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Molecular cloning and characterization of *GhWRKY11,* a gene implicated in pathogen responses from cotton

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Abstract

WRKY transcription factors are key regulators in signaling networks that modulate many plant defense processes. Although the functions of WRKY proteins have been well studied in model plants, their roles in cotton pathogen defense mechanism are still unknown. In the present study, we cloned a cotton group IId WRKY transcription factor gene, designated as *GhWRKY11*, which has only one copy in cotton genome and was targeted to the nucleus. Promoter sequence analysis revealed various *cis*-acting elements related to plant defense responses. Furthermore, semi-quantitative RT-PCR analysis indicated that *GhWRKY11* was induced by pathogen (*Colletotrichum gossypii*) attack, wounding treatment and certain defense-related molecules, including salicylic acid (SA), methyl jasmonate (MeJA), ethylene (ET) and hydrogen peroxide (H_2O_2). In addition, overexpression of *GhWRKY11* in *Nicotiana benthamiana* resulted in an elevated resistance potential to *cucumber mosaic virus* (CMV) compared to the wild-type, following the enhanced transcript levels of SA associated genes (*PR1* and *NPR1*) and reduced H_2O_2 accumulation. These results suggest that *GhWRKY11* may play important roles in regulating plant defense responses through SA- and reactive oxygen species (ROS)-mediated signal pathways.
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Keywords: Cotton; *GhWRKY11*; Defense response; SA; ROS

1. Introduction

Living plant tissues are host to various pathogens. To cope with these threats, plants develop a wide array of plant defense mechanisms in a highly extensive and temporal manner. These sophisticated mechanisms are regulated by phytohormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). SA plays a positive role against biotrophic pathogens, while JA appears to be vital in the case of necrotrophic pathogens (Mur et al., 2006; Thomma et al., 2001). Both the SA and JA/ET mediated signaling pathways require the expression of a large number of genes including pathogenesis-related (PR) genes (Bohnert et al., 1995). The regulation of PR genes is mainly achieved by enforcement of a network of various transcription factors (Chen and Zhu, 2004). By now, many plant transcription factors have been shown to contribute to this regulation, such as ethylene-responsive-element-binding protein (EREBP), basic Leucine Zipper (bZIP), MYB proteins, homeodomain and WRKY transcription factors (Rushton and Somssich, 1998).

WRKY proteins are key zinc finger transcription factors (Eulgem et al., 2000). Since the first *WRKY* gene (*SPF1*) was identified from sweet potato in 1994, an increasing number of WRKY proteins have been found throughout the green lineage (green algae and land plants) (Ishiguro and Nakamura, 1994). To date, there are 74 identified WRKY proteins in *Arabidopsis* and nearly 200 members in soybean (Rushton et al., 2010; Ulker and Somssich, 2004). WRKY proteins share common features of transcription factors, such as nuclear localization signal (NLS) and transactivation capability (Ulker and Somssich, 2004), while

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its defining feature is the DNA-binding domain called WRKY domain, which is identified by the almost invariant WRKYGQK amino acid sequence at its N-terminus and an atypical zinc-finger motif at its C-terminus. The zinc-finger motif structure is either $Cx_{4-5}Cx_{22-23}HxH$ or $Cx_7Cx_{23}HxC$ (Rushton et al., 1996). According to the number of conserved WRKYGQK sequence and the structure of zinc-finger motif, WRKY proteins can be categorized into three distinct groups (I, II and III) and each could be further classified into subgroups based on the additional short conserved structural motif (Rushton et al., 2010). In addition, this WRKY domain generally binds to the W-box (C/TTGACT/C) present in the promoters of a large number of plant defense-related genes (Maleck et al., 2000; Rushton et al., 1995).

A large body of evidence suggests that WRKY transcription factors play a vital role in modulating genes associated with plant defense responses (Pandey and Somssich, 2009; Eulgem and Somssich, 2007). As reported, the majority of WRKY proteins from various species could be induced by pathogen attack. In *Arabidopsis*, 49 out of 72 tested *WRKY* genes respond to an avirulent strain of a bacterial pathogen *Pseudomonas syringae* (Dong et al., 2003). In rice, among the 45 tested *WRKY* genes, the transcript abundance of 15 genes changed significantly following *Magnaporthe grisea* challenge (Ryu et al., 2006). In canola, transcript abundance of 13 *BnWRKY* genes changed significantly following pathogen challenge (Yang et al., 2009). Considering the amount of WRKY transcription factors involved in plant defense response, more and more reports are focusing on multiple roles of WRKY proteins in regulating plant defense response, including both the negative and positive transcript functions. *AtWRKY33* conferred increased susceptibility to two necrotrophic fungi, and silencing of *CaWRKY1* in chili pepper leaves enhanced the resistance to *Xanthomonas axonopodis* pv. *vesicatoria* (Oh et al., 2008; Zheng et al., 2006). Meanwhile, *Arabidopsis* overexpressing *AtWRKY41* showed enhanced resistance to the *P. syringae* pv. *tomato* DC3000 (*Pto*) and *OsWRKY13* expression was regulated by multiple factors to achieve disease resistances (Cai et al., 2008; Higashi et al., 2008). Interestingly, it appears that a given WRKY protein affects different signaling pathways. AtWRKY62 acts as a positive regulator in SA-dependent defense response and negative regulator in JA signaling pathway (Mao et al., 2007). In addition, WRKY transcription factors have been reported to be involved in the regulation of reactive oxygen species (ROS) accumulation. The levels of *MusaWRKY71* and *GhWRKY3* transcript were significant increased in the case of hydrogen peroxide (H_2O_2) treatment (Guo et al., 2011; Shekhawat et al., 2011).

Among the various WRKY proteins, group IId transcription factors are a group of important protein family in plant defense. The common feature of this subgroup is the C-region, which bounds calcium ions known to act as a second messenger (Park et al., 2005). Dong et al. had reported that this group of proteins could be induced by pathogen attack and SA treatment, which generate calcium ions (Dong et al., 2003). In *Arabidopsis*, there are 7 group IId transcription factors. Among these genes, AtWRKY11 and AtWRKY17 act as negative regulators in JA-dependent resistance (Journot-Catalino et al., 2006). AtWRKY7 plays a negative role in defense responses to *P. syringae* (Kim et al., 2006). However, the knowledge about other group IId genes is rather limited, especially in cotton.

Cotton is an important economic crop and used widely in the textile industry. However, it is suffering threats from various pathogens. To cope with those threats, genetic engineering was performed to improve cotton resistance, in which WRKY transcriptional factors play a critical role. Until now, a limited number of reports about the identification of WRKY transcription factors from cotton significantly conceal their biological application in cotton planting. In this study, we isolated a cotton WRKY transcriptional factor, termed as *GhWRKY11*, that conformed well to the general features of group IId WRKY superfamily. Expression analysis indicated that *GhWRKY11* expression is up-regulated through partial defense signals. Furthermore, *GhWRKY11*-overexpressing plants displayed an enhanced resistance to virus challenge through SA-dependent signaling pathway, followed by reduced H_2O_2 accumulations. Thus, we speculated that *GhWRKY11* may play a significant role in regulating plant pathogen defense responses.

2. Materials and methods

2.1. Plant materials and growth conditions

Cotton (*Gossypium hirsutum* L. cv. Lumian 22) was kept at 28 °C in a growth room programmed with 16 h light/8 h dark cycle. The following treatments were performed on seven-day-old cotton seedlings. For the treatment with various signaling molecules, seedling leaves were sprayed with methyl jasmonate (MeJA, 100 μM), SA (2 mM), H_2O_2 (10 mM) and ethylene released from 5 mM ethephon, respectively. Meanwhile, seedling leaves were cut with scissors for wounding treatment. For pathogen-infection treatment, the fungal pathogen *Colletotrichum gossypii* (*C. gossypii*) were cultivated at 28 °C on potato dextrose agar (PDA) medium for 15 d, and then fungal colonies were transferred into 1% glucose solution for conidia harvest. The conidial suspension (10^5 conidia mL^{-1}) was used to inoculate cotton seedlings with dip method. Then the challenged cottons were placed in a moist chamber under growth room conditions. All the samples were immediately frozen in liquid nitrogen at the appropriate time and stored at −80 °C for later use.

Nicotiana benthamiana (*N. benthamiana*) were cultivated at greenhouse condition at 26 ± 1 °C with a 16 h light/8 h dark cycle. For virus treatment, transgenic and wild-type plants were inoculated with 100 μL *cucumber mosaic virus* (CMV) suspensions (CMV in 50 mM phosphate buffer, pH 7.2) through wiping the fully expanded true leaves with CMV, and harvested at the appropriate time.

2.2. RNA isolation, cDNA synthesis and DNA preparation

Total RNA was extracted from cotton seedlings prepared above using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. All the RNA samples were treated

with RNase-free DNaseI (Promega, USA) to remove the potential genomic DNA contamination and then stored at − 80 °C for future cDNA synthesis. Each RNA extraction was performed with pooled materials from at least three plants. RNA extracted above was used for the first-strand cDNA synthesis with reverse transcriptase (TransGen Biotech, China) in accordance with the manufacturer's instruction. Extraction of genomic DNA from cotton seedlings was carried out according to the CTAB method described by Porebski et al. (1997).

2.3. Primers

Primers used in the present study were listed in Table 1.

2.4. Cloning of GhWRKY11 gene

To obtain the internal conservative fragment of *GhWRKY11*, degenerate primers WP1 and WP2 were designed and synthesized (Sangon, China) based on the conserved amino acid

sequence of WRKY11 from other species. Reverse transcription-PCR (RT-PCR) was performed using cotton cDNA as the template with the following conditions: 94 °C for 10 min followed by 35 cycles of amplification (94 °C for 40 s, 53 °C for 40 s and 72 °C for 50 s); 72 °C for 10 min.

For 5′ RACE, purified cDNA polyadenylated at its 5′ end with dCTP using terminal deoxynucleotidyl transferase (TaKaRa, China) was used as the template for primary PCR amplified with specific primers 5W1 and Abridged Anchor Primer (AAP). Then the primary PCR products and the 5W2 as well as Abridged Universal Amplification Primer (AUAP) were employed in the nested PCR. Both PCR were performed under the following conditions: denaturation at 94 °C for 10 min, followed by 35 cycles of amplification: 94 °C for 30 s, 55/ 54 °C for 30 s and 72 °C for 40 s with a final extension at 72 °C for 10 min. For 3′ RACE, the specific primer 3W1 and universal primer B26 were used in the primary PCR reaction with purified cDNA as the template. Then the nested PCR was carried out with the primary PCR products together with nested

Table 1
Primers for PCR.

Abbreviation	Primer sequence (5′–3′)	Description
W1	AGYATGGARCAYYGATYCG	Degenerate primer, forward
	(Y=C, T; R=A, G; H=A, T or G; B=G, T or C)	
W2	TTCCTHGCBGGRCAYCCTCT	Degenerate primer, reverse
5W1	GATTGAACGAAGCTAGAAGGACTG	5′ RACE reverse primer, outer
5W2	GGGAAGATGAGGAAGAAAGGG	5′ RACE reverse primer, inner
3W1	GGTGAAGAAAGTAATAAGGGTTCC	3′ RACE forward primer, outer
3W2	GATATTCCACCAGACGAGTATTCATGG	3′ RACE forward primer, inner
AAP	GGCCACGCGTCGACTAGTAC(G)14	Abridged Anchor Primer
AUAP	GGCCACGCGTCGACTAGTAC	Abridged universal amplification primer
B26	GACTCTAGACGACATCGA(T)18	3′ RACE universal adaptor primer
B25	GACTCTAGACGACATCGA	3′ RACE universal primer
WQC1	CTCTTTGCCTTTCATCTTCATCC	Full-length cDNA sequence primer, forward
WQC2	TATACATCCAATGCCCCAG	Full-length cDNA sequence primer, reverse
WG1	GGATCCCTCTTTGCCTTTCATCTTCATCCC	Subcellular localization primer, forward
WG2	CTCGAGGATTGATATTTGCTTATTATCACG	Subcellular localization primer, reverse
WP1	GGATCCCTCTTTGCCTTTCATCTTCATCCC	Expression vector construction primer, forward
WP2	GAGCTCTATACATCCAATTGCCCCAG	Expression vector construction primer, reverse
WQD1	CAACCTCTTGTACCGCCATCTG	I-PCR inner primer, reverse
WQD2	CAGATCCACCGCCATTTTGAGGTT	I-PCR outer primer, reverse
WE1	CACCTGATCCGTCTCCTTTC	I-PCR inner primer, forward
WE2	GACTGCACCGACCTCGCC	I-PCR outer primer, forward
WQ1	CAGTAGCGTTAGAGGGTGCCC	q-PCR primer, forward
WQ2	CGAAGACCAGTCCATTCCTG	q-PCR primer, reverse
RQ1	CGGAGGAGGGACACAACACC	q-PCR primer, forward
RQ2	TCCACAAGAAACTCAACACCAGAAG	q-PCR primer, reverse
WR1	GATGGCGGTACAAGAGGT	Semi-quantitative RT-PCR primer, forward
WR2	TCGCAGCAGAAGTTAGAGTTAG	Semi-quantitative RT-PCR primer, reverse
SSU1	AACTTAAAGGAATTGACGGAAG	Cotton standard control primer, forward
SSU2	GCATCACAGACCTGTTATTGCC	Cotton standard control primer, reverse
ACTIN1	CCTTGAAGTATCCCATTGAGCAT	*N. benthamiana* standard control primer, forward
ACTIN2	GCCCATCTGGTAACTCATAGC	*N. benthamiana* standard control primer, reverse
PR1-1	GGTGTAGAACCTTTGACCTGG	Semi-quantitative RT-PCR primer, forward
PR1-2	GAACCCTAGCACATCCAACAC	Semi-quantitative RT-PCR primer, reverse
PR4-1	CAGAACATTAACTGGGATTTGAGAG	Semi-quantitative RT-PCR primer, forward
PR4-2	CTCCATTGCTGCATTGATCTACT	Semi-quantitative RT-PCR primer, reverse
NPR1-1	GCAGCAGACGATGTAATGATGG	Semi-quantitative RT-PCR primer, forward
NPR1-2	TCCACAAGCCTAGTGAGCCTC	semi-quantitative RT-PCR primer, reverse
CMV-1	CGGTCGTATTGCTTCCTTCTTT	Semi-quantitative RT-PCR primer, forward
CMV-2	CAACAGGCGAGCGACAGCTGAC	Semi-quantitative RT-PCR primer, reverse

primer 3W2 and universal primer B25. Both PCR conditions were as follows: predenaturation at 94 °C for 10 min, 35 cycles of 94 °C for 30 s, 50/52 °C for 30 s, 72 °C for 40 s, and final extension at 72 °C for 10 min.

GhWRKY11 full-length cDNA was amplified by PCR with specific primers WQC1 and WQC2, which were designed according to the deduced full-length cDNA. The PCR condition was programmed as below: predenatured at 94 °C for 10 min, followed by 35 cycles of amplification (94 °C for 40 s, 50 °C for 40 s, 72 °C for 90 s), and then followed by extension for 10 min at 72 °C. Genomic DNA for GhWRKY11 was so amplified with cotton genomic DNA as the template.

Inverted PCR (I-PCR) was used to amplify the GhWRKY11 promoter sequence. The genomic DNA was completely digested with EcoR1 and then was self-linked with the aid of T4 DNA ligase (TaKaRa, China) to form circles and then was used as the template. Outer primers (WQD1 and WE1) and inner primers (WQD2 and WE2) were respectively used to perform the first and second round PCR reactions. Both PCR conditions were as follows: predenaturation at 94 °C for 10 min, 35 cycles of 94 °C for 30 s, 53/55 °C for 30 s, 72 °C for 1 min, and final extension at 72 °C for 10 min.

All the PCR products were cloned into the pMD18-T vector (TaKaRa, China) and then transformed into Escherichia coli competent cells (E. coli DH5α) for sequencing.

2.5. Estimating the copy number of GhWRKY11 in cotton

The copy number of GhWRKY11 in cotton detected by primers WQ1 and WQ2 was estimated using quantitative real-time PCR following the method described by Mason et al. (2002). Meanwhile, GhRDR6, was used as a control, which was determined by primers RQ1 and RQ2, shown as a single copy in cotton through southern blot analysis (Wang et al., 2012).

2.6. Bioinformatics analysis

Sequence alignment was performed using DNAman software 5.2.2 and BLAST software online (http://www.ncbi.nlm.gov/blast). The phylogenetic tree was constructed using MEGA4.1. The nuclear-localization signals were predicted by PSORT program (http://psort.ims.u-tokyo.ac.jp). In addition, identification of the putative cis-acting elements in the promoter region of GhWRKY11 was performed using PlantCARE databases (http://bioinformatics.psb.ugent.be/webtools/plantcare/) and PLACE (Higo et al., 1999; http://www.dna.affrc.go.jp/PLACE/).

2.7. Generation of the fusion protein and subcellular localization analysis

The coding region of GhWRKY11 without termination codon was obtained using specific primers WG1 and WG2, containing an upstream BamH I site and a downstream Xho I site. The resulting fragment was fused into the N-terminus of GFP expression vector under the control of cauliflower mosaic virus (CaMV) 35S promoter. Then the recombined 35S-GhWRKY11:: GFP construct and 35S-GFP plasmid served as a control were

transferred into living onion epidermal cells, respectively, using the particle bombardment method described previously (Varagona et al., 1992). After incubation on 1/2 MS agar medium at 25 °C for 12 h, the nuclei were stained with 100 μg/mL of 4′, 6diamidino-2-phenylindole (DAPI) (Solarbio, China) for 10 min. The expression of gene was observed using a laser scanning microscope (LSM 510 META, ZEISS, Germany).

2.8. Semi-quantitative RT-PCR analysis

To detect the transcript accumulation of GhWRKY11, 18S ribosomal RNA (18S rRNA) was used as the internal reference and detected by primers SSU1 and SSU2. Meanwhile, specific primers WR1 and WR2 were designed to determine the expression of GhWRKY11. To analyze the expression levels of pathogen-related genes and the CMV-CP protein contents in N. benthamiana, β-actin was used as a loading control to ensure the equal cDNA amounts with primers (ACTIN1 and ACTIN2). Meanwhile pathogen-related gene specific primers were designed, including PR1-1and PR1-2 (specific for PR1), PR4-1 and PR4-2 (specific for PR4), NPR1-1 and NPR1-2 (specific for NPR1). Primers CMV-1 and CMV-2 were used to detect the transcript levels of CMV-CP proteins. The PCR procedure started with an initial denaturation step of 10 min at 94 °C, followed by cycling of 30 s at 94 °C, 30 s at 52 °C and 30 s at 72 °C, terminated by extension for 10 min at 72 °C. The optimal number of PCR cycles was determined for each template.

2.9. Vector construction and plant transformation

To express the GhWRKY11 in N. benthamiana, BamH I and Sac I restriction sites were added, respectively, at the C- and N-terminal of GhWRKY11 by PCR with primers WP1 and WP2. The modified full-length fragment was inserted into the BamH I and SacI restriction sites of pBI121 vector under the control of 35S promoter. Then the resulting construct was introduced into the Agrobacterium tumefaciens strain LBA4404. GhWRKY11-overexpressing N. benthamiana was obtained using leaf disk method described by Horsch et al. (1985). Transgenic progeny was selected on 1/2 MS agar medium containing kanamycin (100 mg/L) and then grown in soil under greenhouse conditions. All transgenic plants used in this study are T_2 lines.

2.10. Histochemical H_2O_2 staining

Leaves were infiltrated with 1 mg/mL DAB (3, 3′-diamino-benzidine) solution (pH 3.8) for 12 h at 25 °C in the dark to detect H_2O_2. Then the leaves were decolorized by boiling in ethanol (96%) for 10 min.

3. Results

3.1. Isolation of GhWRKY11 from cotton

Due to the importance of WRKY proteins in regulating plant disease resistance, a fragment of pathogen-induced cDNA at

the length of 879 bp was obtained using the degenerate primers. Then RACE technique was performed to achieve the full-length cDNA consisting of 1306 nucleotides with a 5′ untranslated region (UTR) of 111 bp, a 3′ UTR of 142 bp and a 1053 bp open reading frame (ORF). The putative clone exhibited high sequence similarity with AtWRKY11 (GenBank accession number: NM_179228) from *Arabidopsis thaliana*. Therefore the cloned cDNA was named as *GhWRKY11* (HQ828074).

3.2. Characterization and molecular evolution analysis of GhWRKY11

The entire ORF of *GhWRKY11* encodes a protein of 373 amino acid residues with a predicted molecular weight of 37.79 kDa and an isoelectric point of 10.35. Similar to the other WRKY transcription factors, the putative GhWRKY11 protein contains a typical DNA binding domain, the WRKY domain. It contains a WRKYGQK motif followed by a putative zinc finger structure ($Cx_{4-5}Cx_{22-23}HxH$), both of which comply with the WRKY consensus. Meanwhile, the deduced protein processed a putative NLS and a typical C domain which is a specific conserved domain of group IId WRKY superfamily (Park et al., 2005). In addition, multi-alignment analysis revealed that GhWRKY11 is highly related to group IId WRKY proteins among different species, sharing a homology of 59.04% to AtWRKY11, 67.81% to RcWRKY11 (XM_002515307) from *Ricinus communis*, 59.15% to BnWRKY11 (EU912390) from *Brassica napus* and 63.13% to PtWRKY11 (XM_002324346) from *Populus trichocarpa* (Fig. 1A). Thus we classified GhWRKY11 as a WRKY group IId protein.

For further investigation, a phylogenetic tree was constructed using MEGA4.1, revealing that GhWRKY11 was more closely related to group IId WRKY members (Fig. 1B). We concluded that GhWRKY11 is a group IId WRKY protein.

3.3. Genomic sequence analysis and copy number determination of GhWRKY11

To characterize the *GhWRKY11* gene on the DNA level, specific primers were used to amplify the 1905 bp full-length fragment from cotton genomic DNA (HQ828083). Alignment analysis of the genomic and cDNA sequence of *GhWRKY11* indicated the presence of two introns (837–1349 bp and 1476–1561 bp in the genomic clone, respectively), both of the locations were found to be conserved among closest relatives.

In addition, the copy number of *GhWRKY11* was determined using real-time PCR. *GhRDR6*, validated as a single copy per haploid *G. hirsutum* genome, was used as the reference to estimate the copy number of *GhWRKY11* in cotton. The average correlation coefficients (R^2) of the two standard curves were 0.999 and 0.997, respectively, indicating a high level of accuracy and robustness in estimating absolute amounts of the two genes based on the standard curves (Fig. 2). Two batches of real-time PCR analysis were conducted with three replicates. The average cycle threshold (Ct) values were used to estimate

the copy number (Table 2). The results showed that *GhWRKY11* existed as a single copy in cotton genome.

3.4. Subcellular localization of GhWRKY11

A prediction program for protein is applied to reveal the subcellular localization of GhWRKY11. The results showed that GhWRKY11 protein was localized in the nucleus. Next, the GFP fusion with GhWRKY11 controlled by 35S promoter was used to further confirm this prediction. Meanwhile, *35S-GFP* was used as a control (Fig. 3A). Both *35S-GhWRKY11::GFP* and *35S-GFP* constructs were introduced into onion epidermal cells using the particle bombardment method, respectively. GFP fluorescence detected by laser scanning microscope indicated that the GhWRKY11::GFP fusion protein was mainly localized in the nucleus, whereas GFP control displayed throughout the whole cell (Fig. 3B). These results suggested that GhWRKY11 protein was localized to the nucleus.

3.5. GhWRKY11 promoter analysis

With the help of I-PCR, we obtained a 1025 bp fragment of *GhWRKY11* 5′flanking region (JQ822293). The PlantCARE and PLACE databases revealed various putative *cis*-acting elements (Table 3) involved in defense responses in the promoter region of *GhWRKY11*, including CGTCA-motif and TGACG-motif (present in the MeJA-responsiveness), ERE (ethylene-responsive element), TCA-element (present in SA responsiveness), TC-rich repeats (present in defense and stress responsiveness) and W-box (WRKY transcription factor binding site). The presence of these *cis*-acting elements suggests a role of GhWRKY11 in defense responses through multiple signal pathways.

In addition, other important *cis*-acting elements were also found in *GhWRKY11* promoter sequence, such as MSA-like (involved in cell cycle regulation), TGA-element (auxin-responsive element) as well as skin-1 motif (required for endosperm). Thus the possibility that GhWRKY11 may be a critical transcriptional factor in regulating various aspects in cotton should be considered.

3.6. GhWRKY11 expression is induced by pathogen infection and partial defense-related molecules

Considering the presence of *cis*-acting elements responding to defense-related plant hormones in *GhWRKY11* promoter, we firstly analyzed the response of *GhWRKY11* to SA, JA and ET. Semi-quantitative RT-PCR analysis showed a strong induction of *GhWRKY11* by SA, MeJA and ET (Fig. 4A–C). The increased expression levels of *GhWRKY11* were detected within 6 h after MeJA treatment and reached a peak at 8 h, then declined slowly. Under the ET treatment, the expression of *GhWRKY11* was dramatically increased after 2 h and reached a maximum after 4 h. However, a comparative increased expression level of *GhWRKY11* was observed until 24 h with SA treatment. We concluded that *GhWRKY11* might participate
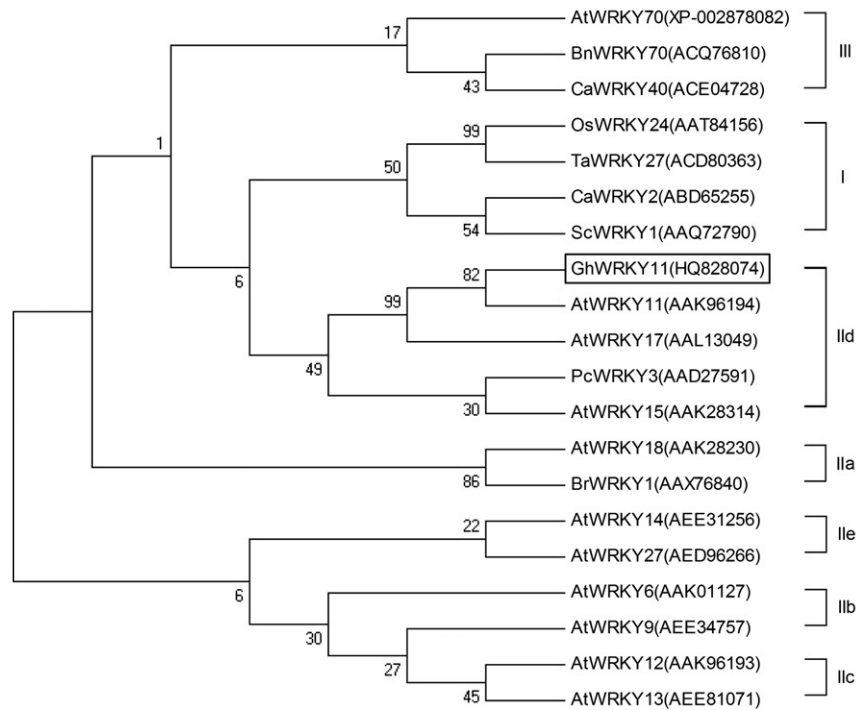
A
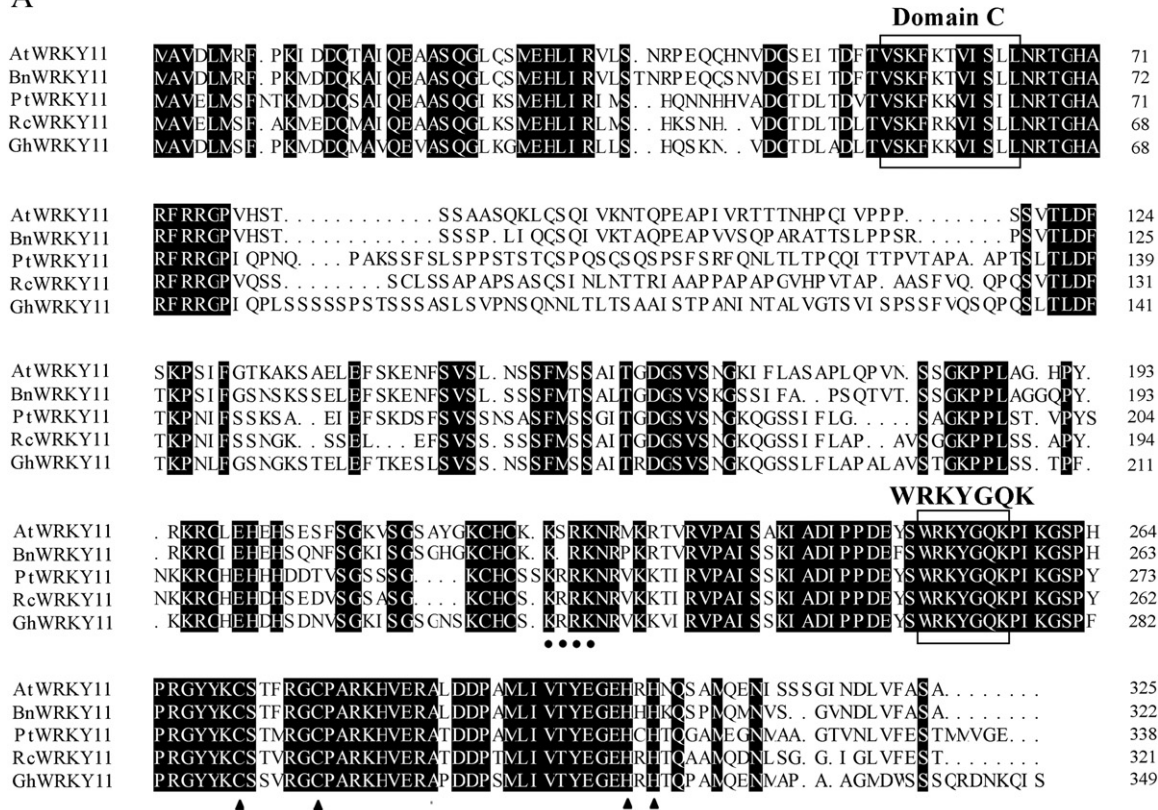
B

Fig. 1. Comparison of GhWRKY11 with plant WRKY proteins. (A) Alignment of GhWRKY11 amino acid sequence with AtWRKY11, BnWRKY11, PtWRKY11 and RcWRKY11. Identical amino acids are shown in white on a black background. The protein domains are shown in frames. The cysteine and histidine residues of the putative zinc finger motif were indicated by arrowheads (▲) and the putative NLS was marked by dots (●). (B) The phylogenetic relationship between GhWRKY11 and other plant WRKY proteins. GhWRKY11 was shown in a frame. Numbers above or below branches indicate bootstrap values (>50%) from 500 replicates. The gene name is followed by the protein ID.
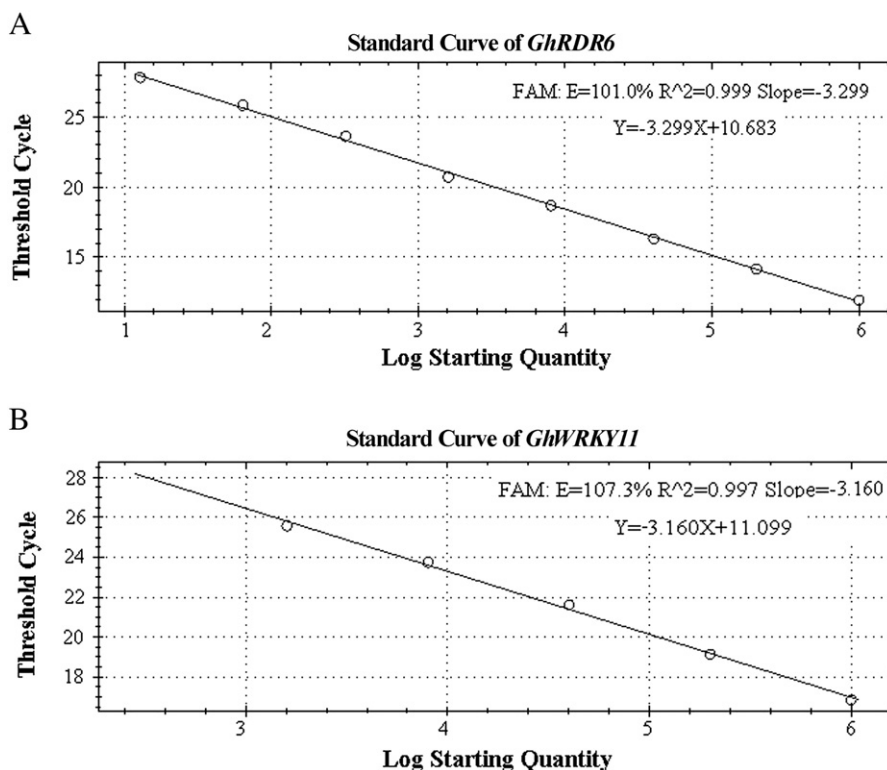
A

B

Fig. 2. Standard curves of *GhRDR6* and *GhWRKY11*. (A) and (B) are standard curves of *GhRDR6* and *GhWRKY11* genes from the amplification of five five-fold serial dilutions of plasmids. Correlation coefficient and slope values are indicated. The calculated threshold cycle values were plotted versus the log of each starting quantity.

in defense responses in SA and JA/ET mediated signal pathways.

To further test this hypothesis, the response of *GhWRKY11* to exogenous H_2O_2 and wounding treatment was detected. As expected, *GhWRKY11* responded to both conditions (Fig. 4D, E). Under wounding treatment, *GhWRKY11* was up-regulated at 2 h and reached the maximum at 3 h, then recovered at 5 h. After H_2O_2 treatment, *GhWRKY11* transcript accumulation commenced at 0.5 h and reached a maximum at 1 h before declining at 10 h. Noticeably, the expression of GhWRKY11 was transiently induced in each case. These results indicated a role of GhWRKY11 in defense response.

Finally, direct evidence supporting our hypothesis was carried out by expression analysis of *GhWRKY11* response to pathogens. A fungal pathogen, *C. gossypii*, was inoculated to cotton seedlings. As expected, the level of *GhWRKKY11* transcript increased gradually from 4 d (Fig. 4F). Above all, we speculated that *GhWRKY11* may be involved in both SA and JA/ET mediated plant defense responses.

3.7. GhWRKY11-overexpressing plants display improved virus resistance through an SA-dependent signaling pathway

To study the role of GhWRKY11 in defense response, *N. benthamiana* was transformed with *35S-GhWRKY11* construct using *A. tumefaciens*-mediated transformation method. Transgenic lines were selected by kanamycin and then confirmed by PCR. Consequently, two independent lines (OE1 and OE2) showing relatively high expression of *GhWRKY11* were further used for functional analysis.

Analyses of both overexpressing *N. benthamiana* lines exhibited no difference in growth and morphology from those of wild-type lines. Six-week old wild-type and transgenic plants were inoculated with CMV and cultured for 12 days, both of which showed stunting and distortion of leaves. However, wild-type plants displayed more severe disease symptoms than that in *GhWRKY11*-overexpressing plants (Fig. 5A). Furthermore, semi-quantitative RT-PCR analysis was used to reveal the CMV coat protein (CP) gene expression

Table 2
Ct value of *GhRDR6* and *GhWRKY11* genes and copy number of *GhWRKY11* gene in cotton.

Samles of *Gossypium hirsutum* L.	*GhRDR6*		*GhWRKY11*		*GhWRKY11/GhRDR6*	Copy number
	Ct values	Calculation result	Ct values	Calculation result		
1	23.20	−3.68	21.88	−3.14	0.93	1
2	25.99	−4.49	24.59	−4.27	0.95	1
3	29.09	−5.40	26.08	−4.74	0.87	1

A

35S- GFP　[CaMV35S Pro] [GFP] —[NOS-ter]

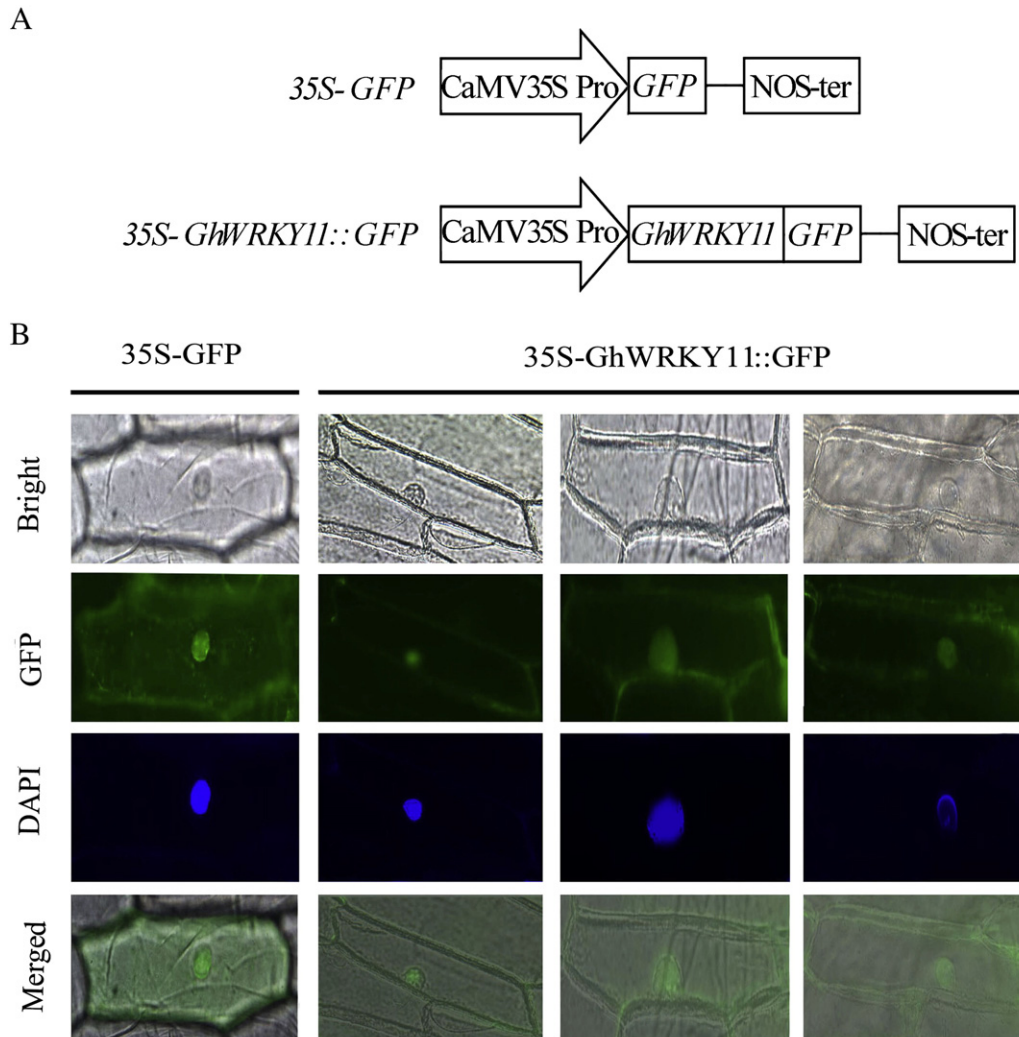*35S- GhWRKY11::GFP*　[CaMV35S Pro] [GhWRKY11 | GFP] —[NOS-ter]

B

Fig. 3. Subcellular localization of GhWRKY11-GFP fusion protein in onion epidermal cells. (A) Schematic view of *35S-GhWRKY11::GFP* and *35S-GFP* construction. (B) Transient expression of *35S::GhWRKY11-GFP* fusion protein and 35S-GFP protein in onion epidermal cells. Cells were observed using confocal laser scanning microscope.

levels. Accordingly, we observed lower accumulation of CMV in transgenic plants than that in wild-type plants (Fig. 5C). These results indicated that *GhWRKY11* might enhance the resistance to CMV in *N. benthamiana*.

To reveal the signal pathways associated with the *GhWRKY11*-dependent CMV resistance, we examined the transcript accumulation of partial pathogen-related genes in

Table 3
Partial putative *cis*-acting elements of the promoter of *GhWRKY11*.

Cis-element	Position [a]	Sequence (5′–3′)
CGTCA-motif	−356 (+)	CGTCA
TGACG-motif	−356 (−)	TGACG
ERE	−377 (+)	ATTTCAAA
TC-rich repeats	−426 (+)	ATTTTCTTCA
W-box	−115 (+)	(TTGACC/T)
MSA-like	−917 (−)	(T/C)C(T/C)AACGG(T/C)(T/C)A
TGA-element	−215 (+)	AACGAC
Skin-1 motif	−976 (+)	GTCAT

[a] Position of the *cis*-element with respect to the putative transcription initiation site. Strands are indicated as: (+), forward; (−), complement.
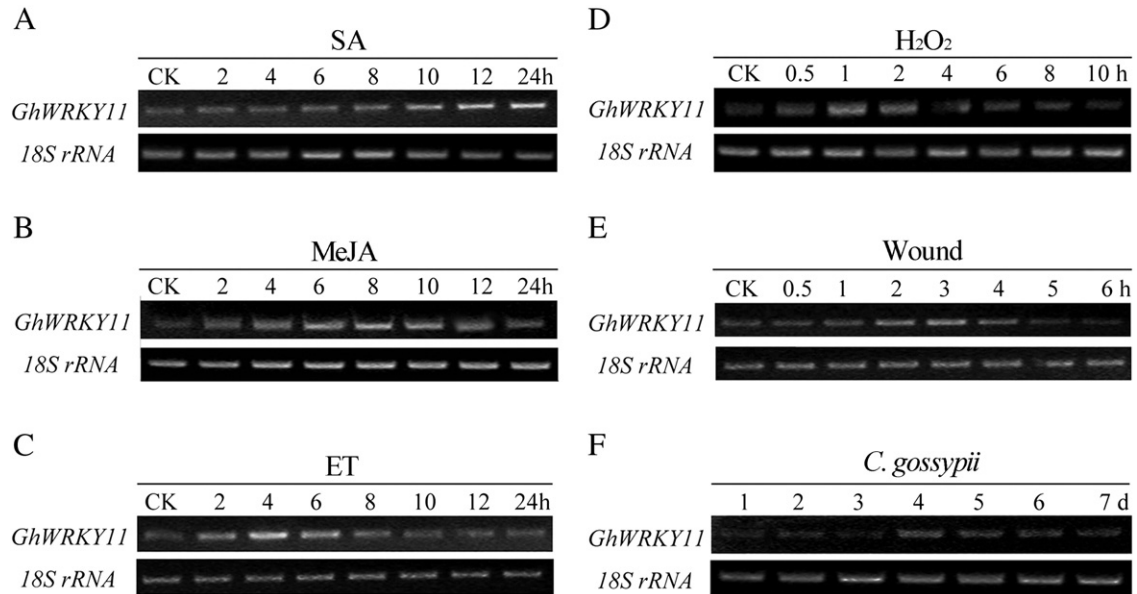
both wild-type and transgenic lines after CMV inoculation. In all transgenic lines, *PR1* and *NPR1* display higher accumulation level than in wild-type lines (Fig. 5C). However, no obvious difference was detected for *PR4* transcript between transgenic and wild-type plants. Those results displayed that *GhWRKY11* enhanced the expression of *PR1* and *NPR1* but not *PR4* in *N. benthamiana* after CMV infection. Since previous studies revealed that *PR1* and *NPR1* are marker genes in SA-mediated pathway, and *PR4* is a marker gene in JA signaling pathway, so we deduced that GhWRKY11 might enhance *N. benthamiana* resistances to CMV through SA-mediated signaling pathway.

3.8. GhWRKY11 reduce the accumulation of ROS in transgenic plants during virus attack

Pathogen attack can induce a series of defense responses, such as the generation of ROS in plants. As a ROS effecter, H_2O_2 can pass through plant cell membranes and thus directly function in cell-to-cell signaling (Apel and Hirt, 2004; Chen et al., 2002). To explore the relationship between the *GhWRKY11*-enhanced

Fig. 4. Expression analysis of *GhWRKY11*. Induction of *GhWRKY11* expression under various conditions, including SA (A), MeJA (B), ET (C), H_2O_2 (D), wounding (E) and *C. gossypii* (F). *18S rRNA* was used as standard control to equal the cDNA amounts used in each reaction.

CMV resistance and the ROS accumulation, we performed a DAB staining assay to detect H_2O_2 accumulation with upper systemic leaves. After we inoculated wild-type and transgenic plants with CMV for 12 days, we found a higher accumulation of H_2O_2 in wild-type tobacco than in transgenic plants (Fig. 5B), suggesting that overexpression of *GhWRKY11* can reduce the generation of H_2O_2 or sweep the redundant H_2O_2.

4. Discussion

Although there are numerous links between WRKY proteins and plant defense mechanisms, information about the biological roles of WRKY transcription factors in economic crops is still very limited. Especially the potential functions of a large number of WRKY proteins in cotton need to be explored extensively.

In this study, we report the isolation and characterization of a cotton WRKY transcription factor gene named *GhWRKY11* existing as a single copy in cotton genome. The deduced protein possesses a WRKYGQK sequence and a zinc finger motif, consistent with the features of WRKY proteins. What's more, both the existence of typical C domain existing in group IId WRKY proteins and the phylogenetic analysis results indicated GhWRKY11 was a group IId WRKY factor, with RcWRKY11 as its closest homologue. In addition, an NLS was found in the GhWRKY11 sequence by using the PSORT program, which indicated that it may function in the nucleus, similarly as VpWRKY1 and VpWRKY2 (Li et al., 2010). Subcellular localization assays further confirmed this hypothesis. Therefore, we speculated that GhWRKY11 may function as a transcription factor in nucleus, presumably through a common mechanism shared with group IId WRKY proteins.
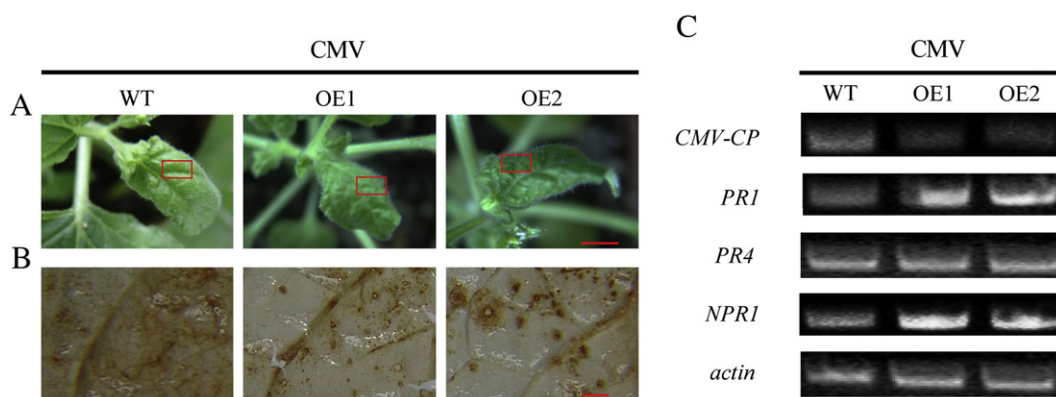

Fig. 5. Enhanced resistance of *GhWRKY11*-overexpressed lines to virus (CMV) infection. (A) Six-week old WT and *GhWRKY11* overexpressing *N. benthamiana*s were inoculated with CMV and the symptoms of top systemic leaves are shown at 12 days postinoculation. The *bar* is 1 cm. (B) The accumulations of H_2O_2 in CMV-treated *N. benthamiana*s indicated by DAB staining. The *bar* is 2 mm. (C) Expression analysis of pathogen-related genes and CMV-CP genes at 12 days postinoculation by semi-quantitative PCR. *Actin* was applied as a standard control. WT, wild-type.

Previous studies showed that WRKY proteins participate in plant defense responses. AtWRKY27 negatively influences symptom development of a vascular pathogen (Mukhtar et al., 2008). OsWRKY6 functions as a positive transcriptional factor of the plant defense response (Hwang et al., 2011). In our study, the *GhWRKY11* transcript is induced following infection by the pathogen fungus *C. gossypii* and wounding treatment, suggesting that GhWRKY11 may be involved in plant defense responses. SA, JA and ET are three important signal molecules involved in two major defense signaling pathways against different types of pathogens: the SA-dependent and JA/ET-dependent defense mechanisms (Dong, 1998; Kunkel and Brooks, 2002). Like *GhWRKY3*, *GhWRKY11* could be induced by all the three molecules (Guo et al., 2011). Along with the existence of *cis*-acting elements (response to SA, JA and ET, respectively) in *GhWRKY11* promoter sequence, it is reasonable to speculate that *GhWRKY11* might act as a key transcriptional factor modulating both SA- and JA/ET-dependent signaling pathways. However, the function of *GhWRKY11* in plant defense responses through SA- and/or JA/ET-mediated pathways remains to be explored.

Direct evidences came from functional analysis of over-expressed *GhWRKY11* in *N. benthamiana*s indicating that GhWRKY11 may participate in plant defense response through SA-mediated signaling pathway. We observed that the enhanced resistance of transgenic plants to CMV was associated with enhanced expression of *PR1* and *NPR1*. The important defense related proteins, *PR1* and *NPR1* are defined as marker genes in SA signal pathway. Thus, *GhWRKY11* possesses a potential to regulate the virus defense resistance through the SA signaling pathway. However, it should be noted that the expression level of *PR4* was not obviously different between transgenic and wild-type plants. As *PR4* is known as the marker gene in JA signaling pathway it is probable that *GhWRKY11* enhanced CMV resistance through SA-mediated signaling pathway rather than JA. As reported before, an *Arabidopsis* WRKY factor, AtWRKY7, demonstrated a similar expression pattern induced by both *P. syringae* attack and SA treatment (Kim et al., 2006). However, AtWRKY11 together with AtWRKY17 participate in the plant disease resistance through JA-dependent signal pathway (Journot-Catalino et al., 2006). Thus, the different members of IId WRKY subfamily may participate in different signaling pathways to resist disease attack.

A large number of reports indicate that plants challenged with pathogens are often exposed to the accumulation of ROS, which is implicated in the damaging effects under stresses (Lamb and Dixon, 1997). As the expression analyses indicated, GhWRKY11 could be induced by both pathogen attack and H_2O_2 treatment, implying that GhWRKY11 might participate in defense responses through ROS-mediated signaling mechanisms. In addition, *GhWRKY11* overexpressing lines displayed less H_2O_2 accumulation compared to wild-type plants when challenged with CMV, which is consistent with its role in ROS-mediated defense response. According to previous studies that revealed the strong interconnection of SA and ROS signaling pathways, *GhMPK7* was shown to be involved in both SA-regulated and ROS-mediated defense responses under pathogen attack (Shi et al., 2010). Like *GhMAPK7*, *GhWRKY11* may be involved in both SA and ROS mediated signaling pathways.

In summary, our results demonstrated that *GhWRKY11* encodes a novel cotton WRKY transcriptional factor targeted to the nucleus that may play important roles in regulating plant defense responses through SA- and ROS-mediated pathways. However, further investigation is still needed to explore the putative roles of *GhWRKY11* in the intertwined signaling pathways that manipulate pathogen defense responses.

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