Novelty statement:

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

Title of the article:

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

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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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60

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 *pG1300*
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 *pG1300* 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 μ M (mock) and 5 μ 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 μ M CaCl₂, and 10mM MES, pH 6.1)
196 for 2 hours followed by incubation in stomatal opening solution supplemented with varying ABA
197 concentrations (0 μ M, 0.1 μ M, 1 μ M, 10 μ 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 H₂O₂ 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 (1mg 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 H₂O₂, ABA
208 treatment were load with 50 µM 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 *MeGRX232mP₆₅G₇₅* was produced by replacing P₆₅AVFIGGILVG₇₅ to
218 A₆₅AVFIGGILVA₇₅. 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 MatchmakerTM GAL4 two-hybrid system 3 manual (Clontech). The *MeGRX232* GSH binding
221 site mutant DNA construct *MeGRX232mP₆₅G₇₅: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 *MeGRX232mP₆₅G₇₅: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 β -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 *pG1300*
 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

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

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

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

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

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

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

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

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

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

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

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

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

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

For water loss mainly depends on stomatal regulation, we infer that *MeGRX232* affect stomatal density or movement therefore increase water loss in transgenic *Arabidopsis*. However, there are no obviously stomatal density differences between vector and *MeGRX232-OE* plants (Fig. 6E). But the stomata apertures (width/length) of *MeGRX232-OE* plants are higher than that of vector plants (Fig. 6F), suggesting *MeGRX232* may affect stomatal closure during drought stress. Thus, we performed ABA-induced stomatal closing assays to test our hypothesis. Without ABA (mock), the *MeGRX232-OE* lines showed similar stomatal aperture as vector plants (Fig. 6G, H). This indicates that *MeGRX232* overexpressing did not affect stomatal aperture in *Arabidopsis* under normal conditions. When treated with 0.1 μ M ABA, the stomata of vector plants displayed a significant closing, whereas the stomata of *MeGRX232-OE* lines open widely as that in mock (Fig. 6G, H). Furthermore, with increasing concentrations of ABA to 1 μ M and 10 μ M, the stomatal aperture of vector plant exhibited dramatically reducing. On the contrary, the *MeGRX232-OE* lines performed relatively less closing of stomata (Fig. 6G, H). It suggests that overexpression of *MeGRX232* resulted impairment of ABA-dependent stomatal closing. Therefore, the more rapid water loss observed in *MeGRX232-OE* lines should mainly be ascribed 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 H₂O₂ promotes leaf stomatal closure acting downstream of ABA (Pei *et al.* 2000). H₂O₂
389 accumulation in guard cells was measured by a fluorescence dye, 2', 7'
390 '-dichlorodihydrofluorescein diacetate (DCFH-DA) under exogenous ABA and H₂O₂ treatment.
391 The *MeGRX232-OE* guard cells show obvious H₂O₂ accumulation, in contrast, no H₂O₂
392 accumulation in vector plants guard cells have been detected (Fig. 7C, D). After treated by H₂O₂
393 for three hours, both *MeGRX232-OE* and vector plant guard cells show significant H₂O₂
394 accumulation, but the H₂O₂ 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 H₂O₂ have been induced
396 by ABA in *MeGRX232-OE* guard cells compared to vector plant guard cells, and the H₂O₂ 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- α -gal on SD/-Trp / X- α -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- α -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:MeGRX232mP₆₅G₇₅ were pairwise co-transformed into yeast Y187,
 419 respectively. Yeast cells that harboring both AD:TGA and BD:MeGRX232mP₆₅G₇₅ pair plasmids
 420 were grown on SD/ -Trp/ -Leu/ X- α -gal medium (Fig. 8B). The yeast cells containing pairwise
 421 plasmids AD:TGA5/BD:MeGRX232mP₆₅G₇₅ and AD:MeTGA074/BD:MeGRX232mP₆₅G₇₅
 422 activated X- α -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

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

Discussion

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

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

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

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

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

The interaction with TGA factors is necessary for CC-type GRX functions in plants (Li *et al.* 2009; Li *et al.* 2011; Zander *et al.* 2012). In *Arabidopsis*, TGA factors have been classified to five subgroups, clade I, II, III, IV, and V. TGA2, 5, 6 are members of clade II TGAs, which are essential activators of jasmonic acid/ethylene-induce defense responses (Kesarwani *et al.* 2007; Stotz *et al.* 2013; Zander *et al.* 2010; Zander *et al.* 2012) and act as a key regulator role in plant responses of abiotic stresses such as drought, cold, and oxidative stress (Sham *et al.* 2014). *Arabidopsis* CC-type GRX GRX480/ROXY19 could interact with TGA2, 5, 6 (Zander *et al.* 2012). TGA2 could interact with GRXS13, and act as repressors of GRXS13 expression in response to biotic stress (La Camera *et al.* 2011). Here, we found that *MeGRX232* nuclear interacted with TGA5 in *Arabidopsis* and MeTGA074 in cassava respectively (Fig. 8). In *Arabidopsis*, GRX480 regulated the expression of ERF (Ethylene Response Factor) factors through interaction with TGA2/5/6 (Ndamukong *et al.* 2007; Zander *et al.* 2012). We found that nearly two hundred transcription factors including ERF factors had been affected by *MeGRX232-OE* in *Arabidopsis* (Fig. 9B, Table S5). A nuclear export signal (NES) should be tag 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 μ 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 H₂O₂ 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. MeGRX232mP₆₅G₆₅ indicate mutant in MeGRX232 GSH binding site. (d) Analysis of interaction between MeGRX232 mP₆₅G₆₅ 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 ($p\text{-value} < 10^{-5}$). 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

Legend: ■ CDS ■ upstream/downstream — intron

B

Figure 3

A

B

C

Figure 4

Figure 5

Figure 6

Figure 7

A

B

C

D

Figure 8

A

B

C

Bar = 20 μ m

Figure 9

A

B

C