Functional analysis of *Gossypium hirsutum* NAC transcription factor, GhNAC4 in abiotic stress responses

Thesis submitted for the award of the degree of

Doctor of Philosophy

By

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This is to declare that I, **V. S. Trishla**, have carried out the research work embodied in the present thesis entitled "**Functional analysis of *Gossypium hirsutum* NAC transcription factor, GhNAC4 in abiotic stress responses**" and submitted for the degree of Doctor of Philosophy under the supervision of **Prof. (Retired) P. B. Kirti**, Department of Plant Sciences, School of Life Sciences, University of Hyderabad. I declare to the best of my knowledge that no part of this thesis was earlier submitted in part or in full, for the award of any research degree or diploma from any University or Institution. A report on plagiarism statistics from the University Library is enclosed.

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   ii. **Trishla VS**, Kirti PB (2020) Structure-function relationship of *Gossypium hirsutum* NAC transcription factor, GhNAC4 with regard to ABA and abiotic stress responses. BioRxiv https://doi.org/10.1101/2020.05.28.121665

B. Presented in the following conferences:
   i. **Trishla VS** and Kirti PB (Poster). Engineering cotton (*Gossypium hirsutum* L.var Durga) for drought and salinity tolerance (2013). Presented at InterDrought-IV, Perth, Australia.
   ii. **Trishla VS** and Kirti PB (Talk). Characterization of GhNAC4 in budding yeast (2015). Presented at 7th Plant Sciences Colloquium, University of Hyderabad.
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- **Trishla VS**, Marriboina S, Boyidi P, Gudipalli P, Kirti PB (2020) Characterization of a vascular bundle localizing *Gossypium hirsutum* NAC4 transcription factor promoter for its role in environmental stress responses. **BioRxiv** https://doi.org/10.1101/510578
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"Gurur-Brahmaa Gurur-Vissnnur Gururdevo Maheshvarah; Gurureve Param Brahma Tasmai Shrii-Gurave Namah". I bow down to my Guru (Shri Vijay Shanti Sureshwar ji) who is God manifested in human form.

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I apologize if I have missed any one, it was unintentional.

-Trishla

Abbreviations

µg	Microgram
µl	Microliter
µmoles	Micromoles
4-MU	4-Methylumbelliferone
4-MUG/MUG	4-Methyl-umbelliferyl-β-d-glucuronide
aa	Amino acids
ABA	Abscisic acid
Ade	Adenine
BAP	6-Benzyl aminopurine
bp	Base pairs
BSA	Bovine serum albumin
CaMV	Cauliflower mosaic virus
cDNA	Complementary deoxy ribonucleic acid
CDS	Coding sequence
CTAB	Cetryl trimethyl ammonium bromide
d	Days
DNA	Deoxy ribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FW	Fresh weight
g	Gram
GA_3	Gibberellic acid
GFP	Green fluorescent protein

GUS	Beta-glucuronidase
h	Hours
His	Histidine
IAA	Indole-3-acetic acid
JA	Jasmonic acid
kb	Kilobases
l	Liter
LA	Luria Bertani Agar
LB	Luria Bertani Broth
Leu	Leucine
M	Molar
MDA	Malondialdehyde
MeJA	Methyl jasmonic acid
min	Minutes
ml	Milliliter
MPa	Mega Pascals
MS	Murashige and Skoog
MV	Methyl viologen
NAA	Naphthalene acetic acid
NAC	NAM ATAF1/2 CUC
NACRS	NAC recognition sequence
ng	Nanogram
nM	Nanomolar

^{o}C	Celsius degree
OD	Optical density
ONPG	o-nitrophenyl-β-galactopyranoside
P	Probability
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pmol	Picomoles
ppm	Parts per million
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	Revolutions per minute
SA	Salicylic acid
SD	Synthetic dropout
SDS	Sodium dodecyl sulfate
SE	Standard error
TCA	Trichloroacetic acid
TF	Transcription factor
TR-domain	Transcriptional regulatory domain
Tris	Tris(hydroxymethyl) aminomethane
Ura	Uracil
WT	Wild type

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Chapter 1

Introduction

1.1 Abiotic stress

Plants live in a constantly changing environment that is frequently adverse for growth and development. In 2007, FAO reported that 96.5 % of the world's arable land is affected by some type of environmental constraints. These unfavorable environmental conditions can be abiotic or biotic in nature. Biotic stress factors include herbivore attack and pathogen infection. Abiotic stress factors include heavy metal toxicity, salinity, nutrient deficiency, drought, cold, and high-temperature. High salinity, extreme temperatures, and drought are the primary abiotic stress factors that affect plant productivity and, thus, food security. Furthermore, climate change is expected to exacerbate these effects.

(Lipiec et al., 2013) defined that drought stress occurs in a plant when there is an imbalance between evapo-transpiration flow and water intake from the soil. Compared to high salinity, drought stress is even more severe and economically damaging (Boyer, 1982). Dehydration stress results in the disruption of photosynthesis, nutrient and water relations, assimilate partitioning, and growth, which ultimately lead to a decline in crop yield (Farooq et al., 2009).

High-temperature stress is defined by an elevation of air and soil temperature for a minimum amount of time beyond a threshold such that an irreversible damage occurs in the plant because of the changed climate (Zhao et al., 2017). High-temperature stress often leads to severe damage to the synthesis and conformation of proteins, membrane disruption, and inactivation of enzymes. It can also affect cell division (Fahad et al., 2017). The reproductive developmental phase is shown to be most sensitive to heat stress, thus directly affecting crop yield (Lamaoui et al., 2018).

High salinity stress has threefold effects viz., sodium ion toxicity, reduction in water potential, and oxidative damage (Zhu, 2011). A decrease in the growth rate is seen due to a plant's inability to take up water from the soil due to the presence of salt. Additionally, ion toxicity occurs due to the presence of excessive amounts of salts in the transpirational stream. The detrimental effects are seen

at all stages of plant growth (Parihar et al., 2015). Abiotic stresses also lead to the production of reactive oxygen species (ROS) in the plant resulting in tissue damage that leads to decreased growth, fertility, and yield (Krasensky and Jonak, 2012).

Freezing or chilling stress occurs when plants are subjected to sub-optimal temperatures. Freezing stress leads to severe growth retardation, chlorosis, wilting, and necrosis. Cold stress during the reproductive stage leads to sterile flowers. The primary physiological effect of cold stress is severe membrane damage and ice formation in the apoplastic region leading to cellular dehydration (Lynch, 1990).

1.2 Abiotic stress signaling and response

To mitigate the harmful effects of abiotic stresses, plants have evolved specific growth responses to avoid stress or adaptation mechanisms. The response to abiotic stress is complex ranging from being dynamic and reversible to long term and irreversible (Bartels & Sunkar, 2005). Responses involve cell division regulation, modification of cell wall architecture, adjustments to the membrane systems and reduction of excess ROS (Cramer et al., 2011). To maintain cellular turgor pressure and stabilize the protein structures, compatible solutes, such as proline and raffinose are produced (Szabados & Savouré, 2010). Adaptation to abiotic stress also involves activation of the signal transduction pathway, regulation of transcription factors (TFs), hormonal crosstalk, and production of detoxifying enzymes, transporters, and osmoprotectants (Osakabe & Osakabe, 2012).

High salinity, extreme temperatures, and drought result in rapid activation of mitogen-activated protein (MAP) kinase modules (Sinha et al., 2011). These modules typically consist of a combination of at least three different types of protein kinases. They are a MAPK, a MAPKK (MAP2K), and a MAPKKK (MAP3K). The phosphorylation-dependent sequential activation of the MAP kinase cascade results in activation or repression of TFs and phospholipases, which eventually leads to

regulation of genes required for abiotic stress tolerance (Popescu et al., 2009). MAPK 3, 4, and 6 are observed to be rapidly activated during abiotic stresses (de Zelicourt et al., 2016).

Ion homeostasis that is required to mitigate salt stress involves the SOS pathway (Zhu, 2000) as depicted in Fig. 1.1. High salinity induces a calcium signal, which is recognized by Salt Overly Sensitive 3 (SOS3). SOS3 is a calcium-binding myristoylated protein that binds to and activates SOS2, which is a threonine/serine protein kinase. The complex of SOS2-SOS3 activates a plasma-membrane based Na^+/H^+ antiporter, SOS1. Activated SOS1 extrudes Na^+ ions into the apoplast thereby reducing the concentration of Na^+ ion in the cytosol. Large quantities of Na^+ that enter the cytosol are compartmentalized into the vacuoles by the activity of NHX1, a Na^+/H^+ vacuolar antiporter protein (Apse et al., 2003). The SOS2 kinase regulates the activity of NHX1 antiporter (Qiu et al., 2004). By compartmentalizing the Na^+ into the vacuoles, plants can avert ion toxicity from the cytosol and also maintain turgor using the vacuolar Na^+ concentration as osmoticum. The proton motive force required for driving the vacuolar transporters is provided by the H^+-p yrophosphatase and the V-type H^+-ATPases (Sze et al., 1999).

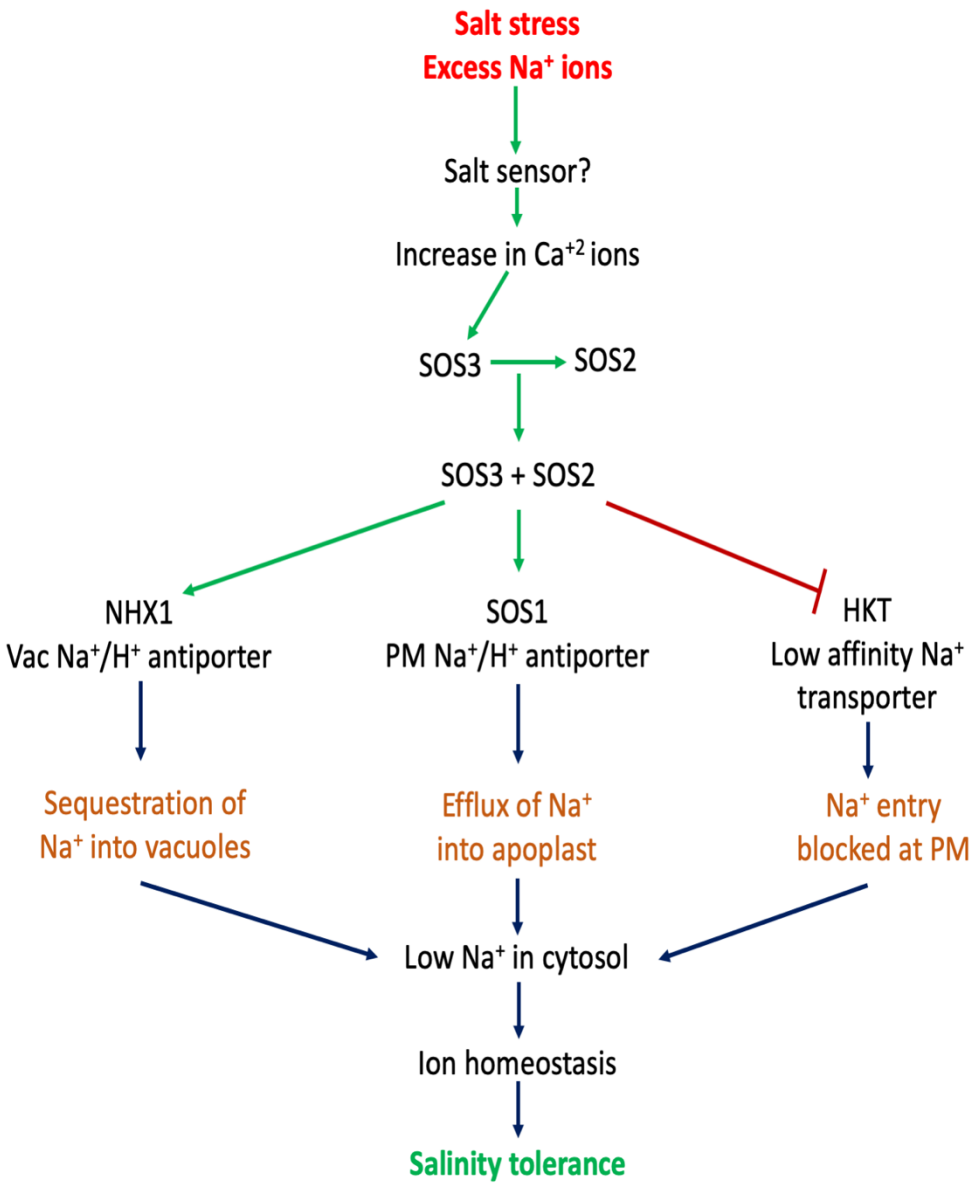


Figure 1.1 Regulation of SOS pathway for ion homeostasis during salt stress in plants.

High concentration of Na^+ ions is perceived by an unknown protein present at the plasma-membrane, which leads to an elevation in concentration of free cytosolic Ca^{+2} ions. Ca^{+2} binds to and activates SOS3, which in turn binds to a protein kinase, SOS2. This causes activation of SOS2 by auto-phosphorylation. Activated SOS3-SOS2 complex phosphorylates to activate a Na^+/H^+ antiporter, SOS1 leading to excess Na^+ ion efflux into the apoplast. The SOS3-SOS2 complex also inhibits the activity of a low affinity Na^+ transporter, HKT, thereby preventing the penetration of Na^+ ions into the cell. A vacuolar Na^+/H^+ exchanger, NHX1 is also activated by SOS2 leading to the accumulation of Na^+ ions into vacuoles, thereby contributing to salt tolerance. *PM* plasma membrane; *Vac* Vacuolar.

To prevent protein denaturation caused by heat/high-temperature stress, plants induce the expression of molecular chaperones such as HSPs (Heat Shock Proteins). These proteins are regulated by HSFs (Heat Stress transcription Factors) to maintain cellular homeostasis. Under normal conditions, the HSFs are bound and inhibited by HSP90 and HSP70 chaperone proteins. Under heat stress, HSP chaperones bind to misfolded proteins, which results in the release and activation of HSFs. These activated HSFs thereby activate the high-temperature stress response pathway (Scharf et al., 2012). HSP gene expression is modulated by the MAPK pathway, activated by calcium signaling and changes in membrane fluidity due to high-temperature stress (Sangwan et al., 2002). This is crucial for thermotolerance.

1.3 Abscisic acid signaling

Growth and development processes such as cell growth and flowering time are severely affected by abiotic stress. This suggests a crosstalk between plant growth and environmental stress signals. Phytohormones play an essential role in these crosstalk events. They act as central players that integrate the complex stress-responsive pathways with developmental signaling cascades. Abscisic acid is the key regulator that causes growth inhibition and activation of adaptive mechanisms to counter abiotic stress, especially drought (Santiago et al., 2012). ABA is also involved in the regulation of seed dormancy development of seeds, delay in seed germination, embryo morphogenesis, induction of stomatal closure and leaf senescence. ABA promotes seed germination and embryo development only when the external environment is conducive to growth. Promotion of stomatal closure under water-stressed prevents water loss and therefore helps in drought stress adaptation (Swamy and Smith, 1999).

The ABA signaling pathway consists of three core components. PYR/PYL/RCAR (Pyrabactin Resistance/Pyrabactin resistance-like/Regulatory Component of ABA Receptor) PP2C (Protein Phosphatase 2C) group A family and SnRK2 (SNF1-Related Protein Kinases type 2) (Fujii et al., 2009).

PYR/PYL/RCAR is the ABA receptor, PP2C phosphatases are negative regulators, and SnRK2 kinases are positive regulators. Without any external stimuli and ABA, PP2C phosphatases constantly deactivate SnRK2 kinases by physical interaction and dephosphorylation of the kinase activation loop. This effectively prevents SnRK2 kinases from phosphorylating its downstream targets. In the presence of ABA, the conformation of PYR/PYL/RCAR receptors is changed by ABA binding and leads to binding of the receptors to the catalytic site of PP2C phosphatases. In turn, it leads to the activation of SnRK2 kinases by auto-phosphorylation (Cutler et al., 2010).

SnRK2 family of kinases are known to regulate various downstream target genes such as transporters, channel proteins, and TFs like AREB/ABF. ABA strongly activates SnRK2.6, SnRK2.3, and SnRK2.2 kinases, which in turn, phosphorylate RbohF, a plasma membrane NADPH oxidase. When activated, RbohF produces O_2^- ion, which eventually produces H_2O_2 (Sirichandra et al., 2009). The ROS generated acts as a stimulus that mediates stomatal closure. The guard cell anion channel proteins (SLAC1) are also substrates of ABA-activated Open Stomata 1 (OST1/SRK2E/SnRK2.6) (Geiger et al., 2009). Activated SLAC1 proteins cause stomatal closure, which eventually causes decreased transpirational water loss during drought stress. In tissues other than guard cells, such as seeds and leaves, SRK2D/SnRK2.2, and SRK2I/SnRK2.3 play a role in rapidly transmitting ABA signal (Fujii et al., 2009). The ABA signaling core module also activates a MAP kinase signaling module comprising MAP3K17/18, MKK3, and the MPK1/2/7/14 (de Zelicourt et al., 2016). These regulate downstream effectors through phosphorylation.

SnRK2 kinases activate the basic-domain leucine zipper (bZIP) TFs, AREB/ABF (ABA Response Element Binding Factor) by phosphorylation of several conserved Serine/ Threonine R-X-X-S/T motifs (Furihata et al., 2006). They belong to group A subfamily and bind to a conserved *cis*-acting motif ABA-responsive element (ABRE; PyACGTGG/TC) in the 5’ upstream region of ABA-activated genes. Among the bZIP TF families, ABF2/AREB1, ABF4/ABREB2, and ABF3 are highly induced by abiotic

stresses and ABA. Under drought stress, AREBs activate various classes of LEA (Late Embryogenesis Abundant) proteins, group-Ab PP2Cs, and various TFs (Takuya Yoshida et al., 2010). Thus, an increase in endogenous levels of ABA results in the activation of ABA-dependent signaling cascade, which eventually leads to enhanced tolerance as depicted in Fig. 1.2.

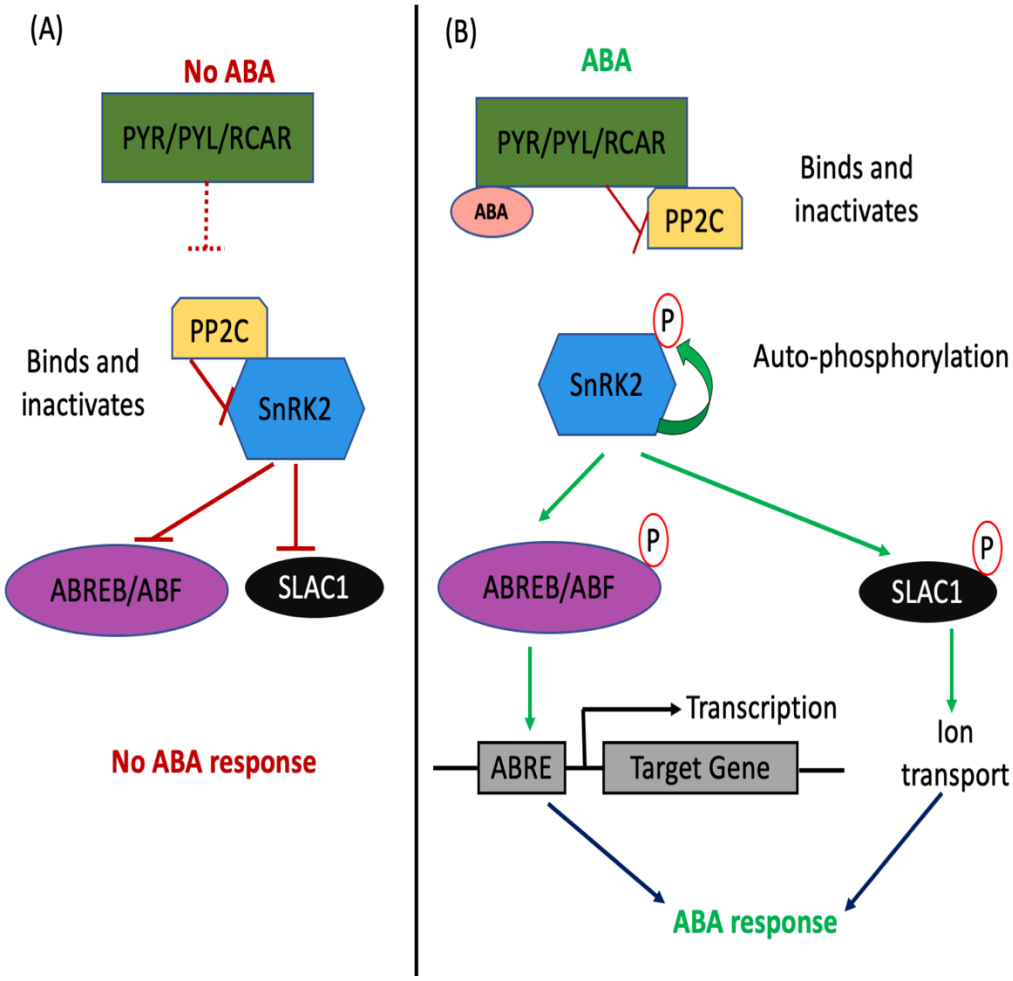


Figure 1.2 Abscisic acid core signaling module.

(A) In the absence of ABA, PYR/PYL/RCAR, the ABA receptors remain inactive and the PP2C phosphatases inhibit SnRK2 kinases. (B) In the presence of ABA, the PYL proteins bind to ABA and interact with PP2C to inhibit its activity. This, in turn, allows the activation of SnRK2 by auto-phosphorylation. Subsequently, SnRK2 phosphorylates ABA-responsive proteins like ABREB/ABF TFs and ion channel proteins like SLAC1. ABFs bind to ABRE motif in the promoter of downstream genes and modulate ABA-responsive gene expression.

1.4 ABA-dependent and independent pathways

It was observed that not all drought-responsive genes were induced by ABA suggesting the existence of a different pathway, the ABA-independent signaling cascade. Although drought and high salinity imposed osmotic stress are regulated through both the pathways, i.e., ABA-dependent and independent pathways, freezing/cold stress adaptation is mainly modulated through the ABA-independent signaling cascade (Shinozaki and Yamaguchi-Shinozaki, 2000).

Osmotic stress-inducible genes that are activated by ABA contain a motif, which is recognized by ABREB/ABF TFs as mentioned in section 1.3. In contrast, osmotic stress-responsive genes that are not induced by ABA contain a motif called DRE/CRT (Dehydration Responsive Element/C-Repeat). DRE/CRT motifs consist of a conserved 9-bp core sequence of 5'-TACCGACAT-3' (Yamaguchi-Shinozaki and Shinozaki, 1994). This sequence is recognized by DREB/CBF (DRE Binding protein/CRT Binding Factor). They belong to the ERF/AP2 TF family and consist of two sub-classes CBF/DREB1 and DREB2. Cold stress induces the expression of *Arabidopsis* DREB1 TFs while DREB2 TFs are upregulated by dehydration, heat, and salt stresses (Ito et al., 2006) in an ABA-independent manner.

DREB1A activates various stress-responsive genes such as phospholipase C, LEA, osmoprotectant biosynthetic proteins, sugar transporters, and RNA-binding proteins. Promoters of several downstream target genes carry one or more copies of DRE/CRT or related *cis*-acting elements (Maruyama et al., 2004). Transcription factors like C2H2 zinc finger-type proteins are also the targets of DREB1A TF suggesting the presence of DRE/DREB regulon regulating gene expression under abiotic stress in an ABA-independent manner.

Freezing stress activates SnRK2.6/OST1 kinases independent of ABA. These activated kinases phosphorylate and interact with ICE1 (Inducer of CBF Expression 1) TF, which is a basic helix–loop–helix MYC-type TF. ICE1 binds to the MYC recognition sequence in the promoters of DREB1/CBF3 TF (Ding et al., 2015). The activated CBF3 binds to the DRE/CRT motif in the promoters of downstream

cold-inducible genes leading to cold acclimation as depicted in Fig. 1.3. The downstream genes of CBF TFs include genes involved in membrane transport, osmolyte biosynthesis, phosphoinositide metabolism, ROS detoxification, and TFs involved in cold adaptation (Chinnusamy et al., 2007).

Freezing stress also induces cytosolic calcium signal, which activates the MAP kinase signaling cascade via the CRLK1 (Calcium/calmodulin Regulated Receptor-Like Kinase) protein. CRLK1 binds to and activates MAP3K kinase, MEKK1 (T. Yang et al., 2010). The freezing stress-activated MEKK1 phosphorylates and activates MAP2K kinase, MKK2, which in turn activates MAPK kinases, MPK4, and MPK6 through phosphorylation leading to cold acclimation by activation of cold-inducible genes (Teige et al., 2004).

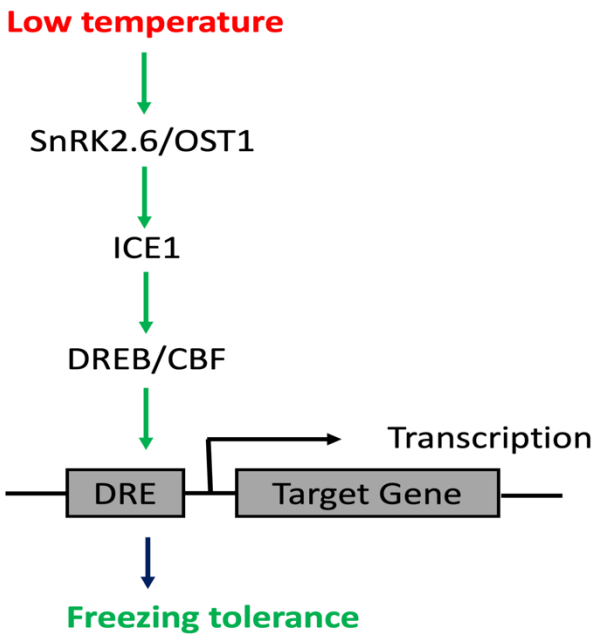


Figure 1.3 Cold stress signaling pathway.

Low temperature is sensed at the plasma membrane, which induces the activation of OST1/SnRK2.6 kinases. Activated OST1 phosphorylates proteins like DREB/CBF TFs. CBFs bind to DRE motif in the promoter of target genes and modulate cold-responsive gene expression in an ABA-independent manner.

The temporal expression of stress-inducible genes is modulated by the combination of TFs and their corresponding recognition sequence. The early response to abiotic stress involves the DRE/CRT motif,

while the ABRE motif functions in the later stages after the ABA synthesis and accumulation (Yamaguchi-Shinozaki and Shinozaki, 2006).

Various studies indicate that ABA-dependent and independent pathways do not have a distinct line of demarcation and often exhibit crosstalk and convergence in the signaling cascade. The combination of TFs and their corresponding recognition sequences are essential for the crosstalk in various stress signaling cascades. DRE/CRT motifs act as a coupling element of ABA, which give rise to cross talk between ABA-independent and ABA-dependent signaling cascade during transcriptional regulation (Yamaguchi-Shinozaki and Shinozaki, 2006). An excellent example of genes regulated by both ABA-dependent and independent pathways is *rd29A* (Response to Dehydration 29A) gene. The promoter of *rd29A* consists of three DREs and one ABRE *cis*-acting elements. It can thus integrate stimuli from freezing, dehydration, salt stress, and ABA signaling pathways (Narusaka et al., 2003). A very good candidate for mediating crosstalk is calcium, which is a secondary messenger for various abiotic stress responses (Mahajan and Tuteja, 2005).

Chapter 2

Review of literature

2.1 NAC transcription factors in plants

To adapt to abiotic stresses, plants activate tolerance mechanisms such as stress perception, signal transduction either through ABA-dependent and independent signaling cascade, expression of stress-inducible genes that promote activation of metabolic and physiological responses leading to stress tolerance (Chaves et al., 2002).

Genomic and molecular analyses have identified many stress-responsive genes, which can be broadly divided into two categories. The first category of genes encodes functional and metabolic proteins like LEA proteins, detoxification enzymes, transporters, channel proteins, and osmoprotectants, which are directly involved in cellular adaptation to abiotic stress. The second category of genes encodes various regulatory proteins like TFs and kinases, which are involved in the signaling pathway and regulation of gene expression (Shinozaki et al., 2003).

Transcription factors play critical roles in converting signal perception to modulating of stress-inducible gene expression. Several analyses have revealed that various different families of TFs and their interacting motifs play a role in the stress-inducible regulation of gene expression. These TFs often are members of large gene families like NAC, bZIP, bHLH, MYB, MYC, WRKY, ERF/AP2 families (Golldack et al., 2014) as depicted in Fig. 2.1.

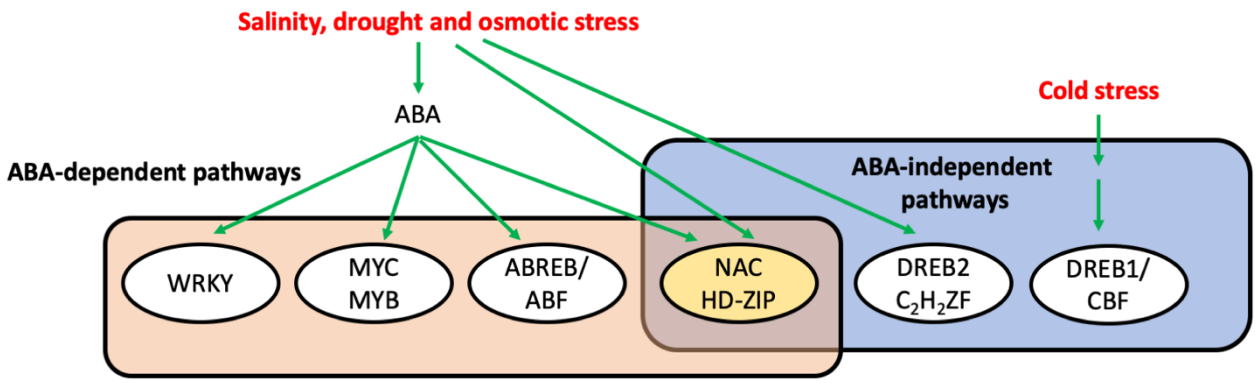


Figure 2.1 Transcription factors associated with abiotic stress pathway.

Salinity and drought signal lead to the activation of both ABA-dependent and independent pathways. In ABA-dependent pathway, accumulation of ABA modulates the activities of WRKY, MYC, MYB, ABREB/ABF, HD-ZIP and NAC TFs. NAC and HD-ZIP TFs, along with DREB2 and C2H2 ZF TFs, are also involved in the ABA-independent gene regulation of drought and salt stress. Cold stress leads to activation of DREB1/CBF TFs in an ABA-independent manner. Various nodes of cross-talks have been reported between the ABA-dependent and independent pathways.

NAC TFs are one of the largest TF families and are specific to plants. The NAC acronym is deduced from the characterization of three proteins consisting of a characteristic domain, now called the NAC domain. These proteins are petunia NAM (No Apical Meristem), and *Arabidopsis* ATAF1-2 and CUC2 (Cup-shaped Cotyledon) (Aida et al., 1997; Souer et al., 1996). Availability of a large number of complete plant genome sequences aided by extensive investigations of EST sequences have led to the identification of numerous NAC TFs in almost 114 plant species so far. For example, 117 and 151 NAC members in *Arabidopsis*, rice (Nuruzzaman et al., 2010a), 163 in Populus (Hu et al., 2010), 152 in soybean (Le et al., 2011), 147 in foxtail millet (Puranik et al., 2013), and 110 have been identified in potato (A. K. Singh et al., 2013).

Table 2.1 Total number of NAC members in various species

Species	Common name	No. of NAC TFs	References
Nicotiana tabacum	Tobacco	152	Rushton et al., 2008
Arabidopsis thaliana	Thale Grass	117	Nuruzzaman et al., 2010
Oryza sativa	Rice	151	Nuruzzaman et al., 2010
Populus trichocarpa	Poplar	163	Hu et al., 2010
Glycine max	Soybean	152	Le et al., 2011
Vitis vinifera	Grape	74	Wang et al., 2013
Setaria italica	Foxtail millet	147	Puranik et al., 2013
Malus domestica	Apple	180	Su et al., 2013
Solanum tuberosum	Potato	110	Singh et al., 2013
Musa acuminata	Banana	167	Cenci et al., 2014
Cajanus cajan	Pigeon pea	88	Satheesh et al., 2014
Brassica rapa	Chinese cabbage	204	Liu et al., 2014
Zea mays	Maize	152	Shiriga et al., 2014
Cicer arietinum	Chickpea	71	Ha et al., 2014
Manihot esculenta Crantz	Cassava	96	Hu et al., 2015
Brachypodium distachyon	Purple false brome	101	You et al., 2015
Jatropha curcas	Physic nut	100	Wu et al., 2015
Solanum lycopersicum	Tomato	104	Su et al., 2015
Camellia sinensis	Tea	45	Wang et al., 2016
Cucumis melo	Melon	82	Wei et al., 2016
Citrullus lanatus	Watermelon	80	Lv et al., 2016
Medicago truncatula	Medicago	97	Ling et al., 2017
Raphanus sativus	Radish	172	Karanja et al., 2017
Triticum turgidum	Durum wheat	168	Saidi et al., 2017
Panicum virgatum	Switchgrass	251	Yan et al., 2017
Cucumis sativus	Cucumber	82	Zhang et al., 2017
Gossypium arboreum	Diploid cotton	147	Sun et al., 2018
Gossypium barbadense	Tetraploid cotton	267	Sun et al., 2018

Gossypium hirsutum	Tetraploid cotton	283	Sun et al., 2018
Gossypium raimondii	Diploid cotton	149	Sun et al., 2018
Sesamum indicum	Sesame	87	Zhang et al., 2018
Capsicum annuum	Pepper	104	Diao et al., 2018
Fragaria x ananassa	Strawberry	112	Moyano et al., 2018
Pyrus pyrifolia	Asian pear	185	Ahmad et al., 2018
Prunus mume	Chinese plum	113	Zhuo et al., 2018
Ananas comosus	Pineapple	73	He et al., 2019
Sorghum bicolor	Sorghum	131	Sanjari et al., 2019
Fagopyrum tataricum	Tartary buckwheat	80	Liu et al., 2019
Chenopodium quinoa	Quinoa	140	Li et al., 2019
Pyrus bretschneideri	White pear	183	Gong et al., 2019
Triticum aestivum	Wheat	488	Guérin et al., 2019
Betula pendula	Birch	114	Chen et al., 2019
Apium graveolens	Celery	111	Duan et al., 2020
Panicum miliaceum	Broomcorn millet	180	Shan et al., 2020
Theobroma cacao	Cacao	102	Shen et al., 2020
Osmanthus fragrans	Sweet Osmanthus	119	Yue et al., 2020
Medicago sativa	Alfalfa	113	Min et al., 2020

Their functions reflect the remarkable large size and diversification of NAC TFs across the plant kingdom in a variety of biological processes such as shoot apical meristem development (Souer et al., 1996), seed development (Sperotto et al., 2009), leaf senescence (Guo and Gan, 2006), flower development (Sablowski and Meyerowitz, 1998), fiber development (Ko et al., 2007a), abiotic (Hu et al., 2006) and biotic stress responses (Nakashima et al., 2007). Furthermore, the large size of NAC TF superfamily leads to complications in understanding their regulatory roles. These functions are mediated through diverse processes such as homo and heterodimerization with multiple interacting proteins, alternative splicing, miRNA-mediated regulation and post-translational modifications

(Welner et al., 2016). However, only a few members of NAC TF superfamily have been characterized in detail to date, and many others have not been studied yet (Shao et al., 2015).

2.2 Structure of NAC transcription factors

As discussed earlier, TFs are sequence-specific DNA-binding proteins that modulate the expression of genes through activation or repression. A typical TF is composed of a DNA-binding domain (DBD) and a transcriptional regulatory domain (TRD) (Lindemose et al., 2013). They are classified into different families based on the DBD structure. The DBD of TFs bind in a sequence-specific manner, thus, targeting only the promoters carrying the recognition sequence (Luscombe and Thornton, 2002).

The TRD usually lacks a well-defined three-dimensional structure and comprises a higher percentage of intrinsically disordered or low-complexity regions. They tend to have flexible protein segments, thus, providing an advantage of binding to several different targets aiding in the regulation of TF. The TRD is commonly classified based on the composition of amino acid. They are often rich in amino acids such as serine, threonine, glutamine, and also acidic amino acids (Dyson and Wright, 2005).

A typical NAC TF comprises two domains- a conserved N-terminal domain (DBD) and a highly divergent C-terminal domain (TRD). The N-terminal NAC domain is usually 150-160 amino acids in length and can be sub-divided into five conserved sub-domains (A to E) (Addie Nina Olsen et al., 2005) as depicted in Fig. 2.2.

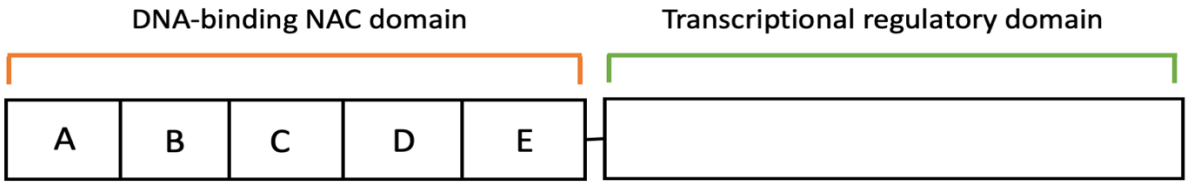


Figure 2.2 Schematic representation of a typical NAC protein.

The NAC-domain is the DNA binding domain and is typically present at the N-terminal end of the protein, which is divided into five domains (A-E). The C-terminal transcriptional regulatory domain is highly variable and consists of intrinsically disordered regions. It is a transcription regulatory domain. The C-terminal region of few NAC proteins also comprises a transmembrane motif.

The structure of the ANAC019 N-terminal NAC domain has been resolved to 1.9 A^{o} using X-ray crystallography. The NAC DBD lacks the classical/typical helix–turn–helix motif. However, it has a novel TF fold of 7-stranded twisted antiparallel β-sheets surrounded by α-helical elements. The N-terminus of NAC DBD has a poorly defined structure and is in an extended conformation (Ernst et al., 2004). Additionally, the NAC-domain of rice SNAC1 (Stress-responsive NAC 1) crystal structure has been resolved to 2.5 A^{o}. It shares 64 % similarity with ANAC019 NAC-domain. The TF fold of both domains are very similar and represent the canonical NAC-domain structure (Q. Chen et al., 2011) as depicted in Fig. 2.3a.

Modulation of DNA-binding specificity of TFs is most commonly achieved by the dimerization of DBDs (Müller, 2001). Size-exclusion chromatography of ANAC019 NAC-domain by Olsen et al., (2004) demonstrated that it mostly exists as a dimer in solution. The diffraction studies revealed the near-perfect non-crystallographic two-fold symmetrical axes and extensive contacts between the monomers involving conserved residues (Ernst et al., 2004). Interactions between the NAC-domain monomers involve two salt bridges formed by highly conserved Arginine and Glutamate at 19 and 26th positions. Also, at the dimer interface, a small antiparallel β-sheet is formed apart from several hydrophobic interactions, involving conserved residues. The core β-sheet is connected to the dimer

interface by a hinge structure; this grants flexibility to the dimer (Welner et al., 2016) as depicted in Fig. 2.3b. Although the interaction area is small (approximately 800 A^{o}), the multiple contact network between the various conserved residues strongly suggests that the dimerization is the preferred functional state and is a biologically active form (Ernst et al., 2004). This was indeed the case, as the ANAC019 dimer was found bound to an oligonucleotide (Welner et al., 2012).

The interaction surface of the ANAC019 NAC-domain was found to particularly positively charged regions suggesting it is well-suited for binding to DNA (Ernst et al., 2004). This was verified by Welner et al. (2012), who resolved the ANAC019 crystal structure with double-stranded DNA to 4.1 A^{o}. The crystal structure revealed that the NAC-domain inserts the outer β3 strand of its core β-sheet into the major groove of DNA helix, at approximately perpendicular axis leaving it undistorted. There is limited flexibility of the NAC-domain dimer leading, indicating its importance in recognizing the sub-optimal DNA binding sequence (Welner et al., 2012). Biochemical solution and low-resolution X-ray crystallography studies have revealed that ANAC019 NAC-domain binds to the major groove of DNA via the β-sheet with is highly conserved WKATGKD recognition motif forming a tight ANAC019-DNA complex (Zhu et al., 2014) as depicted in Fig. 2.3c. Interestingly, both WRKY and GCM (Glial Cell Missing) TF families bind to DNA using the central β-sheet with similar topology suggesting evolutionary conservation of TF fold (Welner et al., 2012).

The NAC TF NAC1 dimer was shown to bind to 35S CaMV promoter fragment to a 21-bp AS1-like motif by gel mobility shift assay (Xie et al., 2000). Later, the NAC TF recognition sequence (NACRS) was identified as the CACGCATGTG motif with CACG core in the ERD1 (Early Response to Dehydration 1) promoter (Simpson et al., 2003). NACRS was shown to be conserved binding sequence among many other NAC TFs (Hu et al., 2006; Jensen et al., 2010). Nevertheless, a few exceptions do exist; for instance, CBNAC (calmodulin-binding NAC TF) binds to a motif with GCTT core sequence (Ho et

al., 2007) and iron-deficiency responsive NAC TF, IDEF2 bound to IDE2 motif core sequence, CA(A/C)G(T/C)(T/C/A)(T/C/A) (Ogo et al., 2008).

The C-terminal region of NAC TFs is highly divergent and is involved in transcriptional regulation of NAC TFs. It can act as an activator or repressor of transcription (Addie Nina Olsen et al., 2005). The C-terminal region is rich in low-complexity amino acids such as serine, threonine, glutamine, and acidic amino acids and is comprised of sub-group specific motifs (Jensen et al., 2010). They do not possess a fixed three-dimensional structure but have low-complexity intrinsically disordered (ID) regions (Uversky et al., 2000).

The ID regions grant the NAC TF flexibility to interact with multiple proteins. For example, *Arabidopsis* NAC TF, CBNAC, can interact with calmodulin, a Ca^{+2}-binding protein (Ho et al., 2007). The over-expression of the C-terminal region (TRD) mirrored the hypersensitive response to ABA, as the full-length ANAC019 protein indicating that NAC TF C-terminal TRD can function independently (Jensen et al., 2010). The C-terminal regions of a few NAC TFs also possess a transmembrane domain, which helps in their localization to plasma-membrane or endoplasmic reticulum. Such NAC TFs are named NTLs and are larger than those of non-membrane anchored NACs and possess an α-helical trans-membrane domain (Kim et al., 2007). These membrane-anchored NAC TFs are activated by proteolytic cleavage, which liberates the NTLs from there transmembrane domain and allows them to translocate to the nucleus for modulation of gene expression. One such example is NTL8, which is proteolytically activated by environmental stress (Kim et al., 2007).

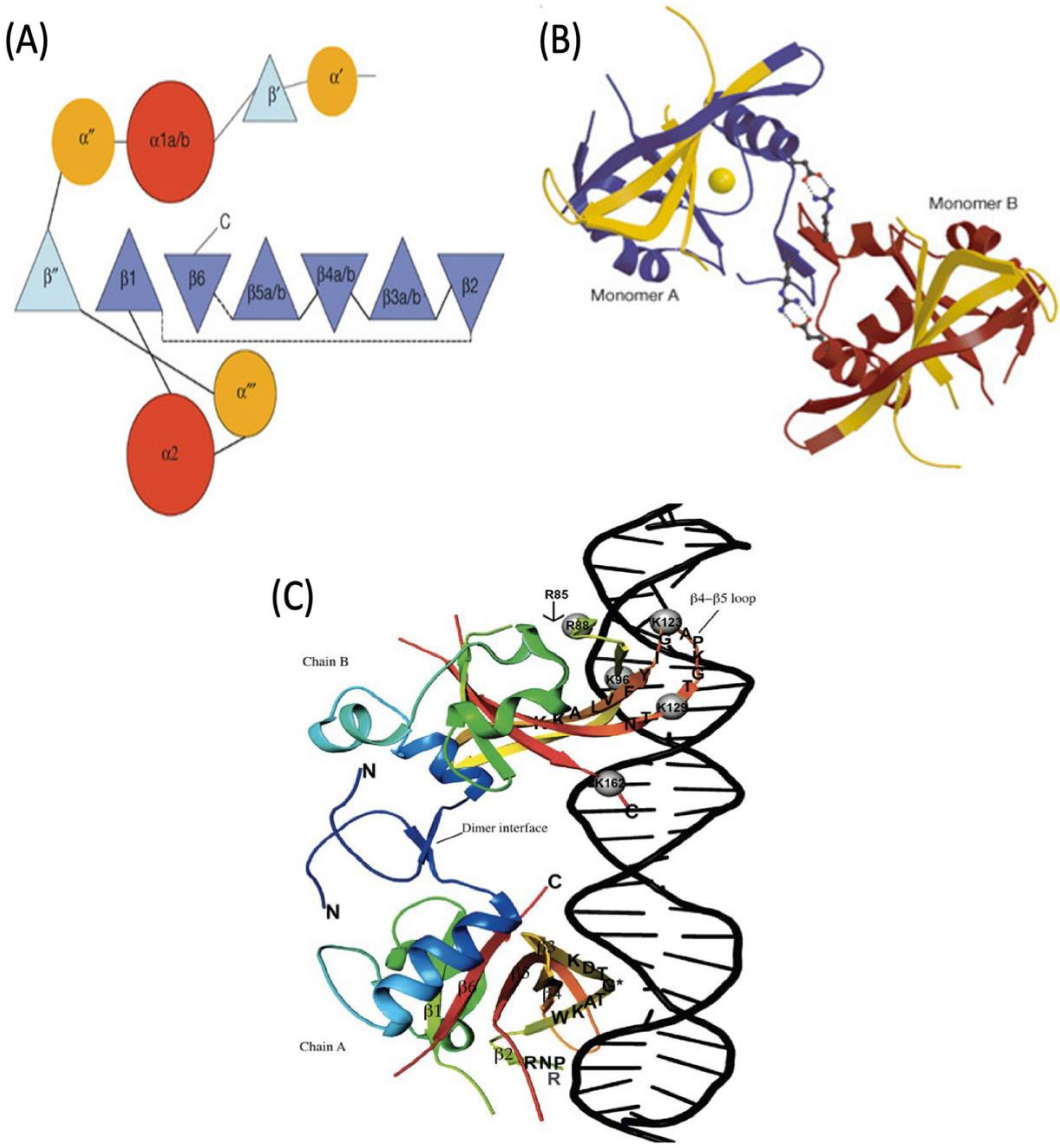


Figure 2.3 Structural features of NAC TFs.

(A) Topology diagram of ANAC019 TF. Red circles and dark blue triangles represent the main helices and twisted antiparallel β-sheet, respectively, while the orange circles and light blue triangles represent the secondary structures (Ernst et al., 2004).

(B) Functional dimer of ANAC019 NAC TF. The two NAC domains are represented in blue and red and the DNA binding region is depicted in yellow. The salt bridges between the conserved residues are also illustrated (Ernst et al., 2004).

(C) Crystal structure of NAC TF and dsDNA complex. The dimer is illustrated as binding to 26 bp DNA and the interaction surface between the two proteins is also represented in dark blue.

The recognition motif and the region predicted to bind DNA are highlighted as a one-letter code. Furthermore, amino acids predicted to bind to dsDNA are shown as gray spheres (Welner et al., 2016).

2.3 Expression of NAC transcription factors during abiotic stresses

Several NAC family members are induced by environmental stresses suggesting that they function in regulating stress responses. Due to the large number of NAC TFs, it is very challenging to identify their functions. However, genome-wide expression analysis has aided in identifying of various NAC TFs that re likely to play a role in environmental stress regulation. Differential expression of 33 NAC TFs was observed in *Arabidopsis* roots under salt stress (Jiang & Deyholos, 2006). Le et al. (2011) identified that dehydration stress treatment resulted in up and downregulated of 25 and 6 NAC members, respectively, in soybean. In rice, 45 NAC members were by upregulated various abiotic stresses, and 11 genes were induced by at least three stress treatments (Nuruzzaman et al., 2010). Similarly, 48 were induced out of the total 136 NAC TFs, in potato by either heat, mannitol or salt stress, and four members were upregulated by all the three treatments (A. K. Singh et al., 2013). Gong et al. (2019) identified 109 and 113 NAC TFs were differentially expressed under drought and cold stress treatments, respectively in white pear. Dehydration and flooding stress treatments strongly induced 18 and 31 NAC genes, respectively in sesame (Zhang et al., 2018). Abiotic stresses induced the expression of five NAC TFs out of 37 significantly in woodland strawberry (Zhang et al., 2018). Zhuo et al. (2018) identified 15 NAC members in the plum plant that showed differential expression to cold stress treatment. In melon, expression levels of 11 NAC genes from a total of 82 were upregulated by high salinity (Wei et al., 2016). Taken together, this strongly indicates that a significant portion of NAC TFs may significantly contribute to environmental stress regulation. However, substantial work is still needed to identify the distinct function of each NAC TF.

2.4 Identification of motifs in promoters of NAC transcription factor

The transcripts of NAC TFs are regulated at the transcription level by the presence of various *cis*-acting elements in the promoter regions. Motifs responsive to various phytohormones, environmental stress and tissue specificity were detected in the promoters of NAC TFs. Fang et al.

(2008) identified that 17 different motifs were enriched in the promoters of NAC TFs from rice. Motif responsive to auxin, SA, and GA, light, cold and seed-specific expression were observed. In the promoters of stress-inducible NAC TFs, motifs such as ABRE, DRE, and LTRE (Low-temperature responsive element) were also predicted. In a similar study, several motifs for phytohormones such as ABA, SA, auxin and JA apart from stress-responsive *cis*-acting elements such as MYBRS (MYB recognition sequence), HSE (Heat shock response element), and TC-rich repeats (defense-responsive motif) were predicted in the promoters of 36 pigeonpea stress-responsive NAC TFs (Satheesh et al., 2014). This suggests a vital role for promoter motifs in the stress-responsive expression of NAC TFs.

Genome-wide analysis of NAC TF promoters in upland cotton was enriched in HSE, TC-rich repeats, GT-1 box (salt and pathogen responsive element) and circadian motifs (developmental regulation). This suggest NAC TFs from cotton are likely to function in the developmental and stress signaling pathway (Sun et al., 2018). In another study, promoters of grain-specific NAC TFs from barley contained various seed and endosperm-specific motifs such as EBOXBNAPA, P-BOX2, RYREPEAT, AACA motif 2, MYBCORE, and MYB1AT suggesting the crucial role of promoter motifs in tissue-specific expression (Murozuka et al., 2018). Promoter analysis of poplar NAC TFs showed enrichment of phytohormone and stress-responsive *cis*-acting elements such as ABRE, ERE (ethylene response element), TC-rich repeats, TCA element (SA response element), CGTCA-motif (wound and JA responsive element), W-box (wound and SA response motif) in varying combination numbers (Wang et al., 2019). The promoters of watermelon NAC TFs exhibited several hormone and stress-responsive motifs like ARE (anaerobic induction responsive element), ABRE, TGACG and CGTCA-motifs (JA responsive elements), LTRE, TCA-element, ERE, HSE, and MYBRS (Lv et al., 2016).

Apart from the genome-wide promoter analyses of NAC TFs, characterization of promoters of individual genes has also been carried out. For example, the promoter of maize NAC TF, *ZmNAC55* was analyzed for *cis*-acting elements. Motifs such as ABRE, DRE, LTRE, MYCRS (MYC recognition

sequence) and MYBRS were predicted (Mao et al., 2016). *Arabidopsis ORE1/ANAC092/AtNAC2* (Oresara1) promoter contained motifs such as W-box, GT-1 box, HSE, and HD-ZIP recognition sequence while the *ORS1* (Oresara1 sister1) promoter had W-box, ASF1 element (auxin/SA response element), ATMYB2 (water stress response element) and WRKY TF recognition sequence. These *cis*-acting elements were also conserved in orthologous ORE1 and ORS1 promoters (Balazadeh et al., 2011).

Promoter sequence of rice *OsNAC6* exhibited different *cis*-acting elements that are known to be involved in responses to abiotic and biotic stresses such as ABRE, MYCRS, MYBRS, GCC-box (ERF TF binding sequence, pathogen response element), as-1 (osmotic stress-responsive element), and W-box (Nakashima et al., 2007). Another rice NAC TF, *ONAC066* promoter region was predicted to have motifs such as ABRE, CGTCA motif, GCC- box, TCA element, TC-rich repeats, and HSE (Yuan et al., 2019). Similarly, chickpea NAC TF, *carNAC4* promoter region exhibited several stress-responsive motifs like ABRE, LTRE, DRRE, MYCRS, MYBRS, PRE (proline and oxidative stress-responsive element), and GT-1 box suggesting the possible role of carNAC4 in environmental stress regulation (Yu et al., 2016). Taken together, this suggests the involvement of NAC TFs in various phytohormone, stress and developmental signaling pathways.

2.5 Characterization of NAC transcription factor promoters

Characterization of promoters is a useful technique to understand the spatial and temporal regulation of genes. However, promoters of only a few NAC TFs, mainly from *Arabidopsis* and rice, have been characterized so far for their induction and localization patterns. *OsNAC6* promoter-GUS fusion was induced by ABA, JA and various environmental stresses (Nakashima et al., 2007). *OsNAC5* promoter was induced by ABA and localized to roots and leaves under ABA and NaCl treatments (Takasaki et al., 2010). The expression of *SNAC1* promoter was observed in callus, root, leaf, guard cells, ligule, stamen, and pistil (Hu et al., 2006) while the promoter activity of *SNAC2* was observed

only in roots and internodes (Hu et al., 2008). The promoter-GUS fusion *of NST1* (NAC secondary wall thickening promoting factor 1) exhibited localization to the anthers, filaments of stamens, carpels and vascular bundles of the leaf, while *NST2* was mostly localized to the anther wall and pollen grains (Mitsuda, 2005).

The expression patterns of *ORE1/ANAC092/AtNAC2* and *ORS1* promoters were observed in cotyledons, senescent leaves, roots, mature floral tissues and pollen grains. Furthermore, the *ORS1* promoter was also salt and wound-inducible (Balazadeh et al., 2010, 2011). *ANAC012* was mainly localized to the vascular bundles, especially in the (pro)cambium region, xylem parenchyma cells along with shoot apical meristem (Ko et al., 2007). The promoter-GUS fusion of *VNI2* (VND interacting 2) was detected in the young leaves and roots apart from abscission zones of cauline leaves and axils of bracts (Yang et al., 2011). The promoter of *NTL4* (NAC with transmembrane motif 1-like 4) expression was localized to mature parts that exhibited symptoms of senescence and in roots. It is also strongly induced by air-drying (Lee et al., 2012), while the *NTL2* promoter was strongly induced by heat stress in leaves (Lee et al., 2014). *Arabidopsis ANAC102* promoter:GUS fusion was localized to the lateral root cap, vascular bundle, endodermis, radicle and was weakly induced by low-oxygen treatment (Christianson et al., 2009).

Grapevine *GvNAC042_5* is a NAC-like TF and was localized to vascular bundles, trichomes, shoot apical meristem, developing leaves and siliques, and the promoter was also induced by powdery mildew infection (Toth et al., 2016). Banana NAC TF, *MaNAC1* promoter was induced by cold stress and ethylene treatment, and was predicted to contain corresponding *cis*-elements (Shan et al., 2014). This confirms the multiple functions of NAC TFs ranging from environmental stress-responsive signaling to regulation of developmental processes like leaf senescence and possibly act as a node integrating multiple signaling pathways.

2.6 Domain analysis of NAC transcription factors

Although several studies have investigated the function of NAC TFs, very few studies have aimed at dissecting the structure-function relationships of NAC TFs. Jensen et al. (2010) identified ANAC019 as a positive regulator of ABA responses and demonstrated that the ectopic expression of full-length, N-terminal NAC-domain or C-terminal transcription regulatory (TR)-domain of ANAC019 alone conferred hypersensitivity to exogenous ABA.

Both the full-length and the individual domains of ANAC019 were able to induce the expression of various stress-inducible genes.

To understand the structure-function relationship of ANAC019 for ABA responsiveness, Jensen et al. (2010) developed chimeric proteins by swapping the NAC-domain and TR-domains with analogous regions from other NAC TFs. They revealed that replacing the NAC-domain of ANAC019 with the corresponding domain of other NAC TFs, the chimeric NAC protein still retained the ABA hypersensitivity. However, replacing the TR-domain of ANAC019 with equivalent TR-domain from other NAC TFs completely abrogated the ABA-responsiveness. Domain-swapping of ANAC019 demonstrated that the biochemical and functional specificity is associated with both the NAC and the TR-domains. They also determined that NAC-domains from various sub-groups were able to bind to the NAC TF recognition sequence (NACRS) with varying affinities. Moreover, most of the NAC-domains were dependent on the C-terminal TR-domain for the transactivation.

In another study, Taoka et al. (2004) investigated the domain specificity of *Arabidopsis* CUC TFs, which play an essential role in shoot apical meristem formation. Furthermore, over-expression of CUC1 or CUC2 resulted in adventitious shoot formation on the calli (Daimon et al., 2003). They demonstrated that TR-domains from three different NAC TFs could be fused to the NAC-domain of CUC2, which could still retain the shoot induction functionality of CUC2. However, replacing the CUC1 NAC-domain with ATAF NAC-domain resulted in the loss of shoot induction functionality. Domain

swapping of CUC TFs revealed that the NAC-domain is essential for the functionality of the CUC TFs. However, the structure-function relationship of NAC TFs, particularly concerning abiotic stress tolerance, remains largely unclear.

2.7 Role of NAC transcription factors in abiotic stress tolerance

NAC TFs play a significant role in diverse developmental and stress signaling pathways and are known to act as nodes integrating several regulatory pathways. Tomato NAC TF, *SlNAC1*, is activated by phytohormones such as ABA, gibberellins, methyl jasmonate (MeJA), salicylic acid (SA), and ethylene. *SlNAC1 is* also responsive to abiotic and biotic stresses like chilling, salinity, high-temperature stress, and wounding, indicating SlNAC1 functions as a node in diverse signaling modules (Ma et al., 2013).

Heat shock transcription factors (HSFs) primarily regulate gene expression associated with heat tolerance. Transcription factors such as HsfA1b, HsfC1, HsfA6b, and HsfA7a play pivotal roles in the upregulation of heat-inducible genes (Yoshida et al., 2011). A NAC TF, ANAC019, binds to the promoter of these HSFs and upregulates their expression during heat stress. ANAC019 serves as a regulatory hub integrating multiple transcription factor signaling networks (Guan et al., 2014).

ABA and abiotic stresses activate plasma membrane-bound NAC TF NTL6, and SnRK2.8 phosphorylates it. Phosphorylation of activated NTL6 is required for its nuclear transport leading to the induction of stress response. NTL6 directly integrates SnRK2.8 signaling pathway with ABA regulated abiotic stress adaptation (Kim et al., 2012).

Leaf senescence is a highly modulated developmental mechanism and is profoundly influenced by various environmental stress conditions. VNI2 (VND Interacting 2) is an ABA-responsive NAC TF, which integrates environmental stress responses into the regulation of leaf senescence. It also plays a negative role in the formation of xylem vessels (Yang et al., 2011). JUB1 (Jungbrunnen 1, ANAC042) is induced by H_2O_2 and plays a pivotal role in the senescence regulation. JUB1 is also responsive to various abiotic stresses and can transactivate DREB2A TF (A. Wu et al., 2012).

An ATAF subfamily NAC TF, OsNAC6, is upregulated by drought, chilling, high salt, wounding, ABA, and jasmonic acid (Ohnishi et al., 2005). Over-expression of OsNAC6 exhibited tolerance to abiotic and biotic stresses in rice. However, the transgenic plants also showed growth retardation and decreased yields (Nakashima et al., 2007), suggesting a possible role of OsNAC6 in integrating environmental stress signaling pathways with the regulation of growth and development.

A membrane-bound NAC TF, NTL6, is activated by cold exposure by proteolytic cleavage and enters the nucleus to directly interact and activate the expression of cold-inducible PR (Pathogenesis Related) genes, PR1, PR5, and PR2. Transgenic plants over-expressing an active NTL6 protein showed enhanced disease resistance at low temperatures, while NTL6 RNAi plants were highly susceptible to infection. This suggests that NTL6 acts as a molecular link in the adaptive process that connects freezing stress signal with pathogen resistance signaling cascade, thus, protecting plants against pathogens in the cold season (Seo et al., 2010). Furthermore, NTL6 is also activated by ABA. Transgenic plants over-expressing NTL6 active form exhibited ABA hypersensitivity and salinity stress tolerance at the seed germination stage. Taken together, the above observations suggest that NTL6 acts as a node integrating plant responses to abiotic and biotic stress signaling pathways (Seo and Park, 2010).

Another membrane-associated NAC TF, NTL8, plays an essential role in integrating external stress stimuli with the regulation of developmental processes such as flowering time. Membrane-associated NTL8 exists as a dormant form, and upon salt stress, it is processed into a nuclear transcriptionally active form. *Arabidopsis* over-expressing the active form of NTL8 displayed delayed flowering and growth retardation with curled leaf phenotype. The expression levels of the FT (Flowering locus T) gene and its target downstream genes were downregulated in NTL8 transgenic plants. Furthermore, the FT gene is highly repressed by salinity stress. This suggests that NTL8

functions as a salt-responsive regulator of membrane-mediated signaling of flowering time via the FT gene in *Arabidopsis* (Kim et al., 2007).

Additionally, NTL8 also is involved in the inhibition of seed germination by salinity stress via the gibberellic acid (GA) pathway (Kim et al., 2008). High salinity represses the GA biosynthetic gene, GA3 oxidase, and induces NTL8 expression in an ABA-independent way. Increased expression of NTL8 leads to inhibition of seed germination. This suggests an adaptive process of delaying seed germination during salt stress (Kim et al., 2008).

ERD1 (Early Response to Dehydration 1), an ATP binding subunit of ClpA protease (Caseinolytic ATP-dependent protease) is rapidly induced by dehydration in an ABA-independent manner and also during dark-induced senescence and natural senescence (Simpson et al., 2003). An investigation of the ERD1 promoter revealed that it has two separately located discrete motifs responsible for the induction during drought and senescence. Sequence integrity between these two motifs is required for gene expression of *ERD1* during drought and senescence (Simpson et al., 2003). The MYC-like sequence is bound by NAC TFs, ANAC019, ANAC055, and ANAC072, and the other *rps*1 site-like sequence is bound by ZFHD1 (Zinc-Finger Homeo Domain 1) TF. Transcriptional activation of *ERD1* requires both NAC and ZFHD TFs during dehydration stress (Tran et al., 2007).

NAC TF, *RD26* (Response to Dehydration 26) is highly upregulated by ABA, dehydration, and salt stress. Four ABREs were predicted in the *RD26* promoter region. Stress and ABA-responsive genes were induced in RD26-overexpressed plants and downregulated in RD26-repressed plants. This indicates that RD26 may regulate the expression of stress-inducible genes in an ABA-dependent manner (Fujita et al., 2004). ANAC096 also involved in the ABA-dependent modulation of dehydration stress by physically interacting with ABRE binding factors, ABF2 and ABF4. ABF2 and ANAC09 synergistically activate the transcription of RD29A during abiotic stress (Z. Y. Xu et al., 2013). *Arabidopsis* anac096 mutant is insensitive to ABA and exhibited enhanced water loss and reduced

ABA-induced closure of stomata under dehydration stress treatment. Moreover, *Arabidopsis* triple mutant anac096 abf2 abf4 exhibited increased sensitivity to osmotic stresses compared to double mutant abf2 abf4 and single mutant anac096 suggesting a synergistic relationship with ABF TFs in ABA-dependent activation of abiotic stress-responsive genes (Z. Y. Xu et al., 2013).

A NAC TF, SNAC1 (Stress-responsive NAC TF 1) from rice is shown to improve yield during severe drought stress in field conditions. Under drought stress, the expression is localized in the guard cells, and *SNAC1* over-expression caused ABA-induced stomatal closure. Transgenic rice expressing *SNAC1* also exhibited dehydration and salinity stress tolerance at all stages of plant development (Hu et al., 2006). This indicates a positive role for SNAC1 in seed setting under abiotic stress conditions.

All NAC TFs do not function as positive regulators of environmental stress adaptation. *Arabidopsis* ATAF1 was among the first few identified NAC TFs and was strongly induced by ABA, dehydration but was downregulated by water treatment. The ataf1 mutant exhibited a higher recovery rate than the wild-type plants during osmotic stress. Expression levels of various stress-responsive genes were induced in ataf1 mutant during drought stress. This indicates that ATAF1 is a negative regulator in the abiotic stress pathway (Lu et al., 2007).

The above increasing molecular evidence indicates that NAC TFs play significant roles in abiotic stress-mediated modulation of gene expression and function as nodes integrating various signaling cascades. They also participate in the complex cross-talk between ABA-dependent and independent signaling modules as depicted in Fig. 2.4.

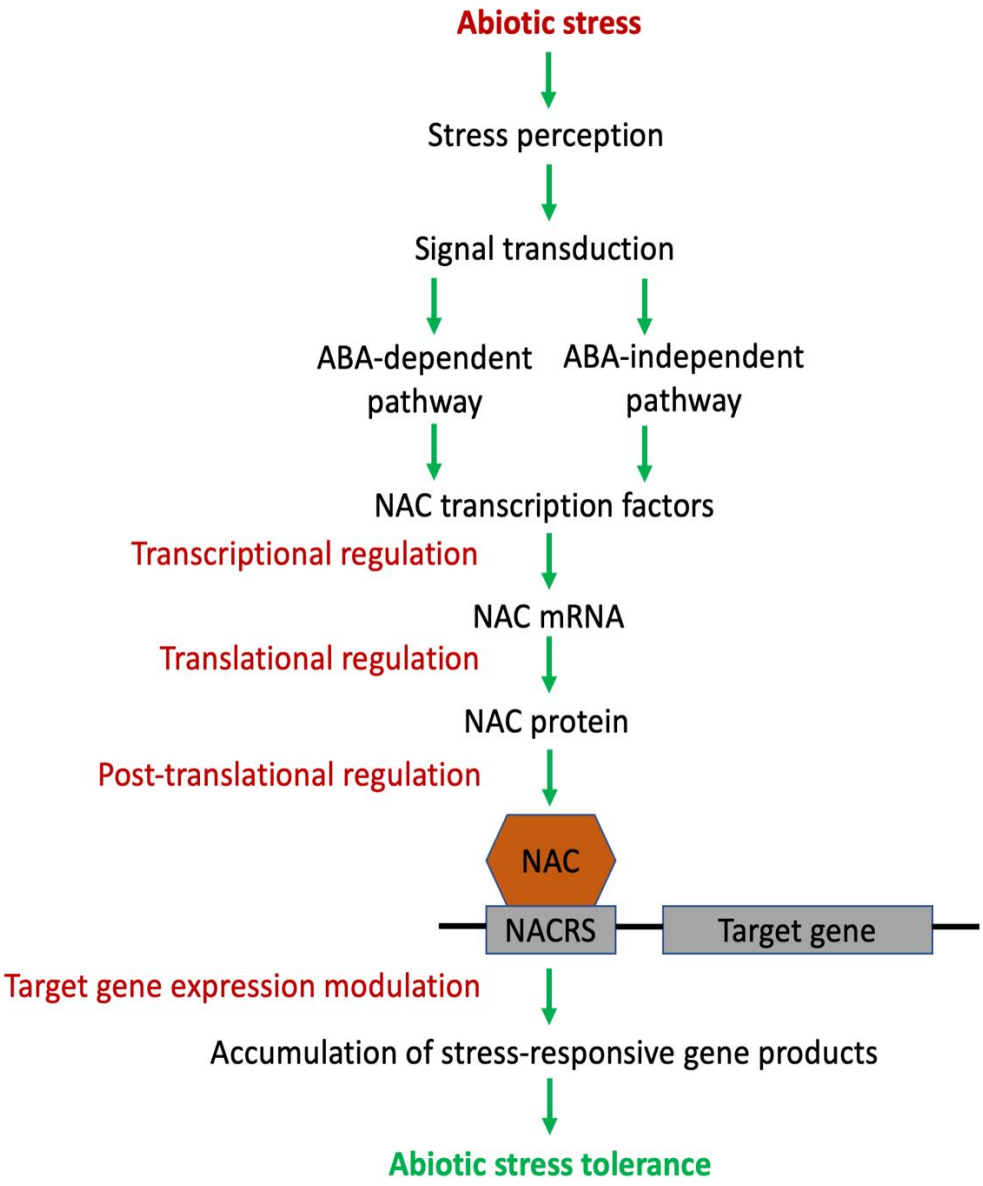


Figure 2.4 Diagram depicting NAC TFs as key component of abiotic stress regulatory pathway (Shao et al., 2015).

2.8 Objectives of the present study

1. Expression analysis of *GhNAC4* under various phytohormones and environmental stress conditions and prediction of *cis*-acting elements in the *GhNAC4*

2. Analysis of spatio-temporal localization and induction of *GhNAC4* promoter by phytohormones and environmental stresses

3. Structure-function relationship of GhNAC4 with regard to ABA and abiotic stress responses.

Chapter 3

Materials and methods

3.1 Plant growth conditions and stress treatments

3.1.1 Plant material and growth conditions

Leaves from two weeks-old cotton plants (*Gossypium hirsutum* var. JK Durga) were used for isolation of promoter, full-length gene or the regions encoding NAC and TR-domains in *GhNAC4*. Leaves from *in vitro* grown two weeks-old tobacco (*Nicotiana tabacum* cultivar Samsun) were used to generate transgenic plants expressing full-length *GhNAC4* or the regions encoding individual domains. The plants were maintained in a growth chamber at 26 ± 2 °C with 16 /8 h of light/dark.

3.1.2 Seed Sterilization

Cotton seeds were surface sterilized with 70 % ethanol for 2 min, followed by 4 % Sodium hypochlorite for 15 min. The seeds were then rinsed four times with sterilized water and soaked for 5-6 h. Subsequently, they were germinated and grown for two weeks on sterile blotting paper placed on top of 0.5x MS media without any growth regulators. The same methodology was followed for the germination of tobacco seeds.

3.1.3 Phytohormonal and stress treatments

For the expression analysis of the *GhNAC4* gene under various phytohormones and environmental stresses, treatments were given as described by Wang et al. (2005) with minor modifications. For hormonal treatments, the filter paper on which the seedlings were grown was moistened with either 100 µM abscisic acid (ABA), 100 µM methyl jasmonic acid (MeJA), 100 µM salicylic acid (SA), 20 µM 6-benzyl aminopurine (BAP), 20 µM gibberellic acid (GA_3) or 20 µM indole-3-acetic acid (IAA) and incubated for 24 h prior to sampling. As controls, untreated seedlings and filter paper moistened with water having the same quantity of sodium hydroxide or ethanol used for dissolving the hormones were also used. Ethylene treatment was carried out for 24 h by placing the seedlings on filter paper in a sealed container. Ethephon was added to the box and diluted to a final concentration of 10 ppm in distilled water. Seedlings in a similar container having air were used as a control.

High salt and osmotic stresses were induced by moistening the filter paper with 0.3 M NaCl and 0.3 M mannitol, respectively, and the seedlings were allowed to grow for 24 h. Oxidative and drought stresses were induced by moistening the filter paper with 10 µM methyl viologen and 15 % (w/v) polyethylene glycol (PEG) 8000, respectively, followed by incubation for 24 h. Air drying stress was carried out by placing the seedlings on the surface of dry filter paper for 30 min (Reference). Flooding stress was achieved by immersing the seedlings in distilled water for 24 h. Wounding stress was provided by squeezing the leaves with a forcep and harvesting them after 30 min. A combination of dark and cold treatments was induced by wrapping the seedlings in aluminum foil and incubating at 4 ^{o}C for 24 h, while the dark treatment was carried out at 25 ^{o}C for 24 h. High-temperature stress was achieved by subjecting the seedlings to 42 ^{o}C for 12 h. Following all the treatments, the leaves were quickly frozen in liquid nitrogen to analyze the expression levels of the *GhNAC4* gene.

To determine the degree of promoter activation by fluorometric GUS activity measurement, homozygous T_2 tobacco seedlings were subjected to different hormonal treatments and environmental stresses following the same methodology as applied to cotton seedlings.

3.2 Cloning of DNA fragments

3.2.1 Plasmids used in the study

Name	Purpose	Primary selection marker	Secondary selection marker	Reference
pTZ57R/T	TA cloning and blue-white screening	Ampicillin	-	Thermo Scientific (Catalogue no. K1213)
pRT100	Fusion of 35S CaMV promoter and polyadenylation signal with transgene	Ampicillin	-	Töpfer et al., 1987

pCAMBIA 2300	Plant expression vector for overexpression of transgene	Kanamycin	Kanamycin	CAMBIA (http://www.cambia.org)
pCAMBIA 1381Z	Promoter-less GUS gene for promoter localization	Kanamycin	Hygromycin	CAMBIA (http://www.cambia.org
pEGAD	N-terminal fusion of eGPF with transgene	Kanamycin	Basta	Cutler et al., 2000
pGBDU	Fusion of transgene with GAL4 DNA-binding domain	Ampicillin	Uracil	James et al., 1996
pGAD	Fusion of transgene with GAL4 activation domain	Ampicillin	Leucine	James et al., 1996

3.2.2 Genomic DNA isolation

Genomic DNA was isolated from the leaves by the CTAB (Cetyltriethylammonium bromide) method, as described by Murray and Thompson (1980) with minor modifications. Approximately 100 mg of leaf tissue was ground to a fine powder with liquid nitrogen and added to 1 ml of pre-warmed extraction buffer. The extraction buffer was composed of 1 % CTAB, 0.7 M NaCl, 50 mM Tris-HCl (pH 8.0), 1 % 2-mercaptoethanol, and 10 mM EDTA. The leaf tissue powder was thoroughly suspended in the extraction buffer and was incubated at 60 ^{o}C for 20-30 min with intermittent mixing. An equal volume of chloroform/isoamyl alcohol (24:1) was added to the extract and gently emulsified, followed by centrifugation at 12,000 rpm for 10 min at room temperature. The upper aqueous was collected, and the above step was repeated to clarify the extract further.

To precipitate the DNA, two volumes of ice-cold ethanol was added to the extract and incubated at -20 ^{o}C for 30 min before centrifugation at 12,00 rpm for 15 min at 4 ^{o}C. The supernatant was discarded, and the pellet was carefully rinsed twice with cold 70 % ethanol. The pellet was air-dried before dissolving in 50 µl of water at 37 ^{o}C.

3.2.3 Isolation and cloning of *GhNAC4* promoter from cotton

The full-length CDS of *GhNAC4* (GenBank accession number EU706342.1) was used as a query to retrieve the 5'-upstream sequence from Phytozome (https://phytozome.jgi.doe.gov/). Cotton genomic DNA was used as a template for PCR amplification of a DNA fragment, in the range of -1492 bp to +119 bp (relative to the transcription start site). The primer sequences (GhNACPRO-F, GhNACPRO-R) used for the amplification are mentioned in Table 3.9.1. The amplicon was cloned into pTZ57R/T (ThermoFisher Scientific, USA), and the accuracy was verified by sequencing. The fragment was excised and sub-cloned into *Bam*HI and *Pst*I restriction sites of the promoter-less vector pCAMBIA 1381Z (http://www.cambia.org) to generate a fusion gene having 5' upstream region of *GhNAC4* and *uidA* gene (pPRO$_{GhNAC4}$:GUS). This construct was used for *Agrobacterium tumefaciens* (strain EHA105) transformation by using the freeze-thaw method.

3.2.4 Total RNA isolation and cDNA synthesis

Total RNA was extracted from the leaves by the CTAB extraction procedure as described by Chang et al. (1993) with minor modifications. Approximately 3 g of leaf tissue was ground to a fine powder with liquid nitrogen and added to 15 ml of pre-warmed extraction buffer. The extraction buffer was composed of 2 % CTAB, 2 M NaCl, 2 % PVP (polyvinyl pyrrolidinone), 100 mM Tris-HCl (pH 8.0), 1 % 2-mercaptoethanol, and 25 mM EDTA. The leaf tissue powder was thoroughly suspended in the extraction buffer and was incubated at 60 ^{o}C for 20-30 min with intermittent mixing. An equal volume of chloroform/isoamyl alcohol (24:1) was added to the extract and gently emulsified, followed by centrifugation at 10,000 rpm for 10 min at room temperature. The upper aqueous was collected, and the above step was repeated to clarify the extract further. To precipitate the RNA, 0.25 volume of 10 M LiCl was added to the extract, incubated overnight at 4 ^{o}C, and later centrifuged at 12,000 rpm for 20 min at 4 ^{o}C. The pellet was resuspended gently in 500 µl of SSTE buffer (1 M NaCl, 0.5 % SDS, 10

mM Tris-HCl pH 8.0, and 1 mM EDTA). The suspension was clarified once with an equal volume of chloroform/isoamyl alcohol (24:1).

To precipitate the RNA, two volumes of ice-cold ethanol was added to the upper-aqueous phase and incubated at -20 ^{o}C for 30 min before centrifugation at 12,00 rpm for 15 min at 4 ^{o}C. The supernatant was discarded and the pellet was carefully rinsed twice with cold 70 % ethanol. The pellet was air-dried before dissolving in 100 µl of water at 37 ^{o}C.

To avoid DNA contamination, total RNA was treated with RNase free DNase1 (Epicentre Biotechnologies, USA) by incubating at 37 ^{o}C for 15 min. One µg of total RNA was used for synthesizing the first-strand cDNA using RevertAid 1st strand cDNA synthesis kit (Thermo Fischer Scientific, USA) following the manufacturer's instructions. An oligo-dT_{18} primer was used in the reverse transcription reaction.

3.2.5 Isolation and cloning of full-length and domains of *GhNAC4 gene*

The full-length cds of *GhNAC4* (80-1120, GenBank Accession no. EU706342.1), NAC-domain (80-496) and TRD (497-1120) were amplified using reverse-transcribed cDNA as a template. The primers used are mentioned in Table 3.9.1. The amplicons were cloned into pRT100 vector (GenBank no. A05521.1, Töpfer et al., 1987) for transcriptional fusion with the CaMV 35S promoter and polyadenylation signal. The expression cassettes were further cloned in to plant binary vector pCAMBIA 2300 (http://www.cambia.org) for constitutive expression of transgenes and *nptII* (Kanamycin resistance) selection gene in transgenic tobacco.

3.2.6 Sub-cellular localization of GhNAC4

Full-length *GhNAC4* CDS was fused N-terminally to EGFP present in pEGAD vector (Cutler et al., 2000) at the *Sma*I and *Bam*HI sites using primers listed in Table 3.9.1. The pEGAD:*GhNAC4* fusion construct was transfected into onion (*Allium cepa*) epidermal peels by agroinfiltration as described by Xu et al.

(2014). The peels were analyzed by laser scanning confocal microscopy (TCS SP2, Leica Microsystems, Germany). The experiment was performed in triplicates.

3.3 Bacterial and plant transformation

3.3.1 Bacterial strains used in the study

Escherichia coli strain, DH5α, was used for cloning and maintenance of vectors and *Agrobacterium tumefaciens* strain, EHA104, was used for tobacco transformation.

3.3.2 *Escherichia coli* competent cell preparation

For the preparation of *E.coli* competent cells, a single colony of DH5α plated on a Luria Agar (LA) was inoculated in 5 ml of Luria Broth (LB) medium. It was incubated overnight with continuous shaking at 200 rpm and 37 ^{o}C. An aliquot of 100 µl of the primary culture was inoculated in 100 ml of LB medium and incubated at 37 ^{o}C and 200 rpm till the optical density at 600 nm (OD_{600}) reached 0.5-0.6. Later, the secondary culture was cooled on ice for 30 min before centrifuged at 5,000 rpm for 10 min at 4 ^{o}C. The supernatant was discarded, and the pellet was gently resuspended in 100 ml of ice-cold 100 mM $CaCl_2$ and incubated on ice for 1 h with occasional gentle mixing. After the incubation period, the culture was centrifuged at 5,000 rpm for 10 min at 4 ^{o}C, and the supernatant was discarded. The pellet was finally resuspended in a freezing mixture consisting of 1.5 ml of ice-cold 100 mM $CaCl_2$ and 500 µl of ice-cold 10 % glycerol (prepared in 100 mM $CaCl_2$). Aliquots of 100 µl of the above suspension were prepared and frozen in liquid nitrogen before storing at -70 ^{o}C.

3.3.3 *Escherichia coli* transformation

For *E.coli* transformation, 50-100 ng/µl plasmid DNA or 10 µl of ligation mixture was added to an aliquot of DH5α competent cells and gently mixed before incubating on ice for 30 min. The cells were then subjected to heat shock by incubating at 42 ^{o}C for 90 s followed by ice for 20 min. 0.8 ml of LB medium was added to the treated cells and incubated at 37 ^{o}C and 200 rpm for 1 h. After the incubation period, the culture was centrifuged at 5,000 rpm at room temperature. The supernatant

was discarded and the pellet was resuspended in 200 µl of fresh LB medium. The transformed cells were plated on selection plates (LA supplemented with selection agent) and incubated overnight at 37 ^{o}C.

3.3.4 *Agrobacterium* competent cell preparation

Agrobacterium EHA104 competent cells were prepared in a similar fashion as *E.coli* cells as mentioned in section 3.3.2 with the following modifications. A single colony was incubated in LB medium supplemented with 100 mg/l Rifampicin at 28 ^{o}C for 48 h. Secondary culture was achieved by inoculating 100 µl of primary culture in 100 ml of LB medium supplemented with 100 mg/l Rifampicin at 28 ^{o}C till the OD_{600} reaches 0.6-0.8. The subsequent steps required for *Agrobacterium* competent cell preparation are same as *E.coli*.

3.3.5 *Agrobacterium* transformation

For *Agrobacterium* transformation, 1 µg of plasmid DNA was added to an aliquot of EHA104 competent cells and gently mixed before incubating on ice for 30 min. The cells were then subjected to freeze-thaw treatment by freezing in liquid nitrogen for 5 min, followed by incubating in 37 ^{o}C water bath for 5 min. One ml of LB medium was added to the treated cells and incubated at 28 ^{o}C and 200 rpm for 4 h. After the incubation period, the culture was centrifuged at 5,000 rpm at room temperature. The supernatant was discarded, and the pellet was resuspended in 200 µl of fresh LB medium. The transformed cells were plated on selection plates (LA supplemented with 100 mg/l Rifampicin and selection agent) followed by incubation at 28 ^{o}C for 3 days.

3.3.6 Generation of transgenic tobacco plants

Transgenic tobacco plants carrying the transgene or empty vector were generated using the standard *Agrobacterium*-mediated leaf disc transformation method as described by Horsch et al. (1985) with minor modifications. *in vitro* grown three weeks old *Nicotiana tobacum* cv. Samsun leaves were used as explants. Leaf discs were co-cultivated with *Agrobacterium* on a co-cultivation medium (MS salts

with 2 mg/l 6-benzyl aminopurine (BAP), 0.1 mg/l 1-naphthaleneacetic acid (NAA) and 3 % sucrose pH 5.8) for 48 h in dark and later transferred to the shoot induction medium (MS salts with 2 mg/l BAP, 0.1 mg/l NAA, 3 % sucrose, 10 mg/l Hygromycin or 100 mg/l Kanamycin and 250 mg/l Cefotaxime pH 5.8) with 16/8 h light/dark photoperiod at 24 ± 2 ^{o}C. The regenerated shoots were sub-cultured on shoot elongation medium (MS salts with 1 mg/l BAP, 0.05 mg/l NAA, 3 % sucrose, 15 mg/l Hygromycin or 125 mg/l Kanamycin and 250 mg/l Cefotaxime pH 5.8). The selection marker resistant shoots were further sub-cultured on rooting medium (0.5x MS salts with 0.1 mg/l NAA and 20 mg/l Hygromycin or 150 mg/l Kanamycin pH 5.8). Plants with well-developed roots were transferred to sterile soil in small plastic cups for acclimatization. Well-established plants were shifted to pots and allowed to grow to maturity in a greenhouse and set seeds.

The putative primary tobacco transformants (T_0) were screened by genomic PCR for the presence of transgene and selection marker gene (Hygromycin or Kanamycin gene, primers mentioned in Table 3.9.1) and allowed to self-pollinate and set seeds. The T_1 seeds from selected plants were germinated on 0.5x MS medium supplemented with 25 mg/l Hygromycin or 150 mg/l Kanamycin. Copy number of the DNA integration was determined by selection marker segregation test to select the single copy integration events that give a ratio (similar to the monohybrid ratio) of resistant (green) and susceptible (yellow) seedlings. The resistant seedlings were later transferred to soil in the greenhouse after 2-3 weeks and allowed to set seeds.

The T_2 progeny were germinated on 0.5x MS medium supplemented with 25 mg/l Hygromycin or 150 mg/l Kanamycin to identify the homozygous single-copy integration T_2 lines that exhibit 100 % germination on the selection medium. The constitutively expressing tobacco transgenics of the *GhNAC4* promoter were labeled as Pro$_{GhANC4}$:GUS. While the constitutively expressing tobacco transgenics of full-length *GhNAC4*, NAC-domain and TR-domain were named as GhNAC4, GhNAC4-N, GhNAC4-C genotypes, respectively.

3.4 Expression analysis of *GhNAC4* gene

To assess the transcript levels of GhNAC4 in cotton seedlings, quantitative real-time PCR (qPCR) was employed. For this, two weeks old seedlings of cotton were treated with various phytohormones and environmental stresses, as mentioned in section 3.1.3. For the real-time quantitative PCR, cDNA was diluted to 100 ng/µl and was mixed with SYBR master mix (Kapa Biosystems, USA), and amplification was carried out following the manufacturer's protocol in the StepOne Plus machine (Applied Biosystems, USA).

The constitutively expressing Ubiquitin gene (*GhUBQ7*, GenBank accession no. DQ116441) from cotton was used as an internal reference gene. The primer sequences used to amplify the internal regions of *GhNAC4* (NAC4-RTF and NAC4-RTR), and *GhUBQ7* (UBQ7-RTF and UBQ7-RTR, Kuppu et al., 2013) are mentioned in Table 3.9.2. To ensure gene specificity, the amplicons obtained using these primers were confirmed by sequencing. The fold change in the *GhNAC4* transcripts levels relative to *GhUBQ7* transcripts was determined using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001). The experiments were performed in triplicates, and two independent biological replicates were used in the analyses.

3.5 *GhNAC4* promoter analysis and characterization

3.5.1 Bioinformatic analysis of *GhNAC4* promoter sequence

The transcription start site was predicted by the Softberry database (Shahmuradov et al., 2017, www.softberry.com), using the default settings. GC composition was analyzed by BioEdit (Hall 1999, http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and the percentage of sequence similarity was analyzed using LALIGN (https://www.ebi.ac.uk/Tools/psa/lalign/). A search for the putative *cis*-acting regulatory elements in the promoter sequence was conducted using the PlantPAN 2.0 (Chang et al., 2008, http://plantpan2.itps.ncku.edu.tw/), PLACE (Higo et al., 1999,

https://www.dna.affrc.go.jp/PLACE/) and PlantCARE (Lescot et al., 2002, http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) databases. Only statistically significant motifs (P-value > 0.9) were selected.

3.5.2 Histochemical localization of GUS activity

β-glucuronidase(GUS) activity was assayed as described by Jefferson et al. (1989) with minor modifications. For histochemical staining, the tissues were vacuum infiltrated with the solution containing 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-b-D-glucuronide), 1 mM Potassium ferrocyanide, 1 mM Potassium ferricyanide, 1 mM EDTA and 0.1 % Triton X-100 in 50 mM phosphate buffer (pH 7.0) and were incubated at 37 ^{o}C for 12-14 h in the dark. After staining, the tissues were fixed in a solution containing 4 % formaldehyde in 50 mM phosphate buffer (pH 7.0) for 12 h at 4 ^{o}C and subsequently cleared in 70 % ethanol at room temperature. Photographs were taken using M165 FC and DM6B microscopes (Leica Microsystems, Germany).

3.5.3 Fluorometric measurement of GUS activity

For the fluorometric assay, the tissue was homogenized in 400 µl GUS extraction buffer containing 10 mM EDTA and 0.1 % Triton X-100 in 50 mM phosphate buffer (pH 7.0). After centrifugation at 12,000 rpm (4 ^{o}C) for 15 min, 5 µl of homogenate was diluted with 95 µl of extraction buffer and mixed with 100 µl of extraction buffer having 2 mM 4-methyl-umbelliferyl-β-D-glucuronide (4-MUG, Duchefa, Netherlands) and incubated at 37 ^{o}C for 1 h. The reaction was terminated by the addition of 1.8 ml of 200 mM sodium carbonate. Total protein concentration in the homogenate was assessed by the Bradford method (Bradford 1976) with Bovine Serum Albumin (BSA) as standard. Fluorescence (excitation 363 nm, emission 447 nm) was determined by Infinite 200 plate reader (Tecan, Switzerland) and GUS activity was expressed as pmol of 4-methyl-umbelliferone (4-MU, Sigma, USA) per µg protein per min. 4-MU in the range of 20 nM - 100 µM was used to generate a standard curve. Each MUG assay was performed in triplicate and repeated three times.

3.6 Yeast-based assays

3.6.1 Yeast competent cell preparation and transformation

Competent cell preparation and transformation of yeast using the polyethylene glycol-lithium acetate method was followed as described by Gietz and Schiestl (2007) with minor modifications. A single colony of *Saccharomyces cerevisiae* strain pJ694a (James et al., 1996) was inoculated in yeast extract peptone dextrose broth (YPDB) medium and incubated overnight at 30 ^{o}C and 200 rpm. An aliquot of 100 µl of the primary culture was inoculated in 100 ml of YPDB medium and incubated at 30 ^{o}C and 200 rpm till the optical density at 600 nm (OD_{600}) reached 0.5-0.6. The secondary culture was centrifuged at 3,000 rpm at 20 ^{o}C, and the supernatant was discarded. The pellet was resuspended in 25 ml of water and centrifuged as above. The wash step was repeated with 25 ml of 0.1 M lithium acetate, and the pellet was transferred to a 1 ml microfuge tube. The pellet was rinsed again with 1 ml of 0.1 M lithium acetate at 13,000 rpm for 30 s (room temperature). The pellet was resuspended in 1 ml of 0.1 M lithium acetate, and aliquots of 100 µl were prepared. The cells were centrifuged again at 13,000 rpm for 30 s (room temperature) and the supernatant was discarded.

An aliquot of 360 µl of transformation mix was added to each tube containing the pellet. The transformation mix comprised of 240 µl of PEG 3500 (50 % W/V), 36 µl of 1 M lithium acetate, 50 µl of single-stranded salmon sperm carrier DNA (2 mg/ml), 33 µl of water and 1 µl of plasmid DNA (100-200 ng/µl). The carrier DNA was heat-denatured for 5 min in boiling water bath and immediately placed on ice before added to the transformation mix. The cells were thoroughly resuspended in the transformation mix by vortexing and incubated at 42 ^{o}C for 40 min. After the incubation period, the transformed cells were pelleted at 13,000 rpm for 30 s (room temperature), and the supernatant was thoroughly removed. The pellet was resuspended in 50-100 µl of sterile water and plated on selection plates followed by incubation at 30 ^{o}C for 2-3 days.

3.6.2 Site-directed mutagenesis

Point mutations and deletion constructs of *GhNAC4* CDS were developed following the site-directed mutagenesis protocol of Edelheit et al. (2009). The primers for inducing various point mutations were designed with the help of PrimerX software (https://www.bioinformatics.org/primerx/) and are listed in Tale 3.9.3.

3.6.3 Transcription activation assay

Full-length *GhNAC4* CDS and a series of deletion derivatives were cloned separately into the GAL4 DNA-binding domain vector, pGBDU (James et al., 1996) using primers listed in Table 3.9.1. The constructs were transformed pJ694a strain and plated on Synthetic Dropout (SD) medium lacking uracil (Ura^-) for 3 d at 30 ^oC. For the qualitative growth assay, three colonies were picked, and overnight grown cultures in SD Ura^- media were diluted to 1.0 OD units. They were then serially diluted 10-fold, and 5 µl was spotted on SD Ura^- media and SD Ura^- media either lacking histidine or adenine ($Ura\ His^-$/ $Ura\ Ade^-$). The transcriptional activity was evaluated based on the growth after 3 d at 30 ^oC. The empty vector pGBDU was used as a negative control. The experiment was repeated with three independent transformations. For the quantitative assay, β-galactosidase activity was measured by the O-nitrophenyl-β-galactopyranoside (ONPG) assay method.

3.6.4 Yeast two-hybrid assay

Various deletion constructs of *GhNAC4* CDS were cloned into the GAL4 DNA-binding domain (pGBDU) and the activation domain (pGAD) vectors (James et al., 1996) using primers listed in Table 3.9.1. The constructs were transformed into the yeast strain, pJ694a and plated on SD medium lacking uracil and leucine ($Ura\ Leu^-$) for 5 d at 30 ^oC. For the qualitative growth assay, three colonies were picked and overnight grown cultures in SD $Ura\ Leu^-$ media. They were then serially diluted 10-fold, and 5 µl was spotted on SD $Ura\ Leu^-$ media and SD $Ura\ Leu^-$ media either lacking histidine or adenine ($Ura\ Leu\ His^-$/ $Ura\ Leu\ Ade^-$). The protein-protein interaction was evaluated based on the growth after 5 d at

30 ^{o}C. The empty vectors pGBDU and pGAD were used as negative controls. The experiment was repeated with three independent transformations. For the quantitative assay, β-galactosidase activity was measured by the ONPG assay method.

3.6.5 Beta-galactosidase assay

Beta-galactosidase activity was essentially assayed following Miller (1972) with the following modifications. Single yeast colony was grown in selective media (Ura^{-} or Ura Leu^{-}) overnight, and secondary cultures were grown to an absorbance (OD_{600}) of 0.8-1.0. A 5 ml sample of the secondary culture was pelleted by centrifugation at 2,000 x g and resuspended in 5 ml of Z-buffer and OD_{600} was again recorded. The composition of the Z-buffer is 60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4 and 50 mM β-mercaptoethanol pH 7.0. To an 800 µl aliquot of the culture, 50 µl each of 0.1 % sodium dodecyl sulphate and chloroform was added and the mixture was vortexed briefly for permeabilization. After 5 min incubation, 160 µl of ONPG solution (4 mg/ml in Z-buffer, HiMedia) was added and vortexed briefly before incubating at 30 ^{o}C for 2 min to 4 h. The reaction was terminated by the addition of 400 µl of 1 M Na_2CO_3. The sample was centrifuged for 2 min at 16000 x g, and the absorbance of the supernatant read at 420 nm and 550 nm. The enzyme activity was expressed in Miller units. Mean values and standard error were calculated from four measurements obtained from three independent yeast transformants.

3.7 Domain analysis of GhNAC4 in tobacco seedlings

3.7.1 Seed germination assay

For the seed germination assay, seeds of WT, GhNAC4, GhNAC4-N, GhNAC4-C genotypes were harvested at the same time and stored under the same conditions. They were surface-sterilized in 70 % (v/v) ethanol for 2 min, followed by 15 min in 4 % (w/v) in aqueous Sodium hypochlorite and rinsed five times with sterile distilled water. Seeds were sown on half-strength salts of Murashige-Skoog (0.5x MS) media (Murashige and Skoog 1962) containing 0.6 % Gelzan (Sigma-Aldrich) pH 5.7,

supplemented with or without 150 mM NaCl and 2 µM ABA (mixed isomers, Sigma-Aldrich). The -0.5 MPa PEG 8000 (HiMedia) infused plates were prepared according to van der Weele et al (2000). Germination rates were scored on the 7 d for radicle emergence or presence of green cotyledons. The experiment was conducted in triplicate with 80-100 seeds for each genotype and experiment.

3.7.2 Root elongation assay

For the post-seed germination growth assay, 7 days old seedlings germinated on 0.5x MS media were transferred to media containing 200 mM NaCl and 10 µM ABA (mixed isomers, Sigma-Aldrich). The -0.7 MPa PEG 8000 (HiMedia) infused plates were prepared according to van der Weele et al (2000). All the plates were placed upright in the growth chamber. The primary root lengths were measured on 15 d post-transfer. The experiment was conducted in triplicate with at least 18-21 seedlings for each genotype and experiment.

3.7.3 Stomatal bioassay

Epidermal strip assay was performed as described by McAinsh et al (1996) with slight modifications. Epidermal strips from the abaxial side were peeled and floated in opening buffer (10 mM MES-KOH and 50 mM KCl, pH 6.1) under a photon flux density of 200 µmol m^2 s^{-1} for 3 h. Subsequently, 10 µM ABA (dissolved in 95 % ethanol) and an equal volume of ethanol (used as control) were added to the buffer. After 150 min of incubation, the peels were imaged using the light microscope (CX21, Olympus). The width of the stomatal aperture was measured using the pre-calibrated ocular micrometer of ProgRes CapturePro image analyzer (Jenoptik, Germany). Approximately 60 stomatal apertures were measured for each genotype and the experiment was repeated three times.

3.7.4 Water loss assay

Leaves from well-watered one-month-old WT, GhNAC4, GhNAC4-N and GhNAC4-C plants were excised and weighed immediately. The excised leaves were left at room temperature and weighed every 1 h (Li et al., 2019). Water loss was calculated as a percentage of loss in fresh weight over a

period of time. Six leaves from separate plants for each genotype were used and the experiment was repeated three times.

3.7.5 Leaf disc assay

The third leaf from the top of one-month-old plants of WT, GhNAC4, GhNAC4-N and GhNAC4-C genotypes was used for the leaf disc assay. Using a cork borer, leaf discs of 1 cm diameter were punched and floated on 200 mM NaCl and 15 % PEG 8000 for 4 d at 24 ± 2 ^{o}C. The experiment was repeated three times.

3.7.6 Measurement of total chlorophyll content

The 100 mg samples of treated leaf discs were used for extracting total chlorophyll using 80 % acetone, according to Arnon (1949). Total chlorophyll content was expressed as mg/g FW. This experiment was repeated three times.

3.7.7 Measurement of the extent of lipid peroxidation

The treated leaf discs (100 mg) were homogenized in 1 ml of 0.1 % trichloroacetic acid (TCA) and centrifuged at 10,000x g for 10 min. To the supernatant, 5 ml of Thiobarbituric acid (0.5 % in 20 % TCA solution) was added, and the mixture was boiled for 25 min at 100 ^{o}C. The reaction was terminated by quick cooling on ice, followed by centrifugation at 10,000x g for 5 min. The supernatant was used to measure Malondialdehyde (MDA) content, according to Heath and Packer (1968). The MDA content was expressed as nmoles/g FW. The experiment was repeated three times.

3.7.8 Measurement of proline content

The treated leaf discs (100 mg) were homogenized in 0.5 ml of 3 % sulfosalicylic acid and centrifuged at 10,000 x g for 10 min. To 100 µl of supernatant, a mixture of 100 µl of 3 % sulfosalicylic acid, 200 µl of glacial acetic acid and 200 µl of acid Ninhydrin were added. The mixture was boiled for 60 min at 100 ^{o}C, and the reaction was terminated by quick cooling on ice. The samples were extracted with toluene, and the upper organic phase was used for measuring the proline content, according to Bates

(1973). The proline content was expressed as µmoles/g FW. The experiment was repeated three times.

3.7.9 Expression analysis of stress marker genes in tobacco

To assess the transcript levels of ABA and stress-responsive genes in tobacco seedlings, quantitative real-time PCR (qPCR) was employed. For this, two weeks old seedlings of wild-type, GhNAC4, GhNAC4-N, GhNAC4-C genotypes were treated with 200 mM NaCl and 15 % PEG 8000 for 24 h. Total RNA was extracted using Trizol reagent (Invitrogen, USA), and DNA contamination was removed by treating it with RNase-free DNase I (Takara Bio, China). Approximately 1 µg of total RNA was used for the first-strand cDNA synthesis using RevertAid cDNA synthesis kit (Thermo Fischer Scientific, USA) according to the manufacturer's instructions. For the qPCR, cDNA was diluted to 100 ng/µl and was mixed with Green Premix Ex Taq II (Takara Bio, China), and amplifications were carried out following the manufacturer's protocol.

The constitutively expressing Ubiquitin gene (*NtUBI1*, GenBank Accession no. U66264.1) from tobacco was used as an internal reference gene. The stress-responsive genes used in the study are *NtAPX* (U15933.1), *NtCAT1* (U93244.1), *NtERD10C* (AB049337.1), *NtERF5* (AY655738.1), *NtDREB3* (EU727157.1), *NtMnSOD* (AB093097.1), *NtNCED3* (JX101472.1), *NtSOS1* (XM_009789739.1), and *NtSUSY* (AB055497.1). The primers of stress-associated genes are mentioned in Table 3.9.4. The fold change was determined using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001). The experiments were performed in triplicates, and two independent biological replicates were used for the analyses.

3.8 Statistical analysis

All experiments were repeated at least three times, and the data were expressed as the mean ± SE. Error bars shown are the standard errors of the experimental data. Data were analyzed by one-way analysis of variance (ANOVA) using Sigma Plot 11.0 software. *** $P<0.001$, ** $P < 0.01$ and * $P < 0.05$

represent significant differences at 0.1, 1 and 5 % level respectively. 'ns' represents no significant difference.

3.9 Primers used in the study

Table 3.9.1 Primers used for cloning of *GhNAC4* promoter, *GhNAC4* full-length cds, NAC-domain (GhNAC4-N) and transcriptional regulatory domain (GhNAC4-C)

Gene	Primer name	Primer sequence
GhNAC4 promoter (-1492 bp to +119 bp)	GhNACPRO-F	5' TATCTGGATCCGGCCTCATAATTGCATCAC 3' *Bam*HI
	GhNACPRO-R	5' TACATACTGCAGGATTCTAAAGTTTTCTTGCCG3' *Pst*1
GhNAC4 full length (80-1120)	GhNAC4- pRT100-F	5' ATCTCGAGATGGGAGTGCCGGAAACTG 3' *Xho*I
	GhNAC4- pRT100-R	5' GCCGGATCCTTATTGTCTAAACCCAAATCC 3' *Bam*HI
	GhNAC4-pEGAD-F	5' ATTCCCGGGATGGGAGTGCCGGAAAC 3' *Sma*I
	GhNAC4-pEGAD-R	5' GCCGGATCCTTATTGTCTAAACCCAAATCC 3' *Bam*HI
	GhNAC4-yeast-F	5' ATTCCCGGGATGGGAGTGCCGGAAAC 3' *Sma*I
	GhNAC4-yeast-R	5' GCCGGATCCTTATTGTCTAAACCCAAATCC 3' *Bam*HI
GhNAC4-N (80-496)	GhNAC4-N-pRT100-F	5' ATTCCCGGGATGGGAGTGCCGGAAAC 3' *Sma*I
	GhNAC4-N- pRT100-R	5'ATAGGATCCTTATCGATATTCATGCATAATC 3' *Bam*HI
	GhNAC4-N-yeast-F	5' ATTCCCGGGATGGGAGTGCCGGAAAC 3' *Sma*I
	GhNAC4-N-yeast-R	5'ATAGGATCCTTATCGATATTCATGCATAATC 3'

		*Bam*HI
GhNAC4-C (80-496)	GhNAC4-C-pRT100-F	5’ ATTCCCGGGATTGAAACTTCTCGTAAAAG 3’ *Sma*I
	GhNAC4-C- pRT100-R	5’ GCCGGATCCTTATTGTCTAAACCCAAATCC 3’ *Bam*HI
	GhNAC4-C-yeast-F	5’ ATTCCCGGGATTGAAACTTCTCGTAAAAG 3’ *Sma*I
	GhNAC4-C-yeast-R	5’ GCCGGATCCTTATTGTCTAAACCCAAATCC 3’ *Bam*HI
nptII	nptII-F	5’ AGATGGATTGCACGCAGGTTCTC 3’
	nptII-R	5’ ATCGGGAGCGGCGATACCGTA 3’
HptII	HptII-F	5’ TATTTCTTTGCCCTCGGACGA 3’
	HptII-R	5’ ATGAAAAAGCCTGAACTCACC 3’

Table 3.9.2 Primers used for expression analysis of *GhNAC4* in cotton seedlings

Gene	Primer name	Primer sequence
GhNAC4 (EU706342.1)	NAC4-RTF	5’ TTCTCGTAAAAGTGGTAGCTCC 3’
	NAC4-RTR	5’ TCCAGTTGTGAAGAAGACGATG 3’
GhUB7 (DQ116441)	UBQ7-RTF	5’ AGAGGTCGAGTCTTCGGACA 3’
	UBQ7-RTR	5’ GCTTGATCTTCTTGGGCTTG 3’

Table 3.9.3 Primers used for generating GhNAC4 deletion constructs and point mutations for yeast-based assays

Deletion variant	Primer name	Primer sequence
GhNAC4 1-346	GhNAC4-F	5’ ATTCCCGGGATGGGAGTGCCGGAA 3’ *Sma*I
GhNAC4 Δ1-320-C1	CDOM-R1	5’ ATACTGCAGTTATTGTCTAAACCCAAATCCACC 3’ *Pst*1

GhNAC4 Δ1-290-C2	CDOM-R2	5’ GTACTGCAGTTAGTTCAACACATTCGAGTTTTG 3’ *Pst*1
GhNAC4 Δ1-260-C3	CDOM-R3	5’ GTACTGCAGTTAACCAATCTTATTTGTCGACAC 3’ *Pst*1
GhNAC4 Δ1-229-C4	CDOM-R4	5’ GTACTGCAGTTACTGACTCTGAGTTTGAGTTTG 3’ *Pst*1
GhNAC4 Δ1-199-C5	CDOM-R5	5’ ATACTGCAGTTAACCCAGACT TTGAAACCCC 3’ *Pst*1
GhNAC4 Δ1-169-C6	CDOM-R6	5’ ATACTGCAGTTAGGGCAATGACTCAAGCATG 3’ *Pst*1
GhNAC4 Δ169-189-D1	20 Del-F	5’ GTCAAAAACCTCAACTGGATGACAT3’
	20 Del-R	5’ ATGTCATCCAGTTGAGGTTTTTGAC3’
GhNAC4 Δ245-261-D2	15 Del-F	5’ TTAGCTCGGTGCCTGGGATTACTAGTTATGGAAATAGTAA 3’
	15 Del-R	5’ TTACTATTTCCATAACTAGTAATCCCAGGCACCGAGCTAA 3’
GhNAC4 Δ294-305-D3	11 Del-F	5’ TAACTCGGTGGAAGCTGATAACTCG 3’
	11 Del-R	5’ CGAGTTATCAGCTTCCACCGAGTTA 3’
GhNAC4 19RΔA	19 R/A-F	5’ GCTTGCCGCCGGGGTTTGCTTTTTATCCAACTGATG 3’
	19 R/A-R	5’ CATCAGTTGGATAAAAAGCAAACCCCGGCGGCAAGC 3’
GhNAC4 26RΔA	26 R/A-F	5’ CCAACTGATGAAGCTCTTTTAGTGCAATATTTATGC 3’
	26 R/A-R	5’ GCATAAATATTGCACTAAAAGAGCTTCATCAGTTGG 3’

Table 3.9.4 Primers used for expression analysis of stress-responsive genes in tobacco seedlings

Gene	Primer name	Primer sequence
NtAPX (U15933.1)	NtAPX-F	5’ GTTTGGGCTTTTCTCCTCGAC 3’
	NtAPX-R	5’ GGAGCATAAGAGGAGCGCAA 3’
NtCAT1 (U93244.1)	NtCAT1-F	5’ GGCCGCTACAACTCTCTCTTT 3’
	NtCAT1-R	5’ ACAGGACCTCTTGCACCAAC 3’
NtERD10C (AB049337.1)	NtERD10C-F	5’ AAAGCCAACTCATGCCCAAG 3’
	NtERD10C-R	5’ AGAGCTGCTACTTGATCGATGG 3’

NtERF5 (AY655738.1)	NtERF5-F	5’ GGATTGTCTCCTGCTGCTGT 3’
	NtERF5-R	5’ GCTCTTCTAATAACTCAGCACCC 3’
NtDREB3 (EU727157.1)	NtDREB3-F	5’ ATGGCTTGGCACTTTCCCTT 3’
	NtDREB3-R	5’ ATATTCTTGGCGTCGGAGGA 3’
NtMnSOD (AB093097.1)	NtMnSOD-F	5’ TCCCCTACGACTATGGAGCA 3’
	NtMnSOD-R	5’ CGGTATGCAATTTGGCGACG 3’
NtNCED3 (JX101472.1)	NtNCED3-F	5’ TGTCTGAAATGATCCGGGGC 3’
	NtNCED3-R	5’ AGTTTCCGGCTCTTCCCAAG 3’
NtSOS1 (XM_009789739.1)	NtSOS1-F	5’ CAAATGTTATCCCCCGAAAGC 3’
	NtSOS1-R	5’ CGGAGAACCTGAGGAAATGTGA 3’
NtSUSY (AB055497.1)	NtSUSY-F	5’ CACGGATATTTCGCCCAGGA 3’
	NtSUSY-R	5’ GCAGCAGCCGAGTAGCAATA 3’
NtUBI1 (U66264.1)	NtUBI1-F	5’ GAGTCAACCCGTCACCTTGT 3’
	NtUBI1-R	5’ ACATCTTTGAGACCTCAGTAG ACA 3’

Chapter 4

Expression analysis of *GhNAC4* under various phytohormones and environmental stress conditions and prediction of *cis*-acting elements in the *GhNAC4* promoter

4.1 Introduction

Transcription factors (TFs) function as regulatory trans-acting proteins that bind to specific *cis*- acting elements in the promoters of target genes and lead to the activation or repression of gene expression. Hence, the expression pattern of a given gene is a direct consequence of the promoter function, and *cis*-acting elements are important molecular switches that play a crucial role in transcriptional regulation (Yamaguchi-Shinozaki and Shinozaki, 2005). They control a complex and dynamic network of gene expression, which affects biological processes. Accurate functional analysis of *cis*-acting elements would help understand the complex gene regulatory networks underlying plant adaptation to stress (Yamaguchi-Shinozaki and Shinozaki, 2005). In this study, the transcript levels of *GhNAC4* under various phytohormones and environmental stresses was investigated. Further, the *GhNAC4* promoter was isolated and the *cis*-acting motifs were predicted.

4.2 Results and discussion

4.2.1 Expression analysis revealed the responsiveness of *GhNAC4* gene to various phytohormones and environmental stresses

To gain an insight into the impact of environmental stresses and phytohormones on the expression of *GhNAC4*, real-time expression analysis was carried out by subjecting cotton seedlings to various treatments. Figs. 4.1 and 4.2 demonstrate that *GhNAC4* responded differentially to several phytohormones and environmental stresses. GA3 and BAP enhanced its expression by ~ 11.5 and 10.4 folds, respectively. The *GhNAC4* transcript levels were also up-regulated by MeJA and ABA to ~ 7.8 and 6.9 folds, respectively. IAA enhanced the expression by ~ 5.4 folds. However, other hormones like ethylene and SA triggered only a slight up-regulation of *GhNAC4* expression (~ 4 folds).

ABA has been shown to be involved in modulating plant abiotic stress responses, MeJA, SA and ethylene play central roles in their biotic stress responses. Role of auxin and cytokinin during

environmental stress response is also emerging (Bielach et al., 2017). Cross-talk between various phytohormones results in antagonistic or synergistic interactions which are necessary for a plant's response to environmental stress (Peleg and Blumwald, 2011). Induction of expression of *GhNAC4* by these hormones suggests that the expression of *GhNAC4* is a possible node connecting multiple hormone pathways in the formation of a complex environmental stress signaling web.

The polyethylene glycol (PEG) induced drought stress treatment resulted in very high up-regulation of *GhNAC4* expression (~ 184 folds). Other abiotic stress treatments like high salinity and osmotic stress also led to high up-regulation of its expression (~ 43.6 and 58.7 folds respectively). Robust up-regulation of *GhNAC4* transcripts was also observed in air-drying and methyl viologen treatments (~ 10.2 and 14.5 folds). Methyl Viologen (MV) is known to generate ROS such as H_2O_2, which are important signaling molecules during environmental stress tolerance (Bhattacharjee, 2005). Disease responses and ABA signaling pathway induce the production of ROS (Laloi et al., 2004). Upregulation of *GhNAC4* by MV suggests that it might be a regulator of ROS-scavenging mechanism and plays a possible role in stress signaling pathway.

Dark treatment caused ~ 6.2 folds increase in gene expression and when this treatment was supplemented with low temperature, the *GhNAC4* expression was enhanced by ~ 19.4 folds suggesting an additive effect of the combination of two treatments. Darkness is known to induce leaf senescence (Liebsch and Keech, 2016) and several NAC TFs play an important role in leaf senescence (Balazadeh et al., 2011; Kim et al., 2014; Yang et al., 2011). High temperature and wounding stress treatments also increased *GhNAC4* expression by ~ 6.1 and 5.7 folds, respectively. However, submergence stress treatment caused only a marginal increase in the level of *GhNAC4* transcripts. The above expression profile suggests that finetuning of *GhNAC4* induction may require elaborate cross-talk between various signaling pathways via the involvement of phytohormones in the cells.

Transcription factors are known to play important roles in phytohormonal and stress responses (Singh et al., 2002; Vishwakarma et al., 2017). Several NAC TFs are shown to be up-regulated by phytohormones and stress treatments. Furthermore, they are known to function in diverse signaling pathways. *Arabidopsis* NAC genes *ANAC019, ANAC055* and *ANAC072,* showed up-regulation after drought, high salinity and ABA treatments (Tran et al., 2004). The expression of rice *OsNAC6* is induced by ABA, JA, cold, drought, high salinity treatments. It is also rapidly induced by wounding (Ohnishi et al., 2005). Expression of wheat *TaNAC4* was up-regulated by ethylene, MeJA and ABA, cold, wounding and high salinity treatments (Xia et al., 2010).

Expression of potato *StNAC016* and *StNAC059* was induced under both BAP and GA_3 treatments and *StNAC059* was also induced by salt stress treatments (Singh et al., 2013). Transcript levels of fox-tail millet NAC gene, *SiNAC* were enhanced by *s*alinity, dehydration, MeJA, and ethephon treatments (Puranik et al., 2011). Auxin induces *AtNAC1* expression during lateral root formation (Xie et al., 2000). Upregulation of *GhNAC4* by multiple stresses such as drought, salinity stress, osmotic stress, ROS, and wounding suggests that it might play a potential role in both biotic and abiotic stress signaling pathway, which is similar to, RD26/ANAC072 that is induced by drought, ABA, MeJA, salinity stress, H_2O_2 and pathogen infections (Fujita et al., 2004; Zimmermann et al., 2004).

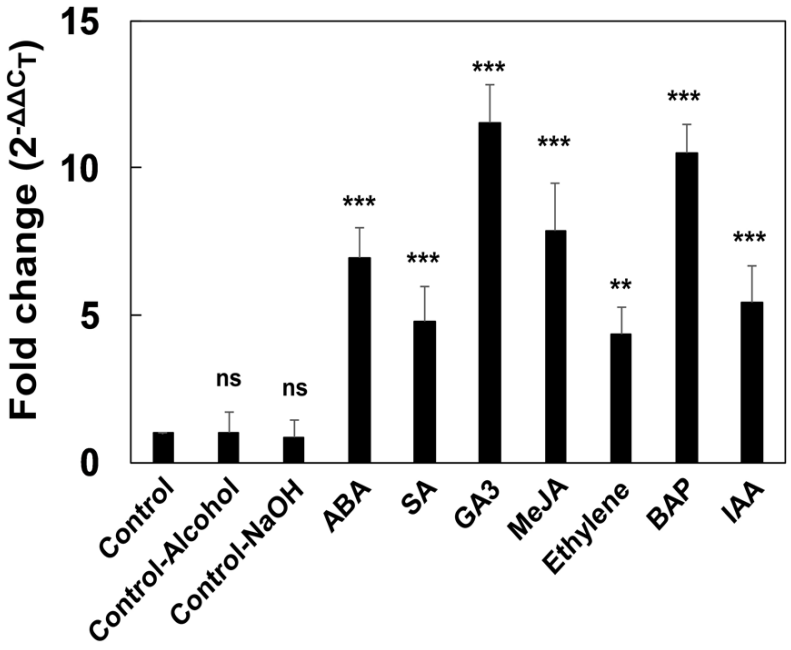


Figure 4.1 Expression patterns of *GhNAC4* transcript in response to various phytohormones.

qRT-PCR expression analysis of the *GhNAC4* gene in *G. hirsutum* leaves after ABA, MeJA, SA, 6-BAP, GA_3, IAA, or Ethephon treatment. Two weeks-old cotton seedlings incubated for 24 h under the treatment, were used for analysis. mRNA levels of *GhNAC4* gene were normalized to that of Ubiquitin gene, *GhUBQ7*. The data are shown as the means ± SE (n=3). A statistical analysis with one-way ANOVA indicates significant differences (** $P < 0.01$, *** $P < 0.001$, ns - not significant). ABA, abscisic acid; SA, salicylic acid; GA3, 3-gibberellic acid; MeJA, methyl jasmonic acid; BAP, 6-benzyl aminopurine; IAA, indole-3-acetic acid.

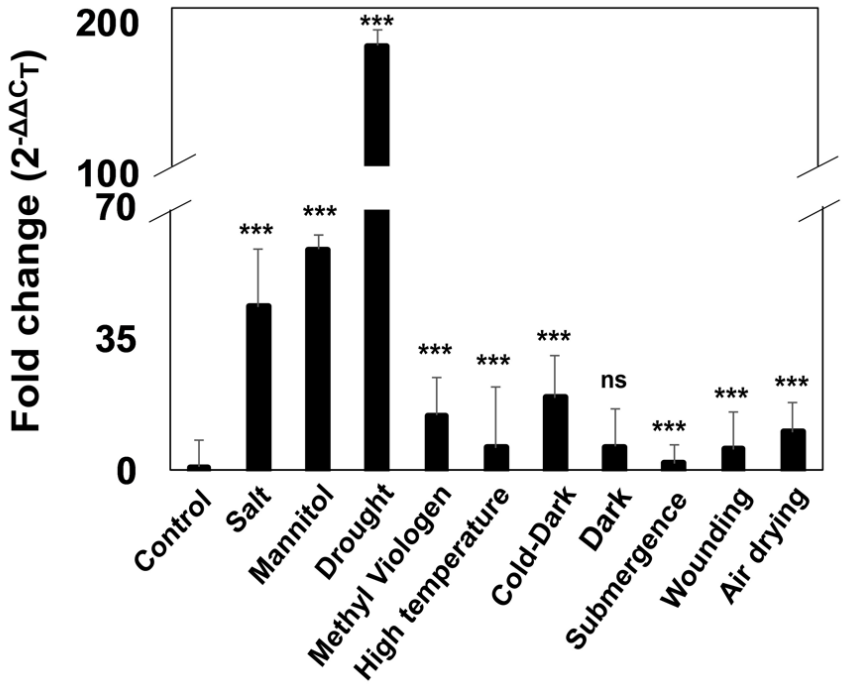


Figure 4.2 Expression patterns of *GhNAC4* transcript in response to various environmental stresses. qRT-PCR expression analysis of the *GhNAC4* gene in *G. hirsutum* leaves after salt, mannitol, drought, methyl viologen, high temperature, air drying, submergence, wounding, dark or combination of dark and cold treatment. Two weeks-old cotton seedlings were used for analysis. mRNA levels of *GhNAC4* gene were normalized to that of Ubiquitin gene, *GhUBQ7*. The data are shown as the means ± SE (n=3). A statistical analysis with one-way ANOVA indicates significant differences (*** $P < 0.001$, ns - not significant).

4.2.2 Sequence analysis of *GhNAC4* promoter

A total of 1612 bp (-1492 bp to +119 bp) was amplified from *G. hirsutum* (Var. JK Durga) and was sequenced. Sequence similarity of 91.6 % (LALIGN, https://www.ebi.ac.uk/Tools/psa/lalign/) was observed between the promoter region of *G. raimondii* and *G. hirsutum* of *NAC4* gene. No significant difference was observed between the predicted motifs in the two sequences. The composition of GC content of *GhNAC4* promoter was 29.9 % (BioEdit, Hall, 1999) which is in accordance with the observed range (Joshi, 1987) for a plant promoter. The putative transcription start site (TSS) predicted by Softberry database (www.softberry.com) using the default settings, was located 119 bp upstream of the ATG translation start codon, which was consistent with the features of a eukaryotic

promoter as shown in Fig. 4.3. The predicted TATA box was located 16 bp upstream of TSS, and a CAAT box was located 179 bp upstream of TATA box. Several other CAAT boxes were also predicted in the entire length of the sequence.

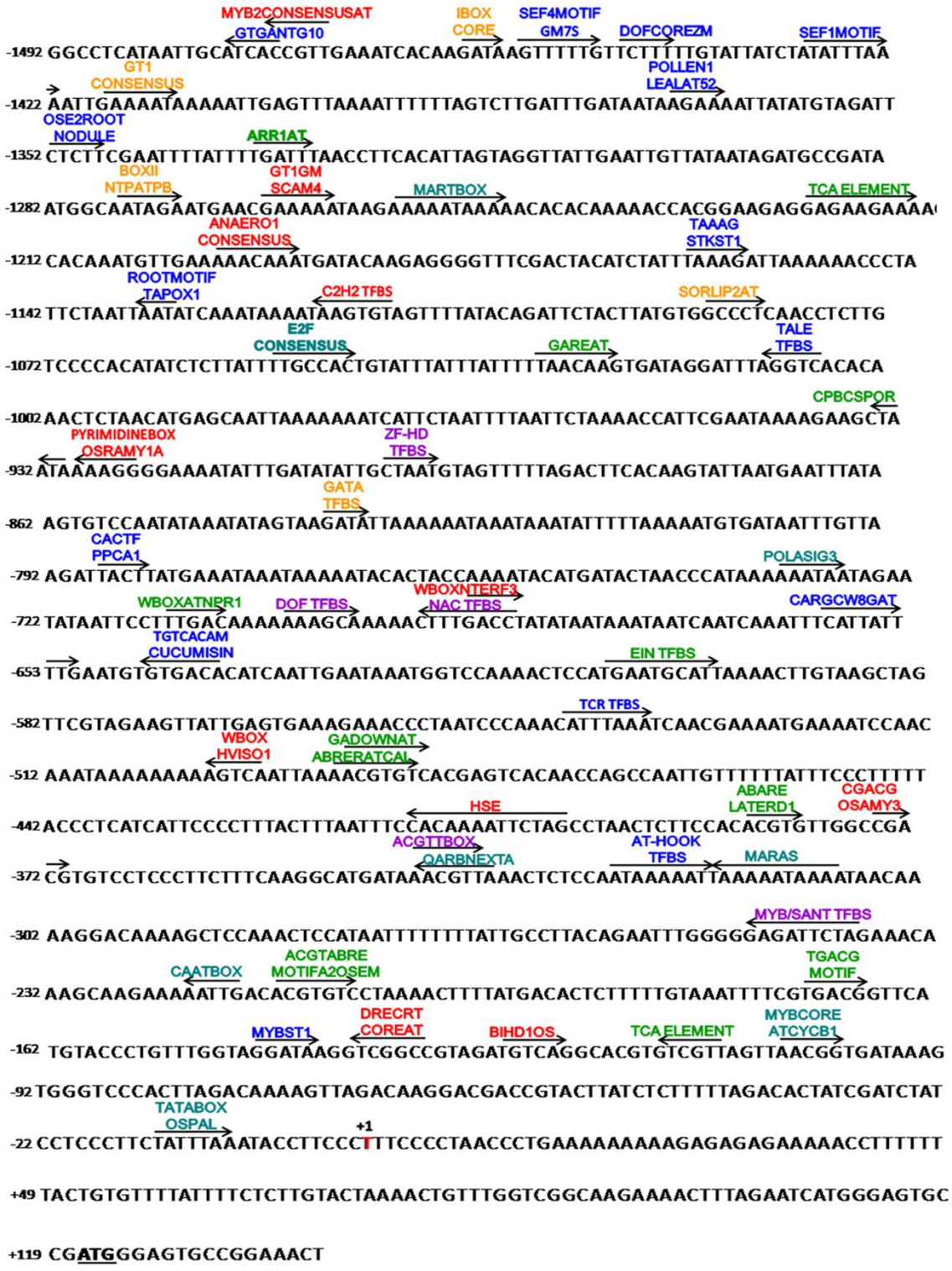


Figure 4.3 Physical map of *GhNAC4* promoter. Nucleotide sequence of the 1492 bp upstream region of *GhNAC4* gene from *G. hirsutum*.

The putative transcription start site is in bold and is designated as +1. The translation start site is bolded and underlined. The numbers on the left side indicate the distance from the transcription start site. The sequence was analyzed by PLACE, PlantCARE and PlantPAN2.0 programs. All the predicted motifs are indicted by arrow and their names are mentioned above. '→' and '←' indicates that the predicted motif is on positive (5'-3') and negative (3'-5') strand respectively. Stress inducible motifs are represented in red, phytohormone responsive motifs are in green, tissue specificity motifs are in blue, light inducible motifs are in orange, transcriptional related motifs in purple and transcription factors binding sites are in teal color.

4.2.3 Multiple hormone-related, environmental stress-related and tissue-specific *cis*-elements were predicted on the *GhNAC4* promoter.

Putative *cis*-acting regulatory elements and their location were searched using the PlantPAN 2.0, PlantCare and PLACE software tools. Only statistically significant motifs (P value >0.9) were selected. The resulting putative *cis*-acting regulatory elements were grouped into six classes, as shown in Table 4.1, including phytohormone- responsive motifs, stress-responsive motifs, light responsive, basic transcription elements, tissue-specific elements, and other TF binding sites. A relatively high proportion of tissue-specific motifs and transcription factors binding sites followed by light specific, hormone responsive and stress responsive elements were observed (Fig. 4.4).

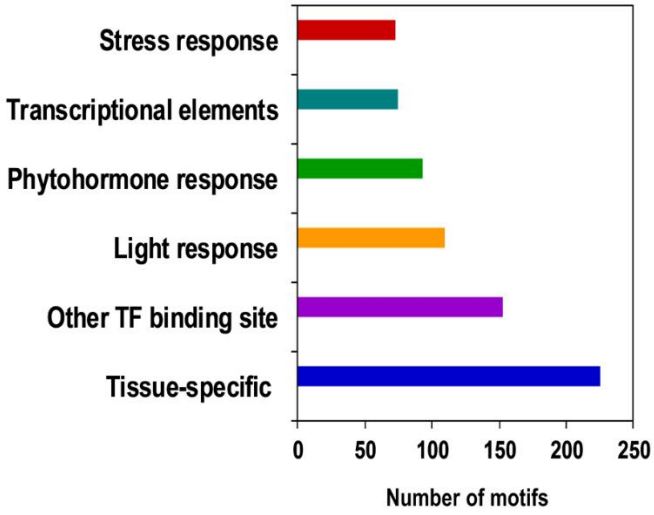


Figure 4.4 Functional classification of *cis*-elements present in the *GhNAC4* promoter.

The motifs in the GhNAC4 promoter were classified into five functional categories. There are a relatively high proportion of tissue-specific motifs and transcription factors binding sites.

4.2.4 Phytohormone-responsive elements predicted in *GhNAC4* promoter

Several motifs responding to phytohormones were revealed in the *GhNAC4* promoter. Various ABA-responsive elements such as ABRELATERD1, ACGTABREMOTIFA2OSEM, and ABRERATCAL (found upstream of Ca^{+2} responsive genes) were observed in the promoter sequence. Auxin-responsive motifs such as one AUXREPSIAA4, one TGA element, one GGTCCCATGMSAUR, and two CATATGGMSAUR motifs, were predicted. Salicylic acid responsive motifs like TCA element and WBOXATNPR1 (recognition site of SA-induced WRKY TFs) were also identified. TGACG and CGTCA motifs, required for jasmonic acid induction were found in the sequence. Gibberellins responsive elements such as GARE1OSREP1, GAREAT, and GADOWNAT were found. ARR1AT TF, a cytokinin response regulator, and CPBCSPOR motif, critical for cytokinin enhanced expression were also observed.

The *cis*-elements known to play a role in ethylene induction such as ERELEE4 and binding sites of EIN/EIL TF, a positive regulator in the ethylene response pathway were also identified in the

promoter sequence. Binding sites of BES1 TF, key TFs in the brassinosteroids signaling pathway were also observed in the putative promoter sequence. Furthermore, several recognition sites of AP2/ERF TFs were also found in the sequence, which are key regulators in integrating various hormone signals and play a role in stress response.

4.2.5 Environmental stress-responsive elements predicted in *GhNAC4* promoter

A scan of the motifs showed that some potential elements, such as the MYB recognition sites like MYB1AT and MYB2CONSENSUSAT that are stress-induced regulatory motifs found in the promoters of the dehydration-responsive gene were identified. *cis*-Elements such as DRECRTCORE, HSE, CBFHV, and a binding site for CSD and HSF TFs, known to regulate responses to cold shock and heat stress were predicted. The binding site for bZIP (ACGTTBOX), WRKY (WBBOXPCWRKY1) and MYB (MYB1LEPR) TFs that were known to play important roles in stress response were found. *GhNAC4* promoter sequence also contained a *cis*-element for nutrient deficiency response like the GMHDLGMVSPB, which is a binding site of homeodomain- Leucine zipper protein, which is found in the phosphate response domain.

Various motifs and target sites of TFs, vital for biotic stress response were also predicted in the promoter sequence. Many fungal elicitor response elements like BOXLCOREDCPAL, TC- RICH REPEATS, and GT1GMSCAM4 were observed. Numerous binding sites of BELL homeodomain TF, BIHD1OS, known to play a vital role in disease response were also observed in the sequence. Hypersensitive response element like HSRENTHSR203J was also observed. Motifs involved in wounding response such as T/GBOXATPIN2 and WBOXNTERF3 were also found. ANAERO1CONSENSUS, a motif found upstream of anaerobic induced genes was observed.

4.2.6 Sugar-responsive elements predicted in *GhNAC4* promoter

cis-Elements involved in sugar repression like PYRIMIDINEBOXOSRAMY1A, UP2ATMSD and WBOXHVISO1 (SUSIBA2 binds to W-box element) TFs were identified; they are also known to plays a role in auxiliary bud outgrowth after stem decapitation.

4.2.7 Light-responsive elements predicted in *GhNAC4* promoter

Several motifs essential for light-regulated transcriptional activation like SORLIP2AT, IBOXCORE, GT1CONSENSUS (that activate and stabilize the transcription machinery), GATA (that also plays a role in vascular-specific expression), and CGACGOSAMY3 (G Box/BOXII) were identified in the putative promoter sequence suggesting that *GhNAC4* might be highly regulated by light. Motifs important for plastid-specific expression such as BOXIINTPATPB, -10PEHVPSBD, and PRECONSCRHSP70A were also present in the *GhNAC4* promoter.

4.2.8 Tissue-specific elements predicted in *GhNAC4* promoter

The specific expression is crucial for genes functioning at particular stages and in a particular tissue(s). A plethora of motifs essential for tissue-specific expression was predicted in the *GhNAC4* promoter sequence. Motifs important for embryo maturation and seed development such as SEF4MOTIFGM7S, SEF1MOTIF, and CANBNNAPA were found. Binding sites for TALE TFs, that are known to play to a role in meristem function and involved in maintaining cells in an undifferentiated state, were also observed. Motifs involved in root specific expression like OSE2ROOTNODULE, ROOTMOTIFTAPOX and recognition sites for MYBST1 were identified.

Several copies of CACTFTPPCA1, which is an important motif for mesophyll expression, were found. Numerous binding sites of DOFCOREZM involved in regulation of carbon metabolism and for shoot and leaf-specific expression were identified. Binding sites of WOX TF, known to be involved in several key developmental processes was also observed. TAAAGSTKST1, a motif known to play a role

in controlling guard cell-specific gene expression, was also found. Numerous binding sites for TFs significant for vascular-specific expression like DOF, GATA, and AT hook TFs were observed.

Many target sites for TCR, SBP and MYBPZMTFs and motifs like CARGCW8GAT and TGTCACACMCUCUMISIN significant for flower and fruit development were present. Motifs for pollen specific expression such as POLLEN1LELAT52 and GTGANTG10 were also identified.

4.2.9 Cell cycle-specific elements predicted in *GhNAC4* promoter

cis-Elements involved in gene expression induction in actively dividing cells such as MYBCOREATCYCB1, E2FCONSENSUS and CELLCYCLESC were predicted in the sequence. Motifs such as MARARS, MARABOX1, and MARTBOX are known to play a role in scaffold attachment region were also found.

4.2.10 Transcription regulatory elements predicted in *GhNAC4* promoter

Basal motifs that play a critical role in the core transcription initiation were found in the promoter of *GhNAC4*. Several TATA box motifs, essential for accurate initiation of transcription and synergistic enhancement of transcription were found. CAAT-box motifs that are universal enhancer elements in promoters were also predicted. In addition to basal regulatory elements, a number of transcriptional regulatory elements were found. Several enhancer elements, for example, CTRMCAMV35S, QARBNEXTA, QELEMENTZMZM13, and 5' UTR - PYRIMIDINE STRETCH and binding sites of NF-Y TF were identified. Interestingly, a repressor element like RE1ASPHYA3 was also found in the promoter. These elements suggest that *GhNAC4* promoter is functional *in situ*.

4.2.11 Transcription factor binding sites predicted in *GhNAC4* promoter

Several potential binding sites for transcription factors, such as NAC, MYB/SANT, ZF-HD, bHLH, Storekeeper, bZIP, GRAS, and TCP were also identified.

The presence of these motifs' hints that regulation of *GhNAC4* gene expression is complex and it may play a possible role during plant growth and is responsive to phytohormones, environmental stresses, and light.

Table 4.1 Putative motifs in the *GhNAC4* promoter identified by *in silico* analysis.

DB ID represents the PLACE database accession number. PC represents the motifs identified by PlantCare database

MOTIFS/TFs RELATED TO HORMONE RESPONSE					
S.No	**MOTIF/TF NAME**	**No.**	**DB ID**	**SEQUENCE**	**EXPECTED FUCTION**
1	TGA-ELEMENT	1	PC	AACGAC	*cis*-Acting regulatory element involved in auxin responsiveness
2	GGTCCCATGMSAUR	1	S000360	GGTCCCAT	
3	CATATGGMSAUR	2	S000370	CATATG	
4	AUXREPSIAA4	1	S000026	KGTCCCAT	Auxin responsive element
2	ABRERATCAL	7	S000507	MACGYGB	ABARE related sequence, found upstream of Calcium ion responsive genes
3	CE3	1	PC	GACGCGTGTC	Involved in ABA and VP1 response
4	ABREOSRAB21	1	S000012	ACGTSSSC	Motifs involved in ABA responsiveness
5	ABRELATERD1	8	S000414	ACGTG	
6	CGTGT SPHZMC1	1	S000294	CGTGTCGTC CATGCAT	
7	ACGTABREMOTIFA2OSEM	6	S000394	ACGTGKC	ACGT-core of motif A in ABRE
8	WBOXATNPR1	4	S000390	TTGAC	Recognition site of salicylic acid (SA)-induced WRKY TFs
9	TCA ELEMENT	1	PC	GAGAAGAATA	Involved in salicylic acid response

10	CPBCSPOR	1	S000491	TATTAG	Critical for Cytokinin-enhanced expression
11	ARR1AT	16	S000454	NGATT	Binding site of ARR1, a cytokine response regulator
12	TGACG-MOTIF	1	PC	TGACG	Involved in jasmonate (JA) induction, plays a role in wounding response
13	T/GBOXATPIN2	1	S000458	AACGTG	
14	PYRIMIDINEBOXOSRAMY1A	4	S000259	CCTTTT	
15	CGTCA-MOTIF	1	PC	CGTCA	
16	GADOWNAT	5	S000438	ACGTGTC	Motif for down-regulation of expression by GA
17	GARE1OSREP1	2	S000419	TAACAGA	Gibberellins-responsive element (GARE)
18	GAREAT	4	S000439	TAACAAR	
19	EIN-EIL TF	4	-	ATGCA	Positive regulator in the ethylene response pathway
20	BES1 TF	7	-	CACGTG	Key TFs in brassinosteroids signaling pathway
21	AP2/ERF TF	3	-	GCCGAC	Key regulator integrating various hormone signals, also plays a role in stress response
MOTIFS/TFs RELATED TO STRESS RESPONSE					
S.No	**MOTIF/TF NAME**	**No.**	**DB ID**	**SEQUENCE**	**EXPECTED FUCTION**
1	ANAERO1CONSENSUS	2	S000477	AAACAAA	Motifs found upstream of anaerobic induced genes
2	UP2ATMSD	2	S000472	AAACCCTA	Plays a role in auxiliary bud outgrowth after stem decapitation
3	SREATMSD	1	S000470	TTATCC	*cis*-Elements involved in sugar repression
4	CGACGCOSAMY3	16	S000205	CGACG	May function as a coupling element for the G-box motif

5	WBOXHVISO1	2	S000442	TGACT	SUSIBA2 bind to W-box element, plays a role in sugar signaling
6	GMHDL GMVSPB	1	S000372	CATTAATTAG	Binding site of the soybean homeodomain leucine zipper proteins (GmHdl56, GmHdl57); Found in the phosphate response domain of the soybean *VspB* promoter
7	MYB2 CONSENSUSAT	2	S000409	YAACKG	MYB recognition site found in the promoters of the dehydration-responsive gene
8	MYB1AT	4	S000408	WAACCA	
9	HSE	1	PC	AGAAAATTCG	Involved in heat stress responsiveness
10	C-REPEAT/DRE	1	PC	TGGCCGAC	Essential for cold and dehydration stress response
11	CBFHV	3	S000497	RYCGAC	
12	DRECRT COREAT	3	S000418	RCCGAC	
13	CSD TF	8	-	AATAAA	Plays a role in cold shock response
14	HSF TF	4	-	CTNGAANNTTCNAG	Heat shock responsive TF
15	C2H2 TF	11	-	ACACT	Play diverse roles in the plant stress response and the hormone signal transduction
16	BOXLCORE DCPAL	1	S000492	ACCWWCC	Core sequences of box-L-like sequences, involved in elicitor induction
17	WBOX NTCHN48	1	S000508	CTGACY	Involved in elicitor-responsive transcription of defense genes
18	HSRE NTHSR203J	1	S000466	CAAAATTTTGTA	"HSRE (HSR203 responsive element)" responsible for the marked induction during the HR

					(hypersensitive response); HSR203J is specifically activated during the early steps of incompatible plant/pathogen interactions;
19	GT1GMSCAM4	6	S000453	GAAAAA	Plays a role in pathogen- and salt-induced gene expression
20	TC- RICH REPEATS	1	PC	GTTTTCTTAC	Involved in defense and stress response
21	BIHD1OS	6	S000498	TGTCA	Binding site of BELL homeodomain TF, known to play a role in disease response
22	WBOXNTERF3	4	S000457	TGACY	W-box, May be involved in activation by wounding
23	WBBOX PCWRKY1	2	S000310	TTTGACY	W-box, binding site of WRKY TFs
24	MYB1LEPR	1	S000443	GTTAGTT	MYB TF binding site
25	ACGTTBOX	2	S000132	AACGTT	Binding site of bZIP TFs
MOTIFS/TFs RELATED TO TISSUE SPECIFIC LOCALIZATION					
S.No	**MOTIF/TF NAME**	**No.**	**DB ID**	**SEQUENCE**	**EXPECTED FUCTION**
1	SEF1MOTIF	3	S00006	ATATTTAWW	Motifs required for seed specific expression
2	SEF3MOTIFGM	1	S000115	AACCCA	
3	SEF4MOTIFGM7S	12	S000103	RTTTTTR	
4	CANBNNAPA	1	S000148	CNAACAC	Required for seed specificity
5	TALE TF	12	-	TGAC	Plays a role in meristem function; Involved in maintaining cells in an undifferentiated, meristematic state
6	ROOTMOTIF TAPOX1	11	S000098	ATATT	Motif found both in promoters of *rolD*
7	OSE1ROOT NODULE	1	S000467	AAAGAT	Consensus sequence motifs of organ-specific elements (OSE)

8	OSE2ROOT NODULE	10	S000468	CTCTT	characteristic of infected cells of root nodules
9	TELOBOX ATEEF1AA1	1	S000309	AAACCCTAA	Required for expression in the root
10	RHERPATEXPA7	3	S000512	KCACGW	Right part of root hair-specific *cis*-element
11	MYBST1	2	S000180	GGATA	Core motif of MybSt1 (a potato MYB homolog) binding site.
12	CACTFTPPCA1	22	S000449	YACT	Tetranucleotide (CACT) is a key component of Mem1 (mesophyll expression module 1)
13	DOFCOREZM	26	S000265	AAAG	Core site required for binding of Dof proteins; involved in regulation of carbon metabolism
14	MYBPZM	1	S000179	CCWACC	Core of consensus maize P (myb homolog) binding site and specifies red pigmentation of kernel pericarp, cob, and other floral organs
15	SBP TF	8	-	CGTAC	Plays a role in flower and fruit development as well as other physiological processes
16	TCR TF	12	-	-	Plays a role in development of both male and female reproductive tissues
17	CARGCW8GAT	6	S000431	CWWWWWWWWG	Binding site for AGL15 (AGAMOUS-like 15)
18	TGTCACACMCUCUMISIN	4	S000422	TGTCACA	Enhancer element necessary for fruit-specific expression

19	AT hook TF	50	-	AAAAT	Known to play a role in vascular specific expression
20	POLLEN1 LELAT52	9	S000245	AGAAA	One of two co-dependent regulatory elements responsible for pollen specific activation
21	GTGANTG10	13	S000378	GTGA	"GTGA motif"; plays a role in pollen specific expression
22	TAAAGSTKST1	5	S000387	TAAAG	Target site for Dof1 TF controlling guard cell-specific gene expression
23	WOX TF	5	-	TTAAT	Involved in several key developmental processes
MOTIFS/TFs RELATED TO LIGHT RESPONSE					
S.No	**MOTIF/TF NAME**	**No.**	**DB ID**	**SEQUENCE**	**EXPECTED FUCTION**
1	GATA MOTIF	1	PC	GATAGGA	Part of a module for light response, also plays a role in vascular specific expression
2	GATA TF	42	-	GATA	Part of a module for light response
3	IBOXCORENT	3	S000424	GATAAGR	Conserved sequence upstream of light regulated genes
4	IBOX	3	S000124	GATAAG	
5	IBOXCORE	11	S000199	GATAA	
6	GT1 CONSENSUS	23	S000198	GRWAAW	Plays a role in light regulation; Consensus GT-1 binding site, can activate and stabilize the transcription machinery
7	SORLIP1AT	1	S000483	GGGCC	*cis*-Acting elements involved in the phytochrome A regulated gene
8	SORLIP2AT	2	S000482	GCCAC	
9	TBOX ATGAPB	1	S000383	ACTTTG	Involved in light activated transcription
10	3-AF1	1	PC	AAGAGATA TTT	Light responsive element

11	ACE	1	PC	AAAACGTTTA	Part of a module for light response
12	AE-BOX	1	PC	AGAAACAA	
13	AT-1	1	PC	AATTATTTTTTATT	
14	ATC MOTIF	1	PC	AGTAATCT	
15	AT-C	1	PC	AATTATTTTTTATT	
16	RBCS CONSENSUS	1	S000127	AATCCAA	rbcS general consensus sequence
17	-10PEHVPSBD	2	S000392	TATTCT	-10 promoter element, involved in chloroplast expression
18	BOXIINTPATPB		S000296	ATAGAA	Conserved sequence found in the promoters of plastid genes
19	PRECONSCRHSP70A		S000506	SCGAYNRNNNNNNNNNNNNNHD	Consensus sequence of plastid response element (PRE) in the promoters of HSP70A
MOTIFS/TFs RELATED TO CELL CYCLE					
S.No	**MOTIF/TF NAME**	**No.**	**DB ID**	**SEQUENCE**	**EXPECTED FUCTION**
1	E2F CONSENSUS	6	S000476	WTTSSCSS	Motifs involved in gene expression induction at the G1/S transition of the cell cycle in actively dividing cells
2	CELLCYCLESC	1	S000031	CACGAAAA	
3	MYBCORE ATCYCB1	1	S000502	AACGG	Myb core" in the 18 bp sequence which is able to activate expression without leading to M-phase-specific expression
4	MARARS	4	S000064	WTTTATRTTTW	

5	MARTBOX	6	S000067	TTWTWTTWTT	Motifs found in SAR (scaffold attachment region); or matrix attachment region, (MAR)
6	MARABOX1	4	S000063	AATAAAYAAA	
MOTIFS/TFs RELATED TO BASIC TRANSCRIPTION AND EXPRESSION LEVELS					
S.No	**MOTIF/TF NAME**	**No.**	**DB ID**	**SEQUENCE**	**EXPECTED FUCTION**
1	CAATBOX1	19	S000028	CAAT	Common *cis*-element in promoter and enhancer regions
2	TATABOX OSPAL	4	S000400	TATTTAA	Binding site of TATA binding protein (TBP); essential for accurate initiation of transcription and synergistic enhancement of transcription
3	TATABOX3	2	S000110	TATTAAT	
4	TATABOX4	1	S000111	TATATAA	
5	TATABOX2	2	S000109	TATAAAT	
6	TATA BOX	5	PC	TATA	
7	POLASIG3	3	S000088	AATAAT	Consensus sequence for plant polyadenylation signal
8	MRNA STA1CRPSBD	1	S000274	CUCUUTGUTTUU	mRNA stability determinant; Required for the stable accumulation
9	NF-Y TF	5	-	CCAAT	Component of the NF-Y/HAP TF complex. The NF-Y complex stimulates the transcription of various genes by recognizing and binding to a CCAAT motif in promoters
10	CTRM CAMV35S	1	S000405	TCTCTCTCT	*cis*-Acting element conferring high transcription levels
11	5'UTR-PYRIMIDINE STRETCH	1	PC	TTTCTTCTCT	
12	QARBNEXTA	1	S000244	AACGTGT	

13	QELEMENT ZMZM13	2	S000254	AGGTCA	
14	EECCRCAH1	1	S000494	GANTTNC	
15	RE1ASPHYA3	2	S000195	CATGGGCG CGG	RE1 (putative repressor element)
OTHER TFs BINDING SITES					
S.No	**MOTIF/TF NAME**	**No.**	**DB ID**	**SEQUENCE**	**EXPECTED FUCTION**
1	NAC/NAM TF	2	-	CGTR	Plays a role in developmental process and stress responses
2	TCP TF	6	-	GGCCCAW W	Plays a pivotal role in shaping plant morphology and also plays a role in synthesis of bioactive compounds
3	STOREKEEPER TF	3	-	GGTCG	Plays a role in sucrose inducible expression of patatin gene
4	GRAS TF	1	-	GTACGG	Play a role in various processes like GA signaling, root radial patterning and floral development
5	DOF TF	19	-	AAAG	Known to play a role in seed development, photoperiodic flowering, photosynthesis, stress tolerance and cell-specific gene expression
6	MYB/SANT TF	43	-	AGATATTT	Play diverse role in developmental and physiological processes, hormone response and stress tolerance
7	bHLH TF	32	-	CANNTG	Known to play many different functions in essential physiological and developmental process

8	ZF-HD TF	36	-	ATTAT	Plays an important role in developmental processes and stress responses
9	WRKY TF	4	-	GTCAA	Forms an integral part of signaling webs that modulate many plant processes
10	bZIP TF	2	-	ACGT CORE	Play diverse role in developmental and physiological processes

Chapter 5

Analysis of spatio-temporal localization and induction of *GhNAC4* promoter by phytohormones and environmental stresses

5.1 Introduction

In a genome-wide analysis, Sun et al. (2018) identified 283 NAC genes in *Gossypium hirsutum*, 147 in *G. arboreum*, 267 in *G. barbadense*, and 149 in *G. raimondii*. They have analyzed the expression of NAC TFs in *G*. *hirsutum* and identified 38 and 124 NAC TFs that were particularly important in fiber development and stress responses, respectively. They also identified the motifs enriched in these NAC TFs promoters. In another study, the promoters of *GhNAC8–GhNAC17* were isolated using genome walking, and the *cis*-acting elements were predicted (Shah et al., 2013). To the best knowledge of the investigators of the present study, the spatio-temporal localization of NAC TFs has not been carried out so far in cotton species. Hence, a GUS-reporter-aided approach was adopted to elucidate the promoter activity of *GhNAC4,* which is highly up-regulated during drought and salt treatments as a step in this direction. Induction of *GhNAC4* promoter under various environmental and phytohormonal treatments and spatio-temporal localization was investigated.

5.2 Results and discussion

5.2.1 Generation and analysis of tobacco transgenics of PRO$_{GhNAC4}$:GUS

To evaluate the promoter activity of *GhNAC4* gene, a total of 12 hygromycin resistant T_0 tobacco plants were generated and were confirmed by genomic PCR. Out of these, 11 plants exhibited the expected amplification of 1612 bp and 1026 bp DNA fragments corresponding to the size of *GhNAC4* promoter sequence and the *HptII* gene, respectively. To eliminate the effect of gene copy number on GUS activity, only single copy T_1 progenies, P7, P9, and P17 were used for further generation of T_2 seeds. The T_2 progenies of three lines, P7.1, P9.5, and P17.3 were used for histochemical and fluorometric assays.

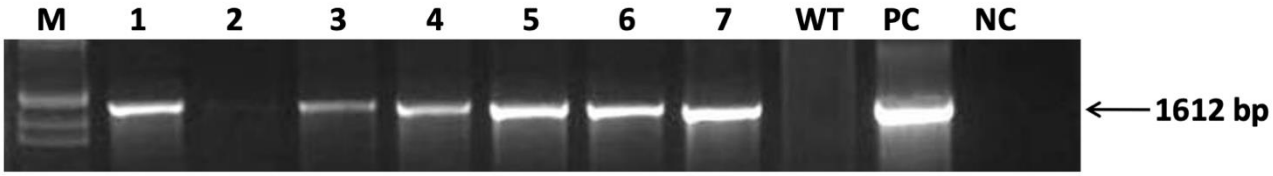


Figure 5.1 Confirmation of GhNAC4 promoter T_0 putative transgenic plants by PCR using tobacco genomic DNA.

A representative gel picture showing 1612 bp amplified product of *GhNAC4* promoter. 'M' represents λ *Eco*RI/*Hin*dIII DNA ladder. 'PC' represents positive control for the PCR using plasmid (Pro_{GhNAC4}:GUS) as a template. 'WT' represents negative control for the PCR using DNA from untransformed plant. 'NC' represents no template control.

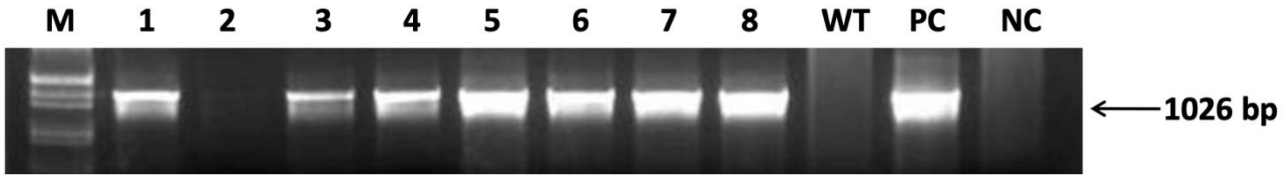


Figure 5.2 Confirmation of GhNAC4 promoter T_0 putative transgenic plants by PCR using tobacco genomic DNA.

A representative gel picture showing 1026 bp amplified product of *HptII*. 'M' represents λ *Eco*RI/*Hin*dIII DNA ladder. 'PC' represents positive control for the PCR using plasmid (Pro_{GhNAC4}:GUS) as a template. 'WT' represents negative control for the PCR using DNA from untransformed plant. 'NC' represents no template control.

5.2.2 GhNAC4 localizes to various tissues during growth and development

To precisely define the spatio-temporal expression patterns of *GhNAC4* promoter, GUS staining analysis of T_2 generation transgenic tobacco plants that express the PRO_{GhNAC4}:GUS fusion was used. Figs. 5.3 and 5.4 show the localization of GUS in vegetative and reproductive tissues in transgenic tobacco plants. These GUS staining images are representative of at least three independent transgenic lines.

In the early stages of tobacco growth (1 d old seedlings), GUS activity was first observed in the emerging radicle (Fig. 5.3a), which was later detected in emerging cotyledons, root tip and shoot

apex of 3 d old seedlings. However, GUS activity was relatively weaker in the hypocotyl tissue (Fig. 5.3b). In 7, and 15 d old transformed tobacco seedlings, GUS expression was detected in leaf veins, petioles, stem, and root (Fig. 5.3c, d). Similar GUS activities were maintained in the one-month-old plants also (Fig. 5.3e).

The main and lateral roots showed GUS expression, which was absent in the root cap region and root hairs (Fig. 5.3f, g). Intense GUS staining was also observed in the midrib and lateral veins, but the leaf lamina showed scanty staining (Fig. 5.3h). GUS activity in guard cells in a close-up photograph was also observed (Fig. 5.3i) and the developing mid rib regions of a young leaf (Fig. 5.3j). Fig. 5.3k-m shows the staining of the floral structures, which revealed that the GUS activity was present in sepals and to a lesser extent in the petal edges. GUS activity was also observed in anthers, pollen grains, and the stigma. However, it was absent in ovary and the pedicel. No staining was detected in seedlings harboring a promoter-less *GUS* gene regardless of the developmental stage (Fig. 5.3n).

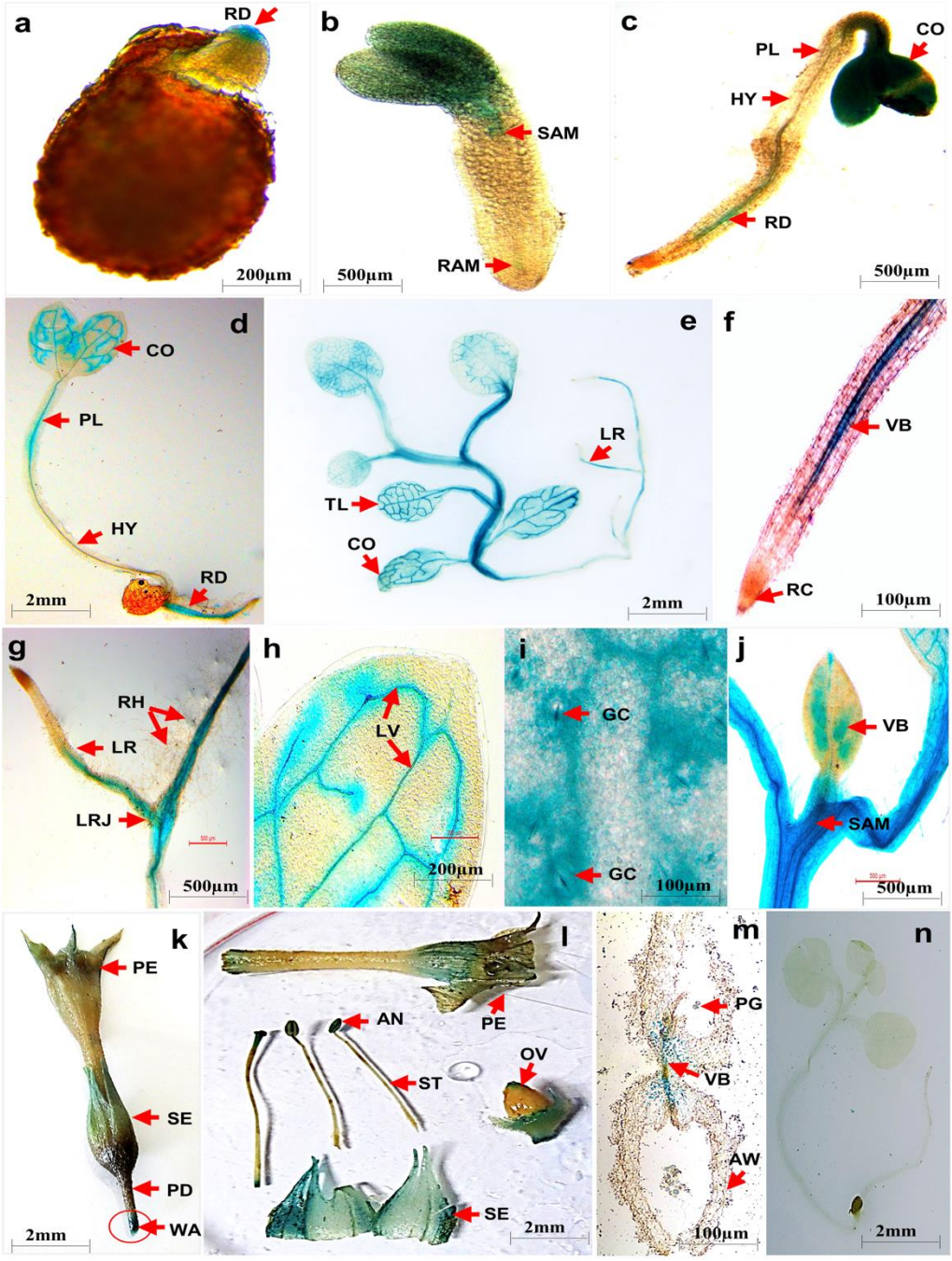


Figure 5.3 Histochemical localization of GUS activity in tobacco transgenic plants containing PRO*GhNAC4*:GUS construct.

(a-e) seedlings grown on MS media with hygromycin at – (a) Day1; (b) Day 3; (c) Day 7; (d) Day 15; (e) Day 30. (f-j) Various tissues of a 30-day old transgenic tobacco plant – (f) Main root; (g) Lateral root; (h) True leaf; (i) Guard cells; (j) Developing leaf. (k-m) Floral structures – (k) Mature flower; (l) Dissected flower showing various tissues; (m) Cross-section of an anther. (n) 15 days old tobacco seedling carrying empty pCAMBIA 1381Z vector. AW, anther wall; CO, cotyledon; GC, guard cell; HY, hypocotyl; LR, lateral root; LRJ, lateral root junction; LV, lateral vein; OV, ovary; PD, pedicel; PE, petal; PG, pollen grains; PL, plumule; RAM, root apical

meristem; RD, radicle; RH, root hairs; SAM, shoot apical meristem; SE, sepal; TL, true leaf; VB, vascular bundle; WA, wounded area. All arrows show strong GUS activity or no activity. Bars of each panel are as shown.

To obtain a better understanding of tissue specificity of GUS activity, thin cross-sections of various tissues were made using a razor blade. Intense GUS staining was detected in the vascular bundles, especially in the phloem of leaf veins, petiole, stem, and root (Fig. 5.4a-e). Both the abaxial and adaxial phloem tissues showed intense GUS staining. Other cell types like the pith, cortex, and epidermis, remained relatively unstained. However, cortex and epidermis also showed intense GUS expression upon wounding (Figs. 5.3k and 5.4f).

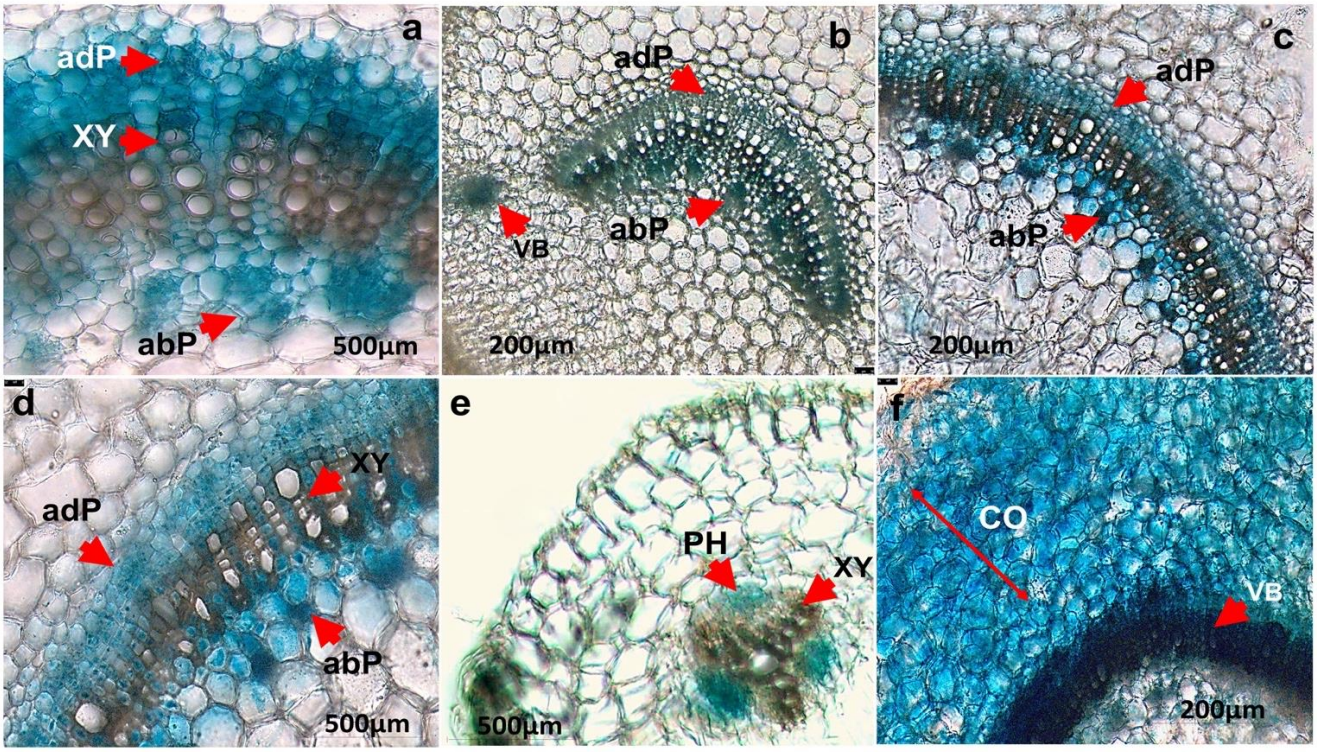


Figure 5.4 GUS activity in free hand cross-sections tobacco transgenic plants containing PRO_{GhNAC4}:GUS construct.

(a) Leaf; (b) Petiole; (c) Stem (d) Magnified view of the stem; (e) Root; (f) Wounded stem. abP, abaxial phloem; adP, adaxial phloem; CO, cortex; P, phloem; VB, vascular bundle; XY, xylem. All arrows show strong GUS activity or no activity. Bars of each panel are as shown.

The occurrence of a unique pattern of *GhNAC4* promoter activity could be corroborated by the presence of several motifs responsible for the tissue-specific expression patterns, as shown in Table 4.1. Several copies of the developing embryo and seed-specific motifs such as SEF1MOTIF, SEF3MOTIFGM, and SEF4MOTIFGM7S motifs were observed in the *GhNAC4* promoter (Lessard et al., 1991). Binding sites for three-amino-acid-loop-extension (TALE) TFs that are known to control the formation and maintenance of meristem are observed in the promoter of *GhNAC4* gene. These homeoproteins provide a gene regulatory link between hormonal stimuli and development of shoot apical meristem (Hamant and Pautot 2010).

Ten copies of OSE2ROOTNODULE are observed in the *GhNAC4* promoter. OSE1ROOTNODULE and OSE2ROOTNODULE motifs are root-specific elements found in the promoter of *Vicia faba* leg-hemoglobin gene (Fehlberg et al., 2005). Eleven copies of the ROOTMOTIFTAPOX1 element are predicted in the *GhNAC4* promoter. These are also found in the promoter of the *rolD* gene of *Agrobacterium rhizogenes,* which has a distinct expression pattern in the root elongation zone and vascular bundle (Elmayan and Tepfer, 1995).

The CACTFTPPCA1 motif is a key component of mesophyll expression module 1 (MEM1) and is sufficient for high mesophyll-specific expression (Gowik et al., 2004). Twenty-two copies of CACTFTPPCA1 motif are observed in the *GhNAC4* promoter region. The TAAAGSTKST1 is a guard cell-specific motif found upstream of K^+ influx channel gene (*KST1*) in potato (Plesch et al., 2001). Five copies of TAAAGSTKST1 motif are also observed in the *GhNAC4* promoter region.

Binding sites for quite a few other TFs known to play roles in organogenesis and tissue-specific expressions were also predicted such as MYBST1, DOFCOREZM, SBP TF, TCR TF, AT HOOK TF, and WOX TF. *GhNAC4* has 26 copies of DOFCOREZM motif, which is a binding site of DOF TFs. They regulate directly or indirectly the processes associated with the establishment and maintenance of the vascular system (Le Hir and Bellini, 2013). Squamosa promoter binding protein (SBP) TFs are

plant-specific TFs that are known to play roles both in vegetative phase change and flower development (Klein et al*.,* 1996). Various motifs such as CARCGW8GAT, TGTCACACMCUCUMISIN and binding sites for MYBPZM and TCR TFs known to be important for flower and fruit development were found in the *GhNAC4* promoter. TGTCACACMCUCUMISIN is a fruit-specific motif found upstream of a subtilisin-like serine protease, a cucumisin gene in melon (Yamagata et al., 2002). MYBPZM is a MYB homolog, P-transcription factor from maize that binds to CCT/AACC sequence and controls red pigmentation in the floral organs by regulating the flavonoid biosynthetic pathway (Grotewold et al., 1994).

Several copies of the two pollen-specific motifs, POLLEN1LELAT52 and GTGANTG10, are also found in the *GhNAC4* promoter. POLLEN1LELAT52 is one of the two co-dependent motifs found upstream of tomato *LAT52* gene that is essential for pollen development (Bate and Twell 1998). The GTGANTG10 motif is important for the pollen-specific expression of tobacco pectate lyase, *g10* gene (Rogers et al., 2001).

GATA motif plays a role in light responsiveness and tissue specificity and is involved in the light-dependent development of phloem tissue (Yin et al., 1997). Forty-two copies of the GATA motif were predicted in the *GhNAC4* promoter region. Fang et al. (2008) carried out a systematic sequence analysis of NAC genes in rice and identified 12 particularly important tissue-specific NAC TFs. The promoters of these genes were predicted to have motifs such as SEF3MOTIFGM, ROOTMOTIFTAPOX1, and GTGANTG10.

5.2.3 *GhNAC4* promoter is induced by various phytohormones

To explore the possible regulation of *GhNAC4* promoter by phytohormones, GUS activity was examined by fluorometric MUG assay, and treating the T_2 transgenic tobacco seedlings expressing the PRO$_{GhNAC4}$:GUS fusion with various hormones. The corresponding results have been depicted in Fig. 5.5. The specific activity of GUS enzyme without any hormone treatments in PRO$_{GhNAC4}$:GUS

seedlings was measured to be 2.4 ± 0.4 pmol μg^{-1} min^{-1}. This is in agreement with the histochemical staining results, where GUS staining was observed even in untreated seedlings. *GhNAC4* promoter was highly induced by BAP and ABA (~ 67.9 % and 63.5 %). IAA and MeJA treatment caused ~ 58.9 % and 54.6 % induction of the promoter activity, respectively. GA_3 and ethylene also enhanced the *GhNAC4* promoter activity by ~ 53.3 % and 45.9 % respectively. The promoter showed no significant induction by SA application as compared with the untreated control samples. The pattern of *GhNAC4* promoter induction observed in phytohormonal treatments in tobacco seedlings is similar to the *GhNAC4* expression in cotton seedlings.

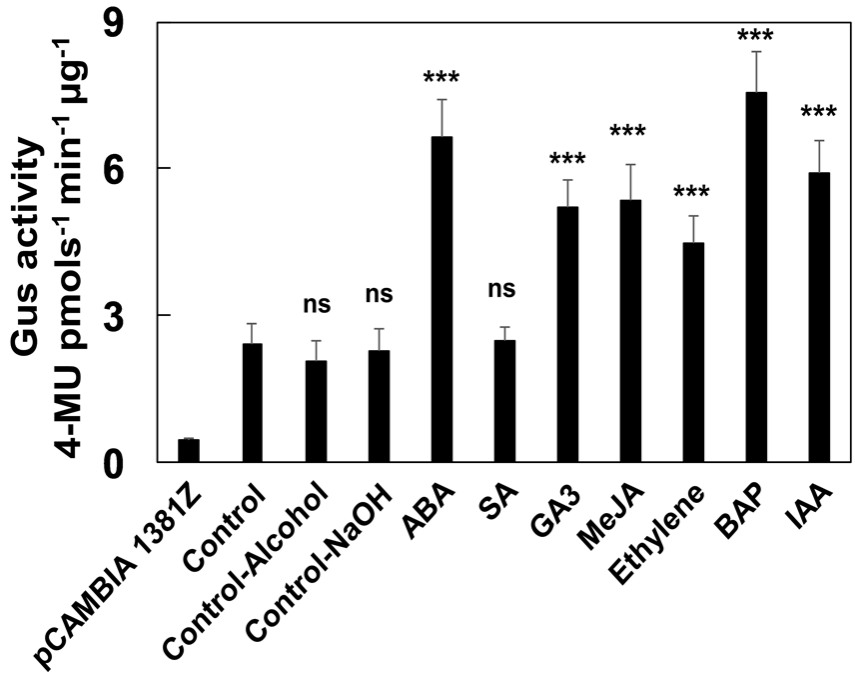


Figure 5.5 Effect of various phytohormones on the GUS activity of PRO$_{GhNAC4}$:GUS tobacco transgenics.

Fluorometric analysis of the GUS in tobacco seedlings after ABA, MeJA, SA, 6-BAP, GA_3, IAA, or Ethephon treatment. Two weeks-old PRO$_{GhNAC4}$:GUS tobacco transgenic seedlings incubated for 24 h under the treatment, were used for analysis. pCAMBIA 1381Z empty vector harboring tobacco seedlings were used as a negative control. The data are shown as the means ± SE (n=3). A statistical analysis with one-way ANOVA indicates significant differences (*** $P < 0.001$, ns - not significant). ABA, abscisic acid; SA, salicylic acid; GA3, 3-gibberellic acid; MeJA, methyl jasmonic acid; BAP, 6-benzyl aminopurine; IAA, indole-3-acetic acid.

Evaluating the *cis*-elements present in the promoter region of the *GhNAC4* gene might allow us a better understanding of the differential up-regulation of *GhNAC4* by various phytohormones. *GhNAC4* promoter was predicted to have one copy of CPBCSPOR motif and 16 binding sites for Authentic Response Regulators1 (ARR1) as mentioned in Table 4.1. CPBCSPOR motif is essential for cytokinin dependent transcriptional activation (Fusada et al., 2005). ARR1 is an important signaling component acting at the head of transcriptional cascade to regulate cytokinin response and is known to be involved in cytokinin-mediated differentiation of protoxylem (Yokoyama et al., 2007).

Several motifs known for ABA responsiveness such as six copies of ACGTABREMOTIFA2OSEM, eight copies of ABRELATERD1 and one copy of ABREOSRAB21 motifs were predicted on the *GhNAC4* promoter. Mutation of the ABRE in the *OsNAC5* promoter led to the abolition of the activation of the promoter by ABA (Takasaki et al., 2010). Auxin responsive motifs such as AUXREPSIAA4, TGA element, GGTCCCATGMSAUR, and CATATGGMSAUR were predicted on the *GhNAC4* promoter. Auxin Response Factors (ARFs) bind to AUXRE and mediate hormone responses (Guilfoyle et al. 1998). *AtNAC1* plays a key role in promoting auxin-mediated lateral root formation (Xie et al., 2000). A plasma membrane-bound NAC TF *NTM2* integrates auxin and salt signaling via the *IAA30* gene during seed germination in *Arabidopsis* (Park et al., 2011).

GhNAC4 promoter region was also predicted to contain JA responsive motifs such as T/GBOXATPIN2, TGACG, and CGTCA. JAMYC/AtMYC2 TF binds to the T/GBOXATPIN2 motif found in the promoter of JA responsive and wound-inducible Protease Inhibitor II (*PIN2*) gene (Boter et al., 2004). ANAC019 and ANAC055 act downstream of AtMYC2 as transcriptional activators to regulate JA-signaled defense responses (Bu et al., 2008).

GhNAC4 promoter was predicted to have four copies GAREAT and two copies of GARE1OSREP1 motifs, which are gibberellin responsive elements, GARE (Skriver et al., 1991). *GhNAC4* promoter region also exhibits four copies of PYRIMIDINEBOXOSRAMY1A motif. Multiple

copies of GARE alone or in association with other motifs such as PYRIMIDINE box or TATCCAC box (forming the Gibberellins Response Complex) are essential for GA induced gene expression at higher levels (Lanahan et al., 1992; Rogers et al., 1994). GADOWNAT motif is a common sequence found in the genes down-regulated by GA, and five copies of this motif are present in the *GhNAC4* promoter. These sequences may function as phytohormonal responsive motifs in the *GhNAC4* promoter. A detailed study of GhNAC4 and its promoter is required to determine the phytohormone signaling pathway associated with the regulation of GhNAC4.

5.2.4 *GhNAC4* promoter is responsive to various environmental stress treatments

As *GhNAC4* gene expression was regulated by various stresses, a study of the promoter activity in transgenic tobacco plants harboring the PRO_{GhNAC4}:GUS fusion by treating them with various environmental stresses was carried out. The GUS activity was measured by the fluorometric MUG assay as shown in Fig. 5.6. Drought stress treatment resulted in high induction of the *GhNAC4* promoter (~ 71 %). Salinity and osmotic stress treatments also enhanced the promoter activity by ~ 68.5 % and 69.5 %, respectively. Oxidative stress and air-drying treatments up-regulated the promoter activity by ~ 68.2 % and 66.6 %, respectively. A combination of low-temperature stress and darkness treatment activated the *GhNAC4* promoter by ~ 59.3 % as compared to darkness treatment alone (~ 43.1 %) suggesting an additive effect of up-regulation of *GhNAC4* promoter induction. High-temperature and wounding stress treatments induced the promoter activity by ~ 42.1 % and 41.7 %, respectively. Flooding stress caused by submerging the seedlings in water also enhanced the promoter activity (~ 49.7 %). The pattern of *GhNAC4* promoter induction observed by environmental stress treatments in tobacco seedlings is similar to the *GhNAC4* expression in cotton seedlings.

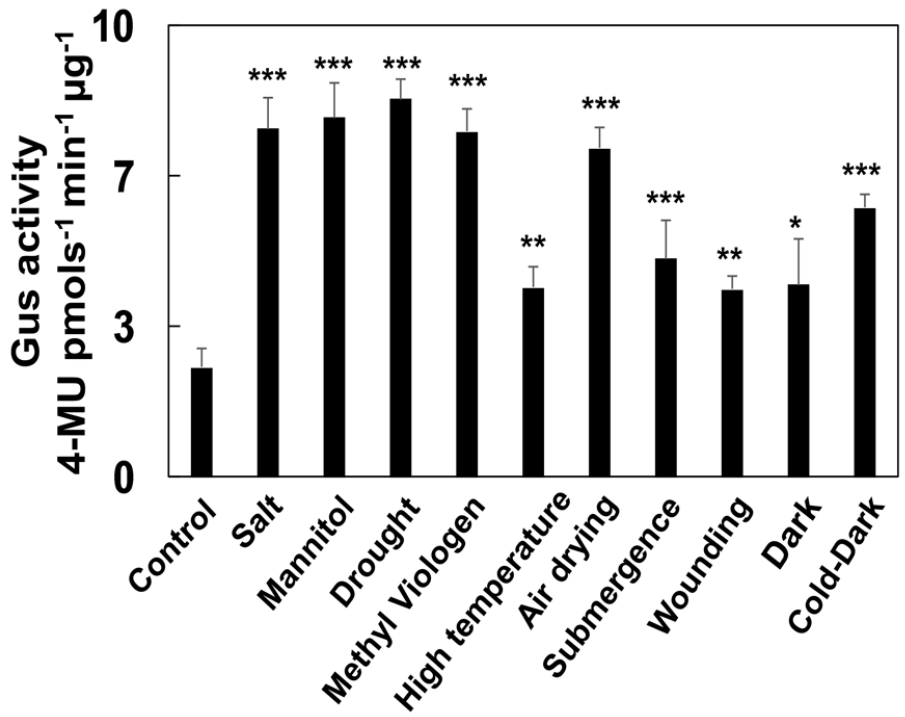


Figure 5.6 Effect of various stresses on the GUS activity of PRO$_{GhNAC4}$:GUS tobacco transgenics.
Fluorometric analysis of the GUS in tobacco seedlings after salt, mannitol, drought, methyl viologen, high temperature, air drying, submergence, wounding, dark or combination of dark and cold treatment. Two weeks-old PRO$_{GhNAC4}$:GUS tobacco transgenic seedlings, were used for analysis. The data are shown as the means ± SE (n=3). A statistical analysis with one-way ANOVA indicates significant differences (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$).

Evaluating the *cis*-elements present in the promoter region of the *GhNAC4* gene might help us with a better understanding of the differential up-regulation of *GhNAC4* by various environmental stress treatments. GhNAC4 promoter region was predicted to contain many motifs like MYB2CONSENSUSAT, C-REPEAT/DRE, DRECRTCOREAT, CSD, and CBFHV TF binding sites required for drought and cold responsiveness, as shown in Table 4.1. Two copies of MYB2CONSENSUSAT motifs are found in the *GhNAC4* promoter, which is the binding site for AtMYB2 TF required for drought inducibility of rd22 gene (Abe, 2003). Cold responsive motif, C-repeat (CRT) is responsible for the regulation of many cold inducible genes in an ABA-independent manner. It is also involved in dehydration responsiveness (Stockinger et al., 1997). A fox-tail millet stress-responsive *SiNAC*

promoter exhibits binding sites for MYB and MYC TFs and also contains CRT motif (Puranik et al., 2011). Three copies of the DRECRTCORE motif are found in the GhNAC4 promoter. CBFHV motif is a binding site of an AP2 domain-containing cold inducible TF, HvCBF1 characterized in barley (Xue, 2002). Three copies of CBFHV motif are found in the GhNAC4 promoter. Eight copies of binding site for cold shock domain (CSD) proteins are also found in the *GhNAC4* promoter, that is highly activated during low-temperature stress. Apart from being important in cold adaptation, these elements are also known to play roles in plant development (Chaikam and Karlson, 2008). Fang et al. (2008) carried out systematic sequence analysis of NAC genes in rice and identified 20 particularly important stress inducible NAC TFs. The promoters of these genes were predicted to have several stress-responsive motifs such as MYB1AT, MYB2CONSENSUSAT, MYCCONSENSUSAT, CBFHV, DRECRTCOREAT, and CRTDREHVCBF2.

Heat stress transcription factors (HSF) bind to the heat stress responsive element (HSE) and modulate transcription during heat stress (Baniwal et al., 2004). Four copies of HSF binding site are present in the *GhNAC4* promoter. Binding sites for C2H2 TF were also identified in the *GhNAC4* promoter region. C2H2 TFs such as ZAT7, ZAT10, and ZPT2 are known to be important in regulating responses to abiotic and biotic stress tolerance (Kiełbowicz-Matuk, 2012). *GhNAC4* promoter exhibited four copies of WBOXNTERF3 motifs. WRKY TF binds to the WBOXNTERF3 motif in the promoter of *ERF3* gene of tobacco and causes its rapid activation upon wounding (Nishiuchi et al., 2004). The promoter of wound-inducible rice NAC gene, *OsNAC6* also exhibits four copies of W-box (Nakashima et al., 2007). Two copies of ANAERO1CONSENSUS, a motif found upstream of anaerobic induced genes was identified (Mohanty et al., 2005). These sequences may function as environmental stress-responsive motifs in the *GhNAC4* promoter. A detailed study of GhNAC4 and its promoter is required to determine the stress-responsive pathway associated with the regulation of GhNAC4.

Chapter 6

Structure-function relationship of *Gossypium hirsutum* NAC transcription factor, GhNAC4 with regard to ABA and abiotic stress responses

6.1 Introduction

Transcription factors (TFs) are key modulators of gene expression patterns leading to the regulation of growth, development and environmental stress responses. A typical TF minimally consists of a DNA binding domain and a transcriptional regulatory domain. The earliest reports of the modular nature of TFs came from the 'domain-swap' experiment with the fusion of DNA binding domain of LexA (*Escherichia coli* repressor protein) and the activation domain of GAL4 TF (*Saccharomyces cerevisiae* transcriptional activator). This resulted in a chimeric transcriptional activator that identified LexA binding site (Brent and Ptashne, 1985). This remarkable modular nature of TFs has been confirmed in many other systems (Graham et al., 1999; Hollenberg and Evans, 1988; Porsch et al., 2005). The exact position of these domains/modules within the chimeric proteins is highly flexible suggesting that each domain represents an independent structural module with an independent function (Frankel and Kim, 1991).

Although several studies have investigated the function of NAC TFs, very few studies have aimed at dissecting the structure-function relationships of NAC TFs. Jensen et al. (2010) identified ANAC019 as a positive regulator of ABA responses and demonstrated that the ectopic expression of full length N-terminal NAC domain or C-terminal TR-domain of *ANAC019* alone conferred hypersensitivity to exogenous ABA. To understand the structure-function relationship of ANAC019 for ABA responsiveness, they developed chimeric proteins by swapping the NAC-domain and TR-domains with analogous regions from other NAC TFs and demonstrated that the biochemical and functional specificity of ANAC019 is associated with both the NAC and the TR-domains.

In another study, Taoka et al. (2004) investigated the domain specificity of *Arabidopsis* CUC TFs, which play an essential role in shoot apical meristem formation. They demonstrated that TR-domains from three different NAC TFs could be fused to NAC-domain of CUC2 and could still retain the shoot induction functionality of CUC2. However, replacing the CUC2 NAC-domain with ATAF NAC-

domain resulted in the loss of shoot induction functionality. Domain swapping of CUC2 TF revealed that the NAC-domain is essential for the adventitious shoot formation. However, the structure-function relationship of NAC TFs, particularly with respect to abiotic stress tolerance remains largely unclear.

In the present study, structure-function relationship of GhNAC4 TF functionality with respect to abiotic stress tolerance and ABA-responsiveness was determined.

6.2 Results

In the chapter 4, it has been shown that *GhNAC4* is highly induced in PEG, NaCl and ABA treatments in cotton (Figs. 4.1 and 4.2). This has prompted us to further examine the role of GhNAC4 protein in abiotic stress responses.

6.2.1 GhNAC4 is a nuclear protein

The recombinant construct carrying the pEGAD:*GhNAC4* translational fusion was agroinfiltrated in onion epidermal cells and visualized through laser scanning confocal microscopy. The GhNAC4:EGFP fusion protein has been identified to localize in the nucleus (Fig. 6.1). The EGFP of the empty pEGAD vector, which was used as a control was observed throughout the whole cell. This result suggests that GhNAC4 is a nuclear-localized protein.

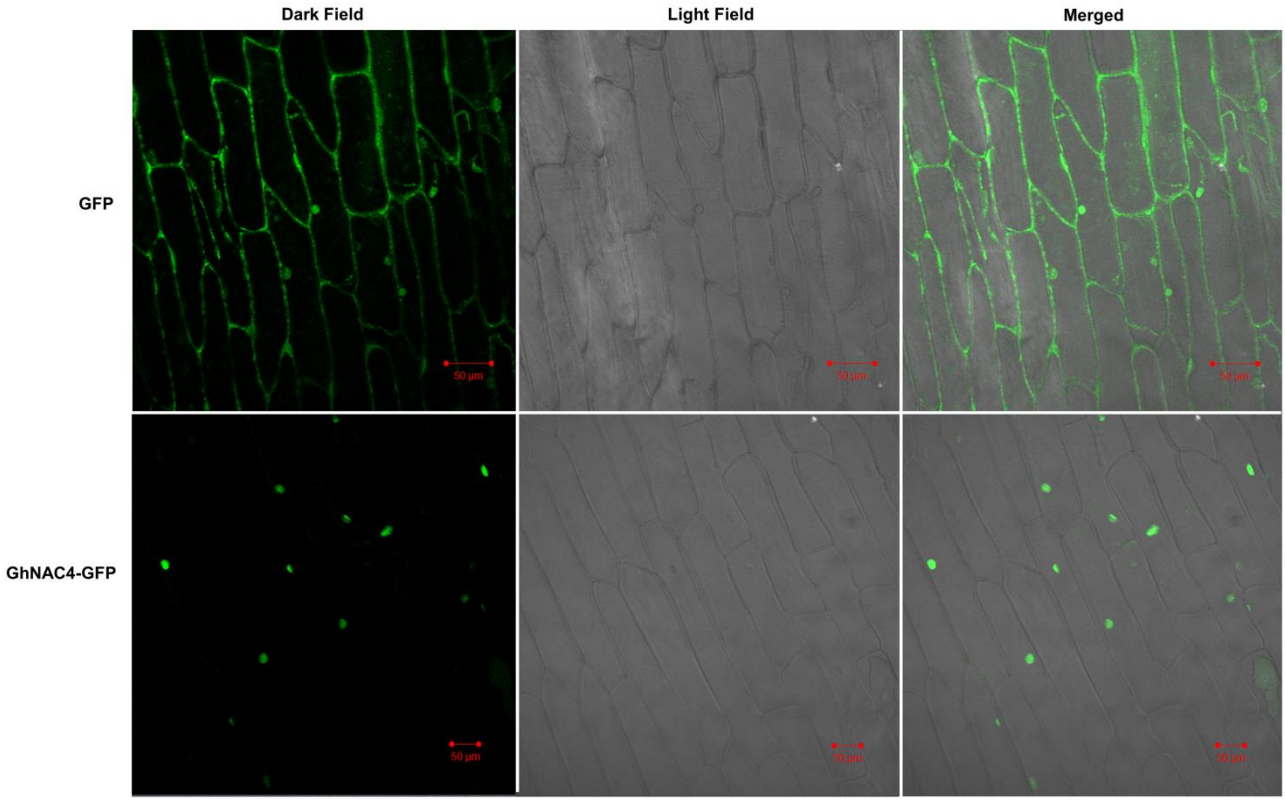


Figure 6.1 Sub-cellular localization of GhNAC4.

Empty pEGAD vector and pEGAD:*GhNAC4* recombinant vectors were agroinfiltrated in onion epidermal peels for transient expression. The fluorescence was analyzed by laser scanning confocal microscopy. The experiment was repeated three times and representative images have been presented here. Scale bar = 50 µM.

6.2.2 GhNAC4 possesses transcription activation property

GhNAC4 has the structure of a typical NAC TF and is predicted to possess an N-terminal NAC-domain and a C-terminal intrinsically disordered transcription regulatory domain (Fig. 6.2). To verify whether GhNAC4 possessed transcription activation property, it was fused to the GAL4 DNA binding domain, as shown in Fig. 6.3. The transcription activation was examined by growing the transformants on Ura^-, Ura His^- and Ura Ade^- media and quantifying the β-galactosidase activity. The full-length GhNAC4 (1-346 aa) and the C-terminal truncated variant, GhNAC4Δ140-346-C showed growth on all media whereas the N-terminal truncated variant, GhNAC4Δ1-139-N failed to grow. It suggests that GhNAC4 is a transcription activator and the C-terminal region of the protein is responsible for this property.

Interestingly, the β-gal assay indicates that the C-terminal variant possesses twice the activity as compared to the full-length protein.

To determine the minimum region essential for the transcription activation property, a progressive series of deletion variants of GhNAC4 were analyzed in yeast. The minimal truncated region that showed transcription activation was from amino acid 140-199 (GhNAC4Δ1-199-C5). Further deletion completely abolished the transcriptional activation (GhNAC4Δ1-169-C6) property. The β-gal assay demonstrated that progressive deletion of 30 amino acids reduced the activity by half, as indicated in Fig. 6.3.

GhNAC4 C-terminal region is predicted to possess three intrinsically disordered regions (Fig. 6.2). To determine the region of the C-terminal domain necessary for the transcription activation property, a stepwise and combination deletions of these low-complexity regions was carried out (Fig. 6.2). Removal of the regions GhNAC4Δ169-189-D1 and GhNAC4Δ245-261-D2 in combination almost abolished the transcription activation. This indicates the importance and dependency of the transcription activation property on the intrinsically disordered regions.

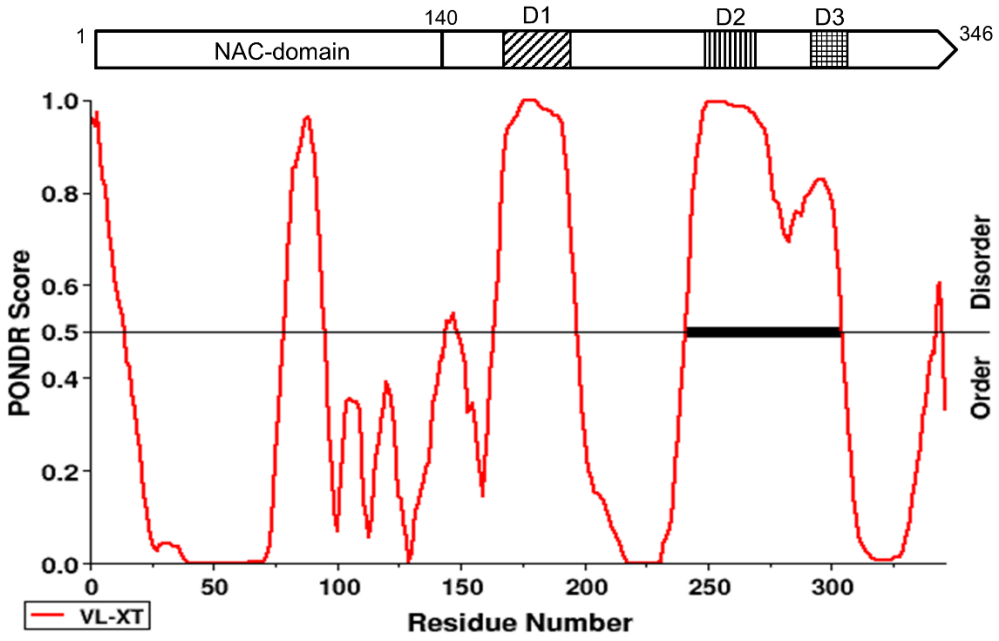


Figure 6.2 Structure of GhNAC4.

Top, Pictorial representation of the predicted structure of GhNAC4 drawn to scale with N-terminal NAC domain and the C-terminal showing three intrinsically disordered regions, D1, D2 and D3. Bottom, prediction of intrinsically disordered regions of GhNAC4 using PONDR (http://www.pondr.com/). The threshold for disordered regions is ≥ 0.5.

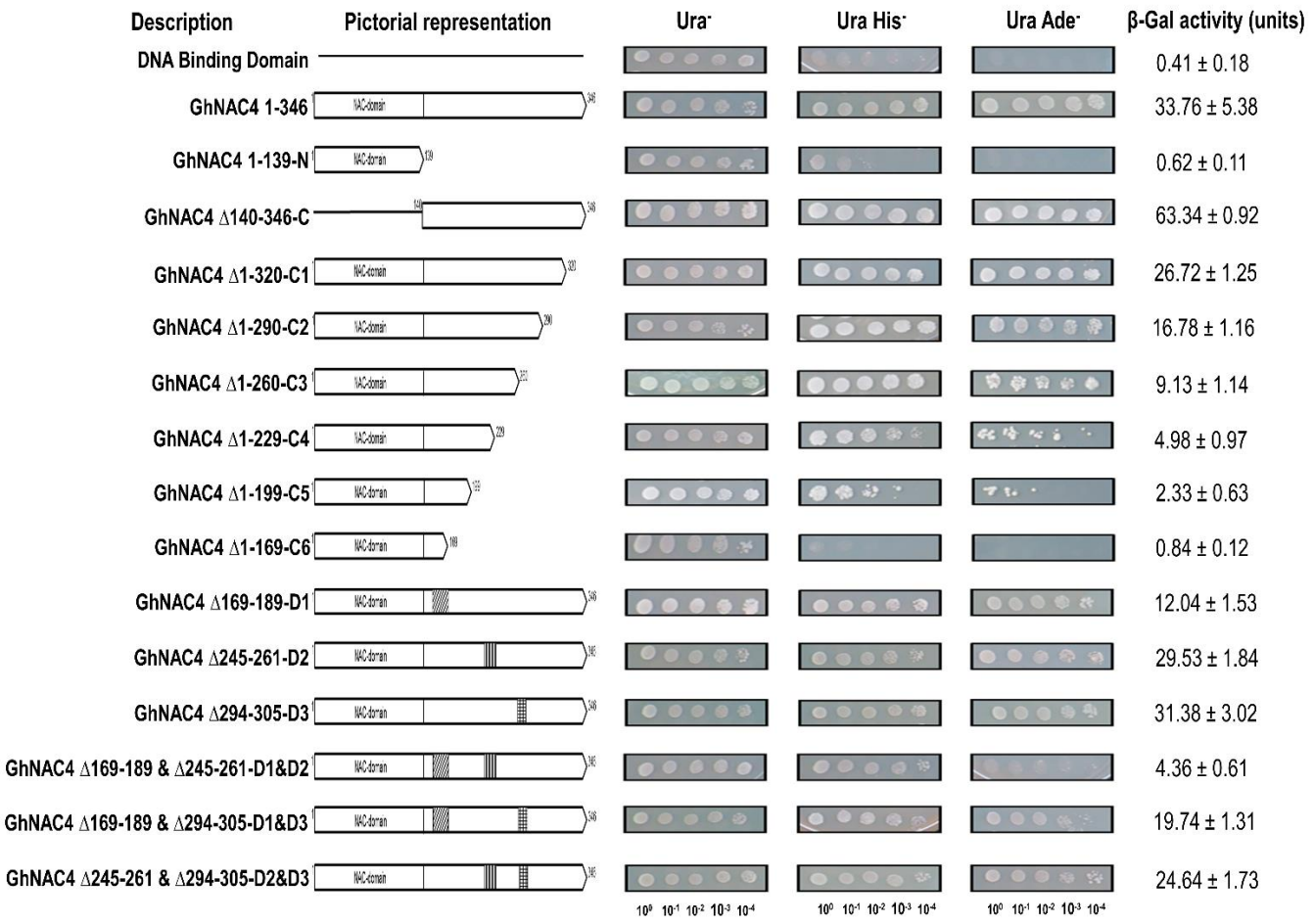


Figure 6.3 Transcription activation of GhNAC4 in yeast and mapping of the activation domain.

Various deletion constructs of *GhNAC4* (left) were fused to the GAL4 DNA-binding domain of pGBDU vector and transformed in yeast strain, pJ694a and screened for transcription activation assay. Transformed yeast cells were serially diluted 10-fold and spotted (right) onto plates lacking uracil (Ura^-) to check for transformation and on plates lacking uracil and histidine ($Ura\ His^-$) and uracil and adenine ($Ura\ Ade^-$) to allow the screening of weak and strong transcription activation activity respectively. Empty pGBDU vector was used as a negative control. β-galactosidase activity (units), represented as mean ± SE from three independent transformations, is indicative of transcription activation (extreme right).

6.2.3 GhNAC4 exists as a dimer

Ernst et al. (2004) reported that ANAC019 exists as a dimer. The formation of two salt bridges by the conserved Arginine 19 and Glutamate 26 are among the prominent interactions between the two dimers. Strong transcription activation capability of the full-length GhNAC4 and the C-terminal domain (GhNAC4Δ140-346-C) prevents protein-protein interaction studies. To assess the homo-dimerization property of GhNAC4, a yeast two-hybrid assay was carried out using the N-terminal

NAC-domain (GhNAC4Δ1-139-N), as shown in Fig. 6.4. The protein-protein interaction was examined by growing the transformants on Ura Leu$^-$, Ura Leu His$^-$ and Ura Leu Ade$^-$ media and quantifying the β-galactosidase activity. The N-terminal domain was able to homo-dimerize although weakly. Any point mutation to disrupt either one or both the salt bridges resulted in the complete abolition of dimerization as shown in Fig. 6.4. This indicates the importance and dependency of the salt bridges for the homo-dimerization property of GhNAC4 protein.

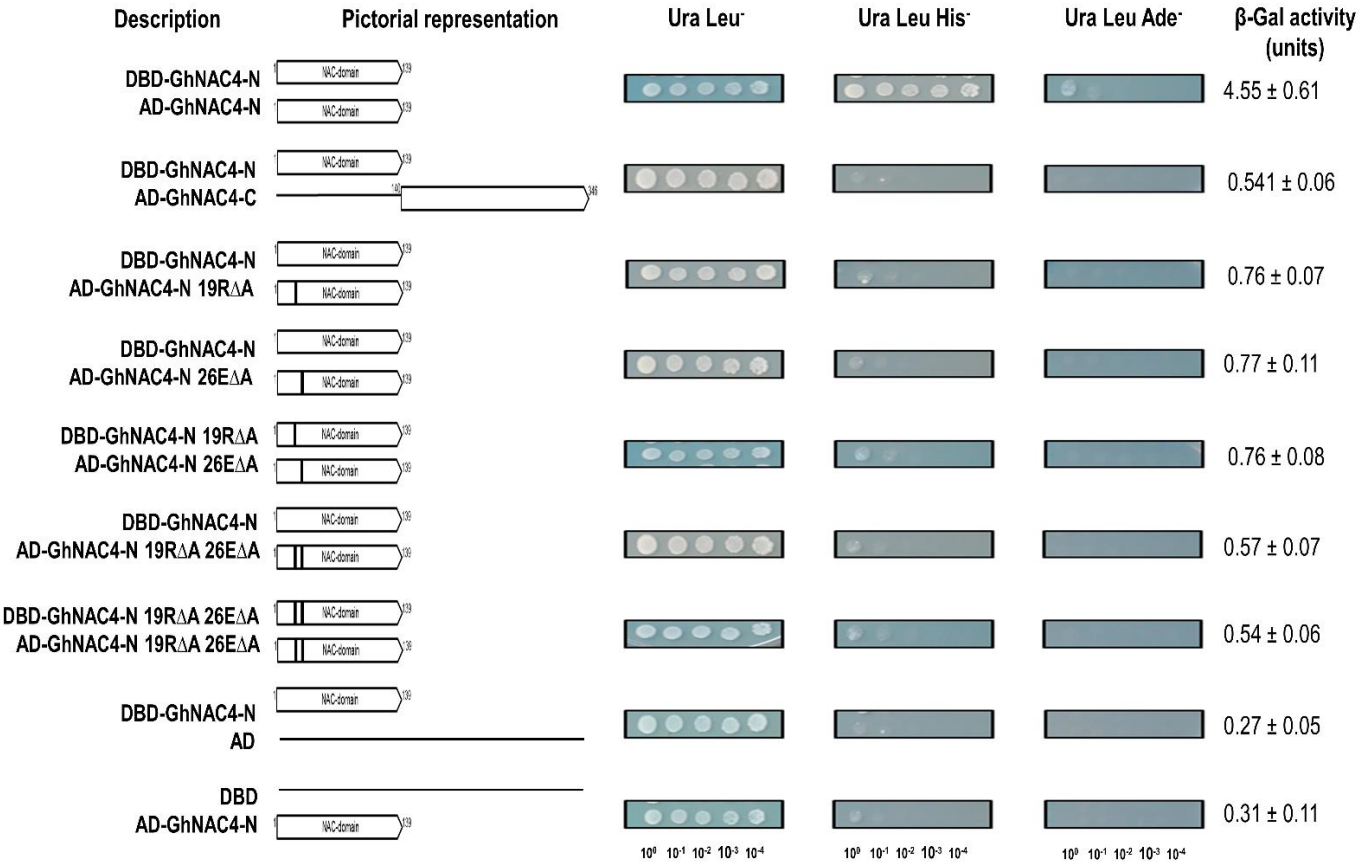


Figure 6.4 Homo-dimerization of GhNAC4.

Various deletion constructs of *GhNAC4* (left) were fused to the GAL4 DNA-binding domain (DBD) of pGBDU vector and GAL4 activation domain (AD) of pGAD vector and transformed in yeast strain, pJ694a to screen for homo-dimerization assay. Transformed yeast cells were serially diluted 10-fold and spotted (right) onto plates lacking uracil and leucine (Ura Leu$^-$) to check for transformation and on plates lacking uracil, leucine and histidine (Ura Leu His$^-$) and uracil, leucine and adenine (Ura Leu Ade$^-$) to allow the screening of weak and strong protein-protein interaction activity, respectively. Empty pGBDU and pGAD vectors were used as negative controls. β-galactosidase activity (units), represented as mean ± SE from three independent transformations, is indicative of protein-protein interaction (extreme right).

6.2.4 The NAC-domain is sensitive to ABA-mediated seed germination and post-germinative growth and both domains are required for stress tolerance

The increased expression of *GhNAC4* in response to exogenous ABA, NaCl and PEG (Figs. 4.1 and 4.2) suggested that GhNAC4 might play a role in ABA signaling and abiotic stress responses. ABA plays a crucial role in abiotic stress response as well as in seed germination and seedling growth (Nakashima and Yamaguchi-Shinozaki, 2013). Thus, to investigate the role of the full-length, NAC- and the TR-domains of GhNAC4 protein in ABA and abiotic stress responses, transgenic tobacco plants overexpressing GhNAC4 were generated and its domains were also expressed independently in transgenic plants (Figures 6.5, 6.6 and 6.7).

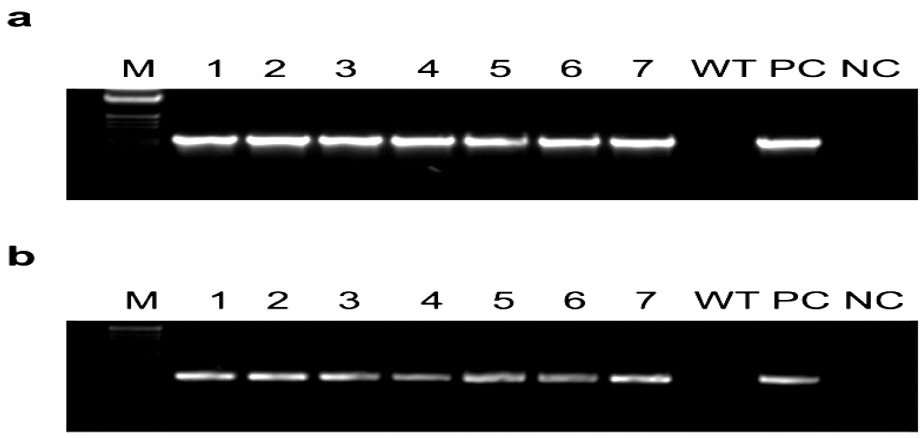


Figure 6.5 Confirmation of GhNAC4 T_0 putative transgenic plants by PCR using tobacco genomic DNA.

A representative gel picture showing **a** 1041 bp amplified product of GhNAC4 cds and **b** 739 bp amplified product of *nptII*. 'M' represents λ *EcoR*I*/Hin*dIII DNA ladder. 'PC' represents positive control for the PCR using plasmid (pCAMBIA2300::GhNAC4) as a template. 'WT' represents negative control.

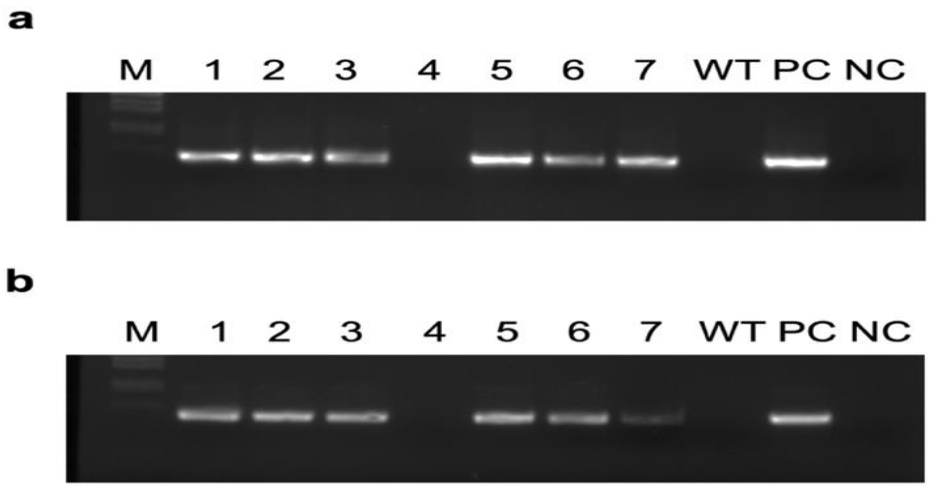


Figure 6.6 Confirmation of GhNAC4 NAC-domain T_0 putative transgenic plants by PCR using tobacco genomic DNA.

A representative gel picture showing **a** 417 bp amplified product of N-terminal NAC-domain of GhNAC4 and **b** 739 bp amplified product of *nptII*. 'M' represents λ *Eco*RI*/Hin*dIII DNA ladder. 'PC' represents positive control for the PCR using plasmid (pCAMBIA2300::GhNAC4-N) as a template. 'WT' represents negative control for the PCR using DNA from untransformed plant. 'NC' represents no template control.

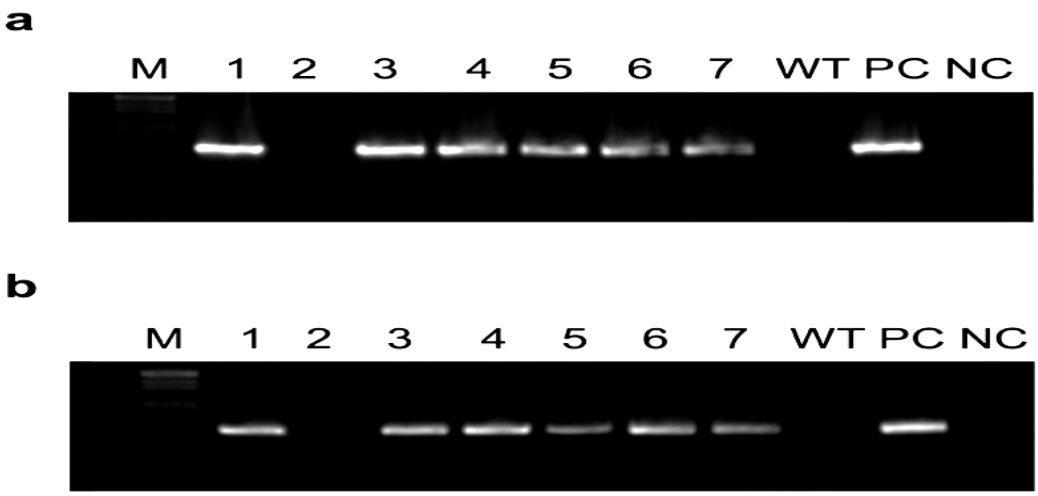


Figure 6.7 Confirmation of GhNAC4 TR-domain T_0 putative transgenic plants by PCR using tobacco genomic DNA.

A representative gel picture showing **a** 624 bp amplified product of C-terminal transcriptional regulatory domain of GhNAC4 and **b** 739 bp amplified product of *nptII*. 'M' represents λ *Eco*RI/*Hin*dIII DNA ladder. 'PC' represents positive control for the PCR using plasmid (pCAMBIA2300::GhNAC4-C) as a template. 'WT' represents negative control for the PCR using DNA from untransformed plant. 'NC' represents no template control.

The germination rates of wild type, GhNAC4, GhNAC4-N, GhNAC4-C T_2 generation seeds were examined under various conditions as shown in Fig. 6.8. Seeds of these genotypes, germinated on 0.5x MS media showed no significant differences in germination rate under normal growth conditions. However, the germination of all genotypes under salinity stress (150 mM NaCl) was severely affected (Fig. 6.8). Wild-type showed less than 15 % germination frequency while GhNAC4-C genotype showed around 28 %. Interestingly, the germination rate of GhNAC4 and GhNAC4-N genotypes reached around 45 % and 38 % respectively, seven days after sowing. Drought stress-induced by PEG (-0.5 MPa) resulted in decreased germination frequency of wild-type and GhNAC4-C genotypes by 20 % and 40 % respectively. However, the germination frequency still reached above 70 % for both the GhNAC4 and GhNAC4-N genotypes, seven days after sowing (Fig. 6.8). This indicates that the stress response of GhNAC4 is associated with both NAC-domain and TR-domain.

The ABA sensitivity of the wild type, GhNAC4, GhNAC4-N, GhNAC4-C genotype during germination was next determined. Treatment with 2 µM ABA severely arrested the growth of GhNAC4-N genotype seeds after the emergence of the radicle suggesting hypersensitivity. Interestingly, GhNAC4-C showed very little inhibition with germination frequency reaching over 95 % suggesting near insensitivity. The germination frequencies of wild-type and GhNAC4 genotypes were also inhibited to a lesser extent (Fig. 6.8). This indicates that the NAC-domain is mainly responsible for the ABA receptivity of GhNAC4.

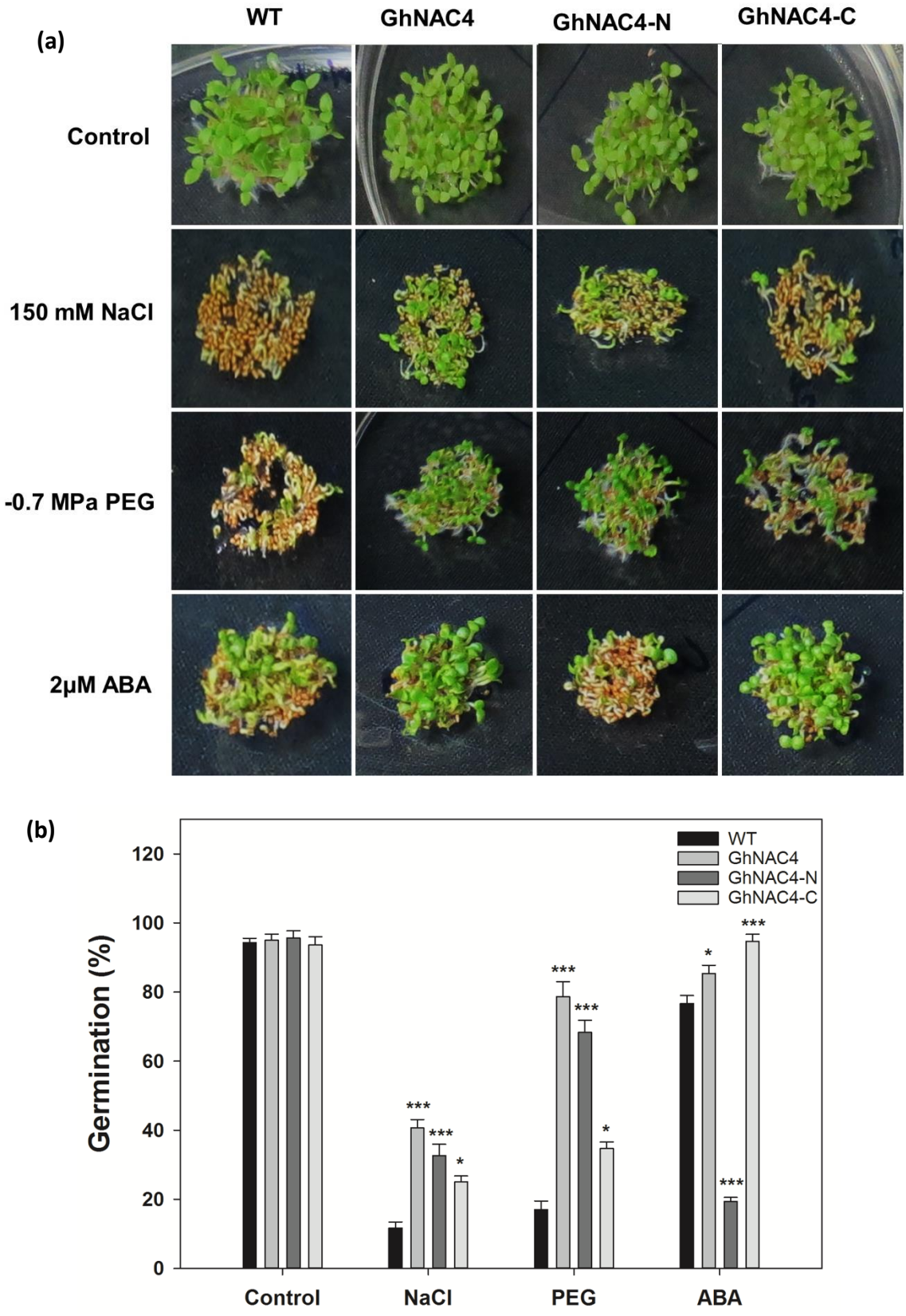


Figure 6.8 Responses of WT, GhNAC4, GhNAC4-N and GhNAC4-C expressing tobacco transgenic plants to NaCl and PEG induced stress at seed germination stage.

a Representative photograph of germination of seeds on the 7 d, sown on control medium (half-strength MS) or media containing 150 mM NaCl, -0.5MPa PEG 8000 or 2 µM ABA. **b** Quantification of the percentage of germinated seeds of each genotype on the 7 day on the indicated treatments as described in **a**. For each experiment, approximately 80-100 seeds were scored per genotype. Data are means ± SE (n=3). A statistical analysis with one-way ANOVA indicates significant differences (*** $P < 0.001$ and * $P < 0.05$).

Subsequently, wild-type, GhNAC4, GhNAC4-N, GhNAC4-C genotype seedlings were assessed for their responses to abiotic stresses and ABA during the post-germinative growth stage as shown in Fig 6.9. Seedlings of these genotypes showed no significant differences in root length under normal growth conditions. However, under salinity stress (200 mM NaCl), the growth of all genotypes was severely affected (Fig. 6.9). Root length in the wild-type reduced to ~ 18 %, while GhNAC4-C genotype around 28 %. The primary root length of GhNAC4 and GhNAC4-N genotypes reached around 77 % and 60 % respectively. In addition, drought-induced by PEG (-0.7 MPa) also caused a reduction in root length of GhANC4, GhNAC4-N and GhNAC4-C genotypes to approximately 90 %, 67 % and 40 % respectively whereas the wild-type seedlings root length reduced to 19 % (Fig. 6.9). This result clearly indicated that GhNAC4 overexpressing plants are tolerant to salinity and drought stress and this property is conferred by both NAC- and TR-domains.

Subsequently, the possible effect of ABA on the root growth was considered. As in the case of seed germination, the GhNAC4-N seedlings showed hypersensitivity to ABA (10 µM) with significantly lower primary root growth (~ 20 %) and whereas the GhNAC4-C seedlings were insensitive (Fig. 6.9). This further confirms the ABA receptivity role of NAC-domain in GhNAC4.

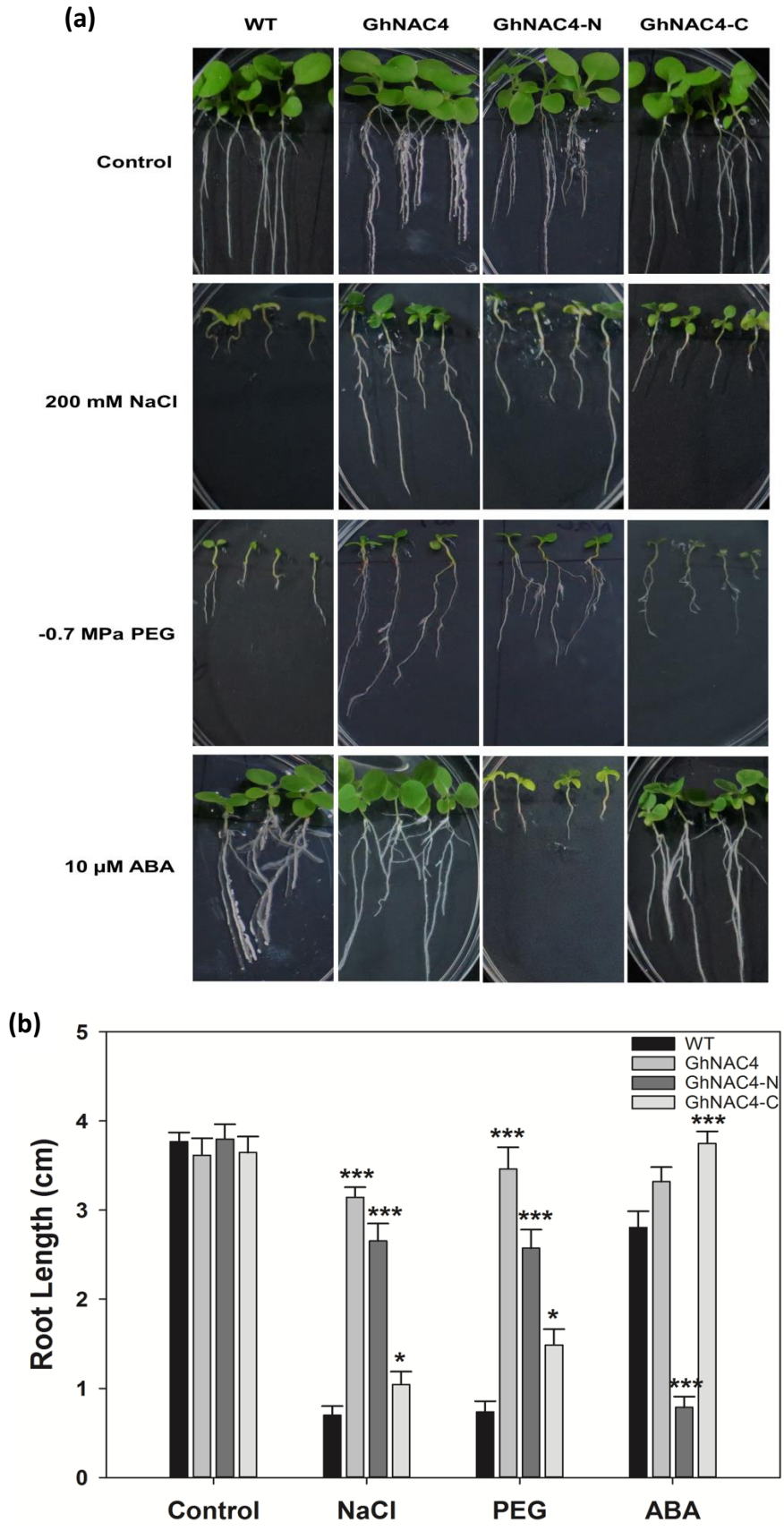


Figure 6.9 Responses of WT, GhNAC4, GhNAC4-N and GhNAC4-C expressing tobacco transgenic plants to NaCl and PEG induced stresses at post-germination stage.

a Representative photograph of seedlings at 15 d after transfer to control medium (half-strength MS) or media containing 200 mM NaCl, -0.7MPa PEG 8000 or 10 µM ABA. Seeds were germinated on half-strength MS for 7 d before transfer. **b** Quantification of root lengths for seedlings treated as described in **a**. For each experiment, approximately 18-21 seedlings were scored per genotype. Data are means ± SE (n=3). A statistical analysis with one-way ANOVA indicates significant differences (*** $P < 0.001$ and * $P < 0.05$).

6.2.5 NAC-domain transgenics showed enhanced stomatal closure under ABA treatment

To understand the physiological roles of the NAC and TR-domains, the behaviour of guard cells under ABA treatment was investigated. As seen in Fig. 6.10 stomatal aperture of GhNAC4-N and GhNAC4-C genotypes, respectively were ~ 20 and 36 % smaller that than that of wild-type plants, under control conditions while there was no significant difference in the stomatal aperture of GhNAC4 plants. ABA is known to induce stomatal closure (Bright et al., 2006) and treatment with 10 µM ABA promoted stomatal closure in all genotypes (Fig. 6.10). However, the leaves of GhNAC4-N genotype showed a greater percentage of stomatal closure (~ 60 %). In GhNAC4-C plants, the same treatment reduced the stomatal aperture by ~ 17 %. Wild type and GhNAC4 plants showed a reduction by ~ 40-46 % (Fig. 6.10). This corroborated the observation that GhNAC4-N genotype shows hypersensitivity to ABA while GhNAC4-C genotype is nearly insensitive and GhNAC4 is a positive regulator of ABA-mediated stomatal closure.

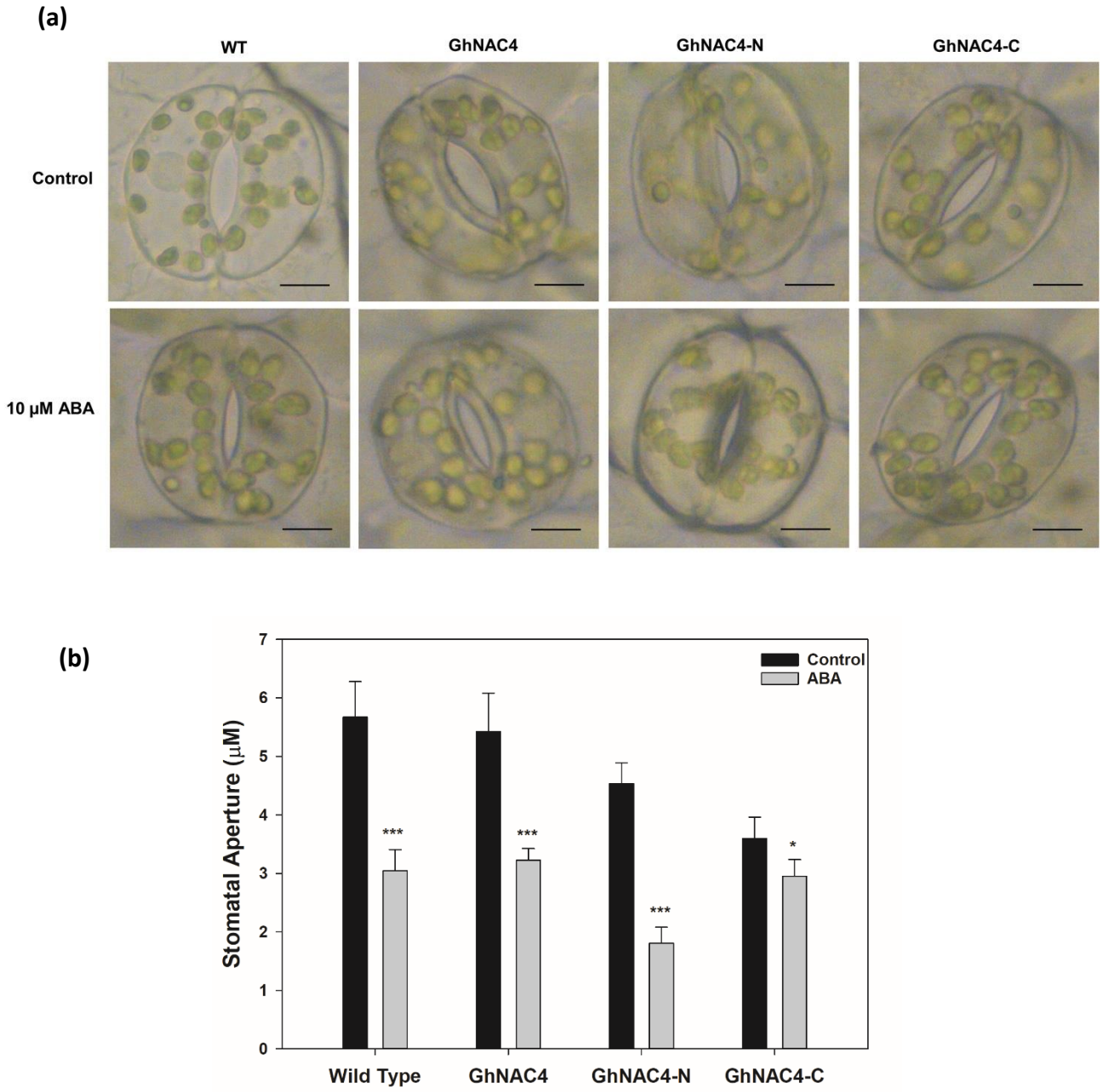


Figure 6.10 Responses of WT, GhNAC4, GhNAC4-N and GhNAC4-C expressing tobacco transgenic plants to ABA-induced stomatal closure.

Epidermal peels prepared from abaxial sides of the leaves were floated in MES-KCl buffer for 3 h under high-light conditions to induce full opening of stomata. Subsequently, they were treated with 10µM ABA for 150 min. **a** A representative image from each genotype is presented. Scale bar = 5µm. **b** Quantification of stomatal aperture treated as described in **a**. Following treatment, the width of the stomatal aperture was measured. The experiment was performed in triplicate with 60 pairs of guard cells per genotype per experiment. Values are means ± SE. A statistical analysis with one-way ANOVA indicates significant differences (*** P<0.001, * P<0.5).

The modulation of the NAC and TR-domains in ABA-mediated stomatal closure in the transgenic plants has again prompted us to examine whether they show differences in the regulation of dehydration responses. To investigate this, leaves of wild-type, GhNAC4, GhNAC4-N and GhNAC4-C genotypes were excised and water loss was examined over a period of time (Fig 6.11). After dehydration for 9 h, the water loss from excised leaves was higher for wild-type plants (~ 75 %) as compared to the GhNAC4 plants (~ 25 %). The excised leaves of GhNAC4-N genotype lost water more slowly (~ 35 %) as compared to GhNAC4-C genotype (~ 55 %) (Fig. 6.11). An increase in water loss of GhNAC4-C plants could be partially due to the insensitivity to ABA-mediated stomatal closure as compared to GhNAC4-N plants.

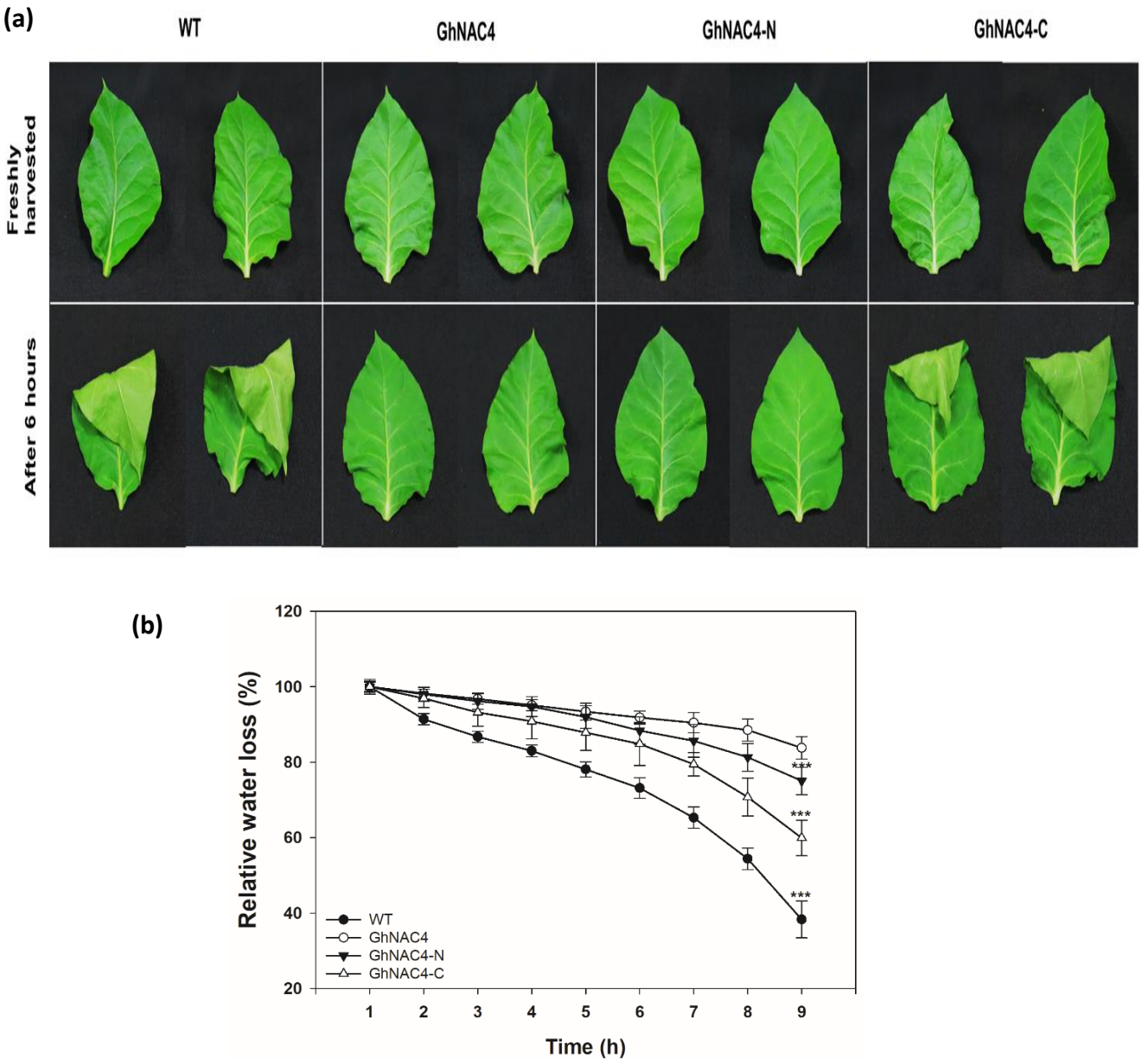


Figure 6.11 Water loss from WT, GhNAC4, GhNAC4-N and GhNAC4-C expressing tobacco transgenic plants.

Leaves from one-month old plants were excised, immediately weighed and kept at room temperature. To measure water loss, fresh weight was weighed at regular intervals. **a** Photograph was taken at 6 h. A representative image from each genotype is presented. **b** Six leaves from separate plants for each genotype were used and the experiment was performed in triplicate. Values are means ± SE. A statistical analysis with one-way ANOVA indicates significant differences (*** $P < 0.001$).

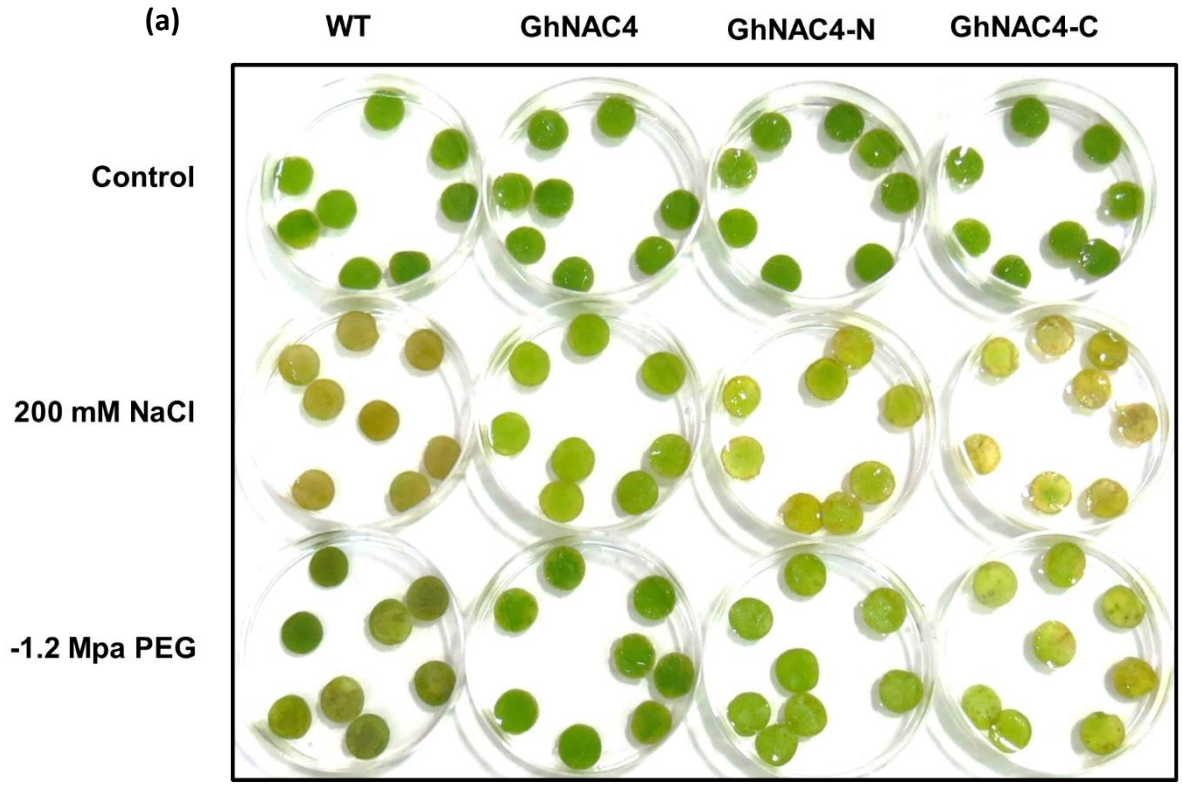
6.2.6 Both NAC and TR-domains are required for stress tolerance during vegetative growth

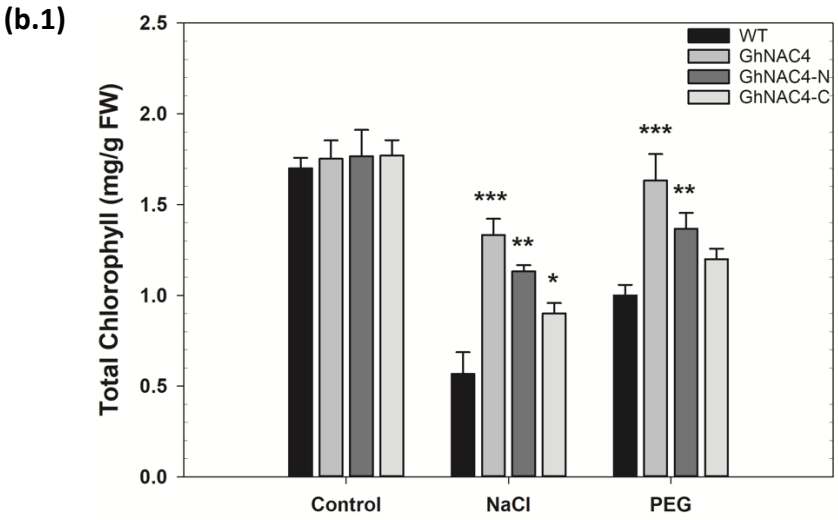
To assess the stress tolerance capabilities of wild-type, GhNAC4, GhNAC4-N and GhNAC4-C genotypes, leaf discs from fully expanded leaves were floated on 200 mM NaCl and 15 % PEG solutions (Fig. 6.12a). After 4 d of incubation, a significant amount of bleaching in the leaf discs of wild-type and GhNAC4-C genotypes as compared to GhNAC4 and GhNAC4-N in both treatments was observed. However, there was no significant difference in the chlorophyll content of all the corresponding leaf discs under control conditions. Under NaCl treatment, leaf discs of wild-type and GhNAC4-C demonstrated ~ 48-53 % bleaching. The total amount of chlorophyll lost from the leaf discs of GhNAC4 and GhNAC4-N was ~ 24-28 % under similar conditions. Dehydration stress caused by PEG treatment resulted in ~ 24-26 % bleaching in the leaf discs of wild-type and GhNAC4-C as compared to GhNAC4 and GhNAC4-N genotypes (~ 6-14 %) as seen in Fig. 6.12b.1.

Salinity and dehydration treatments induce lipid peroxidation as a consequence of increased osmotic stress (Golldack et al., 2014). This is measured by Malondialdehyde (MDA) content using the TBARS assay. There was an increase in the MDA content in all the genotypes under both the treatments. However, GhNAC4 and GhNAC4-N recorded significantly lower content of MDA as compared to wild-type and GhNAC4-C (Fig. 6.12b.2).

Under abiotic stress conditions, plants produce osmolytes such as proline to maintain the redox balance (Szabados & Savouré, 2010). Hence, proline content was measured in leaf discs of wild-type, GhNAC4, GhNAC4-N and GhNAC4-C genotypes floated on salt and PEG solutions. As compared to wild-type, GhNAC4 had a significantly higher amount of proline. Under similar conditions, GhNAC4-N recorded a significantly higher amount of proline as compared to GhNAC4-C (Fig. 6.12b.3). The above data allowed us to conclude that GhNAC4 is a positive regulator of salinity and drought stress tolerance. Further, both the NAC and TR-domains are likely to be involved in the

adaptive processes of GhNAC4. However, the NAC- domain plays a more significant role in this regard.





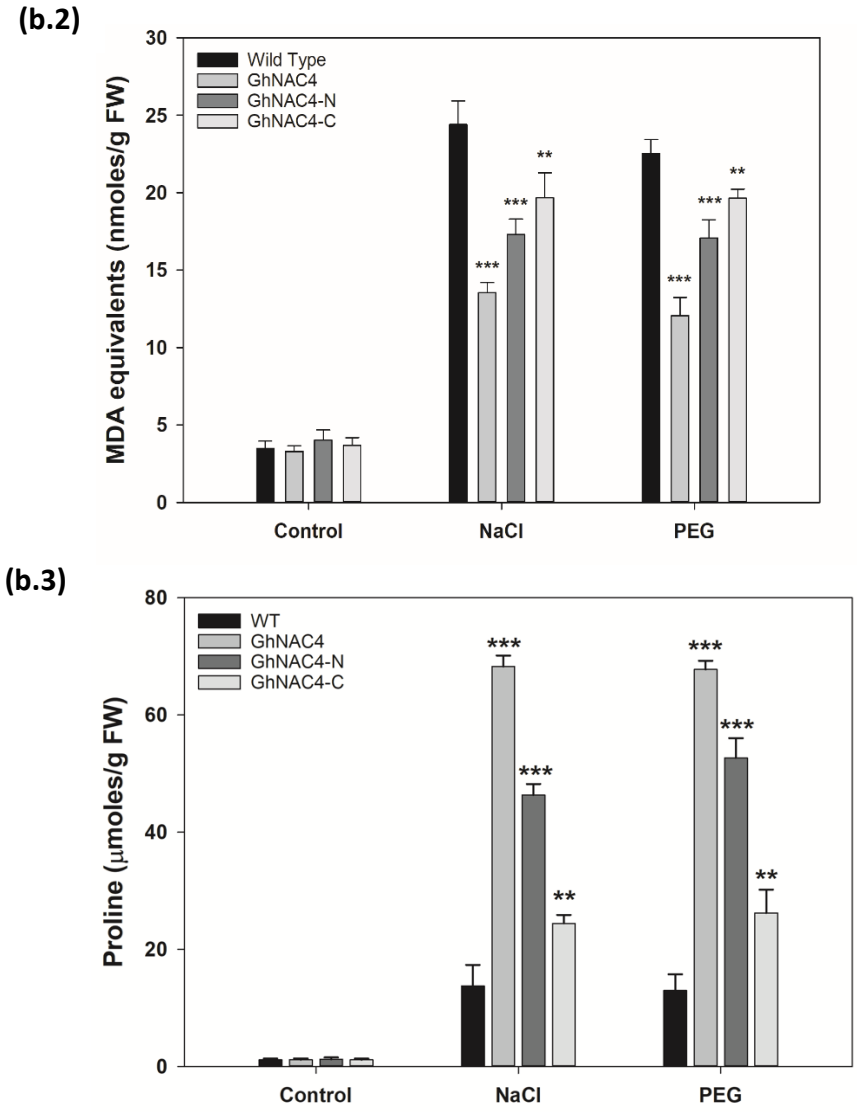


Figure 6.12 Response of WT, GhNAC4, GhNAC4-N and GhNAC4-C expressing tobacco transgenic plants to NaCl and PEG induced stress during vegetative growth.

a Phenotype of leaf discs of wild type, GhNAC4, GhNAC4-N and GhNAC4-Cplants floated on 200 mM NaCl and 15 % PEG 8000 for 4 d. The experiment was repeated three times and representative photograph has been presented here. **b** The leaf discs were analyzed for the effect of NaCl and PEG induced stress on (b.1) total chlorophyll content, (b.2) extent of lipid peroxidation and (b.3) proline accumulation. Values are mean ± SE (n=3). A statistical analysis with one-way ANOVA indicates significant differences (*** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$).

6.2.7 Both NAC and TR-domains regulate the expression of stress-responsive genes

Given the differential role NAC and TR-domains in ABA and abiotic stress responsiveness, it is pertinent to question whether they alter the expression of abiotic stress and ABA-responsive genes.

The expression of nine stress-responsive genes – *NtAPX* (ascorbate peroxidase), *NtCAT1* (catalase 1), *NtERD10C* (early responsive to dehydration 10C), *NtERF5* (ethylene response factor 5), *NtDREB3* (dehydration-responsive element-binding 3), *NtMnSOD* (superoxide desmutase), *NtNCED3* (9-*cis*-epoxycarotenoid dioxygenase), *NtSOS1* (salt overly sensitive 1), and *NtSUSY* (sucrose synthase) under NaCl and PEG treatments in all the genotypes was analyzed. Under control conditions, the expression levels of all the nine genes were very low and no significant differences were observed between wild-type, GhNAC4, GhNAC4-N, GhNAC4-C genotypes. However, under NaCl and PEG treatment, the transcript levels of all the nine genes were up-regulated (Fig. 6.13).

The transcriptional regulatory role of GhNAC4 was supported by the altered expression of many ABA and stress-responsive genes. In the GhNAC4 genotype, the transcript levels of all the nine genes were upregulated to a higher level under both treatments as compared to wild-type, GhNAC4-N and GhNAC4-C genotypes. Notably, a very high upregulation of *NtNCED3* and *NtDREB3* transcripts was observed. This suggests the importance of GhNAC4 in abiotic stress response. It is to be noted that the former gene is involved in ABA biosynthesis whereas the latter is a gene that works in ABA independent pathway.

In GhNAC4-N genotype, under both NaCl and PEG treatments, the expression levels of *NtAPX*, *NtCAT1*, *NtDREB3*, *NtMnSOD*, *NtNCED3*, and *NtSOS1* were upregulated to higher folds as compared to GhNAC4-C, while the expression levels of *NtERD10C*, *NtERF5* showed higher upregulation in GhNAC4-C genotype under both the stresses. This result suggests that both NAC and TR domains play a role in altering the expression of abiotic stress and ABA-responsive genes. Remarkably, GhNAC4 shows an additive effect of upregulation of stress-responsive genes by GhNAC4-N and GhNAC4-C genotypes and is a positive regulator of salinity and drought stress.

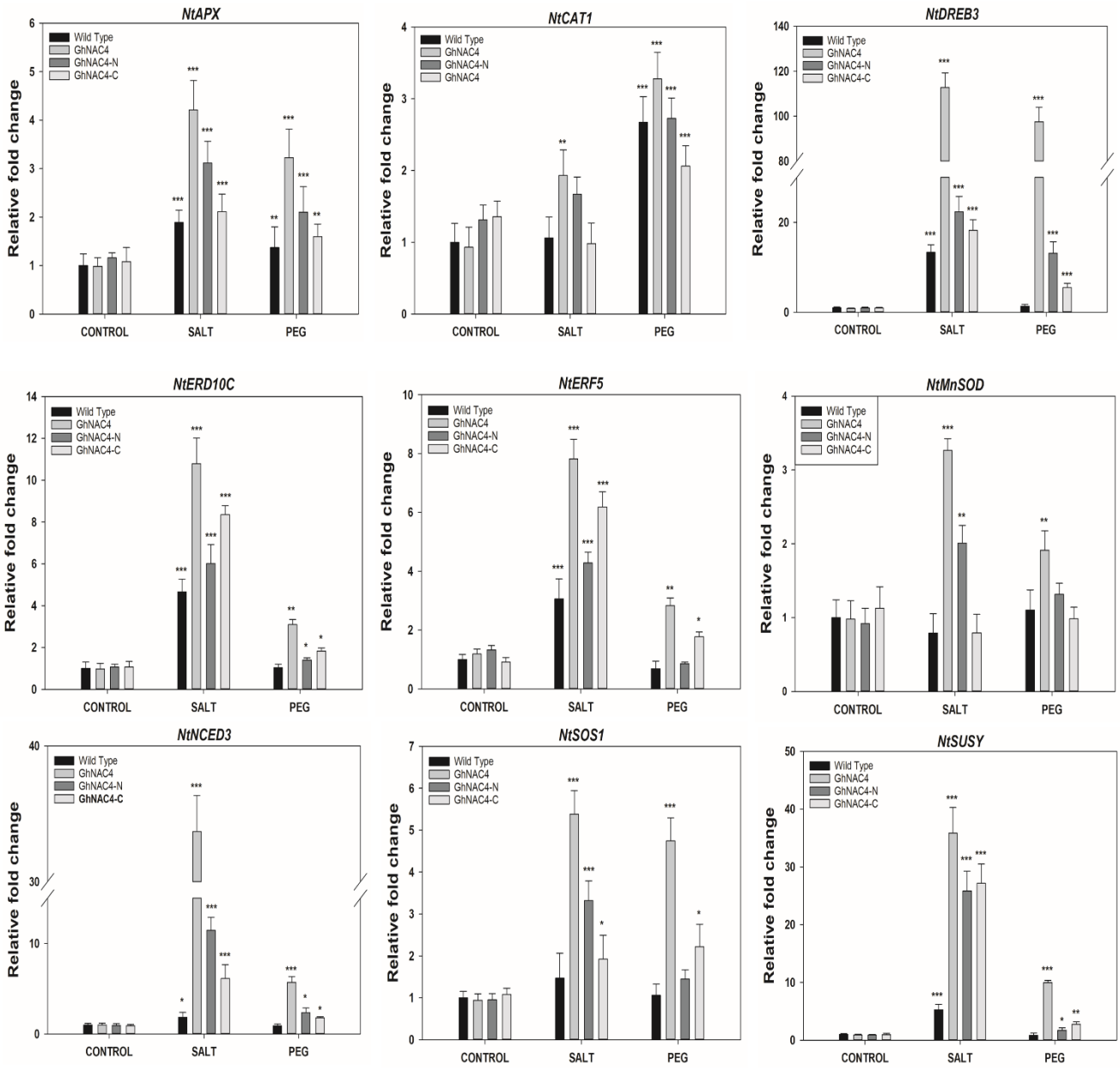


Figure 6.13 Expression patterns of stress responsive genes in WT, GhNAC4, GhNAC4-N and GhNAC4-C expressing tobacco transgenic plants in response to salt and drought treatment.

qPCR expression analyses of *NtAPX*, *NtCAT1*, *NtERD10C*, *NtERF5*, *NtDREB3*, *NtMnSOD*, *NtNCED3*, *NtSOS1*, and *NtSUSY* transcripts after treatment with 200 mM NaCl and 15 % PEG 8000 for 24 h. Two weeks-old tobacco seedlings were used in the analyses. The transcript levels of the stress responsive genes were normalized with that of tobacco Ubiquitin, *NtUBI1*. The data are shown as the mean ± SE (n = 3). A statistical analysis with one-way ANOVA indicates significant differences (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.5$).

Table 6.1 Putative NAC TF recognition sequence (NACRS) in the promoters of tobacco stress-responsive genes identified using PlantPAN 3.0 (http://PlantPAN.itps.ncku.edu.tw).

One kb upstream region was used for analysis. Similarity score > 0.9 used for prediction

Gene	GenBank Accession no.	NACRS
NtDREB3	EU727157.1	4
NtCAT1	U93244.1	12
NtAPX	U15933.1	12
NtERD10C	AB049337.1	7
NtERF5	AY655738.1	11
NtMnSOD	AB093097.1	2
NtNCED3	JX101472.1	10
NtSOS1	XM_009789739.1	9
NtSUSY	AB055497.1	12

6.3 Discussion

In the present study, the domains of a cotton NAC TF, GhNAC4 were investigated to better understand their structure-function relationship. Most NAC TFs have an N-terminal NAC-domain and a C-terminal TR-domain minimally. In this study, the NAC-domain is largely responsible for the ABA responsiveness of GhNAC4 was demonstrated. This conclusion was based on the results showing that GhNAC4-N transgenics exhibited hypersensitivity to exogenous ABA both during seed germination and seedling growth whereas the GhNAC4-C genotype transgenics were insensitive.

6.3.1 Stomatal movement and water loss

It is well known that the stomatal movement is largely controlled by ABA and the measurement of the stomatal aperture is a useful indicator of altered ABA sensitivity. Treatment of epidermal peels with ABA resulted in enhanced closure of stomatal aperture in GhNAC4-N genotype as compared to

GhNAC4-C genotype that exhibited insensitivity towards exogenously applied ABA (as observed by reduced stomatal closure).

Manipulation of genes that modulate stomatal movements has resulted in alteration of stomatal aperture. Members of TF family such as R2R3MYB, ERF/AP2, NF-YA and NAC are known to alter stomatal movements (Cominelli et al., 2010). For instance, *SNAC1*, a stress-responsive NAC TF from rice is predominantly induced in guard cells under drought conditions. Transgenic rice over-expressing *SNAC1* showed enhanced ABA sensitivity and had more rapid stomatal closure under both control and drought conditions as compared to WT plants (Hu et al., 2006). Interestingly, both GhNAC4-N and GhNAC4-C genotypes demonstrated reduced aperture size under normal conditions as compared to wild-type and GhNAC4 genotype. Mutants with altered sensitivity or deficient in ABA also exhibit altered leaf water loss condition. In our experiments, it was observed that GhNAC4-C genotype had faster rate of water loss as compared to the plants of GhNAC4-N genotype corroborating the insensitivity of GhNAC4-C genotype plants to externally applied ABA. Furthermore, the reduced transpiration rate of full length GhNAC4-overexpressing plants and its strong promoter activity in guard cells (Fig. 5.3) suggest the active role of GhNAC4 in stomatal closure.

6.3.2 GhNAC4 expression and stress adaptation

In this study, involvement of GhNAC4 in the adaptation to salinity and drought stress was demonstrated. This conclusion was based on the observation that GhNAC4-overexpressing plants showed increased accumulation of proline, reduced chlorosis and membrane damage under both salinity and drought stress treatments. GhNAC4 plants also had better growth performance as measured by germination rate and seedling growth under stress conditions. Considering the role of GhNAC4 as a TF, it is likely to play a role in regulating gene expression during abiotic stress. Indeed, quite a few stress and ABA-responsive genes (*NtNCED3*, *NtDREB3*, *NtSOS1*, *NtERD10C*, *NtERF5*,

NtAPX, *NtCAT1*, *NtMnSOD,* and *NtSUSY*) were highly upregulated by *GhNAC4* under salt and drought stress conditions. Upregulation of stress-responsive genes suggests that GhNAC4 may act as a transcriptional regulator for the downstream modulators of stress response, thereby further suggesting the importance of GhNAC4 in abiotic stress response. It is possible that GhNAC4 transcriptionally activates the stress-responsive genes by binding to the NAC recognition sequence (NACRS) in their promoter regions. The presence of several NACRS in the 1 kb promoter region of all the nine genes was observed (Table 6.1). This suggests that GhNAC4 may act as a possible direct upstream regulator of abiotic stress responsive genes.

Interestingly, a very high upregulation of *NtNCED3* and *NtDREB3* transcripts was observed in GhNAC4-overexpressing plants under salinity and drought stress. Higher levels of NCED3 (9-*cis*-epoxycarotenoid dioxygenase 3), an ABA biosynthetic enzyme (Iuchi et al., 2001) points towards increased levels of endogenous ABA in GhNAC4-overexpressing plants under abiotic stress treatment. This suggests that GhNAC4 is able to directly or indirectly modulate the biosynthesis of ABA. In contrast, DREB3 (dehydration responsive element-binding protein 3) is an AP2 domain containing TF known to regulate expression of several stress-inducible gene in an ABA-independent manner (Lata and Prasad, 2011). High induction of both *NtNCED3* and *NtDREB3* transcripts in GhNAC4-overexpressing plants under stress treatment suggests that GhNAC4 is likely to be involved in both ABA-dependent and independent pathways for stress signalling. This is partially supported by the fact that the promoter region of GhNAC4 contains several copies of ABA-response element (ABRE, binding site for ABRE binding proteins) and dehydration-response element (DRE, binding site for DREBs) *cis*-acting elements (Table 4.1). In response to abiotic stress, ABRE and DRE are involved in regulation of gene expression in ABA-dependent and independent manner, respectively (Shinozaki and Yamaguchi-Shinozaki, 2007).

The stress tolerance ability of GhNAC4 is associated with both the domains, NAC- and TR. Tobacco transgenics expressing both GhNAC4-N and GhNAC4-C constructs independently were tolerant to salt and drought treatments as compared to wild-type was demonstrated. However, GhNAC4-N genotype plants were more tolerant compared to GhNAC4-C expressing plants. GhNAC4-N genotype plants showed reduced chlorosis and lipid peroxidation and a higher proline content under abiotic stress. Also, GhNAC4-N plants had better growth performance under salinity and drought stress conditions.

6.3.3 GhNAC4 domains and associated upregulation of stress related genes

The transcripts of ABA and stress-responsive genes were also upregulated in GhNAC4-N and GhNAC4-C expressing tobacco transgenic plants. A higher level of expression of *NtNCED3* was observed in GhNAC4-N plants as compared to GhNAC4-C plants. This suggests the enhanced *de novo* ABA biosynthesis in GhNAC4-N plants. This also supports the notion that NAC-domain of GhNAC4 is largely responsible for the ABA-mediated signaling. Also, it can possibly explain the hypersensitivity of GhNAC4-N plants to exogenous application of ABA. The SOS1 (Salt Overly Sensitive 1) encodes a plasma membrane Na^+/H^+ antiporter and plays a critical role in controlling the transport of Na^+ from root to shoot, thus being essential for maintaining Na^+ and K^+ homeostasis. Increased expression of *SOS1* is reported to improve salt tolerance in *Arabidopsis* (Shi et al., 2003). A higher level of induction of *NtSOS1* transcripts was observed in GhNAC4-N plants as compared to GhNAC4-C plants. Salinity and drought treatments impose osmotic stress, which leads to the production of reactive oxygen species (ROS). It is critical to scavenge and decrease ROS levels to maintain cellular homeostasis (Krasensky and Jonak, 2012). There is a positive correlation between abiotic stress tolerance and activity of ROS scavenging antioxidant enzymes (Wang et al., 2009). Again, a level of higher induction of ROS-scavenging genes such as catalase 1 (*NtCAT1*), ascorbate peroxidase (*NtAPX*), and superoxide

dismutase (*NtMnSOD*) was observed in GhNAC4-N as compared to GhNAC4-C genotype. This can presumably explain the reduced extent of lipid peroxidation and increased stress tolerance capabilities of GhNAC4-N genotype.

Transcripts of early response to dehydration 10C (*NtERD10C*) and ethylene response factor 5 (*NtERF5*) were upregulated to a higher extent in GhNAC4-C genotype as compared to GhNAC4-N genotype under both salinity and drought stress treatments. ERD10C is an osmo-protectant hydrophilic protein that is known to play a critical role in maintaining cell homeostasis (Siqueira and Gomes, 2013). Higher induction of ERD10C suggests that the plants can be better equipped with protecting macromolecules and stabilizing labile enzymes (Chakrabortee et al., 2007). ERF5 plays a regulatory role in hormone cross-talk and redox signaling under environmental stress (Pan et al., 2012). This suggests that the C-terminal TR-domain is a transcriptional activator and does contribute to the enhanced stress tolerance capabilities of GhNAC4. The functional specificity of both the NAC and TR-domains may be explained by their ability to interact with other proteins of the transcriptional complex.

6.3.4 Functional significance of individual domains of GhNAC4

A characteristic feature of NAC TFs is that they can form homo- and heterodimers. The NAC-domain contains highly conserved Arginine and Glutamate residues at positions 19 and 26 respectively, which form the two prominent salt bridges essential for dimer formation (Olsen et al., 2005b). Consistent with this observation, the conserved amino acids were present in the GhNAC4 sequence and GhNAC4 also forms dimers in yeast. It was observed that the N-terminal NAC-domain is essential for dimer formation and alteration of the conserved amino acids abolished the dimerization.

Another feature of NAC TFs is that they act as transcriptional activators or repressors. The C-terminal TR-domain have a high degree of intrinsic disorder essential for transcriptional regulation

(Jensen et al., 2010). Consistent with this observation, the C-terminal TR-domain is essential for the transcriptional activation of GhNAC4 and interestingly had a higher degree of β-galactosidase activity as compared to the full-length protein. The intrinsically disordered regions generally contain a higher proportion of charged and polar amino acids. These regions are associated with low mean hydrophobicity and high net charge (Uversky et al., 2000). A similar pattern was observed in GhNAC4, where the two C-terminal regions (D1 and D2) necessary for the transcription activation property are rich in polar amino acids such as serine, glutamine and threonine.

Chapter 7

Summary

7.1 Major Findings from the present study

1. The expression of *GhNAC4* was up-regulated by abscisic acid, cytokinin, methyl jasmonic acid, gibberellic acid, auxin, and ethylene.
2. *GhNAC4* expression was also highly induced by drought, osmotic stress, oxidative stress, salinity, high and low-temperature stress, and wounding.
3. These observations were supported by a detailed bioinformatic analysis of the *GhNAC4* promoter for identifying the *cis*-acting elements that are associated with the regulation of gene expression in a tissue-specific and induced manner.
4. The promoter of *GhNAC4* was cloned and fused it transcriptionally to the *uidA* (GUS) gene for expression in transgenic tobacco.
5. Fluorometric GUS analysis of the transgenic plants revealed that the is also induced by various phytohormones and environmental stresses.
6. The spatio-temporal analysis of the *GhNAC4* promoter revealed that GUS expression was active in all stages of plant development including the reproductive organs. In the mature plant, the *GhNAC4* is expressed at a higher level in vascular bundles and guard cells. Intense expression in other cells upon wounding was also observed.
7. Over-expression of *GhNAC4* in transgenic tobacco conferred tolerance to salinity and drought treatments with associated enhanced expression of several stress-responsive marker genes.
8. GhNAC4 is a nuclear protein that exhibits transcriptional activation property, and the C-terminal transcriptional regulatory (TR) domain is responsible for it. GhNAC4 also forms homo-dimers and the N-terminal NAC-domain is essential for this activity.
9. Transgenic tobacco plants overexpressing the GhNAC4 NAC-domain and the TR-domain separately were developed.

10. NAC-domain transgenics showed hypersensitivity to exogenous ABA while TR-domain transgenics exhibited reduced sensitivity.
11. Abiotic stress assays indicated that transgenic plants expressing both the domains separately were more tolerant than wild-type plants with the NAC-domain transgenics showing increased tolerance as compared to TR-domain transgenics.
12. Expression analysis revealed that various stress-responsive genes were upregulated in both NAC-domain and TR-domain transgenics as compared to wild-type under salinity and drought treatments.
13. These results suggest that the stress tolerance ability of GhNAC4 is associated with both the component domains while the ABA responsiveness is largely associated with N-terminal NAC-domain.

7.2 Conclusion

In the present study, the *GhNAC4* expression responsive to phytohormones like ABA, JA, CK, and auxin and is up-regulated under external stimuli like drought, oxidative stress, osmotic stress, salinity and cold was showed. *GhNAC4* promoter is a vascular-specific promoter by histochemical assay and thin sectioning was also demonstrated. Further analysis of GUS activity showed that GhNAC4 promoter could be induced by multiple plant hormones and environmental stresses. Bioinformatic analysis predicted the presence of various *cis*-regulatory elements associated with tissue specificity, phytohormone responsiveness, stress inducibility, light inducibility and sugar responsiveness, suggesting that GhNAC4 TF may be a common regulator of the molecular mechanism controlling plant development and stress responses. This study also elucidates that GhNAC4 can form homo-dimers and is a transcriptional activator. The significantly enhanced salinity and drought tolerance of

GhNAC4 is associated with both the N-terminal NAC-domain and the C-terminal TR-domain. The ABA-responsiveness of GhNAC4 is largely associated with the NAC-domain.

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ORIGINAL ARTICLE

GUS-reporter based analysis of the promoter activity of *Gossypium hirsutum* NAC transcription factor, *GhNAC4* that is induced by phytohormones and environmental stresses

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Abstract

The expression analysis of *GhNAC4*, a NAC (*NAM*, *ATAF1-2*, and *CUC2*) domain-containing transcription factor of the upland cotton, *Gossypium hirsutum* was carried out. Its expression was up-regulated by abscisic acid, cytokinin, methyl jasmonic acid, gibberellic acid, auxin, and ethylene. Its expression was also highly induced by drought, osmotic, oxidative, salinity, high and low-temperature stresses, and wounding. To corroborate these observations, we cloned the promoter of *GhNAC4* and fused it transcriptionally with *uidA* (GUS) gene for studies in transgenic tobacco. Fluorometric GUS analysis of the transgenic plants revealed that it is also induced by various phytohormones and environmental stresses. The spatio-temporal analysis of the *GhNAC4* promoter revealed that GUS expression was active in all stages of plant development including the reproductive organs. In the mature plant, the *GhNAC4* is expressed at a higher level in vascular bundles and guard cells. We also observed intense expression in other cells upon wounding. These observations were supported by a detailed bioinformatic analysis of the *GhNAC4* promoter for identifying the *cis*-acting elements that are associated with the regulation of gene expression in a tissue-specific and induced manner.

Key message

GhNAC4 gene is induced by phytohormones and environmental stress treatments in cotton. It is expressed throughout a mature plant, including the reproductive organs, vascular bundles, and guard cells.

Keywords Fluorometric assay · Histochemical assay · Motif search analysis · Transgenic tobacco · Vascular bundle · Wounding

Abbreviations

4-MU	4-Methylumbelliferone
4-MUG/MUG	4-Methyl-umbelliferyl-β-D-glucuronide
ABA	Abscisic acid
BAP	6-Benzyl aminopurine
GA	Gibberellic acid
GUS	B-glucuronidase
IAA	Indole-3-acetic acid
JA	Jasmonic acid
MeJA	Methyl jasmonic acid
MV	Methyl viologen
NAC	NAM ATAF CUC
PEG	Polyethylene glycol
SA	Salicylic acid
TF	Transcription factor

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Introduction

NAC (*NAM, ATAF1-2*, and *CUC2*) transcription factors (TFs) constitute one of the largest plant-specific TF superfamilies with more than 100 members in *Arabidopsis*, rice (Nuruzzaman et al. 2010), Populus (Hu et al. 2010), soybean (Le et al. 2011) and foxtail millet (Puranik et al. 2013).

A typical NAC TF carries two domains—a conserved N-terminal domain and a highly divergent C-terminal domain (Olsen et al. 2005). The N-terminal domain is essential for the DNA binding property of NAC TFs (Ernst et al. 2004), while the C-terminal domain is vital for the trans-activating property of NAC TFs (Stender et al. 2015).

Previous studies have shown that NAC TFs play essential roles in regulating a wide variety of biological processes such as shoot apical meristem development (Souer et al. 1996), seed development (Sperotto et al. 2009), leaf senescence (Guo and Gan 2006), flower development (Sablowski and Meyerowitz 1998), fiber development (Ko et al. 2007), abiotic (Hu et al. 2006) and biotic stress responses (Nakashima et al. 2007).

Promoters of only a few NAC TFs from *Arabidopsis* and rice have been characterized for their induction and localization patterns so far. *OsNAC6* promoter-GUS fusion was induced by abscisic acid (ABA), jasmonic acid and various environmental stresses. Its promoter sequence exhibited different *cis*-acting elements that are known to be involved in responses to environmental stresses (Nakashima et al. 2007). *OsNAC5* promoter was induced by ABA and was localized to roots and leaves under ABA and NaCl treatments (Takasaki et al. 2010). The expression patterns of the *SNAC1* promoter was observed in callus, root, leaf, guard cells, ligule, stamen, and pistil (Hu et al. 2006). The promoter-GUS fusion of *NST1* localized to the anthers, filaments of stamens, carpels and vascular bundles of the leaf, while *NST2* was mostly localized to anther wall and pollen grains (Mitsuda et al. 2005). *ANAC012* was mainly localized to the vascular bundles, especially in the (pro) cambium region, xylem parenchyma cells along with shoot apical meristem (Ko et al. 2007).

In a genome-wide analysis, Sun et al. (2018) identified 283 NAC genes in *Gossypium hirsutum*, 147 in *G. arboreum*, 267 in *G. barbadense*, and 149 in *G. raimondii*. They have analyzed the expression of NAC TFs in *G. hirsutum* and identified that 38 and 124 NAC TFs were particularly important in fiber development and stress responses, respectively. They also identified the motifs enriched in these NAC TFs promoters. In another study, the promoters of *GhNAC8–GhNAC17* were isolated using genome walking and the *cis*-acting elements were predicted (Shah et al. 2013). To the best of our knowledge, the spatio-temporal localization of NAC TFs has not been carried out so far in cotton species. Hence, a GUS-reporter-aided approach was adopted to elucidate the promoter activity of *GhNAC4*, a NAC TF which is highly up-regulated during drought treatment (Meng et al. 2009) as a step in this direction. The promoter region was cloned, and the *cis*-acting elements were predicted. The histochemical analysis of *GhNAC4* expression was undertaken through promoter-GUS fusion in tobacco, which showed that it is active in most parts of the transgenic plant including reproductive tissues. In the mature plant, the expression was also seen at higher levels in vascular bundles and guard cells. The expression analysis of *GhNAC4* and induction of *GhNAC4* promoter under various environmental and phytohormonal treatments showed that the gene is differentially regulated suggesting a unique expression pattern during cotton growth and development.

Materials and methods

Plant material

Cotton (*Gossypium hirsutum* var. JK Durga) seeds were surface sterilized, germinated and grown for 2 weeks on sterile filter paper (Whatman no. 1) placed on top of half-strength Murashige and Skoog (1962) medium, denoted as 0.5 × MS salts.

Phytohormonal and stress treatments

Treatments were given as described by Wang et al. (2005) with minor modifications. For hormonal treatments, the filter paper (Whatman no. 1) on which the seedlings were grown was moistened singly with 100 µM abscisic acid (ABA), 100 µM methyl jasmonic acid (MeJA), 100 µM salicylic acid (SA), 20 µM 6-benzyl aminopurine (BAP), 20 µM gibberellic acid (GA_3) or 20 µM indole-3-acetic acid (IAA) and incubated for 24 h prior to sampling. As controls, untreated seedlings on filter paper moistened with water having the same quantity of sodium hydroxide or ethanol used for dissolving the hormones were also used. Ethylene treatment was carried out for 24 h by placing the seedlings on filter paper in a sealed container. Ethephon (Ethrel 40%, HiMedia Laboratories, India) was added to the box and diluted to a final concentration of 10 ppm in distilled water. Seedlings in a similar container having air were used as control.

High salt and osmotic stresses were induced by moistening the filter paper (Whatman no. 1) with 0.25 M NaCl and 0.3 M mannitol separately and the seedlings were allowed to grow for 24 h. Oxidative and drought stresses were induced by moistening the filter paper with 10 µM methyl viologen

(MV) and 15% (w/v) polyethylene glycol (PEG) 8000 individually, followed by incubation for 24 h. Air drying stress was carried out by placing the seedlings on the surface of dry filter paper for 30 min. Flooding stress was achieved by immersing the seedlings in distilled water for 24 h. Wounding stress was provided by squeezing the leaves with a forceps and harvesting them after 30 min. Combination of dark and cold treatments was given by wrapping the seedlings in aluminum foil and incubating at 4 °C for 24 h, while the dark treatment was carried out at 25 °C for 24 h. High-temperature stress was achieved by subjecting the seedlings to 42 °C for 12 h. Following all the treatments, the leaves were quick-frozen in liquid nitrogen to analyze the expression levels of *GhNAC4* gene.

To determine the degree of promoter activation, T_2 tobacco seedlings were subjected to different hormonal treatments and environmental stresses following the same methodology as applied to cotton seedlings.

RNA isolation and real-time quantitative PCR

Total RNA was extracted from the leaves of control and treated cotton seedlings by the CTAB (Cetyltriethylammonium bromide) extraction procedure as described by Chang et al. (1993). One µg of total RNA was used for synthesizing the first-strand cDNA using RevertAid first strand cDNA synthesis kit (Thermo Fischer Scientific, USA) following the manufacturer's instructions. The constitutively expressing Ubiquitin gene (*GhUBQ7*, GenBank Accession No. DQ116441) of cotton was used as an internal reference gene. The primer sequences used to amplify the internal regions of *GhNAC4* (NAC4-RTF and NAC4-RTR) and *GhUBQ7* (UBQ7-RTF and UBQ7-RTR, Kuppu et al. 2013) are mentioned in Supplementary Table 1. The fold change was determined using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001). The experiments were performed in triplicates, and two independent biological replicates were used in the analyses.

Isolation and cloning of *GhNAC4* promoter from cotton

The full-length CDS of *GhNAC4* (GenBank Accession Number EU706342.1) was used as a query to retrieve the 5′-upstream sequence from Phytozome (https://phytozome.jgi.doe.gov/). Genomic DNA was used as a template for PCR amplification of the DNA fragment. The primer sequences (GhNACPRO-F, GhNACPRO-R) used for the amplification are mentioned in Supplementary Table 1. The amplicon was cloned into the promoter-less vector pCAMBIA 1381Z (Cambia, Australia) to generate a fusion gene having 5′ upstream region of *GhNAC4* and *uidA* gene (pPRO$_{GhNAC4}$:GUS). This construct was used for *Agrobacterium tumefaciens* (strain EHA105) mediated tobacco transformation.

Generation of transgenic tobacco plants

Transgenic tobacco plants carrying the PRO$_{GhNAC4}$:GUS or empty pCAMBIA 1381Z (vector control) were generated using the standard *Agrobacterium*-mediated leaf disc transformation method as described by Horsch et al. (1985).

Histochemical localization and fluorometric measurement of GUS activity

β-glucuronidase(GUS) activity was assayed as described by Jefferson (1989) with minor modifications. For histochemical staining, the tissues were vacuum infiltrated with the solution containing 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide), 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 1 mM EDTA and 0.1% Triton X-100 in 50 mM phosphate buffer (pH 7.0) and incubated at 37 °C for 12–14 h in the dark. After staining, they were fixed in a solution containing 4% formaldehyde in 50 mM phosphate buffer (pH 7.0) for 12 h at 4 °C and subsequently cleared in 70% ethanol. Photographs were taken using M165 FC and DM6B microscopes (Leica Microsystems, Germany).

For the fluorometric assay, the tissue was homogenized in 400 µL GUS extraction buffer containing 10 mM EDTA and 0.1% Triton X-100 in 50 mM phosphate buffer (pH 7.0). After centrifugation at 12,000 rpm (4 °C) for 15 min, 5 µL of homogenate was diluted with 95 µL of extraction buffer and mixed with 100 µL of extraction buffer having 2 mM 4-MUG (4-methyl-umbelliferyl-β-D-glucuronide, Duchefa, Netherlands) and incubated at 37 °C for 1 h. The reaction was terminated by adding 1.8 mL of 200 mM sodium carbonate. Total protein concentration in the homogenate was assessed by Bradford method (Bradford 1976) with bovine serum albumin as standard. Fluorescence (excitation 363 nm, emission 447 nm) was determined by Infinite 200 plate reader (Tecan, Switzerland) and GUS activity was expressed as pmol of 4-MU (4-methyl-umbelliferone, Sigma, USA) per µg protein per min. 4-MU in the range of 20 nM to 100 µM was used to generate a standard curve. Each MUG assay was performed in triplicate and repeated three times.

Bioinformatics analysis of *GhNAC4* promoter sequence

The transcription start site was predicted by the Softberry database (Shahmuradov et al. 2017, www.softberry.com) using the default settings. A search for the putative *cis*-acting regulatory elements in the promoter sequence was conducted

using the PlantPAN 2.0 (Chang et al. 2008, http://plantpan2.itps.ncku.edu.tw/), PLACE (Higo et al. 1999, https://www.dna.affrc.go.jp/PLACE/) and PlantCARE (Lescot et al. 2002, http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) databases. Only statistically significant motifs (P value > 0.9) were selected.

Statistical analysis

All experiments were repeated at least three times, and the data were expressed as the mean ± SE. Data were analyzed by one-way analysis of variance (ANOVA) using SigmaPlot 11.0 software. ***P < 0.001, **P < 0.01 and *P < 0.05 represent significant differences at 0.1, 1 and 5% level respectively. 'ns' represents no significant difference.

Results and discussion

Responsiveness of *GhNAC4* gene to treatments with phytohormones and environmental stresses

To gain an insight into the impact of environmental stresses and phytohormones on the expression of *GhNAC4*, real-time expression analysis was carried out. Figures 1 and 2 demonstrate that *GhNAC4* responded differentially to several

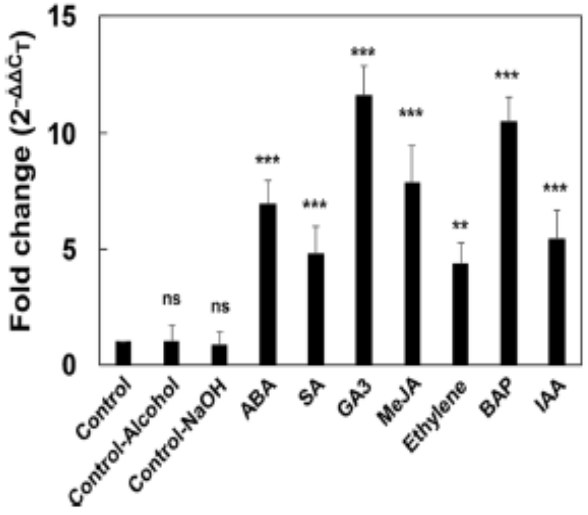


Fig. 1 Expression patterns of *GhNAC4* transcript in response to various phytohormones. qRT-PCR expression analysis of the *GhNAC4* gene in *G. hirsutum* leaves after ABA, MeJA, SA, 6-BAP, GA_3, IAA, or Ethephon treatment. Two weeks-old cotton seedlings incubated for 24 h under the treatment were used in the analysis. The mRNA levels of *GhNAC4* gene were normalized with that of Ubiquitin gene, *GhUBQ7*. The data are shown as the mean ± SE (n = 3). A statistical analysis with one-way ANOVA indicates significant differences (**P < 0.01, ***P < 0.001, *ns* not significant). *ABA* abscisic acid, *SA* salicylic acid, *GA3* 3-gibberellic acid, *MeJA* methyl jasmonic acid, *BAP* 6-benzyl aminopurine, *IAA* indole-3-acetic acid

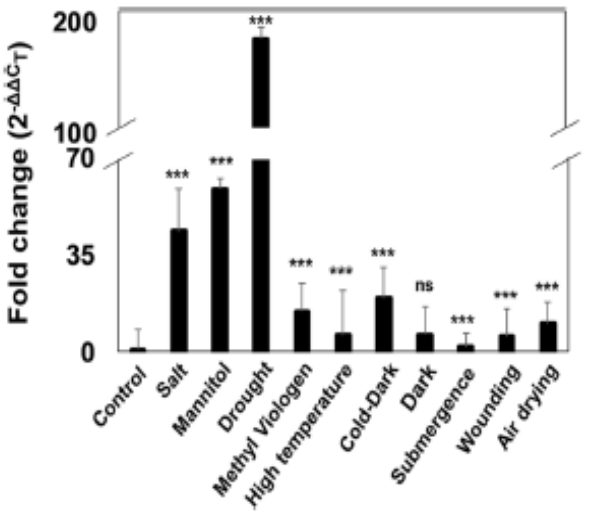


Fig. 2 Expression patterns of *GhNAC4* transcript in response to various environmental stresses. qRT-PCR expression analysis of the *GhNAC4* gene in *G. hirsutum* leaves after salt, mannitol, drought, methyl viologen, high temperature, air drying, submergence, wounding, dark or combination of dark and cold treatment. Two weeks-old cotton seedlings were used in the analysis. The mRNA levels of *GhNAC4* gene were normalized with that of Ubiquitin gene, *GhUBQ7*. The data are shown as the mean ± SE (n = 3). A statistical analysis with one-way ANOVA indicates significant differences (***P < 0.001, *ns* not significant)

phytohormones and environmental stresses. GA3 and BAP enhanced its expression by ~ 11.5 and 10.4 folds, respectively. The *GhNAC4* transcript levels were also up-regulated by MeJA and ABA to ~ 7.8 and 6.9 folds, respectively. IAA enhanced the expression by ~ 5.4 folds. However, other hormones like ethylene and SA triggered only a low level up-regulation of *GhNAC4* expression (~ four folds). ABA modulates plant abiotic stress responses, while MeJA, SA and ethylene play central roles in biotic stress responses. Role of auxin and cytokinin during environmental stress response is also emerging (Bielach et al. 2017). Cross-talk between various phytohormones results in antagonistic or synergistic interactions, which are necessary for plant response to environmental stress (Peleg and Blumwald 2011). Induction of expression of *GhNAC4* by these hormones suggests that the expression of *GhNAC4* is a possible node connecting multiple hormone signaling pathways.

The polyethylene glycol (PEG) induced drought stress treatment resulted in very high up-regulation of *GhNAC4* expression (~ 184 folds). Other abiotic stress treatments like high salinity and osmotic stresses also led to its high up-regulation (~ 43.6 and 58.7 folds respectively). Robust up-regulation of *GhNAC4* transcripts was also observed in air-drying and methyl viologen (MV) treatments (~ 10.2 and 14.5 folds). MV is known to generate ROS such as H_2O_2, which are important signaling molecules during environmental stress tolerance (Bhattacharjee 2005). Upregulation

of *GhNAC4* by MV suggests that it might be involved in the regulation of ROS-scavenging mechanism, playing a possible role in stress signaling pathway. Dark treatment caused ~6.2 folds increase in its expression and when this treatment was supplemented with low temperature, the expression was enhanced by ~19.4 folds suggesting an additive effect of the combination of two treatments. High temperature and wounding also increased *GhNAC4* expression by ~6.1 and 5.7 folds, respectively. The above expression profile suggests that fine-tuning of *GhNAC4* induction may require elaborate cross-talk between various signaling pathways via the involvement of phytohormones in cells.

Several NAC TFs are up-regulated by phytohormones and stress treatments. Furthermore, they function in diverse signaling pathways (Erpen et al. 2018). Auxin induces *AtNAC1* expression during lateral root formation (Xie et al. 2000). The expression of fine-stem stylo NAC TFs, *SgNAC1* and *SgNAC2* showed significant upregulation during cold stress (Zhan et al. 2018). The expression of rice *OsNAC6* is induced by ABA, JA, cold, drought, high salinity treatments and wounding (Ohnishi et al. 2005). Expression of tomato *SlNAC11* is induced by heat, dehydration and cold (Wang et al. 2017). Expression of wheat *TaNAC4* is up-regulated by ethylene, MeJA and ABA, cold, wounding and high salinity treatments (Xia et al. 2010). Various NAC TFs such as *SlNAC3* and *SbSNAC1* are also induced by drought and salinity (Al-Abdallat et al. 2015; Lu et al. 2013). Upregulation of *GhNAC4* by multiple stresses such as drought, salinity stress, osmotic stress, ROS, and wounding suggests it might play a potential role in multiple environmental stress signaling pathways.

Sequence analysis of *GhNAC4* promoter

A DNA fragment of 1612 bp corresponding to the upstream regulatory region (− 1492 bp to + 119 bp) of the *GhNAC4* was amplified from *G. hirsutum* genome. The composition of the GC content of *GhNAC4* promoter was 29.9%, which is as per the observed range for a plant promoter (Joshi 1987). The putative transcription start site (TSS) was located 119 bp upstream of the ATG translation start codon, which was consistent with the features of a eukaryotic promoter as shown in Supplementary Figure 1. The predicted TATA box was located 16 bp upstream of TSS, and a CAAT box was located 179 bp upstream of the TATA box.

Generation and analysis of tobacco transgenics of PRO_{GhNAC4}:GUS

To evaluate the promoter activity of *GhNAC4* gene, a total of 12 hygromycin resistant T_0 plants were generated and were confirmed by genomic PCR (GhNACPRO-F, GhNACPRO-R and HptII-F, HptII-R; Supplementary Table 1). To eliminate the effect of gene copy number on GUS activity, only single copy T_1 progenies, P7, P9, and P17 were used for further generation of T_2 seeds. The T_2 progenies of three lines, P7.1, P9.5, and P17.3, were used for histochemical and fluorometric assays.

GhNAC4 expresses in various tissues during growth and development

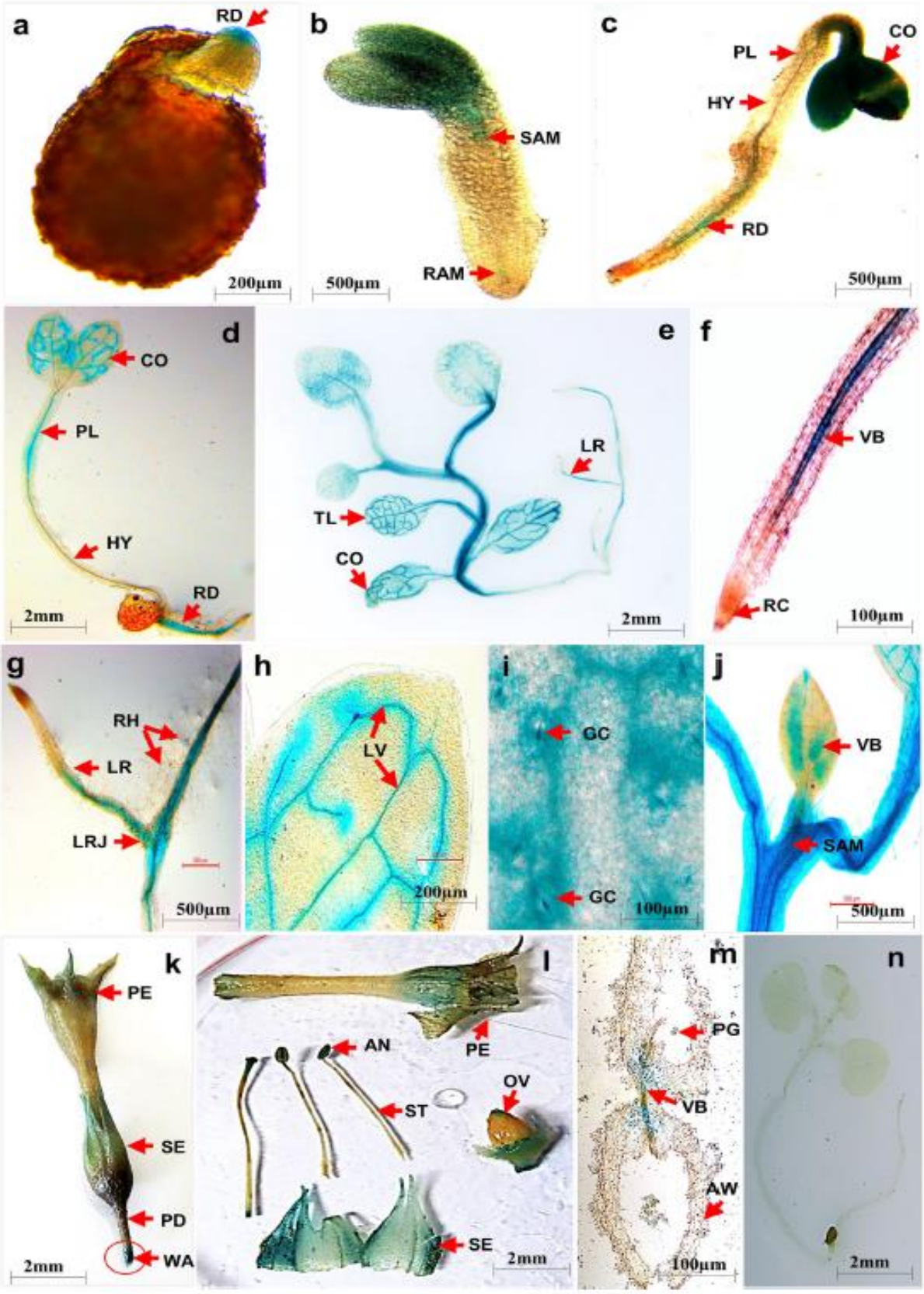
To precisely define the spatio-temporal expression patterns of *GhNAC4* promoter, we have used GUS staining analysis of PRO_{GhNAC4}:GUS transgenic tobacco plants. Figures 3 and 4 show the localization of GUS in vegetative and reproductive tissues. These GUS staining images are representative of at least three independent transgenic lines.

In early stages of tobacco growth (1 day old seedlings), GUS activity was first observed in emerging radicle (Fig. 3a), which was later detected in emerging cotyledons, root tip and shoot apex of 3 day old seedlings. However, GUS activity was relatively weaker in hypocotyl tissue (Fig. 3b). In 7, and 15 days old transformed tobacco seedlings, GUS expression was detected in leaf veins, petioles, stem, and root (Fig. 3c, d). Similar GUS activities were maintained in 1-month-old plants also (Fig. 3e).

The main and lateral roots showed GUS expression, which was absent in the root cap region and root hairs (Fig. 3f, g). Intense GUS staining was also observed in midrib and lateral veins, but leaf lamina showed scanty staining (Fig. 3h). We also observed GUS activity in guard cells (Fig. 3i) and developing mid rib regions of a young leaf (Fig. 3j). Figure 3k–m shows the staining of floral structures, which revealed that the GUS activity was present in sepals and to a lesser extent in the petal edges. GUS activity was also observed in anthers, pollen grains, and the stigma. However, it was absent in ovary and pedicel. No staining was detected in seedlings harboring a promoter-less GUS gene regardless of the developmental stage (Fig. 3n).

To obtain a better understanding of tissue specificity of GUS activity, thin cross-sections of various tissues were made using a razor blade. Intense GUS staining was detected in vascular bundles, especially in phloem of leaf veins, petiole, stem, and root (Fig. 4a–e). Both the abaxial and adaxial phloem tissues showed intense GUS staining. Other tissue types like pith, cortex, and epidermis, remained relatively unstained. However, cortex and epidermis also showed intense GUS expression upon wounding (Figs. 3k and 4f). The expression pattern of Passiflora *PmNAC1* transcripts was observed in root, shoot apex, floral bud and pro-vascular tissue especially the xylem cell specification (Rosa et al. 2013).

The occurrence of a unique pattern of *GhNAC4* promoter activity could be corroborated by the presence of several motifs responsible for tissue-specific expression


a
RD
200µm
b
SAM
RAM
500µm
c
CO
PL
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RD
500µm
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CO
PL
HY
RD
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◂**Fig. 3** Histochemical localization of GUS activity in tobacco transgenic plants containing PRO_{GhNAC4}:GUS construct. **a**–**e** seedlings grown on MS media with hygromycin at **a** day1; **b** day 3; **c** day 7; **d** day 15; **e** day 30. **f**–**j** Various tissues of a 30-day old transgenic tobacco plant **f** main root; **g** lateral root; **h** true leaf; **i** guard cells; **j** developing leaf. **k**–**m** floral structures **k** mature flower; **l** dissected flower showing various tissues; **m** cross-section of an anther. **n** 15 days old tobacco seedling carrying empty pCAMBIA 1381Z vector. *AW* anther wall, *CO* cotyledon, *GC* guard cell, *HY* hypocotyl, *LR* lateral root, *LRJ* lateral root junction, *LV* lateral vein, *OV* ovary, *PD* pedicel, *PE* petal, *PG* pollen grains, *PL* plumule, *RAM* root apical meristem, *RD* radicle, *RH* root hairs, *SAM* shoot apical meristem, *SE* sepal, *TL* true leaf, *VB* vascular bundle, *WA* wounded area. All arrows show strong GUS activity or no activity. Bars of each panel are as shown

patterns, as shown in Supplementary Table 2. Several copies of the developing embryo and seed-specific motifs such as SEF1MOTIF, SEF3MOTIFGM, and SEF4MOTIFGM7S motifs were observed in the *GhNAC4* promoter (Lessard et al. 1991) as also OSE1ROOTNODULE and OSE2ROOTNODULE motifs, which are root-specific elements (Fehlberg et al. 2005). Eleven copies of the ROOTMOTIFTAPOX1 element are predicted in the *GhNAC4* promoter, which has a distinct expression pattern in the root elongation zone and vascular bundle (Elmayan and Tepfer 1995). The CACTFTPPCA1 motif is a key component of mesophyll expression module 1 (MEM1) and is sufficient for high mesophyll-specific expression (Gowik et al. 2004). The TAAAGSTKST1 is a guard cell-specific motif and five copies were observed (Plesch et al. 2001).

Binding sites for quite a few other TFs known to play roles in organogenesis and tissue-specific expressions are also predicted such as GATA, MYBST1, DOFCOREZM, SBP TF, TCR TF, AT HOOK TF, and WOX TF. GATA motif plays a role in light responsiveness and tissue specificity and is involved in light-dependent development of phloem tissue (Yin et al. 1997). *GhNAC4* has 26 copies of DOFCOREZM motif, which is a binding site of DOF TFs. They regulate directly or indirectly the processes associated with the establishment and maintenance of vascular system (Le Hir and Bellini 2013).

Various motifs such as CARCGW8GAT, TGTCACACMCUCUMISIN and binding sites for MYBPZM and TCR TFs known to be important for flower and fruit development are found in the *GhNAC4* promoter. Several copies of the two pollen-specific motifs, POLLEN1LELAT52 (Bate and Twell 1998) and GTGANTG10 (Rogers et al. 2001) are also found in the *GhNAC4* promoter. Fang et al. (2008) carried out a systematic sequence analysis of NAC genes in rice and identified 12 particularly important tissue-specific NAC TFs. The promoters of these genes are predicted to have motifs such as SEF3MOTIFGM, ROOTMOTIFTAPOX1, and GTGANTG10.

GhNAC4 promoter is induced by various phytohormones

To explore the possible regulation of *GhNAC4* promoter by phytohormones, GUS activity was examined by fluorometric MUG assay. The corresponding results have been depicted in Fig. 5. The specific activity of GUS enzyme without any hormone treatments in PRO_{GhNAC4}:GUS seedlings was measured to be 2.4 ± 0.4 pmol μg^{-1} min^{-1}. This is in agreement with the histochemical staining results, where GUS staining was observed even in untreated seedlings. *GhNAC4* promoter was highly induced by BAP and ABA (~ 67.9% and 63.5%). IAA and MeJA treatment caused ~ 58.9% and 54.6% induction of the promoter activity, respectively. GA_3 and ethylene also enhanced the *GhNAC4* promoter activity by ~ 53.3% and 45.9% respectively. The pattern of *GhNAC4* promoter induction observed in phytohormonal treatments in tobacco seedlings is similar to the *GhNAC4* expression in cotton seedlings.

Evaluating the *cis*-elements present in the promoter region of the *GhNAC4* gene might allow us a better understanding of the differential regulation of *GhNAC4* by various phytohormones. *GhNAC4* promoter was predicted to have one copy of CPBCSPOR motif and 16 binding sites for Authentic Response Regulators1 (ARR1) as mentioned in Supplementary Table 2. CPBCSPOR motif is essential for cytokinin dependent transcriptional activation (Fusada et al. 2005). ARR1 is an important signaling component known to be involved in cytokinin-mediated differentiation of protoxylem (Yokoyama et al. 2007). Several motifs known for ABA responsiveness such as six copies of ACGTABREMOTIFA2OSEM, eight copies of ABRELATERD1 and one copy of ABREOSRAB21 motifs were predicted on the *GhNAC4* promoter. Mutation of the ABRE in the rice *OsNAC5* promoter led to the abolition of the activation of the promoter by ABA (Takasaki et al. 2010). Auxin responsive motifs such as AUXREPSIAA4, TGA element, GGTCCCATGMSAUR, and CATATGGMSAUR were predicted on the *GhNAC4* promoter. *Arabidopsis AtNAC1* plays a key role in promoting auxin-mediated lateral root formation (Xie et al. 2000). A plasma membrane-bound NAC TF, *NTM2* integrates auxin and salt signaling via the *IAA30* gene during seed germination in *Arabidopsis* (Park et al. 2011).

GhNAC4 promoter region is also predicted to contain JA responsive motifs such as T/GBOXATPIN2, TGACG, and CGTCA. JAMYC/AtMYC2 TF binds to the T/GBOXATPIN2 motif found in the promoter of JA responsive and wound-inducible Protease Inhibitor II (*PIN2*) gene (Boter et al. 2004). ANAC019 and ANAC055 act downstream of AtMYC2 as transcriptional activators to regulate JA-signaled defense responses (Bu et al. 2008). *GhNAC4* promoter is predicted to have four copies GAREAT and two copies of GARE1OSREP1 motifs, which are gibberellin responsive

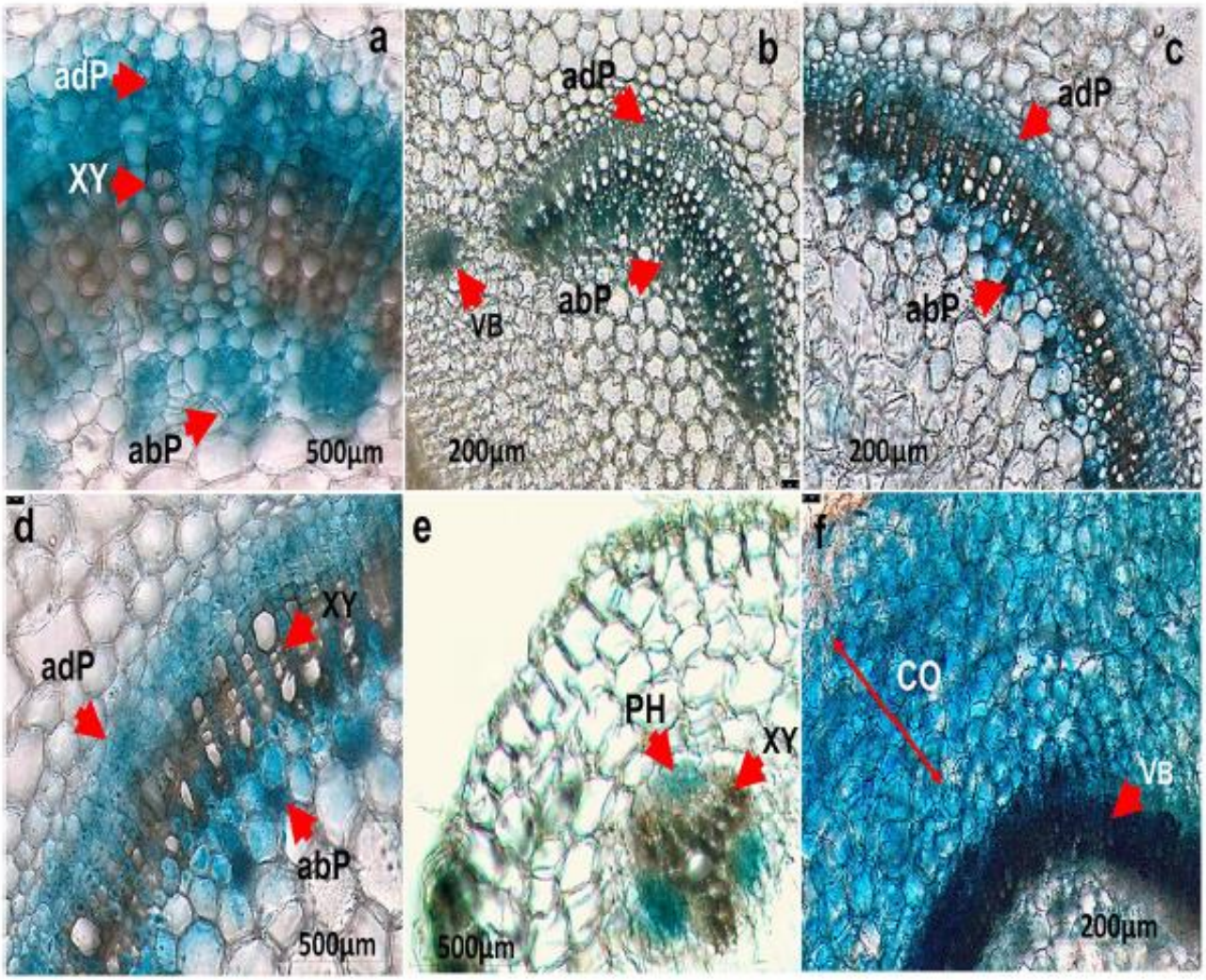

Fig. 4 GUS activity in free hand cross-sections tobacco transgenic plants containing PRO_{GhNAC4}:GUS construct. **a** leaf; **b** petiole; **c** stem **d** magnified view of the stem; **e** root; **f** wounded stem. *abP* abaxial phloem, *adP* adaxial phloem, *CO* cortex, *P* phloem, *VB* vascular bundle, *XY* xylem. All arrows show strong GUS activity or no activity. Bars of each panel are as shown

elements, GARE (Skriver et al. 1991). The above-mentioned sequences may function as phytohormone responsive motifs in the *GhNAC4* promoter.

GhNAC4 promoter is responsive to various environmental stress treatments

As we have observed that *GhNAC4* gene expression was regulated by various stresses, we studied the promoter activity by the fluorometric MUG assay as shown in Fig. 6. Drought stress treatment resulted in high induction of the *GhNAC4* promoter (~71%). Salinity and osmotic stress treatments also enhanced the promoter activity by ~68.5% and 69.5%, respectively. Oxidative stress and air-drying treatments up-regulated the promoter activity by ~68.2% and 66.6%, respectively. A combination of low-temperature stress and darkness treatment activated the *GhNAC4* promoter by ~59.3% as compared to darkness treatment alone (~43.1%) suggesting an additive effect of up-regulation of *GhNAC4* promoter induction. High-temperature and wounding stress treatments induced the promoter activity by ~42.1% and 41.7%, respectively. Flooding stress caused by submerging the seedlings in water also enhanced the promoter activity (~49.7%). The pattern of *GhNAC4* promoter induction observed by environmental stress treatments in tobacco seedlings is similar to the *GhNAC4* expression in cotton seedlings.

Evaluating the *cis*-elements present in the promoter region of the *GhNAC4* gene might help us with a better understanding of the differential regulation of *GhNAC4* by various environmental stresses. *GhNAC4* promoter region is predicted to contain many motifs like

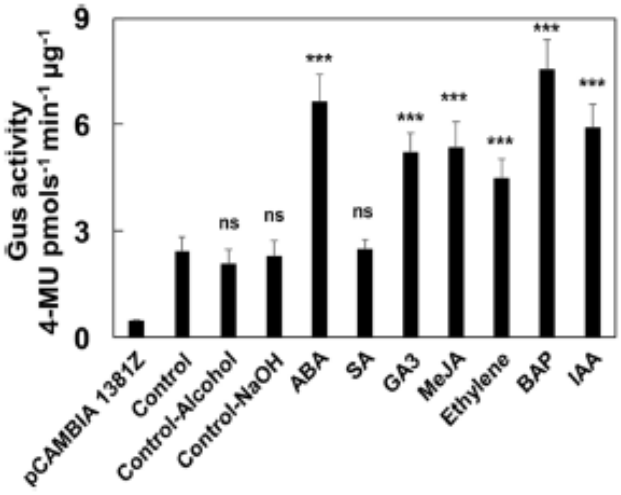

Fig. 5 Effect of various phytohormones on the GUS activity of PRO_{GhNAC4}:GUS tobacco transgenics. Fluorometric analysis of the GUS in tobacco seedlings after ABA, MeJA, SA, 6-BAP, GA_3, IAA, or Ethephon treatment. Two weeks-old PRO_{GhNAC4}:GUS tobacco transgenic seedlings incubated for 24 h under the treatment were used in the analysis. pCAMBIA 1381Z empty vector harboring tobacco seedlings were used as negative controls. The data are shown as the mean ± SE (n = 3). A statistical analysis with one-way ANOVA indicates significant differences (***P < 0.001, *ns* not significant). *ABA* abscisic acid, *SA* salicylic acid, *GA3* 3-gibberellic acid, *MeJA* methyl jasmonic acid, *BAP* 6-benzyl aminopurine, *IAA* indole-3-acetic acid

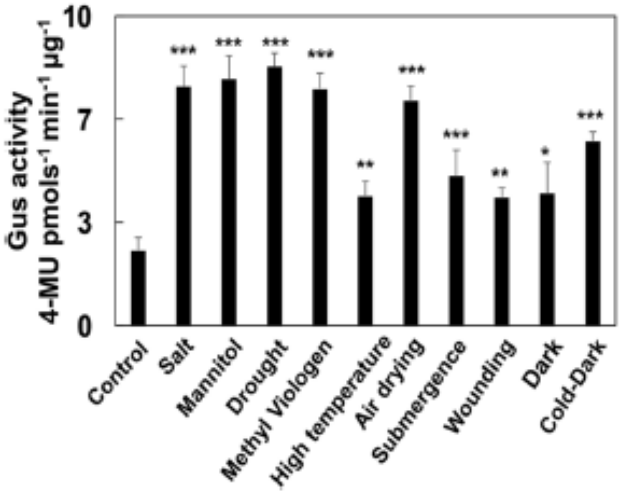

Fig. 6 Effect of various stresses on the GUS activity of PRO_{GhNAC4}:GUS tobacco transgenics. Fluorometric analysis of the GUS in tobacco seedlings after salt, mannitol, drought, methyl viologen, high temperature, air drying, submergence, wounding, dark or combination of dark and cold treatment. Two weeks-old PRO_{GhNAC4}:GUS tobacco transgenic seedlings, were used in the analysis. The data are shown as the mean ± SE (n = 3). A statistical analysis with one-way ANOVA indicates significant differences (*P < 0.05, **P < 0.01, ***P < 0.001)

MYB2CONSENSUSAT, C-REPEAT/DRE, DRECRTCOREAT, CSD, and CBFHV TF binding sites required for drought and cold responsiveness, as shown in Supplementary Table 2. Two copies of MYB2CONSENSUSAT motifs are found in the *GhNAC4* promoter, which is the binding site for AtMYB2 TF required for drought inducibility of *rd22* gene (Abe et al. 2003). Cold responsive motif, C-repeat (CRT) is responsible for the regulation of many cold-inducible genes in an ABA-independent manner. It is also involved in dehydration responsiveness (Stockinger et al. 1997). A fox-tail millet stress-responsive *SiNAC* promoter exhibits binding sites for MYB and MYC TFs and also contains CRT motif (Puranik et al. 2011). Three copies of the DRECRTCORE motif are found in the *GhNAC4* promoter. CBFHV motif is a binding site for an AP2 domain-containing cold-inducible TF, HvCBF1 characterized in barley (Xue 2002). Three copies of CBFHV motif are found in the *GhNAC4* promoter. Eight copies of binding site for cold shock domain (CSD) proteins are also found in the *GhNAC4* promoter that is highly activated during low-temperature stress. Promoter analysis of *MusaNAC68* by Negi et al. (2016) revealed the presence of various stress responsive motifs such as ACGTATERD1, MYB1AT, CBFHV and MYCCONSENSUSAT. Fang et al. (2008) carried out a systematic sequence analysis of NAC genes in rice and identified 20 particularly important stress-inducible NAC TFs. The promoters of these genes were predicted to have several stress-responsive motifs such as MYB1AT, MYB2CONSENSUSAT, MYCCONSENSUSAT, CBFHV, DRECRTCOREAT, and CRTDREHVCBF2.

Heat stress transcription factors (HSF) bind to the heat stress responsive element and modulate transcription during heat stress (Baniwal et al. 2004). Four copies of HSF binding site are present in the *GhNAC4* promoter. Binding sites for C2H2 TF are also identified in the *GhNAC4* promoter region. C2H2 TFs such as ZAT7, ZAT10, and ZPT2 are known to be important in regulating responses to abiotic and biotic stress tolerance (Kiełbowicz-Matuk 2012). *GhNAC4* promoter exhibited four copies of WBOXNTERF3 motifs. WRKY TFs binds to the WBOXNTERF3 motif in the promoter of *ERF3* gene of tobacco and causes its rapid activation upon wounding (Nishiuchi et al. 2004). The promoter of wound-inducible rice NAC gene, *OsNAC6* also exhibits four copies of W-box (Nakashima et al. 2007). The above-mentioned sequences may function as environmental stress-responsive motifs in the *GhNAC4* promoter.

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Author contributions VST and PBK conceived and designed the experiments. VST, SM, and PB performed the experiments. VST analyzed the data. VST and PBK wrote the manuscript.

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Data availability All the data presented in the present manuscript are freely available to the interested researchers and would be made available upon request to the Corresponding author-VST (email- strishlajain@gmail.com).

Compliance with ethical standards

Conflict of interest The authors declare they have no conflict of interest.

Ethical approval This investigation does not necessitate the use of animals and hence, does not need the approval from the Animal Ethics Committee.

Consent to participate The present manuscript is the result of the participation of all the authors listed in it.

Consent for publication The authors of the manuscript hereby give their consent for publication of the manuscript in PCTOC.

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Structure-function relationship of *Gossypium hirsutum* NAC transcription factor, GhNAC4 with regard to ABA and abiotic stress responses

Vikas Shalibhadra Trishla, Pulugurtha Bharadwaja Kirti

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Abstract

Our previous study demonstrated that the expression of *GhNAC4*, a NAC transcription factor from cotton, was induced by abiotic stresses and abscisic acid (ABA) treatment. In the present study, we investigated the molecular mechanisms underlying ABA and stress response of GhNAC4. Over-expression of *GhNAC4* in transgenic tobacco conferred tolerance to salinity and drought treatments with associated enhanced expression of several stress-responsive marker genes. GhNAC4 is a nuclear protein that exhibits transcriptional activation property, and the C-terminal transcriptional regulatory (TR) domain is responsible for it. GhNAC4 also forms homo-dimers and the N-terminal NAC-domain is essential for this activity. The structure-function relationship of NAC transcription factors, particularly with respect to abiotic stress tolerance remains largely unclear. In this study, we investigated the domains essential for the biochemical functions of GhNAC4. We developed transgenic tobacco plants overexpressing the GhNAC4 NAC-domain and the TR-domain separately. NAC-domain transgenics showed hypersensitivity to exogenous ABA while TR-domain transgenics exhibited reduced sensitivity. Abiotic stress assays indicated that transgenic plants expressing both the domains separately were more tolerant than wild-type plants with the NAC-domain transgenics showing increased tolerance as compared to TR-domain transgenics. Expression analysis revealed that various stress-responsive genes were upregulated in both NAC-domain and TR-domain transgenics as compared to wild-type under salinity and drought treatments. These results suggest that the stress tolerance ability of GhNAC4 is associated with both the component domains while the ABA responsiveness is largely associated with N-terminal NAC-domain.

Key Message NAC and transcriptional regulatory domains are responsible for the abiotic stress tolerance ability of the cotton NAC transcription factor GhNAC4 while the ABA-responsiveness is largely associated with the NAC-domain.

Competing Interest Statement

The authors have declared no competing interest.

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