were investigated. There were changes in the expression of genes related to 63 metabolic pathways in response to drought stress ($P < 0.05$), including genes involved in carbohydrate, amino acid, energy, lipid, secondary metabolite, co-factor and vitamin, terpenoid and polyketide metabolism. Based on these results it may be deduced that common millet has a new energetic and developmental equilibrium under drought stress.

To further evaluate the completeness of our transcriptome libraries and the effectiveness of the annotation processes, previously annotated sequences similar to the identified Unigenes were found and annotated according to COG classifications (Table 8). Of the 8,304 annotated Unigenes, 290 sequences had COG classifications. Among the 25 COG categories, none of the Unigenes were assigned to the "RNA processing and modification", "Nuclear structure" or "Extracellular structures" groups. The cluster for "Translation, ribosomal structure and biogenesis" represented the largest group (56; 19.31%), followed by the ''General function prediction only'' (39; 13.45%) and "Post-translational modification, protein turnover, chaperones" groups (19; 6.55%). The "Chromatin structure and dynamics" (2; 0.69%) and "Intracellular trafficking, secretion and vesicular transport" (1; 0.34%) groups were the smallest. Of the Unigenes, 13 (4.48%) were assigned to gene groups related to drought tolerance, such as the "Signal transduction mechanisms" and "Transcription".

According to biological functions, the genes identified as drought-tolerant were classified into three types: drought-responsive transcriptional regulation by WRKY and NAC transcription factors; post-transcriptional protein modifications including phosphorylation / dephosphorylation such as SnRK2; osmoprotectant metabolism such as NCED (9-cis-epoxycarotenoid dioxygenase) and SAMS (S-adenosylmethionine synthetase; Yang *et al.*, 2010). Unigenes involved in all three of the above categories were identified.

The Unigenes comp36254_c0 and comp31983_c0 (Table S1) were assigned to drought-responsive transcriptional regulation. Zinc finger protein (ZFP) transcription factors are best known for their involvement in the regulation of plant growth, development and response to biotic stress. Many studies on ZFPs, including the genes OsZFP252 and DST, have demonstrated the role of ZFPs in drought-tolerance of rice (Xu *et al*., 2008; Huang *et al*., 2009). The Unigenecomp36254_c0 was putatively homologous to the *Setaria italica* zinc finger CCCH domain-containing protein 35-like (LOC101757530) that is involved in the biological process of transcription regulation. bZIPs is a transcription factor family and performs function in ABA signal conduction. Many bZIP transcription factors involved in drought-tolerance have been isolated from thale cress (*Arabidopsis thaliana*) (Jacoby *et al*., 2002; Oh *et al*., 2005). In this study, the Unigene comp31983_c0 was putatively homologous to the *Setaria italica* bZIP transcription factor TRAB1-like (LOC101758999). Although the function of the Unigene comp31983_c0 is unknown, it may perform a specific role in common millet drought-tolerance.

The Unigenes comp35578_c1 and comp42802_c0 (Table S1) were classified into the biological process of protein phosphorylation. Some transcription factors (TFs), such as AREB1, need to be phosphorylated by protein kinases in order to become active (Furihata *et al.*, 2006). Several protein kinases, especially a subset of 604 thale cress receptor-like kinases, have been implicated in osmotic stress responses based on their transcriptional responses to different stress (Boudsocq and Laurière, 2005; Chae *et al.*, 2009). We found that the Unigene comp42802_c0 encoded the hypothetical protein SORBIDRAFT_02 g035930 and that Unigene comp35578_c1 was putatively homologous to *Setaria italica* adenylate kinase chloroplastic-like (LOC101763558).

Finally, Unigenes comp42838_c1 and comp41401_c0 (Table S1) were assigned to osmoprotectant metabolism. NCED is a drought-inducible and a rate-limiting enzyme that plays a key role in ABA biosynthesis (Yang *et al.*, 2010). NCED improves drought and salt tolerance by increasing the endogenous ABA levels (Iuchi *et al*., 2001). The Unigene comp41401_c0 that was putatively homologous to *Setaria italica NCED1*, was also up-regulated in response to drought stress. SAM, a methyl donor during the biosynthesis of ethylene and polyamine, participates in the plant stress response and is catalyzed and synthesized by SAMS. The SAMS2 gene is induced by NaCl, ABA and drought in *Suaeda salsa* (Ma *et al.*, 2003). The gene *PmSAMS* found in broomcorn millet is involved in response to drought and rehydration, and may be one of the key genes responsible for drought-tolerance and water use efficiency (Lin *et al.*, 2008). We also found that the Unigene comp42838_c1 was putatively homologous to *Setaria italica SAMS1-like* (LOC101756508) protein, which was up-regulated in response to salt and drought stress.

In order to validate the RNA-Seq results, 9 identified DEGs (Table S1), including the seven mentioned above (comp31983_c0, comp36254_c0, comp31450_c0, comp35578_c1, comp42802_c2, comp42838_c1 and comp41401_c0) and two others (comp31317_c0 and comp30715_c0) obtained in this drought experiment were selected for qRT-PCR analysis. The relative expression levels as measured by log2FC of each the nine genes was > 1 (see Figure 3). As expected, the relative expression levels of the nine Unigenes were

up-regulated, corresponding to the results produced by RNA-Seq (Table S1). As for the other 2 DEGs (comp31317_c0 and comp30715_c0), they were both found to be homologous to the *Setaria italica* genes including dehydrin DHN1-like (LOC101760090) and plasma membrane ATPase 1-like (LOC101762504), which are involved in drought-tolerance (Molina *et al.*, 2008; Li *et al.*, 2014; Yang *et al.*, 2012). *Setaria italica* is a grain crop well-adapted to drought and its genome has been sequenced (Bennetzen *et al.*, 2012). Genes identified here should be helpful to better understand the molecular mechanisms related to drought-tolerance in common millet.

In conclusion, using an NGS approach, we identified new transcriptome sequences potentially involved in drought-tolerance of common millet. We generated 9.40 Gb of sequence that assembled into 42,240 Unigenes, including 701 DEGs. Most of the above genes showed similarity to already annotated genes. The above genomic resources should prove very useful in understanding the molecular mechanisms of drought-tolerance in common millet, which has the potential to eventually enhance crop yields.

We also identified polymorphisms (SSRs and SNPs), that can be used for developing common millet-specific molecular markers that may be useful for drought-tolerant breeding programs. We are currently evaluating polymorphisms that can be used for molecular marker assisted selection to screen genes responsive to abiotic stress, specifically drought-tolerance. Newly developed common millet-specific molecular markers will accelerate the cultivation of drought-tolerant varieties in the near future.

Our research herein focused only on the leaf response to drought-tolerance. The molecular mechanisms of drought tolerance in other tissues such as the root or flower remains unstudied. Future transcriptome analysis of roots and flowers may lead to the discovery of more DEGs, helping to further identify key genes involved in drought-tolerance.

Table 1. Details of the primers of 9 differential expression genes (DEGs) and tubulin (an internal standard) for qRT-PCR analysis.

Tubulin /DEG	FP (Forward Primer)	RP (Reverse Primer)
Tubulin	5′-GGAGATCCTCCACATCCAG-3′	5′-CAGAAAGGGTAGCATTGTAAG-3′
comp41401_c0	5′-ACCGAAGCCCCTCTTAACCC-3′	5′-GTATGGCTGACACCATCACC-3′
comp36254_c0	5′-TGGTGCTGGACAAGGAGAAG-3′	5′-CAGGTGGAAGCAGAAGCAGTC-3′
comp31450_c0	5′-GGATGATCTAGGCGAACAGCT-3′	5′-AGACCCACGAGTGCCACATC-3′
comp31317_c0	5′-GTAGTAGTACGGTTGGTCGAAGTCC-3′	5′-TGGCGATGGCGATGCACCAC-3′
comp42838_c1	5′-AGGTGGGACGGAGAACCTCT-3′	5′-GAACCCAGCAGGAATAATAAAGTG-3′
comp30715_c0	5′-GTTGATGAGGTCGGACACCC-3′	5′-CCTCAACCTCGGGCTCATCG-3′
comp42802_c2	5′-AAGAAGGATTTCGGGAGGGC-3′	5′-CTTCCGCGAGCTGGTTGAGT-3′
comp35578_c1	5′-GCCAGGCTGCTGAATGTCTC-3′	5′-ACATCTGCTCCCGGAAGACC-3′
comp31983_c0	5′-GGTCCGAGAATGGGAAGCAG-3′	5′-TCCAACCACCCTTTCAACAA-3′

Table 2. Length distribution of the ORFs detected from the samples (ZY and HM).

Length range of ORF	Unigene ORF	
	Total number	Percentage (%)
0-300 (bp)	24,794	59.1
300-500 (bp)	5,743	13.69
500-1000 (bp)	5,959	14.2
1000-2000 (bp)	4,117	9.81
2000+ (bp)	1,339	3.19
Total number	41,952	
Total length (bp)	20,057,046	
N50 length (bp)	954	
Mean length (bp)	478.1	

Table 3. SSRs identified in the samples (ZY and HM).

Searching item	Number
Sequences examined	11,187
Total size of examined sequences (bp)	22,326,234
SSRs identified	2,301
Number of SSR containing sequences	1,959
Sequences containing more than 1 SSR	280
SSRs present in compound formation	103
Mono-nucleotide repeat sequence	649
Di-nucleotide repeat sequence	386
Tri-nucleotide repeat sequence	1,204
Tetra-nucleotide repeat sequence	52
Penta-nucleotide repeat sequence	10

Table 4. SNPs detected in the ZY and HM common millet accessions.

Type	Homo[a]		Hete[b]		
	ZY[c] homo / HM[d] homo	ZY hete / HM homo	ZY homo / HM hete	ZY hete / HM hete	
ZY_vs_HM	831	95,367	101,153	1249797	
Total	831		1,446,317		1,447,148

[a]Homo=homozygous genotype.
[b]Hete=heterozygous genotype.
[c]ZY=sensitive genotype of common millet.
[d]HM=tolerant genotype of common millet.

Table 5. Quantitative assessment of the Illumina ESTs.

Sample	Total reads	Total bases (nt)	GC (%)	N (%)	Q20 %	Cycle Q20 %	Q30 %
ZY	22,719,320	4,588,853,514	52.77	0.04	90.01	100	80.05
HM	23,800,178	4,807,194,304	53	0.04	90.02	100	80.01

Table 6. Results of the *De novo* assembly.

Length range	Contigs		Transcripts		Unigenes	
	Total	Percentage (%)	Total	Percentage (%)	Total	Percentage (%)
0-300	2,870,124	98.72	15,443	16.69	12,439	29.45
300-500	14,054	0.48	15,405	16.65	10,195	24.14
500-1000	11,399	0.39	19,689	21.28	8,419	19.93
1000-2000	8,140	0.28	25,523	27.59	7,086	16.78
2000+	3,640	0.13	16,463	17.79	4,101	9.71
Total number	2,907,357		92,523		42,240	
Total length (nt)	158,915,393		109,298,129		35,221,928	
N50 length (bp)	49		1,825		1,450	
Mean length (bp)	54.66		1,181.31		833.85	

Table 7. Annotated Unigenes and DEGs.

Annotated databases	Annotated Uingenes			Annotated DEG
	Number of annotated sequences	300≤length<1000	Length≥1000	
COG	8,304	2,775	4,649	236
GO	21,556	8,623	9,165	461
KEGG	5,535	2,045	2,546	149
SwissProt	18,289	7,345	8,059	452
TrEMBL	27,599	11,753	10,596	596
NR	27,372	11,589	10,576	595
NT	29,869	12,694	10,668	591
Total	31,564	13,647	10,884	3,080

Table 8. Unigenes involved in the COG classifications.

Class ID	Class Name	Unigenes	
		Numbers	Percentage (%)
A	RNA processing and modification	0	0
B	Chromatin structure and dynamics	2	0.69
C	Energy production and conversion	14	4.83
D	Cell cycle control, cell division, chromosome partitioning	3	1.03

E	Amino acid transport and metabolism	14	4.83
F	Nucleotide transport and metabolism	4	1.38
G	Carbohydrate transport and metabolism	18	6.21
H	Coenzyme transport and metabolism	8	2.76
I	Lipid transport and metabolism	14	4.83
J	Translation, ribosomal structure and biogenesis	56	19.31
K	Transcription	13	4.48
L	Replication, recombination and repair	17	5.86
M	Cell wall/membrane/envelope biogenesis	17	5.86
N	Cell motility	0	0
O	Posttranslational modification, protein turnover, chaperones	19	6.55
P	Inorganic ion transport and metabolism	7	2.41
Q	Secondary metabolites biosynthesis, transport and catabolism	18	6.21
R	General function prediction only	39	13.45
S	Function unknown	7	2.41
T	Signal transduction mechanisms	13	4.48
U	Intracellular trafficking, secretion and vesicular transport	1	0.34
V	Defense mechanisms	4	1.38
W	Extracellular structures	0	0
Y	Nuclear structure	0	0
Z	Cytoskeleton	3	1.03
Total		290	

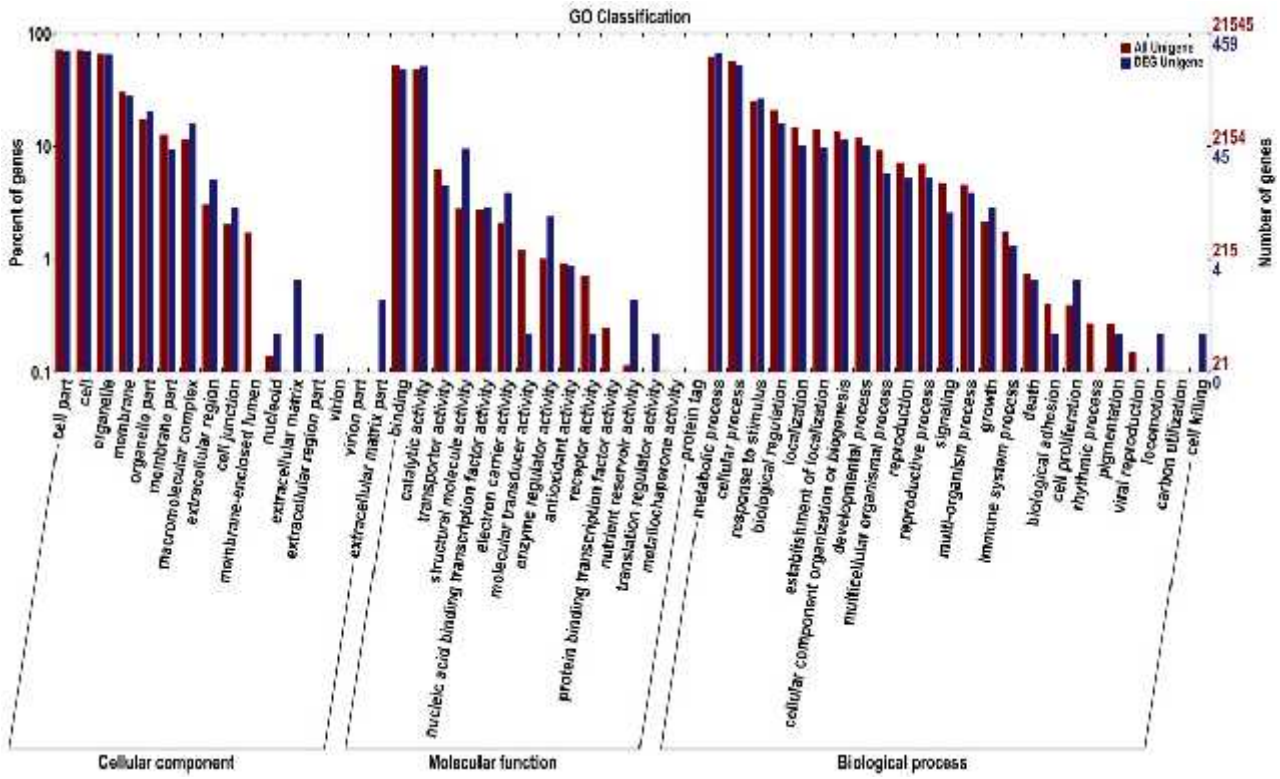

Figure 1. Length distribution of the Unigene ORFs in common millet. X-axis: length range of the Unigene ORF (bp); Y-axis: number of Unigene ORFs.

(TIF)

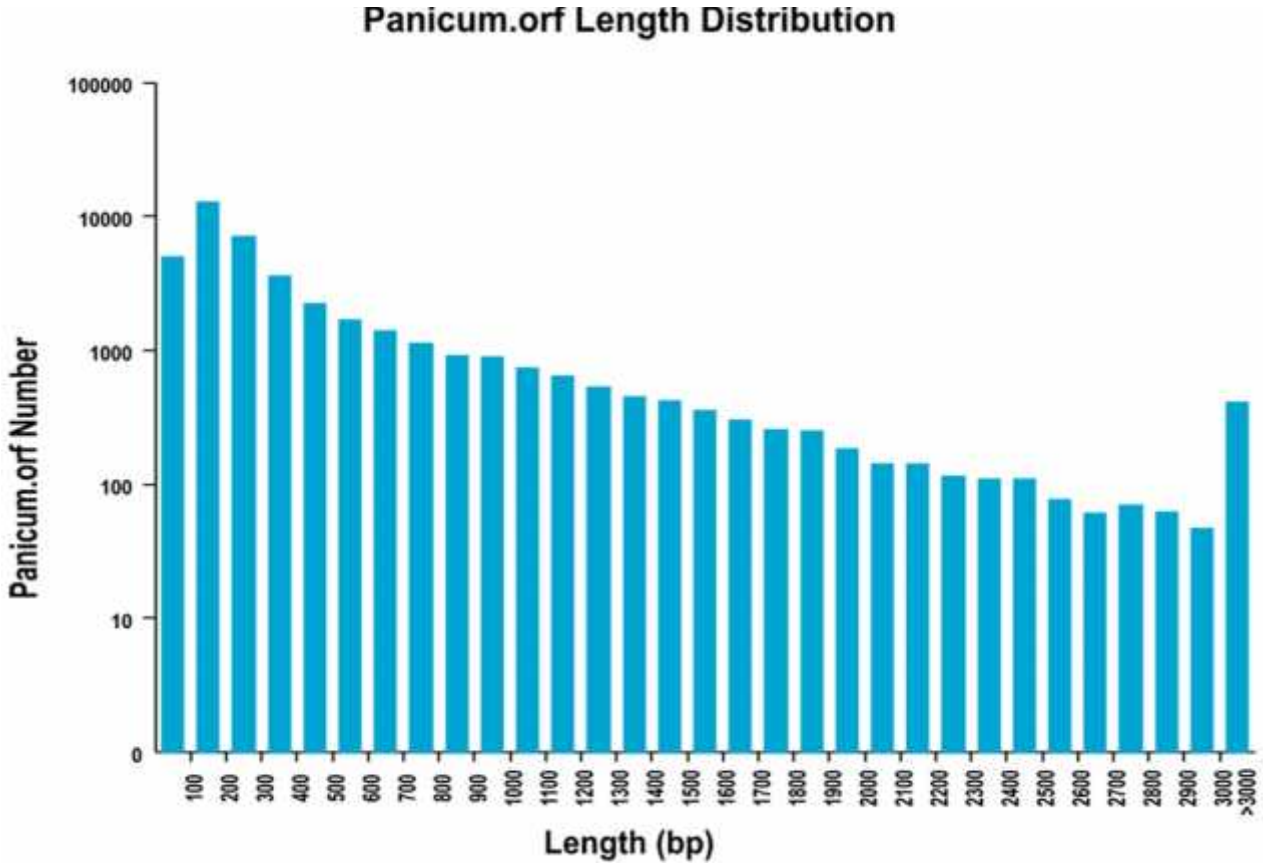

Figure 2. GO categories for the entire genome and the DEG Unigenes. X-axis: three main categories (Cellular, Molecular and Biological functions); Right y-axis: number of Unigenes and DEGs; Left y-axis: percentage of specific categories of Unigenes in the main category.

(TIF)

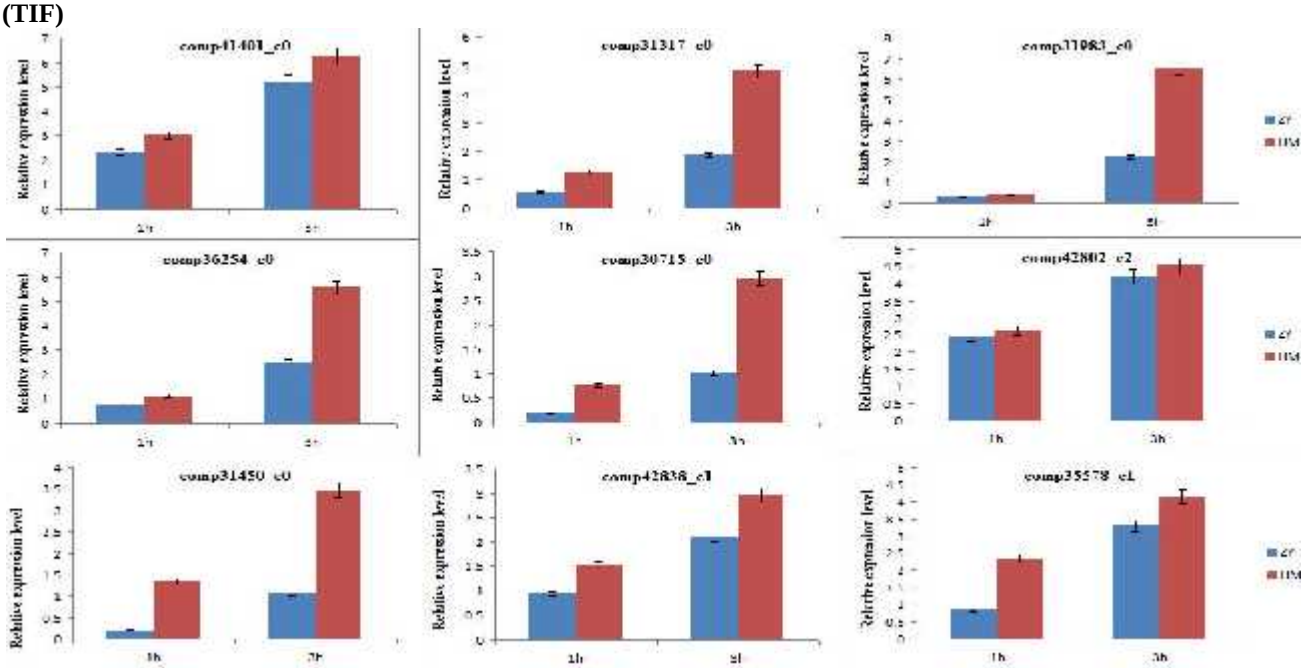

Figure 3. Quantitative RT-PCR validation of 9 DEGs in HM and ZY. X-axis represented the time point of leaves treated by PEG stress. (TIF).

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