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# The chaperone MeHSP90 recruits MeWRKY20 and MeCatalase1 to regulate drought stress resistance in cassava

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## Summary

- The 90 kDa heat shock protein (HSP90) is widely involved in various developmental processes and stress responses in plants. However, the molecular chaperone HSP90-constructed protein complex and its function in cassava remain elusive.
- In this study, we report that HSP90 is essential for drought stress resistance in cassava by regulating abscisic acid (ABA) and hydrogen peroxide ($H_2O_2$) using two specific protein inhibitors of HSP90 (geldanamycin (GDA) and radicicol (RAD)). Among 10 *MeHSP90s*, the transcript of *MeHSP90.9* is largely induced during drought stress. Further investigation identifies MeWRKY20 and MeCatalase1 as MeHSP90.9-interacting proteins. *MeHSP90.9*-, *MeWRKY20*-, or *MeCatalase1*-silenced plants through virus-induced gene silencing display drought sensitivity in cassava, indicating that they are important to drought stress response.
- MeHSP90.9 can promote the direct transcriptional activation of MeWRKY20 on the W-box element of *MeNCED5* promoter, encoding a key enzyme in ABA biosynthesis. Moreover, MeHSP90.9 positively regulates the activity of MeCatalase1, and *MeHSP90.9*-silenced cassava leaves accumulate more $H_2O_2$ under drought stress.
- Taken together, we demonstrate that the MeHSP90.9 chaperone complex is a regulator of drought stress resistance in cassava.

## Introduction

Cassava (*Manihot esculenta*) is one of the most important food and energy crops in the world. Due to its relatively high resistance to drought and poor nutrient environment, it can be widely naturalized in arid regions, such as Africa, and it will not compete for soils resources with other crops. However, persistent drought also strongly affects the growth and development of cassava plants and subsequently restricts its production (El-Sharkawy, 2004). Previous studies have revealed that the drought-resistant feature of cassava involves the deep root system, which is highly sensitive to air humidity and basal leaf abscission to limit water loss (El-Sharkawy & Cock, 1987; Alves & Setter, 2000; El-Sharkawy, 2004). To date, the key regulators and underlying mechanisms of cassava drought stress response remain elusive.

As a highly conserved molecular chaperone, 90 kDa heat shock protein (HSP90) occurs widely in animals and plants (Pearl & Prodromou, 2000; Queitsch *et al.*, 2002). Because of their function in facilitating client protein refolding, HSP90s are widely involved in stress signal transduction, such as temperature, drought, salt stress, and pathogen infection (Hubert *et al.*, 2003, 2009; Bao *et al.*, 2014; Tillmann *et al.*, 2015; Bui *et al.*, 2016). Overexpression of *AtHSP90.2*, *AtHSP90.5*, and *AtHSP90.7* results in altered plant sensitivity to salt and drought stresses in *Arabidopsis* (Song *et al.*, 2009), and overexpression of *GmHSP90s* compromises abiotic stress-induced damages in *Arabidopsis* (Xu *et al.*, 2013b). Meanwhile, AtHSP90 has been reported to modulate plant growth and development under elevated temperature (Wang *et al.*, 2016). Moreover, genetic analysis has revealed that HSP90 is required for *resistant* (*R*) gene-mediated immune responses in plants (Hubert *et al.*, 2009; Huang *et al.*, 2014; Xie *et al.*, 2015). In addition, chloroplast-targeted HSP90.5 interacts with vesicle-inducing protein in plastids 1 (VIPP1) and has essential function in plastid development and embryogenesis in *Arabidopsis* (Feng *et al.*, 2014). Moreover, HSP90 modulates brassinosteroid (BR) signaling via interacting with the BR signaling components, including BRASSINAZOLE RESISTANT 1 (BZR1), BRASSINOSTEROID INSENSITIVE 1-EMS-SUPRESSOR 1 (BES1) and BRASSINOSTEROID INSENSITIVE 2 (BIN2) (Samakovli *et al.*, 2014; Shigeta *et al.*, 2015). Recently, Margaritopoulou *et al.* (2016) revealed that HSP90 interacts with major flowering regulators, including SUPPRESSOR OF CONSTANS OVEREXPRESSOR 1

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(SOC1), AGAMOUS LIKE 24 (AGL24), LEAFY (LFY) and SHORT VEGETATIVE PHASE (SVP), resulting in a molecular scaffold mechanism to progress flowering. AtHSP90 is also a chaperone for the auxin receptor transport inhibitor response 1 (TIR1) and buffers auxin-responsive phenotypes (Wang *et al.*, 2016; Watanabe *et al.*, 2016). Except for mutant analysis, two protein inhibitors (geldanamycin (GDA) and radicicol (RAD)) are also widely used to reveal the functions of HSP90s in plants (Baro *et al.*, 2014; Queitsch *et al.*, 2002; Samakovli *et al.*, 2014; Wang *et al.*, 2016; Watanabe *et al.*, 2016). So far, the HSP90 gene family has been reported on a genome-wide basis in several plant species, including *Arabidopsis* (Krishna & Gloor, 2001), *Populus trichocarpa* (Zhang *et al.*, 2013), tomato (Zai *et al.*, 2015), chickpea and pigeonpea (Agarwal *et al.*, 2016), foxtail millet (Singh *et al.*, 2016), and *Brachypodium distachyon* (Zhang *et al.*, 2017). However, cassava *HSP90* genes have not been identified and their functions remain to be further characterized.

The WRKY transcription factor (TF) family is one of the largest gene families in plants (Rushton *et al.*, 2010; Chen *et al.*, 2012; Hu *et al.*, 2013a; Liu *et al.*, 2014; Lei *et al.*, 2017; Hu *et al.*, 2018; Li *et al.*, 2018). WRKY is named after the highly conserved WRKYGQK motif within the WRKY domain of *c.* 60 amino acids (Eulgem *et al.*, 2000; Rushton *et al.*, 2010). A total of 72 WRKY proteins have been identified from the genome databases of *Arabidopsis* (Eulgem *et al.*, 2000; Dong *et al.*, 2003). In rice, Ross *et al.* (2007) reported 98 and 102 WRKYs in *japonica* and *indica* rice, respectively. Moreover, 133 WRKYs have been identified in soybean (Yin *et al.*, 2013), and 116 WRKYs have been identified in cotton (Dou *et al.*, 2014). Ma *et al.* (2017) identified 188 TaWRKYs and analyzed their evolutionary history. In our previous study, we identified 85 MeWRKYs and analyzed their expression profiles in response to various abiotic stresses (Wei *et al.*, 2016b). Current studies have indicated that WRKY TFs are widely involved in regulating plant abiotic stress responses, such as drought, salt stress, and ABA signaling (Rushton *et al.*, 2012; Liu *et al.*, 2014; Gong *et al.*, 2015; Kayum *et al.*, 2015; Singh & Laxmi, 2015; He *et al.*, 2016). As a WRKY TF, abscisic acid (ABA) overly sensitive 3 (ABO3) modulates ABA-mediated stomatal closure and drought stress resistance in *Arabidopsis* (Ren *et al.*, 2010). Overexpression of *TaWRKY1* and *TaWRKY33* (He *et al.*, 2016), *TaWRKY146* (Ma *et al.*, 2017), and *VaWRKY14* (Zhang *et al.*, 2018) results in enhanced drought stress resistance in *Arabidopsis*. Our previous data also suggested that some *MeWRKYs* exhibit differential expression levels in response to drought stress, indicating the possible involvement of *MeWRKYs* in drought stress resistance in cassava. However, the upstream and downstream elements of WRKY-modulated plant drought stress response need to be further investigated.

Abscisic acid is essential for plant drought stress resistance (MacRobbie, 2000; Lim *et al.*, 2015; Cai *et al.*, 2017). Under drought stress, endogenous ABA content increases rapidly, which results in stomatal closure to retain water in the plant and induction of stress-responsive genes to cope with water deficiency (Xie *et al.*, 2006; Zhang *et al.*, 2014). Carotenoids and xanthophylls are the precursors of ABA biosynthesis (Taylor *et al.*, 2000). The 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) catalyze the oxidative cleavage of 9-*cis*-neoxanthin into xanthoxin (Schwartz *et al.*, 1997), which is thought to be the rate-limiting step in the ABA biosynthesis pathway (Xiong & Zhu, 2003). In addition, the transcripts of *NCED* genes have been reported to be induced upon drought stress treatment in many plants, such as maize, tomato, bean, *Arabidopsis*, cowpea, and avocado (Xiong & Zhu, 2003; Xian *et al.*, 2014).

In this study, the function of HSP90 in drought stress resistance in cassava is demonstrated. Moreover, the links between the chaperones MeHSP90, MeWRKY20 and MeCatalase1 in drought stress response in cassava are revealed, highlighting the direct regulation of ABA and hydrogen peroxide ($H_2O_2$). The identification of such a complex and module provides a novel mechanism underlying drought stress response in cassava.

## Materials and Methods

### Plant materials and growth conditions

Segments were cut from South China 124 (SC124) cassava variety and cultured in pots with high-quality soil substrates. Briefly, vermiculite : nutrient soil substrates (v/v = 1 : 1) (pH 6.0) (00075320; Pindstrup Mosebrug A/S, Ryomgaard, Denmark) was used as soil, which had a water-holding capacity of *c.* 205.3% (w/w). The same volume (4 l for white pots and 1.85 l for red pots) of soil was placed in every plant pot. The cassava plants were grown in the glasshouse with a 16 h : 8 h, light : dark photoperiod at 26°C. For the drought stress treatment, water was withheld from the cassava plants in soil on assigned days.

### Yeast two-hybrid

The full-length coding sequences (CDS) of *MeHSP90.9*, *MeWRKY20*, and *MeCatalase1* were cloned to pGBKT7, pGADT7, and pGADT7 vectors, respectively and confirmed by sequencing. The specific primers were listed in Supporting Information Table S1. Then, the recombinant bait and prey plasmids were used for yeast two-hybrid assays according to the Matchmaker™ Gold yeast Two-Hybrid System User Manual protocol (Clontech, San Francisco, CA, USA). Briefly, the plasmids were transformed into AH109 by the LiAc/polyethylene glycol method and the positive clones were selected on selective SD medium at 30°C.

### Bimolecular fluorescence complementation (BiFC)

The full-length CDS of *MeHSP90.9*, *MeWRKY20*, and *MeCatalase1* were cloned to pFGC-nYFP, pFGC-cYFP, and pFGC-cYFP (Kim *et al.*, 2008), respectively, and verified by sequencing. The sequencing-confirmed plasmids were then transformed into the *Agrobacterium tumefaciens* GV3101 strain, which, containing the recombinant plasmids or peroxisome marker px-ck (CD3-977) (Nelson *et al.*, 2007), were infiltrated into *Nicotiana benthamiana* leaves for transient expression as previously described (Sparkes *et al.*, 2006). At 2 d post-infiltration,

the yellow fluorescence, peroxisome signals (CFP), and 4′,6-diamidino-2-phenylindole (DAPI)-stained cell nuclei were detected under confocal laser-scanning microscope (TCS SP8; Leica, Heidelberg, Germany).

### Pull-down assay

The CDSs of *MeHSP90.9*, *MeWRKY20*, and *MeCatalase1* were cloned into pET28a and confirmed by sequencing. Thereafter, the recombinant proteins of *MeHSP90.9*-Myc-pET28a, *MeWRKY20*-HA-pET28a, and *MeCatalase1*-HA-pET28a were induced and purified using BeyoGold™ His-tag Purification Resin (P2218; Beyotime, Shanghai, China). The pull-down assay was performed as previously described (Hu *et al.* 2013b). Briefly, hemagglutinin (HA)-fused MeWRKY20 and MeCatalase1 were immunoprecipitated using anti-HA mouse monoclonal antibody (AH158; Beyotime), and Myc-fused MeHSP90.9 was detected using anti-Myc mouse monoclonal antibody (AM926; Beyotime). For Western blotting, about 20 μg protein was loaded onto 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. After protein electrophoresis, the protein samples were transferred to the polyvinylidene fluoride (PVDF) membrane, which was further blocked in 5% (w/v) skim milk powder in 20 mM Tris-HCl (pH 7.5) buffer with 150 mM NaCl and 0.1% Tween 20 at 4°C overnight. The PVDF membrane was then probed using anti-Myc mouse monoclonal antibody (AM926; Beyotime) and horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (H + L) (A0216; Beyotime), and the protein band was examined using Beyo electrochemiluminescence Star Kit (P0018AS; Beyotime) in an ImageQuant LAS 4000 mini-chemiluminescence imaging analyzer (GE Healthcare, Fairfield, CT, USA).

### Quantification of malondialdehyde and relative electrolyte leakage

Malondialdehyde (MDA) contents and relative electrolyte leakage (EL) in cassava leaves were quantified as previously described (Wei *et al.*, 2016a). Briefly, MDA was extracted with chilled thiobarbituric acid reagent, and then the MDA contents were calculated by subtracting the absorbance at 450 and 600 nm from that at 532 nm. The relative EL represented the ratio of initial conductivity to the maximum conductivity according to previous description (Wei *et al.*, 2016a).

### Luciferase (LUC) assay

For the reporter construction, the promoter of *MeNCED5* was cloned to pGreenII0800-LUC to form *35S::REN-pMeNCED5::LUC*. The effector of MeWRKY20-pEGAD has been described previously (Yan *et al.*, 2017). After verification by DNA sequencing, the plasmids were transformed into plant mesophyll protoplasts as previously described (Yoo *et al.*, 2007). The relative LUC was assayed using the Dual Luciferase Reporter Gene Assay Kit (RG027; Beyotime), according to the manufacturer's instructions.

### Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

RNA isolation, cDNA synthesis, and quantitative real-time PCR were performed as described by Wei *et al.* (2017, 2018). Briefly, the gene expression assay of all genes was performed with at least three independent biological replicates, and normalized to *MeEF1a* as the reference gene using the comparative ΔΔCT method. The primers are listed in Table S2.

### Subcellular localization analysis

The CDSs of *MeHSP90.9*, *MeCatalase1*, and *MeNCED5* were amplified and cloned to pEGAD (Cutler *et al.*, 2000) to generate *35S::GFP-MeHSP90.9*, *35S::GFP-MeCatalase1*, and *35S::GFP-MeNCED5*. After the plasmids were verified by DNA sequencing, the green fluorescence of the corresponding plasmid was visualized in transient *N. benthamiana* leaves as previously described (Sparkes *et al.*, 2006; Wei *et al.*, 2017).

### Virus-induced gene silencing (VIGS) in cassava

The partial coding region of *MeHSP90.9*, *MeNCED5*, and *MeCatalase1* were amplified and cloned to pTRV2 (Liu *et al.*, 2002) and confirmed by sequencing. The plasmids were then transformed into the *Agrobacterium tumefaciens* GV3101 strain, and VIGS was performed as previously described (Zeng *et al.*, 2019). Briefly, the positive GV3101 stains were cultivated at 28 to obtain fresh bacterial solution, and further resuspended in 10 mM MES solution with 10 mM $MgCl_2$ and 150 μM acetosyringone. After adjusting $OD_{600}$ to about 1, the bacterial solution of pTRV1 and various pTRV2 constructs were mixed to 1 : 1 and syringe infiltrated into cassava leaves using a 1 ml needle; the cassava plants were then cultivated in the glasshouse.

### Chromatin immunoprecipitation (ChIP)

The ChIP assay was performed as described by Wei *et al.* (2017). Briefly, the native chromatin extraction in the protoplasts with *35S::GFP-MeWRKY20*, nuclease digestion, and native-ChIP (N-ChIP) were performed as described by O'Neill & Turner (2003) and Wei *et al.* (2017). The IgG (A7028; Beyotime) and anti-GFP antibody (AG281; Beyotime) were used for immunoprecipitation of the MeWRKY20-DNA complex. Relative enrichment was then performed with at least three independen biological replicates and determined using the comparative ΔΔCT method. The primers are listed in Table S2.

### Electrophoretic mobility shift assay (EMSA)

The EMSA assay was performed as described by Wei *et al.* (2017). Briefly, the recombinant protein of MeWRKY20-pET28a was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and expressed in *Escherichia coli* BL21. Then the recombinant protein of MeWRKY20-pET28a was mixed with

the probes at 25°C for 2 h and analyzed in the agarose gel as previously described (Ream *et al.*, 2016).

### Quantification of endogenous ABA

For ABA quantification, plant leaves were suspended in 80% methanol (v/v) with 0.5 g $l^{-1}$ citric acid and 100 mg $l^{-1}$ butylated hydroxytoluene overnight at 4°C as previously described (Xiong *et al.*, 2001). Then the endogenous ABA in the supernatant was quantified using an ABA enzyme-linked immunosorbent assay kit (Jianglai Biotechnology, Shanghai, China), according to the manufacturer's instructions. Briefly, the supernatant was added to a 96-well plate and combined with HRP-labeled ABA antibody. After complete washing, 3,3′,5,5′-tetramethylbenzidine (TMB) was added for the reaction, and sulfuric acid solution was then added to terminate it. The absorbance was recorded at 405 nm and the concentration was calculated according to the standard curve.

### Quantification of catalase activity and endogenous $H_2O_2$

Catalase activity (EC 1.11.1.6) and endogenous $H_2O_2$ concentration were quantified as previously described (Shi *et al.*, 2014). Briefly, the exaction and quantification of total protein in cassava leaves and catalase activity assay were performed using the Catalase Assay Kit (S0051; Beyotime). Briefly, catalase activity in the supernatant was determined by quantifying the decomposition of $H_2O_2$. Endogenous $H_2O_2$ concentrations in plant leaves were quantified using 0.1% titanium sulfate in 20% $H_2SO_4$ (v/v) at the absorbance of 410 nm.

### Statistical analysis

All results are shown as the average values and standard deviations (SDs). Data were analyzed by Student's *t*-test and Duncan's range test using SAS v.9.4 software. Different letters indicate significant differences in comparison to the control at $P < 0.05$.

### Accession numbers

Gene accession numbers were listed as follows: *MeHSP90.1* (Me.01G003800), *MeHSP90.2* (Me.14G022300), *MeHSP90.3* (Me.06G078500), *MeHSP90.4* (Me.14G091600), *MeHSP90.5* (Me.02G017200), *MeHSP90.6* (Me.05G203300), *MeHSP90.7* (Me.12G061400), *MeHSP90.8* (Me.06G152800), *MeHSP90.9* (Me.18G012200), *MeHSP90.10* (Me.18G012500), *MeWRKY20* (Me.15G118300), *MeCatalase1* (Me.05G130500), *MeCatalase2* (Me.05G130700), *MeCatalase3* (Me.07G024500), *MeCatalase4* (Me.18G004500), *MeCatalase5* (Me.18G004400), *MeCatalase6* (Me.01G154400), *MeCatalase7* (Me.02G113300), *MeNCED1* (Me.15G122400), *MeNCED2* (Me.17G071200), *MeNCED3* (Me.03G083500), *MeNCED4* (Me.15G102000), *MeNCED5* (Me.03G150400), *MeNCED6* (Me.15G050500), *MeEF1a* (Me.15G054800).

## Results

### MeHSP90 is essential for drought stress resistance in cassava

To investigate whether HSP90 is required for drought stress response in cassava, we examined the effects of two specific protein inhibitors of HSP90 (GDA and RAD) on drought stress resistance. Under drought stress conditions, GDA- and RAD-pretreated cassava plants had more wilted leaves and exhibited more serious dehydration phenotype than control cassava plants (Fig. 1a). In accordance with the phenotype, GDA- and RAD-pretreated cassava leaves showed lower relative leaf water content, higher MDA concentrations and increased relative EL than control cassava leaves (Fig. 1b–d). Moreover, GDA- and RAD-pretreated cassava leaves exhibited less endogenous ABA but more $H_2O_2$ compared with control cassava leaves under both control and drought stress conditions (Fig. 1e,f). These results indicated that MeHSP90 is required for drought stress resistance in cassava.

### MeHSP90.9 physically interacts with MeWRKY20 and MeCatalase1

To reveal the mechanism of HSP90-modulated plant drought stress resistance in cassava, we first analyzed the transcripts of 10 *MeHSP90s* in response to drought stress. We found that the transcript of *MeHSP90.9* was largely induced by drought stress (Fig. S1). Then we cloned the coding sequence of *MeHSP90.9* into the pGBKT7 vector, and performed a yeast two-hybrid assay to identify its interacting proteins by screening a cassava cDNA library. Our results suggested that MeWRKY20 and MeCatalase1 might be potential interacting proteins. The coding sequences of MeWRKY20 and MeCatalase1 were then cloned into pGADT7 vector and coexpressed with MeHSP90.9-pGBKT7 in yeast to confirm the interaction of MeHSP90.9 with MeWRKY20 and MeCatalase1 (Fig. 2a). Meanwhile, pull-down assays were also performed with purified proteins to further detect the direct interactions *in vitro* (Fig. 2b). Moreover, we conducted a BiFC assay in *N. benthamiana* leaves to investigate the *in vivo* interactions. The colocalization of yellow fluorescence and DAPI-stained cell nuclei showed that MeHSP90.9 interacted with MeWRKY20 in nuclei in tobacco leaves (Fig. 2c), which is consistent with the localization of MeHSP90.9 (Fig. S2) and MeWRKY20 (Yan *et al.*, 2017). Additionally, the colocalization of yellow fluorescence and peroxisome signal of px-ck (CD3-977) (Nelson *et al.*, 2007) indicated that MeHSP90.9 interacted with MeCatalase1 in peroxisome in tobacco leaves (Fig. 2c), where MeHSP90.9 and MeCatalase1 colocalized within the cells (Fig. S2). These *in vitro* and *in vivo* assays suggested that MeHSP90.9 could physically interact with MeWRKY20 and MeCatalase1.

### Transcriptional profiles of MeHSP90.9, MeWRKY20, and MeCatalase1 in response to drought stress

To determine the involvement of *MeHSP90.9*, *MeWRKY20*, and *MeCatalase1* in drought stress response, we first monitored their

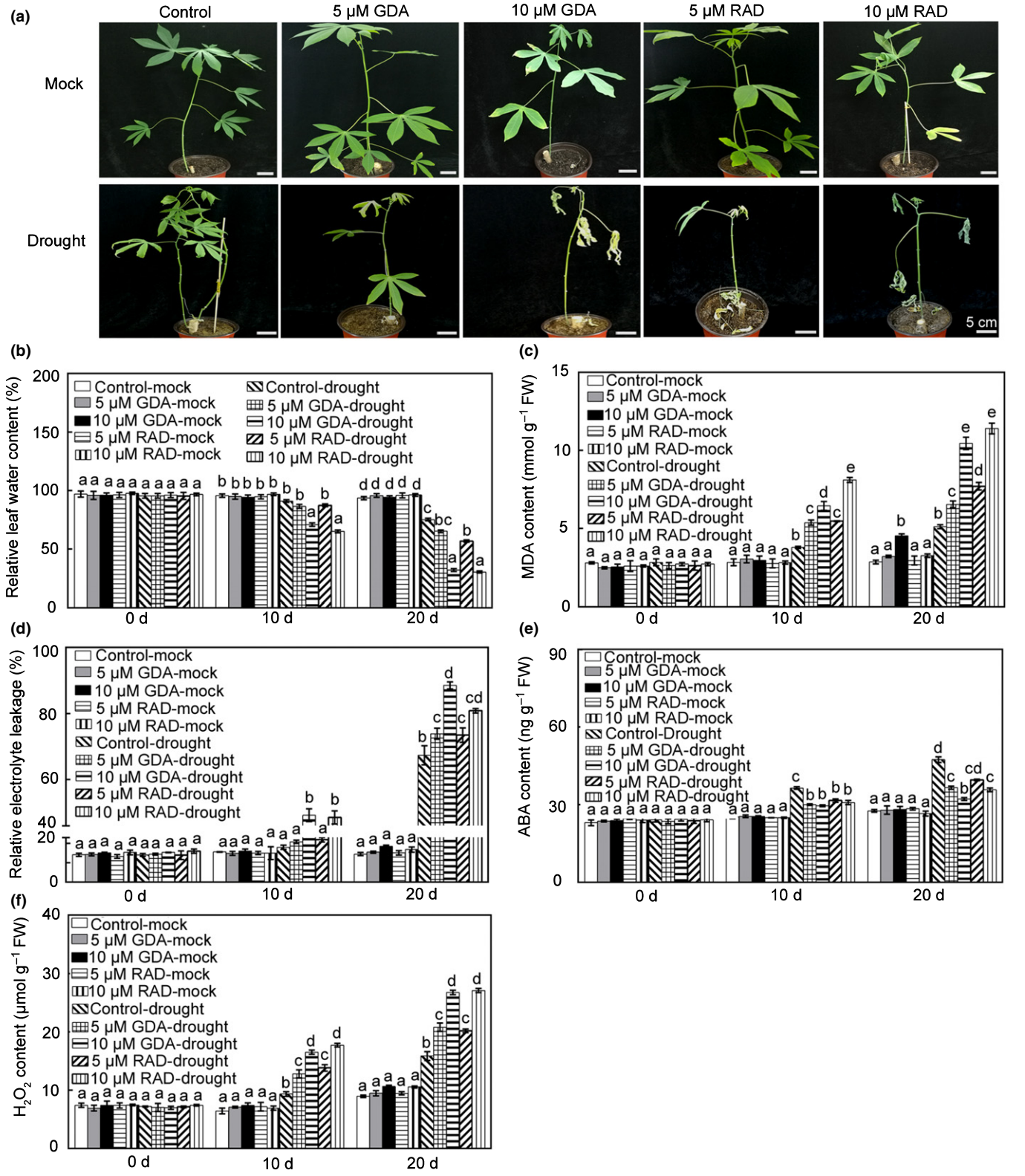


**Fig. 1** MeHSP90 is required for drought stress resistance in cassava. (a) The effects of geldanamycin (GDA) and radicicol (RAD) on drought stress resistance in cassava. Phenotypes of the control, GDA- and RAD-pretreated plants after withholding water for 35 d. Bars, 5 cm. (b–f) Relative leaf water content (b), malondialdehyde (MDA) concentration (c), relative electrolyte leakage (d), endogenous abscisic acid (ABA) level (e) and $H_2O_2$ level (f) in control, GDA- and RAD-pretreated plant leaves under control and drought stress conditions. Error bars are ± SD. Different letters above the bars indicate significant differences at $P < 0.05$ (Duncan's range test).

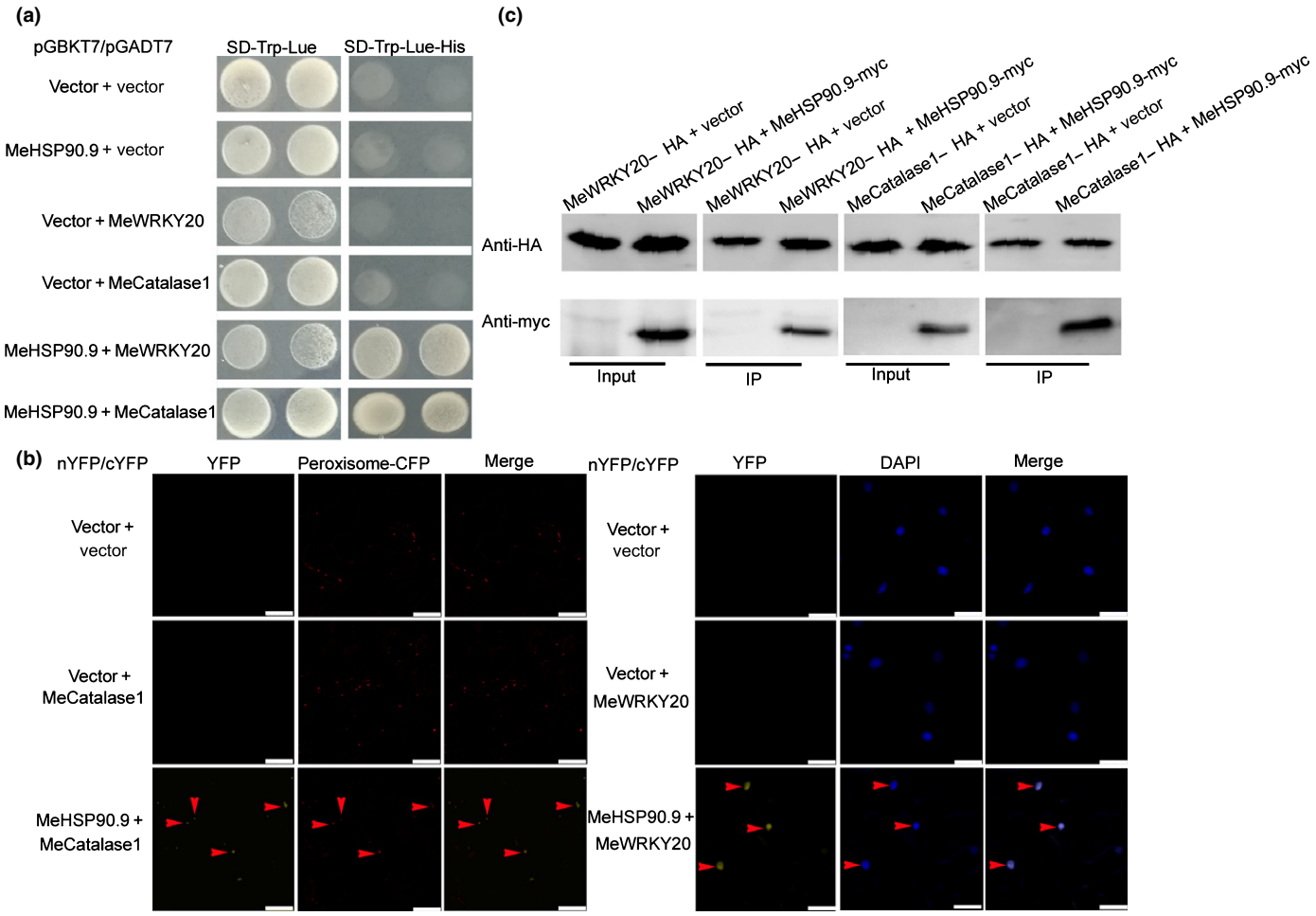


**Fig. 2** MeHSP90 physically interacts with MeWRKY20 and MeCatalase1. (a) MeHSP90.9 interacts with MeWRKY20 and MeCatase1 in yeast two-hybrid assay. (b) Bimolecular fluorescence complementation showing the *in vivo* interaction of MeHSP90.9 with MeWRKY20 and MeCatalase1 in *Nicotiana benthamiana* leaves. Peroxisome marker px-ck signal (CFP) and 4′,6-diamidino-2-phenylindole (DAPI)-stained cell nuclei were examined for colocalization analysis. Bars, 25 μm. (c) Pull-down assay showing *in vitro* interaction of MeHSP90.9 with MeWRKY20 and MeCatalase1. Briefly, hemagglutinin (HA)-fused MeWRKY20 and MeCatalase1 were immunoprecipitated using anti-HA antibody, and Myc-fused MeHSP90.9 was detected using anti-Myc antibody. IP, immunoprecipitation.

expression levels in cassava leaves every 5 d after drought stress. In general, all three genes were significantly upregulated upon drought stress (Fig. 3). The expression of *MeHSP90.9* in cassava leaves was elevated at least 10-fold at each time-point as compared with that in untreated cassava leaves (control), and the expression of *MeWRKY20* and *MeCatalase1* also had a maximum increase of *c.* 500% (Fig. 3). The expression data suggested that *MeHSP90.9*, *MeWRKY20* and *MeCatalase1* may be involved in the drought stress response in cassava.

## MeHSP90.9, MeWRKY20 and MeCatalase1 are required for drought stress resistance in cassava

To reveal the function of *MeHSP90.9*, *MeWRKY20*, and *MeCatalase1* in cassava drought stress resistance, we first constructed the VIGS-mediated gene silencing vectors and transformed into cassava leaves. For TRV-based VIGS in cassava (Wei *et al.*, 2017), the viral transcripts of TRV1 and TRV2 were examined by RT-PCR (Fig. S3a), and the transcripts of the corresponding genes were confirmed by real-time PCR (Fig. S3b), ensuring that silencing of the corresponding genes was induced by TRV. Gene expression analysis indicated that the transcript abundance of the corresponding gene in *MeHSP90.9*-, *MeWRKY20*-, and *MeCatalase1*-silenced cassava leaves reduced to about half of that in control cassava leaves (Fig. 4a). Upon drought stress, relative water content decreased, while MDA accumulated and relative EL increased in cassava leaves with time (Fig. 4b–d). Importantly, as compared with vector-transformed plant leaves, *MeHSP90.9*-, *MeWRKY20*- and *MeCatalase1*-silenced cassava leaves accumulated less relative water content, a greater amount of MDA and had higher relative EL at 16 and 32 d after drought stress (Fig. 4b–d), suggesting there is severe cell damage triggered by drought stress. Consistently, *MeHSP90.9*-, *MeWRKY20*- and *MeCatalase1*-silenced cassava plants had more wilted leaves and exhibited more serious dehydration phenotype than did vector-transformed plants (Fig. 4e). These data indicated that gene silencing of *MeHSP90.9*, *MeWRKY20* or *MeCatalase1* leads to enhanced drought sensitivity in cassava.

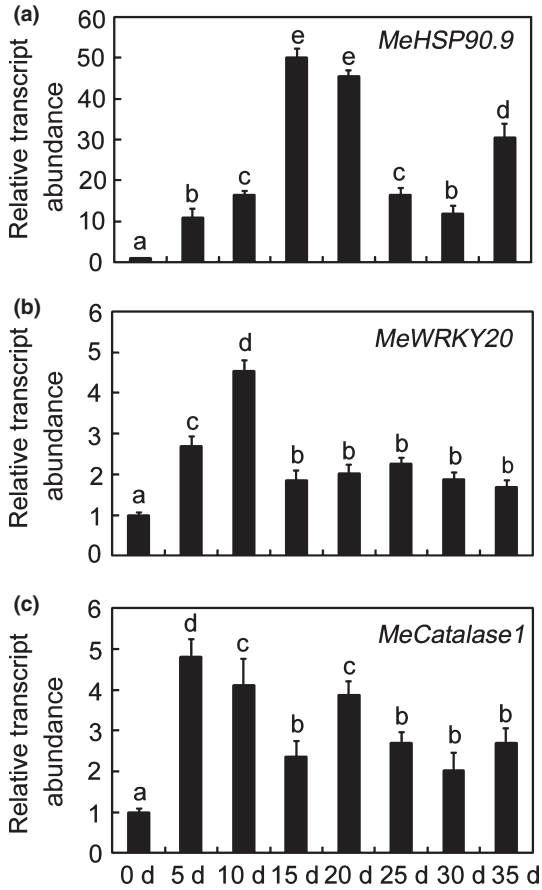


**Fig. 3** Transcript abundance of (a) *MeHSP90*, (b) *MeWRKY20* and (c) *MeCatalase1* in response to drought stress. Water was withheld from 30-d-old cassava plants for 0, 5, 10, 15, 20, 25, 30 and 35 d, and the leaves were collected for gene expression analysis at each time point. Error bars are ± SD. Different letters above the bar indicate significant differences in comparison to 0 d at $P < 0.05$ (Duncan's range test).

## MeHSP90.9 and MeWRKY20 regulate the expression of MeNCED5 and ABA accumulation

Previous studies have revealed that the phytohormone ABA is essential for plant drought response (Xiong *et al.*, 2006). Thus, we wondered whether and how ABA is involved in MeHSP90-mediated plant drought stress resistance in cassava. As WRKY family genes have been reported to regulate plant drought stress in an ABA-dependent manner (Ren *et al.*, 2010; Rushton *et al.*, 2012; Chu *et al.*, 2015), we first detected the expression levels of ABA biosynthesis genes in *MeWRKY20*-silenced cassava leaves. Notably, real-time PCR results indicated that among the six *MeNCED* family genes we examined, only *MeNCED5* was significantly downregulated (Fig. S4). Additionally, *MeNCED5* was dramatically upregulated in response to drought stress in cassava leaves, and its protein was located in cytoplasm and nuclei (Fig. S5a,b). Based on this, we next examined the expression level of *MeNCED5* in *MeHSP90.9*- and *MeWRKY20*-silenced cassava leaves under normal and drought stress conditions. Although *MeNCED5* was induced in all plant leaves upon drought stress, vector-transformed cassava leaves had higher gene expression level than did *MeHSP90.9*- or *MeWRKY20*-silenced cassava leaves (Fig. 5a). In addition, a higher ABA concentration was detected in vector-transformed cassava leaves than in *MeHSP90.9*- or *MeWRKY20*-silenced leaves (Fig. 5b). According to these observations, *MeHSP90.9* and *MeWRKY20* modulate ABA accumulation during drought stress, probably through targeting the ABA biosynthesis gene *MeNCED5*.

## MeWRKY20 is a direct transcriptional activator of MeNCED5

Interestingly, we found that the promoter of the *MeNCED5* gene contains a W-box element (5′-TTGACC-3′), which has been reported to be a direct target of WRKY transcription factors (Ulker & Somssich, 2004). Thus, we investigated whether MeWRKY20 could promote *MeNCED5* transcription by directly binding to its promoter. We first overexpressed *MeHSP90.9* and *MeWRKY20* in cassava leaf protoplasts (Fig. S6), and found that the transcript abundances of *MeNCED5* (Fig. 6a) were significantly increased in comparison to vector-transformed cassava leaf protoplasts Moreover, co-overexpression of *MeHSP90.9* and *MeWRKY20* resulted in even higher transcript abundance of *MeNCED5* (Fig. 6a) as compared with overexpression of single gene. These data suggested that MeWRKY20 positively regulates *MeNCED5* transcription and MeHSP90.9 promotes this effect. In addition, LUC reporter gene assay showed that MeWRKY20 and MeHSP90.9 stimulated the activity of *MeNCED5* promoter (Fig. 6b). Consistently, ChIP-qPCR assay indicated that the W-box element in the *MeNCED5* promoter was dramatically enriched in MeWRKY20-bound DNA fragments (Fig. 6c). Moreover, EMSA further suggested that MeWRKY20 could directly bind to the wild-type (P1) but not the W-box mutated (P1m) promoter fragment of *MeNCED5* (Fig. 6d). Based on these results, we might conclude that MeWRKY20 is a direct transcriptional activator of *MeNCED5*.

## MeHSP90.9 regulates drought stress resistance and ABA accumulation in cassava

The data in the previous sections showed that *MeHSP90.9*- and *MeWRKY20*-silenced plant leaves accumulated lower ABA (Fig. 5b) and compromised drought stress resistance (Fig. 4). To further clarify the relationship between ABA concentration and drought stress resistance in *MeHSP90.9*-modulated drought response in cassava, we carried out an exogenous application assay. Compared with mock, *MeHSP90.9*-, *MeWRKY20*-, *MeNCED5*-, *MeHSP90.9*/*MeWRKY20*- and *MeHSP90.9*/*MeNCED5*-silenced plant leaves showed lower relative water content, and higher MDA and EL under drought stress conditions as well as decreased drought stress resistance (Fig. 7a–e). Although the drought stress treatment decreased the relative water content and increased the MDA content and relative EL in cassava leaves, exogenous application of ABA significantly compromised these effects (Fig. 7a–d) and partially restored the dehydration phenotype caused by drought stress (Fig. 7e) in *MeHSP90.9*-, *MeWRKY20*-, *MeNCED5*-, *MeHSP90.9*/*MeWRKY20*- and

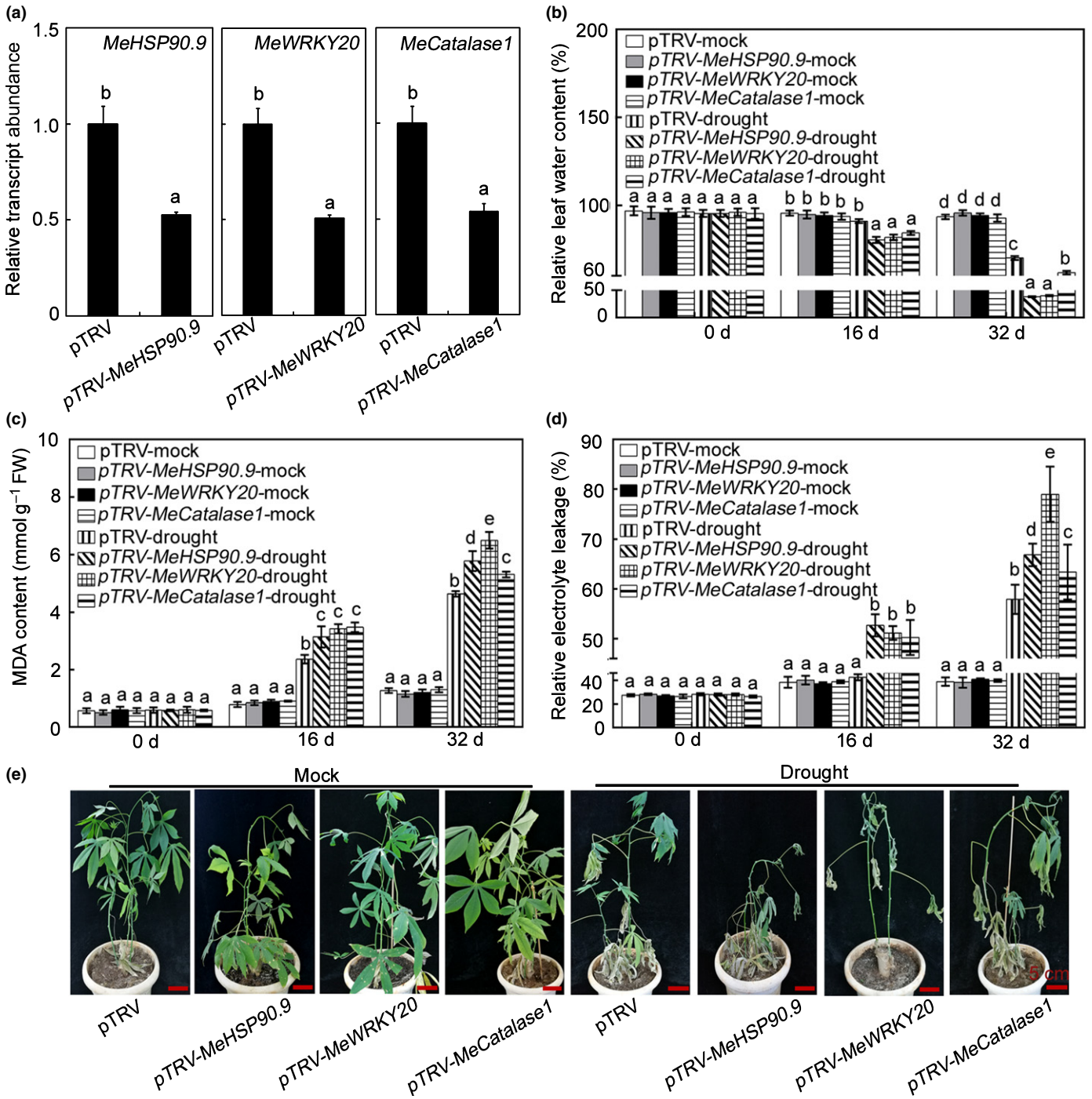


**Fig. 4** *MeHSP90*-, *MeWRKY20*- and *MeCatalase1*-silenced plants display drought sensitivity in cassava. (a) Transcript abundances of corresponding genes in the virus-induced gene silencing (VIGS) plant leaves. At 14 d after *pTRV* syringe infiltration, cassava leaves were used for RNA isolation. (b–d) Relative leaf water content (b), malondialdehyde (MDA) content (c) and relative electrolyte leakage (d) in plant leaves after different lengths of drought stress treatment. (e) Phenotypes of the VIGS plants after withholding water for 35 d. Bars, 5 cm. Error bars are ± SD. Different letters above the bars indicate significant differences between *MeHSP90*-, *MeWRKY20*-, *MeCatalase1*-silenced cassava leaves and vector (pTRV) at $P < 0.05$ (Duncan's range test).

*MeHSP90.9*/*MeNCED5*-silenced plant leaves. More importantly, relative water content, MDA content and relative EL in *MeNECD5*-silenced cassava leaves upon drought stress were returned to the values in vector-transformed plant leaves by exogenous application of ABA, but they were only partially rescued in *MeHSP90.9*-, *MeWRKY20*-, *MeHSP90.9*/*MeWRKY20*- and *MeHSP90.9*/*MeNCED5*-silenced cassava leaves (Fig. 7b–d). In addition, the drought-caused dehydration phenotype in *MeHSP90.9*-, *MeWRKY20*-, *MeHSP90.9*/*MeWRKY20*- and *MeHSP90.9*/*MeNCED5*-silenced cassava

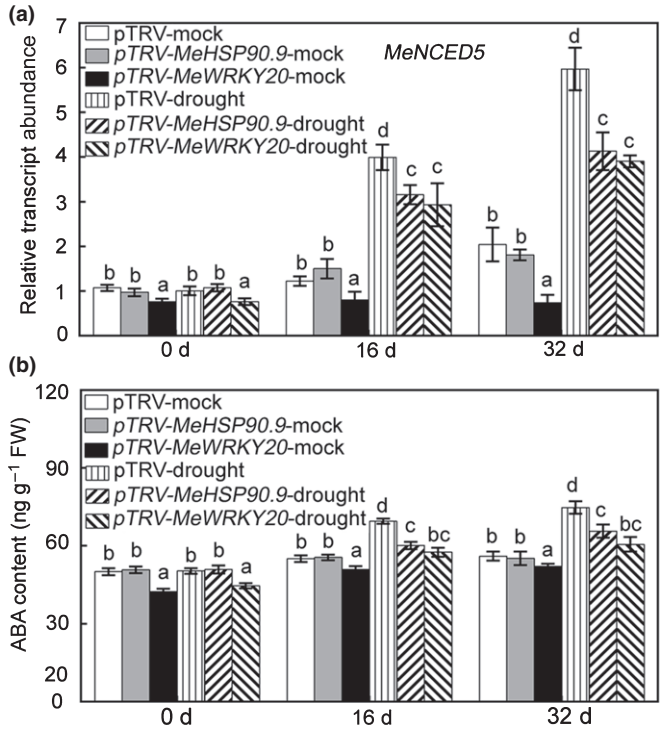


**Fig. 5** MeHSP90 and MeWRKY20 regulate the transcript of *MeNCED5* and abscisic acid (ABA) accumulation. (a, b) Transcript abundances of *MeNCED5* (a) and ABA content (b) in pTRV, *pTRV-MeHSP90-*, and *MeWRKY20*-expressed cassava leaves under normal and drought stress conditions. Error bars are ± SD. Different letters above the bars indicate significant differences between *MeHSP90-* and *MeWRKY20*-silenced cassava leaves and vector (*pTRV*) at $P < 0.05$ (Duncan's range test).

leaves was also just partially rescued by exogenous application of ABA (Fig. 7e). Thus, *MeHSP90.9* and *MeWRKY20* regulated drought stress resistance at least partially through modulation of ABA accumulation.

### MeHSP90.9 and MeCatalase1 regulate catalase activity and $H_2O_2$ concentration

Drought-induced excessive production of reactive oxygen species (ROS), such as $H_2O_2$, leads to progressive oxidative damage and cell death. MeHSP90.9 could physically interact with MeCatalase1 (Fig. 2), which regulates endogenous $H_2O_2$ concentration (Gao *et al.*, 2014; Liu *et al.*, 2017; Yuan *et al.*, 2017). Therefore, we performed additional experiments to assess the relationship between MeHSP90.9 and $H_2O_2$ accumulation. We first monitored the expression of seven *MeCatalase* genes in *MeHSP90.9*-silenced plant leaves by qRT-PCR and found that only *MeCatalase1* was significantly downregulated (Fig. S7). Meanwhile, *MeHSP90.9*-silenced cassava leaves accumulated more $H_2O_2$ after drought stress than did vector-transformed cassava leaves, and *MeCatalase1*-silenced cassava leaves had even higher $H_2O_2$ concentrations (Fig. 8a,c). Consistently, catalase activity in *MeHSP90.9-* and *MeWRKY20*-silenced cassava leaves was relatively lower than that in vector-transformed cassava leaves under drought stress conditions (Fig. 8b). In addition, overexpression of *MeHSP90.9* and *MeWRKY20* enhanced catalase activity in cassava leaves, while co-overexpression of them led to even higher catalase activity (Figs 8d, S8). These results indicated that MeHSP90.9 and MeCatalase1 regulate catalase activity and $H_2O_2$ concentration during drought stress in cassava.

## Discussion

Drought is thought to be the most severe environmental stress that seriously affects plant survival and crop production (Zhu, 2002). Under persistent drought stress conditions, the growth of cassava plants may be greatly restricted, leading to a large number of yield losses (El-Sharkawy, 2004). It is currently believed that the mechanism of cassava resistance to drought environments is 'avoiding drought', by, for example, enabling deep water through its fine root system, which can penetrate nearly 2 m below soil (El-Sharkawy, 2004). Encouraging the mature leaves to reduce water transpiration can help cassava overcome harsh drought conditions (Alves & Setter, 2000). In this study, we reported that MeHSP90.9 is a regulator of plant drought stress resistance in cassava.

HSP90 is a well-known molecular chaperone present in all eukaryotes, which interacts with the downstream functional proteins that are called as client proteins (Borkovich *et al.*, 1989). To date, more than 200 client proteins have been reported (Pratt *et al.*, 2008), confirming diverse biological functions of HSP90. In plants, some client proteins of HSP90 have been substantially investigated. For instance, the well-established HSP90-Mla12 resistance 1 (RAR1)-suppressor of the G2 allele of skp1 (SGT1) chaperone complex is involved in *resistant* (*R*) gene-mediated immune response (Thao *et al.*, 2007; Hubert *et al.*, 2009; Bao *et al.*, 2014). HSP90 can bind to Argonaute (AGO1) and facilitate the assembly of plant RNA-induced silencing complexes (Iki *et al.*, 2010). Although several groups have suggested the involvement of HSP90 in plant drought stress response in different species (Song *et al.*, 2009; Xu *et al.*, 2013b), the underlying regulating mechanism and downstream components remain elusive. In this study, we demonstrated that MeHSP90.9 interacts with MeWRKY20 and MeCatalase1 in nuclei and peroxisome in tobacco leaves, respectively (Fig. 2). Our study not only reported two novel proteins that could be potential HSP90 client proteins, but also revealed the involvement of *MeHSP90.9*, *MeWRKY20*, and *MeCatalase1* in drought stress resistance in cassava.

With the completion of cassava genome sequencing (Wang *et al.*, 2014), a large number of genes related to drought stress resistance have been identified via transcriptome analysis, including ethylene response factor family (ERF) genes (Fan *et al.*, 2016), basic leucine zipper (bZIP) transcription factor family (Hu *et al.*, 2016), homeodomain-leucine zipper (HD-Zip) gene family (Ding *et al.*, 2017), and myeloblastosis (MYB) transcription factor superfamily (Ruan *et al.*, 2017). However, the specific functions of most genes in the drought stress resistance process of cassava remain to be further studied. *MeMYB2-RNAi* transgenic cassava plants are reported to be more resistant to drought stress, and MeMYB2 may recruit other MeMYBs and MeWRKYs during the drought stress response (Ruan *et al.*,

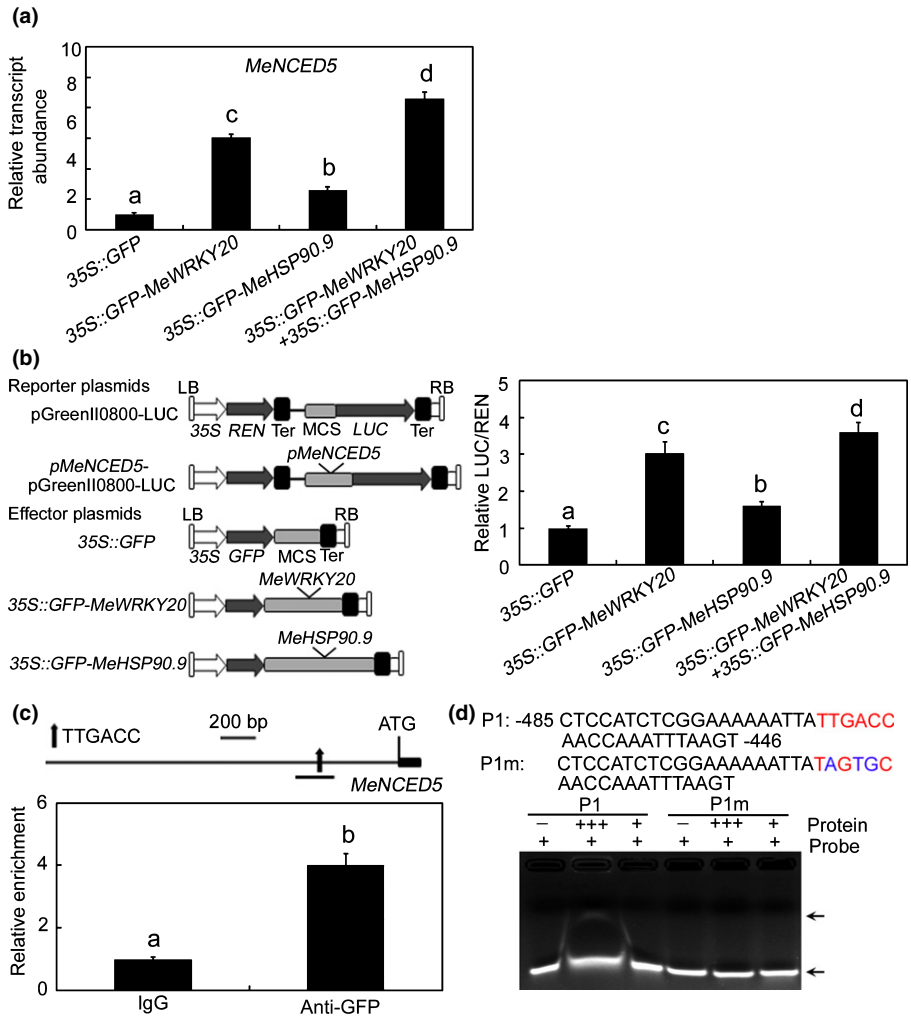


**Fig. 6** MeWRKY20 is a direct transcriptional activator of *MeNCED5*. (a) Quantification of *MeNCED5* transcript abundance in the *35S::GFP*-, *35S::GFP-MeWRKY20*- and *35S::GFP-MeHSP90.9*-expressed cassava leaf protoplasts. (b) Relative luciferase (LUC) activity of *MeNCED5* promoter in cassava leaf protoplasts with transient expression of *35S::GFP*, *35S::GFP-MeWRKY20* and *35S::GFP-MeHSP90.9*. (c) Effect of overexpressing *MeWRKY20* on the enrichment of the promoter fragment in cassava leaf protoplasts using chromatin immunoprecipitation (ChIP)-PCR assay. (d) Effect of *MeWRKY20* on the gel shift of binding of *MeNCED5* promoter probe containing W-box using electrophoretic mobility shift assay. Error bars are ± SD. Different letters above the bars indicate significant differences in comparison to vector at $P < 0.05$ (Duncan's range test).

2017). By contrast, coupled overexpression of *MeCu/ZnSOD* and *MeCatalase* in cassava improves plant drought stress tolerance with higher water retention ability and proline accumulation (Xu *et al.*, 2013a), suggesting that the antioxidative mechanism is involved in the cassava drought stress response. Here, we further found that HSP90 might function as the upstream regulator of ROS accumulation during drought stress. As indicated by exogenous application of two specific HSP90 protein inhibitors (GDA and RAD), HSP90 might be essential for drought stress resistance in cassava by regulating endogenous $H_2O_2$ concentration (Fig. 1). Further investigation indicated that MeHSP90.9 interacted directly with MeCatalase1 and positively regulated catalase activity (Figs 2, 8). Consistently, *MeHSP90.9* or *MeCatalase1*-silenced plants were more sensitive to drought stress in cassava, as evidenced by greater MDA accumulation, higher relative EL, and more serious dehydration phenotype (Fig. 4). Therefore, our study established the crosstalk between HSP90 and ROS accumulation via the MeHSP90–MeCatalase1 interaction.

It is believed that to survive under drought stress conditions, cassava can absorb water in deeper soil via the deep root system, and decrease water loss by closing stomata and shedding older leaves (El-Sharkawy & Cock, 1987; El-Sharkawy, 2004; Zhao *et al.*, 2015). This study confirmed that the interaction between MeHSP90.9 and MeWRKY20 and the module of MeHSP90.9-MeWRKY20-MeNCED5 are involved in regulating ABA biosynthesis in cassava. NCED, one of ABA biosynthetic enzymes, is involved in plant growth, seed dormancy, leaf senescence, ABA accumulation, seed germination, leaf morphology, and multi-abiotic stress respond (Iuchi *et al.*, 2001; Hwang *et al.*, 2010; Frey *et al.*, 2012; Sussmilch *et al.*, 2017; Xu & Cai, 2017; Huang *et al.*, 2018; Pan *et al.*, 2018). In cassava, only a few *NCEDs* were reported to be gradually increased in response to drought stress (Fu *et al.*, 2016; Zhao *et al.*, 2015); however, the

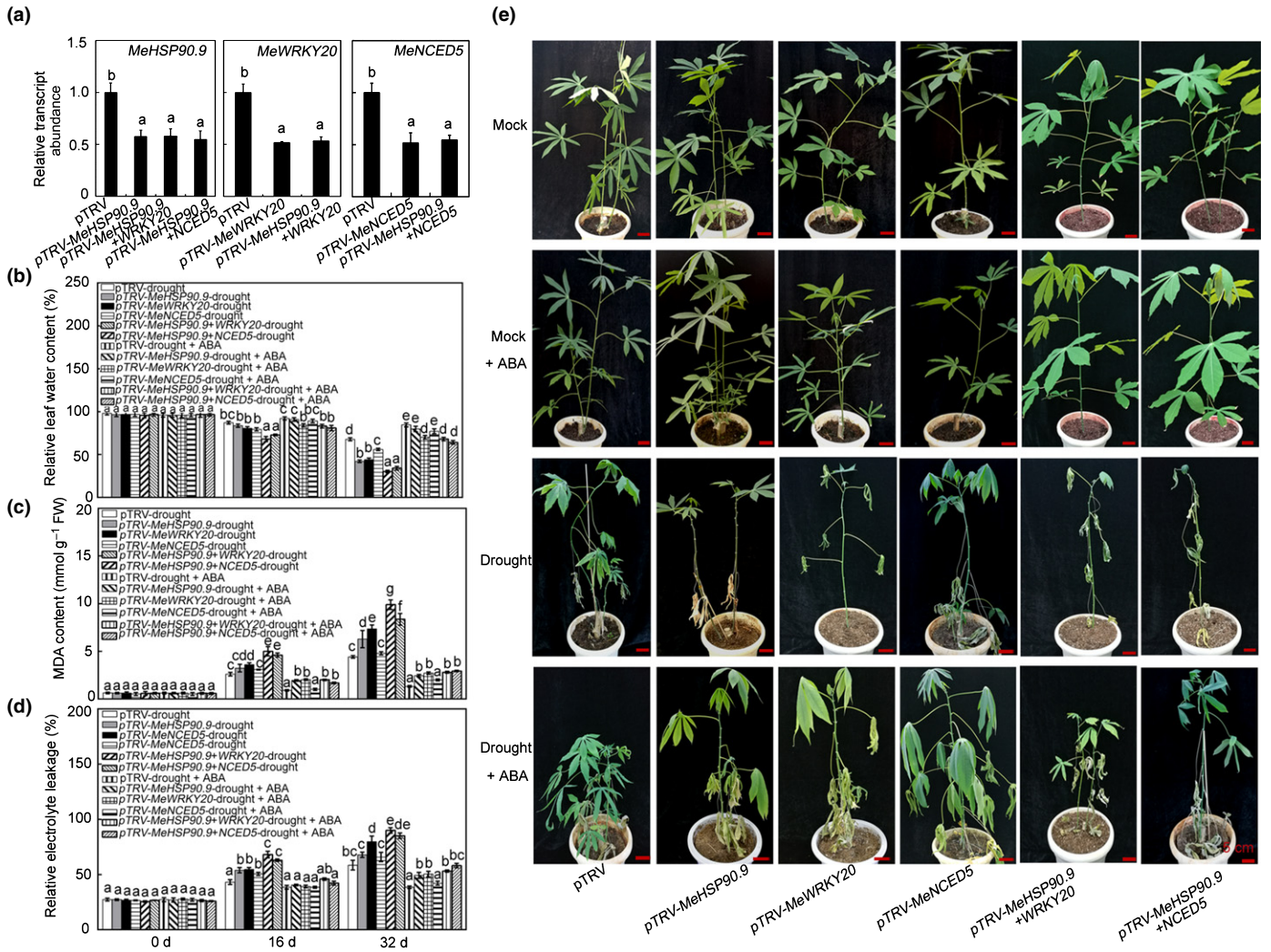


**Fig. 7** Exogenous abscisic acid (ABA) treatment restores the drought sensitivity of *MeHSP90*- *MeWRKY20*- and *MeNCED5*-silenced plants. (a) Transcript abundance of corresponding genes in the virus-induced gene silencing (VIGS) plant leaves. (b–d) Relative leaf water content (b), malondialdehyde (MDA) content (c) and relative electrolyte leakage (d) in VIGS plant leaves after different lengths of drought stress treatment. (e) Phenotypes of the VIGS plants after withholding water for 35 d. Bars, 5 cm. At 14 d after pTRV syringe infiltration, the cassava leaves were used for RNA isolation, and were then sprayed with 100 μM ABA. Two days later, the cassava leaves were used for further assay of drought stress resistance. Error bars are ± SDs. Different letters above the bars indicate significant differences between *MeHSP90*-, *MeWRKY20*-, *MeNCED5*-silenced cassava leaves and vector (pTRV) at $P < 0.05$ (Duncan's range test).

detailed mechanisms are still unclear. In this report, we found that *MeNCED5* was significantly upregulated under drought stress (Fig. S5). In addition, *MeNCED5*-silenced cassava leaves had more MDA and higher relative EL, which were returned to the values in vector-transformed plant leaves by exogenous application of ABA (Fig. 7). These results suggest that *MeNCED5* regulates drought stress resistance by modulating ABA biosynthesis. Similarly, *NCEDs* have also been reported to positively regulate desiccation stress in other species, such as *AtNCED3* and *AtNCED5* (Iuchi *et al.*, 2001; Frey *et al.*, 2012), *OsNCED3* (Huang *et al.*, 2018), *BnNCED3* (Xu & Cai, 2017), and *NtNCED3* (Yang *et al.*, 2018). In *Arabidopsis*, *WRKY33* is associated with plant innate immunity by binding to the W-box in *NCED3/NCED5* promoter, and negatively regulates ABA biosynthesis (Liu *et al.*, 2015). *Arabidopsis thaliana* HD-Zip proteins bind to HD-Zip domain sequences (TAATAATTG) in the *NCED9* promoter region, which is required for *NCED9* expression in testa (Seo *et al.*, 2016). In the current study, we also found that MeWRKY20 directly targets the W-box element of *MeNCED5* promoter and promotes the transcription of *MeNCED5*, which is facilitated by MeHSP90 (Fig. 6). Consistent with the phenotype of drought sensitivity, the ABA concentrations were significantly lower in *MeHSP90.9*- and *MeWRKY20*-silenced plant leaves than in control plant leaves. Notably, exogenous application of ABA could restore relative water content, MDA content and relative EL as well as drought stress resistance in *MeNECD5*-silenced cassava leaves upon drought stress to the values in vector-transformed plant leaves, but only partially rescued them in *MeHSP90.9*-, *MeWRKY20*-, *MeHSP90.9*/*MeWRKY20*- and *MeHSP90.9*/*MeNCED5*-silenced

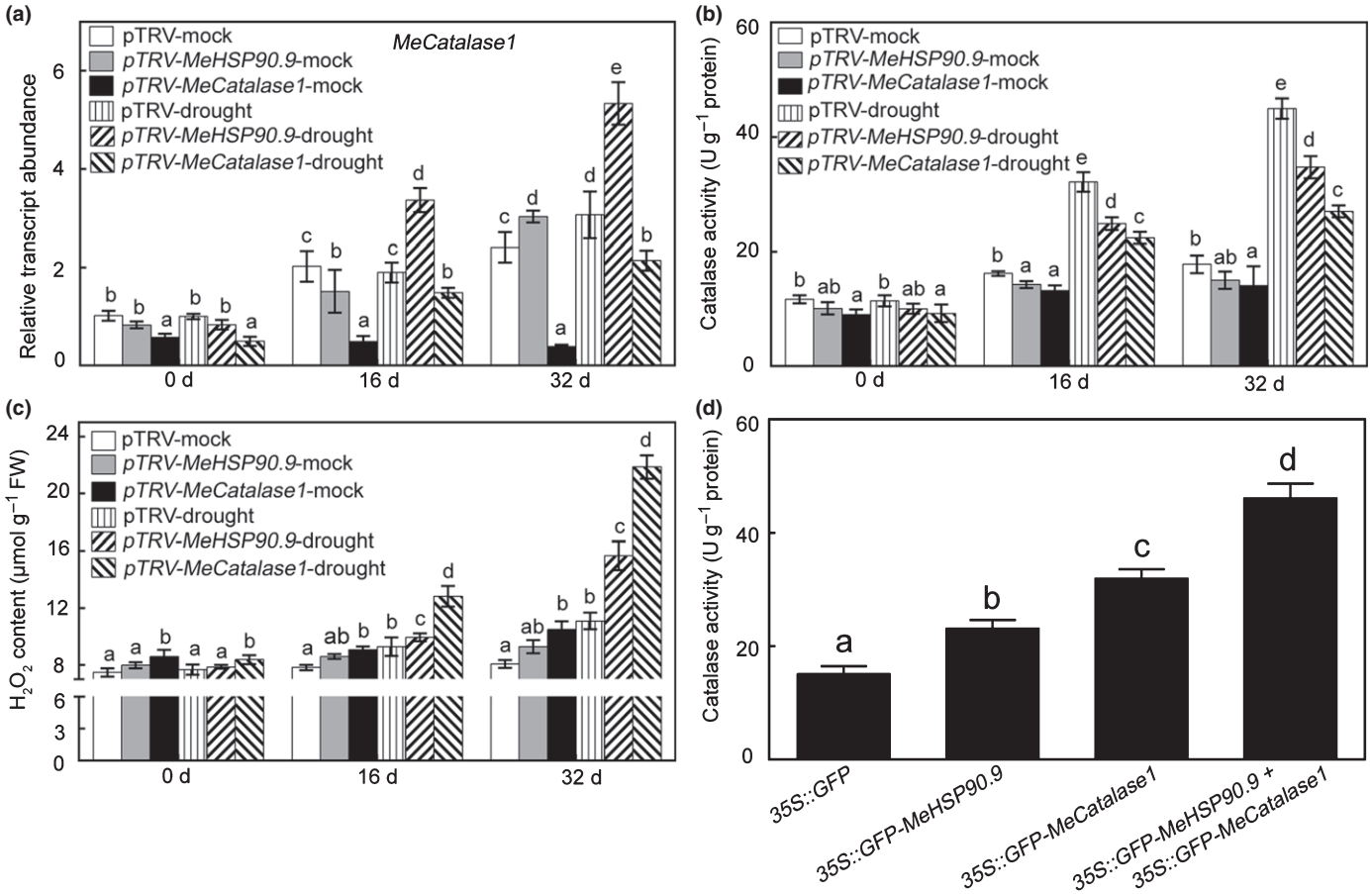


**Fig. 8** MeHSP90 and MeCatalase1 regulate catalase activity and $H_2O_2$ level. (a–c) Gene transcript abundance of *MeCatalase1* (a), catalase activity (b), and $H_2O_2$ concentration (c) in *pTRV-*, *pTRV-MeHSP90.9-* and *pTRV-MeCatalase1*-expressed cassava leaves under normal and drought stress conditions. (d) Catalase activity in cassava leaf protoplasts with transient expression of *35S::GFP*, *35S::GFP-MeHSP90.9* or *35S::GFP-MeCatalase1*. Error bars are ± SD. Different letters above the bars indicate significant differences between *MeHSP90.9-* and *MeCatalase1*-silenced cassava leaves and vector (*pTRV*) at $P < 0.05$ (Duncan's range test).

cassava leaves (Fig. 7). Therefore, the interaction between MeHSP90.9 and MeWRKY20 plants is relevant for the ABA synthesis during drought stress, which is partially contribute to *MeHSP90.9* and *MeWRKY20* regulated drought stress resistance. Besides ABA, the interaction between MeHSP90.9 and MeCatalase1 and their effect on endogenous $H_2O_2$ concentration are also involved in *MeHSP90.9*-regulated drought stress resistance.

Catalase regulates endogenous $H_2O_2$ concentration, seedling establishment and plant response to environments such as light, salt, oxidative stress, and pathogen infection (Willekens *et al.*, 1997; Eastmond, 2007; Verslues *et al.*, 2007; Gao *et al.*, 2014; Liu *et al.*, 2017; Yuan *et al.*, 2017). In tobacco, catalase-deficient plants with lower catalase activity show reduced resistance to salt and oxidative stress compared with the wild-type (Willekens *et al.*, 1997). In *Arabidopsis*, Catalase2 and Catalase3 interact with salt overly sensitive 2 (SOS2), and the interaction is the crucial element of the plant's salt stress response (Verslues *et al.*, 2007). In addition, *Atcatalase2* mutant exhibits increased resistance to *Pseudomonas syringae* pv. *tomato* DC3000 and increased susceptibility to *Botrytis cinerea*, through modulation of salicylic acid repression of auxin and jasmonic acid biosynthesis (Yuan *et al.*, 2017). In cassava, *MeCatalase* positively regulates cassava

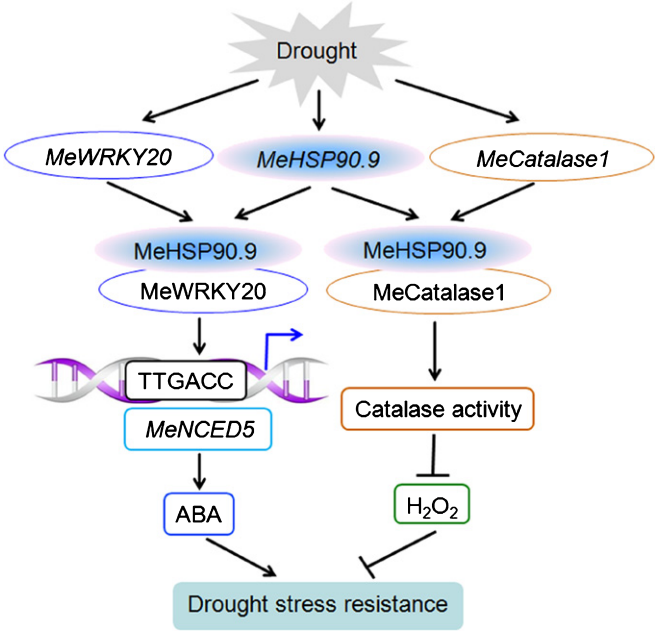


**Fig. 9** Proposed model of MeHSP90-modulated drought stress resistance in cassava. ABA, abscisic acid.

responses to drought stress through modulation of endogenous $H_2O_2$ concentration (Xu *et al.*, 2013a). Here, we further found that MeHSP90.9 could interact with MeCatalase1 and regulate the catalase activity (Figs 2, 8). In addition, the interaction between MeHSP90.9 and MeCatalase1 was involved in regulating endogenous $H_2O_2$ concentration. Thus, our study also provided a novel regulator of Catalase protein and revealed its function in drought stress resistance in cassava (Fig. 9). However, whether MeHSP90 participates in other Catalase-mediated processes needs to be further investigated.

Taken together, our studies demonstrate that HSP90 is essential for drought stress resistance in cassava via its recruitment of MeWRKY20 and MeCatalase1.

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## Author contributions

HS planned and designed the research. YW and YY performed experiments and conducted the fieldwork. YW, WL, WH, YY and HS analyzed the data. YW and WL wrote the manuscript. WH and HS revised the manuscript. YW, WL and WH contributed equally to this work.

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## Supporting Information

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

**Fig. S1** Transcript abundance of MeHSP90s in response to drought stress.

**Fig. S2** Subcellular localization of MeHSP90.9 and MeCatalase1.

**Fig. S3** PCR showing the expression of *MeHSP90.9, MeWRKY20, MeCatalase1, MeNCED5, TRV1*, and *TRV2* in the VIGS plant leaves under drought stress conditions.

**Fig. S4** Transcript abundance of *MeNCEDs* in the *MeWRKY20*-silenced cassava leaves.

**Fig. S5** Expression profile and subcellular localization of MeNCED5.

**Fig. S6** Transcript abundance of *MeWRKY20* and *MeHSP90.9* in the cassava leaf protoplasts that were transformed by *35S::GFP*, *35S::GFP-MeWRKY20*, or *35S::GFP-MeHSP90.9*.

**Fig. S7** Transcript abundance of *MeCatalases* in the *MeHSP90.9*-silenced cassava leaves.

**Fig. S8** Transcript abundances of *MeCatalase1* and *MeHSP90.9* in the cassava leaf protoplasts that were transformed by *35S::GFP*, *35S::GFP-MeCatalase1*, or *35S::GFP-MeHSP90.9*.

**Table S1** Primers used for vector construction in this study.

**Table S2** Primers used for quantitative real time-PCR and RT-PCR in this study.

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