A novel NAP member GhNAP is involved in leaf senescence in *Gossypium hirsutum*

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Abstract

Premature leaf senescence has a negative influence on the yield and quality of cotton, and several genes have been found to regulate leaf senescence. However, many underlying transcription factors are yet to be identified. In this study, a NAP-like transcription factor (GhNAP) was isolated from *Gossypium hirsutum*. GhNAP has the typical NAC structure and a conserved novel subdomain in its divergent transcription activation region (TAR). GhNAP was demonstrated to be a nuclear protein, and it showed transcriptional activation activity in yeast. Furthermore, the expression of GhNAP was closely associated with leaf senescence. GhNAP could rescue the delayed-senescence phenotype of the *atnap* null mutant. Overexpression of GhNAP could cause precocious senescence in *Arabidopsis*. However, down-regulation of GhNAP delayed leaf senescence in cotton, and affected cotton yield and its fibre quality. Moreover, the expression of GhNAP can be induced by abscisic acid (ABA), and the delayed leaf senescence phenotype in GhNAPi plants might be caused by the decreased ABA level and reduced expression level of ABA-responsive genes. All of the results suggested that GhNAP could regulate the leaf senescence via the ABA-mediated pathways and was further related to the yield and quality in cotton.

Key words: Abscisic acid, GhNAP, *Gossypium hirsutum*, leaf senescence, NAP subfamily, transcription factor.

Introduction

Leaf senescence is an accumulative series of physiological and molecular changes that disrupt cellular metabolism (Thomas and Stoddart, 1980; Lim et al., 2007). During leaf senescence, degradation of macromolecules occurs and finally results in its shift from a functionally photosynthetic organ to an actively degenerating and nutrient-recycling tissue (Gan, 2007). Like many other developmental processes, leaf senescence can be regulated by many transcription factors (Guo et al., 2004; Kong et al., 2013). Recently it has been reported that numerous transcription factors, including NAC, WRKY, bZIP, MYB, AP2/EREBP, and C2H2 type zinc finger, are related to leaf senescence (Buchanan Wollaston et al., 2005; Guo and Gan, 2012). In particular, the NAP subfamily (NAC-like, activated by APETALA 3/PISTILLATA) has been genetically and physiologically proved to be an important activator of leaf senescence (Guo and Gan, 2006; Liang et al., 2014).

The NAP members are one of the largest subfamilies of plant-specific NAC (NAM, ATAF1, 2, and CUC2) transcription factors (Duval et al., 2002; Olsen et al., 2005). In the earliest research, the *NAP* gene was found to participate in cell division and expansion of stamens and petals (Sablowski and Meyerowitz, 1998). The NAP subfamily has a highly conserved N-terminal domain (NAC domain) (Aida et al.,...
1997; Ooka et al., 2003) and a highly divergent C-terminal domain in the transcription activation region (TAR) (Fujita et al., 2004; Tran et al., 2004). The NAP subfamily is very important in various biological functions, such as floral development (Sablowski and Meyerowitz, 1998), root morphogenesis (de Zélicourt et al., 2012), seed development (Meng et al., 2007), and stress responses, including salt (Chen et al., 2014), drought (Meng et al., 2009), and H$_2$O$_2$ stress (Peng et al., 2009). In addition, the NAP subfamily is also associated with leaf senescence in Arabidopsis thaliana (Guo and Gan, 2006), Oryza sativa (Li et al., 2014), Crocus sativus (Kalivas et al., 2010), Bambusa emeiensis (Chen et al., 2011), and Triticum aestivum (Uauy et al., 2006). AtNAP was closely linked to the senescence process of A. thaliana rosette leaves, and its corresponding T-DNA insertion knockout lines showed an obvious delay in leaf senescence. In contrast, inducing overexpression of AtNAP in the young leaf led to advanced senescence (Guo and Gan, 2006). The NAP subfamily also improves crop yield and quality through regulating leaf senescence. Reduced OsNAP expression can delay leaf senescence and increase grain yield in rice (Li et al., 2014). Furthermore, NAM-B1, an orthologue of AtNAP, was associated with leaf senescence and grain quality in wheat (Uauy et al., 2006).

Cotton is considered as the most important economic crop in the world. However, premature leaf senescence of cotton has occurred frequently in many cotton-growing countries, and it has become one of the main factors restricting cotton yield and quality (Dong et al., 2006). Leaf senescence is mainly determined by the genetic programme in cotton, but its key regulation mechanisms and corresponding transcription factors remain largely unclear. As is known, the NAP subfamily is related to leaf senescence, but there are limited reports about the NAP-like transcription factors in cotton (Meng et al., 2009; Zhang et al., 2011; Huang et al., 2013; Shang et al., 2013). Although some GhNAPs have been isolated from senescing leaf in cotton, it is still unknown whether or not a NAP-like transcription factor in cotton acts as an important factor for triggering cotton leaf senescence (Kong et al., 2013; Shah et al., 2013; Shah et al., 2014). It is also still unknown how a NAP transcription factor plays a crucial part in leaf senescence in cotton.

To explore the possible regulation pathways for extending the green period in cotton, a novel NAP-like transcription factor, GhNAP, was investigated in upland cotton (Gossypium hirsutum L.) which may be related to leaf senescence. Due to its rapid responses to leaf senescence signals, GhNAP can be identified as an ideal positive senescence marker in cotton. GhNAP could rescue the delayed-senescence phenotype of the atnap null mutant, and overexpression of GhNAP could readily cause precocious senescence in Arabidopsis. On the other hand, reduction of GhNAP expression delayed cotton senescence. Furthermore, GhNAP can mediate abscisic acid (ABA) pathways by regulating several ABA-responsive genes, and the ABA-mediated pathways of GhNAP in senescence may differ from that of AtNAP. In addition, cotton yield and its fibre quality could improve with a reduction of the transcript level of the GhNAP gene in cotton.

**Materials and methods**

**Plant materials and growth conditions**

*Arabidopsis thaliana* seeds of Col-0, atnap null mutants (SALK_005010), and all transgenic lines were sown on Petri dishes containing Murashige and Skoog (MS) salts with 0.7% (w/v) phytoagar. After vernalization at 4 °C for 2 d, the dishes were moved to a growth chamber at 22 °C with 60% relative humidity. After 12 d of germination, seedlings were transplanted to pots containing a peat soil:vermiculite:perlite mixture (3:9:0.5, v/v/v). For dark treatment, the fifth leaves were excised and incubated on wet Petri dishes.

*Gossypium hirsutum* L. cv. Zheda B was used in this research. To induce expression of GhNAP, 100 μM ABA was applied to 1-month-old wild-type seedlings for 12 h. For dark-induced senescence, seedlings were put in a similar environment but in total darkness. GhNAP homozygous lines, together with the wild-type cotton, were also grown in the experimental field of Zhejiang University. A completely randomized block design was employed. Thereafter, the fourth leaf from the apex was used to measure yellowing at the designated times. The cotton bolls were gathered from the above-measured lines in the harvest season to investigate their yield and quality. Fibre quality traits were examined in the Supervision, Inspection and Test Center of Cotton Quality. Leaves from the GhNAPs and wild-type cotton were detached from 1-month-old plants and placed on wet Petri dishes in continuous darkness.

**Isolation, subcellular localization, and transcriptional activation analysis of GhNAP**

*Gossypium hirsutum* expressed sequence tags (ESTs) were downloaded from the NCBI. Through searching and alignment with AtNAP, a unigene was selected to clone the GhNAP gene (Pinheiro et al., 2009; de Oliveira et al., 2011). For subcellular localization, the full-length GhNAP without the stop codon was amplified, and then inserted into the pCHF3-GFP (green fluorescent protein) vector. Then the pCHF3-GFP and pCHF3-GFP-GhNAP vectors were introduced into *Agrobacterium tumefaciens* strain LBA4404, and were transiently expressed in transgenic Nicotiana benthamiana plants expressing red fluorescent protein (RFP)–H2B. The transformed leaves were observed by confocal microscopy. For transcriptional activation analysis, the full-length GhNAP, GhNAP-N (amino acids 1–162), and GhNAP-C (amino acids 163–286) were fused in pGBKTT7 to construct pGBKTT7-GhNAP, pGBKTT7-GhNAP-N, and pGBKTT7-GhNAP-C, respectively. Yeast strains (Clontech) were transformed with the three resulting constructs and the negative control pGBKTT7. The transformants were evaluated on SD–Trp and SD–Trp/X-a-Gal/AbA media.

**Evolutionary analysis**

For phyllogenetic analysis of GhNAP, the amino acid sequences of the NAP subfamily already reported in plants were collected from GenBank. All of the NAP members in this study were aligned using the ClustalX program. Then, MrBayes version 3.1.2 was used to conduct Bayesian analysis (Huelsenbeck and Ronquist, 2001). The conserved motifs among the NAP subfamily were also investigated by the online MEME program (Bailey et al., 2006). The parameters were set as follows: optimum motif width was set to ≥6 and ≤200; maximum number of motifs was set to 15 (Zhao et al., 2011). Sequence logos of the conserved NAC domain and a novel subdomain were generated through the WebLogo program (Crooks et al., 2004).

**Physiological measurement and transcript analysis**

Chlorophyll, the SPAD value, and membrane ion leakage were measured as previously described (Feibo et al., 1998; Woo et al., 2001). The photosynthetic capacity was determined using an LI-6400 Portable Photosynthesis System (Hua et al., 2009) and pulse-modulated...
chlorophyll fluorometer (Chen et al., 2005). The peroxidase (POD) and superoxide dismutase (SOD) activities, and soluble protein and malondialdehyde (MDA) contents were measured using reported methods (Wang et al., 2011). Additionally, the extraction and determination of the endogenous ABA level in the 1-month-old wild-type and GhNAPi cotton was performed as described (Lanoue et al., 2010).

Total RNA was extracted using an RNAprep pure Plant Kit (TIANDZ, China) for cotton and RNAiso Plus (TakaRa) for Arabidopsis. The first-strand cDNA was synthesized from DNase-treated RNA with a PrimerScript 1st Strand cDNA synthesis kit (TakaRa). Quantitative real-time PCR (qRT-PCR) was performed with SYBR premix Extaq (TakaRa) and the CFX96 Realtime System (BioRad). The primers used for qRT-PCR are listed in Supplementary Table S1 available at JXB online. Forty cycles of qRT-PCR were conducted with an annealing temperature of 60 °C. The relative expression levels were calculated by the $2^{-ΔΔCt}$ method assuming 100% primer efficiency (Schmittgen and Livak, 2008). Three biological replications were performed in all reactions.

Plasmid construction and plant transformation

For the complementation test, the promoter region of AtNAP was amplified from Col-0, and subcloned into the pCHF3 vector to replace the 35S promoter. Then the full-length GhNAP was inserted into the ProAtNAP_pCHF3 vector to construct ProAtNAP_pCHF3_GhNAP. For overexpression of GhNAP, the open reading frame (ORF) of GhNAP was amplified and inserted into pCHF3. Primers used for constructing different vectors are given in Supplementary Table S2 at JXB online. In addition, the pCIGhNAPi interference vector was generated by cloning the GhNAP coding region into the RNA interference (RNAi) expression vector pCI. Then the above three vectors were transferred into A. tumefaciens strain LBA4404, which was used to transform A. thaliana via the floral dip method (Clough and Bent, 1998). Putative transgenic plants were selected on MS medium containing 50 mg l$^{-1}$ kanamycin. The antibiotic-resistant T$_1$ transgenic lines were selected, and were further verified by PCR and RT-PCR; phenotypic analyses were performed in the T$_1$ generation and were further confirmed in the T$_2$ generation. Furthermore, G. hirsutum L. cv. Zheda B was transformed with the pCI-GhNAPi vector according to the method of Luo and Wu (1988). The plants were first screened by 500mg l$^{-1}$ kanamycin. Then the positive plants were further confirmed by PCR and RT-PCR. Selected transgenic plants were analysed by Southern blotting using the DIG High Prime DNA Labeling and Detection Strater Kit I (Roche). Homozygous plants were used in all experiments.

Isolation of GhNAP and GhSAG113 promoters

Total genomic DNA was extracted from cotton leaf. The Genome Walking Kit (TakaRa) was used to clone the promoter region of GhNAP and GhSAG113. The gene-specific primers were designed based on the known sequences (Supplementary Table S3 at JXB online), and the specific PCR products were cloned and sequenced. The GhNAP promoter sequence was then searched in the PLACE database to investigate the putative cis-elements.

Yeast one-hybrid assay

The yeast one-hybrid (Y1H) assay was performed using the Matchmaker® Gold Yeast One-Hybrid Library Screening System (Clontech) according to the manufacturer’s instructions. The promoter of GhSAG113 and the ORF of GhNAP were ligated into pAbAi and pGADT7 vectors, respectively, to generate the pAbAi-GhSAG113 and pGADT7-GhNAP vectors, respectively. The pAbAi-GhSAG113 plasmid was linearized and transformed into the Y1H Gold strain. Positive yeast cells were selected on SD/-Ura medium, and then transformed with pGADT7-GhNAP. The transformsants were obtained on SD/-Leu medium, and were evaluated on SD/-Leu/Ab A plates.

Statistical analysis

Data were subjected to PROC GLM using the SAS version 8.0 statistical software designed by the SAS Institute (Cary, NC, USA). The significant differences among different groups were evaluated by least significant difference (LSD) multiple range tests ($P<0.05$).

Results

Isolation and molecular characterization of GhNAP in G. hirsutum

AtNAP was used as the query to search G. hirsutum ESTs. Through comprehensive analysis with AtNAP, a unigene (EV490808, DR455718, DN800623, and CA992692) was selected and tentatively called GhNAP. GhNAP contained the whole ORF, so a pair of PCR primers was designed to clone the GhNAP sequence. Sequencing results showed that GhNAP contained the 861 bp ORF encoding 286 amino acids (Fig. 1A).

To understand the evolutionary history of GhNAP, a phylogenetic tree including some reported NAP proteins and GhNAP was constructed. Through evolutionary analysis, it was found that the NAP subfamily could be further divided into two groups (NAPI and NAPII) (Fig. 1B). The NAPI group contained 11 transcription factors, while the NAPII group had 12 NAP members. Interestingly, both GhNAP and AtNAP belonged to the NAPI group. Furthermore, the motif distribution was consistent with the classification of the phylogenetic analysis (Fig. 1C). Further analysis of the conserved domain revealed that GhNAP shared a high percentage identity (91%) with AtNAP. Therefore, it can be revealed that GhNAP may be a homologue of AtNAP.

Sequence alignment among NAP members revealed that the NAC domain of GhNAP was highly conserved and could be further divided into five typical subdomains. In addition, a novel subdomain in its TAR was relatively conserved within the NAPI group (Supplementary Fig. S1 at JXB online). It was therefore tentatively called subdomain NAPI. Furthermore, the DNA-binding domain (DBD domain) in subdomain C and the nuclear localization signal (NLS) in subdomain D were identified in GhNAP and highly conserved with other NAP members.

Subcellular localization of GhNAP

To determine the subcellular localization of GhNAP, the GhNAP-GFP fusion vector was transiently expressed in RFP–H2B transgenic N. benthamiana. The green fluorescent signal from GhNAP–GFP expression was observed exclusively in the nucleus, which was confirmed with the red fluorescent signal from RFP–H2B, a fusion protein often used to visualize the chromosomal architecture in cells (Fig. 1D). However, the free GFP signal was distributed throughout the cell.
Transcriptional activation activity of GhNAP

To examine whether GhNAP has transcriptional activation activity, the N- and C-terminal fragments as well as the full-length GhNAP were fused to the GAL4 DBD of the pGBK7 vector. The resulting constructs and the negative vector control pGBK7 were transformed into a yeast strain. All of the transformants grew well on SD/–Trp medium, while only the cells containing the pGBK7-GhNAP and pGBK7-GhNAP-C plasmid could grow and simultaneously turned blue on SD/–Trp/X–α-Gal/AbA medium (Fig. 1E).

Expression profile of GhNAP during leaf senescence in cotton

The expression of GhNAP was examined in young leaves (YL), non-senescent leaves (NS), early senescent leaves (ES), and late senescent leaves (LS) (Fig. 2A). Consistent with the leaf...
phenotype, the chlorophyll loss and membrane ion leakage of ES and LS were higher than those of YL and NS (Fig. 2B, C). Moreover, compared with YL and NL, the expression of GhNAP was higher in ES and LS, whereas GhCAB, a negative senescence marker gene, was expressed at a lower level in the same leaves (Fig. 2D, E). During the senescence process (Fig. 2F), more GhNAP expression was detected in the yellow tip (T) than in other parts (Fig. 2G). The present findings were further strengthened by the opposite expression level of GhCAB and GhNAP in the same leaf (Supplementary Fig. S2A, B at JXB online). These results were in line with the decrease in the expression of GhCAB (Supplementary Fig. S2C) and the increase in GhNAP expression (Supplementary Fig. S2D).

**Complementation of Arabidopsis atnap null mutants with GhNAP**

Previous reports have shown that the atnap null mutant can delay leaf senescence (Guo and Gan, 2006). To test whether GhNAP is a functional homologue of AtNAP, the GhNAP ORF, driven by the promoter region of AtNAP, was transfected into the atnap null mutant (Supplementary Fig. S3 at JXB online). The leaves from the GhNAP-complemented lines (GhNAP_RE) senesced in a similar pattern to the leaves from Col-0, but senesced much more quickly than the leaves from...
atnap, both phenotypically (Fig. 3A, B) and in terms of chlorophyll content (Fig. 3C), membrane ion leakage (Fig. 3D), and relative expression of AtSAG12 (Fig. 3E) and AtCAB (Fig. 3F). After dark treatment for 5 d, detached leaves of GhNAP_RE exhibited wild-type-like yellowing (Supplementary Fig. S4).

Ectopic expression of GhNAP in Arabidopsis

The GhNAP coding sequence, driven by the 35S promoter, was introduced into the wild-type Col-0 to overexpress GhNAP (Supplementary Fig. S3 at JXB online). After 20 d growth, the GhNAP overexpressors displayed the senescence phenotype, while the no yellowing phenotype was found in Col-0. After 30 d, the detached leaves were divided into three groups (G1–G3) (Fig. 4A, B). Compared with Col-0, the GhNAP lines showed much lower chlorophyll contents in the G1 and G2 leaves (Fig. 4C), while the GhNAP lines had much higher membrane ion leakage (Fig. 4D). The relative expression level of AtNAP, AtSAG12, and AtCAB also reflected the similar senescing process (Fig. 4E–G). Furthermore, compared with the Col-0 leaves, the GhNAP leaves showed the precocious senescence phenotype after 3 d in darkness (Supplementary Fig. S5).

Delayed-senescence phenotype through down-regulation of GhNAP in cotton

To confirm GhNAP's function further, the GhNAPi transgenic lines were selected through transformation of the pCI-GhNAPi vector (Supplementary Fig. S7 at JXB online). Then

![Image](4674_Fig.3.jpg)
the GhNAPi and corresponding wild-type lines were grown in the field. The GhNAPi lines showed delayed senescence especially during the later stage of growth (Fig. 6).

As the striking phenotypic change, leaf yellowing was measured to investigate the leaf senescence every 15 d from 60 days after planting (DAP) to 150 DAP across the main growth period of cotton. At 120 DAP, leaves of GhNAPi had a much higher chlorophyll content and SPAD value than those of the wild type (Fig. 7A, B). The net photosynthetic rate ($P_n$) and the $F_{v}/F_{m}$ ratio increased at first and gradually declined after 105 DAP, but at 120 DAP the decline of $P_n$ and the $F_{v}/F_{m}$ ratio was significantly slower in GhNAPi than in the wild type (Fig. 7C, E, F). However, the intercellular CO$_2$ concentration ($C_i$) showed the opposite effects (Fig. 7D). Moreover, GhNAP expression was significantly lower at 120 DAP in GhNAPi than in the wild type (Fig. 7G), but GhCAB showed the opposite tendency (Fig. 7H). The MDA content, soluble protein content, SOD activity, and POD activity showed a significant difference at 120 DAP between the wild type and GhNAPi (Supplementary Fig. S8 at JXB online). In addition, the detached leaves were induced by being kept in the dark for 3 d (Supplementary Fig. S9A, B). Higher chlorophyll content and lower membrane ion leakage were measured in GhNAPi than in the wild type (Supplementary Fig. S9C, D). The relative expression level of GhNAP and GhCAB showed similar results (Supplementary Fig. S9E, F).

Effect on cotton yield and quality through reduced expression of GhNAP

The yield and quality of the GhNAPi line were evaluated in the field. There was no significant statistical difference in agronomic traits (plant height, fruit branches, and nodes) and some yield components (boll numbers, seed cotton yield, and boll weight)
However, the lint yield of GhNAPi (32.59 g plant \(^{-1}\)) was higher than that of the wild type (28.19 g plant \(^{-1}\)). The percentage lint was 38.08\% for the wild type, while it is 41.79\% for the GhNAPi cotton. Compared with the wild-type cotton, the lint yield and percentage of GhNAPi were increased by >15\% and 9\%, respectively. Furthermore, fibre quality, especially fibre length, was significantly different between the GhNAPi and wild-type lines (Table 2). Specifically, the GhNAPi cotton produced fibre with significantly shorter length (27.03 mm) than wild-type cotton (29.10 mm). Compared with the wild type, the fibre length of GhNAPi was reduced by >7\%.

Relationship between GhNAP and ABA responses

To elucidate the possible regulatory mechanism, some putative cis-elements were identified in the promoter region of GhNAP (Fig. 8A). Surprisingly, not only the common cis-element (CAAT-box), but also several specific cis-elements related to the ABA responses (ABRE, and recognition sites for MYB and MYC) existed in the promoter region of GhNAP. In addition, qRT-PCR analysis showed that the expression pattern of GhNAP was highly increased after ABA treatment (Fig. 8B). To explore further the function of GhNAP in the ABA responses, the endogenous ABA level was measured in GhNAPi and wild-type leaves. The results showed that the ABA content was significantly lower in GhNAPi than in wild-type leaves (Fig. 8C). Furthermore, the expression levels of ABA-related genes were analysed in GhNAPi and wild-type lines. Compared with the wild-type lines, GhSAG113, GhMYC2, and GhMYB2 exhibited remarkably decreased expression levels in the GhNAPi plants (Fig. 8D–F). In particular, the expression level of GhSAG113 was reduced >70\%
GhNAP in leaf senescence

However, the other four genes did not show a significant difference between GhNAPi and wild-type plants (Supplementary Fig. S10 at JXB online). Then, the Y1H assay was performed to investigate the interaction between GhNAP and GhSAG113 (Fig. 8D). The promoter regions of GhSAG113 (1000 bp) were isolated and introduced into the genome of the Y1H Gold strain as the bait reporter strain. Subsequently, the pGADT7-GhNAP vector was transformed into the bait reporter strain. The transformants exhibited normal growth on SD/-Leu medium, but could not grow on SD/-Leu/AbA medium.

Discussion

GhNAP is a novel NAP member in Gossypium hirsutum

The NAP subfamily are plant-specific transcriptional factors, and are related to plant growth and development, stress responses, and leaf senescence (Sablowski and Meyerowitz, 1998; Guo and Gan, 2006; Uauy et al., 2006). Numerous NAPs have been isolated from different plant species (Meng et al., 2009; Chen et al., 2011; Fan et al., 2014). However, as an important cash crop, cotton lacks systematic research on the NAP subfamily. Thus, studies were focused on cotton in order to find a novel NAP member in G. hirsutum which can regulate leaf senescence.

In the current study, a novel transcription factor GhNAP was successfully identified in G. hirsutum (Fig. 1A). Consistent with other NAP proteins (Meng et al., 2007; Kalivas et al., 2010), GhNAP contained a conserved NAC domain and a divergent TAR (Fig. 1A; Supplementary Fig. S1 at JXB online). Furthermore, the NAC domain can be further divided into five highly conserved subdomains named A–E. The NAC domain, in which WKATGXD was located, mainly acted in DNA binding, while the TAR may determine its specific functions (Ooka et al., 2003). The putative NLS in subdomain D suggested its nuclear localization. Similar to other NAPs (Guo and Gan, 2006; Chen et al., 2014), transient expression in tobacco confirmed that GhNAP was a nuclear protein (Fig. 1D). In addition, transcriptional activation analysis demonstrated that GhNAP and its C-terminus had transcriptional activation activity in yeast (Fig. 1E). These results indicated that GhNAP may function as a transcription factor in cotton. Through the phylogenetic analysis of the NAP subfamily in the plant kingdom, it was shown that the NAP subfamily might consist of two groups, namely NAPI and NAPII (Fig. 1B, C). GhNAP belonged to the NAPI group, and clustered with AtNAP. A complementation test indicated that GhNAP could restore the Arabidopsis atnap null mutant phenotype to the normal wild-type phenotype (Fig. 3). These results collectively indicated that GhNAP is a homologue of AtNAP in cotton. Moreover, GhNAP and other NAP members had a relatively conserved domain in the divergent TAR. The specific subdomain called subdomain NAPI may be specific for NAPI members, indicating certain functions shared by the NAPI group.
Table 1. Comparison of yield components between wild-type and GhNAPi lines

<table>
<thead>
<tr>
<th>Cotton type</th>
<th>Plant height (cm)</th>
<th>Fruit branches (no. per plant)</th>
<th>Fruit nodes (no. per plant)</th>
<th>No. of bolls per plant</th>
<th>Seed cotton yield (g plant⁻¹)</th>
<th>Lint yield (g plant⁻¹)</th>
<th>Boll weight (g)</th>
<th>Percentage lint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>115.08 ± 11.92 a</td>
<td>16.79 ± 1.00 a</td>
<td>53.79 ± 6.69 a</td>
<td>18.00 ± 1.00 a</td>
<td>74.07 ± 3.42 a</td>
<td>28.19 ± 0.90 b</td>
<td>4.12 ± 0.06 a</td>
<td>38.08 ± 0.65 b</td>
</tr>
<tr>
<td>GhNAPi</td>
<td>107.75 ± 7.51 a</td>
<td>17.13 ± 1.19 a</td>
<td>63.46 ± 0.97 a</td>
<td>19.67 ± 1.53 a</td>
<td>78.00 ± 2.36 a</td>
<td>32.59 ± 0.81 a</td>
<td>3.98 ± 0.20 a</td>
<td>41.79 ± 0.24 a</td>
</tr>
</tbody>
</table>

Values in each column followed by different letters are significantly different at P<0.05.
GhNAP is a senescence regulator during leaf development

The NAP subfamily act as transcription factors to regulate leaf senescence in many plants (Guo and Gan, 2006; Chen et al., 2011; Liang et al., 2014). In this study, GhNAP played an important part in leaf senescence in cotton. First, the transcriptional analysis of GhNAP expression from leaves of different ages showed that GhNAP was highly expressed not only in the yellow leaf region, but also in the yellowing part (Fig. 2). Previous studies had reported that the NAC family had a similar expression pattern between natural and dark-induced senescence (Lin and Wu, 2004; Buchanan Wollaston et al., 2005; Fukao et al., 2012). Therefore, leaf senescence was induced by darkness in this study. With the extension of the amount of time in the dark period, leaf senescence gradually became more severe and the expression level of GhNAP increased correspondingly (Supplementary Fig. S2 at JXB online). This result indicated that GhNAP was abundantly expressed under dark-incubated leaf senescence.

GhNAP is a senescence regulator during leaf development

Table 2. Comparison of quality traits between wild-type and GhNAPi lines

<table>
<thead>
<tr>
<th>Cotton type</th>
<th>Fibre length (mm)</th>
<th>Uniformity (%)</th>
<th>Micronaire value</th>
<th>Elongation rate (%)</th>
<th>Strength (cN/tex)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>29.10 ± 0.08 a</td>
<td>85.97 ± 0.80 a</td>
<td>5.13 ± 0.09 b</td>
<td>6.40 ± 0.00 b</td>
<td>28.30 ± 0.35 a</td>
</tr>
<tr>
<td>GhNAPi</td>
<td>27.03 ± 0.50 b</td>
<td>83.67 ± 0.67 a</td>
<td>5.33 ± 0.12 a</td>
<td>6.60 ± 0.00 a</td>
<td>28.43 ± 0.35 a</td>
</tr>
</tbody>
</table>

Values in each column followed by different letters are significantly different at P<0.05.

Fig. 8. Relationship between GhNAP and ABA pathways. (A) Distribution of cis-elements in the promoter region of GhNAP. The main cis-elements are indicated as follows: filled circles, ABRE; filled inverted triangles, MYC recognition site; open triangles, MYB recognition site; open diamonds, CAAT. (B) Effects of ABA on GhNAP expression in cotton. (C) Endogenous levels of ABA in WT and GhNAPi lines. (D–G) Expression of ABA-related genes in wild-type and GhNAPi plants. (G) Interaction between GhNAP and the promoter of GhSAG113 by Y1H assay. (This figure is available in colour at JXB online.)
null mutant to a normal wild-type phenotype during natural or dark-induced leaf senescence (Fig. 3; Supplementary Fig. S4 at JXB online). Overexpression of GhNAP in Arabidopsis caused precocious leaf senescence, and plants exhibited an increase in chlorophyll loss, membrane ion leakage, and the expression level of AtSAG12 and AtNAP, with a decline in the expression level of AtCAB (Fig. 4). After 3 d in darkness, yellowing was much more serious in GhNAP-overexpressing than in wild-type lines (Supplementary Fig. S5 at JXB online). In addition, the GhNAPi cassette (GhNAP interference vector) was ectopically transformed in A. thaliana. The leaf senescence of the GhNAPi transgenic mutant was intermediate between that of the wild type and the atnap null mutant (Fig. 5). The GhNAPi line also showed delayed leaf senescence under a dark environment (Supplementary Fig. S6).

Through the interference with GhNAP expression in cotton, the GhNAPi transgenic cotton showed an obvious delay in leaf senescence especially at the later stage of growth (Fig. 6). The physiological parameters also reflected the similar trend of leaf senescence in GhNAPi lines. In particular, at 120 DAP, the GhNAPi leaves contained a higher chlorophyll content and photosynthetic properties than the corresponding leaves of the wild type, indicating that the reduced expression of GhNAP extended the leaf functional period (Fig. 7A–F; Supplementary Fig. S8 at JXB online). Furthermore, GhNAP was expressed at a much lower level at 120 DAP in GhNAPi than in wild-type plants (Fig. 7G), whereas GhNAPi showed a higher transcript profile of GhCAB (Fig. 7H). Moreover, GhNAPi lines also showed a delay in dark-induced leaf senescence (Fig. S9).

Due to its rapid responses to leaf senescence signals and the sustained high expression patterns during natural and dark-induced leaf senescence, GhNAP can be regarded as an ideal marker to demonstrate leaf senescence in cotton. Taken together, these results suggested that GhNAP played crucial roles in leaf senescence in cotton under both a natural and a dark environment.

GhNAP influences yield and quality in cotton through regulation of leaf senescence

Although leaf senescence is an evolutionarily selected developmental process (Buchanan Wollaston et al., 2003), it may negatively affect crop yield and quality by limiting the growth phase (Lim et al., 2007). Due to the delayed-senescence phenotype in GhNAPi, the lint yield significantly increased, which led further to a percentage gain in lint (Table 1). However, the fibre length declined sharply in the GhNAPi line (Table 2). The decline of fibre length was probably associated with the inhibition of cell elongation. As was previously reported (Sablowski and Meyerowitz, 1998), partial loss of GhNAP function perhaps inhibited cell elongation, and subsequently affected the fibre length. One probable explanation may be that a pulse of GhNAP expression is required for cell expansion. Furthermore, some MYB transcription factors were related to cell expansion in cotton (Walford et al., 2011), and the promoter region of GhNAP contained at least three recognition sites for MYB (Fig. 8A). Thus, these results further confirmed the explanation that a definite level of NAP expression may be necessary for proper cell elongation. In addition, the higher lint yield and lint percentage in this study may be related to the thickened second cell wall in GhNAPi fibre. In previous reports, many MYB members have been shown to regulate secondary cell wall biosynthesis and deposition (Zhong et al., 2007; Sun et al., 2015). Therefore GhNAP might regulate secondary cell wall biosynthesis and deposition, which has been confirmed in other NAC members (Zhong et al., 2006). Furthermore, more fibre cells in GhNAPi probably resulted in the higher lint yield and lint percentage. The endogenous ABA level was significantly lower in GhNAPi than in wild-type lines (Fig. 8C), and ABA is inhibitory to fibre development (Kim and Triplett, 2001). Hence, GhNAPi lines might have more fibre cells through ABA regulation. However, further investigations are still needed to prove these hypotheses.

**GhNAP regulates different ABA pathways during leaf senescence**

ABA is one of the plant hormones that can promote leaf senescence (Zhang, 2014). In this study, GhNAP could be highly induced under ABA treatment (Fig. 8B). Furthermore, various cis-regulatory elements, including ABRE, and recognition sites for MYB and MYC, were identified in the promoter region of GhNAP (Fig. 8A). These cis-elements and their respective transcription factors are very important in ABA responses (Abe et al., 1997). These results suggested a close relationship between GhNAP and ABA pathways in leaf senescence. In addition, the interference with GhNAP expression in cotton led to delayed senescence under a natural or dark environment (Figs 6, 7; Supplementary Figs S8, S9 at JXB online), which was related to the reduction of endogenous ABA content and the decreased expression levels of some ABA-related genes (Fig. 8C–F). All of these results indicated that the delayed yellowing in natural or dark-induced senescence in the GhNAPi transgenic lines might result from the reduced endogenous ABA levels. Hence, GhNAP may regulate leaf senescence through ABA-mediated pathways.

In Arabidopsis, the direct target of AtNAP was reported to be SAG113 (Zhang and Gan, 2012). The unique ABA–AtNAP–SAG113 regulatory chain can control ABA-regulated stomatal movement and water loss specifically during leaf senescence. In this study, qRT-PCR results showed that the expression level of GhSAG113 (the homologue of SAG113) was reduced >70% in the GhNAPi lines (Fig. 8D). In addition, GhNAP is the homolog of AtNAP. Therefore, the interaction between GhNAP and GhSAG113 was investigated (Fig. 8G). The Y1H assay showed that GhNAP did not bind directly to the promoters of GhSAG113, indicating that GhNAP interaction proteins may bind to the promoters of GhSAG113. Thus, the regulation model between GhNAP and ABA in cotton differs from the ABA–AtNAP–SAG113 regulatory chain in Arabidopsis during leaf senescence.

Overall, the results showed that a novel NAP member (GhNAP) in cotton was closely associated with leaf senescence.
senescence via ABA-mediated pathways. Down-regulation of GhNAP in cotton could delay leaf senescence, and affected the yield and fibre quality. The findings presented here open up a new avenue for researchers to investigate further the structure and function of the NAP subfamily in plants, and provided a candidate gene for plant breeding.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. The conserved domain of GhNAP protein.

Figure S2. Physiological and expression analysis of GhNAP in cotton leaves during extended darkness.

Figure S3. Molecular analysis of the GhNAP_RE, GhNAP, and GhNAPi transgenic lines in Arabidopsis thaliana.

Figure S4. Analysis of GhNAP-complemented lines dark treated for 5 d.

Figure S5. Effects of dark treatment for 3 d on detached leaves of GhNAP overexpressors.

Figure S6. Physiological and expression patterns of detached leaves of GhNAPi, atnap, and Col-0 lines under a dark environment for 5 d.

Figure S7. Phenotype and molecular analysis of the GhNAPi transgenic line in cotton.

Figure S8. Changes in content of MDA and soluble protein, and SOD and POD activity of the cotton leaf at the designed times in wild-type and GhNAPi lines.

Figure S9. Effects of dark treatment for 3 d on leaf discs of the wild-type and GhNAPi lines.

Figure S10. Expression of ABA-related genes in wild-type and GhNAPi lines.

Table S1. Primers used for expression analysis by qRT-PCR.

Table S2. Primers used for constructing different vectors.

Table S3. Primers used for isolating the corresponding sequence.

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References


