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The Cotton WRKY Transcription Factor GhWRKY17 Functions in Drought and Salt Stress in Transgenic *Nicotiana benthamiana* Through ABA Signaling and the Modulation of Reactive Oxygen Species Production

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Drought and high salinity are two major environmental factors that significantly limit the productivity of agricultural crops worldwide. WRKY transcription factors play essential roles in the adaptation of plants to abiotic stresses. However, WRKY genes involved in drought and salt tolerance in cotton (*Gossypium hirsutum*) are largely unknown. Here, a group IId WRKY gene, *GhWRKY17*, was isolated and characterized. *GhWRKY17* was found to be induced after exposure to drought, salt, H_2O_2 and ABA. The constitutive expression of *GhWRKY17* in *Nicotiana benthamiana* remarkably reduced plant tolerance to drought and salt stress, as determined through physiological analyses of the germination rate, root growth, survival rate, leaf water loss and Chl content. *GhWRKY17* transgenic plants were observed to be more sensitive to ABA-mediated seed germination and root growth. However, overexpressing *GhWRKY17* in *N. benthamiana* impaired ABA-induced stomatal closure. Furthermore, we found that *GhWRKY17* modulated the increased sensitivity of plants to drought by reducing the level of ABA, and transcript levels of ABA-inducible genes, including *AREB*, *DREB*, *NCED*, *ERD* and *LEA*, were clearly repressed under drought and salt stress conditions. Consistent with the accumulation of reactive oxygen species (ROS), reduced proline contents and enzyme activities, elevated electrolyte leakage and malondialdehyde, and lower expression of ROS-scavenging genes, including *APX*, *CAT* and *SOD*, the *GhWRKY17* transgenic plants exhibited reduced tolerance to oxidative stress compared with wild-type plants. These results therefore indicate that *GhWRKY17* responds to drought and salt stress through ABA signaling and the regulation of cellular ROS production in plants.

Keywords: ABA signaling • Cotton (*Gossypium hirsutum*) • Drought and salt stress • ROS • WRKY transcription factor.

Abbreviations: AbA, aureobasidin A; APX, ascorbate peroxidase; AREB, ABA-responsive element binding; CaMV, *Cauliflower mosaic virus*; CAT, catalase; DAB, 3,3′-diaminobenzidine; DREB, dehydration-responsive element binding; ERD, early responsive to dehydration; GFP, green fluorescent protein; GUS, β-glucuronidase; LEA, late embryogenesis-abundant protein; MDA, malondialdehyde; MS, Murashige and Skoog; MV, methyl viologen; NLS, nuclear localization signal; OE, overexpressing; ORF, open reading frame; PEG, polyethylene glycol; qPCR, quantitative real-time PCR; NBT, nitroblue tetrazolium; NCED, 9-*cis*-epoxycarotenoid dioxygenase; POD, peroxidase; RbohA, RbohB, respiratory burst oxidase homolog; ROS, reactive oxygen species; SA, salicylic acid; SOD, superoxide dismutase; Tu, sodium tungstate; WT, wild type.

The nucleotide sequences reported in this paper have been deposited in GenBank under accession numbers HQ651068 for the GhWRKY17 cDNA sequencem HQ651070 for the GhWRKY17 genomic sequence.

Introduction

Plants are constantly exposed to a variety of environmental stresses, and drought and high salinity are two major stress conditions that impose constraints on plant growth and development. Plants have evolved elaborate and intricate mechanisms to achieve optimal adaption to these unfavorable stress conditions. Stress signaling and transcriptional modulation are vital aspects of the complex genetic and biochemical networks that plants use to respond to stress. As a component of the signaling pathways that are activated in response to environmental stimuli, the phytohormone ABA represents a key signal for regulating a range of plant physiological processes in response to various biotic and abiotic stresses (Zhu 2002, Cutler et al. 2010, Raghavendra et al. 2010). ABA dramatically accumulates under osmotic stress such as drought and high salinity. The increased level of ABA promotes the activity of downstream transcription factors to modulate the expression of various ABA-responsive genes (Fujii et al. 2009, Ma et al. 2009), and stress-responsive transcription factors have been extensively characterized in studies on plant stress tolerance (Cutler et al. 2010).

Among these, the WRKY transcription factors comprise one large family of regulatory proteins. The WRKY transcription factor family is named after a 60 amino acid, conserved region termed the WRKY domain, which is characterized by a

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highly conserved, N-terminal WRKYGQK heptapeptide and a C-terminal zinc finger-like motif (Eulgem et al. 2000, Rushton et al. 2010). The WRKY family of *Arabidopsis thaliana* consists of 74 members, and 104 WRKY proteins have been identified in rice (Eulgem et al. 2000). All of the identified WRKY proteins contain one or two WRKY domains. Based on the number of WRKY domains and the pattern of the zinc finger-like motif, the WRKY proteins are subdivided into three major groups (I–III), and group II is further divided into five distinct subgroups (IIa–IIe) (Eulgem et al. 2000). These proteins regulate the plant responses to pathogenic attack and diverse abiotic stresses by binding to W-box elements [(T)TGAC(C/T)] in target gene promoters to up- or down-regulate their transcription (Eulgem et al. 2000, Eulgem and Somssich 2007, Ren et al. 2010).

Many proteins interact with WRKY transcription factorss, including proteins that direct epigenetic changes, such as histone deacetylases and signaling components [mitogen-activated protein (MAP) kinases, MAP kinase kinases, 14-3-3 proteins, calmodulin, resistance proteins and other WRKY transcription factors] (Rushton et al. 2010). For example, the senescence-related protein AtWRKY53 requires interaction with the MAP kinase kinase kinase MEKK1 for its W-box binding activity (Miao et al. 2007). In addition, interactions between AtWRKY18, AtWRKY40 and AtWRKY60 and the chloroplast/plastid-localized ABA receptor ABAR have been observed (Shang et al. 2010). Moreover, recent reports showed that AtWRKY18, AtWRKY40 and AtWRKY60 interact to form distinct complexes that might differ in their DNA-binding and transcription-regulating activities (Chen et al. 2010).

Previous studies have indicated that the WRKY proteins play roles in the regulation of developmental and physiological processes, including trichomes, seed development and leaf senescence (Hinderhofer and Zentgraf 2001, Johnson et al. 2002, Besseau et al. 2012). However, these proteins perform their major functions as important components in complex signaling processes during plant stress responses (Rushton et al. 2010, Birkenbihl et al. 2012, Jiang et al. 2012). They often act as repressors or activators, and some play roles in the repression and derepression of important plant processes. It is clear that a single WRKY protein might participate in regulating several seemingly disparate processes (Rushton et al. 2010).

A large body of evidence demonstrates that specific WRKY proteins are key positive and negative regulators in basal defense responses and salicylic acid (SA)-mediated defences in Arabidopsis, rice and other plant species (Li et al. 2004, Journot-Catalino et al. 2006, Li et al. 2006, Xu et al. 2006, Shen et al. 2007, Qiu and Yu 2008, Birkenbihl et al. 2012, Matsushita et al. 2012). For example, 49 of 72 Arabidopsis *WRKY* genes are differentially regulated through pathogenic infection or SA treatment (Dong et al. 2003). AtWRKY70 acts as an activator of SA-induced genes to enhance resistance to the fungal biotroph *Erysiphe cichoracerum*, and reduces resistance to the fungal necrotroph *Alternaria brassicicola* (Li et al. 2004, Li et al. 2006). *GhWRKY15*, *GhWRKY11* and *GhWRKY39*, which are three structurally related group IId WRKY members, interact physically and functionally in a complex to provide plant resistance to different pathogens (Yu et al. 2012, Sun et al. 2012, Shi et al. 2014). *CaWRKY27* acts as a positive regulator in tobacco resistance responses to *Ralstonia solanacearum* infection through modulation of SA-, jasmonic acid- and ethylene-mediated signaling pathways (Dang et al. 2014). Furthermore, the promoter regions of many defense-related genes, including several pathogenesis-related (PR) genes and the regulatory non-expresser of PR (*NPR1*) gene, contain W-box elements (Liu et al. 2004, Turck et al. 2004, Lippok et al. 2007).

In contrast, a limited number of studies have aimed to unravel the roles of these proteins in abiotic stress responses. Recently, increasing evidence suggests that many WRKY genes also participate in the regulation of hormones and the abiotic stress response (Miller et al. 2008, Rushton et al. 2011, Chen et al. 2012). For example, ABO3/WRKY63 mediates the plant response to ABA and drought tolerance in Arabidopsis (Ren et al. 2010). As negative regulators, WRKY70 and WRKY54 modulate osmotic stress tolerance in Arabidopsis (Li et al. 2013). Chrysanthemum DgWRKY1 plays a positive regulatory role in salt stress response (Liu et al. 2014). Wang et al. (2014) reported that 15 VvWRKYs are involved in the low temperature-related signal pathways in grape. AtWRKY22 is involved in the dark-induced senescence signal transduction pathway (Zhou et al. 2011). In addition, WRKY25, WRKY26, WRKY33 and WRKY39 play important roles in the plant response to heat stress in Arabidopsis (S.J. Li et al. 2010, Li et al. 2011). However, elucidation of the molecular mechanisms that underlie the plant regulatory response remains a substantial challenge due to the relatively large number of WRKY family members. Moreover, the details of the WRKY genes that are involved in osmotic tolerance during drought and salt stress through the ABA signaling pathway remain largely unclear, particularly in non-model plants, such as economically important crops. *Gossypium hirsutum* L., also known as upland cotton, produces >95% of the annual cotton crop worldwide and provides a model for studying genome-scale evolution and polyploidization (Grover et al. 2004, Zhang et al. 2008).

Here, we isolated an ABA-hypersensitive group IId WRKY gene, *GhWRKY17*, from cotton (*G. hirsutum* L.) and characterized the function and regulation of this protein in ABA signaling. GhWRKY17 interacted with three W-boxes in a yeast one-hybrid assay and transactivated the expression of the downstream β-glucuronidase (GUS) reporter gene in an *Agrobacterium*-mediated transient expression assay. The overexpression of *GhWRKY17* rendered tobacco plants hypersensitive to ABA with respect to seed germination and post-germination growth, but reduced the plant sensitivity to ABA-induced stomatal closure, suggesting that GhWRKY17 functions in ABA-mediated drought stress response pathways. *GhWRKY17* transgenic plants reduced drought tolerance, with elevated electrolyte leakage and malondialdehyde (MDA), reduced proline contents, higher reactive oxygen species (ROS) content, lower expression of ROS-scavenging genes and lower ABA content compared with wild-type plants (WT). Moreover, *GhWRKY17*-overexpressing plants showed increased sensitivity to high salinity and oxidative stress. These data provide new insights into the underlying

mechanism of WRKY proteins in ABA signaling and facilitate the targeted genetic manipulation of crop plants to improve stress tolerance.

Results

Identification of *GhWRKY17* in the cotton genome

A putative WRKY fragment was isolated using reverse transcription–PCR (RT–PCR) and rapid amplification of cDNA ends PCR (RACE-PCR). The full-length cDNA sequence was 1,099 bp and contained a complete open reading frame (ORF) of 957 bp, which encoded a 318 amino acid protein with an estimated molecular mass of 34.773 kDa and an isoelectric point of 9.87. Due to the high degree of homology with *A. thaliana* WRKY17, this new WRKY protein was designated as GhWRKY17 (GenBank accession No. HQ651068). GhWRKY17 exhibited high identities (41.67–54.97%) with its homologous sequences, including AtWRKY17 (AEC07593), AtWRKY15 (NP_179913), StWRKY2 (ABU49721) and NtEIG-D48 (BAB16432). Sequence analysis showed that the deduced protein had a single WRKY DNA-binding domain (WRKYGQK) and a conserved primary C-terminal motif (designated as the C-region) (**Fig. 1A**), which categorized this protein as a group II WRKY superfamily member (Eulgem et al. 2000). In addition, GhWRKY17 contained a nuclear localization signal (NLS) (KRKK, from amino acid 220 to 223) and a conserved structural motif (HARF), which are shared among WRKY group IId proteins. To evaluate the evolutionary relationship of GhWRKY17 within the WRKY protein family, a phylogenetic tree was constructed using the MEGA 4.1 software based on the amino acid sequences of different WRKY members from different species (**Fig. 1B**). The tree substantially supported the classification of WRKY proteins into three groups, with group II being further divided into five subgroups (a, b, c, d and e). GhWRKY17 clustered with the group IId WRKY proteins. These results indicate that GhWRKY17 belongs to group IId.

To study the genomic organization of *GhWRKY17*, we cloned a 1,710 bp genomic fragment of *GhWRKY17* (HQ651070). Comparison of the full-length cDNA sequence with the corresponding genomic DNA sequence suggested that the coding region of *GhWRKY17* was interrupted by two introns (86 and 525 bp). The potential complexity of the *GhWRKY17* locus in the cotton genome was determined using real-time fluorescence quantitative PCR (qPCR) and the single copy *GhRDR6* gene as an internal standard. The standard curves of *GhWRKY17* and *GhRDR6* are shown in **Supplementary Fig. S1**, and their correlation coefficients were 1.000 and 0.999, respectively. These results demonstrate that *GhWRKY17* is organized as a single-copy gene (**Supplementary Table S2**).

Identification of GhWRKY17 as a transcription factor

To verify the intracellular localization of GhWRKY17, the full-length *GhWRKY17* cDNA was fused to the green fluorescent protein (GFP) gene under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter, and this plasmid was transfected into onion epidermal cells. The transiently expressed GhWRKY17:GFP fusion protein was exclusively localized in the nucleus, while the control GFP protein was observed throughout the nucleus and cytoplasm (**Fig. 2A**). These results demonstrate that GhWRKY17 is a nuclear-localized protein.

The WRKY transcriptional activators modulate protein expression by interacting with the *cis*-element W-box to activate transcription. A yeast one-hybrid assay was performed to determine whether the GhWRKY17 protein was able to bind DNA. Three tandem repeats of the W-box (TTGACC) or mW-box (TAGACG) (**Fig. 2B**) were inserted into the pAbAi vector, which harbors the AbA^r reporter gene, and integrated into the genome of the yeast strain Y1HGold. AbA^r basal expression assays showed that 500 ng ml^{-1} aureobasidin A (AbA) could completely suppress the basal expression of the pAbAi-W-box reporter strain in the absence of prey. The full-length coding sequence (CDS) of *GhWRKY17* was subsequently cloned into the yeast expression vector pGADT7, which harbors the GAL4 activation domain. The resulting pGADT7-WRKY17 and pGADT7 constructs were transformed into the yeast strain Y1HGold carrying the pAbAi-W-box or pAbAi-mW-box plasmids. All of the transformed yeast cells grew on leucine (Leu) and uracil (Ura)-deficient synthetic dextrose (SD) medium (SD/-Leu/-Ura), confirming the success of transformation (**Fig. 2C**). As expected, only the yeast clones with pAbAi-W-box and pGAD-GhWRKY17 grew on SD/-Leu containing 500 ng ml^{-1} AbA (**Fig. 2C**), suggesting that GhWRKY17 bound to the W-box element and activated transcription in this yeast system.

To investigate whether GhWRKY17 activates gene expression by interacting with the W-box in plant cells, we further tested the interaction in tobacco leaves using *Agrobacterium*-mediated, transient expression of a GUS reporter gene. The histochemical assay showed that leaves co-transformed with W-box-35S mini-GUS and 35S:GhWRKY17 were stained dark blue and that leaves transformed with only W-box-35S mini-GUS showed a slight blue background (**Fig. 2D**), indicating that the GUS reporter gene was activated in the *N. benthamiana* leaves due to the interaction between GhWRKY17 and the W-box. These results clearly support GhWRKY17 as a transcriptional activator that specifically binds to the W-box.

Transcriptional regulation of *GhWRKY17* by abiotic stress and ABA

To evaluate the role of the GhWRKY17 protein, we analyzed the expression of this protein at the mRNA level. Using qPCR, *GhWRKY17* was found to be differentially up-regulated under drought, salt, H_2O_2 and ABA treatments (**Fig. 3**). For the dehydration treatment, *GhWRKY17* expression was induced after 2 h of treatment, increased and returned to the highest level (1.9-fold induction) at 4 h, and then subsequently dropped (**Fig. 3A**). For salt stress, the *GhWRKY17* transcript rapidly increased upon NaCl treatment, reaching its maximum accumulation (2.5-fold induction) at 6 h and gradually diminishing thereafter (**Fig. 3B**). Abiotic stress consistently accompanies the accumulation of H_2O_2. Therefore, we examined the effect of

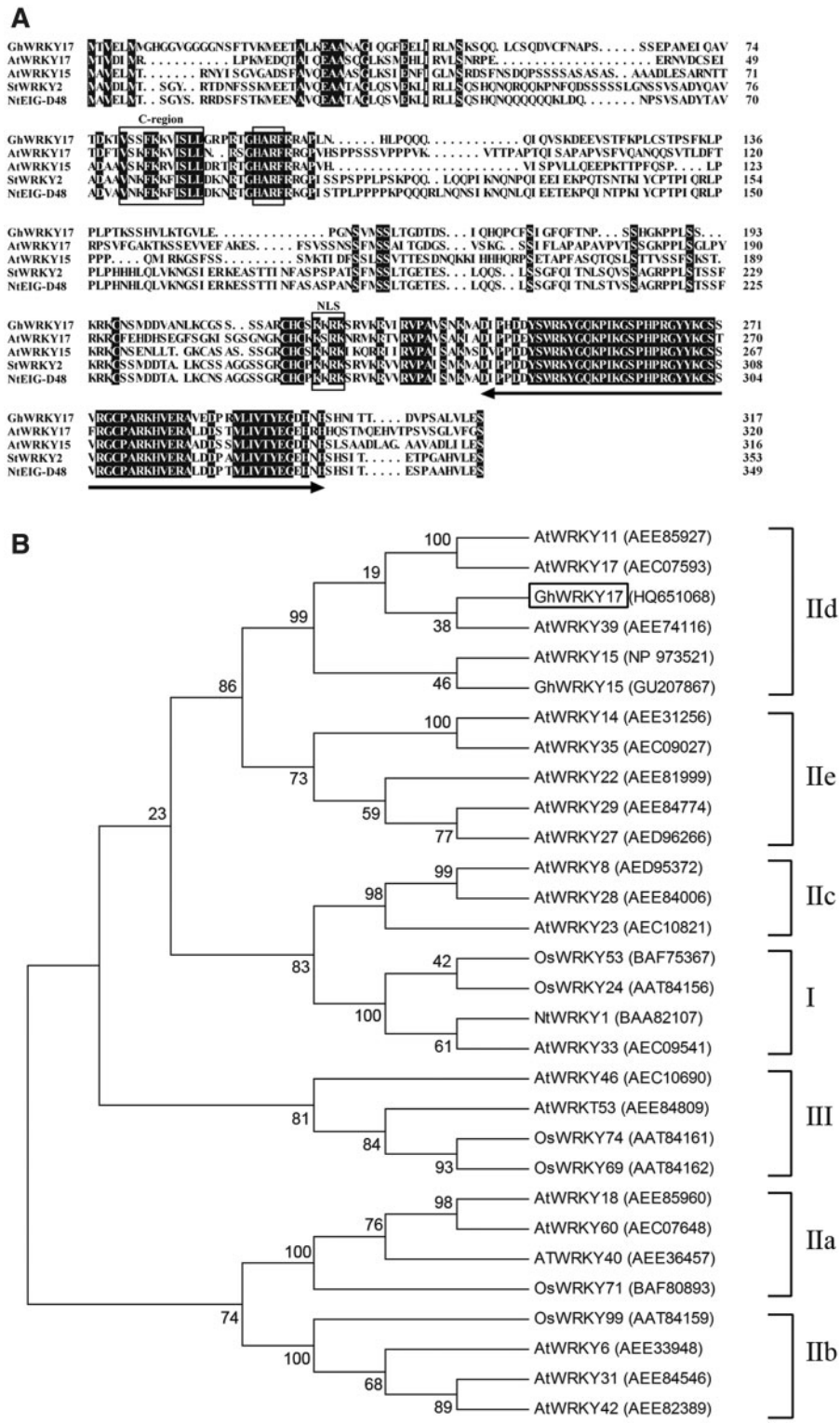

Fig. 1 Sequence and phylogenetic analyses of GhWRKY17. (A) Sequence alignment of the deduced GhWRKY17 protein with its known homologs. Identical amino acids are shaded in black. The 60 amino acid WRKY domain is indicated by a double-headed arrow. The conserved primary sequences of the zinc finger motif, HARF motif and putative NLS are boxed. (B) Phylogenetic analysis of the WRKY proteins from different species. The numbers above or below the branches are the bootstrap values from 1,000 replicates. GhWRKY17 is boxed. The GenBank accession numbers are indicated in parentheses. The species of origin of the WRKYs are indicated by the abbreviation before the gene names: At, *Arabidopsis thaliana*; Gh, *Gossypium hirsutum*; St, *Solanum tuberosum*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*.

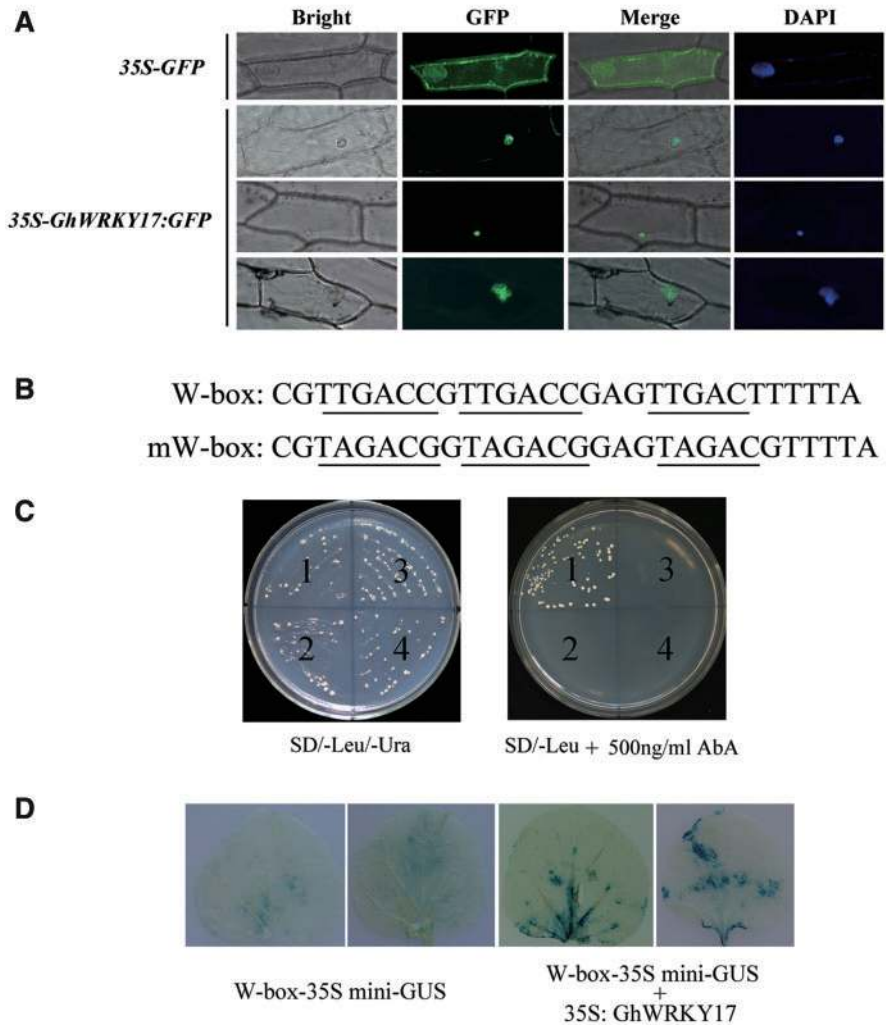

Fig. 2 Characterization of GhWRKY17 as a transcriptional regulator. (A) Subcellular localization of GhWRKY17. The 35S-GFP and 35S-GhWRKY17:GFP fusion proteins were transiently expressed in onion epidermal cells after particle bombardment. The resulting green fluorescence was visualized using laser confocal microscopy. (B) The sequence of the triple tandem repeats of the W-box and mW-box binding elements. (C) Yeast one-hybrid assay using the 3 × W-box or mW-box as bait. Yeast cells carrying pGAD-GhWRKY17 or pGAD7 were grown on SD/-Leu/-Ura or SD/-Leu containing 500 ng ml^{-1} AbA. (1) pAbAi-W-box/pGAD-GhWRKY17; (2) pAbAi-W-box/pGAD7; (3) pAbAi-mW-box/pGAD-GhWRKY17; and (4) pAbAi-mW-box/pGAD7. (D) Histochemical analysis of the transactivation activity of GhWRKY17 in *N. benthamiana* leaves. Fully expanded leaves from 8-week-old *N. benthamiana* were agro-infiltrated with W-box-35S mini-GUS alone or co-transformed with W-box-35S mini-GUS and 35S:GhWRKY17 at a concentration of OD_{600} 0.6. GUS staining was performed 3 d after the transformation.

H_2O_2 on *GhWRKY17* transcription. The expression of the *GhWRKY17* transcript was down-regulated at the 2 h time point and up-regulated by H_2O_2 from 4 to 6 h, and then showed a large reduction (1.5-fold induction) 8 h after the treatment (**Fig. 3C**). In the case of ABA treatment, the expression of *GhWRKY17* was up-regulated and reached the highest level at 4 h (3.1-fold induction) (**Fig. 3D**), so we speculated that *GhWRKY17* was an ABA-hypersensitive gene. These results indicate that *GhWRKY17* might play a predominant role in osmotic stress, and this activity probably depends on ABA.

GhWRKY17 promoter analysis

To elucidate further the mechanism underlying the expression patterns of *GhWRKY17*, a 1,163 bp fragment of the *GhWRKY17* promoter (KC430093) was isolated using inverse PCR (I-PCR). Using the PlantCARE databases, various *cis*-elements associated with the stress response were predicted in this promoter region (**Supplementary Table S3**). Specifically, many abiotic stress-responsive elements and two pathogen/elicitor-related elements were identified. In addition, a number of tissue-specific and development-related elements were found. The results suggest that *GhWRKY17* plays a role in the plant response to environmental stresses and in development.

Ectopic overexpression of *GhWRKY17* in *N. benthamiana* enhances plant sensitivity to ABA-mediated seed germination and post-germinative growth

Transgenic plants overexpressing *GhWRKY17* were generated in *N. benthamiana* to investigate the role of *GhWRKY17* further.

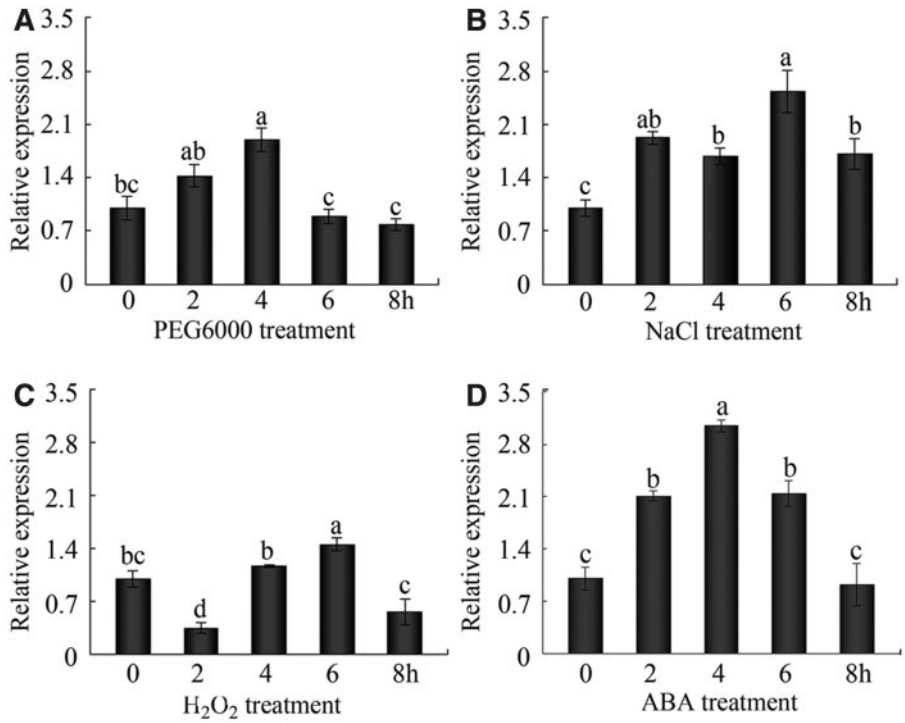

Fig. 3 The expression profiles of *GhWRKY17* under different abiotic stress conditions. Seven-day-old cotton seedlings were subjected to treatment with (A) 15% PEG6000, (B) 200 mM NaCl, (C) 10 mM H_2O_2 or (D) 100 μM ABA. The lanes represent various time points during the treatments. The 18S rRNA was included as an internal control.

Ten independent transgenic lines were obtained using kanamycin selection and PCR analysis (**Supplementary Fig. S2A**). Three representative lines, OE1 (3#), OE2 (6#) and OE3 (8#), were selected using RT–PCR analysis (**Supplementary Fig. S2B**) and showed similar expression to the native endogenous *NbWRKY17* (GenBank accession No. KM085028) gene without gene silencing (**Supplementary Fig. S2C**). Then their T_2 homozygous lines were selected for further analyses.

The increased expression of *GhWRKY17* in response to exogenous ABA suggested that GhWRKY17 might play a role in ABA signaling. Thus, the biological function of *GhWRKY17* in ABA-mediated responses, including the inhibition of seed germination and post-germinative growth, was explored. Under normal conditions, the overexpressing (OE) lines and WT plants showed similar germination rates (**Fig. 4A, B**). However, in medium supplemented with ABA (concentrations ranging from 1 to 5 μM), the OE lines showed a lower germination frequency than the WT plants. At a concentration of 5 μM ABA, the germination rate of the WT seedlings was 50% compared with nearly complete inhibition in the OE lines (**Fig. 4A, B**).

The WT and *GhWRKY17* transgenic plants were also assessed for their responses to ABA during the post-germinative growth stage. The seedlings were germinated on common Murashige and Skoog (MS) medium for 2 d before being transferred to medium supplemented with different concentrations of ABA. In the absence of exogenously applied ABA, the root growth of the OE and WT lines was markedly similar (**Fig. 4C, D**). In the presence of different concentrations of ABA, the shoot and root growth were significantly inhibited. The decrease in the taproot length of the transgenic plants was more remarkable than that in the WT plants. Moreover, for shoot growth, the OE lines exhibited a greater reduction in the whole seedling fresh weight compared with the WT plants (**Fig. 4E**). Together, these data demonstrated that the overexpression of *GhWRKY17* increased the sensitivity of the transgenic plants to ABA at germination and the seedling development stages after germination, which indicated that *GhWRKY17* might act as a negative regulator of ABA signaling during seed germination and post-germinative growth.

Overexpression of *GhWRKY17* in *N. benthamiana* enhances plant sensitivity to drought and salt stress

To investigate whether *GhWRKY17* is involved in drought and salt stress, the seeds of OE and WT plants were sown on MS medium containing 300 mM mannitol or 200 mM NaCl. No difference in germination between the OE and WT plants that were grown on MS medium was observed, but in the presence of mannitol or NaCl, the OE lines consistently germinated much later and showed significantly lower germination rates than the WT plants (**Fig. 5A, B**). The root lengths of the WT and OE lines under drought and salt stress were also calculated. As shown in **Fig. 5C** and **D**, the root growth of the *GhWRKY17* transgenic plants under stress conditions was more seriously inhibited than that in the WT plants.

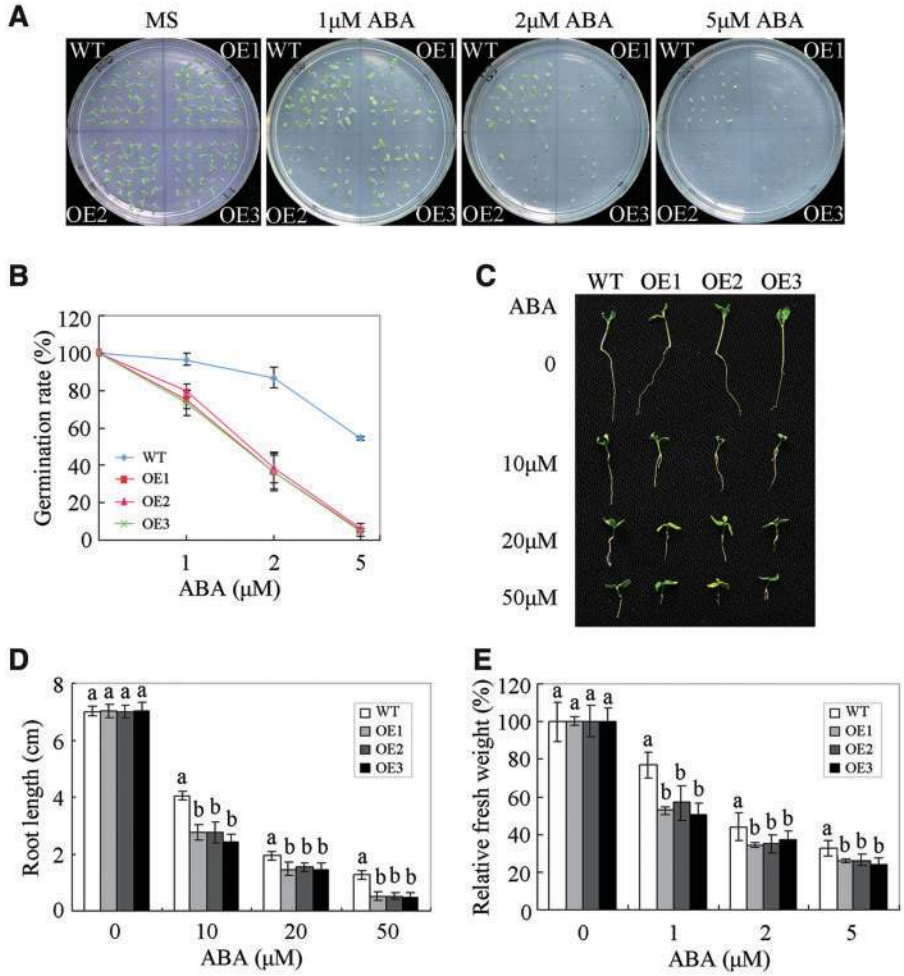

Fig. 4 The overexpression of *GhWRKY17* leads to an ABA-hypersensitive phenotype. (A and B) Comparison and quantitative evaluation of seed germination between the WT and OE lines exposed to 0, 1, 2 and 5 μM ABA. The data represent the means ± SE of three independent experiments, with 30 seeds per genotype and experiment. (C and D) Comparison of the primary root growth of WT and OE seedlings grown on MS plates supplemented with different concentrations of ABA. Three independent experiments were performed with similar results. Each value in (D) represents the mean ± SE of at least 30 seedlings from three independent experiments. (E) Measurement of seedling fresh weight. Seven-day-old germinated seedlings were harvested, and their fresh weights were measured. Their relative fresh weight compared with that of seedlings grown on ABA-free medium is indicated. The different letters above the columns in (B), (D) and (E) indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

To clarify the mechanism of *GhWRKY17* in response to drought and salt stress, sodium tungstate (Tu), a well-known ABA biosynthesis inhibitor, was applied. No significant difference in seed germination was detected between the OE and WT lines grown on MS medium containing 1 mM Tu (**Fig. 5E**). Moreover, when 1 mM Tu was added to medium containing 300 mM mannitol or 200 mM NaCl, the drought and salt sensitivity phenotypes in the OE lines disappeared. These results suggest that the sensitivity of *GhWRKY17* transgenic plants to drought and salt stress is ABA related.

Constitutive expression of *GhWRKY17* reduces drought and salt tolerance during the vegetative stage

To examine further the drought and salt stress tolerance of the transgenic plants, 4-week-old, soil-grown transgenic plants were exposed to drought stress by continuous withholding of water. As shown in **Fig. 6A**, after a 2 week exposure to drought, the transgenic plants became withered, necrotic and bleached, whereas the WT plants displayed mild symptoms. After watering was resumed, <30% of the OE lines survived compared with the 87% survival rate of the WT plants (**Fig. 6B**). Consistent with the drought-sensitive phenotype, the detached leaves of the *GhWRKY17* transgenic plants showed a quicker rate of water loss than those from the WT plants (**Fig. 6C, D**). Furthermore, the transgenic plants showed thinner roots than the WT plants (**Fig. 6C**). ABA-mediated stomatal closure determines the level of transpiration under water-deficit conditions. Thus, we further investigated whether the overexpression of *GhWRKY17* affected the sensitivity of guard cells to ABA treatment. Without ABA treatment, the OE and WT plants showed the same stomatal length:width ratio of fully opened

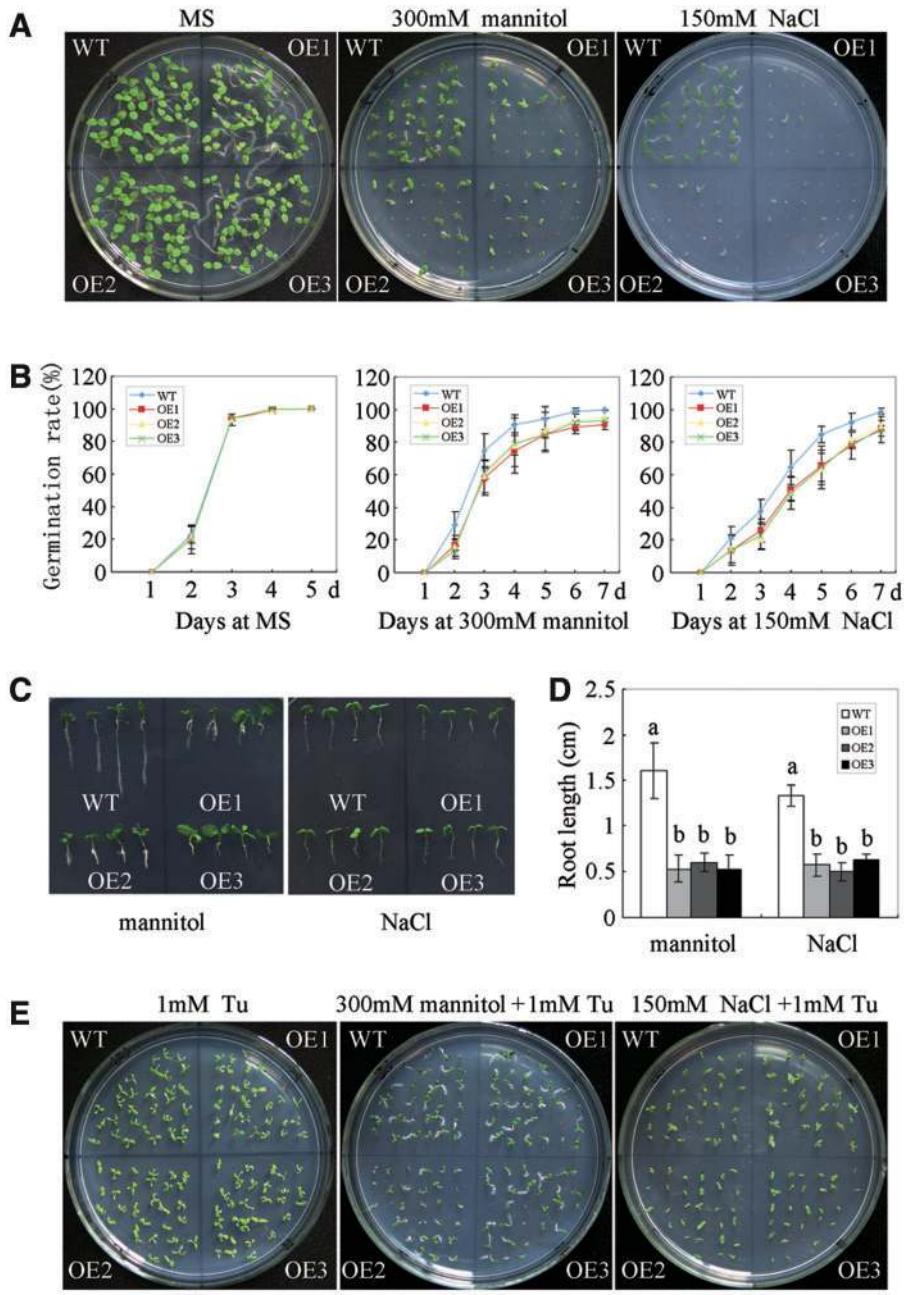

Fig. 5 Analysis of germination and root elongation in WT and transgenic *N. benthamiana* exposed to drought and salt stress. (A) Germination of *N. benthamiana* seeds after treatment with 300 mM mannitol and 200 mM NaCl. (B) Statistical analysis of the seed germination rate in (A). (C) Root elongation of WT and *GhWRKY17* transgenic plants after treatment with 300 mM mannitol and 200 mM NaCl. (D) Statistical analysis of the root length in (C). (E) Germination of WT and transgenic plants grown on MS medium containing 1 mM Tu, 1 mM Tu + 300 mM mannitol or 1 mM Tu + 200 mM NaCl. The data in (B) and (D) represent the means ± SE from three independent experiments. The different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

stomata, but the OE plants showed a lower ratio than the WT plants after ABA treatment (**Fig. 6E, F**). The larger aperture size of the stomata might reflect the observed increase in the water loss rate from the detached leaves of the transgenic plants.

For the salt tolerance assay, the WT and OE lines were exposed to long-term high salinity. Six-week-old plants were irrigated with a 200 mM NaCl solution for 14 d. The growth of the OE plants was somewhat more delayed than that of the WT plants. Furthermore, the signs of stress were more severe in the transgenic lines, which displayed serious chlorosis and wilting (**Fig. 6G**). The total Chl content, which reflects the presence of chlorosis, was reduced to 68, 71 and 70% in the OE lines, whereas the Chl content in the WT plants was 84%, compared with that in the untreated plants (**Fig. 6H**).

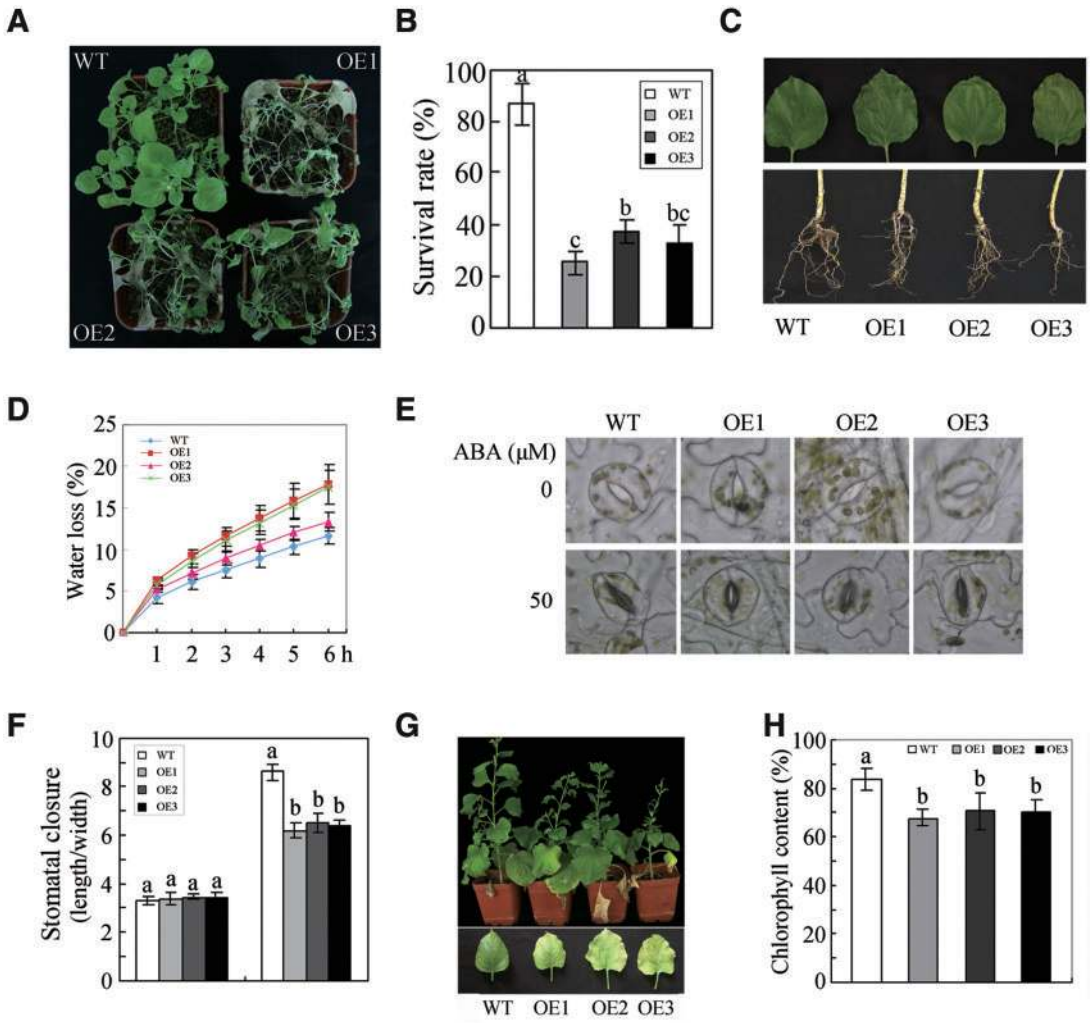

Fig. 6 The constitutive expression of *GhWRKY17* enhances the sensitivity of transgenic *N. benthamiana* to drought and salt stress. (A) Sensitivity of 4-week-old WT and OE plants to drought stress. Drought stress was imposed by withholding water for 2 weeks, and photos were taken 3 d after the plants were re-watered. (B) Survival rates of the plants shown in (A) after re-watering. The SE values (error bars) were calculated from three independent experiments ($n \geq 25$ for each experiment). (C) Leaf (top) and root (bottom) phenotypes of the analyzed plants under the water-deficit condition. (D) Water loss rate of the detached leaves from WT and *GhWRKY17* transgenic plants. The values represent the means ± SE. (E and F) Stomatal closure in response to ABA treatment. The data represent the means ± SE of 40 stomata from three independent experiments. (G) Phenotype of *GhWRKY17*-OE plants exposed to long-term salt stress. (H) Quantification of the Chl content. The values represent the Chl content in the salt-treated plants relative to that of the untreated plants in three independent experiments ($n > 20$). The different letters above the columns in (B), (C), (D), (F) and (H) indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

Taken together, these data suggest that *GhWRKY17* plays an important role, potentially as a negative regulator, in drought and salt stress signaling.

Decreased ABA accumulation and expression of marker genes involved in the ABA signal pathway in *GhWRKY17*-overexpressing plants

The phytohormone ABA serves as an endogenous messenger during stress responses in plants (Raghavendra et al. 2010). To examine the wilt phenotypes in drought-stressed plants, the endogenous ABA contents were measured. All plants were initially grown under normal watering conditions and then subjected to water deficit. Under normal conditions, the ABA level in the OE lines was similar to that in the WT plants. When exposed to drought stress, the ABA level was markedly increased as time went on, and the WT plants accumulated 1.31-fold more ABA than the OE lines at 14 d (**Fig. 7A**). The reduced ABA levels might enhance the drought sensitivity of the OE lines. The results also suggest that the expression of some ABA-responsive genes might be altered in the transgenic plants. To verify this possibility, the expression of ABA-inducible genes, *NbAREB1* (ABA-responsive element binding), *NbDREB* (dehydration-responsive element binding), *NbERD* (early responsive to dehydration), *NbLEA* (late embryogenesis-abundant protein), *NbNCED* (9-*cis*-epoxycarotenoid

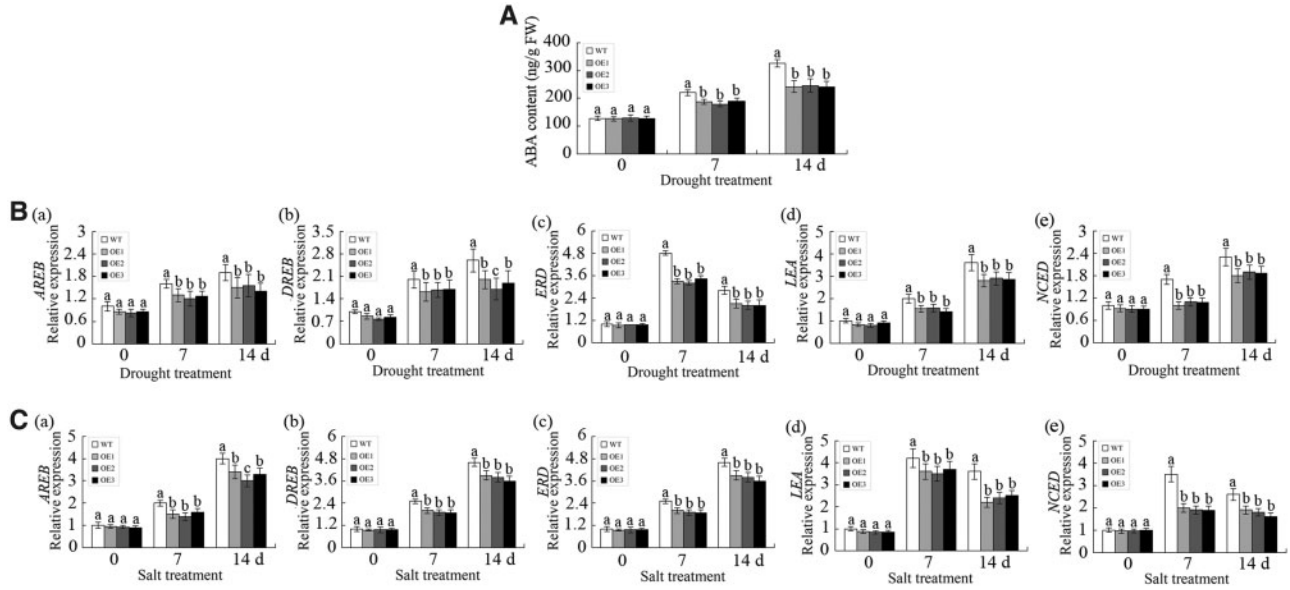

Fig. 7 ABA accumulation and gene expressions in the WT and *GhWRKY17* transgenic plants. (A) Endogenous ABA levels in the leaves of WT and GhWRKY17 transgenic plants under normal and water-deficit conditions. (B and C) The expression levels of ABA-inducible genes in the WT and transgenic plants during the drought and salt stress responses as determined using qPCR. The values represent the means ± SE of three independent experiments. The different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

dioxygenase), which are stress-inducible marker genes that function in the ABA-dependent and ABA-independent pathways (Fujita et al, 2005, Shinozaki and Yamaguchi-Shinozaki 2007, Luo et al, 2012), were analyzed under drought and salt treatments. The results showed that, under the water-deficit condition, the expression of ABA-inducible genes in the transgenic plants was lower than in the WT plants (**Fig. 7B**). Similarly, under salt treatment, the expression levels of ABA-inducible genes in the transgenic plants were decreased compared with those in the WT plants (**Fig. 7C**).

GhWRKY17 transgenic plants display increased ROS levels and enhanced oxidative damage

Drought and high salinity enhance the production of ROS and cause ROS-associated injury (Borsani et al. 2005, Abbasi et al. 2007). To test whether *GhWRKY17* regulates ROS levels in response to drought and salt stress, we compared the ROS levels in the OE lines and WT plants under normal and stress conditions. The accumulation of H_2O_2 and O_2^-, two prominent ROS species that are involved in stress signaling and oxidative injury, was determined using 3,3′-diaminobenzidine (DAB) and nitroblue tetazolium (NBT) staining, respectively. As shown in **Fig. 8A**, under normal conditions, H_2O_2 and O_2^- accumulated at low levels, and no obvious difference was detected between the OE and WT lines. After exposure to drought and salt, the OE lines accumulated more H_2O_2 and O_2^- than the WT plants, indicating that *GhWRKY17* increased the ROS levels under conditions of drought and salinity.

The up-regulation of ROS in the OE lines suggests that *GhWRKY17* enhances ROS-associated oxidative injury. To confirm this idea, electrolyte leakage and MDA content, which reflect membrane injury and membrane lipid peroxidation, respectively, were examined. The basal levels of electrolyte leakage and MDA content in the OE and WT lines were similar in the absence of stress (**Fig. 8B, C**). After drought stress, the electrolyte leakage and MDA levels were markedly higher in the OE lines than in the WT plants. In contrast, the OE lines and WT plants under salt stress showed no significant difference in electrolyte leakage, but the MDA content in the OE lines was 1.2-fold higher than that in the WT plants. Plants accumulate osmotic solutes to regulate their osmotic potential during drought and salinity, which are important for protecting cells against increased levels of ROS under stress (Miller et al. 2010). There is increasing evidence that proline metabolism plays a key role in triggering the signal molecule ROS, and accumulation of proline may participate in scavenging ROS in response to stress (Rejeb et al. 2014). To investigate whether *GhWRKY17* regulates ROS signaling by modulating the production of osmotic solutes under drought and salt stress, the proline content in the plants was measured. Under normal conditions, the proline levels in the WT and OE lines were low (**Fig. 8D**). Drought and salt stress markedly increased the proline content in the leaves of the WT and OE plants, but the OE lines accumulated 11% and 15% less proline than WT plants under drought and salt stress, respectively. Taken together, these results demonstrate that *GhWRKY17* plays a critical role in regulating the cellular levels of ROS and, therefore participates in oxidative stress.

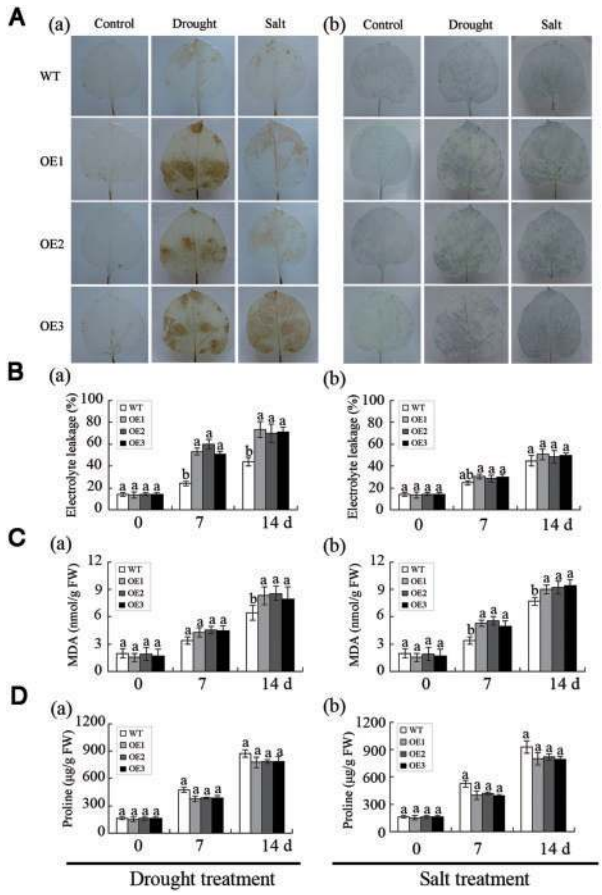

Fig. 8 The constitutive expression of *GhWRKY17* increased ROS production and oxidative damage in *GhWRKY17* transgenic plants under drought and salt stress conditions. (A) Representative photographs showing DAB (a) and NBT (b) staining of H_2O_2 and O_2^{--}, respectively. (B) Electrolyte leakage in the leaves of WT and *GhWRKY17* transgenic plants after drought (a) and salt (b) treatments. (C) MDA content in the WT and OE lines under drought (a) and salt (b) stress. (D) Proline contents in the WT and transgenic plants under drought (a) and salt (b) stress. The data in (B), (C) and (D) represent the means ± SE from three independent experiments. The different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

Overexpression of *GhWRKY17* affects the expression of oxidation-related genes and decreases antioxidant enzyme activities under stress conditions

To explore the underlying mechanism of *GhWRKY17* in ROS accumulation, and thus stress sensitivity, we examined the expression levels of genes that encode ROS-scavenging enzymes [ascorbate peroxidase (*APX*), catalase (*CAT*) and superoxide dismutase (*SOD*)] and ROS producers, i.e. the respiratory burst oxidase homologs (*RbohA* and *RbohB*). After drought and salt treatments, the transcript accumulation of *APX*, *CAT* and *SOD* was lower in OE lines, with the expression levels of *RbohA* and *RbohB* being significantly higher than those in WT plants (**Fig. 9A, B**). Moreover, the total activities of antioxidant enzymes in the transgenic plants exposed to drought stress exhibited reduced activities of 24, 21, 40 and 28% for SOD, peroxidase (POD), CAT and APX, respectively, compared with those in WT plants, whereas the transgenic plants exposed to salt treatment showed reduced activities of 15, 22, 26 and 26%, respectively (**Fig. 9C–F**). The different expression levels of multiple antioxidant enzymes and the reduced antioxidant enzyme activities might reflect the enhanced oxidative damage in transgenic plants under drought and salt stress, suggesting that *GhWRKY17* may be involved in ROS signaling.

Overexpressing *GhWRKY17* reduces the tolerance of the transgenic plants to oxidative stress

The potential role of *GhWRKY17* in oxidative stress was further evaluated by exposing the plants to methyl viologen (MV), which is a herbicide that causes Chl degradation and cell membrane leakage through ROS production (Kurepa et al. 1998). The growth of the transgenic plants was similar to that of the WT on MV-free medium. However, the cotyledon greening rates of the OE plants were significantly lower than those of WT plants grown on MV-containing medium (**Fig. 10A, B**). These results suggest that GhWRKY17 negatively affects oxidative stress tolerance with respect to cotyledon greening during the post-germinative growth phase.

The oxidative stress sensitivity of the *GhWRKY17* transgenic plants was further investigated at the vegetative stage. Leaf discs obtained from 8-week-old plants were incubated in a range of MV solutions (0–100 μM). After treatment for 72 h, bleaching or chlorosis was observed to a more severe extent in the OE lines than in the WT plants (**Fig. 10C**). The Chl content further validated the oxidative damage between the transgenic and WT plants (**Fig. 10D**). These data demonstrate that *GhWRKY17* plays a negative role in oxidative stress.

Discussion

Previously, WRKY transcription factors in plants were widely reported to play key roles in biotic stress responses (Li et al. 2004, Journot-Catalino et al. 2006, Li et al. 2006, Xu et al. 2006, Shen et al. 2007, Qiu and Yu 2008, Matsushita et al. 2012). Over the last several years, growing evidence has shown that WRKY proteins are also involved in plant ABA signaling and abiotic stress (Miller et al. 2008, Jiang and Yu 2009, Ren et al. 2010, Shang et al. 2010, Rushton et al. 2011, Chen et al. 2012, Jiang et al. 2012). Some WRKY proteins have diversified from the WRKY gene family at relatively high rates under evolutionary pressure to acquire unique biological functions (Dong et al. 2003). Here, we isolated a group IId WRKY gene named *GhWRKY17*, which specifically binds to the W-box (**Fig. 2C**) and transactivates the expression of downstream GUS reporter genes in plant leaves (**Fig. 2D**). Overexpression of *GhWRKY17* in *N. benthatiana* reduced plant tolerance to drought and salt stress (**Fig. 5A**). Further experiments revealed that GhWRKY17 contributed to plant drought and salt stress through ABA signaling and the regulation of cellular levels of ROS. Drought and salt, which are two major abiotic factors, directly influence crop productivity. Thus, our data extend

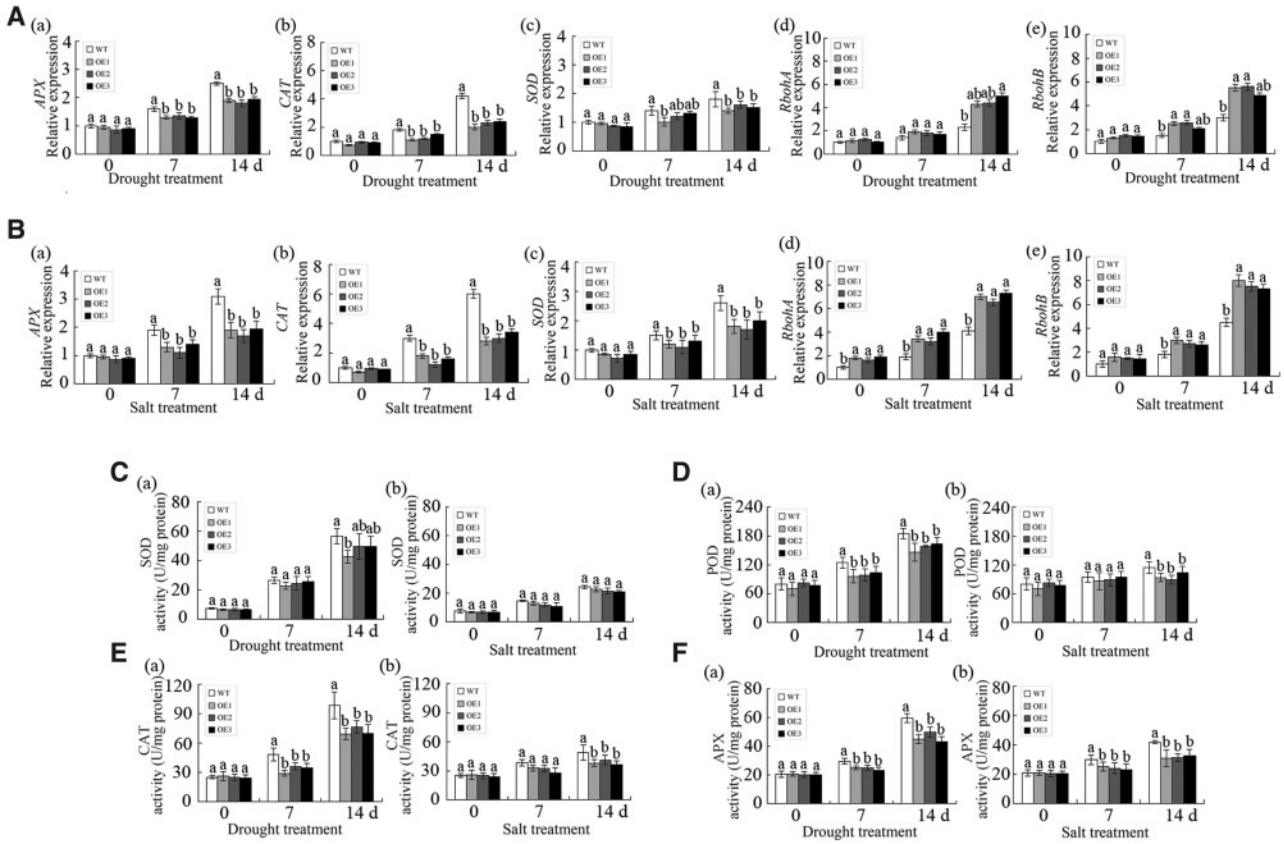

Fig. 9 Overexpression of *GhWRKY17* affects the antioxidant enzyme systems in transgenic plants under drought and salt stress conditions. (A and B) The expression levels of ROS-scavenging and producing genes in WT and OE lines analyzed by qPCR. (C–F) Activities of SOD, POD, CAT and APX, respectively. The values represent the means ± SE of three independent experiments. The different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

our knowledge of the biological function of the WRKY proteins and facilitate the targeted genetic manipulation of crop plants to improve stress tolerance.

GhWRKY17 was found to be present as a single-copy gene (**Supplementary Fig. S1**). Similarly, other group IId WRKY genes, *GhWRKY15*, *GhWRKY11* and *GhWRKY39*, were single-copy genes (Yu et al. 2012, Sun et al. 2012, Shi et al. 2014). Therefore, we speculate that the gene belonging to the group IId WRKY superfamily was a single-copy gene. These findings provide a theoretical basis for further study of the gene, and this characteristic might be related to the evolutionary process. In previous studies, *GhWRKY15* and *GhWRKY11* were shown to play important roles in the response to pathogens, and *GhWRKY39* positively regulates the plant response to pathogen infection and salt stress (Yu et al. 2012, Sun et al. 2012, Shi et al. 2014). These results indicate that the same WRKY subgroup may have redundant/divergent functions.

Previous studies have shown that a large number of genes encoding receptors, kinases, transcription factors and other signaling molecules in plants are induced after exposure to various abiotic stresses (Matsui et al. 2008). In wheat, eight WRKY genes are responsive to low and high temperature, and NaCl or polyethylene glycol (PEG) treatment (Wu et al. 2008). Similarly, *GhWRKY17* is strongly induced at the transcriptional level during the early development stage in cotton upon exposure to PEG6000, NaCl, H_2O_2 or ABA (**Fig. 3**). Analysis of the *GhWRKY17* promoter region identified a MYB recognition site that is also found in the promoter regions of several dehydration-responsive genes that encode known transcriptional activators in ABA signaling (Abe et al. 2003). These observations suggest that GhWRKY17 functions in the plant response to abiotic stress.

ABA is an important phytohormone that regulates plant development and various stress responses. ABA maintains seed dormancy, prevents germination and inhibits seedling growth (Finkelstein et al. 2002). The WRKY proteins are best known for their roles as ABA-inducible repressors of seed germination in the regulation of the ABA response (Jiang and Yu 2009, Chen et al. 2010, Ren et al. 2010). In this study, GhWRKY17 played a negative role in regulating ABA-mediated seed germination and seedling growth (**Fig. 4**). Under mannitol and NaCl exposure, the germination and root growth of *GhWRKY17* transgenic plants were seriously inhibited compared with WT plants (**Fig. 5**). However, when the ABA biosynthesis inhibitor Tu was added, the phenotypes of the WT and OE lines were similar. All the above results indicated that drought and salt stress sensitivity of the transgenic plants were

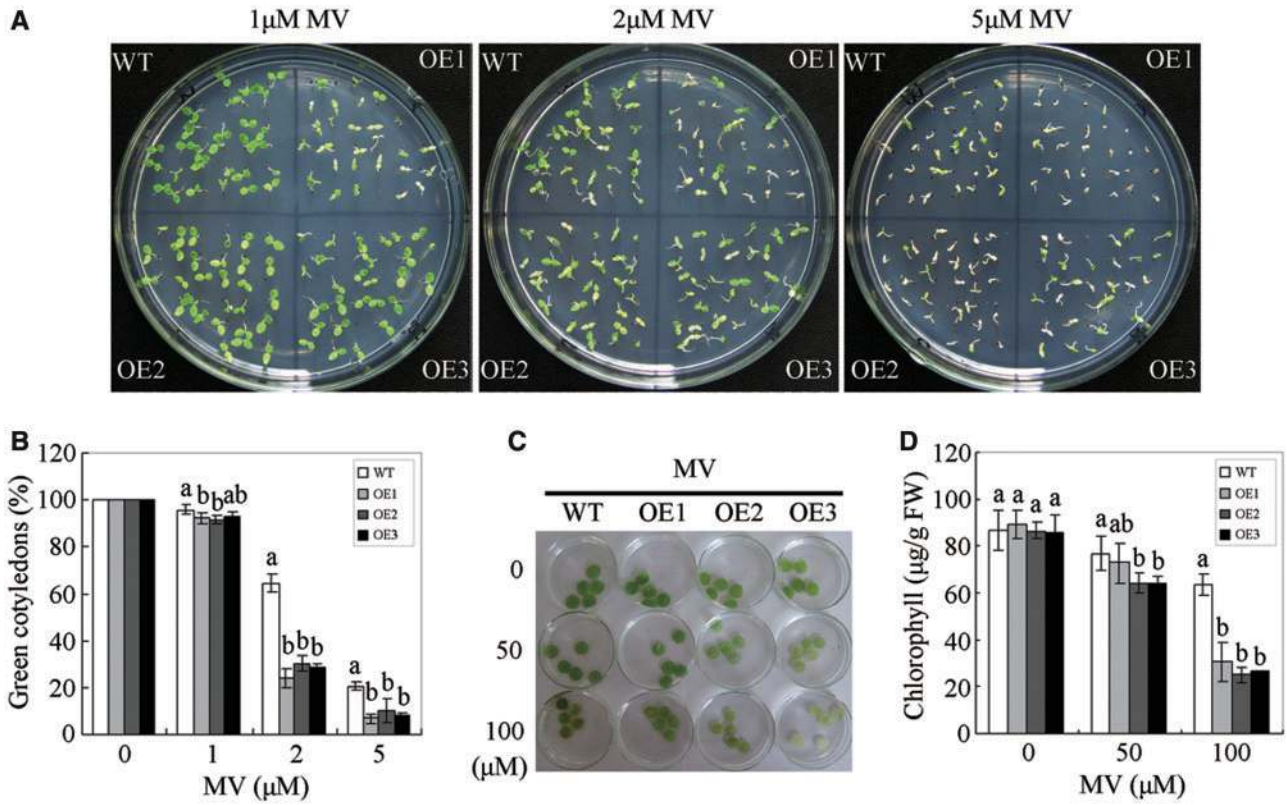

Fig. 10 Oxidative stress sensitivity of the *GhWRKY17* transgenic plants. (A) Phenotypes of young seedlings grown on medium containing 0, 1, 2 or 5 μM MV. (B) Quantification of cotyledon greening in the seedlings described in (A). (C) Leaf discs obtained from 8-week-old WT and OE seedlings were incubated in different concentrations of MV (0, 50 and 100 μM) for 72 h before the photos were taken. (D) Relative Chl content after MV treatment. The Chl content was compared with that of mock-treated samples. The data in (B) and (D) represent the means ± SE of three independent experiments. The different letters above the columns indicate significant differences ($P < 0.05$) according to Duncan's multiple range test.

dependent upon the presence of ABA. Consistent with these results, Zhu (2002) also reported that drought and salt stress conditions can trigger the ABA-dependent signaling pathway.

The constitutive expression of *GhWRKY17* reduced drought and salt stress tolerance in the transgenic plants, as was revealed by the changes that were observed in the physiological parameters of the plants, including the survival rate, leaf water loss and Chl content (**Fig. 6**). ABA-mediated stomatal closure plays a key role in the response to drought and, therefore, increases the capacity for osmotic regulation in plants. Moreover, an increased ABA content is beneficial for plants under stress conditions as a result of ABA-induced changes at the cellular and whole-plant levels (Xiong and Zhu 2003). In addition, *GhWRKY17* transgenic plants were less sensitive to ABA-induced stomatal closure after ABA treatment (**Fig. 6E**). These results indicate that *GhWRKY17* might function in ABA signaling. Similar results were reported for the *abo3/AtWRKY63* mutant (Ren et al. 2010). AtWRKY63 interacts with the W-box in the *AREB1/ABF2* promoter in vitro, and ABA-induced expression of *AREB1/ABF2* is partially impaired in the *AtWRKY63* mutant (Ren et al. 2010). Two recent mutant analyses presented direct evidence that WRKY proteins are components of other ABA-induced signaling networks (Jiang and Yu 2009, Jiang et al. 2012). Drought and salt stress can induce ABA accumulation as a result of the activation of ABA synthesis and inhibition of ABA degradation (Zhu 2002). The OE lines accumulated less ABA under drought stress compared with the WT plants (**Fig. 7A**), which indicated that GhWRKY17 functioned in the ABA response and in ABA accumulation. The role of ABA in drought and salt stress is at least two-fold, as it affects the water balance and cellular dehydration tolerance. While its role in the water balance occurs primarily through guard cell regulation, its role in cellular dehydration tolerance involves the induction of genes encoding dehydration tolerance proteins in nearly all cells (Zhu 2002). Thus, the reduced ABA sensitivity of the guard cells and the lower ABA levels contribute to the increased water loss and reduced drought tolerance capacity. The accumulation of ABA triggered by drought and salt stress, in turn, induces the expression of stress-related genes through ABA-dependent and ABA-independent regulatory systems. *AREB*, *DREB*, *ERD*, *LEA* and *NCED* were reported to function in the ABA-dependent and ABA-independent pathways, respectively (Fujita et al. 2005, Shinozaki and Yamaguchi-Shinozaki 2007, Luo et al. 2012). In the ABA-dependent pathway, AREB can bind to and activate the expression of ABRE-binding genes, and it serves as a major ABA-responsive element. In the ABA-independent pathways, DREB is known to regulate the expression of many stress-inducible genes. *NbERD*, *NbLEA* and *NbKIN* genes were target genes of *NbAREB* and *NbDREB* genes (Shinozaki et al. 2003, Fujita et al.

2005). The gene encoding NCED, a key enzyme of ABA biosynthesis, is a known participant in ABA-mediated responses (Shinozaki et al., 2003, Barrero et al. 2006). In this study, under drought and salt stress, *NbAREB* and *NbDREB* were down-regulated in transgenic plants when compared with WT plants. Therefore, it is likely that *GhWRKY17* is involved in drought and salt stress through ABA-dependent and ABA-independent pathways.

Drought and salinity impose osmotic stress, which leads to the production of ROS (Krasensky and Jonak 2012). ROS, which mainly consist of O_2^- and H_2O_2, act as important signal transduction molecules mediating tolerance to various stresses (Bhattacharjee 2005). It is critical to modulate ROS to an appropriate level, as they cause damage when they accumulate excessively. Although cellular ROS levels are regulated through the ABA-triggered regulation of ROS-producing and ROS-scavenging genes (Mittler et al. 2004, Abbasi et al. 2007), the mechanisms that control ROS signaling through ABA during drought and salt stress remain unclear. In the present study, drought and salt stress in plants overexpressing *GhWRKY17* enhanced ROS accumulation, as indicated by histochemical staining by DAB and NBT, electrolyte leakage and the MDA content (**Fig. 8**). Further study revealed that, under drought and salt stress, the expression of ROS-scavenging genes, including *APX*, *CAT* and *SOD*, was down-regulated in the OE lines, while the expression levels of ROS-producing genes (*RbohA* and *RbohB*) were significantly higher than in the WT plants (**Fig. 9A, B**). These results suggest that GhWRKY17 might contribute to drought and salt stress by regulating the cellular levels of ROS and being involved in ROS signaling. ROS signaling is an integral part of the acclimation response of plants to drought or salinity (Miller et al. 2010). It has been reported that ABA can increase generation of ROS, induce the expression of antioxidant genes encoding SOD, CAT and APX, and enhance the activities of these antioxidant enzymes in plant tissues (Hu et al. 2005). Water deficit stress-induced ABA triggers the activation of the antioxidant defense system, which includes enzymatic and non-enzymatic activities. The up-regulation of the activities of ROS-scavenging enzymes protects plants against oxidative damage due to ROS. In addition, proline plays an important role in protecting cells against increased levels of ROS under stress (Miller et al. 2010). In a previous study, transgenic wheat plants that accumulated higher proline levels than WT plants were shown to exhibit less lipid peroxidation of their membranes during drought, indicating a role for proline in reducing ROS damage during drought (Vendruscolo et al. 2007). However, the activities of antioxidant enzymes, including SOD, POD, CAT and APX, and the proline content in the *GhWRKY17*-OE plants were lower than those in the WT plants under conditions of drought and high salinity (**Fig. 9C–F**), which contributed to the enhanced sensitivity of the transgenic plants. The increased sensitivity of GhWRKY17 after exposing the plants to MV during seed germination and vegetative growth further confirmed the involvement of *GhWRKY17* in ROS signaling.

Based on these data, we conclude that GhWRKY17 exhibits important physiological functions in the drought and salt response through ABA signaling and regulation of ROS. The physiological mechanisms of GhWRKY17 in governing the plant response to drought and salt stress show a high degree of similarity, most probably because salt stress is often accompanied by drought stress, and both stresses induce water deprivation through ABA-dependent and ABA-independent pathways (Zhu 2002).

Materials and Methods

Plant materials and treatments

Cotton (*G. hirsutum* L. cv lumian 22) seeds were grown in a growth chamber under greenhouse conditions at 28°C and a 16 h light/8 h dark cycle (light intensity of 200 μmol m^{-2} s^{-1}; relative humidity of 60–75%). For the drought and salinity treatments, 7-day-old seedling roots were placed in a solution containing 15% PEG6000 (w/v) or 200 mM NaCl, respectively. For the H_2O_2 and ABA treatments, the seedlings were sprayed with 10 mM H_2O_2 or 100 μM ABA. The treated cotyledons were harvested at the appropriate times as indicated, frozen in liquid nitrogen and stored at −70°C for RNA extraction. Each treatment was repeated at least twice.

Cloning of *GhWRKY17*

The full-length cDNA, genomic sequence and promoter fragment of *GhWRKY17* were obtained as previously described (Shi et al. 2010). The primer sequences used in this study are listed in **Supplementary Table S1**. Homologous GhWRKY17 protein sequences were retrieved from the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned using the DNAman software 5.2.2 (Lynnon Biosoft). A phylogenetic tree was generated using the Neighbor–Joining method in the MEGA 4.1 program. The PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) and PLACE (http://www.dna.affrc.go.jp/PLACE/faq.html) programs were used to analyse the *GhWRKY17* promoter sequence.

Estimating the copy number of *GhWRKY17* in the cotton genome

The copy number of *GhWRKY17* in the cotton genome was estimated using qPCR according to previously described methods (Mason et al. 2002). *GhRDR6*, a single-copy gene in cotton determined by Southern blot analysis (Wang et al. 2012), was used as a standard. The primer sequences are listed in **Supplementary Table S1**.

Subcellular localization of GhWRKY17

The coding region of *GhWRKY17* was amplified using specific primers containing the *Xba*I and *Xho*I restriction enzyme sites (**Supplementary Table S1**). The fragment was fused to the 5′-terminal end of the GFP gene to generate pBI121-GhWRKY17-GFP, which is driven by the CaMV 35S promoter. The pBI121-GhWRKY17-GFP construct and the positive control were transformed into onion epidermal cells using the Biolistic PDS-1000/He system (Bio-Rad). The resulting green fluorescence was observed using a confocal microscope (LSM 510 META; Carl Zeiss AG).

Binding assay using a yeast one-hybrid system

The Matchmaker Gold Yeast One-Hybrid Library Screening System (Clontech) was used to examine protein binding. An oligonucleotide sequence containing three tandem copies of the W-box (TTGACC) was synthesized, annealed and inserted into the pAbAi vector. The resultant plasmid was introduced into the yeast strain Y1HGold, forming a W-box-specific reporter strain. The background AbA^r expression of the reporter strain was tested. The ORF of *GhWRKY17* was fused in-frame with the GAL4 activation domain of the one-hybrid vector pGADT7, forming pGAD-GhWRKY17. The W-box-specific reporter strain was transformed with pGADT7 or pGAD-GhWRKY17, and a mutant W-box (mW-box) (TAGACG) was used as a negative control. The

detailed, yeast one-hybrid procedure was performed according to the manufacturer's protocol (Clontech).

Transactivation assay

To construct the reporter vector, a sequence containing three tandem W-box sequences fused to the CaMV 35S minimal promoter (W-box-35S mini) was synthesized, annealed and substituted for the CaMV 35S promoter in pBI121GUS (Clontech) to generate the W-box-35S mini-GUS plasmid. For 35S:GhWRKY17, the ORF of *GhWRKY17* was inserted into pBI121, replacing GUS. The W-box-35S mini-GUS and 35S:GhWRKY17 plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101. The *Agrobacterium*-mediated transient assay was performed as previously described (Yang et al. 2000). GUS histochemical staining was detected using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) as the substrate. The samples were fixed in ice-cold 90% (v/v) acetone for 10 min and incubated in GUS staining solution [10 mM Na_2EDTA, 100 mM NaH_2PO4, 0.5 mM $K_4Fe(CN)_6{\cdot}3H_2O$, 0.1% Triton X-100 and 0.5 mg l^{-1} X-gluc, pH 7.0] overnight at 37°C. Chl was removed using several changes of 70% ethanol.

RNA extraction and qPCR

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. To eliminate genomic DNA contamination, each 2 μg sample of RNA was treated with 2 U of DNase I at 37°C for 1 h. First-strand cDNA was then synthesized using EasyScript First-Strand cDNA Synthesis SuperMix (TransGen), and qPCR was performed using the SYBR Premix Ex Taq (TAKARA) in a 25 μl volume and a CFX96™ Real-time Detection System (Bio-Rad). The PCR program was as follows: pre-denaturation at 95°C for 30 s; 40 cycles of 95°C for 30 s, 53°C for 15 s and 72°C for 15 s; and a melt cycle from 65 to 95°C. The *G. hirsutum* 18S rRNA gene (*18S rRNA*) and *N. benthamiana β-actin* genes were used as internal references. The primers used in the qPCR analysis are shown in **Supplementary Table S1**.

Generation of transgenic overexpression lines and stress tolerance assays

The 35S:GhWRKY17 construct that was generated for the transactivation assay was introduced into *A. tumefaciens* LBA4404 using electroporation. *Nicotiana benthamiana* transformation was performed using the leaf disc method, as previously described (Horsch et al. 1985). The transformants were selected using kanamycin resistance (50 μg ml^{-1}), and the presence of the transgene in the regenerating plantlets was further confirmed using PCR. The transgenic T_2 lines were used in the experiments.

For the germination assay, the seeds were surface sterilized and planted on 1/2 MS medium containing different concentrations of ABA, mannitol or NaCl or 1 mM Tu, and the germination rates were measured daily. For the root elongation assay, the seeds were germinated on MS medium for 3 d. When the radicles emerged, the plants were transferred to MS medium supplemented with ABA, mannitol or NaCl and placed upright in the chamber. The primary root lengths were measured after the treatments.

For the drought treatment, 4- and 6-week-old *GhWRKY17*-OE and WT plants were sown in pots and adequately watered. Subsequently, water was withheld for approximately 14 d, and the survival rates were recorded after re-watering for 1 week. To measure water loss, fully expanded leaves were cut from 8-week-old plants and weighed immediately (fresh weight). The leaf samples were placed with the abaxial side up on the bench (humidity, 45–50%, 20–22°C) and weighed at the designated time intervals (desiccated weight). The rate of transpirational water loss was calculated relative to the initial fresh weights.

For the salt stress treatment, soil-grown plants were treated daily with 200 mM NaCl (200 ml) every day and maintained under the same growth conditions as described above. The Chl content was measured according to Lichtenthaler and Wellburn (1983). To examine the ABA response, a stomatal aperture assay was performed essentially as previously described (Chen et al. 2006). Epidermal peels were incubated in a buffer solution under high humidity for 12 h to open the stomata fully. Subsequently, the peels were treated with 50 μM ABA for 3 h to assess the stomatal response. The ratio of stomatal length to width indicated the degree of stomatal closure.

For oxidative stress treatment, the seeds were germinated on 1/2 MS medium supplemented with different concentrations of MV (0, 1, 2 and 5 μM), and the cotyledon greening rates were calculated. Leaf discs (1.3 cm in diameter) were detached from the fully expanded leaves of 8-week-old plants and incubated in an MV solution (0, 50 and 100 μM) for 72 h before the Chl contents were measured.

Quantification of endogenous ABA content

Fresh leaves were ground in liquid nitrogen and homogenized in 80% (v/v) methanol containing 1 mM butylated hydroxytoluene overnight at 4°C to extract the ABA. After centrifugation at 4,000 × *g* for 20 min, the supernatant liquid was eluted through a Sep-Pak C_{18} cartridge (Waters) and dried in N_2. The residues were dissolved in phosphate-buffered saline (PBS; 0.01 M, pH 7.5), and the endogenous ABA content was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Yansheng) according to the manufacturer's instructions.

Histochemical detection of ROS and measurement of electrolyte leakage, MDA and proline content

A histochemical staining procedure was used to detect the accumulation of H_2O_2 and O^{2-} using DAB and NBT, respectively. Detached leaves were incubated in DAB (1 mg ml^{-1}, pH 3.8) or NBT (0.1 mg ml^{-1}) overnight at room temperature in the dark. The stained leaves were then boiled in 95% ethanol to remove the Chl before imaging.

Electrolyte leakage was determined using relative conductivity as previously described (Cao et al. 2007). Lipid peroxidation was estimated as the MDA content using a previously described method (Cui and Wang 2006), and the free proline content was measured as previously described (Shan et al. 2007).

Antioxidant enzyme activity assays

The antioxidant enzyme activities of SOD, POD, CAT and APX in the leaves were estimated as previously described (Q.Li et al. 2010). Each assay was replicated at least three times per sample.

Statistical analysis

The data were analyzed using Duncan's multiple range test and analysis of variance (ANOVA) using the Statistical Analysis System (SAS) version 9.1 software. The results were expressed as the means ± SE of triplicate experiments ($n = 3$). Comparisons showing significant differences are shown.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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