Xu et al. Journal of Cotton Research (2024) 7:27
https://doi.org/10.1186/s42397-024-00188-9

Journal of Cotton Research

REVIEW

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Roles of NAC transcription factors in cotton

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Abstract

Climate deterioration, water shortages, and abiotic stress are the main threats worldwide that seriously affect cotton growth, yield, and fiber quality. Therefore, research on improving cotton yield and tolerance to biotic and abiotic stresses is of great importance. The NAC proteins are crucial and plant-specific transcription factors (TFs) that are involved in cotton growth, development, and stress responses. The comprehensive utilization of cotton NAC TFs in the improvement of cotton varieties through novel biotechnological methods is feasible. Based on cotton genomic data, genome-wide identification and analyses have revealed potential functions of cotton NAC genes. Here, we comprehensively summarize the recent progress in understanding cotton NAC TFs roles in regulating responses to drought, salt, and Verticillium wilt-related stresses, as well as leaf senescence and the development of fibers, xylem, and glands. The detailed regulatory network of NAC proteins in cotton is also elucidated. Cotton NAC TFs directly bind to the promoters of genes associated with ABA biosynthesis and secondary cell-wall formation, participate in several biological processes by interacting with related proteins, and regulate the expression of downstream genes. Studies have shown that the overexpression of NAC TF genes in cotton and other model plants improve their drought or salt tolerance. This review elucidates the latest findings on the functions and regulation of cotton NAC proteins, broadens our understanding of cotton NAC TFs, and lays a fundamental foundation for further molecular breeding research in cotton.

Keywords Cotton, NAC transcription factor, Stress, Regulatory network

Background

Cotton, as an important economic crop and strategic resource, is cultivated around the world. Cotton production is closely related to national economies and individual daily lives, and it is considered as the leading commercial crop in many countries, such as China, USA, India, Pakistan, and Brazil (Billah et al., 2021). Cotton fiber is the major natural raw material for textiles, cottonseed and cotton stalk are also important agricultural product. Due to the wide uses, cotton is in great demand in various industries. China is a large cotton consumer and producer, and many areas in China, especially Xinjiang, are suitable for cotton growth. Along with global climate change, the growth and production of cotton in China are significantly affected by water deficit, soil salinization, high temperatures, and other extreme environmental conditions (Ibrahim et al., 2019). Additionally, high-quality fiber is in seriously shortage, and most of the fiber with high strength and length are imported. Thus, improving fiber yield, fiber quality and stress tolerance are the main goals of cotton production in China. Breeding excellent cotton varieties with synergistically improving multiple traits is the key solution to addressing the above mentioned issues in cotton production in China. Traditional cotton breeding mainly relies on breeders' experience and is both time and resource consuming (Kushanov et al., 2021). At present, by taking advantage of modern biotechnology, including molecular maker-assisted breeding, transgenic

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technology, and genome editing, the process and precision of the breeding have been remarkablely accelerated. The expanded knowledges of gene functions and regulatory mechanisms will facilitate the future integration of traditional cotton breeding and modern molecular-designed breeding.

Transcription factor (TF) genes are important constituents in plant genomes and represent 7% of the coding areas of plant transcriptomes (Singh et al., 2021). There are more than 80 tgroups of TFs have been identified in plants, which directly bind to the *cis*-elements in promoters or interact with other proteins to temporarily and spatially regulate the target gene expression. NAC, MYB, bZIP, and WRKY families are widely existed in cotton species and have been well-studied. Among them, NAC TFs, a large family, control growth, development, morphogenesis, stress tolerances, and fiber formation in cotton. This review summaries the latest findings regarding the functions and regulatory mechanisms of NAC TFs.

NAC transcription factors

The NAC TFs are plant-specific, and widely exists in plant species. The NAC domain was named from three previously identified genes, *No Apical Meristem* (*NAM), Arabidopsis Transcription Activation Factor (ATAF) and Cup-Shaped Cotyledon (CUC)* (Aida et al., 1997; Sablowski and Meyerowitz, 1998; Souer et al., 1996). The first NAC TF was identified in embryo of Petunia. With the explosive increase of plant genomic data, more NAC members were identified in other plants. There have been 105 NAC TFs members predicted in *Arabidopsis thaliana*, 75 in *Oryza sativa*, 488 in *Triticum aestivum*, 148 in *Zea mays*, 139 in *Glycine max*, and 283 in *Gossypium hirsutum* (Sun et al., 2018a; Ooka et al., 2003; Guerin et al., 2019; Peng et al., 2015; Hussain et al., 2017). Despite the diverse number of NAC members among different plant species, the structure of the NAC protein domains are evolutionarily conserved. A classic NAC protein is characterized by the highly homologous DNA-binding domain at the N-terminal and the highly variable transcriptional regulatory region at the C-terminal (Puranik et al., 2012). The crystal structure of the NAC domains in the ANAC019 and ANAC092 in Arabidopsis and SNAC1 in rice revealed by X-ray crystallography is consisted of a central semi-β-barrel formed by seven twisted anti-parallel β-strands surrounded by three α-helices on one side and the open side, respectively (Chen et al., 2011; Chun et al., 2023; Ernst et al., 2004). The NAC domain located at the N-terminal, which contains approximately 150–160 amino acids, is divided into five subdomains, named after A to E (Ooka et al., 2003). Subdomain A may be involved in the formation of functional homodimers or heterodimers. Subdomains C and D, as DNA-binding sites, carry the positive charge and are evolutionarily conserved. Arginine (Arg) 85 of ANAC019, Arg91 of ANAC, and Arg88 of SNAC1 in subdomain C are crucial for longer sequence recognition and DNA interactions. The subdomain D contains nuclear localization signals, which has been identified by lysine residues. Subdomains B and E are probably related to various function of NAC proteins. The C-terminal of NAC proteins are highly divergent among species. As it is a transcriptional regulatory region, C-terminal regulates the transcription of downstream genes. Several C-terminal of NAC proteins contain transmembrane motifs, which might localize the proteins to the plasma membrane or endoplasmic reticulum (Seo et al., 2010b). A handful of NAC members designated as NTM1-Like have been known as membrane-bound dormant forms. Under certain environmental or developmental induction, they are triggered and regulated by a intramembrane proteolysis mechanism (Hoppe et al., 2001; Kim et al., 2006). And the activated NTM1-Likes can enter the nucleus and in turn elicit the expression of target genes (Seo et al., 2010a).

The introduction of cotton NAC transcription factors

The genome sequence data of *Gossypium* have provided an opportunity for the genome-wide investigation of the NAC gene family. In recent years, comprehensive and systematic genome-wide analyses of the NAC TFs family have been performed. Cheng et al. (2021) has identified 150, 153, and 299 *NAC* genes from *Gossypium arboreum* (Institute of Cotton Research version), *Gossypium raimondii* (United States Department of Energy Joint Genomics Institute version) and *Gossypium hirsutum* (Zhejiang University version), respectively, using HMM (Hidden Markov model) and blastp searches. The 602 NAC TF members have been divided into eight groups (Cheng et al., 2021). Bai et al. (2022) has performed a genome-wide survey of the NAC TFs family and identified 271 *NAC* genes using the reference genome of *G. hirsutum* (Nanjing Agricultural University version). Based on the sequence similarity, *GhNAC* members have been classified into 12 groups (Bai et al., 2022). Sun et al. (2018a) has identified non-redundant and complete NAC genes in four cotton genomes, including 147 in *G. arboreum*, 149 in *G. raimondii*, 267 in *G. barbadense*, and 282 in *G. hirsutum*, which have been clustered in seven subfamilies. These identified NAC family members are not completely the same, owing mainly to the utilization of different version of reference genomes, or different selection methods and parameters.

Full-length GhNAC proteins range from approximately 168 to 1219 amino acids. The molecular weights and isoelectric points range from 20.1 kDa to 135.6 kDa, and

4.39–9.80, respectively. The gene structure of the majority of NAC family members includes three exons and two introns. Among them, the lengths of the first two exons are relatively conserved, whereas the third exon is highly diverse. The distribution of *NAC* members on chromosomes have been examined, and the result suggests that all the chromosomes of the A and D sub-genomes possess more than one *NAC* gene. However, the distribution of *NAC* members is unbalanced across the chromosomes in cotton. Chromosome A05, A09, A11, D05, and D11 contain more *GhNAC* genes than other chromosomes, whereas chromosome D10 contains less *GhNAC* genes. The majority of *GhNAC* members cluster within a short distance at the start or the end of chromosomes. Less *GhNACs* are located in the central regions of chromosomes especially the centromere and pericentromere regions (Bai et al., 2022). Expansion mechanism analyses of *GhNAC* members have indicated that both the segmental and tandem duplications occur during *GhNAC* gene expansion. However, the segmental duplication has been found more often than the tandem duplication, making segmental duplication the dominant duplication pathway. Most of the non-synonymous (d_N) to synonymous (d_S) substitution ratios of *NAC* genes within and between cotton species are less than 1, indicating that purifying selection has been the main selective pressure in their evolution. The single nucleotide polymorphism (SNP) density analysis in different regions of *GhNAC* genes, including coding, upstream, and downstream regions, has shown that SNP density of wild cotton is higher than that of cultivated cotton, which perhaps due to the purifying selection and domestication (Sun et al., 2018a).

Additionally, in the promoter regions of *NAC* genes, there are various *cis*-elements including light-responsive and development-associated (GT1-motifs and circadian), defense- and stress response-associated (stress responsive element, heat stress-response element, TC-rich repeats, and G-boxes), plant hormone response-associated [abscisic acid (ABA)- and ethylene-responsive element]. The *cis*-element compositions of *GhNAC* promoter regions and expression patterns of *GhNACs* indicate that NAC domain proteins as regulators of the transcription play a vital role in the whole cotton growth and development process, and in abiotic and biotic stress responses.

Functions of Cotton NAC TFs

Drought stress

Water deficit severely hinders the growth and yield of cotton. Due to the global warming, the earth is more parched and hotter, and the water deficit impact on cotton is likely to be exacerbated in the future (Gupta et al., 2020). As central regulators of functional gene expression, TFs play a key role in plant responses to drought stress. Various NAC TFs have been identified in cotton responses to drought stress (Fig. 1).

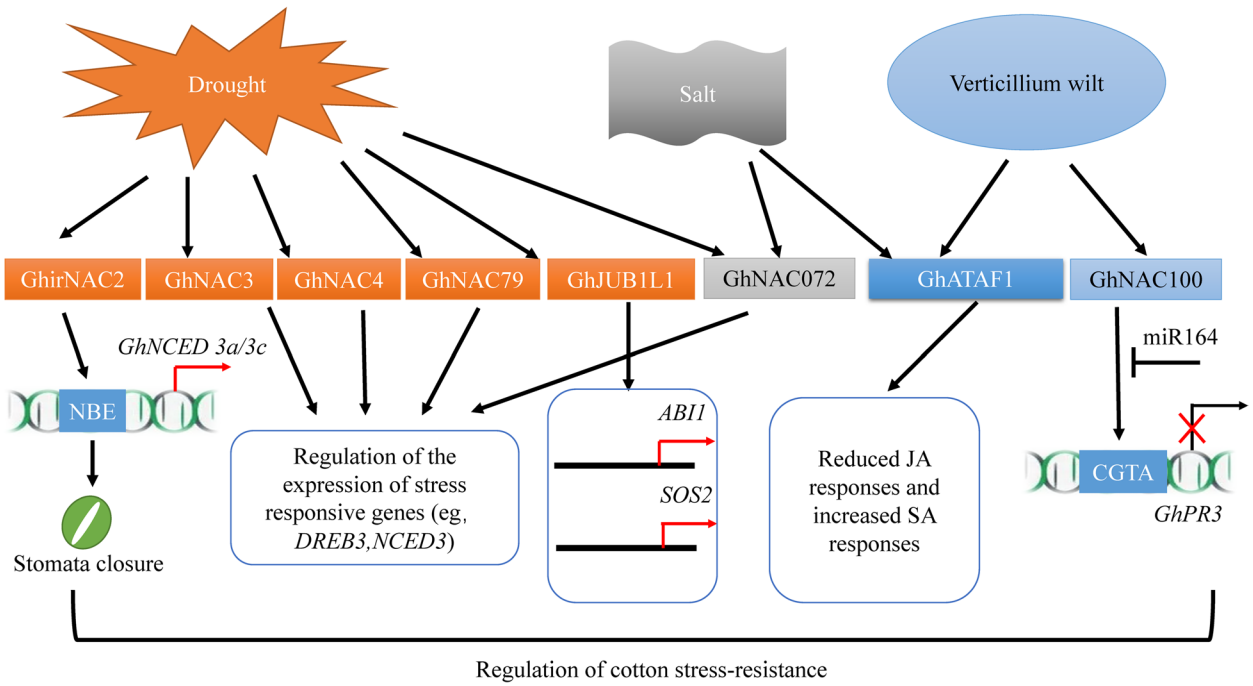

Fig. 1 The role of NAC transcription factors in cotton response to abiotic and biotic stresses

Shang et al. (2020) have isolated *GhNAC2* from Jinmian 19, which is a drought-tolerance germplasm line, and has investigated its expression pattern. The expression level of *GhNAC2* has been significantly induced at 8 h after a 15% PEG treatment in leaves compared with normal watering treatment, and the up-regulated expression level has been maintained for at least 24 h. Co-suppression cotton lines of *GhNAC2* have shown reduced drought tolerance, with higher water-loss rates, reduced stomatal closure levels, lower ABA contents, higher malondialdehyde (MDA) contents, lower catalase activities, lower photosynthetic rate, lower stomatal conductance, and lower transpiration rates under water deficit conditions. GhNAC2 binds to the promoters of *GhNCED3a-At/Dt* and *GhNCED3c-At/Dt* in vitro based on the electrophoretic mobility shift assay (EMSA), and activates the expression of *GhNCED3a-At/Dt* and *GhNCED3c-At/Dt* in vivo as determined by transient expression analyses of *β-Glucuronidase* (*GUS*) and *Luciferase* (*LUC*) (Shang et al., 2020). Overexpression of *GhNAC2* in tobacco has improved drought tolerance and resulted in the increase of seed germination rate, better root growth, and a higher survival rate under drought-stress. *GhNAC2*, as a positive regulator, improves cotton drought tolerance by modulating the expression level of *GhNCED 3a/3c* and stomatal closure under drought-stress conditions (Shang et al., 2020). Similarly, *GheNAC2* from *G. herbaceum* also plays an essential role in response to drought stress. Overexpression of *GheNAC2* increases root length and growth in both transgenic *Arabidopsis* and cotton under normal and stress conditions. GheNAC2 activates the ABA/jasmonic acid (JA) pathway and suppresses the ethylene pathway to help the plants for drought stress through the regulation of stomatal closure, decreasing transpiration, and inhibiting growth-retarding factors (Gunapati et al., 2016).

Xia et al. (2023) have indicated that *GhNAC3* plays a key role in cotton's response to abiotic stress. *GhNAC3* is expressed in major tissues, especially in root, and is rapidly up-regulated under drought treatment. The ectopic expression of *GhNAC3* in *Arabidopsis* has resulted in a much higher seed germination rate and longer root lengths compared with wildtype controls on the medium containing 500 mmol·L^{-1} mannitol. *GhNAC3* silenced cotton using virus induced gene silencing (VIGS) technology has shown significantly reduced resistance level to drought. In addition, the MDA and hydrogen peroxide contents in *GhNAC3* silenced plants are remarkably increased, whereas the water contents in leaves are decreased under drought stress (Xia et al., 2023). Chen et al., (2021) have cloned *GhJUB1L1* (Gh_D06G2096), a NAC TF, from Jimian14 and have found that the transcript level of *GhJUB1L1* is higher in stem and leaf. *GhJUB1L1* silenced cotton seedlings wilt and undergo yellow, with reduced relative water content in leaves, and greater ion leakage. This indicates that silencing *GhJUB1L1* results in reduced drought tolerance and retarded secondary cell wall (SCW) development in cotton. Silencing *GhJUB1L1* significantly down-regulated expression levels of drought-related and SCW synthesis-related genes, including *CBP1, P5CS, ABI1, SOS2, 4CL1, CCoAOMT1, IRX9*, and *IRX14*. Using the dual-luciferase assay, GhJUB1L1 has been verified to directly bind the promoters of *GhABI1, GhSOS2, GhCCoAOMT1, GhCesA7*, and *GhIRX14* genes and activate their expression (Chen et al., 2021).

GhNAC4 is also found to be induced by abiotic stresses, and its overexpression in tobacco remarkably increases tolerance to drought by up-regulating the expression levels of a series of stress-responsive genes and stomatal movement. The domains essential for the functions of GhNAC4 have been studied biochemically, in which both N and C terminals of GhNAC4 have been shown to involve in the abiotic stress tolerance, and the N-terminal is largely associated with ABA responsiveness (Trishla et al., 2021). *GhNAC072* (GH_D01G0514) has been selected through a weighted gene co-expression network analysis and RT-qPCR. *GhNAC072* silenced cotton shows increased water loss and ion leakage, reduced relative water, and chlorophyll contents in detached leaves than the wildtype controls during drought stress. The activities of catalase and superoxide dismutase in GhNAC072 silenced cotton seedlings decreases and the hydrogen peroxide and MDA contents increases under both regular and drought conditions. Thus, *GhNAC072* is part of the drought tolerance regulatory mechanism in cotton (Mehari et al., 2021). Guo et al. (2017) have isolated *GhNAC79* from cotton cultivar CCRI10 and verified that GhNAC79 is a candidate gene for drought-stress response in cotton. *GhNAC79* is highly expressed in cotyledon and fibers at 20–25 days post anthesis (DPA) in fiber development, while its expression is lower in the stem and in the initial stage of fiber development. Transgenic expressing *GhNAC79* in Arabidopsis shows enhanced drought tolerance, while *GhNAC79*-silenced cotton seedlings have a drought-sensitive phenotype (Guo et al., 2017). Together, NAC proteins play a positive role in cotton drought tolerance and have enormous potential for genetic improvement of drought tolerance in cotton (Table 1).

Salt stress

Although cotton is a moderately salt-tolerant plant, salt stress is a destructive environmental stress limiting cotton growth and fiber production worldwide (Ahmad et al., 2022). The two most abundant neutral salts causing stress in soil are sodium chloride (NaCl) and sodium sulfate (Na_2SO_4). Because of irrigation with water containing trace amounts of NaCl or seawater is used, salt is

Table 1 Function of NAC transcription factors in cotton

Genes	Source	Functions	Expression	Method	Ref
GhNAC2	Jinmian 19	Drought tolerance	Drought: up-regulated in leaf	Co-suppression, ectopic expression in tobacco	Shang et al., 2020
GheNAC2	Vagad	Drought tolerance	ABA, ethylene, salt stress, and drought stress: up-regulated in root.	Overexpression, ectopic expression in *Arabidopsis thaliana*	Gunapati et al., 2016
GhNAC3	Jin668	Drought tolerance	Drought and salt stress: up-regulated	VIGS, ectopic expression in *Arabidopsis thaliana*	Xia et al., 2023
GhJUB1L1	Jimian 14	Drought tolerance	Drought: up-regulated in stem	VIGS	Chen et al., 2021
GhNAC4	JK Durga	Drought and salt tolerance	ABA and abiotic stresses: up-regulated in leaf	Ectopic expression in tobacco	Trishla et al., 2021
GhNAC072	Marie-galante 85, Latifolium-40, CRI-12	Drought and salt tolerance	Drought and salt stress: up-regulated in leaf and root	VIGS, ectopic expression in *Arabidopsis thaliana*	Mehari et al., 2022; Mehari et al., 2021
GhNAC79	CCRI10	Drought tolerance	Drought and ethylene: up-regulated in leaf and root	VIGS, ectopic expression in *Arabidopsis thaliana*	Guo et al., 2017
GhATAF1	YZ1	Salt tolerance, Verticillium susceptibility	MeJA, SA and *Verticillium dahlia* treatment: up -regulated in root	Overexpression	He et al., 2016
GhNAC061		Salt tolerance	Salt stress: up-regulated in leaf		Sun et al., 2018a; Sun et al., 2018b
GhNAC100	Jihe713	Verticillium susceptibility	Verticillium dahlia treatment: up-regulated in root	Overexpression, knockdown	Hu et al., 2020
GhFSN5	Coker312	SCW biosynthesis		Ectopic expression in *Arabidopsis thaliana*	Sun et al., 2020
GhSND2s	Jimian 14	SCW biosynthesis		VIGS	Wang et al., 2021a; Wang et al., 2021b
GhXND1	Coker312	SCW biosynthesis		Ectopic expression in *Arabidopsis thaliana*	Li et al., 2014
GhFSN1	Coker312	SCW biosynthesis		Overexpression, RNAi	Zhang et al., 2018
GhNAC82	CCRI 10	Leaf senescence		Ectopic expression in *Arabidopsis thaliana*	Wang et al., 2022
GhNAP	Zheda B	Leaf senescence	ABA: up-regulated in leaf	RNAi, Ectopic expression in *Arabidopsis thaliana*	Fan et al., 2015
GhNAC12	CCRI10	Leaf senescence		Ectopic expression in A*rabidopsis thaliana*	Zhao et al., 2016
GbiNTL9	G. bickii	Gland development		VIGS	Sun et al., 2023
GhCGF2	Stoneville 7 A	Gland development		VIGS	Janga et al., 2019

accumulated in soils (Deinlein et al., 2014). Soil salinization leads to growth inhibition, developmental changes, metabolic disturbance in plants via destroying plant ion homeostasis and osmotic homeostasis (van Zelm et al., 2020). Multiple salt-stress signaling pathways including ionic and osmotic homeostasis signaling pathways, detoxification response pathways, and plant growth regulatory pathways establish the salt resistance (Zhu, 2002; Yang et al., 2018). *GhSOS1* and *GhNHX1* are associated with excretion of excessive Na^+ from the cytoplasm and restoration of the ionic homeostasis, and are up-regulated in cotton under salt stress (Guo et al., 2020). Additionally, various NAC TFs have a prominent role in responding to salt stress in cotton (Fig. 1).

Mehari et al. (2022) have transformed *GhNAC072* which is a hub gene in the co-expression network analysis in *Arabidopsis thaliana*, and has shown lower leaf water loss and ion leakage levels, but higher relative leaf water and chlorophyll contents in transgenic plants than the wildtype under salt stress. *GhNAC072* overexpression lines have enhanced germination rates and root lengths in the 200 $mmol{\cdot}L^{-1}$ NaCl treatment. And stress responsive genes, including *APF4*, *RAB18*, *RD22*, and *SOS1* are markedly up-regulated in the *GhNAC072* transgenic plants under salt stress in comparison to the wildtype. *GhATAF1*, a cotton NAC TF, is up-regulated by ABA and salt stress. The expression level of *GhATAF1* is higher in roots, leaves, and stems than in ovules and fibers. Overexpression

of *GhATAF1* in cotton shows a better salt tolerance in 0.2 mol·L^{-1} NaCl treatment than wildtype seedlings. The Na^+ content and Na^+/K^+ ratio are obviously reduced in transgenic cotton, although the K^+ content does not change under salt-stress conditions. Similarly, stress-related genes (*GhAVP1*, *GhRD20*, *GhRD22*, *GhDREB2A*, *GhLEA3*, and *GhLEA6*), ABA-responsive gene (*GhABI4*), and transporter gene (*GhHKT1*) are up-regulated in transgenic cotton compared with the wildtype after salt treatment (He et al., 2016). Sun et al. (2018b) have performed a genome-wide association study based on the salt-tolerance phenotype of a natural population at the seedling stage using the Illuminia Infinium Cotton SNP63K array and has identified two possible candidate genes for salt tolerance, including *Gh_D09G0943* and *Gh_D09G0950*, which are the orthologs of *NAC061* and *NAC089*, respectively. The expression level of *Gh_D09G0943* under salt stress conditions has been validated by RT-qPCR in five salt tolerant and salt sensitive varieties. *Gh_D09G0943* has a higher relative expression level in salt tolerant varieties than in salt sensitive varieties, indicating that this gene is associated with salt tolerance in cotton (Sun et al., 2018b).

Together, NAC genes are involved in the regulation of cotton responses to salt stress and are important for salt resistance improvements in cotton.

Verticillium wilt

Verticillium wilt caused by the fungus *Verticillium dahlia* is the most devastating soil-borne vascular disease, and leads to dramatic cotton yield losses worldwide (Shaban et al., 2018). Cotton Verticillium wilt is difficult to control and prevent mainly because the wide distribution and destructiveness of *V. dahlia,* and the shortage of cotton germplasm with high resistance toward *V. dahlia* (Zhang et al., 2020a). NAC TFs are closely involved in Verticillium wilt disease responses, and related studies were summarized below (Fig. 1).

Hu et al. (2020) have suggested that *GhNAC100* expression is differentially regulated by miR164 in cotton after inoculation with *V. dahlia* compared with mock treatment (Hu et al., 2020). *GhNAC100* silenced cotton shows fewer wilting leaves, less stunted growth, decreased fungal biomass, reduced disease index (DI) values in 21 days post infection (DPI) than mock treatment. *GhPR3* and *GhPDF1.2* expression levels in the *NAC100* transiently silenced plants are up-regulated compared with the control at 13 DPI. GhNAC100 represses *PR3* expression by binding to the CGTA-box element in the *PR3* promoter. Thus, GhNAC100 plays a negative role in plant resistance to *V. dahlia* (Hu et al., 2020). He et al. (2016) have indicated that *GhATAF1* overexpression decreases the tolerance to *V. dahlia* in cotton. Overexpression of *GhATAF1* in cotton results in more wilt and yellow leaves and higher DI values compared with the wildtype controls at 10 DPI. Additionally, the JA biosynthesis and response pathway-related genes including *GhLOX1*, *GhMYC2*, *GhPR3*, and *GhPR4*, are down-regulated in the *GhATAF1* overexpression cotton lines, whereas the *GhNDR1-1*, *GhNPR1*, *GhPR1*, and *GhPR5* involved in SA pathway, are up-regulated in comparison with that in the control. GhATAF1 is a negative regulator of the response to *V. dahlia* that acts through an antagonistic interaction between the JA-mediated and SA-mediated defense signals (He et al., 2016).

2020Fiber and xylem development

Cotton fibers are an important source for apparel, home furnishings, and durable paper money. Cotton fiber development is a complex process and can be divided into five distinct but partially overlapping stages, including initiation, primary cell wall formation (elongation), transition, SCW thickening (cellulose biosynthesis), dehydration and maturation (Haigler et al., 2012). NAC TFs involved in the SCW thickening have been extensively studied in model plants. NAC SECONDARY WALL THICKENING PROMOTING FACTOR 1 (NST1) and NST3 are key regulators of the formation of SCWs. And XYLEM NAC DOMAIN1 (XND1), as a NAC TF and NST-interacting protein, modulates NST1 activity and is a negative regulator of xylem SCW formation (Zhang et al., 2020b; Mitsuda et al., 2007). Ohashi-Ito et al. (2010) have indicated that VASULAR-RELATED NAC-DOMAIN6 (VND6) is involved in SCW formation by inducing many downstream genes, such as cellulose synthases (*CESA4, CESA7*, and *CESA8*), *MYB46*, and *MYB83*. Many studies have showed that NAC TFs are involved in SCW thickening in cotton fiber development (Fig. 2).

Sun et al. (2020) have shown that the cotton NAC domain TF GhFSN5 negatively regulates SCW biosynthesis in transgenic *Arabidopsis thaliana*. *GhFSN5* is highly expressed in 15 DPA fibers and hypocotyls, and it is co-expressed with multiple genes involved in fiber SCW thickening and regulators associated with SCW biosynthesis. The cell wall thicknesses of the xylem and interfascicular fibers in transgenic GhFSN5 Arabidopsis are significantly thinner than in controls, and the cell wall component contents including crystalline cellulose and lignin are reduced. And the relative expression levels of genes associated with cellulose synthase (*CESA4*, *CESA7*, and *CESA8*), xylan biosynthesis (*IRX9* and *IRX10*), lignin biosynthesis (*C4H*, *4CL1*, *CCoAOMT*, *CCR*, and *COMT*), and TF genes (*NST1*, *NST2*, *NST3*, *MYB46*, and *MYB83*), are down-regulated in *GhFSN5* overexpression lines (Sun et al., 2020). Shang et al. (2016) have suggested that over 80%

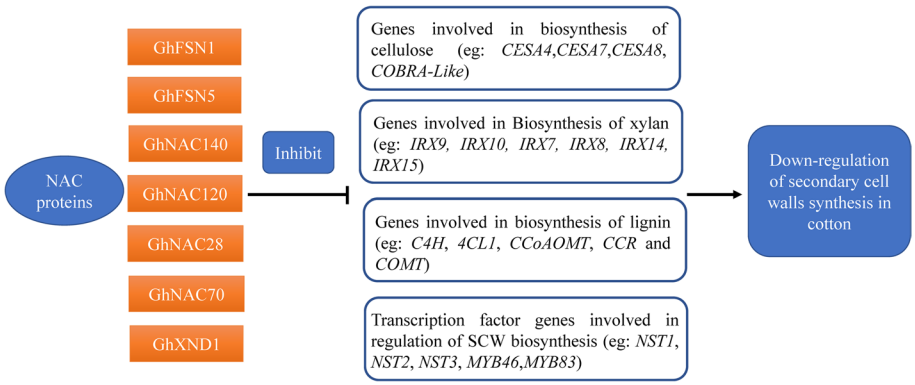

Fig. 2 The downstream target genes of cotton NAC transcription factors related to SCW formation

of the identified NAC TF genes from diploid cotton are expressed during fiber development, which indicates that the encoded TFs may play a key role during cotton fiber development.

A wide range of SCW-related NAC proteins including VND1, VND4, NST1, secondary wall associated NAC domain protein 2 (SND2), and their homologs, interact with DELLA proteins and mediate GA signaling involved in the SCW formation in cotton stems. *GhSND2S* silenced cotton reduces lignification and SCW development in stems. And the expression levels of genes associated with cellulose and hemicellulose biosynthesis and lignin polymerization are down-regulated, while the monolignol biosynthetic genes are not differencially expressed in *GhSND2S* silenced lines compared with the controls (Wang et al., 2021b). A dynamic transcriptome analysis on the chromosome 15 substitution line (SL15) and its recurrent parent LMY22 during fiber cell development has revealed that the key agronomic traits of SL15 are similar to those of LMY22. However, the lint percentage and single fiber weight of SL15 are significantly reduced. According to the transcriptome results, four NAC genes (*GH_A03*G1732, *GH_A04G1218*, *GH_D02G1891*, and *GH_D04G1551*) are highly expressed in SL15 at 25 DPA compared with in LMY22. *NAC83* is the only one of the five NST1 to have increased its expression level in SL15 at 25 DPA. *XND1* is significantly higher expressed in SL15 at 25 DPA, whereas *NAC74* expression is down-regulated compared with LMY22. Additionally, SCW-related MYB TF genes and cell wall biosynthesis genes are differentially expressed during fiber development in SL15 and LMY22. Thus, the different expression levels of SCW-related genes and the NAC_MYB_CESA network during fiber formation may result in the decreased lint percentage and wall thickness in SL15 (Gao et al., 2021). Li et al. (2014) have identified a gene encoding NAC TF and designated it as *GhXND1,* which is a negative regulator in plant xylem development. qRT-PCR results indicated that *GhXND1* is dominantly expressed in the vegetative tissues, such as cotyledons, petals, roots, stems, and hypocotyls, low expressed in leaves and anthers, but rarely expressed in ovules and fibers. Ectopic expression of *GhXND1* in *Arabidopsis thaliana* results in a significant decrease in the number of vessel cells in the stem and a thinner cell layer in the interfascicular fibers. The expression levels of genes related to SCW biosynthesis (*CESA4*, *CESA7*, *CESA8*, *IRX9*, *FRA8*, *F5H*, and *CCoAOMT*) in stems are remarkably down-regulated in transgenic plants compared with in the control. *GhFSN1* (Fiber SCW-related NAC1) is a fiber-specific expressing *NAC* gene in cotton which shares a high sequence similarity with *AtNST1/2/3*. The expression level of *GhFSN1* is the highest in 15–28 DPA fibers and is lower in other tissues in cotton. *GhFSN1* overexpression in cotton lines shows a significant decrease in fiber length and increase in average cell wall thickness of 30 DPA fibers, whereas *GhFSN1* RNAi lines show no obvious differences in fiber lengths and thicknesses compared with the wildtype. GhFSN1 regulates a series of fiber SCW-related genes including *GhFSN1*, *GhKNL1*, *GhMYBL1*, *GhGUT1*, *GhDUF231L1*, and *GhIRX12*, by directly binding to their promoters. The results suggest that GhFSN1 acts as a positive regulator in cotton fiber development (Zhang et al., 2018).

Fang et al. (2020) have identified and characterized cotton secondary wall NACs (SWNs) subfamily genes in upland and sea-island cotton. It has been shown that cotton SWNs are clustered into two subgroups, SND1 and NST1s subgroup, which coincide with the cluster analysis in *Arabidopsis*. Specifically silencing SND1 or NST1 subgroup genes alone produce no obvious phenotypes in cotton, whereas silencing of all SWNs leads to a dramatic decrease in the fiber strength, and cellulose and lignin contents of stems, This indicates that NST1- and SND1

subgroup genes exhibit functional redundancy in the regulation of SCW formation. SCW synthesis-associated genes (*CESA4*, *CESA7 CESA8*, and *COBL4*), and xylan and lignin biosynthetic-related genes (*IRX 7/8/9/10/14/15*, *C4H*, *4CL1*, *C3H1*, *HCT*, *CCoAOMT1*, and *F5H*) are remarkably down-regulated in silenced cotton lines, which is consistent with previous studies. The ectopic expression of *GhNAC140* (*NAT1*), *GhNAC28* (*NAT1*), *GhNAC70* (*SND1*), and *GhNAC120* (*SND1*) in tobacco results in obvious curling and lignification phenotypes, and the cellulose and lignin content in leaves of transgenic lines are higher than that in the wildtype. Additionally, several GhSWNs up-regulate the transcription of *GhMYB46* and *GhMYB83*, which are genes closely related to SCW development by directly binding the SWN binding elements at promoters of target gene (Fang et al., 2020).

Leaf senescence

Leaf senescence is a natural termination and internally programmed degradation process that features the visible yellowing of leaves and has a crucial influence on agriculture production (Hortensteiner, 2006). A series of biological events occur at the physiological, biochemical, and molecular levels in this phase, including degradation of cellular structures (chlorophyll), hydrolysis of macromolecules (protein, lipids, RNA, and carbohydrates), decrease in cytoplasmic volume, and decline in photosynthetic and cellular metabolic activities (Rivero et al., 2007; Watanabe et al., 2013; Yang et al., 2022). Leaf senescence is a vital and active process for plant survival and reproduction because nutrients from the dying leaves are remobilized and relocated into developing seeds, fruit or other storage tissues (Woo et al., 2013). Cotton NAC TFs also play a vital role in leaf senescence.

Wang et al. (2022) analyze the function of *GhNAC82* in the premature senescence traits (in cv. CCRI 10, and have found that *GhNAC82* is involved in the negative regulation of premature leaf senescence. The *GhNAC82* expression level is higher in root, petals, 10 DPA ovules, 20 DPA fibers, and 25 DPA fibers, but lower in torus, 5 DPA ovules, and 20 DPA ovules. During cotyledon senescence, the expression level of *GhNAC82* peaks at 21 days after the cotyledons emerged, and is gradually decreased in CCRI 10, while the expression level of *GhNAC82* is relatively stable in the normal senescence variety (cv. CCRI 50). *GhNAC82* overexpressing *Arabidopsis thaliana* exhibit earlier leaf senescence and a shorter root length phenotype compared with the wildtype. Additionally, the senescence-related genes, such as *AtUBQ7*, *AtCAT2* are higher expressed in transgenic plants (Wang et al., 2022). There are 10 NAC genes (*GhNAC8-GhNAC17*) involved in leaf senescence have been reported in cotton, and those NAC proteins are cluster into three groups. There are NAP subfamily (GhNAC9 and GhNAC12), which are involved in senescence, the NAM subfamily (GhNAC8, GhNAC10, GhNAC11, and GhNAC17), and the ATAF subfamily (GhNAC13, GhNAC14, GhNAC15, and GhNAC16), which function in stress responses, respectively. The expression levels of *GhNAC8-GhNAC17* are significantly enhanced during the early phase of ABA-induced leaf senescence, only *GhNAC10-GhNAC16* are remarkably up-regulated by ethylene-induced leaf senescence. During natural leaf senescence, *GhNAC8-GhNAC17* were have been induced, except *GhNAC13*. The comprehensive study of senescence-related *GhNAC* genes during induced and natural leaf senescence indicates that *GhNAC* regulates the process of leaf senescence in cotton (Shah et al., 2013). Kong et al. (2013) have obtained gene expression profiles of main-stem leaves of early senescence and late senescence cotton lines at 110 day after planting, and have identified 11 NAC genes such as *GhNAC4-GhNAC6*, which are up-regulated in early senescence. Fan et al. (2015) have identified a novel NAP member GhNAP, in NAC TFs family, acts as a crucial factor for triggering leaf senescence in cotton. Higher amounts of *GhNAP* transcripts have been found in early senescent leaves and late senescent leaves compared with young leaves and non-senescented leaves, and the expression level of *GhNAP* is remarkablely higher in the yellow tips of leaves than in other areas of leaves. The senescence process in leaves of *nap* null Arabidopsis mutants has been complemented by the *GhNAP*, and the complementary line shows wildtype phenotype, indicating that *GhNAP* is a functional homolog of *AtNAP*. Additionally, the ectopic expression of *GhNAP* in the wildtype Arabidopsis results in a premature senescence phenotype compared with the wildtype, along with higher membrane ion leakage and a lower chlorophyll content. The GhNAPi transgenic cotton lines show delayed senescence, and significantly higher chlorophyll contents and SPAD values in GhNAPi leaves at 120 days after planting than those of the wildtype. The main agronomic traits and some yield components, such as the number and weights of bolls, as well as seed cotton yields of GhNAPi lines, are similar to those of wildtypes whereas the lint yield increases and the fiber length significantly decreases in GhNAPi plants compared with the wildtype (Fan et al., 2015). *GhNAC12*, belonging to the NAM subfamily, was isolated from CCRI 10 and is up-regulated at 35 days after the cotyledons emerged. The *GhNAC12* expressing *Arabidopsis* line shows earlier leaf senescence than the wildtype. And the relative expression levels of senescence-related genes including *ETHYLENE-INSENSITIVE3* (*EIN3*), *WRKY53*, and *NAC029* are significantly higher in 50 day old *GhNAC12* overexpressing plants than that in controls (Zhao et al., 2016). Thus, *NAC* genes appear to play an important role in the regulation of leaf senescence in cotton.

Gland development

Pigment and gossypol glands are specialized cavity structures of *Gossypium* spp. that form from lysigenous intercellular space. These glands store high concentrations of gossypol and various terpenoid aldehydes. Gossypol, a phytoalexin, provides a cotton defense against pests and pathogens, but is poisonous to humans and other monogastric animals (Sunilkumar et al., 2006; Gadelha et al., 2014). To better use cotton by-products and improve cotton's commercial value, the cultivation of cotton varieties that contain glands in the plant body but produce glandless seeds is necessary. NAC TFs participate in gland developmental regulation. *The Cotton Gland Formation* (*CGF*) *2* gene, encoding a NAC TF has been identified using RNA-seq analysis of embryos from glandular and glandless cotton. *GhCGF2* has higher expression level in glandular embryos at 14 DPA and 16 DPA than in glandless embryos. However, there are no dramatic decreases in the numbers of pigment glands in *GhCGF2* silenced VIGS lines, although the color intensity and structures of glands from silenced lines are qualitatively different from that of normal glandular cotton. Similarly, the glands of a knockout mutant of *GhCGF2* are smaller and abnormal compared with those of the wildtype, and the terpenoid levels in leaves of the *GhCGF2* mutant is obviously reduced (Janga et al., 2019). Cheng et al. (2021) identified six *NAC* genes using an expression correlation analysis with GhMYC2-like, which is a master regulator of gland development, as the bait. *GhNAC5*, *GhNAC85*, *GhNAC86*, *GhNAC153*, *GhNAC235*, and *GhNAC236* exhibit low levels of transcripts in ovules before 10 DPA and high levels of transcripts in ovule after 20 DPA, which is consistent with the gland development time. Additionally, silencing of the gland related NACs results in the decrease in gland numbers in cotton (Cheng et al., 2021). The trajectory of pigment gland formation and development has been constructed with *G. bickii* seeds at 48 h after imbibition using single cell RNA-seq, and NAC TRANSCRIPTION FACTOR-LIKE 9 (NTL9) is found to affect pigment gland development. The density and gossypol contents of pigment glands from leaves and stems in *GbiNTL9* silenced cotton seedlings are lower than the control, which indicates that *GbiNTL9* is involved in the transcriptional regulatory network of pigment gland development in *G. bickii* (Sun et al., 2023).

Signaling pathways associated with NAC TFs

Cotton, as a sessile organism is often exposed to a wide range of detrimental environmental stresses and accordingly, has evolved a series of complex signal transduction mechanisms to survive. Plant hormones especially ABA and JA, play a crucial role in regulating developmental processes and signaling networks, and NAC TFs are regulatory elements in hormone-mediated signaling pathways.

ABA signaling pathway

ABA is an important phytohormone, which is involved in the regulation of plant growth, development, and stress responses (Ali et al., 2021; Cao et al., 2011). A model for ABA action has been proposed, including three types of essential components, PYrabactin resistance (PYR)/ PYR-like (PYL)/ Regulatory component of ABA receptors, protein phosphatase 2c (PP2C), and sucrose nonfermenting 1-related protein kinase 2 (SnRK2). In the absence of ABA, PYLs are not bound to PP2Cs which prevents the activities of SnRK2s. When plants encounter stresses, ABA rapidly accumulates and inhibits PP2Cs by binding to the PYLs, which allows the accumulation of phosphorylated SnRK2s. Then, SnRK2s phosphorylate ABA-responsive element binding factors activate the expression of ABA-responsive genes. Thus, ABA regulates the expression of multiple related genes by utilizing various kinases and TFs in the signaling pathways. Among them, NAC TFs also act as regulators in the ABA-mediated pathway.

The expression of *GhNAC2* in cotton leaves is rapidly induced by ABA, and under drought treatment, the endogenous ABA content of leaves from *GhNAC2* co-suppression lines are lower than in wildtype. GhNAC2 binds to and activates the promoters of *GhNCED3a-At/Dt* and *GhNCED3c-At/Dt*, which are key genes for ABA biosynthesistic genes, verified in vitro through EMSA and in vivo through transient expression analyses of *GUS* and *LUC*. GhNAC2 modulates ABA biosynthesis by regulating the expression of *GhNCED3*, thereby improving the drought tolerance of cotton (Shang et al., 2020). Similarly, *GheNAC2* from G. *herbaceum* is also strongly and rapidly induced by ABA treatments. The ABA receptor gene *AtPYL8* is up-regulated, whereas negative regulator genes of ABA (*AtPP2C*, *AtCYP7073A3*, and *AtWRKY18*, *AtWRKY33*, *AtWRKY40*, and *AtWRKY70*) are down-regulated in *GhNAC2* transgenic *Arabidopsis* (Gunapati et al., 2016). *GhNAC3* is up-regulated at 4 h after ABA treatment, drought, or salt stresses. *GhNAC3* silenced cotton seedlings show decreases in ABA contents in leaves, and ABA synthesis-related genes, including *GhZEP*, *GhABA2*, and *GhAAO3*, are obviously down-regulated in silenced cotton seedlings (Xia et al., 2023). Under both NaCl and PEG treatments, very high up-regulations of *NtNCED3* transcripts and hypersensitivity to exogenous ABA are observed in transgenic *GhNAC4* tobacco, indicating that *GhNAC4* is associated with ABA-dependent abiotic stress-related pathways. Additionally, stomatal aperture is affected in *GhNAC4* transgenic lines after ABA

treatments, consistent with the mechanism of stomatal movement regulation by ABA (Trishla et al., 2021). There are multiple specific *cis*-elements related to the ABA responses in the *GhNAP* promoter and the expression of *GhNAP* is highly induced by ABA treatment. It has been suggested that GhNAP is involved in ABA responses by altering the endogenous ABA content and regulating ABA-related gene expression (Fan et al., 2015).

MeJA- and SA- signaling pathways

MeJA and SA also play a vital role in cotton responses to various stresses. *GhATAF1* expression is highly induced by MeJA treatment. And under 5 μmol·L^{-1} MeJA treatment, hypocotyl elongation is promoted in *GhATAF1* overexpression cotton. Genes associated with JA biosynthesis (*GhLOX1*) and JA responses (*GhMYC2*, *GhPR3*, and *GhPR4*) are suppressed in transgenic cotton lines, indicating that *GhATAF1* functions in the JA signaling responses (He et al., 2016). *GhATAF1* expression is quickly and stablely up-regulated at high level in cotton roots by exogenous SA treatments. The SA-signaling pathway-related genes, including *GhNDR1-1*, *GhNPR1*, *GhPR1*, and *GhPR5* are up-regulated in *GhATAF1* transgenic cotton lines, and the survival rate after 4 mmol·L^{-1} SA treatment for 5 days is remarkably lower in transgenic lines than in the control, suggesting that *GhATAF1* acts as the regulator to activate SA-mediated signaling pathways (He et al., 2016). *GhNAC82* expression is positively induced by MeJA and ABA treatments, whereas it is negatively induced by indoleacetic acid and ethylene treatments indicating that *GhNAC82* plays a key role in the regulation of hormone responses (Wang et al., 2022).

NAC protein regulatory network in cotton

NAC proteins are involved in plant responses to various environments by directly or indirectly regulating the expression of a series of relevant downstream genes or interacting with related proteins (Diao et al., 2020). NAC TFs regulate many types of downstream genes, including stress resistance related genes (*CAD8B*, *CAD9B*, *DERB2A*, and *WRKY62*) (Yuan et al., 2019; Yuan et al., 2023a, b; Wang et al., 2021a), and hormone signal transduction-related genes (*NCED3* and *AREB1*) (Jensen et al., 2013; Sakuraba et al., 2015), by recognizing the *cis*-elements, known as NAC recognition sites and core DNA-binding sequences. NAC genes, in turn, are regulated by other TFs. For instance, OsbZIP23 binds to the promoter of NAC028 to modulate rice resistance to sheath blight disease (Yuan et al., 2023a). Additionally, other related proteins interact with NAC proteins (Takasaki et al., 2010), the complex regulatory networks of NAC proteins in cotton are summarized as follow.

Shang et al. (2016) suggest that GhNAC2 directly binds to the promoter of *GhNCED3a/3c* and activates the expression of *GhNCED3a/3c*, which modulates ABA biosynthesis. GhSWNs bind to the promoters of *GhMYB46* and *GhMYB83*, and positively regulate the expression of target genes to control SCW formation (Fang et al. 2020). Chen et al. (2021) have verified that GhJUB1L1 is involved in responses to drought stress and SCW development through direct binding to the promoters of *GhABI1, GhSOS2, GhCCoAOMT1, GhCesA7*, and *GhIRX14* genes and activating their expression. GhFSN1 regulates several downstream SCW biosynthesis-related genes, including *GhFSN1*, *GhKNL1*, *GhMYBL1*, *GhGUT1*, *GhDUF231L1*, and *GhIRX12* by binding to the SWN-binding element motifs in their promoters (Zhang et al., 2018). Hu et al. indicated that *NAC100* is the target gene of ghr-miR164 and that NAC100 represses the expression of *PR3* by binding to its promoter (Hu et al., 2020). Through the comprehensive application of yeast two-hybrid and BiFC technology, SCW-related NAC proteins, including GhVND1-4D, GhVND4-3A, GhVND4-5D, GhFSN1A, GhFSN2D, GhSND2-1A, GhSND2-1D, GhSND2-2A, GhSND2-2D, and GhSND2-3A, have been shown to interact with GhGA1D (DELLA protein in cotton stem), thereby influencing SCW formation in cotton stems (Wang et al., 2021b). GhORE1, a senescence-associated NAC TFs interacts with PevD1 to participate in *V. dahlia*-induced leaf senescence (Zhang et al., 2021). In summary, the NAC TFs of cotton play a key role in plant growth, development, and stresses signaling pathways by regulating a series of downstream genes and interacting with related proteins.

Conclusion

The NAC proteins, as important plant-specific TFs, are involved in cotton growth and development. This review emphasized the latest findings on the roles of the cotton NAC TF family in responses to drought, salt, and Verticillium wilt stress, as well as in leaf senescence and the development of fibers, xylem, and glands. The signaling pathways and regulatory network associated with NAC TFs in cotton were also highlighted. The NAC TF members have been identified based on the cotton genomic data, the function of cotton NAC proteins have been further studied and validated thoroughly by molecular biotechniques. However, compared with NAC TFs of *Arabidopsis* and rice, NAC TFs of cotton need further study. The downstream genes and respective interaction of cotton NAC proteins have not been fully identified, and the molecular mechanism involved in cotton NAC TF responses to various stresses need detailed analysis. The specific functions of NAC TFs in cotton may be further explored using the latest developed methods, such

as gene-editing technology. Additionally, the comprehensive utilization of cotton NAC TFs in improvement of cotton varieties improvement and ultimately apply in cotton production are important research directions. This review laid the foundation for further analysis of NAC TFs in cotton.

Acknowledgements
Not applicable.

Authors' contributions
All authors contributed to the review design. The first draft of the manuscript was written by Xu YW and Zhao P. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding
This work was supported by the Young Scientists Fund of the National Natural Science Foundation of China (32101797) and Central Public-interest Scientific Institution Basal Research Fund (No. 1610162023020).

Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Received: 19 March 2024 Accepted: 20 June 2024
Published online: 02 August 2024

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