

# *GhWRKY44*, a WRKY transcription factor of cotton, mediates defense responses to pathogen infection in transgenic *Nicotiana benthamiana*

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**Abstract** WRKY transcription factors play essential roles in mediating various stress responses in plants. Although numbers of researches have investigated the functional mechanisms of WRKY TFs, relative to the research progress in model plants, rather limited numbers of WRKY TFs have been functionally characterized in cotton. In this study, we isolated and characterized *GhWRKY44*, a group I WRKY gene from cotton (*Gossypium hirsutum*). Subcellular localization indicated that GhWRKY44 was localized to the nucleus. Additionally, a group of *cis*-acting elements associated with the response to environmental stresses were predicted in the promoter. The expression of *GhWRKY44* can be induced by pathogen injection, abiotic stresses and diverse signaling molecules. Furthermore, the overexpression of *GhWRKY44* in *N. benthamiana* exhibited enhanced resistance to bacterial pathogen *R. solanacearum* and fungal pathogen *R. solani* compared with wild-type plants. Importantly, several

defense-related genes were induced in the transgenic plants, including *PR-1*, *PR-2*, *PR-5* and *NPR1* for SA signaling, and *PR-4* for JA signaling. The transgenic plants also exhibited lower levels of ROS ( $H_2O_2$  and  $O_2^-$ ) accumulation than wild-type plants following pathogen infection. Taken together, these results suggest that *GhWRKY44* positively regulates pathogen induced plant disease resistance, and these findings will expand our knowledge on the functions of WRKY TFs in multiple signal transduction pathways of disease resistance.

**Keywords** *GhWRKY44* · Cotton · Disease resistance · ROS · Defense signaling

## Abbreviations

CTAB	Cetyltrimethyl ammonium bromide
DAB	3,3'-Diaminobenzidine
DAPI	4',6-Diamidino-2-phenylindole
ET	Ethylene
GFP	Green fluorescent protein
Gh	<i>Gossypium hirsutum</i>
hiTAIL-PCR	High-efficiency TAIL-PCR
MeJA	Methyl jasmonate
MES	2-( <i>N</i> -Morpholino)ethanesulfonic acid
MS medium	Murashige and Skoog medium
Nb	<i>Nicotiana benthamiana</i>
NBT	Nitro blue tetrazolium
NPR1	Non-expression of PR1
OE	Overexpression
ORF	Open reading frame
PR	Pathogenesis-related
RACE	Rapid amplification of cDNA ends
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-PCR
<i>R. solanacearum</i>	<i>Ralstonia solanacearum</i>

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<i>R. solani</i>	<i>Rhizoctonia solani</i>
qRT-PCR	Quantitative real-time PCR
SA	Salicylic acid

## Introduction

Pathogen infection is one of the most severe biotic stresses that restrict plant growth and crop production. To reduce the adverse effects of pathogen attack, plants have evolved a variety of efficient defense mechanisms, such as physiological, morphological and biochemical adaptations (Qiu et al. 2007; Zheng et al. 2007; Zhang et al. 2012). Pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) are two interconnected branches innate immune in plants for defense against pathogens (Meng and Zhang 2013). Extensive defense-related genes expression has been associated with PTI and ETI, and appears to be controlled by complex signaling networks (Pieterse et al. 2009; Tsuda and Katagiri 2010). Transcription factor-mediated transcriptional regulation of defense-related genes is a key step in the activation of various plant defense responses (Yu et al. 2001; Dang et al. 2013). Previous studies have shown that transcription factors (TFs), such as WRKY TFs, play crucial roles in both PTI and ETI (Pandey and Somssich 2009).

The WRKY transcription factor can interact with W-box, a common *cis*-element that is present in the promoter regions of various biotic stress-related genes (Rushton et al. 2010). In plants, WRKY proteins belong to a large family with more than 70 members in *Arabidopsis thaliana* and more than 100 members in rice (Wu et al. 2005; Zhang and Wang 2005). Recently, a genome-wide analysis of the WRKY gene family in cotton (*Gossypium raimondii* and *Gossypium hirsutum*) has been taken, 116 WRKY genes have been identified in *G. raimondii* and 102 WRKY genes have been cloned in *G. hirsutum* (Dou et al. 2014). A common property of the WRKY family is their DNA-binding domain, which is composed of a highly conserved peptide sequence (WRKYGQK) at its N-terminal end together with a zinc-finger-like motif (Eulgem et al. 2000). Based on the domain structures, WRKY families are divided into three major groups (I, II, and III) and various subgroups (e.g. IIa, IIb, etc.) (Eulgem et al. 2000).

To date, WRKY TFs appear to function in the regulation of defense responses to biotic and abiotic stresses (Jiang and Deyholos 2009; Rushton et al. 2010; Li et al. 2011; Guo et al. 2011; Shi et al. 2014). Increasing evidences suggest that many WRKY genes can positively or negatively regulate the response to pathogens (Kim et al. 2008; Pandey and Somssich 2009; Lai et al. 2011; Mao et al. 2011; Dang et al. 2013). In *Arabidopsis*, 49 of 72 *AtWRKY*

genes are differentially regulated by pathogen infection or SA treatment (Dong et al. 2003). *AtWRKY70* was a positive regulator of disease resistance to pathogens. Meanwhile, *AtWRKY70* has been shown to regulate the cross-talk between SA- and JA-mediated signaling by promoting SA-dependent and suppressing JA-dependent defense responses (Li et al. 2004; Knoth et al. 2007). Overexpression of *AtWRKY41* in *Arabidopsis* exhibited enhanced resistance to *Pseudomonas syringae*, but increased susceptibility to *Erwinia carotovora* (Higashi et al. 2008). In pepper, overexpression of *CaWRKY40* enhanced resistance to *R. solanacearum*. In contrast, silencing of *CaWRKY40* enhanced susceptibility to *R. solanacearum*. These results suggest that *CaWRKY40* played a positive role in the pathogen infection (Dang et al. 2013). However, *CaWRKY58* acts as a transcriptional activator of negative regulators in the resistance to *R. solanacearum* infection (Wang et al. 2013). Furthermore, WRKY TFs frequently regulate the expression of several defense-related genes, via specific binding to the W-box sequences in the promoter regions, to increase the defense response (Yu et al. 2001). *AtWRKY18* increased resistance to defense responses in *Arabidopsis* and enhanced the expression of defense-related genes, such as *PR-1*, *PR-2* and *PR-5* (Chen and Chen 2002). Ectopic overexpression of *GhWRKY15* in tobacco plants enhanced the resistance to viral and fungal infections and up regulated the expression of *NPRI* (*NON-EXPRESSOR OF PRI*) and several *PR* (pathogen-related) genes (Yu et al. 2012). Overall, the above findings indicated that the WRKY TFs play an important role in plant disease resistance.

Cotton (*G. hirsutum*) is one of the most important fibre and oil crops around the world. Various environmental factors have been shown to inhibit its growth and yield (Sunilkumar et al. 2006). Previous researches have suggested that WRKY TFs were involved in defense-related response (Dong et al. 2003; Eulgem and Somssich 2007; Lai et al. 2011). The study of WRKY TFs in cotton will have great potential to help cotton improving the growth and yield. However, only a small number of WRKY proteins have been characterized in cotton (Yu et al. 2012; Shi et al. 2014; Wang et al. 2014; Yan et al. 2014). In this study, *GhWKRY44*, a group I WRKY gene from cotton, was isolated and characterized. The *GhWKRY44* expression patterns under various biotic and abiotic stresses were investigated. Ectopic expression of *GhWKRY44* in *N. benthamiana* enhanced the plant resistance to bacterial and fungal infections. In addition, *GhWKRY44*-overexpressing plants increased ability to scavenge reactive oxygen species (ROS). These results suggest that *GhWRKY44* may participate in multiple mechanisms by which plants respond to pathogen defense.

## Materials and methods

### Plant materials, growth conditions and stress treatments

The cotton (*G. hirsutum* L. cv. Lumian 22) seedlings were grown in a growth chamber at 25 °C with 16 h light/8 h dark cycle (relative humidity of 60–75 %). Seven-day-old cotton seedlings were used for various treatments. Seedlings treated with sterile water were chosen as the control. For the tissue-specific expression analysis, the roots, stems and leaves were harvested from the same plant. For the hormone treatments, the seedling leaves were sprayed with SA (2 mM), MeJA (100 µM), ET (5 mM), 6-BA (20 µM), respectively. For the salt and drought treatments, the seedlings were performed by irrigating the seedlings with NaCl (200 mM) and PEG6000 (15 %). For wounding treatment, the seedling leaves were cut with scissors. For the pathogen treatments, the roots of seedlings were dipped into the bacterial pathogen *Ralstonia solanacearum* (*R. solanacearum*) suspensions (OD<sub>600</sub> = 0.5–0.8) and the fungal pathogens *Rhizoctonia solani* (*R. solani*) conidial suspensions (10<sup>6</sup> spores/ml), respectively. Each treatment was repeated at three times. All the samples were harvested for RNA extraction.

*Nicotiana benthamiana* (*N. benthamiana*) seeds were surface-sterilized and planted on Murashige–Skoog (MS) medium for germination, then transplanted in soil and maintained at 25 °C with a 16 h light/8 h dark cycle. Eight-week-old *N. benthamiana* seedlings were used for the disease resistance assays.

### Cloning and sequence analysis of *GhWRKY44*

The full-length *GhWRKY44* cDNA was amplified by reverse transcription-PCR (RT-PCR) and RACE-PCR (Shi et al. 2011). HiTAIL-PCR was performed to obtain the promoter sequence (Liu and Chen 2007). The primers are shown in Table S1. The PCR products were cloned into the pMD18-T vector (TaKaRa, China) after being purified and transformed into *Escherichia coli* competent cells (*E. coli* DH5α) for sequencing.

The *GhWRKY44* nucleic acid sequences were translated into amino acids using the DNAMAN 6.0 program. The amino acids sequences were compared with those in the GenBank using BLAST on the NCBI's (<http://www.ncbi.nlm.nih.gov/>) homepage. The phylogenetic tree was constructed in MEGA 5.1 program (Tamura et al. 2011) using the neighbor-joining method.

### Vector construction and genetic transformation

The entire *GhWRKY44* cDNA was inserted downstream of the Cauliflower Mosaic Virus (CaMV) 35S promoter in the binary expression vector pBI121 via *SalI* and *SacI* sites.

The recombinant plasmid was then electroporated into *Agrobacterium tumefaciens* (strain LBA4404), and transformed into *N. benthamiana* by the leaf disc method (Horsch et al. 1985). The transgenic T<sub>3</sub> lines were selected for further studies.

### Subcellular localization of the 35S-GhWRKY44::GFP fusion protein

A full-length *GhWRKY44* ORF without the termination codon was amplified using the specific primers WG1/WG2 (Table S1). The PCR amplification products and the pBI121-GFP binary vector (driven by the constitutive CaMV35S promoter) were both digested with *XhoI* and *XbaI*, and the fragment was inserted into the pBI121-GFP to yield the expression vector 35S-GhWRKY44::GFP. The 35S-GFP was used as a control. *A. tumefaciens* strain GV3101 transformed with 35S-GhWRKY44::GFP or 35S-GFP confusion constructs were grown overnight, pelleted, re-suspended in infiltration buffer (10 mM MES pH 5.7, 10 mM MgCl<sub>2</sub>, and 200 mM Acetosyringone) and adjusted to the final OD<sub>600</sub> of 0.8. Then the mixtures were incubated for 3 h at room temperature. Fully expanded leaves of 5-week-old *N. benthamiana* were infiltrated with the *Agrobacterium* suspension using a 1 ml syringe. Leaf samples were collected after 2 days infiltration. Images were performed using a confocal laser scanning microscope (LSM 510 META, ZEISS, Germany).

### RNA isolation and quantitative real-time PCR analyses

Total RNA was extracted from cotton seedling leaves by modified CTAB method (Lu et al. 2013). While TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate Total RNA from *N. benthamiana* leaves according to the manufacturer's instructions. The first-strand cDNA was synthesized by a reverse transcriptase system (Transgen, Beijing, China). Quantitative RT-PCR was used to analyze the expression levels of *GhWRKY44* under different treatments. Fragment of *GhWRKY44* gene was amplified using the primer pairs QRT1/QRT2 (Table S1). Quantitative real-time PCR (qRT-PCR) was performed using the SYBR PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China) on a CFX96™ Real-time System (Bio-Rad, Hercules, CA, USA). The PCR program was as follows: pre-denaturation at 95 °C for 30 s; 40 cycles of 95 °C for 30 s, 55 °C for 15 s and 72 °C for 15 s; and a melt cycle from 65 °C to 95 °C. The polyubiquitin gene (*UBI*) gene and *N. benthamiana* β-actin gene was used as the standard control. The results were analysed with the CFX Manager software program (version 1.1) using the 2<sup>-ΔΔCt</sup> comparative CT method (Livak and Schmittgen 2001). The data were examined by multiple comparisons

using one-factor analysis of variance (ANOVA). The significant differences were determined according to Duncan's multiple range test using Statistical Analysis System (SAS) version 9.1 software.

### Pathogen infection

Bacterial and fungal cultures were performed as described previously (Lu et al. 2013). Detached uniform leaves from 8-week-old T<sub>3</sub> transgenic and wild-type plants were inoculated with suspensions of *R. solanacearum* bacteria (OD<sub>600</sub> = 0.5–0.8) or *R. solani* spores (10<sup>6</sup> spores/ml) using a syringe with a needle. The respective fourth leaves were harvested a transparent culture dish under glasshouse condition for the preparation of RNA or histochemical staining. In addition, the infection was confirmed using the trickle irrigation method to observe disease symptoms (Li et al. 2014). The inoculated plants with roots in soil were kept in a moist chamber at 25 °C for 24 h in the dark and transferred glasshouse condition at 25 °C. Bacterial growth was monitored by performing serial dilutions onto King's B agar medium. The disease resistance analysis was repeated three times.

### 3,3'-Diaminobenzidine (DAB), nitro blue tetrazolium (NBT), and trypan blue staining assays

Uniform leaves from 8-week-old T<sub>3</sub> transgenic and wild-type plants were inoculated by *R. solanacearum* bacteria (OD<sub>600</sub> = 0.5–0.8) and *R. solani* spores (10<sup>6</sup> spores/ml) for staining assays. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was detected in the leaves of the plants using DAB staining. The infection leaves were incubated in a DAB solution (1 mg/ml, pH 3.8) for 12 h at 25 °C in the dark. After staining, the leaves were soaked overnight in 95 % ethanol to remove chlorophyll (Thordal-Christensen et al. 1997). Superoxide anion (O<sub>2</sub><sup>-</sup>) was measured in the leaves of the plants via NBT staining. The infection leaves were incubated in a NBT solution (0.1 mg/ml) for 24 h at 25 °C in the dark. After staining, the leaves were also destained overnight in 95 % ethanol (Jabs et al. 1996). The cell death was detected in the leaves using trypan blue staining. After treatment, leaves were stained with lactophenol-trypan blue solution for 5 min, soaked in chloral hydrate (2.5 g/ml) for 12 h to remove chlorophyll (Zhang et al. 2012).

## Results

### Sequence analysis of *GhWRKY44*

Based on the vital function of the WRKY motif in the activity of the WRKY family members, we designed pair

primers to isolate a cDNA fragment from cotton cotyledons. A predicted full-length cDNA sequence of WRKY gene was successfully obtained by the homology cloning strategy. The deduced full-length sequence of cDNA fragment consisted of 1,716 nucleotides, including a 188 bp 5'-untranslated region (UTR), a 127 bp 3'-UTR, and a 1,401 bp open reading frame (ORF). The cDNA encodes a polypeptide of 467 amino acids with a putative molecular weight of 51.39 kDa and an isoelectric point of 8.76. The putative WRKY clone from cotton exhibits high sequence similarity to *Arabidopsis thaliana AtWRKY44* (NM\_129282), so the cotton WRKY gene was designated as *GhWRKY44* (GenBank accession no. KJ871340).

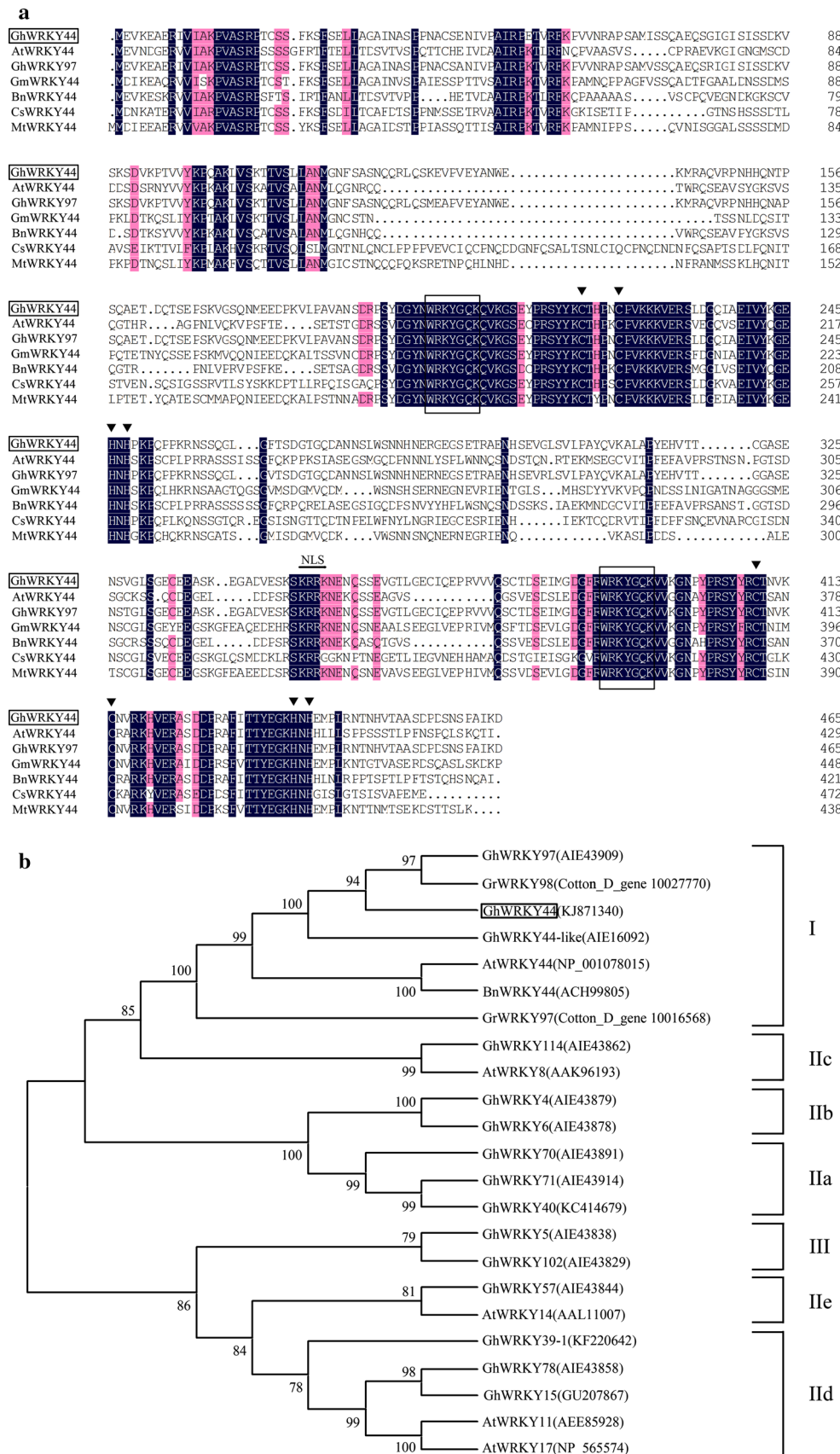
Alignment analysis of GhWRKY44 protein sequence with related sequences revealed that it shared 45.1, 97.4, 60.54, 42.3, 48.5 and 58.6 % identity with AtWRKY44 (NP\_001078015), GhWRKY97(AIE43909), GmWRKY44 (XP\_003520637), BnWRKY44 (ACH99805), CsWRKY44 (NP\_001267637) and MtWRKY44 (XP\_003625561), respectively (Fig. 1a). Similar to the other members of WRKY group I, the putative GhWRKY44 protein contains two typical DNA binding domain that possess the highly conserved amino acid sequence WRKYGQK and two putative zinc finger motifs (C-X<sub>4</sub>-C-X<sub>22-23</sub>-H-X<sub>1</sub>-H) (Fig. 1a). It suggests that the GhWRKY44 represent a member of WRKY group I. A phylogenetic tree was also constructed to determine the evolutionary relationship between GhWRKY44 and the other WRKY proteins (Fig. 1b). Following the standard of previous classification, GhWRKY44 protein was closely related to group I WRKY family members, including AtWRKY44, GhWRKY44-like, GhWRKY97, GrWRKY98, BnWRKY44 and GrWRKY97. These results strongly indicate that GhWRKY44 belongs to group I WRKY family.

### Subcellular localization of GhWRKY44

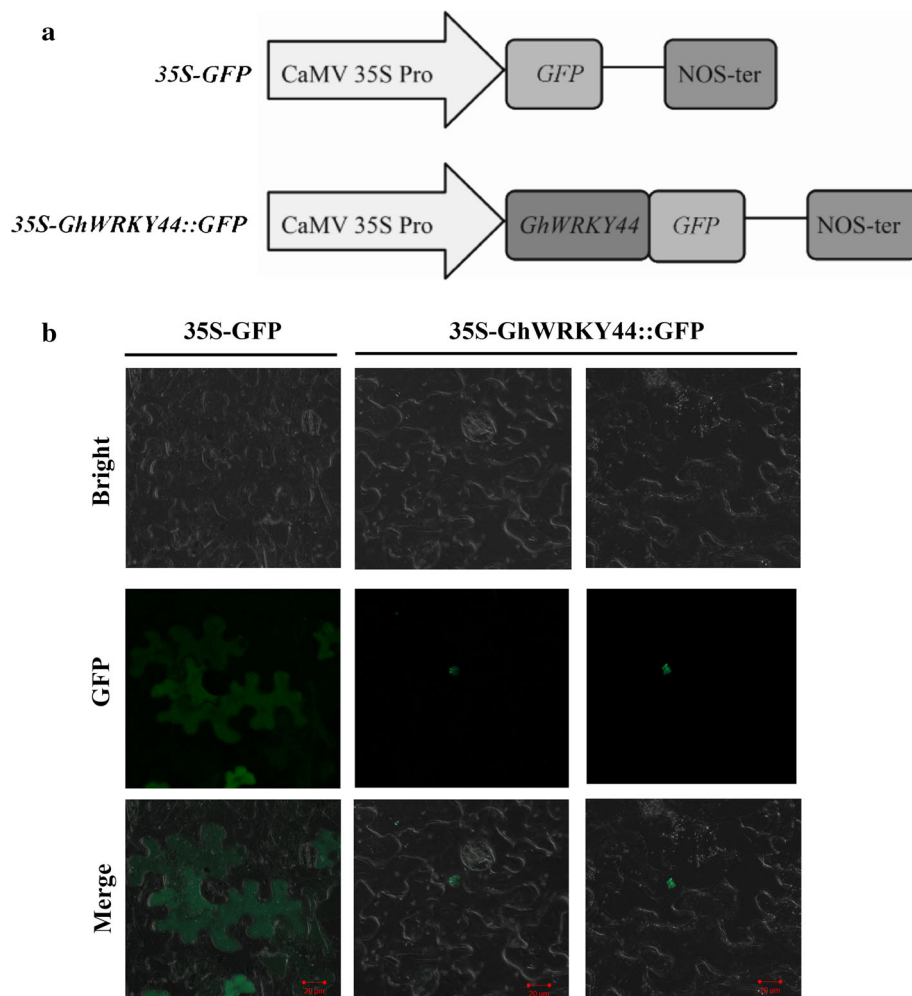
Using a prediction program for protein subcellular localization, PSORT (<http://psort.ims.u-tokyo.ac.jp>), it was predicted that GhWRKY44 might be localized in the nucleus due to the existence of the putative nuclear localization signal (KRRK). To further confirm this prediction, we constructed the 35S-GhWRKY44::GFP over-expression vector driven by the constitutive CaMV35S promoter. As shown in Fig. 2b, the tobacco leaf cell carrying the 35S-GhWRKY44::GFP emitted fluorescence only in nucleus, whereas 35S::GFP control were present in multiple subcellular compartments, including the cytoplasm and nuclei. These results indicate that the GhWRKY44 is a nuclear protein, which might function in the nucleus.



**Fig. 1** Characterization and sequence analysis of GhWRKY44. **a** Sequence alignment of the deduced GhWRKY44 protein with BnWRKY44 (ACH99805), GhWRKY97 (AIE43909), GmWRKY44 (XP\_003520637), BnWRKY44 (ACH99805), CsWRKY44 (NP\_001267637) and MtWRKY44 (XP\_003625561). Identical and similar amino acids were shaded in dark blue and pink, respectively. The WRKY domains are shown in frames. The cysteine and histidine residues of the putative zinc finger motif were indicated by arrowheads (▼). The putative NLS was indicated by straight line. **b** Phylogenetic analysis of GhWRKY44. The phylogenetic tree was constructed using the software MEGA 5.1. Neighbor-joining method was used and bootstrap analysis was performed with 1,000 replications. Bootstrap values above 50 were indicated at branch points. The species of origin is indicated by the abbreviation before the gene names: At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Gh, *Gossypium hirsutum*; Gr, *Gossypium raimondii*. (Color figure online)



**Fig. 2** Subcellular localization of GhWRKY44 fusion protein in tobacco leaf epidermal cells. **a** Schematic diagram of the 35S-GhWRKY44::GFP fusion construct and the 35S-GFP construct. **b** Transient expression of the 35S-GhWRKY44::GFP and 35S-GFP constructs in tobacco leaf epidermal cells



Analysis of partial putative *cis*-acting elements in the 5'-flanking region of *GhWRKY44*

To elucidate the mechanism underlying the expression patterns of *GhWRKY44*, a 1,418 bp fragment of 5'-flanking region of *GhWRKY44* was obtained by hiTAIL-PCR method. The Plant CARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) was used to analyze the putative *cis*-acting elements of this region. All of the identified *cis*-elements are shown in Fig. 3. Several putative *cis*-acting elements may be involved in defense responses, including CGTCA-motif involved in the MeJA-responsiveness, TCA-element involved in salicylic acid responsiveness, TC-rich repeats involved in defense and stress responsiveness and ethylene-responsive element ERE. In addition, abiotic stress responsive *cis*-acting elements were also identified, such as the HSE element which involved in heat stress responsive, MBS representing the MYB binding site involved in drought-inducibility, gibberellin-responsive element GARE-motif and so on. These

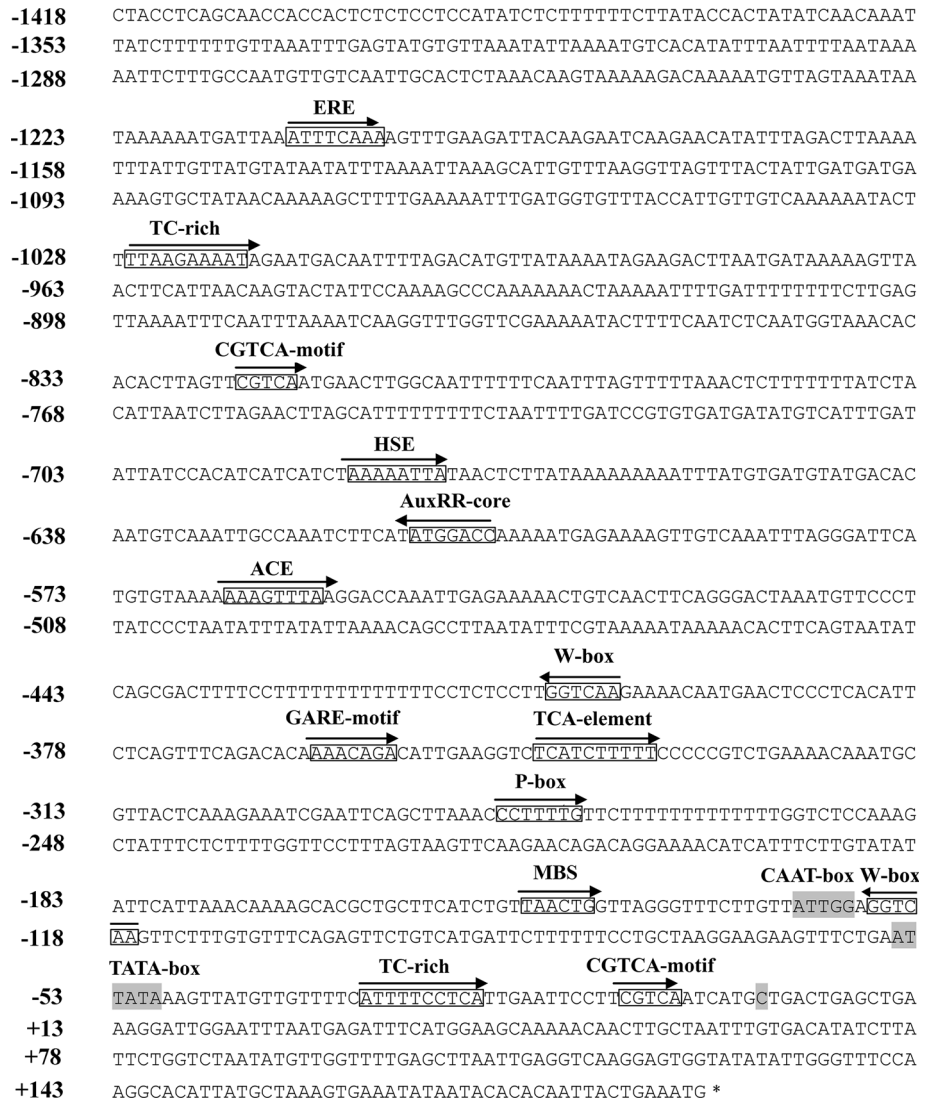
data suggest that *GhWRKY44* may play a role in the response to various environmental stresses.

Expression patterns of *GhWRKY44* mRNA

To further identify the expression patterns of *GhWRKY44* gene, quantitative RT-PCR was performed using 7-day-old cotton seedlings. As shown in Fig. 4a, the expression of *GhWRKY44* was higher in leaves than that in roots and stems.

To explore whether *GhWRKY44* expression is triggered by biotic stresses, the cotton seedlings were infected with *R. solanacearum* and *R. solani*. As shown in Fig. 4b, *GhWRKY44* transcripts was induced in 1d and noticeably elevated on 2d after *R. solanacearum* treatment. Under *R. solani* treatment, the expression of *GhWRKY44* was rapidly enhanced in 1d, then decreasing gradually to the level of the control (Fig. 4c). These results indicated that *GhWRKY44* might play crucial roles in the plant pathogen defense response.

**Fig. 3** Nucleotide sequence of the promoter of *GhWRKY44*. The predicted transcription initiation site is highlighted in grey (C). The start codon is marked with an asterisk, and the putative core promoter consensus sequences (TATA-box and CAAT-box) are highlighted in grey. The W-box and putative *cis*-acting elements are indicated by boxes and their corresponding names are given above each element. Arrows indicate the direction of the *cis*-acting elements



In addition, we also examined the expression of *GhWRKY44* under abiotic stresses. For wounding treatment, the expression of *GhWRKY44* was induced and reached maximal level at 2 h (Fig. 4d). As shown in Fig. 4e, the transcript level of *GhWRKY44* was increased at 2–12 h after PEG6000 treatment, with a highest level at 2 h. After NaCl treatment, a slight increase of *GhWRKY44* transcripts appeared at 4 h, while the induction phenomenon is not obvious (Fig. 4f). The drought and salinity results fit well with earlier research results of *GhWRKY97* (Dou et al. 2014). These results showed that the expression levels of *GhWRKY44* can be regulated by abiotic stresses.

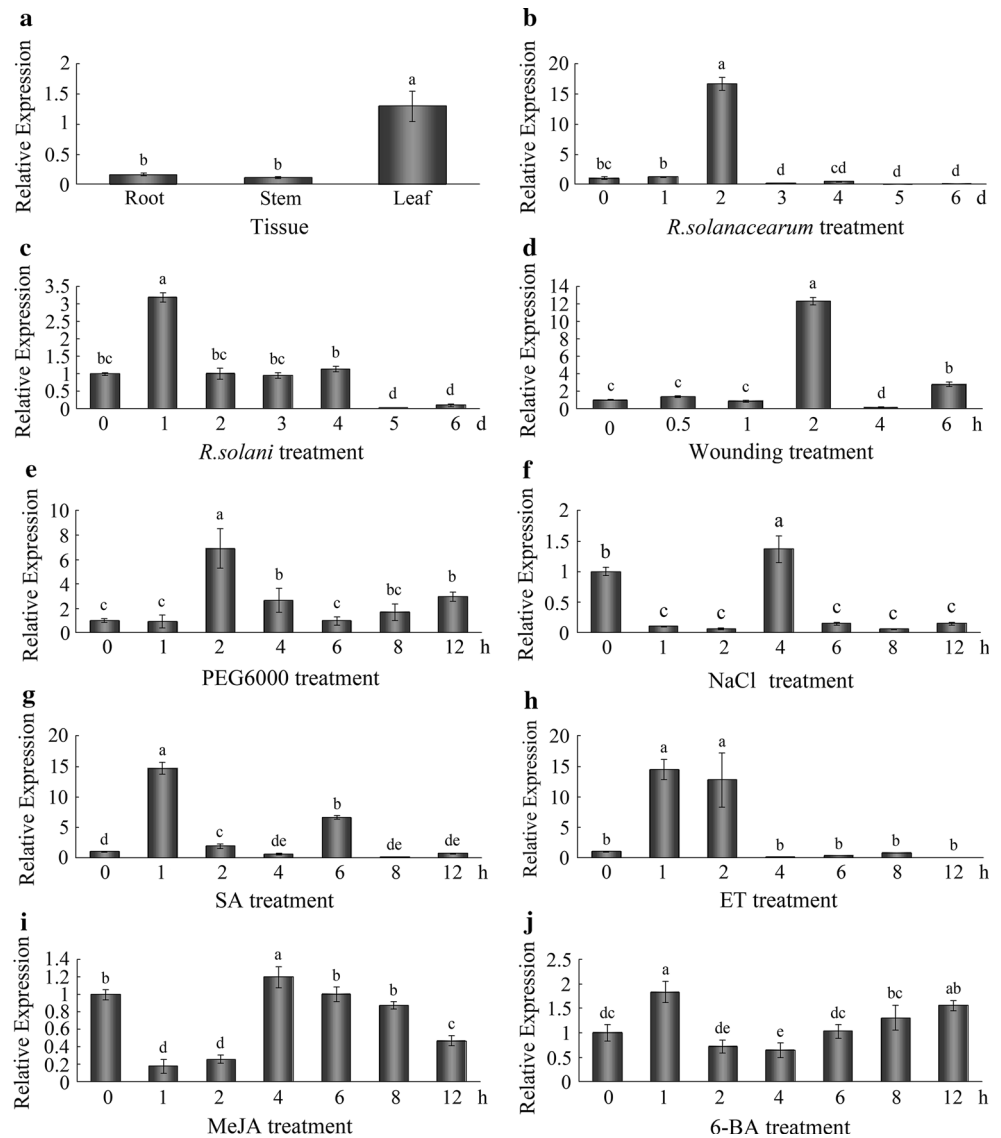
To explore the molecular mechanism underlying the responsiveness to various stresses, cotton seedlings were inoculated with signaling molecules, including SA, ET, MeJA and 6-BA. As shown in Fig. 4g, h, the expression levels of *GhWRKY44* were drastically elevated after SA

and ET treatments at the same time point. However, the transcript level of *GhWRKY44* was slightly induced through MeJA treatment (Fig. 4i). For 6-BA treatment, the *GhWRKY44* expression was also increased (Fig. 4g). Based on these results, we deduced that *GhWRKY44* might be involved in complex signaling pathways and played crucial roles in controlling the expression of downstream defense genes.

Overexpression of *GhWRKY44* exhibit enhanced pathogen resistance

The characterization of promoter and endogenous expression implied that the *GhWRKY44* might be involved in plant defense responses. In order to further analyze the putative function of *GhWRKY44* in plant, full-length *GhWRKY44* was cloned into the binary vector pBI121 under

**Fig. 4** Expression patterns of *GhWRKY44* in different tissues and under different stress conditions. **a** Tissue-specific expression of *GhWRKY44* was detected with quantitative RT-PCR. Total RNA were extracted from roots, stems and cotyledon leaves of 7-day-old cotton seedlings. **b–j** The expression patterns of *GhWRKY44* in cotton seedlings were analysed with quantitative RT-PCR following treatments with *R. solanacearum* (**b**), *R. solani* (**c**), wounding (**d**), 15 % PEG6000 (**e**), 200 mM NaCl (**f**), 2 mM SA (**g**), 5 mM ET (**h**), 100  $\mu$ M MeJA (**i**), 20  $\mu$ M 6-BA (**j**). The lanes represent various time points during the treatments. The *ubiquitin* gene was used as an internal control. Different letters above the columns indicate significant differences ( $P < 0.01$ ) as determined by Duncan's multiple range test. Each experiment was repeated at least three times



control of the CaMV35S promoter and transformed into *N. benthamiana*. In all, 14 independent transgenic lines were obtained by kanamycin resistance selection (data not shown). The expression levels in different lines (10 randomly selected lines) were detected using semi-quantitative RT-PCR (Fig. 5a). Compared to the control, no obvious morphological changes were observed in the transgenic lines (Fig. 5b). However, transgenic lines accumulated different levels of *GhWRKY44* transcripts compared with the wild-type plants. Three representative lines, OE1 (5#), OE2 (8#), and OE3 (9#), exhibiting different expression levels of T<sub>3</sub> progeny were selected for further investigation.

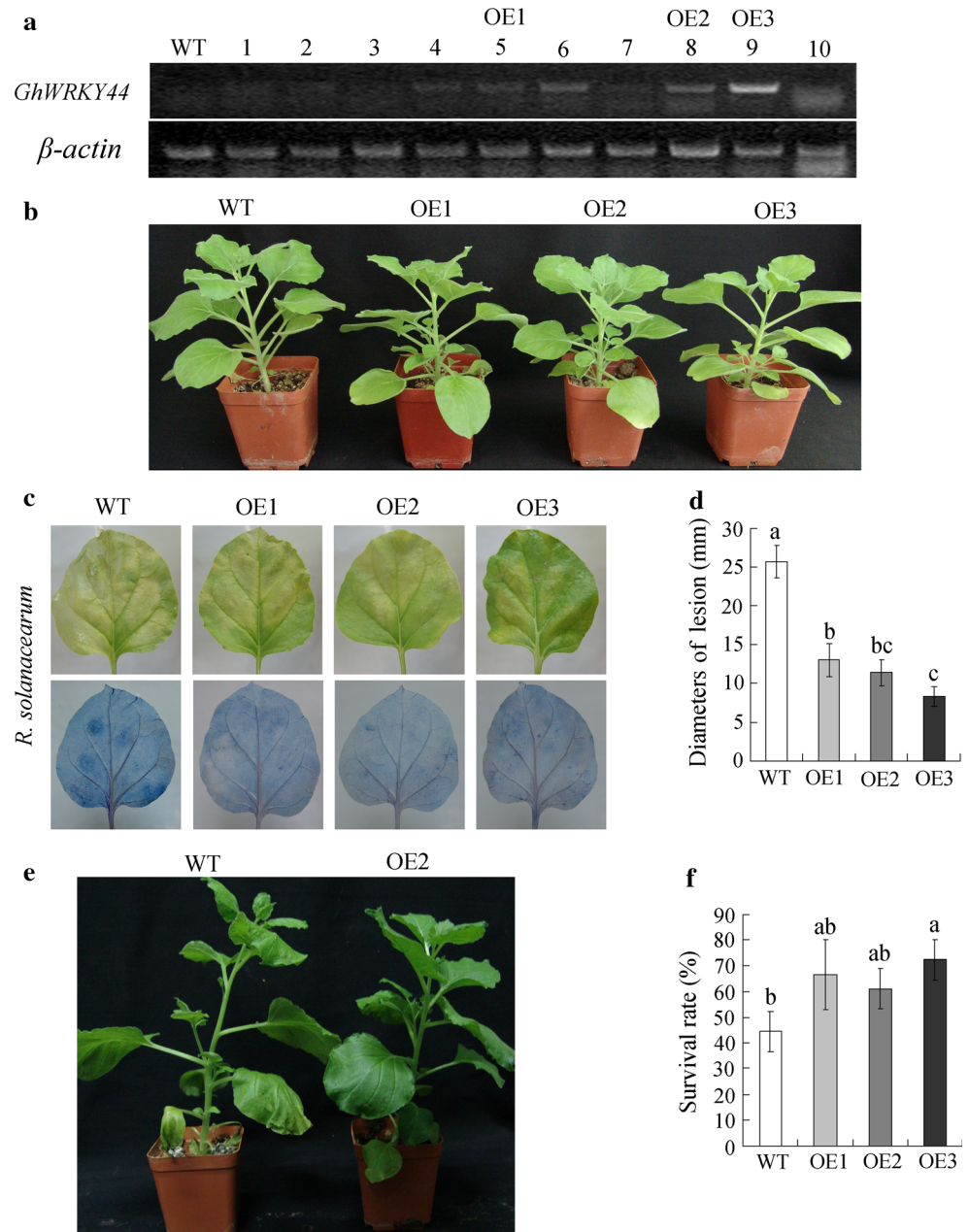
Eight-week-old transgenic plants were used for the resistance tests. When the detached leaves were inoculated with *R. solanacearum* over 6 days, the leaves of the transgenic lines exhibited more resistance than the wild-type plants. It was found that the transgenic lines showed

minor signs of chlorosis and relatively smaller lesion diameters than those of the wild-type plants (Fig. 5c, d). Trypan blue staining showed relatively less staining in the leaves of the transgenic plants than those of the wild-type plants, which indicated that overexpression of *GhWRKY44* decreased the levels of cell death in the transgenic plants (Fig. 5c). The trickle irrigation method was also used to examine the response to the bacterial pathogen. Marked wilting symptoms were observed on the wild-type plants, whereas the transgenic lines exhibited only slight wilting symptoms after 10 days inoculation. In addition, the transgenic lines showed a relatively higher survival rate than the wild-type plants (Fig. 5e, f). These results indicated that *GhWRKY44* could enhance resistance to the bacterial pathogen in transgenic plants.

To investigate whether transgenic plants possess enhanced fungal resistance, 8-week-old plants were



**Fig. 5** Identification of transgenic plants and bacterial pathogen resistance analysis. **a** The expression levels of *GhWRKY44* in the T1 progeny of transgenic plants. **b** The developmental phenotype from 8-week-old T<sub>3</sub> generation transgenic plants. **c** Disease signs on the detached leaves of wild-type and transgenic plants 6 days after *R. solanacearum* infection. The cell death was visualized by trypan blue staining. **d** The lesion diameters on the leaves after inoculation with *R. solanacearum*. **e** The phenotypes of the wild-type and transgenic plants after inoculation with *R. solanacearum* using the trickle irrigation method. **f** The survival rates of wild-type and transgenic plants after inoculation with *R. solanacearum*. Different letters above the columns indicate significant differences ( $P < 0.05$ ) as determined by Duncan's multiple range test. Each experiment was repeated at least three times



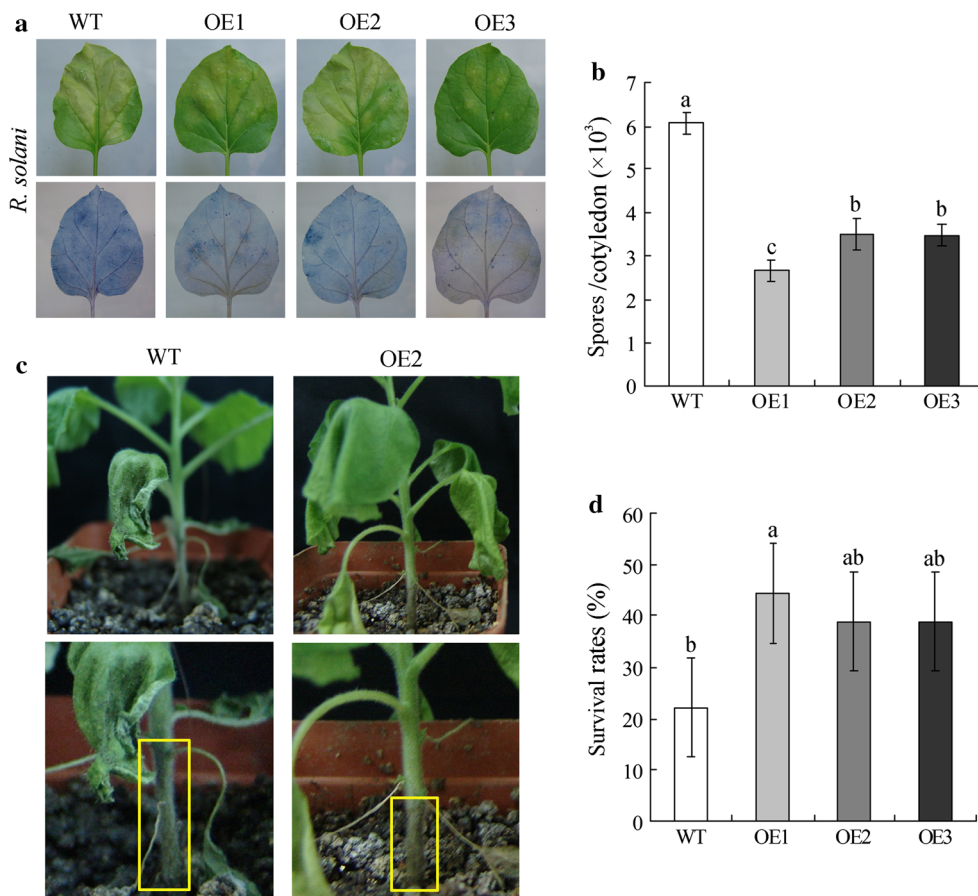
inoculated with *R. solani*. Conidial suspensions spores ( $10^6$  spores/ml) were sprayed on detached leaves. After 7 days inoculation, the increased disease symptoms characterized by extensive chlorosis and many more spores were present in the wild-type leaves than the transgenic leaves (Fig. 6a, b). In addition, Trypan blue staining revealed that the wild-type leaves showed a more serious cell death phenotype than the transgenic lines leaves. Similarly, the trickle irrigation method was also used to examine the response to the fungal pathogen. After 2-week inoculation, the transgenic lines displayed less severe disease symptoms. The wilting symptoms on the transgenic plants were slighter than those on the wild-type leaves, with a higher survival rate than the

wild-type plants (Fig. 6c, d). These results demonstrated that *GhWRKY44* may be a positive regulator of plant defense against the fungal pathogens.

#### *GhWRKY44* regulates the accumulation of ROS

Generally, pathogens invasion in plant often result in the generation of ROS, including hydrogen peroxide ( $H_2O_2$ ), singlet oxygen, superoxide anion ( $O_2^-$ ), and hydroxyl radicals (Hernández et al. 2004; Yoshioka et al. 2003). To test whether ROS accumulation was related to the pathogens invasion of the transgenic plants, the accumulation of  $H_2O_2$  and  $O_2^-$  were monitored in transgenic and wild-type plants following

**Fig. 6** Enhanced resistance of *GhWRKY44*-overexpressing plants to fungi. **a** Disease signs on the detached leaves of wild-type and transgenic plants 7 days after inoculation with *R. solani*. The cell death was visualized by trypan blue staining. **b** The number of spores per cotyledon at 7 days after *R. solani* infection. **c** The symptoms of wild-type and transgenic plants inoculated with *R. solani* using the trickle irrigation method. **d** The survival rates of wild-type and transgenic plants after inoculation with *R. solani*. Different letters above the columns indicate significant differences ( $P < 0.05$ ) as determined by Duncan's multiple range test. Each experiment was repeated at least three times



exposure to *R. solanacearum* or *R. solani*. The accumulation of  $H_2O_2$  and  $O_2^-$  were detected by 3,3'-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) staining methods, respectively. As shown in Fig. 7a, following exposure to *R. solanacearum* or *R. solani*, the DAB staining was found to be much lighter in the leaves of the transgenic plants, indicating that lower levels accumulation of  $H_2O_2$  compared with wild-type plants. Similar to that observed in DAB staining, NBT staining revealed the leaves of the transgenic plants also accumulated less  $O_2^-$  compared with wild-type plants (Fig. 7b). These results indicated that overexpression of *GhWRKY44* could enhance defense resistance by inhibiting the accumulation of pathogen-induced ROS.

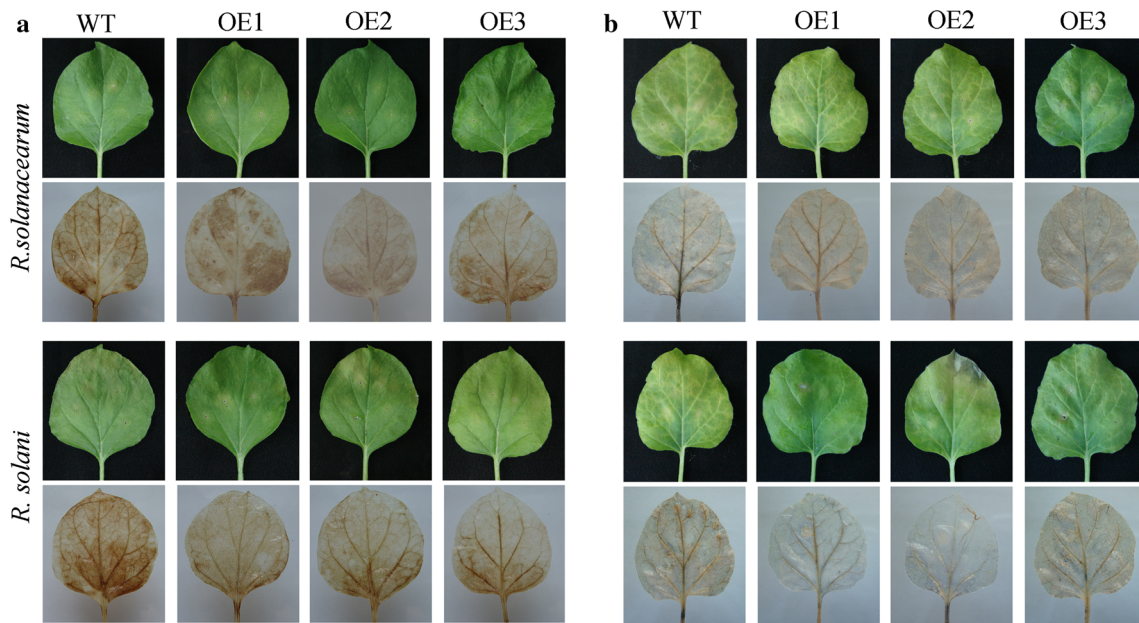
Overexpression of *GhWRKY44* activates the expression of defense-related genes

Generally, the expression levels of *PR* genes and *NPR1* gene, are involved in the defense pathway, such as salicylic acid (SA) and jasmonic acid (JA) signaling pathways (Agrawal et al. 2000; Yu et al. 2001; Pieterse and Van Loon 2004). To explore the possible mechanisms underlying the enhanced pathogen resistance in the transgenic plants, we characterized the expression levels of several *PR*

genes and *NPR1* gene before and after infection with *R. solanacearum* using qRT-PCR. As shown in Fig. 8a, the expression of *PR2* ( $\beta$ -1,3-glucanase) gene was greater in the three transgenic lines than the wild-type plants before infection with *R. solanacearum*. To our surprise, other defense-related genes, including *PR1a*, *PR4*, *PR5* and the *NPR1* showed lower expression levels in the transgenic plants compared with the wild-type plants (Fig. 8a). After infection with *R. solanacearum*, the transcriptional levels of these defense-related genes were all up regulated in transgenic plants (Fig. 8b). Furthermore, the expression of *PR2*, *PR4* and *NPR1* genes was much higher than the wild-type plants after infection with *R. solanacearum*. Notably, *PR1a*, *PR2*, *PR5* and *NPR1* are SA signaling-related genes, and *PR4* is JA signaling-related gene. Thus, it was speculated that the enhanced defense resistance conferred by *GhWRKY44* in transgenic plants might be related to SA- and JA dependent signaling pathway.

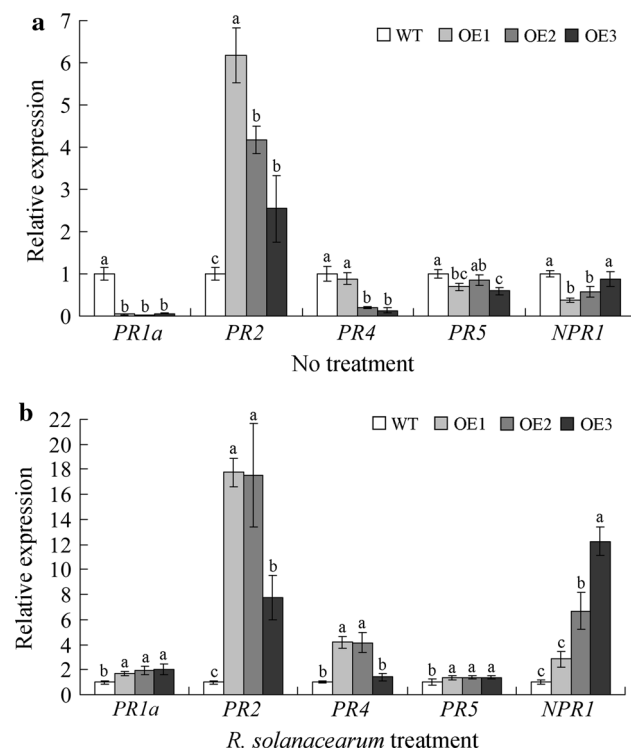
## Discussion

The transcriptional regulation of plant defensive genes plays a pivotal role in the activation of plant defense



**Fig. 7** Analysis of ROS accumulation in wild type and transgenic plants in response to pathogens infection. **a** The accumulation of  $H_2O_2$  using DAB staining after inoculation with *R. solanacearum* and

*R. solani*. **b** The accumulation of  $O_2^-$  using NBT staining after inoculation with *R. solanacearum* and *R. solani*



**Fig. 8** Expression of defense-related genes in wild-type and transgenic plants by qRT-PCR. **a** The expression levels of defense related genes under normal conditions. **b** The expression levels of defense related genes after inoculation with *R. solanacearum*. Different letters for each defense related gene indicate significant differences ( $P < 0.05$ ) as determined by Duncan’s multiple range test

responses (Jing et al. 2009). The significance of WRKY TFs in response to various biotic and abiotic stresses had been described (Eulgem and Somssich 2007; Rushton et al. 2010; Chen et al. 2012). Recently, a large body of evidences suggested that many WRKY genes have been implicated in the regulation of pathogens infection (Oh et al. 2008; Cai et al. 2008; Yu et al. 2012; Shimono et al. 2012). To explore the function of the WRKY transcription factors in cotton, a group I WRKY gene named *GhWRKY44* was isolated from *G. hirsutum* and the 5'-flanking region containing two W-box were identified. W-box is the minimal core element necessary for binding of a WRKY protein to DNA (Rushton et al. 1996, Ciolkowski et al. 2008). And W-boxes can be found in the promoters systemic acquired resistance related (SAR) genes, including *non-expresser of PR genes 1*, and *pathogenesis related 1* (Fu and Dong. 2013). Also, several other putative cis-elements involved in defense response including MeJA, SA, ET were found. These results indicated that *GhWRKY44* may play positive roles in disease defense.

In this study, the *GhWRKY44* transcriptions were induced by diverse signaling molecules and pathogens (Fig. 4). So we speculated that *GhWRKY44* might be involved in defense responses. To test the role of *GhWRKY44* in pathogen infection, transgenic plants were inoculated with bacterial and fungal pathogens, respectively. The overexpression of *GhWRKY44* enhanced the



resistance to bacterial pathogen *R. solanacearum* and fungal pathogen *R. solani* in transgenic plants (Fig. 5, 6). Moreover, the expression of defense related genes showed higher level in transgenic plants than wild-type plants (Fig. 8). These results strongly suggest that *GhWRKY44* functions as a positive regulator to pathogen resistance in transgenic plants. Most of the WRKY TFs in plants reported recently have shown positive roles in pathogen resistance (Zheng et al. 2006; Liu et al. 2007; Peng et al. 2012; Yu et al. 2012; Shi et al. 2014). For example, loss-of-function mutants of *AtWRKY33* caused enhanced susceptibility to the necrotrophic fungal pathogens *Botrytis cinerea* and *Alternaria brassicicola*, while transgenic overexpression of *AtWRKY33* increased resistance to the two necrotrophic fungal pathogens (Zheng et al. 2006). In rice, *OsWRKY45* could positive regulate the disease resistance to rice blast fungus *Magnaporthe grisea* by mediating a signaling pathway downstream of SA (Shimono et al. 2007). These results provide further evidence for WRKY TFs playing positive roles in regulating disease resistance.

Mounting evidences suggest that plants challenged with pathogens are often exposed to the accumulation of ROS (Kotchoni and Gachomo 2006). It is critical to modulate ROS to an appropriate level. Low ROS concentrations can act as signaling molecules to regulate cell growth, while high ROS concentrations induce cellular senescence and lead to oxidative damage (Poli et al. 2004). The overexpression of *GhWRKY44* in transgenic plants resulted in the decreased accumulation of  $H_2O_2$  and  $O_2^-$  than wild-type plants under pathogen infection (Fig. 7). These results suggest that the enhanced resistance to pathogen infection in *GhWRKY44*-overexpressing plants may be associated with the removal of ROS. Recently, it was also reported that overexpression of *GhWRKY39* in *N. benthamiana* enhanced tolerance to pathogen infection and decreased  $H_2O_2$  accumulation than wild-type plants (Shi et al. 2014). Overall, WRKY TFs may play crucial roles in ROS homeostasis, and alleviated the ROS-associated oxidative injury.

SA and JA/ET are two well-studied signaling pathways which play important roles in the regulation of plant defense responses to pathogen infection (Spoel and Dong 2008; Peng et al. 2012). Recent studies have shown that a variety of WRKY genes are involved in different defense signaling pathways (Birkenbihl et al. 2012; Peng et al. 2012; Dang et al. 2013). In *Arabidopsis*, WRKY25 functions as a negative regulator of SA-mediated defense responses to *P. syringae* by T-DNA insertion mutants and transgenic overexpression methods (Zheng et al. 2007). In rice, JA plays an important role in WRKY30-mediated defense responses to fungal pathogens (Peng et al. 2012). In this study, *GhWRKY44* could be induced by SA, MeJA and ET, which suggested that *GhWRKY44* might be

involved in SA or JA/ET signaling pathway. In addition, the expression of the marker genes for different signaling pathways (*PR1a*, *PR2*, *PR5* and *NPR1* for SA signaling; *PR4* for JA signaling) were significantly increased in *GhWRKY44*-overexpressing plants under *R. solanacearum* infection (Fig. 8). Similarly, overexpression of *OsWRKY03* enhanced the resistance to bacterial blight pathogen, and induced the expression of several pathogenesis-related genes in transgenic plants. Overall, *OsWRKY03* is located upstream of *OsNPR1* as a transcriptional activator in SA-dependent or JA-dependent defense signaling pathways (Liu et al. 2005). Thus, it is reasonable to speculate that *GhWRKY44* may be involved in the crosstalk between the SA- and JA-mediated pathogen defense pathways.

In conclusion, we isolated and characterized a group I WRKY member, *GhWRKY44*, in cotton. The results of the present study strongly suggest that overexpression of *GhWRKY44* in *N. benthamiana* enhanced their resistance to bacterial and fungal pathogens. The enhanced resistance may be associated with the SA-, JA- and ROS-mediated defense mechanism. The study provides evidence for the positive regulatory functions of *GhWRKY44* resistance against pathogenic infection. Further studies on the function of *GhWRKY44* in transgenic cotton should be elucidated and loss-of function data in cotton are particularly needed.

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