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Full Length Research Paper

GhNAC18, a novel cotton *(Gossypium hirsutum L.)* NAC gene, is involved in leaf senescence and diverse stress responses

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***GhNAC18* is a novel NAC gene that was isolated from cotton (*Gossypium hirsutum* L.). The full-length cDNA was 1511 bp including an open reading frame of 1260 bp in length and encodes a protein of 419 amino acids. With qRT-PCR analysis, *GhNAC18* was downregulated during natural and dark-induced senescence, implicating this gene as anti-aging gene in cotton. Analysis of its promoter revealed a group of putative *cis*-acting elements especially, the light and stress responsive elements, indicating that it may have a potential role in leaf development. Additionally, *GhNAC18* was found to have transcriptional activation activities on its C-terminal region and by bioinformatics analysis, *GhNAC18* was localized in the nucleus. Tissue specific expression analysis indicated that *GhNAC18* is constitutively expressed in roots, stems, earlier stages of senescing leaves, fibers and flower parts with high expression levels registered in the young leaves and cotyledon leaves. *GhNAC18* was up-regulated by exogenous application of various phytohormones including salicylic acid (SA), methyl Jasmonate (MeJA) and ethylene (ET) but downregulated with abscisic acid (ABA). Moreover, the gene was induced by drought (PEG6000), H_2O_2, cold (4°C) and wounding but was inhibited by high salinity. These results indicated that *GhNAC18* is a transcriptional activator that is involved in leaf development, especially inhibition of leaf senescence and plant stress responses in cotton. This study provides fundamental information on understanding the function of *GhNAC18* gene in cotton leaf senescence and stress tolerance and thereafter its manipulation for breeding of late-aging and stress-tolerant cultivars.**

Key words: *GhNAC18*, stress response, senescence, *Gossypium hirsutum* L. NAC.

INTRODUCTION

Plants face survival challenges posed by ever varying adverse environmental conditions which include but not limited to abiotic stress such as cold, high salinity, drought and extreme temperatures (Fujita et al., 2004; Ning et al., 2010; Nakashima et al., 2011). Biotic attacks such as infectious pathogens also complicate the sessile habit of plants. In this regard, plants adapt to these dynamic conditions by evoking responses at physio-logical, biochemical and molecular levels (Nakashima et al., 2011; Fan et al., 2015) including regulation of genes enhancing survivability (Nuruzzaman et al., 2013). The immune response in plants is triggered by pathogen

infection that is characterized by activation of multiple defense responses including expression of defense-related genes, regulated by different types of transcription factors (TFs).

TFs play important roles in regulating plant development and stress responses. They can be grouped into different families on the basis of conserved structural domains involved in DNA binding to *cis*- acting elements in the promoters of target genes, or other functional modular structures. Many TFs belong to NAC (Puranik et al., 2012), ERF, MYB/MYC (Christian et al., 2010), WRKY (Eulgem and Somssich, 2007), DREB/CBF, AP2/EREBP (Dietz and Viehhauser, 2010) and bZIP families.

NAC (for NAM, ATAF1,2 and CUC2) is a plant-specific family of transcription factors which share the N-terminal DNA-domain with a varying C-terminus that regulates transcription (Hao et al., 2011). The diverse C-terminal sequences among NACs are putative transcriptional activation domains which either activate or repress downstream of target genes. NACs are widely distributed in land plants and comprise one of the largest transcription factor families (Olsen et al., 2005). Since the first NAC gene denoted as NAM for no apical meristem was isolated from petunia (Yamasaki et al., 2013), many NACs have been reported to contribute to various developmental processes such as shoot apical meristem development (Nuruzzaman et al., 2013; Yamasaki et al., 2013), lateral root development, senescence, flowering and secondary wall formation. Moreover, NACs have also been associated with plant responses to biotic and abiotic stresses such as fungus infection, drought, cold, and high salinity (Sefyan et al., 2013; Hao et al., 2011; Xingwang et al., 2014). For instance, *OsNAC6* improves stress tolerance to dehydration and salinity in rice (Nakashima et al., 2007).

GhNAP regulate leaf senescence via the ABA-mediated pathways and has been associated with improved yield and quality in cotton (Fan et al., 2015; Mauch-Mani and Mauch, 2005). Age mediated senescence genes have been reported to be upregulated during the process (Zhao et al., 2015). Weaver et al. (1998) demonstrated that several SAGs are internally induced while others are elicited by external factors, however some SAGs may inhibit senescence (Weaver et al., 1998). Worthy to note is that some NACs simultaneously play multiple roles in regulating plant development and responses to exogenous stimuli (Shah et al., 2014). In arabidopsis *AtNAC2*, a transcription factor in the downstream of ethylene and auxin signaling pathways is simultaneously involved in salt stress response and lateral root development (Cao et al., 2005). Other NAC genes have been found to be upregulated during senescence (Shah et al., 2014) or by wounding and bacterial infection (Boller et al., 2001). Further, NAC proteins have been shown to mediate viral resistance. Apparently NAC family members play various roles not only in plant development but also in the recognition of environmental stimuli.

Upland cotton (*Gossypium hirsutum* L.) is the most important and widely cultivated crop in the world because of its fiber. The challenges posed by environmental stress and competition for land area by food crops call for short season stress tolerant varieties to tackle these challenges. Cotton short-season varieties are accompanied by premature leaf senescence which affects yield quality and quantity. In order to understand the molecular mechanism of cotton leaf senescence and stress responses, we selected a short-season variety CCRI-10 for this study. CCRI-10 exhibit early aging traits. Although, some *GhNAC* genes have been isolated and classified, there is no information available for specific functions of *GhNAC* genes in cotton stress responses and leaf senescence (Shah et al., 2013, 2014). This study reports the characterization of a novel *GhNAC18* gene that could play crucial role in cotton leaf senescence and stress responses. *GhNAC18* as transcriptional activator is downregulated by both natural and dark-induced senescence. Its rapid response to abiotic stress and induction by signal molecules validate *GhNAC18* as novel gene which could be involved in developmental processes and stress responses in cotton. These results taken together, demonstrate that *GhNAC18* could be involved in the regulation of leaf senescence and stress response in cotton.

METHODS AND MATERIALS

Plant materials and growth conditions

Cotton (*G. hirsutum* L. cv CCRI-10) seedlings were grown in a growth chamber at 25°C under a 16 h light and 8 h dark photoperiod. Seedling leaves were harvested at three and four leaf stages, frozen in liquid nitrogen and stored at -80°C for RNA extraction. For tissue specific expression analysis, cotyledon leaves, true leaves, stems, roots, flower parts and fiber tissues were collected from field plants and stored at -80°C for later use. Seven-day-old cotton seedlings were used for the various treatments.

For exogenous application of hormone treatments, leaves of uniformly developed seedlings were irrigated with 2 mM salicylic acid (SA), 100 µM abscisic acid (ABA), 100 µM methyl jasmonate (MeJA) and 100 µM ethylene (ET), respectively.

For salinity and drought treatments, the seedlings were treated with 200 mM NaCl or 15% (w/v) PEG6000, respectively. For hydrogen peroxide (H_2O_2) and wounding, seedlings were sprayed with 20 mM H_2O_2 and wounds inflicted by injuring three leaves from the top. Control plants were sprayed with sterile distilled water. After each treatment, samples from treated and control were frozen in liquid nitrogen and stored at -80°C for further analysis.

For analysis of natural leaf senescence, CCRI10 seeds were field

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grown under natural conditions during the summer of 2015 in Anyang (Henan province, China). Leaves from these plants were harvested at five leaf senescence stages defined by severity of visible symptoms, from non-senescent leaf stage (NS) to completely senescent stage (CS) approximately 90% yellowing of the leaf surface (Shah et al., 2014). For dark-induced senescence, detached flag leaves, submerged in water were incubated in dark for three days at room temperature. At each senescence stage and time, RNA was isolated for qRT-PCR analysis. Further, natural senescence was monitored on the cotyledon leaves from one week after germination. Samples of cotyledon leaves for 8 weeks were collected from the field, noting their morphological changes and then frozen in liquid nitrogen for transcript measurement. To enhance reliability of results, three repeats for each experiment mentioned above was conducted.

Total RNA extraction and cDNA synthesis

Total RNA was extracted using hot borate method described by Wan and Wilkins (1994), and treated with DNase I digestion using RNAprep Pure Plant Kit (Tiangen, China) to eliminate potential genomic DNA contamination. The RNA concentration and purity were determined. Only those that met the criterion (260/280 ratio of 1.8-2.1, 260/230 ratio ≥ 2.0) were used for further analyses and stored at -80°C. The cDNA for cloning work was synthesized by using 5X All-In-One RT MasterMix (ABM. Canada) according to manufacturer's protocol .The RT-PCR was set as follows; 25°C for 10 min, 42°C for 50 min and 85°C for 5 min then put in ice for a few minutes. The newly synthesized strand cDNA was stored at -20°C.

Expression analysis of *GhNAC18*

Total RNA extraction from different tissues was performed by using RNAprep Pure Plant Kit (Tiangen China). The cDNA was synthesized from 2 µg of RNA in a 20 reaction volume using ReverTra Ace qPCR RT kit (TOYOBO, Japan) according to the manufacturer's manual. Relative expression levels of genes in each sample were normalized to the expression level of *GhActin1.* For real-time quantitative PCR, the gene-specific primer pairs (Table 2) were used for *GhNAC18, GhCAB*, *GhNAP* and *GhActin1*. PCR products were detected by SYBR Green I fluorescence dye (Takara, China) in the Applied Biosystems 7500/7500 Faster Real-Time PCR system machine. The following thermal cycle conditions were used: 95°C for 2 min, followed by 40 cycles of 95°C for 5 s, products collected at 60°C for 34 s. All reactions were performed in triplicate. Following the PCR, a melting curve analysis was performed. Ct or threshold cycle was used for relative quantification of the input target number. Relative expression fold difference (N) is the number of treated target gene transcript copies relative to that of the untreated gene transcript copies, and is calculated according to Schmittgen and Livak (2001) as follows:

$$N= 2^{\Delta\Delta Ct}=2^{(\Delta Ct\ treated - \Delta Ct\ control)}$$

Where $\Delta\Delta Ct = \Delta Ct$ of the treated sample minus ΔCt of the untreated control sample, and ΔCt is the difference in threshold cycles for *GhNAC18* target and the *GhActin1* internal reference.

Multiple sequence alignment and phylogenetic analysis

The nucleotide sequences from cDNAs were downloaded from NCBI Blast program (http://www.ncbi.nlm.nih.gov/BLAST). Translation of nucleotide sequences was done using Expasy online program (http://www.web.expasy.org/translate/) and alignment was conducted using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2). Phylogenetic analysis was employed to investigate the evolutionary relationships between *GhNAC18* and NAC proteins from other plants. A neighbor joining tree was generated by MEGA6. A bootstrap analysis with 1,000 replicates was performed to assess the statistical reliability of the tree topology.

Bioinformatics analysis

Open reading frame (ORF) and protein prediction were made using NCBI ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The theoretical isoelectric point (pI) and mass values for mature peptides were obtained using the Peptide-Mass program (http://us.expasy.org/tools/peptide-mass.html). Protein subcellular localization was predicted using WoLF PSORT (http://wolfpsort.org/). In order to investigate the Genomic structure of *GhNAC18*, gene structure display server (GSDS) program (http://gsds.cbi.pku.edu.cn/) was used to illustrate exon/intron organization by comparing the cDNA with their corresponding genomic DNA sequences obtained from cotton genome project database (http://cgp.genomics.org.cn/). The structure of the cotton *GhNAC18* protein was analyzed by Motifscan (http://myhits.isbsib.ch/cgi-bin/motif_scan) and (http://www.ebi.ac.uk).

Cloning of *GhNAC18* gene

The RT products were used to amplify the full length of *GhNAC18* using gene specific primer designed by Oligo 7.0 based on the cDNA (Accession no. *KC847195)*. A pair of primers (Table 1) was used with MightyAmp polymerase enzyme (Takara, China) and the PCR products were gel purified. They were then linked to simple T-vector (pMD18-T Vector). The clones were confirmed by sequencing.

Promoter analysis

Total genomic DNA was extracted from cotton leaf using the cetyl trimethylammonium bromide (CTAB) method (Permingeat et al., 1998). *GhNAC18* promoter of approximately 1.5 kb upstream of ATG was amplified by PCR. The gene specific primes were designed based on the known upstream sequence region of the coding region of *GhNAC18*. The PCR products were purified, linked to T-vector and sequenced. The promoter sequence was then searched in the PLACE and PLANT CARE databases to investigate the putative *cis-* acting elements.

Transcriptional activation activity of *GhNAC18*

To investigate whether *GhNAC18* has transcriptional activities, the entire or partial coding regions of *GhNAC18* were obtained by PCR using fragment specific primers (Table 1). The PCR products were inserted into the *Eco*R1 and *Bam*H1 site of pGBKT7 vector, containing the GAL4 DNA binding domain to obtain pGBKT7:*GhNAC18*-F, pGBKT7:*GhNAC18*-N, and pGBKT7: *GhNAC18*-C. Three constructs and pGBKT7 vector (negative control) were transformed into the yeast strain Y187 (clontech China), plated and incubated for three days.

RESULTS

Characterization and cloning of *GhNAC18*

To date, there are 77 cotton *GhNACs* (Zhao et al., 2015).

Table 1. Primers used for isolating corresponding sequences of *GhNAC18*

Prime name	Primer sequence (5'-3')	Gene/Region
GhNAC18-F	GGAGCTGACATAGTTTCTGGTTAGT	GhNAC18
GhNAC18-R	GTAGGCGCCGCAGTATTTCTTATAT	GhNAC18
pGhNAC18F	TTTATTTCTTCTCGAGTACGCATGG	Promoter
pGhNAC18F	GTTGGTATTATCGTTGGGGTCGTTG	promoter
GhNAC18NF	CGGAATTCCG AAACTAAGAGGGTAGGAGCTCGG	N- terminus
GhNAC18NR	CGGGATCCCG CTCACCACGAAACTGAACGCTAC	N- terminus
FGhNAC18F	CGGAATTCCGGGAGCTGACATAGTTTCTGGTTAGT	Full length
FGhNAC18R	CGGGATCCCGATATAAGAAATACTGCGGCGCCTAC	Full length
GhNAC18CF	CGGAATTCCG ACCACGAAACTGAACGCTACCAA	C-terminus
GhNAC18CR	CGGGATCCCG CCTATTTGGTTCTGGGATTGGGT	C-terminus

Table 2. Primers used for expression analysis by qRT-PCR.

Prime name	Primer sequence (5'-3')	Gene
qPCRGhNAC18-F	CGACGACCTCCACAGACTAGT	GhNAC18
qPCRGhNAC18-R	ACTTGGAATTGCGCTGGGTAG	GhNAC18
qGhNAP-F	GCCCCAATTCACATGACACAGT	GhNAP
qGhNAP-R	TCTCAACATGGTCACCTGTGGT	GhNAP
qGhCAB-F	TGTCCCCGGAAAATGAACAAC	GhCAB
qGhCAB-R	TATGTGCTGCAGAAAATCATGCT	GhCAB
GhACTIN-F	ATCCTCCGTCTTGACCTTG	Actin
GhACTIN-R	TGTCCGTCAGGCAACTCAT	Actin

Genome-wide analysis of these genes as demonstrated by the expression levels in cotton, predict their potential role they play in cotton growth and development (Meng et al., 2007; Shah et al., 2013, 2014; Puranik et al., 2012). *GhNAC12* and *GhNAP* are reported to promote senescence and yield improvement (Zhao et al., 2015; Fan et al., 2015). Our study points out to one of *GhNACs, GhNAC18,* which, unlike others previously reported, is downregulated during leaf senescence and could be involved in cotton stress responses. *GhNAC18* was isolated from upland cotton using gene specific primers (Table 1). The full length of cDNA (GenBank Accession Number KC847195.) was 1511 bp with an open reading frame (ORF) of 1260 bp, encoding 419 amino acids. The relative molecular weight and theoretical isoelectric point of the predicted protein were 48.23 kDa and 6.94, respectively. Using WoLF PSORT program *GhNAC18* was predicted to be located in the nucleus, confirming its role as nuclear transcription factor.

Sequence alignment, phylogenetic analysis and genomic structure

Multiple sequence alignments of the full-length protein sequences from cotton and other known NACs from other plant species, including the highly conserved N-terminal NAM domain and the more divergent C-terminal activation domain, were performed by ClustalW program. To investigate the evolutionary relationship of *GhNAC18* and these other proteins, an unrooted phylogenetic tree was constructed with MEGA 6.0 using the neighbor joining (NJ), minimal evolution (ME) and maximum parsimony (MP) methods and the bootstrap test was carried out with 1000 iterations. Pair wise gap deletion mode was used to ensure that the more divergent C-terminal domains could contribute to the topology of the NJ tree. In this regard, *GhNAC18* belongs to *NAM* subfamily with the NAM domain stretching from 45-192aa of its protein (Figure 1a). *GhNAC18* showed homology with *TaNAC67, ANA036* and *NAM* (Figure 1d). *TaNAC67* is reported to be involved in chlorophyll retention, photosynthetic efficiency and enhanced water retention (Mao et al., 2013). *ANAC036* is highly expressed in the leaf and it is involved in the growth of leaf cells (Kato et al., 2010). This identity with other proteins, imply that *GhNAC18* would have similar functions like these proteins. Moreover multiple sequence alignment resulting to phylogenetic relationship, showed that all the members used, contained A-E subdomains (Figure 1c) (Ooka et al., 2003). This is consistent with previously reported work (Puranik et al., 2012) confirming that *GhNAC18* is a

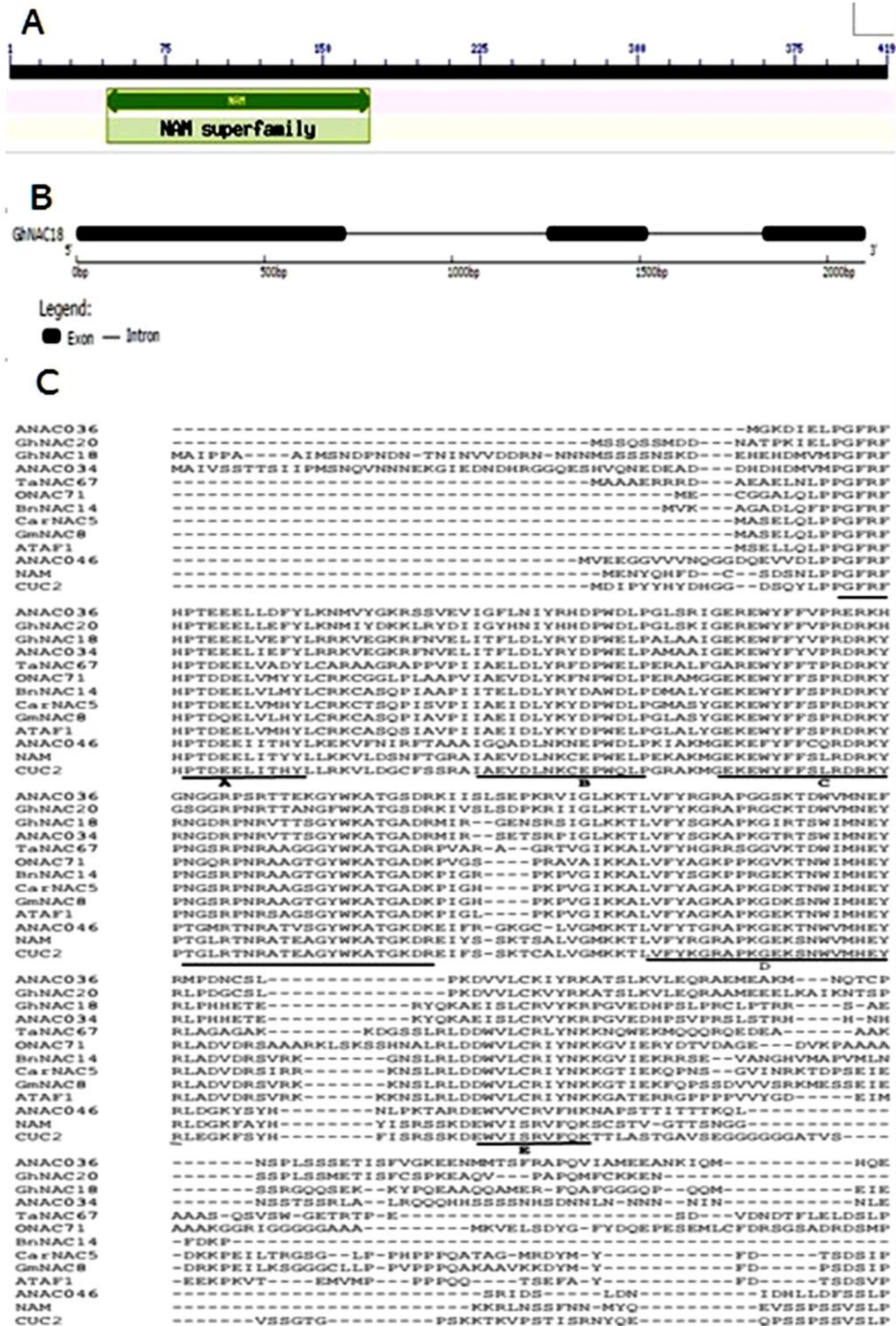


Figure 1. NAM subdomain of NAC domain, genomic organization of *GhNAC18*, Alignment with other related proteins and Phylogenetic tree. ***A***. is a NAC domain (*45-192aa*) of *GhNAC18*, ***B.*** Genomic structure showing exons (*in solid blocks*)and introns (*lines*) of *GhNAC18*, ***C.*** Multiple alignment of putative amino acids sequence of *GhNAC18* and other NAC protein. The location of conserved motifs are underlined and labeled *A-E*. ***D.*** A Phylogenetic tree with *GhNAC18* marked with black circle.

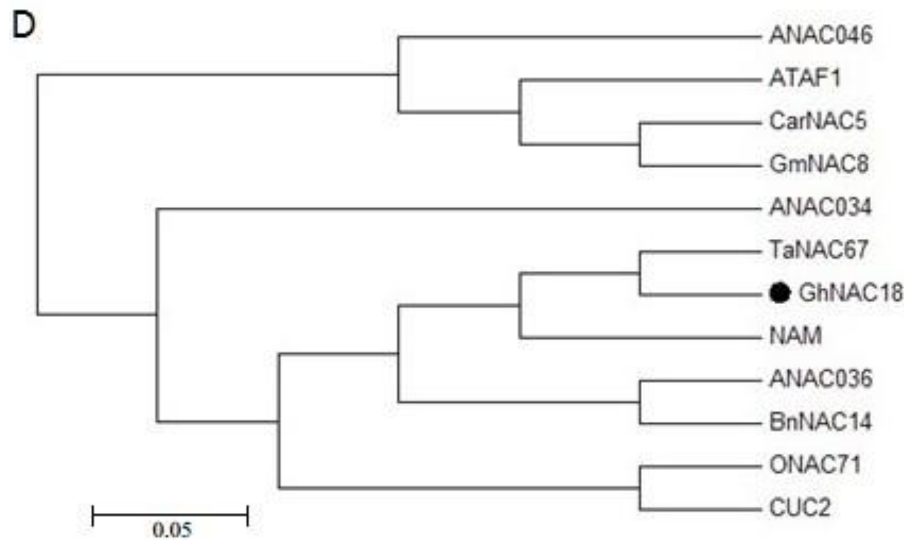


Figure 1. Contd.

member of *NAC* TF family.

Additionally, genomic structure of *GhNAC18* was determined by comparing the genomic sequence and the cDNA sequence. Like most of the NAC family members, *GhNAC18* has two introns and three exons (Meng et al., 2007; Yu et al., 2014). The first two exons encoded the N-terminal domain while the last exon encoded the highly divergent C-terminal region (Figure 1b).

Promoter analysis

Expression of stress-responsive NACs may be tightly regulated by several stress-responsive regulatory elements contained in the promoter region (Puranik et al., 2012). The presences of these cis-acting elements predict some of the roles played by the target gene. *GhNAC18* showed several *cis*- acting elements including 5UTR Py-rich stretch ACE,G-box, MRE(MYB) TC-rich repeats TCA-element, WUN-motif, DREs and LTREs among others. The existence of numerous elements suggested that *GhNAC18* could be involved in regulation of stress responses.

GhNAC18 transcriptional activation activity

To examine whether *GhNAC18* has transcriptional activation activity, the N- and C-terminal fragments as well as the full-length *GhNAC18* were fused to the GAL4 DBD of the pGBKT7 vector. The resulting constructs and the negative vector control (pGBKT7) were transformed into Y187 yeast strain. After three days all of the transformants grew well on SD/–Trp/medium, but only the yeast cells containing pGBKT7-*GhNAC18* and pGBKT7-*GhNAC18*-C plasmid grew and turned blue on SD/–Trp/X-α-Gal/ medium (Figure 2) These results indicated that *GhNAC18*, has trans-activation activity in the C-terminus region.

Tissue-specific expression of *GhNAC18*

To investigate how *GhNAC18* is expressed in cotton, various tissues were harvested from the field at specific period of cotton development (Figure 3). *GhNAC18* was constitutively expressed in all parts investigated except 10 days post anthesis (DPA) fiber and pistil. Strong expression was observed in cotyledon leaves and young leaves. Moderate expression was exhibited in the stem and stamen. Low expression level was detected in the roots, sepal, petal, ovule and senescing leaves (Figure 3). The significant abundance in both cotyledon and young leaf suggested that *GhNAC18* may play an important role in leaf development.

Expression of *GhNAC18* during leaf senescence

With high expression level of *GhNA18* in the young leaves and cotyledon leaves, we decided to further investigate its role in these tissues during natural and dark-induced leaf senescence. For leaf senescence in cotyledons, cotyledon leaves were collected weekly after germination for eight weeks. By qRT-PCR analysis, *GhNAC18* was gradually expressed from week 1 up to week 4 after which there was a steady decline in expression level. The decline in expression level corresponded to the onset of senescence due to aging of the cotyledon leaves (Figure 4a).This indicated that *GhNAC18* is down regulated as senescence is initiated. Morphologically, some cotyledon leaves started to yellow

Figure 2. Transactivation activity of *GhNAC18.* Three constructs: pGBKT7:*GhNAC18*-F, pGBKT7:*GhNAC18*-N and GBKT7-C.) and GBKT7 vector (negative control) were transformed into the yeast strain Y187 plated and incubated for three days. All yeast cells containing the plasmids grew well into white in SD/-Trip medium, but only yeast cell containing pGBKT7:*GhNAC18*-F and pGBKT7:*GhNAC18*-C turned blue in SD/-Trp/-α-Gal medium.

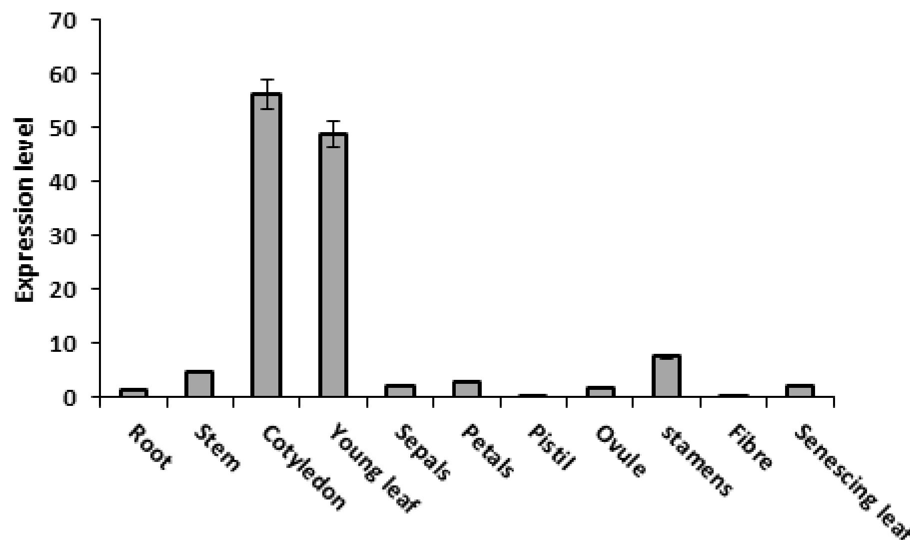


Figure 3. Expression profiling of *GhNAC18*. Tissue-specific expression level of GhNAC18 in cotton (*Gossypium hirsutum* L. CCRI-10).

at 8th week, an indication of senescence (Weaver et al., 1998). Progression of natural senescence on mature leaves was marked by the severity of yellowing in the leaf (Figure 4c).The stages of leaf senescence start from NS leaf to CS leaf, where *NS* is non-senescent leaf, *IS* is initial senescent leaf (15% yellow) *ES* is early senescent leaf (30% yellow), *LS* is late senescent leaf (50% yellow) and *CS* is complete senescent leaf (90% yellow) (Shah et al., 2013). By qRT-PCR analysis, *GhNAC18* expression level decreased from NS to CS (Figure 4b). To verify that senescence took place in these leaves, the expression level of positive and negative marker genes for senescence, *GhNAP* and *GhCAB* respectively for senescence were used on the same leaves as shown in Figure 4b. Chlorophyll content, a measure of senescence, was also measured in these five senescence stages (Figure 4d). For dark-induced senescence, *GhNAC18* was down regulated (Figure 4e). Contrary to early reports

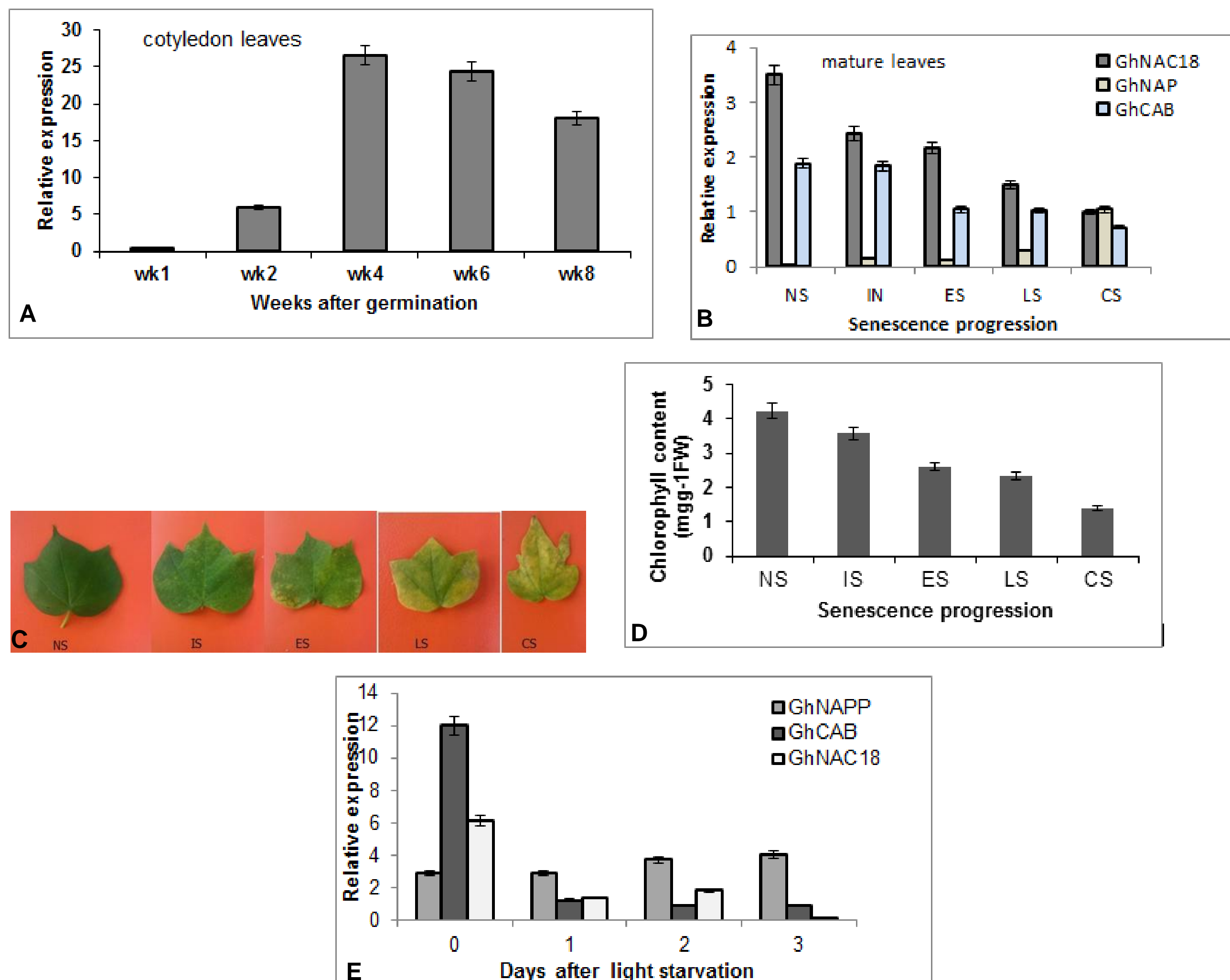


Figure 4. Progression of natural senescence and dark-induce senescence. **A**). Expression level of *GhNAC18* during cotyledon leaves senescence. **B**). Relative expression level of *GhNAC18 GhNAP* and *GhCAB* during progression of natural senescence in mature leaves. **C**) Morphological changes from *NS* leaf to *CS* leaf, where *NS* non-senescent leaf, IS initial senescent leaf (15%yellow) *ES* early senescent leaf(30%yellow), *LS* later senescent leaf (50%yellow) ,and *CS* complete senescent leaf(90%yellow). **D**) Chlorophyll content decrease from *NS* leaf to *CS* leaf. **E**) Relative expression level of *GhNAC18 GhNAP* and *GhCAB* during dark-induced leaf senescence. (*GhNAP* was used as appositive marker gene and *GhCAB* as negative marker gene for senescence during 3days of incubation). Error bars indicate SD.

on senescence associated genes (SAGs), *GhNAC18* was apparently inhibited by dark-induced senescence. Previous studies have generally indicated that *GhNAC*s are involved in leaf senescence, however, this studies show that *GhNAC18* might be required at the onset of senescence (IS-ES), but not on progression of this process.

Effects of phytohormones on *GhNAC18* expression

Plant hormones are implicated in complex signaling pathways and play crucial roles in regulating plant responses to a variety of environmental stresses and developmental processes (Bari and Jones, 2009). In the present study, the effect of phytohormones on *GhNAC18* expression were investigated. ABA, ET, JA and SA solutions were sprayed on cotton leaves and *GhNAC18* transcript levels measured by qRT-PCR. The application of MeJA and ET induced the expression level of *GhNAC18* reaching its maximum at 24 h (Figure 5). SA as a signaling molecule plays a significant task in plant defence and generally involved in the activation of defense responses against biotrophic and hemi-biotrophic pathogens (Lamb and Grant, 2006). Further, SA level accumulates in the pathogen infected tissues of

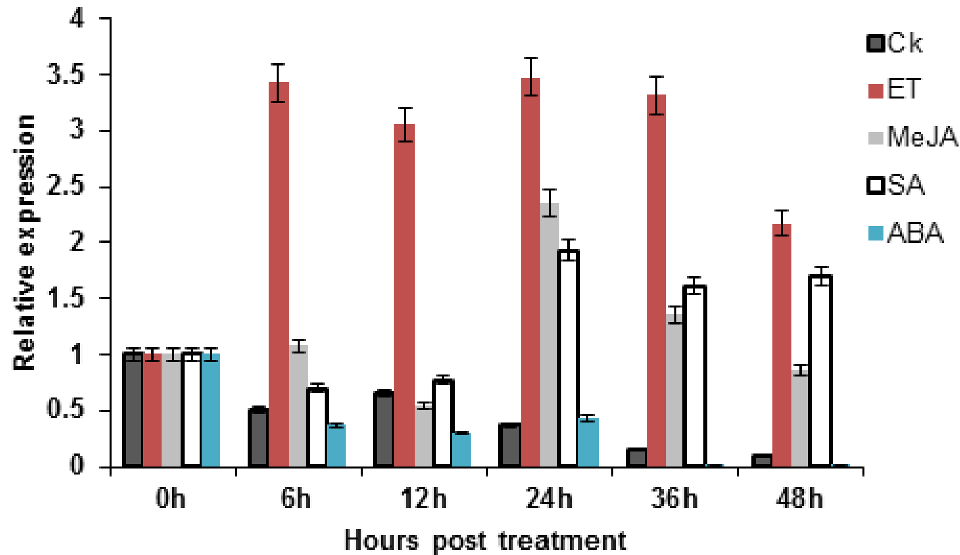


Figure 5. Expression patterns of *GhNAC18* in response to chemical stress during 48h of treatment. Uniformly grown two-week old cotton seedlings were treated with 2mM SA, 100μM ABA, 100μM MeJA, and 100μM ET. Total RNA was isolated at indicated times after treatment for qPCR analysis. *GhActin* was used as standard control in the experiments. Error bars indicate Standard deviations.

plants and exogenous application results to expression of pathogen related genes enhancing tolerance to infections (Bari and Jones, 2009). In this study, application of SA induced the expression of *GhNAC18* suggesting that *GhNAC18* could be involved in early detection of biotic stress triggering stress response network (Figure 5). ABA regulates many aspects of plant growth and development such as leaf senescence, seed germination, embryo maturation, stomatal aperture and adaption to environmental stress. Earlier studies have shown that ABA induces senescence in plants (Becker et al., 1993; Oh et al., 1996). In the present experiment, it was demonstrated that treatment of cotton seedlings with ABA lowered the expression of *GhNAC18* (Figure 5) indicating that *GhNAC18* is antagonistically regulated by ABA pathways that lead to senescence. Overall, activation of GhNAC18 by these phytohormones, indicated that GhNAC18 could be involved in abiotic and biotic responses through signaling pathways.

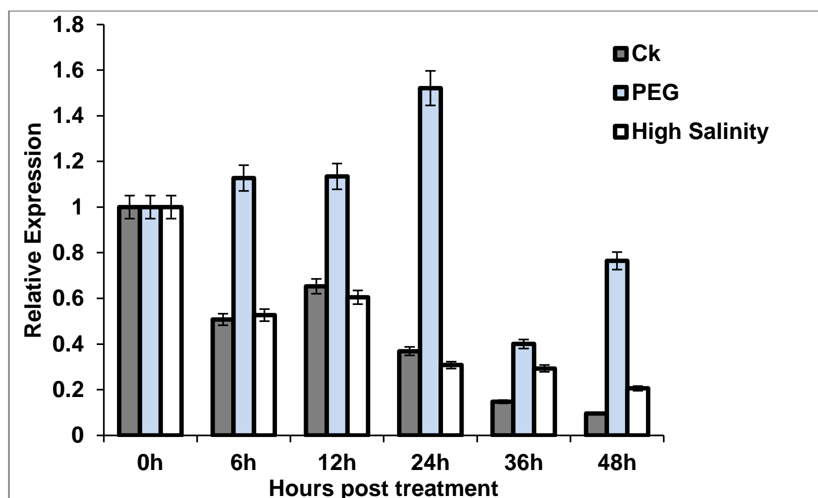
Expression of *GhNAC18* under abiotic stress

To further examine the expression pattern of *GhNAC18* in cotton under various stress conditions, cotton seedlings were subjected to drought (PEG6000), salt (NaCl), cold (4°C), H_2O_2 and wounding. In the analysis, *GhNAC18* was induced by all these treatments, however salt and cold treatments did not have a significant change on its expression (Figure 6a-b). There was steady increase of *GhNAC18* transcripts levels when seedlings were wounded, peaking at 8 h (Figure 6b). *GhNAC18* was sensitive to H_2O_2 treatment at 2 h (Figure 6b) followed by decline in transcript level. Drought treatment upregulated the expression level of *GhNAC18* (Figure 6a). Based on these results, *GhNAC18* might be involved in regulation of abiotic responses in cotton.

DISCUSSION

NAC proteins form the largest transcription factors in plants (Ooka et al., 2003). Their role in regulating plant responses to stresses and plant development cannot be underestimated. To date numerous *NAC* family genes have been isolated and characterized revealing a wide range of functions they play in plant species. This family of genes share common NAC domain (Ooka et al., 2003; Nakashima et ., 2007; Ma et al., 2010; Christiansen et al., 2011; Saad et al., 2013). Like other known NACs, *GhNAC18* has a conserved NAC domain at N-terminal region which can be divided into five subdomains namely A-E (Figure 1a and c). By bioinformatics analysis, *GhNAC18* was located in the nucleus while their transcriptional activation activity was located in the C-terminal region (Figure 2). The results taken together indicated that *GhNAC18* is a nuclear protein with a NAC domain and may function as transcription activator in cotton.

Although, the functions of NAC genes have been analyzed in various plants, not much information is available on the specific function of *GhNACs* in cotton (*G.*



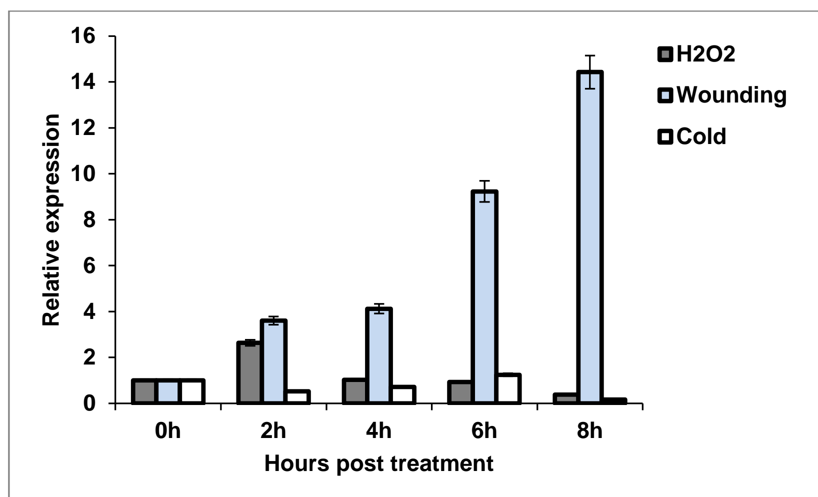


Figure 6. Expression pattern of GhNAC18 under abiotic stress. **A**. Drought stress with15% PEG 6000, Salinity stress with 200 mM NaCl and control (Ck). **B**. Hydrogen peroxide (20mMH2O2), Wounding and cold (4°C) treatments.

hirsutum L) (Meng et al., 2009; Nuruzzaman et al., 2013; Xu et al., 2014). In cotton, two *NAC* genes (*GhNAC12* and *GhNAP*) have been fully functionally characterized, however, many more are yet to be reported (Zhao et al., 2015). In the current study, *GhNAC18* was isolated and characterized during senescence and under stress conditions. During natural and dark-induced senescence, *GhNAC18* was down regulated (Figure 4). Its expression level markedly decreased as the leaf matured and as senescence sets in, suggesting that senescence inhibits the expression of this gene. It has been reported that most SAGs are induced by darkness however, *GhNAC18* was apparently downregulated by absence of light while *GhNAP*, a positive regulator for senescence was upregulated implying that *GhANAC18* could act as a negative regulator for this process. This was also confirmed by comparing it with another known negative regulator gene for senescence (*GhCAB*) (Figure 4e).

Many NACs have been reported to be expressed in specific tissues of plants. For example, *ANAC036* gene is highly expressed in rosette leaves and slightly expressed in seedlings and inflorescence. It is believed to function in leaf cell development due to its abundance but its overexpression caused semi-dwarfism in arabidopsis (Kato et al., 2010). The expression of *GhNAC18* was found to have varied expression levels in different tissues. There was high expression on the young and cotyledon leaves than other parts (Figure 3). This implies

that *GhNAC18* may have a significant role in the young leaves. Although, *NAC*s have been reported to be involved in early and late senescence in upland cotton (Kong et al., 2013), *GhNAC18* seemed to have a different trend during aging process because, its transcript abudance decreases as aging advances. Premature leaf senescence causes poor or low yield in cotton in early maturing cultivars (Wright, 1998). During this process, many SAGs are upregulated with possible regulatory roles. On the contrary, *GhNAC18* was found to be downregulated during leaf senescence, however low expression was observed on the onset of senescence implying that it may not be involved in progression of senescence.

GhNAC18 shared high identity with *TaNAC67* as indicated by phylogenetic analysis. *TaNAC67* is reported to improve abiotic stress tolerances and enhances high chlorophyll content retention in wheat (Mao et al., 2014). Because proteins which align together may have similar functions, there is a possibility that *GhNAC18* may have the same functions like *TaNAC67* in cotton leaves. Promoter analysis also revealed presence of light responsive elements which function in the leaf to enhance photosynthesis, probably a reason why *GhNAC18* was downregulated in the absence of light. These results taken together indicate that *GhNAC18* is non-aging gene that could be important in delaying senescence and increasing the life span of cotton.

Plant defense mechanism triggers molecular, biochemical and morphological changes such as oxidative burst, expression of defence-related genes, production of antimicrobial compounds, and/or damage-limiting mechanisms (Collinge and Bollar, 2001;Van Loon et al., 2006) boosting adaptability. In the current study *GhNAC18* was induced by SA, ET and MeJA. These signaling molecules are reported to be involved in regulating plant defense responses against various biotic and abiotic stresses (Glazebrook, 2001; McGrath et al., 2005). Upon treatment of cotton seedlings with ET and MeJA, transcript level of *GhNAC18* significantly accumulated indicating its sensetivity to responding to these stresses (Oh et al., 2005) therefore it could be involved in cotton defense response, possibly through the ET/MeJA-dependent signal transduction pathway. *OsNAC19* and *CarNAC1* which belong to NAC family are involved in stress tolerance at the same time induced by exogenous application of ET, MeJA and ABA (Lin et al., 2007). The induction of *GhNAC18* by SA and ET further confirm its involvement in biotic stress responses (Figure 5) since both SA and ET are important signaling mediators in biotic stress pathways (Fujita et al., 2004; Peng et al., 2009; Xia et al., 2010). Moreover, transcripts of *GhNAC18* showed significant increase under H_2O_2 treatments (Figure 6b), suggesting that there exists across-talk between abiotic stress and signal transduction pathways. Interestingly, *GhNAC18* was rapidly and transiently induced by wounding (Figure 6b), indicating its potential as an early regulator in the biotic stress response (Tran et al., 2004; Hao et al., 2011). Additionally it has been reported that NAC transcription factors can regulate drought stress response through both ABA-dependent and ABA-independent pathways (Fujita et al., 2004). In this study, the expression of *GhNAC18* is induced by dehydration, but not by ABA (Figure 5), suggesting that this protein may be associated with drought response in an ABA-independent manner.

The functions of some transcription factors are generally involved in plant development and stress responses at one time. For example, the Arabidopsis *ATAF1* gene was induced by wounding, pathogen attack, drought and ABA (Boller, 2001; Jensen et al., 2007; Lu et al., 2007). *AtNAC2,* as a transcription factor downstream of ET and auxin signaling pathways, was simultaneously involved in salt stress (Cao et al., 2005). Five *GhNACs* (*GhNAC2-GhNAC6*) genes were upregulated by drought, cold and salt (Meng et al., 2009). Reactive oxygen species, especially H_2O_2 are important signal transduction molecules, mediating the acquisition of tolerance to various stress. H_2O_2 induced the expression of *RD26* gene which regulates genes involved in defense and senescence (Fujita et al., 2004). This results have shown that *GhNAC18* is responsive to not only plant developmental processes, such as leaf senescence, but also to exogenous stimuli, such as drought and wounding (Figure 6), indicating that the *GhNAC18* transcription factor may be a common regulator of the molecular mechanisms of special plant development and stress responses.

In conclusion, this result suggests that *GhNAC18*, as a transcription activator, is possibly involved in developmental processes and stress responses in cotton. We are currently investigating what effect *GhNAC18* will have on transgenic plants and how *GhNAC18* is integrated into special phytohormone signaling pathway.

Conflict of interest

The authors declare that they have no conflict of interest.

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Abbreviations

SA, Salicylic acid; **MeJA,** methyl Jasmonate; **ET,** ethylene; **ABA,** abscisic acid; **DNA,** deoxyribonucleic acid; **cDNA,** complementary DNA; **PCR,** polymerase chain reaction; **qRT-PCR,** quantitative reverse

transcription PCR; **CTAB,** cetyl trimethylammonium bromide; **NJ,** neighbor joining; **ME,** minimal evolution; **MP,** maximum parsimony; **NS,** non-senescent leaf; **IS,** initial senescent leaf; **ES,** early senescent leaf; **LS,** late senescent leaf; **CS,** complete senescent leaf; **TFs,** transcription factors; **NAC,** a family of transcription factors comprising NAM, ATAF1,2 and CUC; **DREB,** dehydration-responsive element-binding protein; **ERF,** ethylene-responsive factor; ***GhNAC18,* NAC gene of *Gossypium hirsutum* L.**

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