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Transcriptomic studies reveal a key metabolic pathway contributing to a well-maintained photosynthetic system under drought stress in foxtail millet (*Setaria italica* L.)

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Drought stress is one of the most important abiotic factors limiting crop productivity. A better understanding of the effects of drought on millet (*Setaria italica* L.) production, a model crop for studying drought tolerance, and the underlying molecular mechanisms responsible for drought stress responses is vital to improvement of agricultural production. In this study, we exposed the drought resistant F₁ hybrid, M79, and its parental lines E1 and H1 to drought stress. Subsequent physiological analysis demonstrated that M79 showed higher photosynthetic energy conversion efficiency and drought tolerance than its parents. A transcriptomic study using leaves collected six days after drought treatment, when the soil water content was about ~20%, identified 3066, 1895, and 2148 differentially expressed genes (DEGs) in M79, E1 and H1 compared to the respective untreated controls, respectively. Further analysis revealed 17 Gene Ontology (GO) enrichments and 14 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in M79, including photosystem II (PSII) oxygen-evolving complex, peroxidase (POD) activity, plant hormone signal transduction, and chlorophyll biosynthesis. Co-regulation analysis suggested that these DEGs in M79 contributed to the formation of a regulatory network involving multiple biological processes and pathways including photosynthesis, signal transduction, transcriptional regulation, redox regulation, hormonal signaling, and osmotic regulation. RNA-seq analysis also showed that some photosynthesis-related DEGs were highly expressed in M79 compared to its parental lines under drought stress. These results indicate that various molecular pathways, including photosynthesis, respond to drought stress in M79, and provide abundant molecular information for further analysis of the underlying mechanism responding to this stress.

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2 **maintained photosynthetic system under drought stress in foxtail millet**
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16 ABSTRACT

17 Drought stress is one of the most important abiotic factors limiting crop productivity. A better
18 understanding of the effects of drought on millet (*Setaria italica* L.) production, a model crop for
19 studying drought tolerance, and the underlying molecular mechanisms responsible for drought
20 stress responses is vital to improvement of agricultural production. In this study, we exposed the
21 drought resistant F₁ hybrid, M79, and its parental lines E1 and H1 to drought stress. Subsequent
22 physiological analysis demonstrated that M79 showed higher photosynthetic energy conversion
23 efficiency and drought tolerance than its parents. A transcriptomic study using leaves collected
24 six days after drought treatment, when the soil water content was about ~20%, identified 3066,
25 1895, and 2148 differentially expressed genes (DEGs) in M79, E1 and H1 compared to the
26 respective untreated controls, respectively. Further analysis revealed 17 Gene Ontology (GO)
27 enrichments and 14 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in M79,
28 including photosystem II (PSII) oxygen-evolving complex, peroxidase (POD) activity, plant
29 hormone signal transduction, and chlorophyll biosynthesis. Co-regulation analysis suggested that
30 these DEGs in M79 contributed to the formation of a regulatory network involving multiple
31 biological processes and pathways including photosynthesis, signal transduction, transcriptional
32 regulation, redox regulation, hormonal signaling, and osmotic regulation. RNA-seq analysis also
33 showed that some photosynthesis-related DEGs were highly expressed in M79 compared to its
34 parental lines under drought stress. These results indicate that various molecular pathways,
35 including photosynthesis, respond to drought stress in M79, and provide abundant molecular
36 information for further analysis of the underlying mechanism responding to this stress.

37 INTRODUCTION

38 Drought is one of the main abiotic stresses that affect global crop production. It also severely
39 influences metabolism and growth of many crops (Reynolds and Tuberosa, 2008; Zhao and
40 Running, 2011). Foxtail millets (*Setaria italica* L.) is a widely cultivated, dryland crop with
41 superior drought tolerance and higher water use efficiency (WUE) compared to other crops such
42 as corn, sorghum, and wheat (Lata et al., 2013). Foxtail millet has a small genome, fast growth,
43 and rich germplasm resources, making it a model crop for stress tolerance research (Li and
44 Brutnell, 2011; Muthamilarasan and Prasad, 2015).

45 Due to the lack of complete reference genome sequence, previous studies used suppression
46 subtractive hybridization (SSH) and complementary DNA-amplified fragment length
47 polymorphism (cDNA-AFLP) to explore drought-stress response genes in millets (Zhang et al.,
48 2007; Puranik et al., 2011). However, genomic research in millet became easier after whole
49 genome sequencing and annotation of the Zhanggu and Yugu1 varieties are available (Bennetzen
50 et al., 2012; Zhang et al., 2012a). RNA-seq technology has been widely used to study how stress
51 factors affect transcriptome in crops such as maize (Zhang et al., 2013), wheat (Camilios-Neto et
52 al., 2014), rice (Zhou et al., 2016), sorghum (Fracasso et al., 2016), and foxtail millet (Qi et al.,
53 2013; Yadav et al., 2015; Yi et al., 2015; Wang et al., 2016). Using transcriptomic analysis, Qi et
54 al. (2013) identified 2824 genes and 215 miRNAs that respond to osmotic stress; while Yadav et
55 al. (2015) found 55 known and 136 new miRNAs differentially expressed genes in two millet
56 varieties after treating plants with 20% PEG-6000 to induce dehydration stress. Using the
57 parallel analysis of RNA ends (PARE) and RNA-seq, Yi et al. (2015) identified four decay
58 modes of millet mRNA in response to drought stress. Wang et al. (2016) found that the millet
59 variety An04-4783 expressed 81 known miRNAs and 72 new miRNAs under drought stress.
60 These reports provide important information on drought responsive mechanisms and related
61 regulatory networks in millet.

62 Millet is a C4 crop, and photosynthesis is the most important to its carbon metabolisms
63 (Lata et al., 2013). However, drought stress changes the intracellular environment, which results
64 in decreased electron transfer rates, uncoupling of photosynthetic phosphorylation, increased
65 hydrolysis, and reduced chlorophyll biosynthesis and photosynthetic enzyme activity. The
66 chloroplast is the central organelle that produces reactive oxygen species (ROS), whereas
67 accumulation of ROS may cause oxidative damage and inhibit photosynthesis (Gollan et al.,
68 2015; Exposito-Rodriguez et al., 2017). However, crops are capable of coping with stress
69 through various mechanisms, including osmotic adjustment, accumulation of protective proteins,
70 and antioxidant defense systems (Cui et al., 2016; Gürel et al., 2016). These regulatory pathways
71 crosstalk with each other to form a drought-defensive network that allows plants to maintain
72 photosynthesis under drought stress, and ensures biomass accumulation and eventually high
73 yield (Redillas et al., 2012; Hu and Xiong, 2014; He et al., 2016). Therefore, the ability of crops
74 to maintain photosynthesis under drought stress is an important indicator of drought tolerance

75 (Ma et al., 2016). However, how the expression of photosynthetic genes in response to drought
76 stress is related to drought tolerance remains poorly studied (Ambavaram et al., 2014).

77 In this study, we employed RNA-seq to investigate the transcriptomic changes between the
78 hybrid M79 and its parental lines in response to drought stress with the aims at identifying DEGs
79 related to drought tolerance, and understanding the associated molecular mechanisms and
80 metabolic pathways in millet. The results may facilitate establishment of a molecular
81 photosynthesis regulatory network of millet under drought stress to lay a foundation for
82 molecular breeding of drought tolerant millet varieties.

83 MATERIALS AND METHODS

84 Materials and experimental design

85 The materials used in this study were E1 (maternal line), H1 (paternal line) and their F₁ hybrid
86 M79, a drought resistant variety. H1 is a drought-tolerant cultivar released from the Shanxi
87 Academy of Agricultural Sciences, and has been sporadically grown in arid and barren areas
88 during the past decade. E1 developed from the same institute has been an important parental line
89 for many varieties currently grown in China.

90 Millet seeds were surface sterilized with 0.5% NaClO, washed three times with ddH₂O and
91 sown in pots (diameter: 8 cm, height: 10 cm, 15 seeds per pot) filled with peat and nutrient soil
92 (1:1). After the pots were kept in a growth chamber (light/darkness: 16h/8h, temperature:
93 30°C/22°C) for seven days, five healthy plants were maintained in each pot by removing extra
94 plants. After three weeks, the plants at six-leaf stage were treated with drought stress. Each
95 genotype with 100 plants were divided into the control group (M79_CK, E1_CK, and H1_CK)
96 with regular watering, and the drought group (M79_DR, E1_DR, and H1_DR) without watering.
97 For the latter group, the soil gravimetric water content was monitored using a soil moisture
98 analyzer TDR300 (Spectrum, USA). At the 5th, 6th, and 7th days after stopping watering, the soil
99 gravimetric water content dropped to 26.1%, 20.3%, and 14.6% of field capacity, respectively.

100 Samples were collected on the 6th day when the soil water content was about 20% (Tang et
101 al., 2017). At this stage, the water potential in M79 leaves was significantly higher than those of
102 the parental lines (Fig. 1b). Plants from the control group (soil water content about 55%) were
103 also sampled at the same stage. Samples were immediately wrapped in foil and frozen in liquid

104 nitrogen, and then kept in a -80°C freezer. The top second leaf was used as the test materials for
105 all treatments with the upper half for transcriptome sequencing and the lower half for measuring
106 physiological indicators including catalase (CAT) and relative electrolyte leakage (REL). All
107 samples were biologically duplicated three times. The top second leaves from different plants of
108 the same treatments were used for measuring leaf water potential (LWP), photosynthetic rate (A),
109 transpiration rate (E), maximum energy conversion efficiency in PSII centers (Fv/Fm), and
110 quantum yield of PSII electron transport (Φ PSII). The back of each tested leaf was labeled before
111 measuring LWP, A, E, Fv/Fm and Φ PSII to facilitate the next measurement. Each measurement
112 was repeated five times using fully expanded, uninjured leaves.

113 **Measurement of drought-related physiological changes**

114 Plasma membrane damage of millet leaves was determined as previously reported (Cao et al.,
115 2007), and REL was used to measure the extent of damage to the plasma membrane. CAT
116 quantification was performed following Bonnacarrère et al. (2011). LWP was measured using a
117 Psypro plant water potential meter (WESCOR, USA). A, E, Fv/Fm, Φ PSII were measured using
118 a LI-6800 portable photosynthesis system (LI-COR, USA) as described by Lowry et al. (2015).
119 Light intensity, CO₂ concentration, and air flow rate were set to 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 400 $\mu\text{mol mol}^{-1}$,
120 and 500 $\mu\text{mol s}^{-1}$, respectively. Measurements were carried out from 8:30 - 11:30 am on each
121 day. WUE ($\mu\text{mol mmol}^{-1}$) was calculated by: $\text{WUE} = A (\mu\text{mol m}^{-2} \text{s}^{-1}) / E (\text{mol m}^{-2} \text{s}^{-1}) / 1000$.

122 **RNA extraction, cDNA library construction, and transcriptome sequencing**

123 RNA samples were prepared from 18 harvests (2 treatments \times 3 genotypes \times 3 biological
124 replicates) using Trizol[®] reagent (Invitrogen, USA) for subsequent RNA-seq analysis. Quality
125 and concentration of RNA were determined by agarose gel electrophoresis and a NanoDrop 2000
126 spectrophotometer (Thermo Fisher Scientific, USA). A more accurate RNA quantification was
127 performed by using an Agilent 2100 Bioanalyzer RNA Nano chip (Agilent Technologies,
128 Germany).

129 Messenger RNA from each sample was enriched using mRNA Capture Beads and then
130 fragmented at high temperature in the presence of metal ions. Using mRNA as a template, the
131 first strand cDNA was synthesized using random hexamers, followed by synthesis of the second

132 strand cDNA, and then purification of the double-stranded cDNA using VAHTS™ DNA Clean
133 Beads. After end repair and A-tailing, purified double-strand cDNA was ligated to the
134 sequencing adapter and sorted using VAHTS™ DNA Clean Beads to get 300-400 bp fragments.
135 Finally, PCR amplification was performed, and PCR products were purified with VAHTS™
136 DNA Clean Beads to generate the final libraries. Library concentrations were assayed using
137 Qubit 3.0, and the library inserts were subsequently examined using an Agilent Bioanalyzer 2100
138 system (Agilent Technologies, USA) and further quantified using the ABI Step One Plus Real-
139 Time PCR system. Finally, the libraries were pooled and sequenced on a HiSeq X Ten (Illumina,
140 USA) platform using PE150 mode by Nanjing Vazyme Biotech Company Ltd..

141 **RNA-seq data analysis**

142 Clean reads extracted from raw reads using Tophat2 (v2.0.13) were compared with the reference
143 genome *Setaria italica* V2.2 (phytozome.jgi.doe.gov) to get mapped reads (Kim et al., 2013).
144 Based on the available data, we also performed analyses on gene saturation, homogeneity of
145 sequencing, the proportions of mapped reads in genomic exons, introns, and intergenic regions,
146 and correlation analysis between replicates.

147 Gene expression analysis was performed using Cufflinks v2.2.1. Transcriptome reads
148 aligned to the reference genome were quantified and normalized to fragments per kilobase of
149 transcript per million fragments mapped (FPKM), differences between drought-treated, and the
150 control FPKM values were compared using the software Cuffdiff v2.2.1 (Trapnell et al., 2010).
151 The thresholds of DEGs were set as $FDR \leq 0.05$ and $|\log_2 \text{FoldChange}| \geq 1$.

152 **Functional annotation, pathway analysis, clustered heat map, and co-regulation network** 153 **analysis**

154 All DEGs were mapped to terms in the GO database (<http://www.geneontology.org/>), and the
155 number of genes per term was calculated. Based on the GO:: TermFinder, the GO enrichment of
156 the DEGs was performed using a hypergeometric test with a corrected $FDR < 0.05$ as a threshold
157 (Boyle et al., 2004). A biological pathway analysis of DEGs was performed using KEGG
158 (<http://www.genome.jp/kegg/>), and significance was calculated by hypergeometric distribution
159 with a corrected $FDR < 0.05$ as the threshold (Kanehisa et al., 2008).

160 To generate a clustered heat map, expression data were converted using the formula \log_2
161 (FPKM + 1), and the map was drawn using the heatmap 2 function in the R/Bioconductor
162 package gplots (Warnes, 2016).

163 Co-regulation network analysis was conducted by using Cytoscape (v3.4.0) to plot the co-
164 regulation network with the Pearson correlation coefficient setting $| PCC | \geq 0.93$ (Saito et al.,
165 2012).

166 qRT-PCR

167 To verify RNA-seq data, DEGs were confirmed by qRT-PCR following Livak and Schmittgen
168 (2001). Primers were designed (Table S2) based on gene sequences from *Setaria italica* V2.2
169 (phytozome.jgi.doe.gov) using Primer 3 (<http://frodo.wi.mit.edu/>). Quantitative PCR was
170 performed using a SYBR[®] Green PCR Master Mix Kit (Applied Biosystems, GA, USA) and an
171 ABI7900 system. The $2^{-\Delta\Delta CT}$ method was used to calculate relative gene expression. Correlation
172 between RNA-seq and qRT-PCR was analyzed using SPSS 22.0 software (IBM, USA).

173 RESULTS

174 Phenotypes of M79 and its parental lines under drought conditions

175 After drought treatment for five days, seedlings of M79 and its parental lines showed no obvious
176 differences. However, after 6 or 7 days of drought stress, leaves in most E1 and H1 plants
177 exhibited curling and withering, whereas M79 appeared normal (Fig. 1a). Physiological analysis
178 of those plants before and after drought treatment showed that LWP decreased sharply after
179 drought stress. On the 5th day of drought treatment, the water potentials of E1 and H1
180 significantly declined ($P < 0.01$ and 0.05 , respectively) in comparison to M79 (Fig. 1b). We used
181 REL to analyze the amounts of damage after drought. From the 5th day, REL of E1 was
182 significantly higher than that of M79 ($P < 0.01$), while the REL of M79 on the 6th day was the
183 lowest among the three genotypes (20.87%) (Fig. 1c). The CAT activity of M79 was the highest
184 (10.97) on the 6th day (Fig. 1d), which was significantly higher than that of E1 and H1. Although
185 the WUE of all genotypes decreased after drought stress, M79 exhibited a significantly higher
186 WUE on the 6th and 7th days (4.37 and 4.10, respectively) compared to E1 and H1 (Fig. 1e).

187 These results demonstrated that M79 had better tolerance to drought stress than its parental lines
188 as shown by phenotypical and physiological indexes.

189 **RNA-seq data export, quality control, and sequence alignment**

190 Leaves of M79 and its parental lines were sampled for transcriptomic sequencing six days after
191 drought stress. A total of 18 libraries were constructed and sequenced using the HiSeq X Ten
192 sequencing platform, and generated 8.55×10^8 raw reads. After removing the linker and low-
193 quality data, we obtained 8.17×10^8 (95.57%) clean reads, consisting of about 122.58 Gb of
194 clean data, and representing an average of 4.54×10^7 clean reads, i.e., about 6.81 Gb of valid
195 data per sample. Phred mass fraction Q30 (error rate 0.1%) ranged from 86.38 to 88.22%, with
196 an average GC content of 56.30%. We aligned 93.13 to 94.32% of the valid data to the reference
197 genome (Table S1). The FPKM density distribution (Fig. S1a) and FPKM box diagram (Fig. S1b)
198 suggested that the density of the detected genes followed a standard normal distribution. These
199 results indicated high quality and reasonable reproducibility of our sequence data.

200 **Validation by qRT-PCR**

201 To verify the reliability of our transcriptomic sequence data, we selected ten genes from all three
202 lines for qRT-PCR, including genes encoding POD, No Apical Meristem, ATAF1/2, Cup-
203 Shaped Cotyledon 2 (NAC) transcription factor, wax, lipid transfer proteins (LTPL78), Domain
204 of unknown function (DUF538), expansin precursor, PsbP, Psb28, and two oxygen evolving
205 enhancer proteins 3 (Table S2). Positive correlation coefficients between RNA-seq and qRT-
206 PCR results was high and significant ($R^2 = 0.975$, $P < 0.01$, Fig. 2), indicating that the
207 transcriptome sequencing results were accurate and reliable.

208 **Comparative analysis of DEGs between M79 and its parental lines before and after** 209 **drought stress**

210 Comparative analysis of DEGs in non-stressed plants showed that M79 had 1359 and 648 genes
211 up- and down-regulated, respectively, when compared to E1, and had 1496 and 1033,
212 respectively, when compared to H1 (Fig. S2). To explore how these DEGs enhanced drought-
213 resistance, a GO analysis was performed on highly DEGs identified between M79_CK and

214 E1_CK, and between M79_CK and H1_CK. The DEGs found between M79 and E1 were mainly
215 involved in ADP binding, RNA synthesis, post-translational modification, cell recognition, and
216 carbohydrate metabolism (Table S3). In the comparison between M79 and H1, the DEGs were
217 mainly related to protein kinase, iron binding, redox balance, and post-translational modification
218 (Table S4).

219 We analyzed the DEGs between M79_DR and E1_DR, and between M79_DR and H1_DR.
220 5,258 (2739 up-regulated, 2519 down-regulated) DEGs were identified between M79_DR and
221 E1_DR, and 3594 (1795 up-regulated, 1799 down-regulated) DEGs were identified between
222 M79_DR and H1_DR (Fig. S3). GO analysis showed that the DEGs between M79_DR and
223 E1_DR, and between M79_DR and H1_DR were significantly enriched in protein kinase activity,
224 ATP binding, iron ion binding, carbohydrate metabolism, redox balance, and post-translational
225 modification (Tables S5 and S6). KEGG analysis on these DEGs found 18 pathways (FDR <
226 0.05) after the comparison between M79_DR and E1_DR, including metabolism of glutathione,
227 phenylalanine, porphyrin, chlorophyll, arginine, and proline, and biosynthesis of
228 phenylpropanoid, carotenoids, flavonoids, cuticle, suberin, and wax (Fig. 3a). Comparison
229 between M79_DR and H1_DR identified 15 different pathways (FDR < 0.05) including
230 metabolism of glutathione, porphyrin and chlorophyll, and biosynthesis of carotenoids,
231 brassinosteroids, cuticle, suberin and wax, and plant hormone signaling (Fig. 3b).

232 **DEGs analysis of M79, E1 and H1 under drought stress**

233 Compared to untreated plants, 3066, 1895, and 2148 DEGs were identified after drought
234 treatment in M79, E1 and H1, respectively, with 1404, 1116, and 1328 up-regulated genes and
235 1662, 779 and 820 down-regulated genes in corresponding genotypes. Among these DEGs, 288
236 (208 up-regulated and 80 down-regulated) genes were expressed in all three genotypes,
237 accounting for 9.39%, 15.20%, and 13.41% of all DEGs in drought-treated M79, E1 and H1,
238 respectively (Fig. 4). GO analysis showed that these genes were significantly enriched in
239 carbohydrate metabolism, iron ion and heme binding, oxidoreductase, POD, protein kinase
240 activity, and plasma membrane osmoregulation (Tables S7 and S8). Among them, genes known
241 to be involved in drought tolerance included two POD precursors (Seita.5G174100 and
242 Seita.8G015200), two late embryogenesis abundant proteins (LEAs) (Seita.1G015800 and
243 Seita.5G021400), and two aquaporins (Seita.3G082100 and Seita.1G264900). Furthermore,

244 genes involved in photosynthesis, such as one early light-induced protein (Seita.2G053800), one
245 pheophorbide a oxygenase (PaO) (Seita.1G348100), and one senescence-inducible chloroplast
246 stay-green protein 1 (SGR1) (Seita.2G285600) (Table S9) were also found. In addition, many
247 transcription factors including basic leucine zipper (bZIP), NAC, v-myb avian c viral oncogene
248 homolog (MYB) and early responsive to dehydration (ERD) family members, as well as genes
249 related to calmodulin, protein kinase, and hormone (gibberellin (GA), ethylene (ETH))
250 biosynthesis and signaling (Table S9).

251 Further analysis of DEGs that were specifically expressed before and after drought stress
252 detected 908, 475 and 745 up-regulated genes, and 1368, 404, and 497 down-regulated genes in
253 M79, E1 and H1, respectively (Fig. 4). GO analysis showed that the drought-specific DEGs in
254 M79 were significantly enriched in the GO-terms associated with PSII oxygen-evolving complex,
255 carbohydrate metabolism, redox balance, and iron ion binding (Table S10). In contrast, the
256 drought-specific DEGs were mainly enriched in GO-terms associated with iron ion binding,
257 redox balance, and nucleic acid and transcription factor activity in E1 (Table S11), and with iron
258 ion binding, protein kinase activity, and fatty acid synthesis in H1 (Table S12). KEGG analysis
259 showed that the DEGs in drought-stressed M79 were mostly involved in pathways such as
260 phenylalanine biosynthesis, plant hormone signaling, porphyrin, chlorophyll metabolism, cuticle
261 and wax biosynthesis, and arginine and proline metabolism (Table S13). The pathways included
262 phenylalanine biosynthesis, phenylalanine metabolism, plant hormone signaling, linoleic acid
263 metabolism, and glycerophospholipid metabolism in E1 (Table S14), and phenylalanine
264 biosynthesis, plant hormone signaling, and carotenoid biosynthesis in H1 (Table S15). These
265 results suggested that those DEGs enabled the three genotypes to respond differently to drought
266 stress.

267 **Expression and regulation of drought stress-responsive genes in M79**

268 GO enrichment and KEGG pathway analysis indicated that these stress-induced DEGs in M79
269 were widely involved in signal transduction, transcriptional regulation, hormone signaling, redox
270 regulation, osmotic regulation, photosynthesis, and other biological processes (Table S16).

271 Among the signal transduction-related genes that were differentially expressed in M79 after
272 drought stress, 24 genes encode receptor kinases, of which 14 encoded wall-associated kinase
273 receptor-like protein kinase (WAK-RLK) (eight up-regulated and six down-regulated), 27 genes

274 encode protein kinases, of which 10 encode calcium/calmodulin dependent protein kinases
275 (CAMK) (five up-regulated and five down-regulated), and six are Ca²⁺-related genes, including
276 one up-regulated gene that encodes an EF hand family protein, and five genes encoding
277 sodium/calcium exchanger (NCX) protein (three up-regulated and two down-regulated) (Table
278 S16).

279 Ninety-six transcription factors were differentially expressed in M79 in response to drought
280 stress, including 20 NAC, 19 APETALA2 (AP2), 14 transcription factors containing highly
281 conserved protein domain (WRKY), seven bZIP, three ethylene response factor (ERF), and five
282 dehydration-responsive element-binding (DREB) family transcription factors. These
283 transcription factors played essential roles in M79 when it was exposed to drought stress (Table
284 S16).

285 Many genes involved in phytohormone signaling were also responsible for drought
286 tolerance in M79. We identified 17 DEGs that are related to auxin/indole-3-acetic acid (Aux/IAA)
287 regulation, including nine of the *OsIAA* family (one up-regulated and eight down-regulated) and
288 four belonging to the *OsSAUR* family (one up-regulated and three down-regulated). In addition,
289 we identified one up-regulated cytokinin (CTK) dehydrogenase-related gene, ten DEGs involved
290 in the GA regulation pathway, including six gibberellin 20 oxidases (four up-regulated and two
291 down-regulated), three gibberellin 2-beta-dioxygenases (two up-regulated and one down-
292 regulated), and one up-regulated gibberellin receptor *GIDIL2*, and three DEGs related to ETH
293 (two up-regulated and one down-regulated) (Table S16).

294 A total of 63 DEGs in M79 were identified to be involved in redox regulation, including
295 genes encoding superoxide dismutase (SOD), glutathione peroxidase (GPx), oxidoreductase,
296 POD, ascorbate peroxidase (APX), and lipoxygenase (LOX) (Table S16).

297 Forty-four DEGs related to osmotic regulation in M79. Among them, twelve DEGs were
298 involved in proline metabolism, with ten up-regulated genes; seven DEGs were aquaporin genes
299 with three up-regulated genes, and 12 DEGs belonging to ATP-binding cassette, subfamily G
300 (ABCG) transporter family genes with six up-regulated genes (Table S16).

301 Forty-nine photosynthesis-related genes were differentially expressed in drought-treated
302 M79 plants. These were involved in several photosynthetic metabolic pathways, including
303 synthesis and degradation of chlorophyll, light energy absorption and transmission, PSI reaction
304 center electron transfer, PSII reaction center electron transfer, and water oxidation. Among them,

305 one PaO gene, one PsbP gene, one phytoene synthase gene, one scaffold protein in nitrogen
306 fixation system (NifU) gene, and one ferredoxin-NADP+ reductase gene were significantly up-regulated in
307 M79 (Table S16). These genes maintained photosynthesis in M79 under drought.

308 **Co-regulation analysis of drought-responsive DEGs in M79**

309 The co-regulation study generated a regulatory network of 72 genes (Fig. 5), and the related
310 genes were further divided into five groups. Genes in group A were mainly involved in signal
311 transduction, including those encoding receptor kinases, Ca²⁺-related proteins, and protein
312 kinases. Group B contained genes for GA, Aux/IAA, ETH, and CTK signaling. Group C
313 consisted of transcriptional regulatory genes, including 4 bZIP, 1 DREB, and three WRKY
314 family transcription factors. Genes in group D were drought-related, acting downstream of the
315 molecular pathway responsible for drought tolerance in M79, including redox balance regulation
316 genes (POD, GPx, and APX) and osmotic regulation-related genes (ion transporter, aquaporins,
317 and proline synthesis-related genes). Group E contained photosynthesis-related genes, including
318 two genes encoding oxygen evolving enhancer protein, three encoding PsbP proteins, and one
319 encoding a ferredoxin-NADP+ reductase. The regulatory network was involved at all stages, and the drought
320 response pathways in M79 may be essential for higher drought tolerance in M79 than its parental
321 lines.

322 **Responses of drought-treated M79 in photosynthesis-related pathways**

323 As a C₄ crop, the ability for millet to maintain photosynthesis under drought stress is an
324 important indicator of drought resistance (Feng et al., 2015). The net photosynthetic rate of the
325 three cultivars eventually decreased with prolonged drought stress. The net photosynthetic rate of
326 E1 was the highest before drought treatment (16.82) in comparison with H1 (14.57) and M79
327 (13.77). After the 6th and 7th days of stresses, the net photosynthetic rate of M79 was the highest
328 (11.52 and 10.67, respectively), reflecting the smallest decrease among the three cultivars in
329 response to drought stress (Fig. 6a). Under drought stress, both Fv/Fm and ΦPSII showed a
330 decreasing trend, and the parental lines E1 and H1 declined to a greater extent than M79 (Fig. 6b,
331 c). These results demonstrated that the function of PSII was inhibited under drought stress, and
332 that M79 maintained a relatively higher light energy utilization ratio than E1 and H1.

333 Functional annotation, GO enrichment and KEGG analysis identified 49 DEGs involved in
334 the photosynthesis pathway responding to drought stress and showed higher expression levels in
335 M79 than E1 and H1 (Fig. 6d). Further analysis of the photosynthetic pathway in M79 under
336 drought stress showed that a gene encoding ferrochelatase-2 (Seita.4G016600) was up-regulated
337 and involved in absorption and utilization of light energy during photosynthesis. Moreover, two
338 genes encoding oxygen evolving enhancer protein (Seita.J002400 and Seita.1G208500) along
339 with three PsbP genes (Seita.3G333700, Seita.9G561800 and Seita.5G442500) and one gene
340 encoding the PSII reaction center Psb28 protein (Seita.5G446500) were involved in the breakage
341 of water and oxygen release in the PSII reaction center. In addition, the NifU gene
342 (Seita.8G058400) was up-regulated and involved in Fe-S cluster assembly in the PSI reaction
343 center. Finally, two APX genes (Seita.7G023900 and Seita.9G444200) played a role in
344 scavenging ROS (Fig. 6e). In addition, five genes (Seita.2G303000, Seita.5G234700,
345 Seita.5G235200, Seita.6G024800 and Seita.9G545500) could be directly linked to the net
346 photosynthetic rate and Fv/Fm based on the correlation analysis on physiological data and the
347 expression of photosynthesis-related DEGs (Table S17). All of these genes regulated the
348 photosynthetic pathway in M79 in response to drought stress.

349 **DISCUSSION**

350 **The drought responsive pathway and related genes in millet**

351 Previous studies on drought-responsive pathways indicate that drought-inducible genes from
352 different varieties of the same crop likely play a relatively conserved role in their regulatory
353 networks (Yamaguchi-Shinozaki and Shinozaki, 2006). However, drought-induced genes codify
354 not only proteins that directly protect the cell structure and related metabolic pathways, but also
355 regulators with roles in stress signaling, and forming a set of elements responding to
356 environmental stress (Caldana et al., 2011; Vermeirssen et al., 2014). Among them, WAK and
357 CAMK are central in plant responses to abiotic stress (Zhang et al., 2005; Lv et al., 2014).
358 Protein phosphatase 2C (PP2C) belongs to a group of phosphatases involved in ABA signaling,
359 and is a negative regulator of ABA signaling (Zhang and Gan, 2012). Among the DEGs that we
360 identified from three drought-treated cultivars, four genes encoding CAMK kinases were up-
361 regulated, three genes encoding WAK receptor kinases were down-regulated, and seven PP2C

362 genes were up-regulated in all three cultivars (Table S9). Aux/IAA, ETH, and GA are
363 phytohormones playing an important part in maintaining normal plant growth and development,
364 and in reacting to abiotic stresses (Arraes et al., 2015; Gaion et al., 2017; Yu et al., 2017). Our
365 study detected one down-regulated gene encoding for Aux/IAA, two ETH-encoding genes (one
366 up-regulated and one down-regulated) and three GA-encoding genes (one up-regulated and two
367 down-regulated) in all three drought-treated cultivars (Table S9). Transcription factor families
368 such as NAC, MYB, bZIP, and basic helix-loop-helix (bHLH) also have a role in plant responses
369 to both biotic and abiotic stresses (Katiyar et al., 2012; Puranik et al., 2013; Yong et al. 2014).
370 Twelve DEGs belonging to these families were expressed in all three cultivars. They include five
371 up-regulated genes encoding NACs, five genes encoding MYBs (three up-regulated and two
372 down-regulated), an up-regulated gene encoding a bZIP and an up-regulated gene encoding a
373 bHLH transcription factor (Table S9). Many protective proteins are also considered essential for
374 protecting plants from damage caused by drought stress. For example, drought stress induces the
375 expression of LEAs, which reduce water loss in plant cells and increase their WUE under
376 adverse conditions (Wang et al., 2014). Genes encoding for protective proteins that were up-
377 regulated in all three cultivars in our study included three coding for universal stress proteins
378 (USP) and two for LEAs (Table S9).

379 A variety of intrinsic membrane proteins protect plant cells from abiotic stress by regulating
380 the permeability of the plasma membrane (Kasim et al., 2015). Here, we detected two genes
381 encoding aquaporins, one up-regulated and one down-regulated (Table S9). Plant cuticle is a
382 hydrophobic protective layer that prevents water loss and protects plants from abiotic stress, such
383 as those created from exposition to high temperature, drought, and salt (Ma et al., 2015). We
384 detected both up-regulated genes encoding waxes in our experiments (Table S9). The antioxidant
385 defense system in plants under drought stress is composed of ROS scavenging enzymes. Among
386 them, CAT, SOD, APX, and GPx are essential to remove ROS and act synergistically to
387 counteract oxidative damage caused by drought stress (Adriano et al., 2015). Our results
388 indicated that one gene encoding POD was up-regulated and one down-regulated (Table S9). In
389 addition, LTPs play an important role in response to biotic and abiotic stresses. We detected
390 seven LTPL-coding genes up-regulated in all three cultivars, suggesting a positive role for these
391 proteins in the drought defensive pathway (Safi et al., 2015; Table S9). All DEGs identified in
392 drought-treated M79, E1 and H1 were involved in drought-defensive processes, and the

393 transcription of many of them was up-regulated, suggesting that these genes play a positive
394 regulatory role in drought response.

395 **Molecular basis for better drought tolerance in M79 than its parental lines**

396 RNA-seq analysis identified 5258 DEGs between M79 and E1, and 3594 between M79 and H1,
397 indicating that the drought-tolerant cultivar M79 and its parental lines had different
398 transcriptional profiles (Fig. S3). GO analysis of these DEGs showed that they were highly
399 enriched in GO-terms such as membrane, protein kinase activity, transferase activity,
400 carbohydrate metabolism, iron ion binding, ATP binding, heme binding, oxidoreductase activity,
401 phosphorylation, and protein modification (Tables S5, S6). Among them, metal ion binding,
402 electron-carrier activity, and expression of genes related to oxidoreductase synthesis increase the
403 ability of plants to resist drought and high temperature (Rizhsky et al., 2004). In addition,
404 phosphorylation is involved in stress responses in plants. In Arabidopsis, the SUCROSE
405 NONFERMENTING1 (SNF1) kinase homologs 10 and 11 play an essential role in stress
406 responses (Chen and Hoehenwarter, 2015).

407 KEGG analysis found 18 and 15 significant pathways that differentiate between M79_DR
408 and E1_DR (FDR < 0.05), and between M79_DR and H1_DR (FDR < 0.05). These pathways
409 include biosynthesis of secondary metabolites, plant-pathogen interaction, metabolic pathways,
410 carotenoid biosynthesis, glutathione metabolism, amino sugar and nucleotide sugar metabolism,
411 phenylalanine metabolism, phenylpropanoid biosynthesis, porphyrin and chlorophyll metabolism,
412 diterpenoid biosynthesis, monoterpene biosynthesis, arginine and proline metabolism,
413 glycerophospholipid metabolism, ubiquinone and other terpenoid-quinone biosynthesis,
414 limonene and pinene degradation, flavonoid biosynthesis, cutin, suberine and wax biosynthesis,
415 galactose metabolism, brassinosteroid biosynthesis, glycerolipid metabolism, plant hormone
416 signal transduction, and alanine, aspartate and glutamate metabolism (Fig. 3). Among them,
417 glutathione metabolism can reduce and eliminate oxidative damage caused by ROS, and it plays
418 an important role in maintaining redox balance (Hicks et al., 2007). As an important osmotic
419 regulator in plants, proline helps to maintain osmotic pressure, and it stabilizes proteins and
420 cellular structures under drought stress (Vendruscolo et al., 2007). Phenylalanine and flavonoids
421 also play an important role in adapting plants to stress and overcoming stress damage
422 (Hernández et al., 2006; Babst et al., 2014; Pan et al., 2018). The cuticle and wax contribute in

423 protecting plants from biotic and abiotic stresses, and in maintaining plant morphology (Pollard
424 et al., 2008). Using high-throughput Illumina RNA-seq, Wang et al. (2016) identified and
425 quantified DEGs related to flavonoid biosynthesis in drought-stressed plants. Biosynthesis of
426 brassinosteroids, monoterpenoids, porphyrins, chlorophyll, ubiquinone, and other terpenoid-
427 quinones also is instrumental in adapting plants to drought stress and overcoming stress damage
428 (Ksouri et al., 2016; Hajrah et al., 2017; Miao et al., 2017). In addition, KEGG pathway analysis
429 indicated a significant enrichment in amino sugar and nucleotide sugar metabolism, and
430 limonene and pinene degradation in response to abiotic stress (Singh et al., 2017; Zhang et al.,
431 2017).

432 When challenged with drought stress, the transcriptional profile and ability to tolerate
433 drought stress in M79 were significantly different from those of both parental lines.
434 Crossbreeding is a means to promote recombination of parental genes, and is also a prerequisite
435 for breeding superior offspring, that not only inherit desired traits from both parental lines, but
436 also can exhibit novel F₁ phenotypes, heterosis and modified gene expression (Shivaprasad et al.,
437 2012; Bell et al., 2013). However, the role of heterosis in defense mechanism of abiotic stress is
438 still poorly understood (Dong et al., 2006; Korn et al., 2008; Singh et al., 2010; Miller et al.,
439 2015). Korn et al. (2008) showed that there is a strikingly strong correlation between heterosis,
440 freezing tolerance, and flavonol content. The heterotic vigor for SOD, POD and CAT suggests
441 an improvement of stress tolerance level in hybrids compared to the parental lines (Singh et al.,
442 2010). Miller et al. (2015) found that the levels of stress-responsive gene expression in parental
443 lines could be used to predict biomass heterosis in hybrids. We therefore hypothesized that the
444 reason for the higher drought stress tolerance in M79 than its parental lines was due to heterosis.
445 However, this hypothesis needs further examination.

446 **Molecular co-regulatory network for drought tolerance in millet**

447 The millet genome has a complex molecular regulatory network to cope with drought, which can
448 activate specific cell signaling pathways and induce transcriptional regulation that leads to
449 enhanced cellular responses, increased expression of antioxidant-related genes, and accumulation
450 of soluble substances (Lata et al., 2010). We analyzed DEGs in M79 before and after drought
451 stress. Genes that are widely involved in biological processes such as signal transduction,

452 hormonal signaling, transcriptional regulation, redox regulation, osmotic adjustment, and
453 photosynthesis formed a drought-tolerance regulatory network (Table S16).

454 Many signal transduction-related genes were up-regulated after drought treatment, including
455 five genes encoding NCX proteins (Table S16). This gene family is involved in Ca²⁺ signaling,
456 and dehydration induces the expression of *OsNCX3*, *OsNCX10*, and *OsNCX15* in rice (Singh et
457 al., 2015). Therefore, NCXs in M79 may be involved in signal transduction during drought stress
458 to activate the expression of downstream genes. In addition, we found 14 DEGs (eight up-
459 regulated) encoding WAKs, and 10 (five up-regulated) encoding CAMKs (Table S16). WAKs
460 belong to a receptor-like kinase gene family, and respond to abiotic stress acting on the signal
461 transduction between the cell wall and cytoplasm (Zhang et al., 2005), whereas CAMKs have
462 roles in Ca²⁺ signaling and protein phosphorylation (Chen et al., 2017). These up-regulated genes
463 positively regulated drought-related signaling pathways in M79, contributing to drought stress
464 tolerance with a rapid activation of downstream gene expression.

465 ETH, CTK, Aux/IAA, and GA are all involved in plant response to abiotic stresses (Werner
466 and Schmölling, 2009; De Diego et al., 2013; Colebrook et al., 2014; Zwack and Rashotte, 2015;
467 Yang et al., 2015; Yu et al., 2017). CTK and Aux/IAA negatively regulate ABA-induced
468 stomatal closure (Werner and Schmölling, 2009; De Diego et al., 2013). Colebrook et al. (2014)
469 showed that decreased GA content and transcriptional alteration of related genes inhibit plant
470 growth and development under various abiotic stress conditions. Yang et al. (2015) demonstrated
471 that ETH is involved in salt stress-related responses in rice, and that it plays a vital role in
472 regulating biotic and abiotic stress responses. In this study, we found two, one, three, and seven
473 up-regulated genes involved in ETH, CTK, Aux/IAA, and GA pathways, respectively, in M79
474 following drought stress (Table S16). These genes maintain growth and development in plants
475 under drought stress by regulating hormone balance.

476 Transcription factors play a central role in biotic and abiotic stress responses, and in the
477 regulation of various biological processes. AP2/DREB, WRKYs, ERF, bHLHs, bZIP and NAC
478 transcription factor families are involved in transcriptional regulation in response to stress (Lata
479 et al., 2014; Li et al., 2014; Muthamilarasan et al., 2015). Expression analysis of the millet
480 AP2/ERF genes *SiAP2/ERF-069*, *SiAP2/ERF-103*, and *SiAP2/ERF-120* showed that they were
481 all up-regulated under drought stress and therefore may play a positive role in this process (Lata
482 et al., 2014). *SiARDP* belongs to the DREB family of transcription factors and is one of the target

483 genes of *SiAREB*; it participates in the ABA-dependent signaling pathway. Overexpression of
484 *SiARDP* improves drought resistance in millet (Li et al., 2014). In addition, Muthamilarasan et al.
485 (2015) showed that the *SiWRKY* genes *SiWRKY066* and *SiWRKY08* give an essential contribution
486 in response to abiotic stress. In this study, multiple members of these families were differentially
487 expressed after drought stress, including 20 NACs, 19 AP2s, 14 WRKYs, seven bZIPs, three
488 ERFs, and five DREBs, indicating their involvement in the responses to drought stress (Table
489 S16). In addition, Komivi et al. (2016) showed that 90% of heat shock factors (HSFs) respond to
490 drought stress in sesame seedlings, and that two HSF transcription factors are significantly up-
491 regulated after drought stress, suggesting that these genes might contribute to this process (Table
492 S16).

493 Upon drought stress, gene expression is pivotal in protecting plants from oxidative damage.
494 For example, SOD converts superoxide into the less toxic H₂O₂, which is then reduced to H₂O by
495 POD, APX, and GPx (Alscher et al., 2002; Fecht-Christoffers et al., 2006; Islam et al., 2015). In
496 our study, drought stress up-regulated 10 genes encoding PODs, two genes encoding GPx, and
497 one gene encoding APX in M79. These genes function as positive regulators removing ROS and
498 maintaining a redox balance (Table S16).

499 Our study detected a large number of genes related to osmoregulation in M79 after drought
500 stress, including 12 (10 up-regulated) involved in proline metabolism, 12 (six up-regulated)
501 ABCG transporters, seven (three up-regulated) aquaporins, and two (both up-regulated) ATP-
502 binding cassette (ABC) transporters (Table S16). Proline is an important macromolecule serving
503 as an osmotic regulator in stress-defensive responses, since its accumulation can relieve damage
504 caused by osmotic stress under drought (An et al., 2013). ABC transporters utilize ATP
505 hydrolysis to transport osmotic regulators such as amino acids, peptides, carbohydrates, lipids,
506 hormones and metal ions (Jeong et al., 2014). Aquaporin proteins assist the plant to combat
507 abiotic stress by regulating the permeability of the plasma membrane (Kasim et al., 2015).
508 ABCG transporters help to biosynthesize protective cuticles and wax, transporting lipids or to
509 regulate phytohormone homeostasis transporting indole butyric acid and ABA (Yadav et al.,
510 2014). Expression of the above genes can regulate the osmotic potential of M79 cells under
511 drought stress to reduce injury.

512 Co-regulation analysis of these DEGs in M79 revealed a regulatory network consisting of
513 72 genes, which might contribute to the excellent drought resistance of M79. This system

514 includes signal perception and transduction, hormone signaling pathways, transcriptional
515 regulatory factors, and downstream functional genes (including ROS removal factors, ion
516 transporters and osmotic regulators) (Fig. 5). Although the results of our co-regulation analysis
517 remain to be further verified, the co-regulation network provides an important theoretical basis to
518 propose a model for the molecular mechanisms of drought tolerance in millet.

519 **Maintenance of a high photosynthetic rate is an important indicator of drought tolerance** 520 **in crop plants**

521 Photosynthesis is the basic metabolism regulating crop growth and final yield. The maintenance
522 of photosynthetic rates under drought stress is essential for drought tolerance in crops (Galmés et
523 al., 2007; Chaves et al., 2009). We found that photosynthetic rate and light energy utilization in
524 E1 and H1 was significantly lower than in M79 after drought stress, suggesting that M79 can
525 maintain higher photosynthesis under drought (Fig. 6a-c). Therefore, photosynthetic rate under
526 drought stress is not only related to photosynthetic capacity, but also to drought tolerance (Zhang
527 et al., 2016).

528 Photosynthesis in chloroplasts converts light into chemical energy that is used for plant
529 growth and development. Under drought stress, O_2 produced in chloroplasts can receive
530 electrons from the photosynthetic electron transport chain to become O^{2-} , which can cause
531 oxidative damage to photosynthetic pigments and the plasma membranes (Gill and Tuteja, 2010;
532 Exposito-Rodriguez et al., 2017). Previous studies showed that osmoregulation and antioxidant
533 capacity of plants contribute to maintaining photosynthetic capacity (Ramachandra Reddy et al.,
534 2004). Our co-regulatory analysis showed that osmotic regulation and antioxidation played a
535 vital role in the management of drought tolerance in M79 (Fig. 5; Table S16), explaining why
536 M79 was able to maintain a high net photosynthetic rate under drought stress.

537 Our results identified 49 photosynthesis-related DEGs in drought-treated M79. Among
538 them, 11 genes were involved in the photosynthetic pathway, including one gene encoding
539 ferrochelatase-2 (Seita.4G016600), two genes encoding oxygen evolving enhancer proteins
540 (Seita.J002400 and Seita.1G208500), three PsbP-encoding genes (Seita.3G333700,
541 Seita.9G561800 and Seita.5G442500), one gene encoding PSII reaction center Psb28 protein
542 (Seita.5G446500), one NifU-encoding gene (Seita.8G058400), and two APX-encoding genes
543 (Seita.7G023900 and Seita.9G444200) (Fig. 6e). Previous studies showed that ferrochelatase is

544 related to the absorption of light by the light-harvesting complex (LHC) proteins (Suzuki et al.,
545 2002; Espinas et al., 2016). PsbP and Psb28, two subunits of the PSII reaction center, are
546 involved in the water photolysis and oxygen release during photosynthesis (Mabbitt et al., 2014).
547 NifU plays an important role in the synthesis and assembly of the Fe-S cluster in PSI (Yabe et al.,
548 2004). Under drought stress, electron transport and photosynthetic phosphorylation in
549 chloroplasts produce a large amount of ROS, while APX effectively removes them and reduces
550 oxidative damage to plants (Jiang et al., 2016; Exposito-Rodriguez et al., 2017). Up-regulation of
551 these genes enables M79 to maintain a relatively high level of photosynthesis under drought
552 stress, and to resist the damage caused by drought. Through the analysis of the photosynthetic
553 pathway in drought-stressed M79, we provide an initial insight to the molecular mechanism of
554 drought resistance.

555 CONCLUSIONS

556 After the exposure of the F₁ hybrid M79 and its parental lines (E1 and H1) to drought stress
557 treatment, we demonstrated that M79 had higher photosynthetic energy conversion efficiency
558 and better tolerance to drought stress when compared to its parental lines. Transcriptomic study
559 suggested that DEGs in M79 contributed to the formation of a regulatory network involving
560 multiple biological processes and pathways, including photosynthesis, signal transduction,
561 transcriptional regulation, redox regulation, hormonal signaling, and osmotic regulation. We also
562 demonstrated that, upon drought treatment, some photosynthesis-related DEGs were highly
563 expressed in M79 compared to its parental lines. Finally, this study revealed critical molecular
564 pathways, such as photosynthesis, involved in the responses to drought stress in M79, and
565 provided abundant genetic information for further study of the underlying mechanism.

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569 REFERENCES

- 570 **Adriano S, Antonio S, Maria N, Antonella V. 2015.** Ascorbate peroxidase and catalase
571 activities and their genetic regulation in plants subjected to drought and salinity stresses.
572 *International Journal of Molecular Sciences* **16**:13561-13578.
- 573 **Alscher RG, Erturk N, Heath LS. 2002.** Role of superoxide dismutases (SODs) in controlling
574 oxidative stress in plants. *Journal of Experimental Botany* **53**:1331-1341.
- 575 **Ambavaram MMR, Basu S, Krishnan A, Ramegowda V, Batlang U, Rahman L, Baisakh N,**
576 **Pereira A. 2014.** Coordinated regulation of photosynthesis in rice increases yield and
577 tolerance to environmental stress. *Nature Communications* **5**:5302.
- 578 **An Y, Zhang M, Liu G, Han R, Liang Z. 2013.** Proline accumulation in leaves of *Periploca*
579 *sepium* via both biosynthesis up-regulation and transport during recovery from severe
580 drought. *Plos One* **8**:e69942.
- 581 **Arraes FB, Beneventi MA, Lisei de Sa ME, Paixao JF, Albuquerque EV, Marin SR,**
582 **Purgatto E, Nepomuceno AL, Grossi-de-Sa MF. 2015.** Implications of ethylene
583 biosynthesis and signaling in soybean drought stress tolerance. *BMC Plant Biology*
584 **15**:213.
- 585 **Babst BA, Chen HY, Wang HQ, Payyavula RS, Thomas TP, Harding SA, Tsai CJ. 2014.**
586 Stress-responsive hydroxycinnamate glycosyltransferase modulates phenylpropanoid
587 metabolism in *Populus*. *Journal of Experimental Botany* **65**:4191-4200.
- 588 **Bell GD, Kane NC, Rieseberg LH, Adams KL. 2013.** RNA-seq analysis of allele-specific
589 expression, hybrid effects, and regulatory divergence in hybrids compared with their
590 parents from natural populations. *Genome Biology and Evolution* **5**:1309-1323.
- 591 **Bennetzen JL, Schmutz J, Wang H, Percifield R, Hawkins J, Pontaroli AC, Estep M, Feng**
592 **L, Vaughn JN, Grimwood J, Jenkins J, Barry K, Lindquist E, Hellsten U,**
593 **Deshpande S, Wang X, Wu X, Mitros T, Triplett J, Yang X, Ye CY, Mauro-Herrera**
594 **M, Wang L, Li P, Sharma M, Sharma R, Ronald PC, Panaud O, Kellogg EA,**
595 **Brutnell TP, Doust AN, Tuskan GA, Rokhsar D, Devos KM. 2012.** Reference genome
596 sequence of the model plant *Setaria*. *Nature Biotechnology* **30**:555-561.
- 597 **Bonnecarrère V, Borsani O, Díaz P, Capdevielle F, Blanco P, Monza J. 2011.** Response to
598 photooxidative stress induced by cold in *japonica* rice is genotype dependent. *Plant*
599 *Science* **180**:726-732.

- 600 **Boyle EI, Weng S, Gollub J, Jin H, Botstein D, Cherry JM, Sherlock G. 2004.**
601 Go::TermFinder-open source software for accessing Gene Ontology information and
602 finding significantly enriched Gene Ontology terms associated with a list of genes.
603 *Bioinformatics* **20**:3710-3715.
- 604 **Caldana C, Degenkolbe T, Cuadros-Inostroza A, Klie S, Sulpice R, Leisse A, Steinhauser D,**
605 **Fernie AR, Willmitzer L, Hannah MA. 2011.** High-density kinetic analysis of the
606 metabolomic and transcriptomic response of Arabidopsis to eight environmental
607 conditions. *Plant Journal* **67**:869-884.
- 608 **Camilios-Neto D, Bonato P, Wassem R, Tadra-Sfeir MZ, Brusamarello-Santos LC,**
609 **Valdameri G, Donatti L, Faoro H, Weiss VA, Chubatsu LS, Pedrosa FO, Souza EM.**
610 **2014.** Dual RNA-seq transcriptional analysis of wheat roots colonized by *Azospirillum*
611 *brasilense* reveals up-regulation of nutrient acquisition and cell cycle genes. *BMC*
612 *Genomics* **15**:378.
- 613 **Cao WH, Liu J, He XJ, Mu RL, Zhou HL, Chen SY, Zhang JS. 2007.** Modulation of
614 ethylene responses affects plant salt-stress responses. *Plant Physiology* **143**:707-719.
- 615 **Chaves MM, Flexas J, Pinheiro C. 2009.** Photosynthesis under drought and salt stress:
616 regulation mechanisms from whole plant to cell. *Annals of Botany* **103**:551-560.
- 617 **Chen F, Zhang L, Cheng ZM. 2017.** The calmodulin fused kinase novel gene family is the
618 major system in plants converting Ca²⁺ signals to protein phosphorylation responses.
619 *Scientific Reports* **7**:4127.
- 620 **Chen Y, Hoehenwarter W. 2015.** Changes in the phosphoproteome and metabolome link early
621 signaling events to rearrangement of photosynthesis and central metabolism in salinity
622 and oxidative stress response in Arabidopsis. *Plant Physiology* **169**:3021-3033.
- 623 **Colebrook EH, Thomas SG, Phillips AL, Hedden P. 2014.** The role of gibberellin signaling in
624 plant responses to abiotic stress. *Journal of Experimental Biology* **217**:67-75.
- 625 **Cui Y, Wang M, Zhou H, Li M, Huang L, Yin X, Zhao G, Lin F, Xia X, Xu G. 2016.** OsSGL,
626 a novel DUF1645 domain-containing protein, confers enhanced drought tolerance in
627 transgenic rice and *Arabidopsis*. *Frontiers in Plant Science* **7**:2001.
- 628 **De Diego N, Rodríguez JL, Dodd IC, Pérez-Alfocea F, Moncaleán P, Lacuesta M. 2013.**
629 Immunolocalization of IAA and ABA in roots and needles of radiata pine (*Pinus radiata*)
630 during drought and rewatering. *Tree Physiology* **33**:537-549.

- 631 **Dong J, Wu F, Jin Z, Huang Y. 2006.** Heterosis for Yield and some Physiological Traits in
632 Hybrid Cotton Cikangza 1. *Euphytica* 151: 71-77.
- 633 **Espinas NA, Kobayashi K, Sato Y, Mochizuki N, Takahashi K, Tanaka R, Masuda T. 2016.**
634 Allocation of heme is differentially regulated by ferrochelatase isoforms in *Arabidopsis*
635 cells. *Frontiers in Plant Science* 7:1326.
- 636 **Exposito-Rodriguez M, Laissue PP, Yvon-Durocher G, Smirnoff N, Mullineaux PM. 2017.**
637 Photosynthesis-dependent H₂O₂ transfer from chloroplasts to nuclei provides a high-light
638 signalling mechanism. *Nature Communications* 8:49.
- 639 **Fecht-Christoffers MM, Führs H, Braun HP, Horst WJ. 2006.** The role of hydrogen
640 peroxide-producing and hydrogen peroxide-consuming peroxidases in the leaf apoplast of
641 cowpea in manganese tolerance. *Plant Physiology* 140:1451-1463.
- 642 **Feng ZJ, He GH, Zheng WJ, Lu PP, Chen M, Gong YM, Ma YZ, Xu ZS. 2015.** Foxtail
643 Millet NF-Y Families: Genome-Wide Survey and Evolution Analyses Identified Two
644 Functional Genes Important in Abiotic Stresses. *Frontiers in Plant Science* 6:1142.
- 645 **Fracasso A, Trindade LM, Amaducci S. 2016.** Drought stress tolerance strategies revealed by
646 RNA-Seq in two sorghum genotypes with contrasting WUE. *BMC Plant Biology* 16:115.
- 647 **Gaion LA, Monteiro CC, Cruz FJR, Rossatto DR, López-Díaz I, Carrera E, Lima JE,**
648 **Peres LEP, Carvalho RF. 2017.** Constitutive gibberellin response in grafted tomato
649 modulates root-to-shoot signaling under drought stress. *Journal of Plant Physiology*
650 221:11-21.
- 651 **Galmés J, Medrano H, Flexas, J. 2007.** Photosynthetic limitations in response to water stress
652 and recovery in Mediterranean plants with different growth forms. *New Phytologist*
653 175:81-93.
- 654 **Gill SS, Tuteja N. 2010.** Reactive oxygen species and antioxidant machinery in abiotic stress
655 tolerance in crop plants. *Plant Physiology and Biochemistry* 48:909-930.
- 656 **Gollan PJ, Tikkanen M, Aro EM. 2015.** Photosynthetic light reactions: integral to chloroplast
657 retrograde signalling. *Current Opinion in Plant Biology* 27:180-191.
- 658 **Gürel F, Öztürk ZN, Uçarlı C, Rosellini D. 2016.** Barley genes as tools to confer abiotic stress
659 tolerance in crops. *Frontiers in Plant Science* 7:1137.
- 660 **Hajrah NH, Obaid AY, Atef A, Ramadan AM, Arasappan D, Nelson CA, Edris S,**
661 **Mutwakil MZ, Alhebshi A, Gadalla NO, Makki RM, Al-Kordy MA, El-Domyati FM,**

- 662 **Sabir JSM, Khiyami MA, Hall N, Bahieldin A, Jansen RK. 2017.** Transcriptomic
663 analysis of salt stress responsive genes in *Rhazya stricta*. *PLoS One* **12**:e0177589.
- 664 **He GH, Xu YJ, Wang XY, Liu MJ, Li SP, Chen M, Ma YZ, Xu ZS. 2016.** Drought-
665 responsive WRKY transcription factor genes *TaWRKY1* and *TaWRKY33* from wheat
666 confer drought and/or heat resistance in *Arabidopsis*. *BMC Plant Biology* **16**:116.
- 667 **Hernández I, Alegre L, Munné-Bosch S. 2006.** Enhanced oxidation of flavan-3-ols and
668 proanthocyanidin accumulation in water-stressed tea plants. *Phytochemistry* **67**:1120-
669 1126.
- 670 **Hicks LM, Cahoon RE, Bonner ER, Rivard RS, Sheffield J, Jez JM. 2007.** Thiol-based
671 regulation of redox-active glutamate-cysteine ligase from *Arabidopsis thaliana*. *Plant*
672 *Cell* **19**:2653-2661.
- 673 **Hu H, Xiong L. 2014.** Genetic engineering and breeding of drought-resistant crops. *Annual*
674 *Review of Plant Biology* **65**:715-741.
- 675 **Ksouri N, Jiménez S, Wells CE, Contreras-Moreira B, Gogorcena Y. 2016.** Transcriptional
676 Responses in Root and Leaf of *Prunus persica* under Drought Stress Using RNA
677 Sequencing. *Frontiers in Plant Science* **7**:1715.
- 678 **Islam T, Manna M, Reddy MK. 2015.** Glutathione peroxidase of *Pennisetum glaucum* (PgGPx)
679 is a functional Cd²⁺ dependent peroxiredoxin that enhances tolerance against salinity and
680 drought stress. *Plos One* **10**:e0143344.
- 681 **Jeong CB, Kim BM, Lee JS, Rhee JS. 2014.** Genome-wide identification of whole ATP-
682 binding cassette (ABC) transporters in the intertidal copepod *Tigriopus japonicus*. *BMC*
683 *Genomics* **15**:1-15.
- 684 **Jiang G, Yin D, Zhao J, Chen H, Guo L, Zhu L, Zhai W. 2016.** The rice thylakoid
685 membrane-bound ascorbate peroxidase *OsAPX8* functions in tolerance to bacterial blight.
686 *Scientific Reports* **6**:26104.
- 687 **Kanehisa M, Araki M, Goto S, Hattori M, Hirakawa M, Itoh M, Katayama T, Kawashima**
688 **S, Okuda S, Tokimatsu T, Yamanishi Y. 2008.** KEGG for linking genomes to life and
689 the environment. *Nucleic Acids Research* **D480-D484**.
- 690 **Kasim K, Pallavi A, Arti S, Vidhu AS. 2015.** Heterologous expression of two *Jatropha*
691 aquaporins imparts drought and salt tolerance and improves seed viability in transgenic
692 *Arabidopsis thaliana*. *PloS One* **10**: e0128866.

- 693 **Katiyar A, Smita S, Lenka SK, Rajwanshi R, Chinnusamy V, Bansal KC. 2012.** Genome-
694 wide classification and expression analysis of *MYB*, transcription factor families in rice
695 and arabidopsis. *BMC Genomics* **13**:544.
- 696 **Kim D, Perteza G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. 2013.** Tophat2: accurate
697 alignment of transcriptomes in the presence of insertions, deletions and gene fusions.
698 *Genome Biology* **14**:R36.
- 699 **Komivi D, Diaga D, Ndiaga C. 2016.** Genome-wide investigation of Hsf genes in sesame
700 reveals their segmental duplication expansion and their active role in drought stress
701 response. *Frontiers in Plant Science* **7**:1522.
- 702 **Korn M, Peterek S, Mock HP, Heyer AG, Hinch DK. 2008.** Heterosis in the freezing
703 tolerance, and sugar and flavonoid contents of crosses between *Arabidopsis thaliana*
704 accessions of widely varying freezing tolerance. *Plant Cell and Environment* **31**: 813-827.
- 705 **Lata C, Gupta S, Prasad M. 2013.** Foxtail millet: a model crop for genetic and genomic studies
706 in bioenergy grasses. *Critical Reviews in Biotechnology* **33**:328-343.
- 707 **Lata C, Mishra AK, Muthamilarasan M, Bonthala VS, Khan Y, Prasad M. 2014.** Genome-
708 wide investigation and expression profiling of AP2/ERF transcription factor superfamily
709 in foxtail millet (*Setaria italica* L.). *Plos One* **9**:e113092.
- 710 **Lata C, Sahu PP, Prasad M. 2010.** Comparative transcriptome analysis of differentially
711 expressed genes in foxtail millet (*Setaria italica* L.) during dehydration stress.
712 *Biochemical and Biophysical Research Communications* **393**:720-727.
- 713 **Li C, Yue J, Wu X, Xu C, Yu J. 2014.** An ABA-responsive DRE-binding protein gene from
714 *Setaria italica*, *SiARDP*, the target gene of SiAREB, plays a critical role under drought
715 stress. *PLoS One* **65**:5415-5427.
- 716 **Li P, Brutnell TP. 2011.** *Setaria viridis* and *Setaria italica*, model genetic systems for the
717 panicoid grasses. *Journal of Experimental Botany* **62**:3031-3037.
- 718 **Livak KJ, Schmittgen TD. 2001.** Analysis of relative gene expression data using real-time
719 quantitative PCR and the 2(T) (-Delta Delta C) method. *Methods* **25**:402-408.
- 720 **Lowry DB, Hernandez K, Taylor SH, Meyer E, Logan TL, Barry KW, Chapman JA,**
721 **Rokhsar DS, Schmutz J, Juenger TE. 2015.** The genetics of divergence and
722 reproductive isolation between ecotypes of *Panicum hallii*. *New Phytologist* **205**:402-414.

- 723 **Lv DW, Subburaj S, Cao M, Yan X, Li X, Appels R, Sun DF, Ma W, Yan YM. 2014.**
724 Proteome and phosphoproteome characterization reveals new response and defense
725 mechanisms of *Brachypodium distachyon* leaves under salt stress. *Molecular & Cellular*
726 *Proteomics* **13**:632-652.
- 727 **Ma X, Wang P, Zhou S, Sun Y, Liu N, Li X, Hou Y. 2015.** De novo transcriptome sequencing
728 and comprehensive analysis of the drought-responsive genes in the desert plant
729 *Cynanchum komarovii*. *BMC Genomics* **16**:753.
- 730 **Ma X, Xia H, Liu Y, Wei H, Zheng X, Song C, Chen L, Liu H, Luo L. 2016.** Transcriptomic
731 and metabolomic studies disclose key metabolism pathways contributing to well-
732 maintained photosynthesis under the drought and the consequent drought-tolerance in
733 rice. *Frontiers in Plant Science* **7**:1886.
- 734 **Mabbitt PD, Wilbanks SM, Eaton-Rye JJ. 2014.** Structure and function of the hydrophilic
735 photosystem ii assembly proteins: Psb27, Psb28 and Ycf48. *Plant Physiology and*
736 *Biochemistry* **81**:96-107.
- 737 **Miao Z, Han Z, Zhang T, Chen S, Ma C. 2017.** A systems approach to a spatio-temporal
738 understanding of the drought stress response in maize. *Scientific Reports* **7**:6590.
- 739 **Miller M, Song Q, Shi X, Juenger TE, Chen ZJ. 2015.** Natural variation in timing of stress-
740 responsive gene expression predicts heterosis in intraspecific hybrids of *Arabidopsis*.
741 *Nature Communications* **6**: 7453.
- 742 **Muthamilarasan M, Bonthala VS, Khandelwal R, Jaishankar J, Shweta S, Nawaz K,**
743 **Prasad M. 2015.** Global analysis of WRKY transcription factor superfamily in *Setaria*
744 identifies potential candidates involved in abiotic stress signaling. *Frontiers in Plant*
745 *Science* **6**:910.
- 746 **Muthamilarasan M, Prasad M. 2015.** Advances in *Setaria* genomics for genetic improvement
747 of cereals and bioenergy grasses. *Theoretical and Applied Genetics* **128**:1-14.
- 748 **Pan L, Meng C, Wang J, Ma X, Fan X, Yang Z, Zhou M, Zhang X. 2018.** Integrated omics
749 data of two annual ryegrass (*Lolium multiflorum* L.) genotypes reveals core metabolic
750 processes under drought stress. *BMC Plant Biology* **18**:26.
- 751 **Pollard M, Beisson F, Li Y, Ohlrogge JB. 2008.** Building lipid barriers: biosynthesis of cutin
752 and suberin. *Trends in Plant Science* **13**:236-246.

- 753 **Puranik S, Bahadur RP, Srivastava PS, Prasad M. 2011.** Molecular cloning and
754 characterization of a membrane associated NAC family gene, *SiNAC* from foxtail millet
755 [*Setaria italica* (L.)P. Beauv]. *Molecular Biotechnology* **49**:138-150.
- 756 **Puranik S, Sahu PP, Mandal SN, B VS, Parida SK, Prasad M. 2013.** Comprehensive
757 genome-wide survey, genomic constitution and expression profiling of the NAC
758 transcription factor family in foxtail millet (*Setaria italica* L). *PLoS One* **8**:e64594.
- 759 **Qi X, Xie S, Liu Y, Yi F, Yu J. 2013.** Genome-wide annotation of genes and noncoding RNAs
760 of foxtail millet in response to simulated drought stress by deep sequencing. *Plant*
761 *Molecular Biology* **83**:459-473.
- 762 **Ramachandra Reddy A, Chaitanya KV, Vivekanandan M. 2004.** Drought-induced responses
763 of photosynthesis and antioxidant metabolism in higher plants. *Journal of Plant*
764 *Physiology* **161**:1189-1202.
- 765 **Redillas MC, Jeong JS, Kim YS, Jung H, Bang SW, Choi YD, Ha SH, Reuzeau C, Kim JK.**
766 **2012.** The overexpression of *OsNAC9* alters the root architecture of rice plants enhancing
767 drought resistance and grain yield under field conditions. *Plant Biotechnology Journal*
768 **10**:792-805.
- 769 **Reynolds M, Tuberosa R. 2008.** Translational research impacting on crop productivity in
770 drought-prone environments. *Current Opinion in Plant Biology* **11**:171-179.
- 771 **Rizhsky L, Liang H, Shuman J, Shulaev V, Davletova S, Mittler R. 2004.** When defense
772 pathways collide. The response of *Arabidopsis* to a combination of drought and heat
773 stress. *Plant Physiology* **134**:1683-1696.
- 774 **Safi H, Saibi W, Alaoui MM, Hmyene A, Masmoudi K, Hanin M, Brini F. 2015.** A wheat
775 lipid transfer protein (TdLTP4) promotes tolerance to abiotic and biotic stress in
776 *Arabidopsis thaliana*. *Plant Physiology and Biochemistry* **89**:64-75.
- 777 **Saito R, Smoot ME, Ono K, Ruscheinski J, Wang PL, Lotia S, Pico AR, Bader GD, Ideker**
778 **T. 2012.** A travel guide to Cytoscape plugins. *Nature Methods* **9**:1069-1076.
- 779 **Shivaprasad PV, Dunn RM, Santos BA, Bassett A, Baulcombe DC. 2012.** Extraordinary
780 transgressive phenotypes of hybrid tomato are influenced by epigenetics and small
781 silencing RNAs. *EMBO Journal* **31**:257-266.

- 782 **Singh AK, Kumar R, Tripathi AK, Gupta BK, Pareek A, Singla-Pareek SL. 2015.** Genome-
783 wide investigation and expression analysis of sodium/calcium exchanger gene family in
784 rice and Arabidopsis. *Rice* **8**:21.
- 785 **Singh BK, Sharma SR, Singh B. 2010.** Heterosis for superoxide dismutase, peroxidase and
786 catalase enzymes in the head of single cross-hybrids of cabbage (*Brassica oleracea* var.
787 *capitata*). *Journal of Genetics* **89**: 217-221.
- 788 **Singh D, Singh CK, Taunk J, Tomar RSS, Chaturvedi AK, Gaikwad K, Pal M. 2017.**
789 Transcriptome analysis of lentil (*Lens culinaris* Medikus) in response to seedling drought
790 stress. *BMC Genomics* **18**:206.
- 791 **Suzuki T, Masuda T, Singh DP, Tan FC, Tsuchiya T, Shimada H, Ohta H, Smith AG,**
792 **Takamiya K. 2002.** Two types of ferrochelatase in photosynthetic and nonphotosynthetic
793 tissues of cucumber: their difference in phylogeny, gene expression, and localization.
794 *Journal of Biological Chemistry* **277**:4731-4737.
- 795 **Tang S, Li L, Wang Y, Chen Q, Zhang W, Jia G, Zhi H, Zhao B, Diao X. 2017.** Genotype-
796 specific physiological and transcriptomic responses to drought stress in *Setaria italica* (an
797 emerging model for Panicoideae grasses). *Scientific Reports* **7**:10009.
- 798 **Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL,**
799 **Wold BJ, Pachter L. 2010.** Transcript assembly and quantification by RNA-Seq reveals
800 unannotated transcripts and isoform switching during cell differentiation. *Nature*
801 *Biotechnology* **28**:511-515.
- 802 **Vendruscolo EC, Schuster I, Pileggi M, Scapim CA, Molinari HB, Marur CJ, Vieira LG.**
803 **2007.** Stress-induced synthesis of proline confers tolerance to water deficit in transgenic
804 wheat. *Journal of Plant Physiology* **164**:1367-1376.
- 805 **Vermeirssen V, De Clercq I, Van Parys T, Van Breusegem F, Van de Peer Y. 2014**
806 *Arabidopsis* ensemble reverse-engineered gene regulatory network discloses
807 interconnected transcription factors in oxidative stress. *Plant Cell* **26**:4656-4679.
- 808 **Wang M, Li P, Li C, Pan Y, Jiang X, Zhu D, Zhao Q, Yu J. 2014.** *SiLEA14*, a novel atypical
809 LEA protein, confers abiotic stress resistance in foxtail millet. *BMC Plant Biology* **14**:290.
- 810 **Wang W, Xin H, Wang M, Ma Q, Wang L, Kaleri NA, Wang Y, Li X. 2016.** Transcriptomic
811 Analysis Reveals the Molecular Mechanisms of Drought-Stress-Induced Decreases in
812 *Camellia sinensis* Leaf Quality. *Frontiers in Plant Science* **7**:385.

- 813 **Wang Y, Li L, Tang S, Liu J, Zhang H, Zhi H, Jia G, Diao X. 2016.** Combined small RNA
814 and degradome sequencing to identify miRNAs and their targets in response to drought in
815 foxtail millet. *BMC Genetics* **17**:57.
- 816 **Warne GR. 2016.** *gplots: Various R Programming Tools for Plotting Data*. Available at:
817 <https://cran.r-project.org/web/packages/gplots/>
- 818 **Werner T, Schmülling T. 2009.** Cytokinin action in plant development. *Current Opinion in*
819 *Plant Biology* **12**:527-538.
- 820 **Yabe T, Morimoto K, Kikuchi S, Nishio K, Terashima I, Nakai M. 2004.** The Arabidopsis
821 chloroplastic NifU-like protein CnfU, which can act as an iron-sulfur cluster scaffold
822 protein, is required for biogenesis of ferredoxin and photosystem I. *Plant Cell* **16**:993-
823 1007.
- 824 **Yadav A, Khan Y, Prasad M. 2015.** Dehydration-responsive miRNAs in foxtail millet:
825 genome-wide identification, characterization and expression profiling. *Planta* **243**:749-
826 766.
- 827 **Yadav V, Molina I, Ranathunge K, Castillo IQ, Rothstein SJ, Reeda JW. 2014.** ABCG
828 transporters are required for suberin and pollen wall extracellular barriers in *Arabidopsis*.
829 *Plant Cell* **26**:3569-3588.
- 830 **Yamaguchi-Shinozaki K, Shinozaki K. 2006.** Transcriptional regulatory networks in cellular
831 responses and tolerance to dehydration and cold stresses. *Annual Review of Plant Biology*
832 **57**:781-803.
- 833 **Yang C, Ma B, He SJ, Xiong Q, Duan KX, Yin CC, Chen H, Lu X, Chen SY, Zhang JS.**
834 **2015.** *MAOHUZI6/ETHYLENE INSENSITIVE3-LIKE1* and *ETHYLENE INSENSITIVE3-*
835 *LIKE2* Regulate Ethylene Response of Roots and Coleoptiles and Negatively Affect Salt
836 Tolerance in Rice. *Plant Physiology* **169**:148-165.
- 837 **Yi F, Chen J, Yu J. 2015.** Global analysis of uncapped mRNA changes under drought stress and
838 microrna-dependent endonucleolytic cleavages in foxtail millet. *BMC Plant Biology*
839 **15**:241.
- 840 **Yong HY, Zou Z, Kok EP, Kwan BH, Chow K, Nasu S, Nanzyo M, Kitashiba H, Nishio T.**
841 **2014.** Comparative transcriptome analysis of leaves and roots in response to sudden
842 increase in salinity in *Brassica napus* by RNA-seq. *Biomed Research International*
843 467395.

- 844 **Yu C, Zhan Y, Feng X, Huang ZA, Sun C. 2017.** Identification and expression profiling of the
845 auxin response factors in *Capsicum annuum* L. under abiotic stress and hormone
846 treatments. *International Journal of Molecular Sciences* **18**:e2719.
- 847 **Zhang G, Liu X, Quan Z, Cheng S, Xu X, Pan S, Xie M, Zeng P, Yue Z, Wang W, Tao Y,**
848 **Bian C, Han C, Xia Q, Peng X, Cao R, Yang X, Zhan D, Hu J, Zhang Y, Li H, Li H,**
849 **Li N, Wang J, Wang C, Wang R, Guo T, Cai Y, Liu C, Xiang H, Shi Q, Huang P,**
850 **Chen Q, Li Y, Wang J, Zhao Z, Wang J. 2012.** Genome sequence of foxtail millet
851 (*Setaria italica*) provides insights into grass evolution and biofuel potential. *Nature*
852 *Biotechnology* **30**:549-554.
- 853 **Zhang H, Shen J, Wei Y, Chen H. 2017.** Transcriptome profiling of litchi leaves in response to
854 low temperature reveals candidate regulatory genes and key metabolic events during
855 floral induction. *BMC Genomics* **18**: 363.
- 856 **Zhang J, Liu T, Fu J, Zhu Y, Jia J, Zheng J, Zhao Y, Zhang Y, Wang G. 2007.** Construction
857 and application of EST library from *Setaria italica*, in response to dehydration stress.
858 *Genomics* **90**:121-131.
- 859 **Zhang K, Gan SS. 2012.** An abscisic acid-AtNAP transcription factor-*SAG113* protein
860 phosphatase 2C regulatory chain for controlling dehydration in senescing Arabidopsis
861 leaves. *Plant Physiology* **158**:961-969.
- 862 **Zhang LM, Liu XG, Qu XN, Yu Y, Han SP, Dou Y, Xu YY, Jing HC, Hao DY. 2013.** Early
863 transcriptomic adaptation to Na₂CO₃ stress altered the expression of a quarter of the total
864 genes in the maize genome and exhibited shared and distinctive profiles with NaCl and
865 high pH stresses. *Journal of Integrative Plant Biology* **55**:1147-1165.
- 866 **Zhang S, Chen C, Li L, Meng L, Singh J, Jiang N, Deng XW, He ZH, Lemaux PG. 2005.**
867 Evolutionary expansion, gene structure, and expression of the rice wall-associated kinase
868 gene family. *Plant Physiology* **139**:1107-1124.
- 869 **Zhang ZF, Li YY, Xiao BZ. 2016.** Comparative transcriptome analysis highlights the crucial
870 roles of photosynthetic system in drought stress adaptation in upland rice. *Scientific*
871 *Reports* **6**:19349.
- 872 **Zhao M, Running SW. 2011.** Response to comments on “drought-induced reduction in global
873 terrestrial net primary production from 2000 through 2009”. *Science* **333**:1093.

- 874 **Zhou Y, Yang P, Cui F, Zhang F, Luo X, Xie J. 2016.** Transcriptome analysis of salt stress
875 responsiveness in the seedlings of Dongxiang wild rice (*Oryza rufipogon* Griff.). *PLoS*
876 *One* **11**:e0146242.
- 877 **Zwack PJ, Rashotte AM. 2015.** Interactions between cytokinin signalling and abiotic stress
878 responses. *Journal of Experimental Botany* **66**:4863-4871.

Figure 1

Morphological and physiological analysis of M79, E1 and H1 before and after drought treatment.

a: Phenotypes of M79, E1 and H1 seedlings under normal conditions and 5-7 days after drought stress (photo credit: Weiping Shi); b: LWP of M79, E1 and H1 under normal conditions and 5-7 days after drought stress; c: REL in M79, E1 and H1 at 5-7 days after drought stress; d: CAT activities of M79, E1 and H1 under normal condition and 5-7 days after drought stress; e: WUE of M79, E1 and H1 under normal condition and 5-7 days after drought stress. Each column represents the mean \pm SD (5 replicates); * Significance levels in comparison to M79 were determined by t-tests (* $P < 0.05$, ** $P < 0.01$).

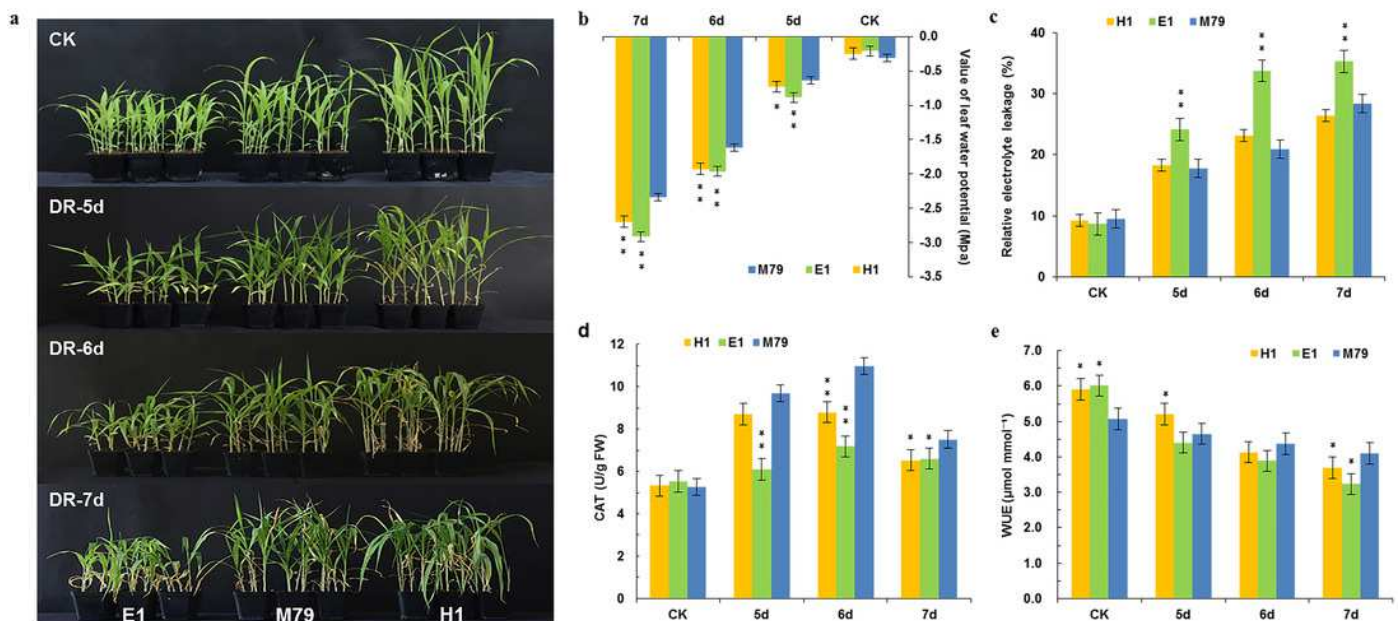


Figure 2

Correlation analysis of RNA-seq and qRT-PCR results.

a-j: Expression levels of 10 DEGs in drought-treated M79, E1 and H1. Values are presented as $\text{Log}_2(\text{Fold Change})$. k: Scatter plots of expression values of 10 DEGs in drought-treated M79, E1 and H1. X and Y axes represent $\text{Log}_2(\text{Fold Change})$ obtained from RNA-seq and qRT-PCR experiments, respectively. ** Gene expression values for RNA-seq and qRT-PCR were significant (** $P < 0.01$).

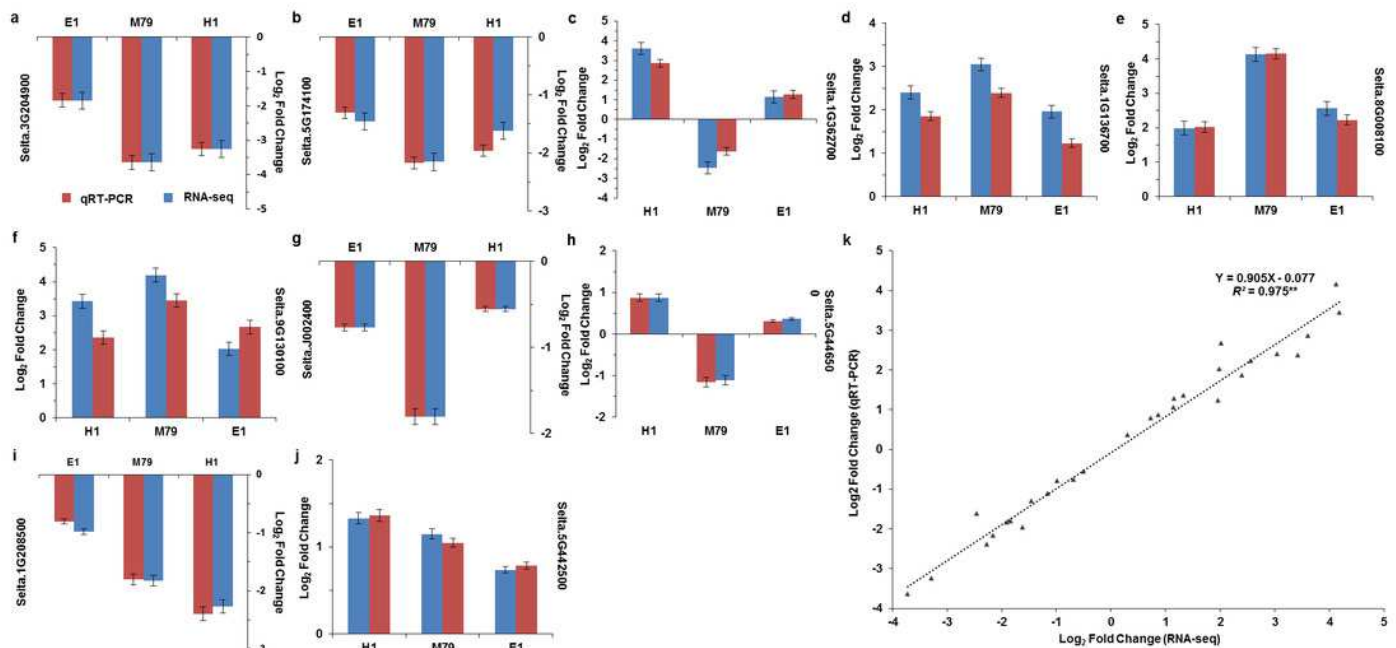


Figure 3

Scatter plots of enriched KEGG functional pathways in response to drought treatment.

a: M79_DR VS E1_DR; b: M79_DR vs H1_DR. The “Rich Factor” shows the ratio of the number of the DEGs to the total gene number in certain pathways.

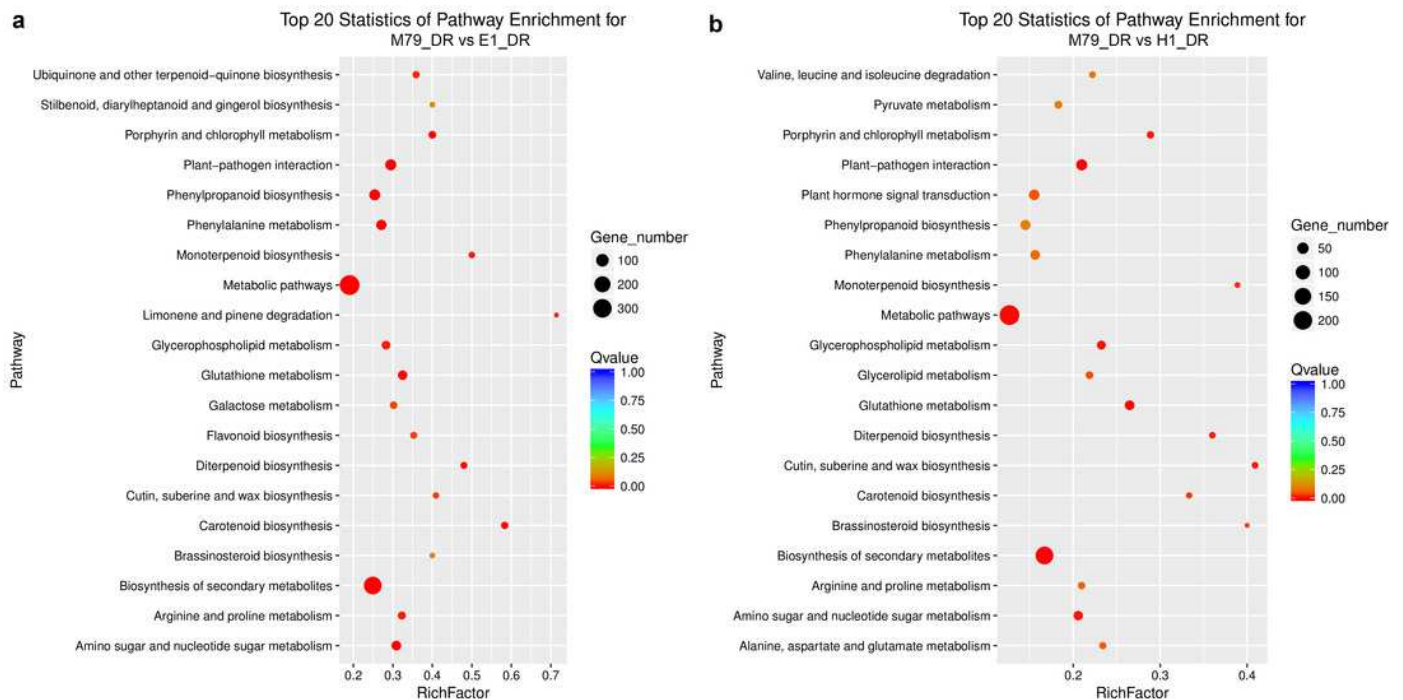


Figure 4

Venn diagrams of drought-responsive DEGs (both up- and down-regulated) in M79, E1 and H1. The DEGs were selected when $FDR \leq 0.05$.

a: Up-regulated DEGs in three genotypes after drought treatment; b: Down-regulated DEGs in three genotypes after drought treatment.

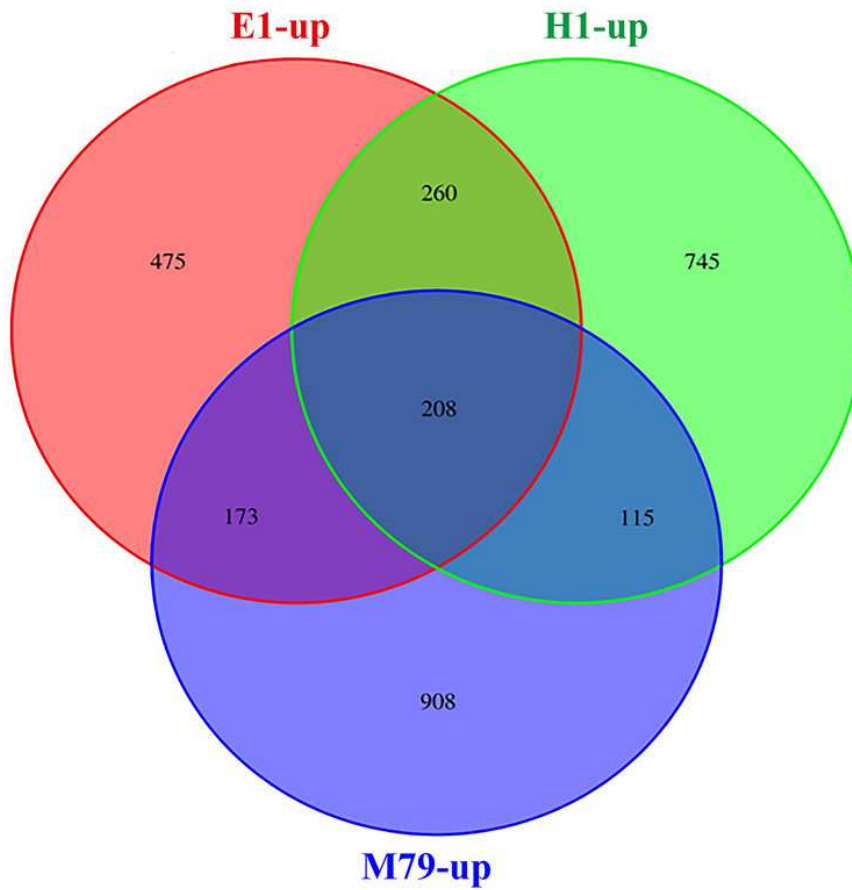
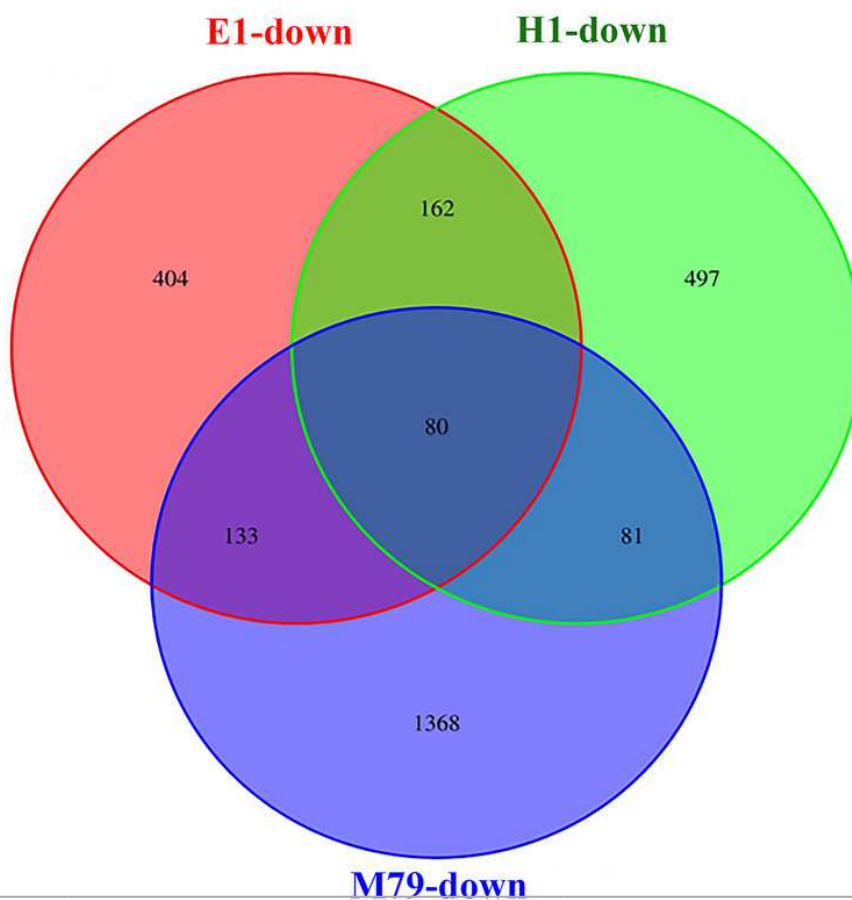
a**b**

Figure 5

A regulatory network consisting of drought-responsive DEGs in M79. Pearson's correlation coefficient $|PCC| \geq 0.93$.

The genes were categorized into five groups, with different colors representing different functional annotations: A: Signal transduction (Pink), B: Phytohormones (green), C: Transcription factors (light pink), D: redox (purple) and osmotic adjustment (dark blue), E: photosynthesis (light blue).

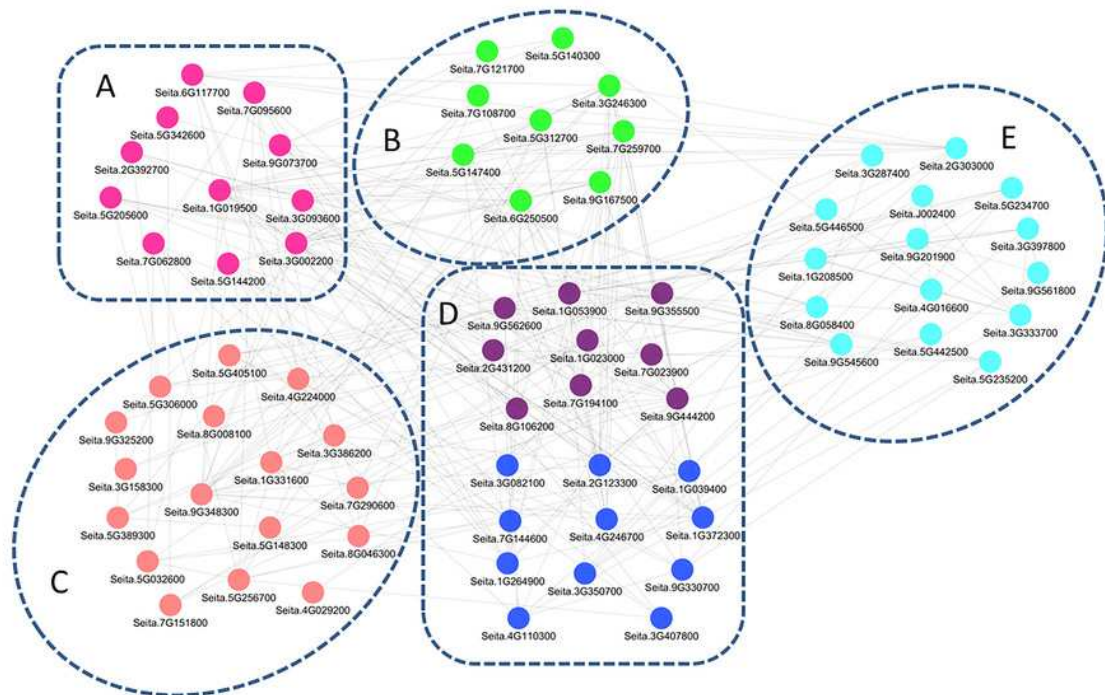


Figure 6

Photosynthetic analysis of drought-stressed M79, E1 and H1.

a: A ($\mu\text{mol m}^{-2} \text{s}^{-1}$) of M79, E1 and H1 under normal conditions and 5-7 days after drought treatment; b: F_v/F_m of M79, E1 and H1 under normal conditions and 5-7 days after drought treatment; c: ΦPSII of M79, E1 and H1 under normal conditions and 5-7 days after drought treatment; d: Clustered heatmap showing photosynthetic DEGs in drought-stressed M79, E1 and H1 e: Photosynthetic pathways in drought-tolerant M79. Red and green indicate the up- and down-regulated DEGs, respectively, in response to drought stress. Data are presented as means \pm SD ($n=5$); * Significance levels in comparisons with M79 were determined by t-tests (* $P < 0.05$, ** $P < 0.01$).

