CC-type glutaredoxin, MeGRXC3, associates with catalases and negatively regulates drought tolerance in cassava (Manihot esculenta Crantz)

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Introduction

Drought stress negatively affects plant growth and development and can, therefore reduce agricultural yield (Boyer, 1982). However, many plants have developed stress response mechanisms to adapt their patterns of growth under drought stress (Claeys and Inze, 2013). One such plant is cassava (Manihot esculenta Crantz), which is the most important staple crop in many tropical and subtropical arid regions of the world that are characterized by alternating wet and prolonged drought periods. These regions include sub-Saharan Africa, subtropical Asia and parts of South America (Hillocks et al., 2002). To support robust drought tolerance in cassava, many cultivars have been developed with differing drought response strategies, such as quick stomatal closure, reduction in photosynthetic proteins levels and photosynthetic capacity, induction of senescence in older leaves and size reduction in epidermal cells of leaves (Alves and Setter, 2004; Zhao et al., 2014). However, drought still seriously affects growth and tuber roots formation of cassava cultivars at seedling stage (Kengkanna et al., 2019). Recently, many drought-responsive genes in cassava have been identified via high-throughput sequencing methods (Ding et al., 2019b; Ruan et al., 2017, 2018; Suksamran et al., 2020; Weng et al., 2021), but only few of these have been analysed. Therefore, the genomic mechanism by which cassava is adapted to drought, especially through human-mediated selection, remained largely unknown.

A prior study (Xu et al., 2013a) showed that drought tolerance in cassava improved under the combined expression of SOD (Cu/Zn-superoxide dismutase) and CAT (catalase), which are enzymatic scavengers of reactive oxygen species (ROS) that become more abundant during drought stress and caused damage to plant cells (Wrzaczek et al., 2013). Moreover, drought-induced leaf abscission in cassava was delayed in transgenic cassava overexpressing SOD and CAT (Liao et al., 2016), while a heat shock protein, MeHSP90, was found to recruit MeWRKY20 and MeCAT1 to regulate drought-induced ROS accumulation in leaves of a cassava cultivar (Wei et al., 2020). In addition, a transcription factor, MeRAV5, interacts with peroxidase (POD), which negatively regulates ROS accumulation under drought stress (Yan et al., 2021a). Silencing MeRAV5 by in leaves virus-induced gene silence yielded drought sensitivity in a cassava cultivar (Yan et al., 2021a). Taken together, these studies highlight the importance of mitigation of ROS activities and...
maintenance of their homeostasis in cassava during drought stress.

The glutathione/glutaredoxin (GSH/GRX) system is essential for ROS signalling and protein redox homeostasis in responses of plants to stress (Meyer et al., 2012). GRXs are classified into five subgroups. The CC-type is specific to land plants and was characterized as the ROXY family in Arabidopsis (Meyer et al., 2012). In Arabidopsis, overexpression of ROXY1 was correlated with a significant increase in ROS accumulation and caused higher susceptibility to fungal infection by Botrytis E. M. Fries, 1832 (Wang et al., 2009). On the contrary, the roxy18 mutant showed higher initial and photo-oxidative-stress-induced ROS accumulation, and therefore caused sensitivity to methyl viologen herbicide and high light (Laporte et al., 2012). These results indicate that ROXYS play antagonistic roles in ROS homeostasis. However, how CC-type GRX regulates ROS homeostasis remained largely unknown.

Direct redox modification of transcription factors is critical for the perception of intracellular ROS (Wrzaczek et al., 2013). For example, redox modification of a basic domain leucine zipper (bZIP) transcription factor, TGACG-BINDING FACTOR 1 (TGA1), affected its transcriptional regulation ability (Lindermay et al., 2010). TGA1 appears to interact with several CC-type GRXs, such as ROXY9, ROXY18 and ROXY19 (Li et al., 2019). Physical interaction with ROXY19 and subsequent redox modification of TGA2 is essential for the function of this transcription factor (Zander et al., 2012). Furthermore, ROXY1 and ROXY2 regulate another development by controlling redox modification of TGA9 and TGA10 in Arabidopsis (Li et al., 2009; Murmu et al., 2010; Xing and Zachgo, 2008).

CC-type GRXs are also involved in abiotic stress and phytohormone responses in plants (Ndamukong et al., 2007; Zander et al., 2012). In rice, the expression of OsGRX8 is induced by auxin and abiotic stress (Sharma et al., 2013). Furthermore, overexpression of OsGRX8 in Arabidopsis enhanced tolerance to abscisic acid (ABA) and abiotic stresses (Sharma et al., 2013). Another CC-type GRX of rice, OsGRX6, undergoes changes in expression levels depending on the availability of nitrate, and overexpression of this gene delays leaf senescence and causes giberellin acid insensitivity (El-Kereamy et al., 2015). These data from model species indicate that CC-type GRXs may be useful in improving the resistance of crops to abiotic stress.

Previously, we screened several CC-type GRXs, which respond to drought and ABA in cassava cultivars (Ruan et al., 2018). For one gene, MeGRXC3, we determined that its overexpression cause mannitol-induced osmotic stress sensitivity in transgenic Arabidopsis (Ruan et al., 2022). However, whether MeGRXC3 is involved in ROS signalling and drought resistance in cassava remains unclear. Here, we found that two SNPs in the promoter of MeGRXC3 are significantly associated with the activity of catalase in mature leaves of 100 cultivars of cassava (McManus et al., 2010). MeGRXC3 was expressed in OE transgenic cassava (Figure 2c,d).

Results

MeGRXC3 is associated with the activity of catalase in cultivars of cassava under drought stress

There are 21 CC-type GRXs in cassava genome (Ruan et al., 2018). MeGRXC3 (Manes.01G215000) is an intronless coding region that is 315 bp in length from 30 421 960 bp to 30 422 275 bp in cassava chromosome 1 (Figure 1a). We performed re-sequencing in 100 cultivars of cassava to investigate whether MeGRXC3 is correlated with drought tolerance, and we investigated the association with drought-related marker-traits that we previously reported (Wang et al., 2017). Re-sequencing of the genomic DNA of MeGRXC3 revealed a total of 44 SNPs. Two SNPs in the promoter were significantly associated with drought tolerance coefficients (DTCs) of catalase activity in mature leaves at P < 0.01 (Figure 1b), suggesting that transcription of MeGRXC3 is correlated with catalase activity in cassava cultivars under drought stress.

Under non-drought conditions, MeGRXC3 was expressed weakly in leaf blades and strongly in petioles and trivially in roots (Figure 1c). Drought and ABA treatments led to up-regulation of MeGRXC3 in leaf blades of cassava cultivar cv.60444 (Figure 1c). Moreover, expression of MeGRXC3 was induced in mature leaves by drought in several cassava cultivars with differing genotypes (Figure 1d). Promoter activity analysis results revealed green fluorescence in leaf epidermal cells, including guard cells, of the MeGRXC3:GFP transgenic Arabidopsis (Figure 1e, f). These indicate that MeGRXC3 may be expressed in guard cells of cassava and play conserved roles across cultivars in response to drought.

MeGRXC3 negatively regulates tolerance to dehydration in vitro in transgenic cassava

Overexpression of MeGRXC3 resulted in sensitivity to drought in transgenic Arabidopsis (Figure S1). Therefore, we produced transgenic plants overexpressing MeGRXC3:GFP (MeGRXC3-OE) or expressing RNAi against MeGRXC3 (MeGRXC3-RNAi) in cv.60444. In total, we successfully generated 13 transgenic cassava lines (Figure S2). Thus, we used two independent lines of MeGRXC3-OE (OE#12, OE#88) and of MeGRXC3-RNAi (RNAi#1, RNAi#17) in subsequent experiments.

We observed no significant phenotypic differences among control plants comprising wild-type and transgenic in vitro plantlets after grew on cassava basic medium (CBM) for 50 days (Figure 2a). However, root development of all plantlets was strongly inhibited by polyethylene glycol (PEG; Figure 2a). Moreover, we found that only the OE plantlets showed dwarf phenotypes (Figure 2a). Under PEG treatment, biomass of the OE plantlets was lower than that of controls; a much greater effect than in the wild-type plantlets and RNAi plantlets. (Figure 2b). These data indicate that MeGRXC3 negatively regulates tolerance to dehydration in cassava. The Western blot and green fluorescence in abaxial epidermal cells including guard cells suggest that the MeGRXC3:GFP protein complex is expressed in OE transgenic cassava (Figure 2c,d).

MeGRXC3 negatively regulates drought tolerance in transgenic cassava seedlings

Prior to drought treatment, there were no obvious phenotypic differences among 90-day-old seedlings of transgenic and wild-
type cassava. At the time of 20 days drought treatment, we observed that the leaves and shoot apexes of the OE and wild-type cassava were withered, but the shoot apexes and several leaves of RNAi cassava remained alive (Figure 3a). After re-watering, plants grew under normal conditions for 7 days before we calculated their survival rates. After 7 days, only a few of OE and wild-type cassava seedlings were survived, while most RNAi cassava seedlings were survived (Figure 3b). These results support that MeGRXC3 negatively regulates drought tolerance in transgenic cassava.

The transcript level of MeGRXC3 in OE cassava leaves was much higher than that in wild-type cassava leaves (Figure 3c). Under drought stress, the expression of MeGRXC3 was dramatically repressed by transgenes in RNAi cassava leaves (Figure 3c). qRT-PCR result showed that the MeGRXC3-RNAi transgenes had no effect on the expression of other drought-responsive CC-type GRXs under non-drought condition, and it did not alter their expression patterns under drought stress (Figure S3). Thus, the drought tolerance improvement of RNAi cassava can be explained by knock-down of MeGRXC3.

We performed stomatal assays by using six mature leaves of these cassava seedlings (Figure S4). Since MeGRXC3:GFP was predominantly localized in the nucleus of guard cells of OE transgenic cassava (Figure S5), stomatal movement assays of transgenic cassava seedlings help to clarify the functions of MeGRXC3. We classified stomatal status as open or closed upon examination by microscopy (Figure S5). Just before drought stress (i.e. 0 day drought treatment), the OE and RNAi plants showed no obvious differences in stomatal status (Figure 3d). After drought stressed for 6 days, most stomata of OE cassava, and half of the stomata of wild-type cassava were opened, while only a few stomata of the RNAi cassava were opened (Figure 3d). Further assays show that transpiration rates of leaves of the OE cassava were higher than that of the wild type (Figure 3e), while transpiration rates of leaves of the RNAi lines were lower than that of the wild type (Figure 3e). No significant difference in stomatal index was observed among the transgenic and wild-type leaves of cassava (Figure 3f). These data indicate that MeGRXC3 negatively regulates drought-induced stomatal closure.

MeGRXC3 regulates expression of MeCAT7 and distribution of drought-induced ROS in epidermal cells in cassava

Abscisic acid and jasmonic acid (JA) are important positive signalling hormones promoting stomatal closure. No obvious difference in endogenous ABA levels among all leaves of cassava was observed under non-drought and drought stress (Figure 4a). Under normal watering conditions, endogenous JA levels of transgenic cassava leaves were slightly lower than that of wild-type leaves (Figure 4b). However, we observed no differences in endogenous JA levels among treated cassava leaves at 6 days drought treatment (Figure 4b). Therefore, ABA and JA biosynthesis in transgenic
cassava leaves under drought stress were not affected by MeGRXC3 transgenes.

Compared with wild type, leaves of both the OE and RNAi cassava exhibited a higher base level of H$_2$O$_2$ under normal conditions (Figure 4c). We observed an increased H$_2$O$_2$ content in the RNAi and wild-type cassava leaves at 6 days drought treatment (Figure 4c). On the contrary, there was a slight decline in H$_2$O$_2$ content in the leaves of OE cassava at 6 days drought treatment. (Figure 4c). These findings indicate that MeGRXC3 plays a role in response to drought by regulating H$_2$O$_2$ homeostasis in leaves of cassava.

Drought led to increased SOD activity in OE leaves of cassava but reduced it in RNAi and wild-type leaves (Figure 4d). Consistently, POD and CAT activities in both OE and RNAi cassava leaves were relatively lower than those in the wild type under normal watering conditions (Figure 4e,f). Reduced POD and CAT activities were detected in all of transgenic and wild-type cassava leaves at 6 days drought treatment (Figure 4e,f) but were much lower in the RNAi lines compared with the other two.

Our qRT-PCR analysis indicated that the relative expression level of MeCAT1 and MeCAT7 in the mature leaves of wild-type cassava was much higher than that of the other five MeCATs under non-drought stress (Figure S6). Expression of MeCAT1 was up-regulated in leaves of OE cassava, while it was down-regulated in leaves of RNAi cassava (Figure 4g). Both MeGRXC3-OE and -RNAi transgenic cassava did not affect drought-induced up-regulation of MeCAT2 in leaves (Figure 4h).

Under normal conditions, MeCAT7 was down-regulated in leaves of OE cassava, while it was up-regulated in leaves of RNAi cassava (Figure 4i), and under drought conditions, RNAi plants maintained higher levels of MeCAT7 than the wild-type and OE cassava (Figure 4i). Nevertheless, MeGRXC3-transgenic cassava did not alter drought-induced down-regulation of MeCAT1 or MeCAT7 in leaves. Taken together, these results suggest that...
MeGRXC3 negatively regulates drought tolerance of cassava

(a) MeGRXC3-OE
#12 #88 WT #1 #17
MeGRXC3-RNAi

(b) Survival Rate (%)

(c) MeGRXC3

(d) MeGRXC3-OE#12 MeGRXC3-OE#88 WT MeGRXC3-RNAi#1 MeGRXC3-RNAi#17

(e) Transpiration Rate

(f) Stomatal Index (%)

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MeGRXC3 may regulate the expression of CAT genes in a complex manner in cassava in response to drought. We stained H$_2$O$_2$ by DAB in abaxial epidermal cells of leaves of transgenic cassava after H$_2$O$_2$ treatment, and this revealed a build-up of H$_2$O$_2$, but it was distributed throughout the epidermis in the OE lines and predominantly in guard cells of the leaves of RNAi cassava (Figure 4j–l). These results indicate that MeGRXC3 negatively regulates H$_2$O$_2$ accumulation in guard cells. MeGRXC3 transgene alters the expression of MeCAT7 and distribution of drought-induced ROS in epidermal cells of cassava leaves. ABA content (a), JA content (b), H$_2$O$_2$ content (c), SOD activity (d), POD activity (e) and CAT activity (f) in mature leaves from transgenic and wild-type cassava under drought conditions. D0: 0 day drought treatment; D10: 10-day drought treatment. Error bars are ±SD (n = 3). The expression levels of MeCAT7 (g), MeCAT2 (h) and MeCAT7 (i) in mature leaves of transgenic and wild-type cassava. Expression levels of these genes were normalized against that in wild-type plants before drought stress. Error bars are ±SD (n = 3). (j) DAB staining of abaxial epidermal cells and guard cells in mature leaves of cassava under H$_2$O$_2$ treatment. DBRW: day before re-watering. (k) Relative value of DAB staining in abaxial epidermal cells. Error bars are ±SD (n = 200). (l) Relative value of DAB staining in guard cells. Error bars are ±SD (n = 200). Different letters indicate differences with $P < 0.05$ (ANOVA test).

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transgenes negatively regulated water loss rate in leaves of cassava (Figure S7). This may partly explain the reduced drought tolerance in OE cassava and increased drought tolerance in RNAi cassava, as ABA-induced stomatal closure dependent on ROS signalling in Arabidopsis (Pei et al., 2000). Our data indicate that MeGRXC3 negatively regulates ABA-induced H₂O₂ accumulation in guard cells of cassava and, therefore, affects stomatal closure (Figure S7).

MeGRXC3 interacts with MeCAT1 and MeCAT2 to regulate the activity of catalase

The total soluble protein extract isolated from mature leaves of OE#88 cassava was checked by Western blot with anti-GFP antibody (Figure 5a, input). Proteins that immunoprecipitated with anti-GFP antibody were loaded in an SDS-PAGE gel (Figure 5a, IP). We identified IPed proteins by LC-MS/MS. From these IPed proteins (Data S1), we found three putative catalases comprising MeCAT1, MeCAT2 and MeCAT7 (Figure 5b), suggesting that MeGRXC3 probably interacts with catalases in vivo.

We screened MeCAT1 and MeCAT2, but not MeCAT7 by applying a MeGRXC3 non-transcriptional activation ability mutant, MeGRXC3P65L, as bait in the Y2H screening. Y2H assay indicates that MeGRXC3 interacts with MeCAT1 and mutant, MeGRXC3P65L, as bait in the Y2H screening. Y2H assay shows MeGRXC3 directly binding to MeTGA2, a cassava TGACG-motif (5'-TGATTACGTCA-3') (Figure 5d). Furthermore, MeMYB63 dramatically enhanced the transcriptional activity of the MeCAT7 promoter based on a LUC/REN dual-luciferase report assay using ProMeCAT7:pGreen0800-luc as a reporter (Figure 7h,i). Moreover, we transiently overexpressed MeMYB63 in leaves of cassava cv.60444 (Figure 7j). Consistently, the result suggests that MeMYB63 promoted the expression of MeCAT7 and catalase activity in the leaves (Figure 7k,l). Thus, it can be concluded that MeMYB63 is a direct transcriptional activator of MeCAT7.

MeMYB63 is negatively regulated by MeTGA2 in cassava through interaction with MeGRXC3

The expression of MeMYB63 was negatively regulated by MeGRXC3 in mature leaves of cassava (Figure 8a). A TGACG-motif (5'-TGATTACGTCA-3') was identified in the MeMYB63 promoter based on the MeTGA2 DAP-seq results (Figure 8b). The yeast one-hybrid assay shows MeTGA2 binding to the promoter of MeMYB63 (Figure 8c). Furthermore, the MeTGA2 protein was expressed in E. coli and the purified (Figure 8d,e). We found MeTGA2 directly binding to P2 (containing the 5'-TGATTACGTCA-3' motif) but not the P2m promoter fragment of MeMYB63 (Figure 8f,g). Subsequently, LUC/REN dual-luciferase report assay showed that MeTGA2 did not affect the activity of the MeMYB63 promoter without MeGRXC3, while MeGRXC3 could reduce the activity of the MeMYB63 promoter without MeTGA2 (Figure 8h,i). Furthermore, the activity of the MeMYB63 promoter was significantly reduced by co-expression of MeTGA2 and MeGRXC3 (Figure 8b). Together, these results suggest that MeGRXC3 negatively regulate the expression of MeMYB63, probably by interacting with MeTGA2.

Discussion

Functional characterization of drought-responsive CC-type GRXs in Arabidopsis provided criteria for choosing MeGRXC3 as a candidate that should be further investigated in transgenic cassava (Ruan et al., 2022). Furthermore, target re-sequencing and association mapping analyses revealed that the genetic variation of the MeGRXC3 promoter was significantly associated with the drought-efficient trait of catalase activity in cassava cultivars under drought stress (Figure 1). Likewise, significant associations between genetic variation in a maize CC-type GRX 2mGRXC1C14 and drought tolerance were found at the seedling stage (Ding et al., 2019a). Thus, taken together, CC-type GRXs may play important roles in regulating drought tolerance of crops. Herein, we demonstrated the function of MeGRXC3 in regulating drought tolerance of cassava cultivar.

Glutaredoxins play antagonistic roles in regulating drought tolerance in plants (Guo et al., 2010; Hu et al., 2017). Overexpression of MeGRXC3 caused hypersensitivity to mannitol-induced osmotic stress in transgenic Arabidopsis (Ruan et al., 2012). Therefore, it may be unsurprising that our Y2H assays showed that the MeGRXC3 protein was able to interact with MeTGA2 in yeast (Figure 6a).

MeMYB63, which is a R2R3-MYB transcription factor that act as a transcriptional activator of the lignin biosynthetic pathway in Arabidopsis (Zhou et al., 2009). Yeast one-hybrid assay showed that MeMYB63 bound to the MeCAT7 promoter and the AC-element with the sequence 5'-'ACCACA-3' (Figure 7b). MeMYB63 was predominantly localized in the nucleus and showed transcriptional activation ability in yeast (Figure 7c,d). The MeMYB63 protein was expressed in E. coli and purified by affinity (Figure 7e). As the Electrophoretic mobility shift assay (EMSA) result indicates, MeMYB63 can directly bind to the wild-type AC-element (P1) but not the mutated sequences (P1m; Figure 7f,g). Furthermore, MeMYB63 dramatically enhanced the transcriptional activity of the MeCAT7 promoter based on a LUC/REN dual-luciferase report assay using ProMeCAT7:pGreen0800-luc as a reporter (Figure 7h,i). Moreover, we transiently overexpressed MeMYB63 in leaves of cassava cv.60444 (Figure 7j). Consistently, the result suggests that MeMYB63 promoted the expression of MeCAT7 and catalase activity in the leaves (Figure 7k,l). Thus, it can be concluded that MeMYB63 is a direct transcriptional activator of MeCAT7.

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et al., 2022), and conferred sensitivity to PEG and drought in transgenic Arabidopsis (Figure S1). In cassava, phenotypic analyses of MeGRXC3 transgenic lines show that this gene negatively regulated PEG and drought tolerance (Figures 2 and 3). These data indicate that MeGRXC3 acts as a negative regulator of drought tolerance in cassava. In fact, repression of MeGRXC3 increased yield of tuber roots in the field in transgenic cassava under drought conditions (Figure S8a,b). Thus, MeGRXC3 has agroeconomic potential for improving yield in cassava under drought stress. In addition, the dry matter rate of tuber roots from MeGRXC3-RNAi transgenic cassava is lower than that of wild type (Figure S8c), which may be because under drought stress, RNAi transgenic cassava has higher water use efficiency and therefore retain more water in its tuber root.

Glutaredoxin regulates stomatal movement by modulating ROS accumulation in guard cells in rice (Hu et al., 2017). It remains

Figure 5 MeGRXC3 interacts with two catalases and regulates their activity. (a) Protein co-immunoprecipitation in MeGRXC3-OE transgenic cassava using Anti–GFP antibody. IP: immunoprecipitation. (b) Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis of IPed proteins predicted three catalases as putative targets of MeGRXC3 in cassava. (c) MeGRXC3 interacts with MeCAT1 and MeCAT2 in yeast two-hybrid assay. (d) Bimolecular fluorescence complementation indicating the in vivo interaction of MeGRXC3 with MeCAT1 and MeCAT2 in tobacco leaves. Red fluorescence of mCherry was detected for colocalization analysis. (e) MeGRXC3 affects catalase activity of MeCAT1 and MeCAT2 in tobacco leaves. Relative catalase activity of tobacco that was co-transformed by two vectors (NYFP + CYFP) was set to 100%. Error bars are ±SD (n = 5), different letters indicate differences with P < 0.05 (ANOVA test).
unclear whether the CC-type GRX can regulate stomatal movement in cassava. However, analysis of promoter activity indicates that MeGRXC3 expresses in leaf epidermal cells including guard cells (Figure 1f), so we infer that MeGRXC3 may be involved in regulation of stomatal movement. When the 35S promoter drove MeGRXC3 to be expressed in guard cells in the MeGRXC3-OE lines, the MeGRXC3 protein was predominantly localized in the nucleus of the transgenic cassava (Figures 2d and 55). Thus, stomatal movement assays of transgenic cassava can lend partial support to the function of MeGRXC3 in cassava under drought conditions (Figure S7). It can be concluded that less water loss might be one of the main reasons to confer drought tolerance in MeGRXC3-RNAi transgenic cassava.

Both ABA and JA are important for stomatal movement (Kollist et al., 2014; Zhu et al., 2020), and ABA biosynthesis is essential for drought tolerance in cassava (Wei et al., 2020). We found that the MeGRXC3 gene did not affect biosynthesis of ABA and JA in cassava under drought conditions (Figure 4a,b). In Arabidopsis, a CC-type GRX gene, GRXS13, was found to be able to negatively regulate base-level and oxidative stress-induced production of ROS (Laporte et al., 2012). ROS production is often induced by ABA, and ROS and ABA operate in tandem as key signals involved in stomatal closure (Lee et al., 2012; Yan et al., 2007; Zhao et al., 2016). Interestingly, in our study, base-level and drought-induced production of H2O2 in leaves was enhanced in both OE and RNAi plants (Figure 4c). However, the MeGRXC3 transgene antagonistically regulated H2O2 accumulation in epidermal cells and guard cells (Figure 4d–l). Similar to
our RNAi plants, repression of OsGRXS17 in rice promoted ABA-induced stomatal closure by modulating ROS accumulation in guard cells (Hu et al., 2017). Our results suggest that MeGRXC3 negatively regulates ABA-induced stomatal closure by modulating H$_2$O$_2$ accumulation in guard cells (Figure 5f), but the main function of MeGRXC3 in stomatal regulation seems to be not only to inhibit stomatal closing because, therefore, it would be contradictory for cassava to induce the expression of MeGRXC3 under drought conditions. A possible explanation is that MeGRXC3 is involved in negative feedback regulation involving ROS to balance photosynthesis under drought (i.e. maintaining some stomatal opening for CO$_2$ entry, even under drought).

MeGRXC3 negatively regulated the activity of catalase in leaves of cassava under drought stress (Figure 4f). Previous studies have demonstrated that catalase activity is essential for drought-induced ROS accumulation in cassava, and it can be regulated either at transcription or post-translation (Wei et al., 2020; Xu et al., 2013a; Yan et al., 2021b). There are seven catalase genes present in the cassava genome, and the transcript level of MeCAT7 is the highest in leaves of cassava (Figure 5e). We found that MeGRXC3 negatively regulated expression of MeCAT7 (Figure 4i), suggesting that MeGRXC3 may regulate the activity of catalase at the transcription level. Furthermore, catalases have been identified as having possible interactions with GRX proteins in plants (Rouhier et al., 2005). In fact, our work shows that MeGRXC3 interacts with MeCAT1 and MeCAT2 (Figure 5a-d) and antagonistically affects activity of these two catalases (Figure 5e). Our study implicates that MeGRXC3 can regulate catalase activity at both transcription and post-translation levels.

In Arabidopsis, the CAT3 Cys343Thr mutant displays increased catalase but decreased transnitrosyl activity, and the S-nitrosylation of Cys-343 is critical to the main activities of CAT3 as a transnitrosylase instead of a catalase (Chen et al., 2020). Cys-343 is a unique and highly conserved residue in CAT3, which is Thr-343 in MeCAT1, MeCAT2 and MeCAT7 (Figure 5f). GRX carries out deglutathionylation of Cys residue through binding with GSH, thus changing the redox state of its target proteins (Gutsche et al., 2015). Although MeGRXC3 may regulate catalase activity of MeCAT1 and MeCAT2 at post-translation level, it remains to be determined whether MeGRXC3 regulates catalase activity by post-modification translation.

MeGRXC3:GFP transiently expressed in tobacco and overexpressed in transgenic Arabidopsis indicates that MeGRXC3 is localized to both the nucleus and cytoplasm (Figures 1f and S10). Surprisingly, MeGRXC3 is predominantly localized in the nucleus as indicated by MeGRXC3-OE transgenic cassava (Figures 2d and S5a). Nuclear activity is critical for the function of some CC-type GRXs in plants. For example, ROXY1 predominantly functions in the nucleus, regulates TGA transcription factors and was found to have both positive and negative activity during petal development in Arabidopsis (Li et al., 2009). We have demonstrated that nuclear activity is required for the function of MeGRXC3 during regulation of mannitol-induced osmotic stress in transgenic Arabidopsis, which is dependent on the interaction with TGA factors (Ruan et al., 2022). In this study, a protein interaction assay indicated that MeGRXC3 interacted with MeTGA2 in the nucleus (Figure 6a,b). MeTGA2 is a typical TGA transcription factor, which binds to a TGACG-motif with the sequence 5’-TGACGTCATCA-3’ in cassava (Figure 6b-d). In Arabidopsis, ROXY19 suppresses AtPDF1.2 transcription by interacting with TGA transcription factors (Ndumukong et al., 2007). It indicated that CC-type GRX can regulate expression of transcription factor through interaction with TGA factors in plant. In prior studies, we also found that MeGRXC3 and MeGRXC15 can regulate expression of several stress-related transcription factors through interaction with TGA factors in transgenic Arabidopsis (Ruan et al., 2018, 2022). Here, via DAP-seq, we identified several stress-related transcription factors as potential targets of MeTGA2 (Figure 6e), including MeDREB1D, which we reported previously (Yang et al., 2016). In brief, together with quantitative real-time PCR (qPCR) results (Figure S11), our data indicate that MeGRXC3 may regulate the expression of these transcription factors by forming a transcriptional regulatory complex with MeTGA2 in the nucleus.

The regulation of MeCAT7 by MeGRXC3 appears somewhat enigmatic. Specifically, the DAP-seq analysis revealed that MeCAT7 is not a direct target of MeTGA2. Therefore, MeGRXC3 may regulate the expression of MeCAT7 through other transcription factors. We did find three AC-elements, which could be bound by the transcription factors AtMYB61 and AtMYB63 (Prouse and Campbell, 2013; Romano et al., 2012; Zhou et al., 2009), in the promoter of MeCAT7 (Figure 7a). MeMYB63 was predicted as a putative target of MeTGA2 (Figure 6e), which could bind to the MeCAT7 promoter and the AC-element in yeast and was identified as a transcription factor (Figure 7b-d). EMSA and dual LUC/REN reporter assays indicate that MeMYB63 directly bound to an AC-element in the MeCAT7 promoter and positively regulated its activity (Figure 7f-h). Additionally, MeMYB63 up-regulated transcription of MeCAT7 and consequently promoted activity of catalase in cassava leaves (Figure 7i-l). These findings indicate that MeMYB63 is a transcriptional activator of MeCAT7.

Previous reports showed that AtMYB61 is a positive regulator of stomatal closure (Li et al., 2005; Romero-Romero et al., 2018). Whether MeMYB63 is involved in stomatal movement in cassava merits further investigation. Nevertheless, this work taken together with prior studies suggests that MeGRXC3 may regulate the expression of MeCAT7 dependent

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**Figure 7** MeMYB63 directly binds to the MeCAT7 promoter and initiates transcription of MeCAT7. (a) Schematic diagram represents AC-elements in the MeCAT7 promoter. (b) MeMYB63 binding to MeCAT7 promoter and AC-elements in yeast one-hybrid assay. (c) Subcellular localization of MeMYB63 in tobacco leaves. Red fluorescence of the MeHistone3:mCherry construct was used as a nuclear localization reference. (d) Transcription activation analysis of MeMYB63 in yeast. (e) Expression of the SUMO-MeMYB63 complex in bacteria (left) and purified SUMO-MeMYB63 (right). 3: negative control; M: protein marker; NPE: the soluble supernatant fraction; DPE: the supernatant of denatured protein; 1, 3: induced at 16 °C; 2, 4: induced at 37 °C. (f) Probes used in the electrophoretic mobility shift assay. (g) MeMYB63 binding to AC-elements in electrophoretic mobility shift assay. (h) Plasmids used in dual-luciferease reporter assay. (i) Relative luciferase (LUC/REN) activity of the MeCAT7 promoter in tobacco leaves with transient expression of MeMYB63. Error bars are ±SD (n = 5). qPCR analysis of MeMYB63 (i) and MeCAT7 (k) in cassava leaves with transient expression of MeMYB63. Error bars are ±SD (n = 3). (l) Relative catalase activity in cassava leaves with transient expression of MeMYB63. Error bars are ±SD (n = 5). Different letters indicate differences with $P < 0.05$ (ANOVA test).
on MeMYB63. Here, we show that MeMYB63 in leaves of cassava was negatively regulated by MeGRXC3 (Figure 8a), and that, when the TGACG-motif with the sequence 5'-TGATTACGTCA-3' in the promoter of MeMYB63 was bound by MeTGA2, the activity of MeMYB63 promoter was diminished in the presence of MeTGA2 and MeGRXC3 (Figure 8b–i). Therefore, it can be logically (although tenuously) concluded that MeGRXC3 negatively regulates the expression of MeCAT7.
by repressing MeMYB63 through interaction with MeTGA2 in cassava.

RNA-seq analysis indicated that MeGRXC3 regulates many drought-responsive genes, including a portion of transcription factors such as members of AP2/ERF, MYB and WRKY in cassava (Figure S12). Together, we summarized our study in a diagram of MeGRXC3 associating with catalases and participating in cassava response to drought, which is shown in Figure 9. Our study expands the knowledge of CC-type GRX in plants and highlights the potential value of MeGRXC3 for improvement of drought tolerance in cassava cultivars.

Experimental procedures
Candidate gene re-sequencing and association mapping
We isolated the genomic DNA sequence of MeGRXC3 in cassava cultivar cv.60444 and identified it according to its locus in cassava genome (https://phytozome.jgi.doe.gov, M. esculenta v8.1). A total of 100 accessions of cassava were chose for re-sequencing from among cassava germplasm resources collected by our laboratory (Wang et al., 2017). We designed primers covering the entire candidate genomic region to amplify the candidate segments (Data S4). SNPs were identified by alignment with the

Figure 8 MeGRXC3-MeTGA2 complex acts as transcriptional repressor of MeMYB63. (a) qPCR analysis of MeMYB63 in transgenic cassava under drought stress. Error bars are ±SD (n = 3). (b) Schematic diagram represents the TGACG-motif in the MeMYB63 promoter. (c) MeTGA2 binding to the MeMYB63 promoter in a yeast one-hybrid assay. (d) SUMO-MeTGA2 expression in bacteria. (e) Western bolt analysis of the purified SUMO-MeTGA2 protein complex. (f) Probes used in the electrophoretic mobility shift assay. (g) MeMYB63 binding to the TGACG-motif of the MeMYB63 promoter in the electrophoretic mobility shift assay. (h) Plasmids used in a dual-luciferase reporter assay. (i) Relative luciferase (LUC/REN) activity of the MeMYB63 promoter in tobacco leaves with transient expression of MeGRXC3 and MeTGA2. Error bars are ±SD (n = 5). Different letters indicate differences with P < 0.05 (ANOVA test).

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MeGRXC3 genomic DNA sequence in the draft cassava genome (Data S5). The association mapping between SNPs and DTCs was performed as described in Appendix S1.

Plant materials and growth conditions

To study drought stress, stems of different cassava genotypes were cultured in pots for 90 days under greenhouse conditions (12 h/12 h of light/dark, 30 °C/25 °C day/night). The plants were kept in pots, which were 16 cm in diameter × 14 cm in height, containing well-mixed soil (soil : vermiculite : pellets, 1 : 1 : 1). We used cassava cultivar cv. 60444 as the wild type for this study because it is growing well-mixed soil (soil : vermiculite : pellets, 1 : 1 : 1). We used cassava cultivar cv. 60444 as the wild type for this study because it can be used as a transgenic acceptor from cassava cultivars, and we produced transgenic cassava plants using this genotype as a background. For Arabidopsis plants for transformation, we used Col-0 as the wild type and grew plants under 12 h light/12 h dark at 20 °C until the primary inflorescences were 5–15 cm tall and a secondary inflorescence appeared at the rosette.

Drought treatment of cassava plants

For drought treatments, we treated eight different cassava cultivars, the wild type and transgenic cassava plants by water withholding. Continuously watered plants were used as controls. In each treatment, we used more than five plants of each line or genotype. We repeated the treatments twice for biological replication. After applying drought stress for 20 days, we re-watered all the treated wild type and transgenic plants. Following 7 days of normal watering, we determined survival rates. Throughout the experiment, we monitored soil moisture content of each pot using a moisture sensor once daily.

Quantitative real-time PCR

We isolated total RNA from tissues of cassava using an RNAprep Pure Plant Kit (TIANGEN, Beijing, China), and we synthesized cDNA with FastQuant RT Kits (TIANGEN). We performed gene expression analysis in cassava by qPCR with gene-specific primers (Data S4). All qPCR reactions were carried out in triplicate. To evaluate the quantity of the amplified qPCR products, we used the comparative ΔΔCT method.

Cassava transformation and molecular analysis of transgenic plants

We generated a hairpin RNA construct and a 35S:MeGRXC3phRNA construct as per the method described in the reference (Ruan et al., 2017). We transformed friable embryogenic calluses of cassava cv.60444 using Agrobacterium strain LBA4404 carrying either DNA construct 35S:MeGRXC3::GFP or 35S:MeGRXC3phRNA, and we selected transgenic cassava plants as previously described by Zainuddin et al. (2012). The transgenic cassava was identified by Southern blot and qPCR as in our previously reported (Ruan et al., 2017). For identification of MeGRXC3-OE transgenic cassava, we performed Western blot by using anti-GFP as an antibody to detect the fused MeGRXC3::GFP protein. We imaged GFP fluorescence to identify subcellular localization of MeGRXC3 in the transgenic lines.

PEG treatments with transgenic cassava in vitro plantlets

For PEG treatment, we prepared two-layered media. The upper layer was a 3-cm-thick CBM, and the lower layer was either CBM (control) or CBM containing 8% PEG6000. We cut ~2 cm shoots of 40-day-old in vitro plantlets of wild-type and transgenic cassava plants and cultured them on the upper layer of the medium. Plantlets were cultured at 26 °C under 12-h light/12-h dark in a versatile environmental test chamber. After growing the plantlets for 50 days, we measured the weight of at least ten plantlets of each line to calculate biomass. Biomass of wild-type plantlets that grew in control medium was set to 100% in the assay.

Determination of stomatal conductance and rate of loss water

Using a microscope, we imaged the abaxial epidermis of mature leaves of cassava during drought stress, and we qualified stomata as being closed or open according to their aperture. We detected transpiration rate by Li-6400XT (LI-COR, Lincoln, NE) in six mature leaves (4th, 5th, 6th, 7th, 8th and 9th from the apical meristem). To calculate and average stomatal index, we used these six leaves from each line. For measurement of rate of water loss, we excised five mature leaves (5th, 6th, 7th, 8th and 9th from the apical meristem) from five unstressed transgenic or wild-type plants and kept them on plastic dishes at room temperature. We performed weight measurement at 2, 4, 8 and 24 h after excision. The rate of water loss was calculated by comparison with the initial fresh weight.

Determination of endogenous ABA and JA contents

We determined endogenous ABA and JA contents by extraction and detection using liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) according to methods described previously (Ross et al., 2004). We extracted 100 mg of mixed leaf sample with 1.5 mL of methanol formic acid solution (Methanol : formic acid : water = 7.8 : 0.2 : 2). Each sample comprised homogenized mature leaf tissue from three plants of each line as one biological replicate. We used three biological replicates for the results and a total of nine plants per line.
Determination of H$_2$O$_2$ quantity and enzyme assays

To measure the H$_2$O$_2$ content and SOD, POD and CAT activity in the mature leaves, we used the appropriate detection kit (H$_2$O$_2$ #BC3595; SOD #BC0175; POD #BC0195, CAT #BC0205, Solarbio, Beijing, China) following the manufacturer’s instructions. Mature leaves from three plants of each line comprised one biological replicate. We used three biological replicates for the results and a total of nine plants per line.

Visualization of H$_2$O$_2$

H$_2$O$_2$ treatment of cassava leaves was performed as described in the reference (Xu et al., 2013b). For cassava leaves, H$_2$O$_2$ was visualized by staining with diamobenzidine (DAB) according to a widely accepted protocol. After infiltration with 2 mL of DAB solution (1 mg/mL DAB, pH 3.8) in an Eppendorf tube for 12 h, the leaves were immersed in 95% (w/v) boiling ethanol for 10 min to decolorize the chloroplasts. Epidermal peels from the abaxial surface of stained leaves were peeled off and imaged by a Zeiss Scope A1 Imaging System. The relative value of DAB staining in guard cells or epidermal cells was calculated using ImageJ. We performed calculations for at least 200 cells of each leaf.

For the ROS accumulation assay in guard cells of ABA-treated leaves, we infiltrated prepared epidermal peels with 50 μl 2,7-dichlorofluorescin diacetate (DCFH-DA) for 30 min. The resulting fluorescence was imaged by a confocal laser scanning microscope with excitation/emission at 488/515 nm. We calculated the fluorescent intensity from at least 50 guard cells of each leaf by FV10-ASW (Olympus, Tokyo, Japan).

Stomatal movement assay

We performed ABA-induced stomatal closing assays using mature leaves from cassava plants as previous reported (Sharma et al., 2015) with slight modification. Prepared epidermal peels of the mature leaves were incubated in stomatal opening solution (10 mM KCl, 100 mM CaCl$_2$ and 10 mM MES, pH 6.1) for 12 h followed by incubation in stomatal opening solution supplemented with varying concentrations of ABA (0, 10, 50 and 100 μM) for eight more hours. We acquired photographs of stomata via an EVOS FL Imaging System and measured the stomatal aperture using the same software. We measured at least 200 stomata of each sampled mature leaf.

Immunoprecipitation and liquid chromatography and mass spectrometry (LC-MS/MS)

We extracted total protein from mature leaves of OE#88 transgenic cassava seedlings by IP lysis solution (#G2038, Servicebio, Wuhan, China). For input analysis, 40 μl transgenic cassava seedlings by IP lysis solution (#G2038, Servicebio, Wuhan, China) performed a Y2H against a cDNA library of cassava mature leaves to screen for proteins potentially interacting with MeGRXC3, we performed a Y2H against a cDNA library of cassava mature leaves based on the Matchmaker Gold Yeast Two-Hybrid System User Manual using the DNA construct of MeGRXC3p65L:pGBK7T7 as bait. We introduced cDNA sequences of putative interacting proteins, MeCAT1, MeCAT2, MeCAT7 and MeTGA2, into the pGADT7 or pGBK7T7 expression vectors. These constructs were pairwise with MeGRXC3p65L:pGBK7T7/MeGRXC3:pGADT7 and co-transformed into yeast strain Y2HGold. We confirmed the presence of transformation by growth on DDO (SD/-Leu/-Trp) plates. We confirmed interactions between MeGRXC3 and MeCAT1/MeCAT2/MeTGA2 based on growth on QDO/X/A medium as described in the Y2H manual.

Bimolecular fluorescence complementation assay

To confirm the interactions between MeGRXC3 and MeCAT1/MeCAT2/MeTGA7, we performed a BIFC assay via a tobacco transient system as previously reported (Ruan et al., 2018). The full-length coding sequence without the stop-codon of MeGRXC3 was fused to the N- or C-terminus of yellow fluorescent protein (YFP) fragments (NYFP/CYFP) in frame to produce 35S:MeGRXC3:NYFP and 35S:MeGRXC3:CYFP. The full-length coding sequence without the stop-codon of MeCAT1, MeCAT2, MeCAT7 and MeTGA3 was fused in frame to CYFP or NYFP, respectively, to produce 35S:MeCAT1:CYFP, 35S:MeCAT2:CYFP, 35S:MeCAT7:CYFP, 35S:MeTGA2:CYFP, 35S:MeCAT1:NYFP, 35S:MeCAT2:NYFP, 35S:MeCAT7:NYFP and 35S:MeTGA2:NYFP. The 35S:mCherry (RFP) plasmid was used as a reference.

DNA-affinity purification sequencing

We performed DAP-seq binding assays as described previously (Bartlett et al., 2017; O’Malley et al., 2016) with modifications as described briefly by Yao et al. (2020). We used BLAST (Altschul et al., 1990) against the cassava genome database (https://phytozome.jgi.doe.gov, M. esculenta v8.1) to identify potential target genes for annotation. Motifs were revealed using the MEME-ChIP suite 5.0.5 (Machanick and Bailey, 2011).

Electrophotoreic mobility shift assay

The MeMYB63 and MeTGA2 were expressed in the Rosetta Escherichia coli strain. The protein extraction and purification were described in Appendix S1. Briefly, the supernatant comprises soluble (NPE) and denatured insoluble sediment (DPE) were determined by SDS-PAGE. Then, the MeMYB63 protein was qualified by SDS-PAGE, and MeTGA2 was checked by Western blot with anti-His as the antibody. We designed and labelled probes with biotin and mixed 2 μg of the purified protein MeMYB63 or MeTGA2 with the assigned probe at 25 °C for 20 min. We performed Western blot with Streptavidin-HRP Conjugate to detect the shift in bands.

Dual-luciferase (LUC/REN) reporter assay

We constructed reporter plasmids by cloning the MeCAT7 and MeMYB63 promoters into pGreenII0800-luc respectively to form proMeCAT7:pGreenII0800-luc and proMeMYB63:pGreenII0800-luc. For analysis of the MeCAT7 promoter, the above-mentioned 35S:MeMYB63:GFP was used as the effector, while 35S: MeTGA2:GFP and 35S:MeGRXC3:GFP were used as effectors in analysis of the MeMYB63 promoter. We performed assays following the reference (Helliens et al., 2005). The relative LUC/REN was analysed using the Dual-Luciferase Reporter Assay System (#0000469205, Madison, Promega, WI).
Statistical analyses

One-way ANOVA followed by Dunnett’s multiple comparisons test was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla CA, www.graphpad.com). In figures, different letters indicate significant differences of P < 0.05.

Accession numbers

Gene accession numbers were listed as follows: MeGRXC3 (Manes.01G215000), MeCAT7 (Manes.05G130500), MeCAT2 (Manes.05G130700), MeCAT4 (Manes.18G004500), MeCAT7 (Manes.02G113300), MeTGA2 (Manes.04G157200), MeTGA9 (Manes.12G140100), MeMYB63 (Manes.06G175200) and MeHs-tone3 (Manes.13G097500). RNA-seq data from this study can be found in the GenBank/EMBL data libraries under following accession number: PRJNA797171.

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Conflicts of interest

The authors declare there are no conflicts of interest.

Author contributions

MR planned and designed the research. MR, XG, XY and MG performed data analyses. MR and XG wrote the manuscript. PZ, LZ and WL carried out the experiments. MR, XG, XY and MG performed data analyses. MR and XG wrote the manuscript. PZ, MP and MR revised the manuscript.

Data availability statement

The data that support the findings of this study are available in the supplementary material of this article.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Alves, A.A. and Setter, T.L. (2004) Response of cassava leaf area expansion to water deficit: cell proliferation, cell expansion and delayed development. Ann. Bot. 94, 605–613.

Bartlett, A., O’Malley, R.C., Huang, S., Galli, M. and Ecker, J.R. (2017) Mapping genome-wide transcription-factor binding sites using DAP-seq. Nat. Protoc. 12, 1659–1672.

Boyer, J.S. (1982) Plant productivity and environment. Science, 218, 443–448.

Chen, L., Wu, R., Feng, J., Feng, T., Wang, C., Hu, J., Zhan, N. et al. (2020) Transmittosylation mediated by the non-canonical catalase ROG1 regulates nitric oxide signaling in plants. Dev. Cell, 53, 444–457.45.

Claeys, H. and Inze, D. (2013) The agony of choice: how plants balance growth and survival under water-limiting conditions. Plant Physiol. 162, 1768–1779.

Ding, S., He, F., Tang, W., Du, H. and Wang, H. (2019a) Identification of maize CC-type glutaredoxins that are associated with response to drought stress. Genes (Basel), 10, 610.

Ding, Z., Wu, C., Tie, W., Yan, Y., He, G. and Hu, W. (2019b) Strand-specific RNA-seq-based identification and functional prediction of lncRNAs in response to melatonin and simulated drought stresses in cassava. Plant Physiol. Biochem. 140, 96–104.

El-Kereamy, A., Bi, Y.M., Mahmood, K., Ranathunge, K., Yaish, M.W., Nambara, E. and Rothstein, S.J. (2015) Overexpression of the CC-type glutaredoxin, OsGRX6 affects hormone and nitrogen status in rice plants. Front. Plant Sci. 6, 934.

Gao, Y., Huang, C., Xie, Y., Song, F. and Zhou, X. (2010) A tomato glutaredoxin gene SGRX1 regulates plant responses to oxidative, drought and salt stresses. Planta, 232, 1499–1509.

Gutsche, N., Thurow, C., Zachgo, S. and Gatz, C. (2015) Plant-specific CC-type glutaredoxins: functions in developmental processes and stress responses. Biol. Chem. 396, 495–509.

Hellens, R.P., Allan, A.C., Friel, E.N., Bolitho, K., Grafton, K., Templeton, M.D., Karunaretanum, S. et al. (2005) Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. Plant Methods, 1, 13.

Hillocks, R.J., Thed, J.M. and Bellotti, A. (2002) Cassava: Biology, Production, and Utilization. Wallingford, Oxon, UK, New York, NY: CABI Pub.

Hu, Y., Wu, Q., Peng, Z., Sprague, S.A., Wang, W., Park, J., Akhunov, E. et al. (2017) Silencing of OsGRXS17 in rice improves drought stress tolerance by modulating ROS accumulation and stomatal closure. Sci. Rep. 7, 15950.

Kengkanna, J., Jakaew, P., Amawan, S., Busener, N., Bucksch, A. and Saengwilai, P. (2019) Phenotypic variation of cassava root traits and their responses to drought. Appl. Plant Sci. 7, e01238.

Kollist, H., Nuhiat, M. and Roelfsema, M.R. (2014) Closing gaps: linking elements that control stomatal movement. New Phytol. 203, 44–62.

Laporte, D., Olatte, E., Salinás, P., Salazar, M., Jordana, X. and Holgué, L. (2012) Glutaredoxin GKS13 plays a key role in protection against photooxidative stress in Arabidopsis. J. Exp. Bot. 63, 503–515.

Lee, S., Seo, P.J., Lee, H.J. and Park, C.M. (2012) A NAC transcription factor NTL4 promotes reactive oxygen species production during drought-induced leaf senescence in Arabidopsis. Plant J. 70, 831–844.

Li, N., Muthreicht, M., Huang, L.J., Thurow, C., Sun, T., Zhang, Y. and Gatz, C. (2019) TGACG-BINDING FACTORS (TGAs) and TGA-interacting CC-type glutaredoxins modulate hyponastic growth in Arabidopsis thaliana. New Phytol. 221, 1906–1918.

Li, S., Gutsche, N. and Zachgo, S. (2011) The ROXY1 C-terminal L**L motif is essential for the interaction with TGA transcription factors. Plant Physiol. 157, 2056–2068.

Li, S., Lauri, A., Ziemann, M., Busch, A., Bhave, M. and Zachgo, S. (2009) Nuclear activity of ROXY1, a glutaredoxin interacting with TGA factors, is required for petal development in Arabidopsis thaliana. Plant Cell, 21, 429–441.

Liang, Y.K., Dubos, C., Dodd, I.C., Holroyd, G.H., Hetherington, A.M. and Campbell, M.M. (2005) AtMYB61, an R2R3-MYB transcription factor controlling stomatal aperture in Arabidopsis thaliana. Curr. Biol. 15, 1201–1206.

Liao, W., Wang, G., Li, Y., Wang, B., Zhang, P. and Peng, M. (2016) Reactive oxygen species regulate leaf pulvinus abscission zone cell separation in response to water-deficit stress in cassava. Sci. Rep. 6, 21542.

Lindermayr, C., Sell, S., Muller, B., Leister, D. and Durner, J. (2010) Redox regulation of the NPR1-TGA1 system of Arabidopsis thaliana by nitric oxide. Plant Cell, 22, 2894–2907.

Machnick, P. and Bailey, T.L. (2011) MEME-Chip: motif analysis of large DNA datasets. Bioinformatics, 27, 1696–1697.

Meyer, Y., Belin, C., Delorme-Hinoux, V., Reichheld, I.-P. and Riondet, C. (2012) Thioredoxin and glutaredoxin systems in plants: molecular mechanisms, crosstalks, and functional significance. Antioxid. Redox Signal. 17, 1124–1160.

Murmu, J., Bush, M.J., DeLong, C., Li, S., Xu, M., Khan, M., Malcolmson, C. et al. (2010) Arabidopsis basic leucine-zipper transcription factors TGA9 and TGA10 interact with floral glutaredoxins ROXY1 and ROXY2 and are
redundantly required for anther development. *Plant Physiol.* **154**, 1492–1504.

Ndamukong, I., Abdallat, A.A., Thourow, C., Fode, B., Zander, M., Weigel, R. and Gatz, C. (2007) SA-inducible Arabidopsis glutaredoxin interacts with TGA factors and suppresses JA-responsive PDF1.2 transcription. *Plant J.* **50**, 128–139.

Ogbonnaya, F.C., Rasheed, A., Okechukwu, E.C., Jighly, A., Makdis, F., Wuletat, T., Hagras, A. et al. (2017) Genome-wide association study for agronomic and physiological traits in spring wheat evaluated in a range of heat prone environments. *Theor. Appl. Genet.* **130**, 1819–1835.

O’Malle, R.C., Huang, S.C., Song, L., Lwesy, M.G., Bartlett, A., Nery, J.R., Galli, M. et al. (2016) Cisrome and epistrome features shape the regulatory DNA landscape. *Cell*, **165**, 1280–1292.

Pei, Z.M., Murata, Y., Benning, G., Thomine, S., Klusener, B., Allen, G.J., Grill, E. et al. (2000) Calcium channels activated by hydrogen peroxide mediate asbic acid signalling in guard cells. *Nature* **406**, 731–734.

Prouse, M.B. and Campbell, M.M. (2013) Interactions between the R2R3-MYB transcription factor, AtMYB61, and target DNA binding sites. *PLoS ONE* **8**, e65132.

Romano, J.M., Dubos, C., Prouse, M.B., Wilkins, O., Hong, H., Poole, M., Kang, K.Y. et al. (2012) AtMYB61, an R2R3-MYB transcription factor, functions as a pleiotropic regulator via a small gene network. *New Phytol.* **195**, 774–786.

Romero-Romero, J.L., Inostroza-Blancheteau, C., Orellana, D., Aquea, F., Reyes-Diaz, M., Gil, P.M., Matte, J.P. et al. (2018) Stomata regulation by tissue-specific expression of the Citrus sinensis MYB61 transcription factor improves water-use efficiency in Arabidopsis. *Plant Physiol. Biochem.* **130**, 54–60.

Ross, A.R., Ambrose, S.J., Cutler, A.J., Feurtado, J.A., Kermode, A.R., Nelson, W., Wang, X., Zou, X., Xie, S., Gu, J. and Wang, Z.Y. (2021) Identification of cassava alternative splicing-related genes and functional characterization of MeSCL30 involvement in drought stress. *Plant Physiol. Biochem.* **160**, 130–142.

Witzczak, M., Brosche, M. and Kangasjärvi, J. (2013) ROS signaling loops – production, perception, regulation. *Curr. Opin. Plant Biol.* **16**, 575–582.

Xing, S. and Zachgo, S. (2008) ROXY1 and ROXY2, two Arabidopsis glutaredoxin genes, are required for anther development. *Plant J.* **53**, 790–801.

Xu, J., Duan, X., Yang, J., Beeching, J.R. and Zhang, P. (2013a) Coupled expression of CuZn-superoxide dismutase and catalase in cassava improves tolerance against cold and drought stresses. *Plant Signal. Behav.* **8**, e24525.

Xu, J., Duan, X., Yang, J., Beeching, J.R. and Zhang, P. (2013b) Enhanced reactive oxygen species scavenging by overexpression of superoxide dismutase and catalase delays postharvest physiological deterioration of cassava storage roots. *Plant Physiol.* **161**, 1517–1528.

Yan, J., Tsuichihara, N., Etioh, T. and Iwai, S. (2007) Reactive oxygen species and nitric oxide are involved in ABA inhibition of stomatal opening. *Plant Cell Environ.* **30**, 1320–1325.

Yan, Y., Wang, P., Lu, Y., Bai, Y., Wei, Y., Liu, G. and Shi, H. (2021a) MeRAV5 promotes drought stress resistance in cassava by modulating hydrogen peroxide and lignin accumulation. *Plant J.* **107**, 847–860.

Yan, Y., Wang, P., Wei, Y., Bai, Y., Lu, Y., Zeng, H., Liu, G. et al. (2021b) The dual interplay of RAV5 in activating nitrate reductases and repressing catalase activity to improve disease resistance in cassava. *Plant Biotech. J.* **19**, 785–800.

Yang, Y., Liao, W., Yu, X., Wang, B., Peng, M. and Ruan, M. (2016) Overexpression of MeDREB1D confers tolerance to both drought and cold stresses in transgenic Arabidopsis. *Acta Physiol. Plant.* **38**, 243.

Yao, J., Shen, Z., Zhang, Y., Wu, X., Wang, J., Sa, G., Zhang, Y. et al. (2020) Populus euphratica WRKY1 binds the promoter of H+-ATPase gene to enhance gene expression and salt tolerance. *J. Exp. Bot.* **0**, 1527–1539.

Zainuddin, I.M., Schlegel, K., Gruissem, W. and Vanderschuren, H. (2012) Robust transformation procedure for the production of transgenic farmer-preferred cassava landraces. *Plant Methods* **8**, 24.

Zander, M., Chen, S., Imkampke, J., Thourow, C. and Gatz, C. (2012) Repression of the Arabidopsis thaliana jasmonic acidicylhydrogen-induced defense pathway by TGA-interacting glutaredoxin depends on its C-terminal AMLW motif. *Mol. Plant* **5**, 831–840.

Zhao, P., Liu, P., Shao, J., Li, C., Wang, B., Guo, X., Yan, B. et al. (2014) Analysis of different strategies adapted by two cassava cultivars in response to drought stress: ensuring survival or continuing growth. *J. Exp. Bot.* **66**, 1477–1488.

Zhao, Y., Chan, Z., Gao, J., Xing, L., Cao, M., Yu, C., Hu, Y. et al. (2016) ABA receptor PYL9 promotes drought resistance and leaf senescence. *Proc. Natl Acad. Sci. USA* **113**, 1949–1954.

Zhou, J., Lee, C., Zhong, R., and Ye, Z.H. (2009) MYB88 and MYB63 are transcriptional activators of the lignin biosynthetic pathway during secondary cell wall formation in Arabidopsis. *Plant Cell* **21**, 248–266.

Zhu, M., Geng, S., Chakravorty, D., Guan, Q., Chen, S. and Assmann, S.M. (2020) Metabolomics of red-light-induced stomatal opening in Arabidopsis thaliana: coupling with asbassic acid and jasmonic acid metabolism. *Plant J.* **101**, 1331–1348.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** Overexpression of *MeGRXC3* confers PEG and drought sensitivity in transgenic *Arabidopsis*.

**Figure S2** Molecular analysis of transgenic cassava.

**Figure S3** Expression analysis of other drought-responsive CC-type GRXs in *MeGRXC3-RNA* transgenic cassava.

**Figure S4** Leaf positions of cassava seedling.

**Figure S5** Cassava stomata.

**Figure S6** Expression analysis of cassava catalase genes in mature leaf.

**Figure S7** The *MeGRXC3* transgene affects ABA-induced stomatal closure.
MeGRXC3 negatively regulates drought tolerance of cassava.

Figure S8 Yield of tuber roots in the field in transgenic cassava under drought conditions.

Figure S9 Alignment of catalases from cassava and Arabidopsis.

Figure S10 Subcellular localization of MeCAT1, MeCAT2, MeCAT7, MeGRXC3 and MeHistone3.

Figure S11 qPCR analysis of six transcription factors in wild-type and transgenic cassava under drought conditions.

Figure S12 RNA-seq analysis of wild-type and transgenic cassava under drought conditions.

Appendix S1 Methods.

Data S1 Target proteins of MeGRXC3 identified by CoIP with LC-MS/MS in MeGRXC3 OE transgenic cassava.

Data S2 DAP-seq results of MeTGA2 in cassava.

Data S3 Candidate targets prediction of MeTGA2 based on DAP-seq analysis.

Data S4 Primers used in this study.

Data S5 SNPs in MeGRXC3 genomic DNA.

Data S6 RNA-seq results of transcription factors, including AP2/ERF, MYB, TCP and WRKY in wild-type and transgenic cassava.