

1 **Novelty statement:**

2 We found that drought and ABA stress induced the transcription of CC-type glutaredoxins (GRXs)  
3 in cassava leaves. Ectopic expression of one of them, *MeGRX232* in *Arabidopsis* affected the  
4 sensitivity to abscisic acid (ABA) and mannitol, and caused drought hypersensitivity by  
5 impairment of ABA-dependent stomatal closure.

6

7 **Title of the article:**

8 A cassava drought inducible CC-type glutaredoxin, *MeGRX232*, negatively regulates  
9 drought tolerance in *Arabidopsis* by inhibition of ABA-dependent stomatal closure

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11 Meng-Bin Ruan<sup>1,2,†,\*</sup>, Yi-Ling Yang<sup>3,†</sup>, Xin Guo<sup>4,†</sup>, Xue Wang<sup>1,2</sup>, Bin Wang<sup>4</sup>, Xiao-Ling Yu<sup>1,2</sup>,  
12 Peng Zhang<sup>5</sup>, Ming Peng<sup>1,2,\*</sup>

13 **Institution address:**

14 <sup>1</sup>Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural  
15 Sciences, Haikou, 571101, China.

16 <sup>2</sup>Key Laboratory of Biology and Genetic Resources of Tropical Crops, Ministry of Agriculture, H  
17 aikou, 571101, China.

18 <sup>3</sup>Crops Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, 510640,  
19 China.

20 <sup>4</sup>Huazhong Agricultural University, Wuhan, 430070, China.

21 <sup>5</sup>National Key Laboratory of Plant Molecular Genetics, CAS Center for Excellence in Molecular  
22 Plant Sciences, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological  
23 Sciences, Chinese Academy of Sciences, Shanghai, 200032, China.

24 <sup>†</sup>These authors contributed equally to this work.

25 <sup>\*</sup>Corresponding author; e-mail: [ruanmengbin@itbb.org.cn](mailto:ruanmengbin@itbb.org.cn) or [pengming@itbb.org.cn](mailto:pengming@itbb.org.cn); fax:  
26 860-898-66890981

27 **Authors to whom correspondence to be addressed:**

28 Name: Ming Peng

29 Address: Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical  
30 Agricultural Sciences, Haikou, China.

31 Name: Yi-Ling Yang, E-mail: [luckystar1221@163.com](mailto:luckystar1221@163.com)

32 Name: Xin Guo, E-mail: [guoxinyxh@gmail.com](mailto:guoxinyxh@gmail.com)

33 Name: Xue Wang, E-mail: [hnwxue@sina.com](mailto:hnwxue@sina.com)

34 Name: Bin Wang, E-mail: [wangbinatt@126.com](mailto:wangbinatt@126.com)

35 Name: Xiao-Ling Yu, E-mail: [lingdang01@126.com](mailto:lingdang01@126.com)

36 Name: Peng Zhang, E-mail: [zhangpeng@sibs.ac.cn](mailto:zhangpeng@sibs.ac.cn)

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38 Running title: *MeGRX232* negatively regulates drought tolerance in *Arabidopsis*.

39 Keywords: Cassava (*Manihot esculenta*), CC-type glutaredoxin, drought tolerance, stomatal  
40 closure, *Arabidopsis*

41

42 **Abstract**

43 CC-type glutaredoxins (GRXs) are a land plant-specific GRX subgroup that evolved from CGFS  
44 GRXs, and participate in organ development and stress responses through the regulation of  
45 transcription factors. Here, genome-wide analysis identified 18 CC-type GRXs in the cassava  
46 genome, of which six (*MeGRX058*, *232*, *360*, *496*, *785*, and *892*) were induced by drought and  
47 ABA stress in cassava leaves. Furthermore, we found that overexpression of *MeGRX232* results  
48 in drought hypersensitivity in soil-grown plants, with a higher water loss rate, but with increased  
49 tolerance of mannitol and ABA in *Arabidopsis* on the sealed agar plates. The ABA induced  
50 stomatal closure is impaired in *MeGRX232-OE Arabidopsis*. Further analysis reveals that the  
51 overexpression of *MeGRX232* leads to more ROS accumulation in guard cells. *MeGRX232* can  
52 interact with TGA5 from *Arabidopsis* and MeTGA074 from cassava *in vitro* and *in vivo*. The  
53 results of microarray assays show that *MeGRX232-OE* affected the expression of a set of drought  
54 and oxidative stress related genes. Taken together, we demonstrated that CC-type GRXs involved  
55 in ABA signal transduction and play roles in response to drought through regulating stomatal  
56 closure.

57 **Keywords:** Cassava (*Manihot esculenta*), drought response, CC-type glutaredoxin, stomatal  
58 closure

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## 61 **Introduction**

62 As a tropical crop, cassava (*Manihot esculenta*) evolved different response to drought stress, such  
63 as quick stomata closure, reduction of photosynthetic proteins levels and photosynthetic capacity,  
64 induction of senescence in older leaves, and size reduction of leave epidermal cells (Alves and  
65 Setter 2004; Zhao *et al.* 2014). Some cassava cultivars display faster senescence in older leaves  
66 than the others (Zhao *et al.* 2014). Senescence in cassava is partly controlled by a reactive  
67 oxygen species (ROS) and ethylene signaling (Liao *et al.* 2016b). Increasing the ROS  
68 scavenging ability in cassava delay leaf senescence under drought stress (Liao *et al.* 2016b; Xu *et*  
69 *al.* 2013a, 2013b). It is therefore necessary to analyze genes involved in these pathways for a  
70 deeper functional characterization.

71 Glutaredoxin (GRX) is one of the most important protein modification system in plants (Rouhier  
72 *et al.* 2006). The glutathione/GRX (GSH/GRX) system is essential for redox homeostasis and  
73 ROS signal in plant cells (Meyer *et al.* 2012). GRX target proteins are involved in all aspects of  
74 plant growth, including basal metabolism, iron/sulfur cluster formation, development, adaptation  
75 to the environment, and stress responses (Meyer *et al.* 2012). GRX are in particular studied for  
76 their involvement in oxidative stress responses (Carroll *et al.* 2006; Kanda *et al.* 2006; Meyer *et*  
77 *al.* 2012). GRXs are classified in five subgroups, among which CC-type GRXs are a  
78 plant-specific subgroup, also known as the ROXY family in *Arabidopsis* (Xing *et al.* 2005;  
79 Ziemann *et al.* 2009). CC-type GRXs likely evolved from the CPYC subgroup and expanded  
80 during land plant evolution (Ziemann *et al.* 2009). There are only two CC-type GRXs in the  
81 basal land plant *Physcomitrella*, but between 15 and 24 members in land plants such as rice,  
82 *Arabidopsis*, *Vitis* and *Populus* (Ziemann *et al.* 2009). However, the number of CC-type GRXs in  
83 cassava remains unclear.

84 CC-type GRXs are characterized by the presence of a redox site CC\*(C/S/G) as well as their  
85 disulfide reductase activity that uses glutathione as cofactor (Couturier *et al.* 2010). The first  
86 CC-type GRX has been identified as a regulator of petal development (Xing *et al.* 2005).  
87 However, CC-type GRXs are also involved in jasmonic acid (JA)/ethylene mediated biotic stress  
88 responses through the interaction with TGA factors in *Arabidopsis* (La Camera *et al.* 2011; Wang  
89 *et al.* 2009; Zander *et al.* 2012). Moreover, some CC-type GRXs are critical in limiting basal and

90 photo-oxidative stress-induced ROS production (Laporte *et al.* 2012). Thus, CC-type GRXs may  
91 play a key role in the crosstalk between ROS and ethylene. CC-type GRX members are also  
92 involved in organ development and biotic stress responses in other plants (El-Kereamy *et al.*  
93 2015; Garg *et al.* 2010; Gutsche *et al.* 2015; Hong *et al.* 2012; R. Sharma *et al.* 2013; Wang *et al.*  
94 2009).

95 During evolution, CC-type GRXs might have gained new functions in high land plants (Rouhier  
96 *et al.* 2006; Wang *et al.* 2009; Ziemann *et al.* 2009). Although several CC-type GRXs have been  
97 characterized in *Arabidopsis* and rice (Gutsche *et al.* 2015; Wang *et al.* 2009), no previous work  
98 profiled them in cassava. As a tropical crop, cassava evolved to be tolerant to intermittent  
99 drought, but hypersensitive to cold (An *et al.* 2012; Xia *et al.* 2014; Zeng *et al.* 2014; Zhao *et al.*  
100 2014). Genome-wide analysis base on high-quality sequencing data and EST predict the presence  
101 of many genes related to abiotic stress responses in cassava (An *et al.* 2012; W. Hu *et al.* 2015b;  
102 W. Hu *et al.* 2015a; Wei Hu *et al.* 2016; Lokko *et al.* 2007; Sakurai *et al.* 2007; Xia *et al.* 2014;  
103 Zeng *et al.* 2014; Zhao *et al.* 2014). Therefore, it is now possible to analyze the expression  
104 pattern of a whole gene family to identify drought stress related members, and to characterize  
105 their functions during abiotic stresses response (W. Hu *et al.* 2015b; W. Hu *et al.* 2015a; Wei Hu  
106 *et al.* 2016; Xia *et al.* 2014; Zeng *et al.* 2014; Zhao *et al.* 2014).

107 In this study, we performed computational and phylogenetic analyses to identify plant specific  
108 CC-type GRXs in the cassava genome. In total, we identified 18 CC-type GRXs in cassava. Due  
109 to the lack of characterization of the function of CC-type GRXs during drought responses in  
110 cassava, we chose this subgroup for systematic and functional analysis. Based on our previously  
111 reported transcriptomic data of cassava cultivars (Wei Hu *et al.* 2016), we identified six CC-type  
112 GRXs (*MeGRX058*, *232*, *360*, *496*, *785*, *892*) that responded to drought using qPCR analysis in  
113 cassava leaves. To investigate potential CC-type GRX-mediated drought stress responsive  
114 pathways, we examined the expression levels of these six genes in leaves under ABA treatment.  
115 Our results showed that CC-type GRXs may functions as component in drought stress in an  
116 ABA-dependent pathway in both Arg7 and SC124 plants. Overexpression of *MeGRX232* induces  
117 insensitivity to ABA and mannitol, and confers drought susceptibility in *Arabidopsis* in  
118 soil-grown condition by inhibiting ABA-dependent stomatal closing. The overexpression of

119 *MeGRX232* caused more ROS accumulation in guard cells. In addition, gene expression analysis  
120 reveals that *MeGRX232* regulated a set of oxidative stress related genes in *Arabidopsis*.

## 121 **Materials and Methods**

### 122 **Plant materials**

123 Stems of cassava Arg7 and SC124 were cultured in pots (36 cm in diameter × 30 cm in height)  
124 containing well-mixed soil (soil: vermiculite: pellets, 1:1:1) for 80 days in greenhouse at the  
125 Institute of Tropical Bioscience and Biotechnology (HaiKou, China). Wild type (Col-0)  
126 *Arabidopsis* plants for transformation were grown in 12 hrs light/12 hrs dark at 20-23 °C until the  
127 primary inflorescence was 5-15 cm tall and a secondary inflorescence appeared at the rosette.

### 128 **Drought and ABA treatments**

129 For drought treatment, watering was interrupted for 14 days. Leaves were collected from three  
130 Arg7 and SC124 plants eight or 14 days from the start of the drought treatment and 24 hours  
131 after re-water at the end of the treatment. Plants watered as normal were used as control. Three  
132 leaves (the second, third and fourth leaf from the top of plant) from each plant were collected.  
133 For ABA treatment, mature leaves with petiole were excised from Arg7 and SC124 plants,  
134 treated by dipping the petioles in water with 20 μM ABA respectively for 30 min before  
135 collection.

### 136 **Bioinformatics analysis**

137 The protein sequences of cassava GRXs were predicted using a TBLASTN search against the  
138 cassava genome database in Phytozome (<https://phytozome.jgi.doe.gov>, *Manihot esculenta* v4.1)  
139 with the protein sequence from *Arabidopsis* GRXs as a query. All *Arabidopsis* GRX protein  
140 sequences were downloaded from GenBank (Supplementary Table S1). Multiple sequence  
141 alignments were conducted using ClustalW (Thompson *et al.* 1994). An unrooted phylogenetic  
142 tree showing cassava GRXs and *Arabidopsis* GRX family was generated via the neighbor joining  
143 method using MEGA5.0 (Tamura *et al.* 2011). Editing of aligned sequences of cassava CC-type  
144 GRXs was performed using AlignX (Vector NTI suite 10.3, Invitrogen).

### 145 **Transcriptome data analysis**

146 For drought responsive CC-type GRXs identification, we used our previously reported RNA-seq

147 data (Wei Hu *et al.* 2016). We used data that included two tissues (leaf and root) under drought  
148 treatment (12 day after water withholding) and a control. Gene expression levels were  
149 normalized using FPKM. We selected CC-type GRX genes, and generated a heat map and  
150 hierarchical clustering using Cluster 3.0. RNA-seq datasets are available at NCBI and the  
151 accession numbers are listed in [Supplementary Table S2](#).

#### 152 **Quantitative real-time PCR (qPCR) analysis**

153 Total RNA was isolated from leaves of cassava plant used a modified CTAB method. cDNA  
154 synthesis was performed with FastQuant RT Kit (TIANGEN). Expression analysis of CC-type  
155 GRXs in cassava leaves after drought and exogenous ABA treatment were performed by qPCR  
156 with gene-specific primers ([Supplementary Table S3](#)). For qPCR analysis in transgenic plants,  
157 total RNA was isolated from wild type, three independent *MeGRX785-OE* and *MeGRX058-OE*  
158 transgenic lines, respectively. All qPCR reactions were carried out in triplicates, with SYBR®  
159 Premix Ex Taq™ II Kit (Takara) on StepOne™ Real-Time PCR system (Applied Biosystems),  
160 and the comparative  $\Delta\Delta$ CT method employed to evaluate amplified product quantities in the  
161 samples.

#### 162 **DNA constructs, protein subcellular localization, and *Arabidopsis* transformation**

163 Full-length coding sequence without stop-codon of *MeGRX232* was isolated from cDNA of  
164 drought stressed leaves by RT-PCR. Fragments were identified by sequencing and fused to *GFP*  
165 behind the *CaMV 35S* promoter in the modified plant expression vector *pGI300*  
166 (*eGFP:pCAMBIA1300*) to make *P35s:MeGRX232:GFP*. The *P35s:MeGRX232:GFP* and vector  
167 construct were transferred into *Agrobacterium LBA4404* respectively. Leaves from  
168 four-week-old *Nicotiana benthamiana* plants were transformed by infiltration of *Agrobacterium*  
169 cells ( $OD_{600}=1.2$ ) harboring appropriate DNA constructs using 5-mL syringe without needle. The  
170 *pGI300* vector (*GFP*) and *P35s:MeHistone3:GFP* (*H3:GFP*) were used as the positive controls.  
171 After three days, infiltrated *N.benthamiana* leaves were imaged for reconstitution of GFP  
172 fluorescence by confocal laser scanning microscope (Olympus FluoView FV1100). *Arabidopsis*  
173 was transformed using the DIP method (Clough and Bent 1998) with *A. tumefaciens* strain  
174 *LBA4404* carrying the DNA constructs *P35s:MeGRX232:GFP*. More than three homozygote lines  
175 of each construct were selected for further phenotype analysis.

176 **ABA and mannitol stress tolerance assays of transgenic *Arabidopsis***

177 To study the response of *MeGRX232-OE* transgenic plants to ABA, 5-d-old seedlings were  
178 transferred to MS medium contained with 0  $\mu$ M (mock) and 5  $\mu$ M ABA grown for 10 days. For  
179 mannitol treatment, 5-d-old seedlings were transferred to MS medium contained with 0 mM  
180 (mock) and 250 mM D-mannitol grown for 15 days. Rosette diameter, primary root length and  
181 later root number were measured. The transgenic plant that contained *pG1300* vector was used as  
182 control.

183 **Drought stress tolerance assays of transgenic *Arabidopsis***

184 Post-germinated seedlings of *MeGRX232-OE* and vector transgenic plants were grown in soil in  
185 one pot for 15 days under normal conditions. For drought stress, the plants were treated by water  
186 withholding for 21 days, then re-watering. Survival rates have been calculated at 5 days after  
187 re-watering. Lipid peroxidation in transgenic *Arabidopsis* leaves was measured in terms of MDA  
188 in the samples as described in reference (Xu *et al.* 2013b) during drought stress. For water loss  
189 rate measurement, excised leaves from 28-d-old unstressed transgenic plants were kept on filter  
190 paper at room temperature. Their weight was measured after every 1 hour, up to 7 hour followed  
191 by calculation of water loss percentage.

192 **Stomatal aperture assays**

193 ABA-induced stomatal closing assays were performed using fully expanded healthy leaves from  
194 28-d-old transgenic plants as previous report (G. Sharma *et al.* 2015). Excised leaves were  
195 incubated in stomatal opening solution (10mM KCl, 100 $\mu$ M CaCl<sub>2</sub>, and 10mM MES, pH 6.1)  
196 for 2 hours followed by incubation in stomatal opening solution supplemented with varying ABA  
197 concentrations (0 $\mu$ M, 0.1 $\mu$ M, 1 $\mu$ M, 10 $\mu$ M) for 2 hrs. With the help of forceps, epidermal peels  
198 from the abaxial surface of treated leaves were peeled off and mounted on glass slides covered  
199 with coverslips followed by observation under Zeiss Scope A1 Imaging System. The ratio of  
200 width and length of stomata was measured using ZEN software. Approximately 30 guard cells  
201 were taken into account in measuring aperture in each sample.

202 **Determination of ROS accumulation**

203 H<sub>2</sub>O<sub>2</sub> was visualized by staining with DAB according to the reference (Thordal-Christensen *et al.*  
204 1997). The untreated leaves of *MeGRX232-OE* and vector *Arabidopsis* were infiltrated with 2

205 mL of DAB solution ( $1\text{mg mL}^{-1}$  DAB, pH 3.8) in Eppendorf tube for 12 hours. Then the leaves  
206 were immersed in 95% (w/v) boiling ethanol for 10 min to decolorize the chloroplast. For the  
207 ROS accumulation assay in guard cells, prepared epidermal peels with or without  $\text{H}_2\text{O}_2$ , ABA  
208 treatment were load with  $50\ \mu\text{M}$  2,7-dichlorofluorescein diacetate (DCFH-DA; Beyotime  
209 Biotechnology) for 30 min. The fluorescence was recorded by confocal microscopy with  
210 excitation at 546 nm. The fluorescence intensity was calculated from at least 30 guard cells by  
211 FV10-ASW (Olympus).

### 212 **Transactivation analysis and yeast two hybrid assay**

213 Before analyzing the interaction between MeGRX232 and TGA factors, an autonomous  
214 transactivation analysis has been performed in yeast strain Y187. The MeGRX232 was in frame  
215 fused to GAL4 BD (binding domain) in *pGBKT7*, and then transformed into yeast Y187.  
216 Because MeGRX232 shows “autonomous transactivation” in yeast, a MeGRX232 GSH binding  
217 site mutant *MeGRX232mP<sub>65</sub>G<sub>75</sub>* was produced by replacing P<sub>65</sub>AVFIGGILVG<sub>75</sub> to  
218 A<sub>65</sub>AVFIGGILVA<sub>75</sub>. Next, for identification the interaction between MeGRX232 and TGA  
219 factors, a yeast two-hybrid assay has been performed in yeast strain Y187 based on the  
220 Matchmaker™ GAL4 two-hybrid system 3 manual (Clontech). The *MeGRX232* GSH binding  
221 site mutant DNA construct *MeGRX232mP<sub>65</sub>G<sub>75</sub>:pGBKT7* was used as bait. The cDNA sequences  
222 of TGA factors from *Arabidopsis* and cassava were introduced into the *pGADT7*, respectively in  
223 frame fused to GAL4 activate domain (AD). The *MeGRX232mP<sub>65</sub>G<sub>75</sub>:pGBKT7* and  
224 *TGA:pGADT7* constructs were pairwise co-transformed into yeast strain Y187. The presence of  
225 transgenes was confirmed by growth on SD/ -Trp/-Leu plates. Interactions between two proteins  
226 were checked by examining  $\beta$ -galactosidase activity as the manual instructed.

### 227 **Bimolecular fluorescence complementation analysis**

228 To confirm the interactions between MeGRX232 and TGA2/MeTGA074 factors, a bimolecular  
229 fluorescence complementation assay has been performed by *N.benthamiana* transient system as  
230 previously report (Pazmino *et al.* 2011). The full-length coding sequence without stop-codon of  
231 *MeGRX232* was in frame fused to N- or C-terminus to yellow fluorescent protein (YFP)  
232 fragments (YN/YC) respectively to produce *Pro35S:MeGRX232:YN:pBiFC* and  
233 *Pro35S:MeGRX232:YC:pBiFC*. The full-length coding sequence without stop-codon of *TGA2*

234 and *MeTGA074* were in frame fused to YC or YN respectively to produce  
235 *Pro35S:TGA2:YC:pBiFC*, *Pro35S:TGA2:YN:pBiFC*, *Pro35S:MeTGA074:YC:pBiFC*, and  
236 *Pro35S:MeTGA074:YN:pBiFC*. The resulting constructs were then introduced into *A.*  
237 *tumefaciens* LBA4404 strains. Constructs were pair-wise transiently expressed in epidermal  
238 cells of tobacco leaves. 3-5 days after transient co-expression of protein pairs, reconstitution of  
239 YFP fluorescence was examined by confocal microscopy using GFP filter. Then the assays were  
240 performed as the method of proteins subcellular localization described. As positive controls,  
241 green fluorescent protein (eGFP) was tagged to the C-terminus of TGA2 and MeTGA074  
242 respectively, transiently expressed in tobacco leaves.

### 243 **Microarray analysis of transgenic *Arabidopsis***

244 Microarray experiments were conducted using Affymetrix *Arabidopsis* ATH1 Genome Array.  
245 Experiments were performed as three biological repeats using cDNAs prepared independently  
246 from three individual homozygote lines of *MeGRX323-OE Arabidopsis* that have been  
247 phenotypic analyzed in plant growth. The transgenic *Arabidopsis* plants that carried the *pGI300*  
248 vector were used as control. The experiments and data analysis were performed under the  
249 instruction of Affymetrix. Total microarray data have been deposited in the NCBI GEO database  
250 with the accession number: GSE81136. Gene ontology (GO) analyses for significant enrichments  
251 of various categories (**Supplementary Table S4**) were performed using MAS 3.0  
252 (<http://bioinfo.capitalbio.com/mas3/>). The Venn diagram is created by online tool  
253 (<http://bioinformatics.psb.ugent.be/webtools/Venn/>).

## 254 **Results**

### 255 **Phylogenetic, gene structure and conserved motif analysis of cassava CC-type GRXs**

256 We predicted a total of 38 putative GRX proteins in the cassava genome using the *Arabidopsis*  
257 ROXYs in a BLAST search against the genome of the cassava cultivar AM560  
258 (<https://phytozome.jgi.doe.gov>, *Manihot esculenta* v4.1). To understand the relationship between  
259 GRX proteins in cassava and *Arabidopsis*, we built a neighbor-joining phylogenetic tree using  
260 MEGA5.0 on the basis of the protein sequences. The results showed that many cassava GRXs  
261 were highly similar to their *Arabidopsis* counterparts (**Fig. 1**). We found that the CC-type  
262 subgroup had the most members among the GRX subgroups in cassava. All putative cassava

263 GRXs are classified in five subgroups as in *Arabidopsis* and rice (Meyer *et al.* 2012; Rouhier *et*  
264 *al.* 2006). CC-type GRXs are plant-specific GRXs, derived from the CPYC subgroup and  
265 expanded from basal to high land plant (Ziemann *et al.* 2009). Our result also demonstrated that  
266 all cassava CC-type GRXs evolved from three cassava CPYC GRXs (Fig. 1). Our analysis  
267 predicted 18 full-length CC-type GRX members of cassava (Table. 1), less than 21 of  
268 *Arabidopsis* (Xing and Zachgo 2008). Cassava CC-type GRX genes we represent on nine  
269 chromosomes (Table. 1) and generally possess no intron (Fig. 2A). Almost all of cassava  
270 CC-type GRXs shared a redox site in N-terminus, an L\*\*LL protein binding motif, and an  
271 ALWL motif at the C-terminus. However, two members, MeGRX954 and MeGRX956, did not  
272 present ALWL motif at C-terminus (Fig. 2B). CC-type GRXs have a distinctive conserved  
273 CC(M/L)(C/S) redox site motif in *Arabidopsis*, whereas this motif is extends to  
274 C(C/G/F/Y/P)(M/L)(C/S/I/A) in rice (Rouhier *et al.* 2006; Wang *et al.* 2009; Xing and Zachgo  
275 2008; Ziemann *et al.* 2009). Most of cassava CC-type GRXs shared a distinctive CCM(C/S)  
276 redox site (Fig. 2B). However, this motif (CDMC) was extended in two CC-type GRXs  
277 (MeGRX785 and MeGRX892) in cassava.

#### 278 **Identification of drought- and ABA-inducible CC-type GRX genes in cassava cultivars**

279 As a tropical crop, cassava is well adapted to interment drought, but hypersensitive to cold (An *et*  
280 *al.* 2012; Okogbenin *et al.* 2013; Xia *et al.* 2014; Zeng *et al.* 2014; Zhao *et al.* 2014). Previous  
281 studies used high quality RNA-seq and iTRAQ-based proteomic datasets to examine genes  
282 responsive to drought resistance in cassava (W. Hu *et al.* 2015b; W. Hu *et al.* 2015a; Xia *et al.*  
283 2014; Zeng *et al.* 2014; Zhao *et al.* 2014). To investigate the role of CC-type GRXs in response  
284 to drought in cassava, we used eight arrays of our previously reported transcriptomic data (Wei  
285 Hu *et al.* 2016). RNA-seq datasets are available at NCBI and the accession numbers are listed in  
286 Table S4. Hierarchical expression clustering (FPKM) showed that the CC-type GRX expression  
287 patterns in response to drought in cassava cultivars Arg7 and SC124 grouped in three clusters  
288 (Fig. 3A). Cluster III included CC-type GRXs induced by drought only in leaves. To detail the  
289 expression of CC-type GRXs in the drought response in cassava leaves, we performed a qPCR  
290 analysis to investigate the expression changes of genes in cluster III under drought and re-water  
291 treatments. For this analysis, we selected six drought-inducible CC-type GRXs (*MeGRX058*, 232,

292 360, 496, 785, and 892) from cluster III on the basis of RNA-seq. We collected leaves from plants  
293 of two cassava cultivars under drought stress for eight (D8) or 14 days (D14), and D14 plants  
294 re-water 24 hours later (RW). We used leaves from well-water cassava plants as control (DC). As  
295 expected, drought stress up-regulated the expression of all six CC-type *GRXs* in both Arg7 and  
296 SC124 leaves (Fig. 3B, C). It indicates that CC-type *GRXs* may play conserved roles in drought  
297 response of cassava different cultivars. The expression of *MeGRX360*, *MeGRX785*, and  
298 *MeGRX892* was the highest in D14 plants. In contrast, the expression of *MeGRX058* and  
299 *MeGRX232* was the highest in D8 plants. These results indicated that CC-type *GRXs* may  
300 regulate drought response through different mechanisms in cassava.

301 The phytohormone ABA regulates many important processes in plants, especially in relation to  
302 environmental stress responses (Nakashima and Yamaguchi-Shinozaki 2013; Sharp and LeNoble  
303 2002; Wilkinson and Davies 2002). Numerous drought-responsive genes have been described as  
304 ABA-inducible (Nakashima and Yamaguchi-Shinozaki 2013). A conserved cis-element,  
305 designated the ABA-responsive element (ABRE), is present in the promoter region of most  
306 ABA-inducible genes (Fujita *et al.* 2011). Analyzing the 1.5 kb up-stream region of our six  
307 drought responsive CC-type *GRXs*, we found ABREs in the promoter region of *MeGRX232*,  
308 *MeGRX360*, *MeGRX496*, *MeGRX785*, and *MeGRX892*. Thus, we inferred that these genes may  
309 respond to ABA. We performed qPCR analysis to confirm this hypothesis. Interestingly, ABA  
310 application up-regulated the expression of these six CC-type *GRXs* in both Arg7 and SC124  
311 leaves (Fig. 4). The data suggest these CC-type *GRXs* may play roles in ABA signal transduction  
312 pathways in cassava.

### 313 **Overexpression of *MeGRX232* in *Arabidopsis* confers seedling development insensitive to** 314 **ABA and affects root architecture under mannitol stress**

315 Most *Arabidopsis* CC-type *GRX* proteins localize in the cytosol or in the nucleus (Couturier *et al.*  
316 2011; Rouhier *et al.* 2007; Xing *et al.* 2005; Xing and Zachgo 2008). The sub-cellular  
317 localization of these proteins is essential for their function (Li *et al.* 2009). We tagged the cDNA  
318 of our six drought-responsive CC-type *GRXs* with GFP at the C-terminus to analyze their  
319 cellular localization (Fig. S1). We imaged our *MeGRX:GFP* fusions in transiently transformed  
320 *Nicotiana benthamiana* leaf epidermis, detecting fluorescence in both the cytosol and the

321 nucleus (Fig. S1). In *Arabidopsis*, the nuclear localization of ROXY1 is required for its function  
322 in petal development (Li *et al.* 2009). Thus, the sub-cellular indicates that these  
323 drought-inducible CC-type GRXs may function in the nucleus during stress responses.

324 To investigate the functions of drought-inducible MeGRXs in plant, we heterologous  
325 overexpressing four of these genes that is *MeGRX058*, *232*, *360*, and *785* in *Arabidopsis* (Fig.  
326 S2). Overexpression of *MeGRX785* caused ABA and mannitol susceptibility of seed germination  
327 in *Arabidopsis*, while in contrast, *MeGRX232-OE* plants was insensitive to ABA and mannitol  
328 (Fig. S2). Three independent lines of *MeGRX232-OE* transgenic *Arabidopsis* have been used in  
329 ABA and osmotic stress analyses. And the transgenic *Arabidopsis* that contained vector (*pG1300*)  
330 were used as control. We found that ABA did not affect the seed germination and seedling  
331 development of *MeGRX232-OE* transgenic plants (Fig. S3). We infer that overexpression of  
332 *MeGRX232* may cause ABA insensitivity in *Arabidopsis*. Next, 5-day-old seedlings of  
333 *MeGRX232-OE* transgenic *Arabidopsis* were grown on MS medium supplement with 0 $\mu$ M ABA  
334 (mock) or 5 $\mu$ M ABA, respectively. After 10 days grown on mock medium, no visible phenotypic  
335 differences between *MeGRX232-OE* and vector plants were observed (Fig. 5A). On  
336 ABA-supplement medium, the growth of vector plants was significantly inhibited, while the  
337 growth of *MeGRX232-OE* plants were less inhibited (Fig. 5A). The rosette diameter of  
338 *MeGRX232-OE* plants was ~50% higher than that of vector plants (Fig. 5B). Also, the primary  
339 root of *MeGRX232-OE* plants was ~48% longer than that of vector plants (Fig. 5C). Our data  
340 address the issue that overexpression of *MeGRX232* caused ABA insensitivity in transgenic  
341 *Arabidopsis*.

342 To analysis mannitol tolerance of transgenic *Arabidopsis*, 5-day-old seedlings of transgenic  
343 plants were grown on MS medium supplement with 0 mM (mock) and 250 mM D-mannitol.  
344 After 15 days, the *MeGRX232-OE* and vector plants show no significant difference on mock  
345 medium (Fig. 5D). While root system of *MeGRX232-OE* plants were dramatically affected by  
346 250 mM D-mannitol (Fig. 5D). The rosette diameter of *MeGRX232-OE* plants was higher than  
347 that of vector plants, ranged from ~25% to ~50% (Fig. 5E). However, the primary root  
348 elongation in *MeGRX232-OE* plants decreased ~36% compared with that in vector plants (Fig.  
349 5F). Under mannitol stress, *MeGRX232-OE* plants have more lateral roots than vector plants. We  
350 found that the lateral root number increased ~4 fold in *MeGRX232-OE* plants, compared to

351 vector plants (Fig. 5G). Collectively, the data indicate that overexpression of *MeGRX232*  
352 affected root architecture in *Arabidopsis* under mannitol stress.

353 ***MeGRX232* confers drought hypersensitivity in soil-grown plants via impairing**  
354 **ABA-dependent stomatal closing**

355 To further investigate the role of *MeGRX232* in drought tolerance, *MeGRX232-OE* and vector  
356 plants grown in soil were used. The vector and three independent lines of *MeGRX232-OE* plants  
357 were grown in one pot under normal conditions. 21-day-old plants in soil have been treated by  
358 water withholding (Fig. 6A). When exposed to water deficit for 21 days, all treated plants  
359 displayed severe wilting (Fig. 6B). After drought treatment, plants were re-watered and cultured  
360 for five more days. The *MeGRX232-OE* lines displayed a significantly lower survival rate than  
361 vector plants (Fig. 6C). It indicates that overexpression of *MeGRX232* caused drought  
362 hypersensitivity in *Arabidopsis* under soil culture conditions. This might result from a more rapid  
363 loss of water in *MeGRX232-OE* plants than in vector plants (Fig. 6D).

364 For water loss mainly depends on stomatal regulation, we infer that *MeGRX232* affect stomatal  
365 density or movement therefore increase water loss in transgenic *Arabidopsis*. However, there are  
366 no obviously stomatal density differences between vector and *MeGRX232-OE* plants (Fig. 6E).  
367 But the stomata apertures (width/length) of *MeGRX232-OE* plants are higher than that of vector  
368 plants (Fig. 6F), suggesting *MeGRX232* may affect stomatal closure during drought stress. Thus,  
369 we performed ABA-induced stomatal closing assays to test our hypothesis. Without ABA (mock),  
370 the *MeGRX232-OE* lines showed similar stomatal aperture as vector plants (Fig. 6G, H). This  
371 indicates that *MeGRX232* overexpressing did not affect stomatal aperture in *Arabidopsis* under  
372 normal conditions. When treated with 0.1  $\mu$ M ABA, the stomata of vector plants displayed a  
373 significant closing, whereas the stomata of *MeGRX232-OE* lines open widely as that in mock  
374 (Fig. 6G, H). Furthermore, with increasing concentrations of ABA to 1  $\mu$ M and 10  $\mu$ M, the  
375 stomatal aperture of vector plant exhibited dramatically reducing. On the contrary, the  
376 *MeGRX232-OE* lines performed relatively less closing of stomata (Fig. 6G, H). It suggests that  
377 overexpression of *MeGRX232* resulted impairment of ABA-dependent stomatal closing.  
378 Therefore, the more rapid water loss observed in *MeGRX232-OE* lines should mainly be ascribed  
379 to impaired stomatal closure.

380 **Overexpression of *MeGRX232* caused more ROS accumulation in guard cells**

381 CC-type GRX GRXS13 is critical for ROS production during photooxidative stress (Laporte *et*  
382 *al.* 2012). To determine whether *MeGRX232* affect cell redox homeostasis, we measured the  
383 MDA content in stress transgenic plants. We found a higher MDA content in *MeGRX232-OE*  
384 plants than in vector plants (Fig. 7A). Furthermore, we found more ROS signals have been  
385 stained by DAB in *MeGRX232-OE* plants under normal conditions (Fig. 7B). Together, these  
386 data indicate the possibility that overexpression of *MeGRX232* caused more ROS accumulation  
387 in transgenic *Arabidopsis* leaves.

388 As H<sub>2</sub>O<sub>2</sub> promotes leaf stomatal closure acting downstream of ABA (Pei *et al.* 2000). H<sub>2</sub>O<sub>2</sub>  
389 accumulation in guard cells was measured by a fluorescence dye, 2', 7'  
390 '-dichlorodihydrofluorescein diacetate (DCFH-DA) under exogenous ABA and H<sub>2</sub>O<sub>2</sub> treatment.  
391 The *MeGRX232-OE* guard cells show obvious H<sub>2</sub>O<sub>2</sub> accumulation, in contrast, no H<sub>2</sub>O<sub>2</sub>  
392 accumulation in vector plants guard cells have been detected (Fig. 7C, D). After treated by H<sub>2</sub>O<sub>2</sub>  
393 for three hours, both *MeGRX232-OE* and vector plant guard cells show significant H<sub>2</sub>O<sub>2</sub>  
394 accumulation, but the H<sub>2</sub>O<sub>2</sub> levels in *MeGRX232-OE* guard cells are much higher than that in  
395 vector plant guard cells (Fig. 7C, D). After treated for three hours, more H<sub>2</sub>O<sub>2</sub> have been induced  
396 by ABA in *MeGRX232-OE* guard cells compared to vector plant guard cells, and the H<sub>2</sub>O<sub>2</sub> have  
397 been accumulated in membrane of *MeGRX232-OE* guard cells (Fig. 7C, D). Thus, we can  
398 conclude that overexpression of *MeGRX232* in *Arabidopsis* caused more ROS production in  
399 guard cells.

400 ***MeGRX232* is interacts with TGA5 and MeTGA074**

401 Most CC-type GRXs play roles in organ development and plant defense via interaction with  
402 TGA factors (Hong *et al.* 2012; Li *et al.* 2009; Li *et al.* 2011; Zander *et al.* 2012). TGA factors  
403 regulate genes that involved in both biotic and abiotic stress (Sham *et al.* 2014). It is necessary to  
404 identify the interactors of *MeGRX232* in *Arabidopsis* and cassava. We fused *MeGRX232* with  
405 the GAL4 DNA-binding domain (BD) sequence in *pGBKT7* (Clontech) and then transformed the  
406 result construct into yeast strain Y187. The *pGBKT7* vector was used as negative control.  
407 However, yeast cells harboring *MeGRX232:pGBKT7* activated X- $\alpha$ -gal on SD/-Trp / X- $\alpha$ -gal  
408 medium (Fig. 8A), suggesting *MeGRX232* has transcriptional activation ability. CC-type GRXs

409 need to interact with GSH to catalyze essential biosynthesis reactions by its redox regulation  
410 (Xing and Zachgo 2008). Therefore we created a MeGRX232 mutant by replacing GSH binding  
411 site. As is shown in Fig. 8c, the MeGRX232 mutant did not activated X- $\alpha$ -gal on the medium. It  
412 suggests that the GSH binding site is required for transcriptional activation ability of  
413 MeGRX232. A possible explanation is that MeGRX232 could binding and modify transcription  
414 factor depending on GSH in yeast.

415 Subsequently, six TGA factors including TGA1, TGA3, TGA4, TGA5, TGA6, TGA7 in  
416 *Arabidopsis* and two TGA factors (MeTGA074 and MeTGA813) in cassava have been fused  
417 with GAL4 activation domain (AD) sequence in pGADT7 (Clontech). The resulted AD:TGA  
418 constructs and BD:MeGRX232mP<sub>65</sub>G<sub>75</sub> were pairwise co-transformed into yeast Y187,  
419 respectively. Yeast cells that harboring both AD:TGA and BD:MeGRX232mP<sub>65</sub>G<sub>75</sub> pair plasmids  
420 were grown on SD/ -Trp/ -Leu/ X- $\alpha$ -gal medium (Fig. 8B). The yeast cells containing pairwise  
421 plasmids AD:TGA5/BD:MeGRX232mP<sub>65</sub>G<sub>75</sub> and AD:MeTGA074/BD:MeGRX232mP<sub>65</sub>G<sub>75</sub>  
422 activated X- $\alpha$ -gal. It suggests that MeGRX232 could respectively interact with TGA5 or  
423 MeTGA074.

424 To further investigate the interactions between MeGRX232 and TGA5/MeTGA074 *in planta*, we  
425 employed BiFC. Nuclear fluorescence co-expression of MeGRX232 and TGA5/MeTGA074 has  
426 been detected in epidermal cells (Fig. 8C). The *in planta* nuclear interactions of MeGRX232  
427 with TGA5/MeTGA074 suggest that this CC-type GRX might functions in *Arabidopsis* and  
428 cassava by nuclear interacting with TGA5/MeTGA074. We created a phylogenetic tree based on  
429 TGA protein sequences in *Arabidopsis* and cassava (Fig. S4). We found that MeTGA074 is a  
430 member of clade II TGA, closely to TGA5. Together, our data suggest that MeGRX232 may  
431 regulate drought response via interaction with TGA5/MeTGA074.

### 432 **MeGRX232 regulates a group of genes involved in stress and redox homeostasis in** 433 ***Arabidopsis***

434 To understand the effects of the MeGRX232 overexpressing on gene expression in *Arabidopsis*, a  
435 microarray analysis has been performed using Affymetrix *Arabidopsis* ATH1 Genome Array.  
436 Three independent lines of MeGRX232-OE and vector *Arabidopsis* grew in soil under normal  
437 conditions were used. We found that transcription levels of 2674 genes were altered significantly

438 (with more than a twofold change; P value < 0.05) in *MeGRX232-OE* lines compared with vector  
439 lines under normal conditions (Table S5). 1264 genes were up-regulated, whereas 1410 genes  
440 were down-regulated. The relative expression levels of these genes were shown by the heat map  
441 (Fig. 9A). Gene ontology (GO) analysis results showed that many stress responsive genes have  
442 been affected by *MeGRX232-OE Arabidopsis* (Fig. 9B). 27 more abundant GO categories  
443 ( $q$ -value <  $10^{-5}$ ) including categories that response to abiotic, biotic stress, and phytohormone  
444 stimulus in *MeGRX232-OE Arabidopsis* were exhibited here. Interestingly, nearly two hundred  
445 transcription factors have been affected by *MeGRX232-OE Arabidopsis* (Fig. 9B). We found that  
446 192 oxidative stress-related, 44 drought related, and 53 ABA related genes were significantly  
447 altered in *MeGRX232-OE* plants. Nevertheless, there was three genes overlap among the genes  
448 in response to drought, oxidative stress and ABA (Fig. 9C), indicating that a specific regulatory  
449 mechanism which dependent on ABA-ROS crosstalk conferred by MeGRX232 is presented in  
450 response to drought. Moreover, there was three drought related and three oxidative stress related  
451 genes were overlapped to the genes that involved in JA/ET signal transduction respectively (Fig.  
452 9C), suggesting the MeGRX232 may play roles in drought response depending on regulation of  
453 JA/ET pathway.

## 454 Discussion

455 CC-type GRXs is a land plant specific subgroup of GRX family, derived from the CPYC  
456 subgroup (Ziemann *et al.* 2009). Phylogenetic data (Fig. 1) showed that cassava CC-type GRXs  
457 developed from two CPYC members (cassava4.1\_018918m and cassava4.1\_024091m). During  
458 land plant evolution, CC-type GRXs gained new functions (Rouhier *et al.* 2006; Wang *et al.*  
459 2009; Ziemann *et al.* 2009). In *Arabidopsis*, CC-type GRXs play an important role in petal  
460 development and biotic stress responses (Ndamukong *et al.* 2007; Xing *et al.* 2005). CC-type  
461 GRXs function in the regulation of ethylene responsive genes through the interaction with TGA  
462 factors (Garg *et al.* 2010; La Camera *et al.* 2011; Meyer *et al.* 2012; Ndamukong *et al.* 2007;  
463 Wang *et al.* 2009; Zander *et al.* 2012). The interaction between these proteins and TGA factors  
464 depends on their C-terminal L\*\*LL and ALWL motifs (Li *et al.* 2009; Li *et al.* 2011; Zander *et al.*  
465 2012). These motifs were also present in almost all cassava CC-type GRXs except MeGRX954  
466 and MeGRX956 (Fig. 2).

467 To date, no CC-type GRX has been identified as a regulator of drought response in cassava.  
468 Based on our previously reported RNA-seq data (Wei Hu *et al.* 2016), we identified six drought  
469 stress inducible CC-type GRXs in leaves of cassava using qPCR analysis (Fig. 3). Under drought  
470 stress, ABA concentrations increases and, in turn, induces gene expression (Huang *et al.* 2011).  
471 In our study, ABA stress up-regulated the expression of these six drought-inducible CC-type  
472 GRX genes in leaves of both Arg7 and SC124 plants (Fig. 4). Thus, we believe that CC-type  
473 GRXs might be playing roles in cassava drought response in an ABA-dependent manner.  
474 Therefore, understanding the molecular mechanisms controlled by CC-type GRXs may provide  
475 an effective method for genetic improvement of drought stress resistance for cassava and other  
476 crops. We over-expressed four drought-inducible CC-type GRX genes (*MeGRX058*, *MeGRX232*,  
477 *MeGRX360* and *MeGRX785*) in *Arabidopsis* under control of the *CMV 35S* promoter (Fig. S1).  
478 Indeed, when we compared the seed germination of and *MeGRX232-OE*, *MeGRX785-OE* and  
479 wild type *Arabidopsis* on MS media supplemental with 2 $\mu$ M ABA, significant inhibition of seed  
480 germination was observed in *MeGRX785-OE* only (Fig. S1), and the seedling development of  
481 *MeGRX232-OE Arabidopsis* was insensitive to ABA and mannitol (Fig. 5), indicating that  
482 members of drought-inducible CC-type GRXs may play different roles in responses to drought in  
483 cassava.

484 Recently, several abiotic stress related CC-type GRXs have been identified in *Arabidopsis* and  
485 rice (El-Kereamy *et al.* 2015; Gutsche *et al.* 2015; La Camera *et al.* 2011; Laporte *et al.* 2012; R.  
486 Sharma *et al.* 2013). A splicing variant of *AtGRXS13 (ROXY19)* involved in the protection  
487 against photo oxidative stress in *Arabidopsis* (Laporte *et al.* 2012). Overexpression of the rice  
488 CC-type GRX *OsGRX8*, which is generally induced by auxin and abiotic stress enhances  
489 tolerance to ABA and abiotic stresses in *Arabidopsis* (R. Sharma *et al.* 2013). Here, the  
490 overexpression of *MeGRX232* in *Arabidopsis* caused tolerance to ABA and mannitol on the seal  
491 agar plates (Fig. 5). However, the *MeGRX232-OE Arabidopsis* showed drought hypersensitivity  
492 in soil-grown condition (Fig. 6). Then we found the drought hypersensitivity is partly resulted by  
493 impairment of ABA-dependent stomatal closure in *MeGRX232-OE Arabidopsis* (Fig. 6G).  
494 Therefore, the inverse phenotypes of *MeGRX232-OE Arabidopsis* were possibly ascribed to the  
495 different stress conditions: in sealed agar plates, the transpiration of seedlings is almost

496 negligible (Verslues *et al.* 2006), whereas in open environment, the *MeGRX232-OE* plants show  
497 higher water loss rate (Fig. 6D).

498 Overexpression of *MeGRX232* resulted in hypersensitivity to drought and caused a higher water  
499 loss rate, which led us to suppose that *MeGRX232* is involved in stomatal movement.  
500 ABA-induced stomatal closing was impaired in *MeGRX232-OE* plants (Fig. 6), suggesting that  
501 *MeGRX232* plays a role in the inhibition of stomatal closing. But the main function of  
502 *MeGRX232* in stomatal regulation seems not to only inhibit stomatal closing, because it is  
503 contradictory for cassava to induce the expression of *MeGRX232* under dehydration conditions  
504 to inhibit stomatal closing. During drought treatment, the high ROS accumulation is essential for  
505 abscission zone initial in cassava petiole (Liao *et al.* 2016a). *MeGRX232* was induced by drought  
506 not only in leaves but also in abscission zone (Fig. S5), and overexpression of *MeGRX232*  
507 caused more ROS accumulation in *Arabidopsis* (Fig. 7), suggesting a potentially role of  
508 *MeGRX232* in ROS accumulation during abscission zone formation in cassava. It will be of  
509 interesting to investigate whether this gene is involved in leaf abscission in cassava.

510 The interaction with TGA factors is necessary for CC-type GRX functions in plants (Li *et al.*  
511 2009; Li *et al.* 2011; Zander *et al.* 2012). In *Arabidopsis*, TGA factors have been classified to  
512 five subgroups, clade I, II, III, IV, and V. TGA2, 5, 6 are members of clade II TGAs, which are  
513 essential activators of jasmonic acid/ethylene-induce defense responses (Kesarwani *et al.* 2007;  
514 Stotz *et al.* 2013; Zander *et al.* 2010; Zander *et al.* 2012) and act as a key regulator role in plant  
515 responses of abiotic stresses such as drought, cold, and oxidative stress (Sham *et al.* 2014).  
516 *Arabidopsis* CC-type GRX GRX480/ROXY19 could interact with TGA2, 5, 6 (Zander *et al.*  
517 2012). TGA2 could interact with GRXS13, and act as repressors of GRXS13 expression in  
518 response to biotic stress (La Camera *et al.* 2011). Here, we found that *MeGRX232* nuclear  
519 interacted with TGA5 in *Arabidopsis* and MeTGA074 in cassava respectively (Fig. 8). In  
520 *Arabidopsis*, GRX480 regulated the expression of ERF (Ethylene Response Factor) factors  
521 through interaction with TGA2/5/6 (Ndamukong *et al.* 2007; Zander *et al.* 2012). We found that  
522 nearly two hundred transcription factors including ERF factors had been affected by  
523 *MeGRX232-OE* in *Arabidopsis* (Fig. 9B, Table S5). A nuclear export signal (NES) should be tag  
524 to *MeGRX232* to eliminate its nuclear localization to investigate whether the *MeGRX232*

525 regulated ERFs through nuclear interaction with TGA5 in *Arabidopsis*. The presence of TGA  
526 binding elements (TGACG) in the sequence of MeGRX232 promoter suggests that members of  
527 TGA factors could directly bind to the promoter (Table S6). It will be of interest to further study  
528 the mechanism by which MeGRX232 respond to drought via interaction with MeTGA074 in  
529 cassava.

530 In summary, we demonstrate that *MeGRX232* plays a key role in regulating stomatal closure. The  
531 expression of *MeGRX232* in cassava leaf can be induced by drought and ABA. Overexpression  
532 of *MeGRX232* results increased ROS accumulation in guard cells, and confers drought  
533 hypersensitivity by inhibition of ABA-dependent stomatal closing in *Arabidopsis*. As a CC-type  
534 GRX, MeGRX232 could interact with *Arabidopsis* TGA2 and cassava MeTGA074 factors, and  
535 regulated the expression of ABA and oxidative stress related genes in *Arabidopsis*. Our study  
536 demonstrates that CC-type GRXs may functions in ABA-ROS signal transduction in drought  
537 response of cassava. It will contribute to an enhanced understanding of the specific mechanisms  
538 that elucidate the roles of CC-type GRXs involved in drought response in cassava.

539

540 **Supplementary data**

- 541 Supplementary figure S1. Protein localization analysis of six drought-responsive CC-type GRXs.
- 542 Supplementary figure S2. Identification and seed germination analysis of *MeGRX058*-OE,
- 543 *MeGRX232*-OE, *MeGRX360*-OE and *MeGRX785*-OE *Arabidopsis*.
- 544 Supplementary figure S3. Germination analysis of *MeGRX232*-OE transgenic *Arabidopsis* on
- 545 MS supplement with ABA.
- 546 Supplementary figure S4. Phylogenic analysis of TGA factors from *Arabidopsis* and cassava.
- 547 Supplementary figure S5. Expression analyses of *MeGRX232* in different tissue from drought
- 548 stressed cassava cultivar Arg7 and SC124.
- 549 Supplementary table S1. Protein sequences of glutaredoxins in *Arabidopsis* and cassava.
- 550 Supplementary table S2. RNA-seq data of CC-type GRXs in drought stressed cassava.
- 551 Supplementary table S3. List of primers used for qPCR analysis.
- 552 Supplementary table S4. GO results of *MeGRX232* regulated genes in transgenic *Arabidopsis*.
- 553 Supplementary table S5. DNA sequence of *MeGRX232* promoter region.

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Tables

Table 1. CC-type glutaredoxins in cassava				
JGI identifier (V4.1)	JGI identifier (V6.1)	Gene Symbol	Annotation	Chromosome location
cassava4.1_018177m	<i>Manes.13G141400.1</i>	<i>MeGRX177</i>	MeGRXS13	Chromosome13:26980309..26981228
cassava4.1_018360m	<i>Manes.17G050200.1</i>	<i>MeGRX360</i>	MeGRXC9	Chromosome17:18791502..18792218
cassava4.1_018918m	<i>Manes.03G049400.1</i>	<i>MeGRX918</i>	MeGRXC10-related	Chromosome03:4265637..4266041
cassava4.1_019954m	<i>Manes.05G067000.1</i>	<i>MeGRX954</i>	MeGRXC13-related	Chromosome05:5123236..5123953
cassava4.1_019956m	<i>Manes.01G214700.1</i>	<i>MeGRX956</i>	MeGRXC13-related	Chromosome01:30392540..30393324
cassava4.1_021286m	<i>Manes.05G066900.1</i>	<i>MeGRX286</i>	MeGRXC11-related	Chromosome05:5120724..5122777
cassava4.1_024091m	<i>Manes.16G081400.1</i>	<i>MeGRX091</i>	MeGRXC10-related	Chromosome16:23848065..23848499
cassava4.1_024232m	<i>Manes.15G015500.1</i>	<i>MeGRX232</i>	MeGRXS2-related	Chromosome15:1265181..1265486
cassava4.1_024597m	<i>Manes.01G214800.1</i>	<i>MeGRX597</i>	MeGRXC11-related	Chromosome01:30394902..30395210
cassava4.1_024608m	<i>Manes.11G083800.1</i>	<i>MeGRX608</i>	MeGRXC7-related	Chromosome11:11464621..11464992
cassava4.1_025892m	<i>Manes.05G066700.1</i>	<i>MeGRX892</i>	MeGRXS1-related	Chromosome05:5107836..5108531
cassava4.1_026496m	<i>Manes.05G015600.1</i>	<i>MeGRX496</i>	MeGRXS10	Chromosome15:1268904..1269746
cassava4.1_027873m	<i>Manes.03G192100.1</i>	<i>MeGRX873</i>	MeGRXS2-related	Chromosome03: 27589330..27589919
cassava4.1_027058m	<i>Manes.01G215100.1</i>	<i>MeGRX058</i>	MeGRXS1-related	Chromosome01:30426232..30427641
cassava4.1_028408m	<i>Manes.15G124000.1</i>	<i>MeGRX408</i>	N/A	Chromosome15:9399566..9399949
cassava4.1_032796m	<i>Manes.13G007200.1</i>	<i>MeGRX796</i>	MeGRXC7-related	Chromosome13:771539..771955
cassava4.1_032936m	<i>Manes.12G007000.1</i>	<i>MeGRX936</i>	MeGRXC7-related	Chromosome12:652588..653004
cassava4.1_033785m	<i>Manes.01G215000.1</i>	<i>MeGRX785</i>	MeGRXS1-related	Chromosome01:30421882..30422775

Figure legends:

Figure 1. Phylogenetic tree of glutaredoxins from cassava and *Arabidopsis*. Multiple sequence alignments were conducted using the ClustalW program. An unrooted phylogenetic tree showing cassava GRXs and *Arabidopsis* GRXs was generated using the neighbor joining method using MEGA5.0. Members of GRXs were classified by their redox activate site. CC-type: CC-type subgroup; CPYC: CPYC subgroup; 4 CXXC: 4×CXXC subgroup; CGFS: CGFS subgroup; CPFC: CPFC subgroup.

Fig. 2. Gene structure and protein sequences alignment of cassava CC-type GRXs. A. The exon-intron structure of cassava CC-type GRXs. Analysis was carried out with GSDS (<http://gsds.cbi.pku.edu.cn>); B. Protein sequences alignment of cassava CC-type GRXs. The editing of aligned sequences among cassava CC-type GRXs was performed using AlignX (Vector NTI suite 10.3 Invitrogen). Black boxes indicate conserved indentify positions, gray box indicate similar positions. The letters above the sequence indicate motif name.

Fig. 3. Figure 3 Expression analysis of CC-type GRXs in cassava Arg7 and SC124. (a) Heat map represent expression of CC-type GRXs in Arg7 and SC124. Hierarchical clustering was performed using Cluster3.0 based on the RNA-seq data from drought stressed Arg7 and SC124 plants. Heat map was built using TreeView1.0.4. (b) qPCR analysis of six drought-inducible CC-type GRXs in drought stressed fifth leaves of Arg7. (c) qPCR analysis of six drought-inducible CC-type GRX genes in drought stressed fifth leaves of SC124. DC: control; D8: 8 day after the start of the drought stress treatment; D14: 14 day after the start of the drought stress treatment; RW: 1 day after re-watering at the ending of drought treatment. Expression levels of the six CC-type GRXs were normalized against DC. Biological triplicates were averaged and significance of differences between treatments and control were analyzed using the Student's t-test (\*\*,  $p \leq 0.01$ ). Bars represent the mean  $\pm$  standard error.

Fig. 4. Expression analysis of cassava CC-type GRXs respond to exogenous ABA in leaves. The expression level of these six CC-type GRX genes were set to 1 in DC. Biological triplicates were

averaged and significance of differences between treatments and control were analyzed using the Student's t-test (\*\*,  $p \leq 0.01$ ; \*,  $0.01 < p \leq 0.05$ ). Bars represent the mean  $\pm$  standard error.

Figure 5 Effects of ABA and mannitol on seedling development of *MeGRX232-OE Arabidopsis*. (a) Post-germinated seedlings development of transgenic plants on MS medium supplemented with 0 (mock) and 5 $\mu$ M ABA, respectively. The plants that contained pG1300 (Vector) were used as control. (b), (c) Rosette diameter and primary root length of transgenic *Arabidopsis* in (a). (d) Post-germinated seedlings development of transgenic plants on MS medium supplemented with 0 (mock) and 250mM D-mannitol, respectively. The plants that contained pG1300 (Vector) were used as control. (e), (f), (g) Rosette diameter, primary root length, and lateral root number of transgenic *Arabidopsis* in (d). Biological triplicates were averaged and significance of difference between treatments and control was analyzed using the Duncan's multiple range tests. Error bars show standard errors for three independent replicates. Different letters represent a significant difference at  $p < 0.05$ .

Figure 6 Drought tolerance analyses of transgenic *Arabidopsis* grown in soil and effect of ABA on transgenic *Arabidopsis* with respect to stomatal aperture. (a), (b), (c) Drought responses of transgenic plants. Survival rate has been calculated from three independent experiments. (d) Water loss rate of transgenic plants. Biological triplicates were averaged. Error bars show standard errors for three independent replicates. (e) Stomatal distribution in leaves of transgenic *Arabidopsis*. (f) Stomatal size of transgenic *Arabidopsis*. (g) Effects of ABA on stomata of abaxial leaf epidermal peels were observed. The abaxial leaves were treated by ABA with different concentrations, respectively. (h) Stomatal aperture measurement after ABA treatment in (g) was carried out by recording width to length ration. Biological triplicates were averaged and significance of difference between treatments and control was analyzed using the Duncan's multiple range tests. Error bars show standard errors for three independent replicates. Different letters represent a significant difference at  $p < 0.05$ .

Figure 7 ROS accumulation analysis in transgenic *Arabidopsis*. (a) MDA content of transgenic *Arabidopsis* during drought treatment. (b) DAB staining of transgenic *Arabidopsis* leaves. (c)

ROS accumulation in guard cells of leaves from transgenic *Arabidopsis*. (d) Quantification of ROS levels in guard cells of transgenic *Arabidopsis*. The fluorescent intensity in *MeGRX232-OE Arabidopsis* after H<sub>2</sub>O<sub>2</sub> treatment was taken as 100%. More than 30 stomata of each leaf were calculated and significance of difference between treatments and control was analyzed using the Duncan's multiple range tests. Error bars show standard errors for three independent replicates. Different letters represent a significant difference at  $p < 0.05$ .

Figure 8 Identification of MeGRX232 interactors in *Arabidopsis* and cassava. (c) Autonomous transactivation analysis of MeGRX232 in yeast. MeGRX232mP<sub>65</sub>G<sub>65</sub> indicate mutant in MeGRX232 GSH binding site. (d) Analysis of interaction between MeGRX232 mP<sub>65</sub>G<sub>65</sub> and TGA factors by yeast two hybrid system. (e) BiFC analysis of the interactions between MeGRX232 and TGAs identified by (d) in transiently transformed *N. benthamiana* leaves. Green fluorescence in nucleus was detected for interactions of MeGRX232 with MeTGA074 and AtTGA2, respectively. As a negative control, co-expression of MeGRX232:YN with free YC, and MeGRX232:YC with free YN failed to reconstitute a fluorescent YFP chromophore. Expression of MeTGA074:GFP and AtTGA2:GFP in transiently transformed *N. benthamiana* as positive controls.

Figure 9 Gene expression profiles in *MeGRX232-OE* transgenic *Arabidopsis* and role of MeGRX232 regulators in drought response. (a) Heat map represent gene expression between *MeGRX232-OE* and control (Vector) plants. The data was processed and normalized as described in Materials and Methods. Hierarchical clustering of significantly expressed genes is displayed by average linkage. The figure was drawn by TreeView software. (b) GO analysis of *MeGRX232-OE* induced genes in *Arabidopsis*. Comparison of GO terms identified from the differentially expression genes identified in SAM analysis. GO tags were selected according to the significance ( $p$ -value  $< 10^{-5}$ ). Numbers on y-axis indicate gene numbers of the GO tag. (c) Venn diagram showing the overlap between MeGRX232-OE regulated genes in response to different stress and signals.

Figure 1

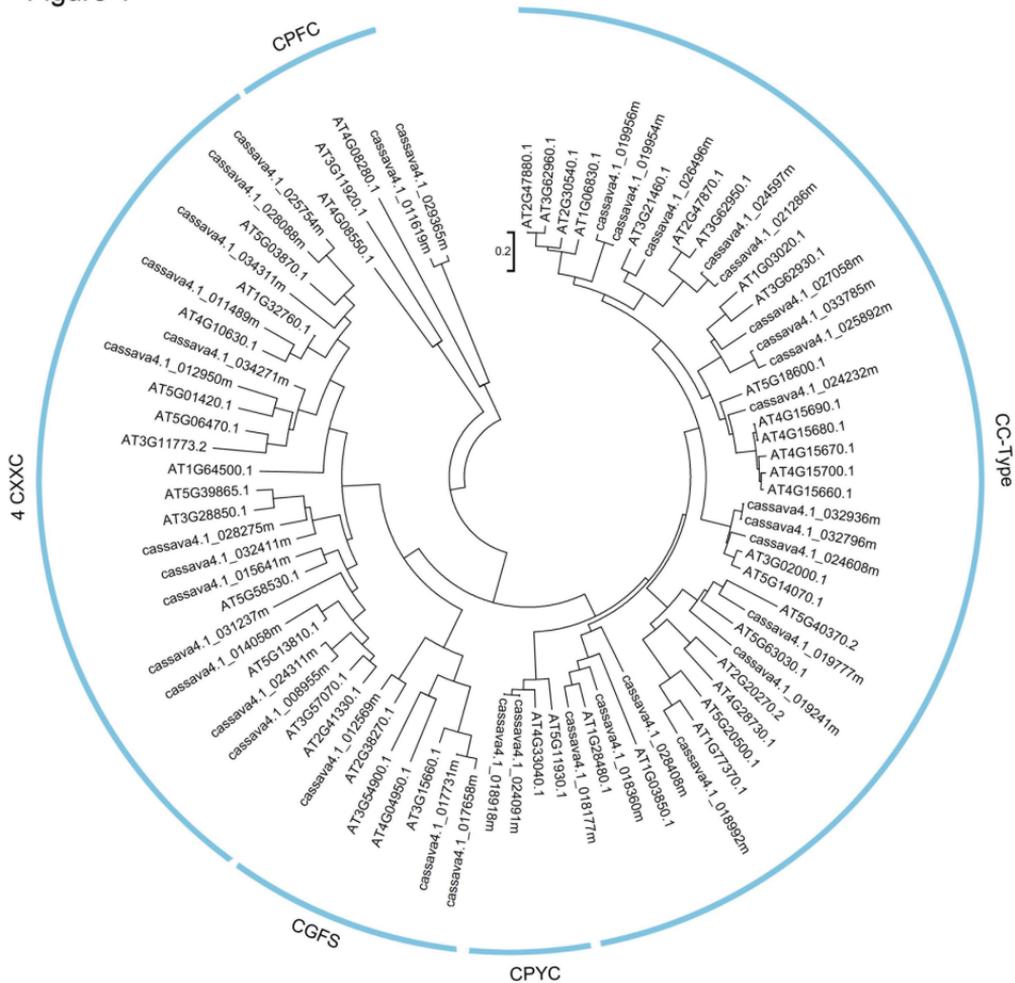
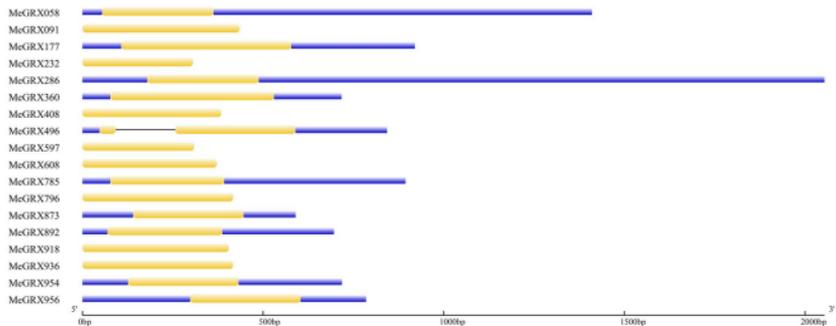


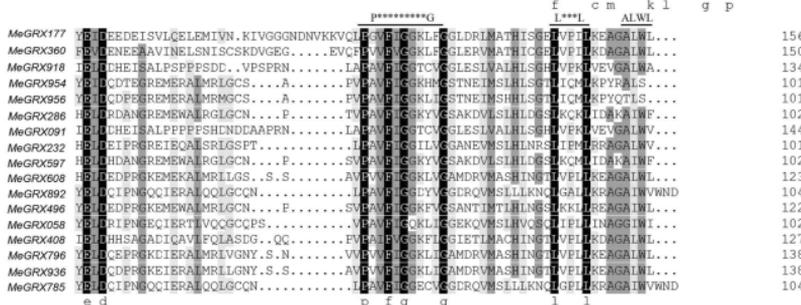
Figure 2

A



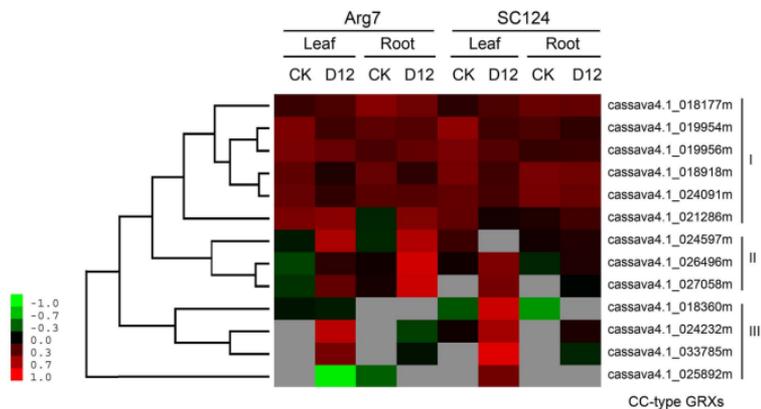
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B

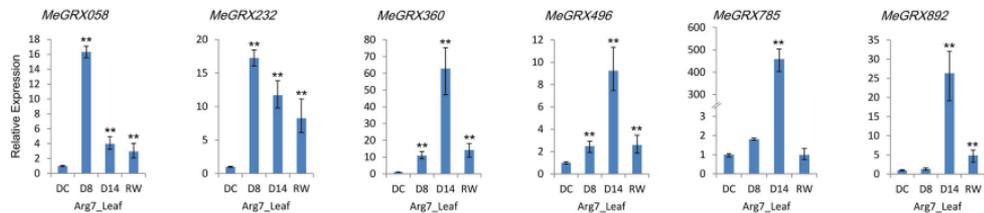


# Figure 3

## A



## B



## C

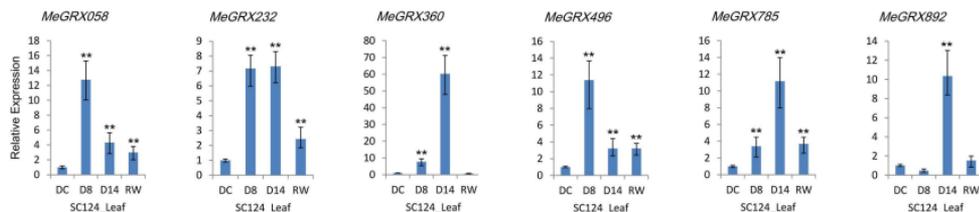


Figure 4

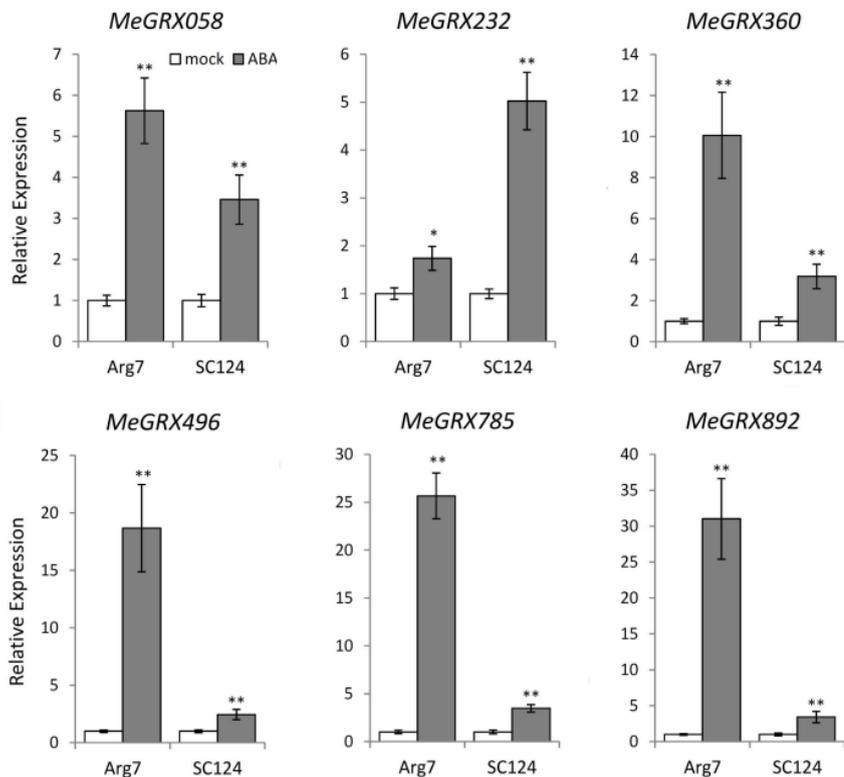


Figure 5

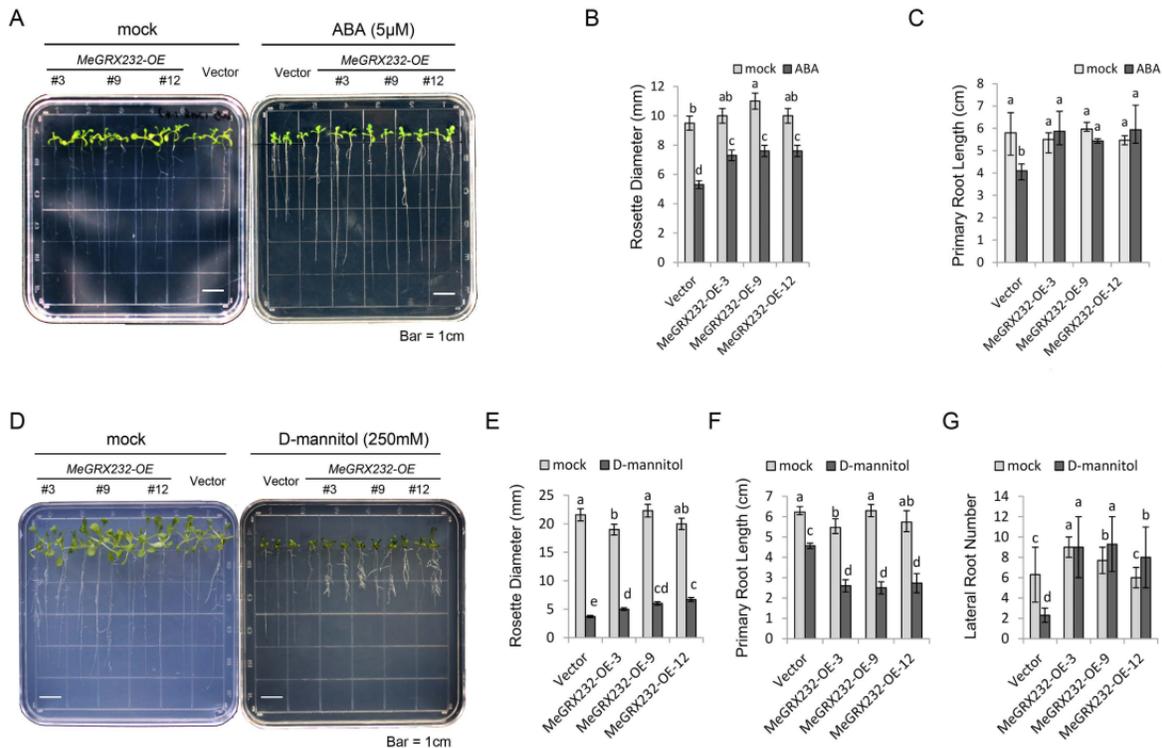


Figure 6

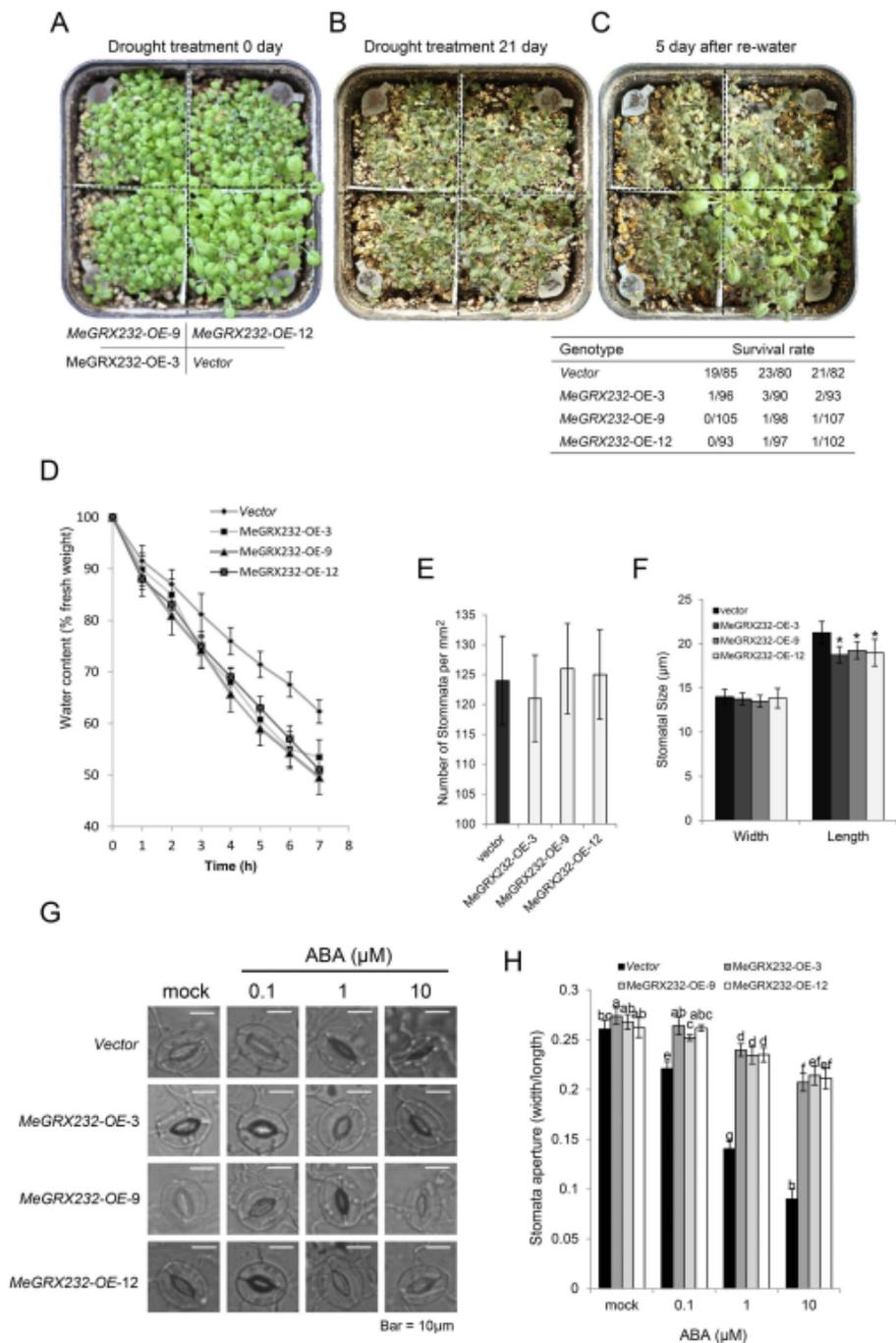
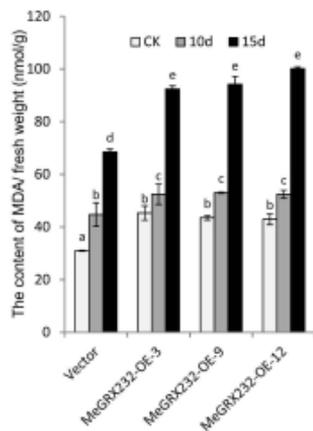


Figure 7

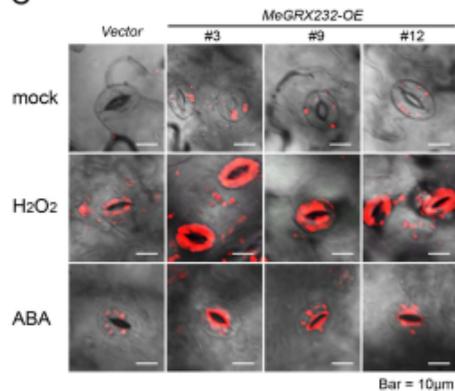
A



B



C



D

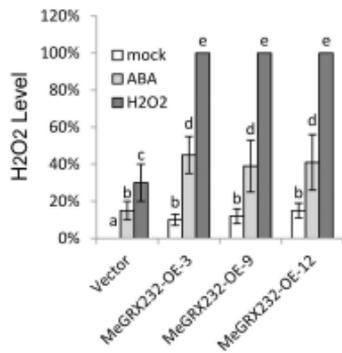
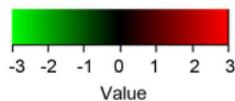
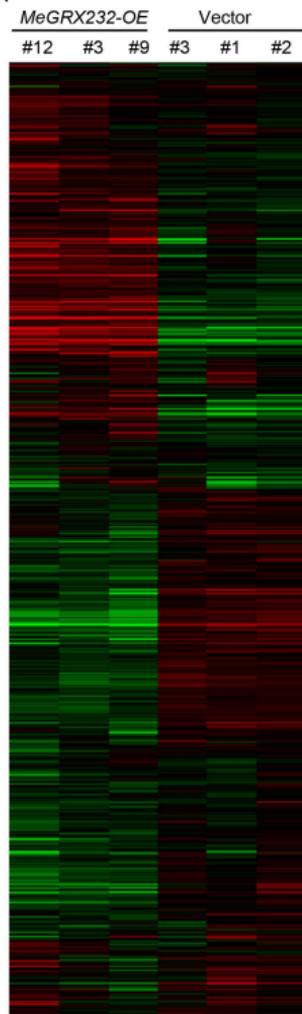


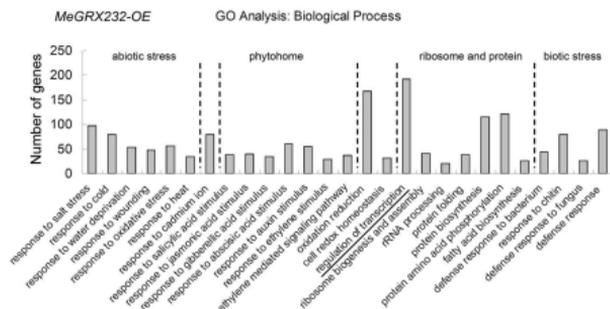


Figure 9

A



B



C

