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Evolutionary progression and functional diversification of NAC family members in pearl millet with comprehensive characterization of *PgNAC103* under drought stress☆

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ABSTRACT

This study investigated the role of NAC transcription factors (TFs) in the stress response of pearl millet, a C4 crop known for its nutritional value and drought tolerance. Phylogenetic and synteny analysis of 155 NAC TFs revealed the contribution of segmental duplication to *NAC* gene evolution. Promoter analysis identified various stress-related *cis*-elements in the upstream regions of these genes. We analysed expression pattern of identified NAC genes under phytohormones (ABA, MeJA, and SA) and abiotic stresses (drought, salinity, and heat). PgNAC103 was found to be a nuclear protein having a C-terminal transactivation domain. Arabidopsis and pearl millet overexpressing the *PgNAC103* showed enhanced stress responses under drought. Transgenic lines showed less sensitivity towards ABA treatment. In transgenic Arabidopsis, the drought stress response was manifested through the upregulation of stress marker genes (*RD22, KIN1, COR15A*) and increased ROS scavenging (SOD, POD and CAT). The transcriptional activity of the *PgNAC103* promoter was induced by drought stress in transgenic plants. These findings suggest that NAC TFs function as positive or negative regulators of the abiotic stress response in pearl millet, with *PgNAC103* specifically acting as a positive regulator of drought stress tolerance. *PgNAC103* represents a promising genetic resource for developing climate-resilient crops.

List of abbreviations

TFs	Transcription factors
CREs	*cis*-regulatory elements
MeJA	Methyl Jasmonate
ABA	Abscisic Acid
SA	Salicylic acid
CIM	Callus induction medium
GFP	Green fluorescent protein
MEGA	Molecular evolutionary genetics analysis
MS	Murashige and Skoog medium
PEG	Polyethylene glycol
Plant CARE	Plant Cis-acting regulatory elements
PCR	Polymerase chain reaction
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RT	Room temperature
POD	Peroxidase
CAT	Catalase
SD	Synthetic dropout
WT	Wild type
SOD	Superoxide dismutase
PgNAC	*Pennisetum glaucum* NAC

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*At*NAC	*Arabidopsis thaliana* NAC
*Si*NAC	*Setaria italica* NAC
*Os*NAC	*Oryza sativa* NAC
*Ta*NAC	*Triticum aestivum* NAC
h	Hour
D	Day

1. Introduction

Extreme climatic conditions severely threaten agricultural productivity (Pereira 2016). Studies suggest that combinations of different abiotic stresses cause greater damage to plants than specific stress alone (Pandey et al., 2017). However, plants have intrinsic molecular mechanisms that allow them to adapt and withstand stressful events. Of these, the interplay between phytohormone signalling pathways and transcription factors (TFs) is crucial for enabling stress responses (Baillo, et al. 2019; Wang, et al. 2016). Phytohormones viz. abscisic acid (ABA), salicylic acid (SA), methyl jasmonate (MeJA), and others have significant importance in different environmental stress responses and in many other physiological steps (Shen, et al. 2020; Verma, et al. 2016). The phytohormone ABA has an intermediatory role in adverse environmental conditions such as cold, salinity, drought, temperature and light (Vishwakarma, et al. 2017). Furthermore, ABA has a crucial role in leaf senescence, stomatal regulation and maintaining root system architecture (Sah, et al. 2016).) Lipid-derived signal molecules, MeJA and Jasmonic acid (JA), play an important part in plant growth and development (Wasternack and Song 2017). JA-mediated salinity tolerance was observed in *Triticum aestivum* (Qiu, et al. 2014). The application of JA precursor 12-OPDA led to improved drought tolerance in *Arabidopsis thaliana* has been reported (Savchenko and Dehesh 2014). Earlier studies showed under salt stress, the genes associated with JA biosynthesis get upregulated in roots (Geng, et al. 2013; Valenzuela, et al. 2016). Similarly, SA is an important signalling molecule for inducing stress responses in plants (Khan, et al. 2015) and regulates plant metabolism (Hayat, et al. 2010). Earlier studies indicated that in order to trigger stress responses, these phytohormone signaling molecules interact with TFs (Chanwala, et al. 2022).

Transcription factors are the key multi-domain mediator protein molecules that regulate the expression pattern of effector genes (Baillo, et al. 2019; Shao, et al. 2015). Among many TFspresent in plants, the NAC [No Apical Meristem (NAM), *A. thaliana* transcription activation factor (ATAF1/2) and cup-shaped cotyledon proteins (CUC2)] TF family is crucial and involved in complex signalling processes during plant stress responses. The conserved NAC/NAM domain, is found in the NAC gene family members (Aida, et al. 1997). The typical structure of NAC proteins contains an N-terminal domain, which is very conserved (160 amino acids) and a highly variable C-terminal transcriptional regulatory region (TRR). The N-terminal conserved domain comprises of 5 sub-domains named A-E; they facilitate protein-DNA binding and dimerization activity. The TRR in the C-terminal region is often found to act as either a transcriptional repressor or activator (Hussey, et al. 2015; Welner, et al. 2016).

The NAC genes have been identified in multiple plants like Arabidopsis (Ooka, et al. 2003), rice (Nuruzzaman, et al. 2010), wheat (Borrill, et al. 2017), maize (Fan, et al. 2014), foxtail millet (Puranik, et al. 2013), Tartary buckwheat (Liu, et al. 2019), Sorghum (Sanjari, et al. 2019) and sugarcane (Ramaswamy, et al. 2017). They are associated with many developmental processes, which include grain-filling (Sperotto, et al. 2009), leaf senescence (Breeze, et al. 2011), formation of flowers (Sablowski and Meyerowitz 1998) and ripening of fruits (Zhu, et al. 2014). NAC proteins also regulate plant defence responses by triggering hypersensitive responses and activating *PR* genes (Jensen, et al. 2008; Kaneda, et al. 2009). NAC TFs-mediated responses against abiotic stress have been studied in plant systems. For instance, overexpression of *ANAC01* and *ANAC055* enhanced the drought tolerance in transgenic plants. Also, *OsNAC52* and *OsNAC42* impart salinity and drought resistance in rice. Similarly, wheat *NAC* genes, Ta*NAC2*, Ta*NAC29* and Ta*NAC67* when overexpressed in Arabidopsis, provided cold, drought and salinity stress tolerance (Nakashima, et al. 2012; Nuruzzaman, et al. 2013; Shao, et al. 2015). The individual NAC TFs in millets have not been well studied yet; only *PgNAC21* was characterized, and it imparted better salinity tolerance to the transgenic Arabidopsis (Shinde, et al. 2019). NAC TFs can also act as negative regulators like *OsNAC2,* increasing the susceptibility of transgenic plants toward many abiotic stresses (Shen, et al. 2017). In apple, NAC TF (*MdNAC29*) negatively regulates drought tolerance (Li, et al. 2023). Also, it has been well-studied that the *NAC* gene expression is modulated by phytohormones such as JA and ABA as well as abiotic stress (Bu, et al. 2008; Yoon, et al. 2020; Yoshii, et al. 2010).

Pennisetum glaucum (pearl millet), a Poaceae family crop, ranked 6th among globally cultivated cereals and 4th among tropical cereals (Satyavathi, et al. 2021). This plant is extensively grown around the African and Indian subcontinent for forage and food, whereas in countries like USA, Australia, and Brazil it is only grown for feeding farm animals. Pearl millet is very nutritious and a good source of energy (Hassan, et al. 2021; Saxena, et al. 2018). It can survive in extreme climates like high salinity, less rainfall, elevated temperature, and marginal soil. It also has greater photosynthetic efficiency and produces high biomass (Singh, et al. 2017; Varshney, et al. 2017). In Africa, pearl millet is cultivated on marginal soil with less annual rainfall (Ausiku, et al. 2020). Despite its multifaceted economic potential, the genomic information of this crop is lagging in comparison to rice, wheat, maize, and soybean. In this context, recent developments in pearl millet genome study could appear as a genomic resource for advanced research (Varshney, et al. 2017; Yan, et al. 2023).

Previously a study had identified 151 NAC genes from pearl millet and studied their expression under salt and drought conditions (Dudhate, et al. 2021). However, current study comprehensively analyzed 155 pearl millet NAC transcription factors, examining their evolutionary progression, gene duplication events, cis-elements, and expression patterns to validate their functional diversity, particularly in phytohormone-mediated stress responses. The role of *PgNAC103* in drought response was investigated through overexpression in transgenic Arabidopsis. Stress gene expression and biochemical assays were performed to elucidate the underlying mechanisms. *PgNAC103* shows promise for developing stress-resilient plants.

2. Materials and methods

2.1. Plant growth condition

Pearl millet germplasm PRLT 2/89–33 (drought-tolerant) and H77/833–2 (drought-sensitive), were received from ICRISAT Patancheru, India (International Crops Research Institute for Semiarid Tropics). Pearl millet seeds were grown in red and black soil mixture with a 1:1 ratio in a 16 h of light and 8 h of dark cycle at 28 °C (± 2). For induction of calli, seeds of H77/833–2 were sterilized and germinated on MS media (Himedia) supplemented with 2,4-dichloro phenoxyacetic acid (2,4-D) and kinetin (Sahoo, et al. 2011). The seeds of wild-type and transgenic lines of Arabidopsis (Col-0) were surface sterilized and germinated on half-strength MS medium plate for seedling development. Subsequently, seedlings were transferred in plastic pots having a soil mixture of vermiculite soil, red soil, and soil-rite mix. Seedlings were allowed to grow at 22 °C (± 2) in a growth chamber, light period of 16 h light and 8 h of dark cycle and with a relative humidity of 70–75 %.

2.2. *Identification of PgNAC genes*

The nucleotide sequences and proteome dataset were retrieved from the genome database (v1.1) of pearl millet (http://cegsb.icrisat.org/ipmgsc/) (Varshney, et al. 2017). The reference NAC family protein sequences of rice, Arabidopsis and foxtail millet were obtained from

Oryzabase (Kurata and Yamazaki 2006), GRASSIUS (Grass Regulatory Information Server) (Yilmaz, et al. 2009) and Phytozome (Goodstein, et al. 2012). From Pfam site the HMM profiles of NAM (PF02365) and NAC (PF01849) were downloaded (Sonnhammer, et al. 1998) and used for identification of NAC domain from pearl millet proteome by HMMER tool v3.2 with default parameters (Finn, et al. 2011). Further, the NAC and NAM domains in the identified NAC members were verified by employing SMART, HMMscan, and CDD. The identified sequences having complete NAC or NAM domains were selected for further analysis. The protein features of NAC protein were analysed by ProtParam tool on the ExPASy server (Gasteiger, et al. 2005). The WoLFPSORT was utilized to identify subcellular localization sites of PgNACs (Horton, et al. 2007).

2.3. *Phylogenetic analysis and in-silico features of PgNAC*

The MUSCLE was used to perform multiple sequence alignment of NAC sequences of *P. glaucum* with *A. thaliana, O. sativa & S. italica* with default parameters (Edgar 2004). For the construction of phylogenetic tree, the neighbor-joining method (with 1000 bootstrap replicates) with default parameters was employed in the MEGA V10.0 program (Kumar, et al. 2018).

The gene duplication events and collinearity relationship between identified *PgNAC* and *NAC* members from *A. thaliana, O. sativa* and *S. italica* were determined with the help of MCScanX (Wang, et al. 2012) and visualized with AccuSyn software. The duplicated and orthologous NAC sequences were aligned by using EMBOSS Needle program. For calculation of synonymous (Ks) and nonsynonymous (Ka) substitution rates, PAL2NAL web-server was employed (Suyama, et al. 2006). Finally, for each individual gene pair, the Ka/Ks ratio was determined to understand the selection pressure. The 2000 bp upstream sequences (putative promoter) of *PgNAC*s were extracted and uploaded into PlantCARE server to find out the presence of *cis*-regulatory elements (Rombauts, et al. 1999).

2.4. *Abiotic stresses and phytohormone treatment*

A total number of ten (10) pearl millet (PRLT 2/89–33) plants were grown under greenhouse conditions, and samples were collected from three biological replicates for each experiment. The samples from different parts of pearl millet seedlings (root, stem, and leaf) were harvested under normal conditions for determining the expression pattern of *PgNAC* genes in tissues. Abiotic stresses and phytohormonal treatments were conducted on 4-week-old pearl millet seedlings (PRLT 2/89–33). For imparting heat treatment, the seedlings were kept in 45 °C incubator for 12 h and then returned to greenhouse condition for recovery. Samples were collected at 0 h, 3 h, 12 h and 24 h (Sun, et al. 2020). To perform drought stress, watering was stopped for 8 consecutive days. After that, on 9th day seedlings were rewatered for recovery, samples were collected at the interval of 2 days (Chanwala, et al. 2020). For inducing salinity stress, plants were dipped in a nutrient solution (Hoagland solution) having 250 mM NaCl. Also, the pearl millet seedlings were placed into same nutrient solution without NaCl for control. After that, samples were collected at 0 h, 3 h (early) and 24 h (late) (Chanwala, et al. 2020).

The ABA, MeJA and SA were sprayed on the pearl millet seedlings at a concentration of 100 μM each for phytohormonal treatment (Puranik, et al. 2013). All the treated samples were harvested at different time points of 0 h, 3 h and 24 h All the samples harvested at different time points were flash-frozen using liquid nitrogen and then stored at −80 °C.

2.5. *RNA extraction and qRT-PCR assay*

Total RNA was extracted using the Spectrum Plant Total RNA kit. RNA quality was assessed on a 1.2 % agarose gel containing 18 % formaldehyde. RNA purity and yield were determined using a NanoDrop ND-2000 spectrophotometer. Samples with a 260/280 nm ratio between 2.0 and 2.1 were used for downstream applications. DNAse-I (Sigma-Aldrich, USA) treatment was done for removing DNA contaminants. cDNA was prepared using first-strand cDNA synthesis kit (Thermo Scientific, MA, USA). Quantitative RT-PCR of candidate *PgNAC*s was performed with QuantStudio™ 5 (Applied Biosystems, Foster City, CA, USA). The procedure and reaction mixture composition are same as we reported earlier (Jha, et al. 2024). PrimerQuest™ tool of IDT was employed for designing the primers used for this study (Owczarzy, et al. 2008). For data normalization, *GAPDH* and *Actin* (Reddy, et al. 2015) were used. The relative expression pattern of *PgNAC*s was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The mean values and standard deviation of three biological and two technical of each experiment were used for data presentation.

2.6. *Cloning and bioinformatic assay of PgNAC103 gene and its promoter*

The cDNA prepared from pearl millet leaf and root was used for the isolation of *PgNAC103* gene with specific primers. The pGEM®-T-Easy-Vector-Systems (Promega) was used to clone the obtained PCR product. Sequencing was done to check the sequence integrity from start to stop codon. The sequences of NAC protein from different plants were extracted and aligned with PgNAC103 using Clustal-omega (Sievers and Higgins 2018) and alignment was visualized through GeneDoc (KB 1997). Phylogeny was generated with aligned sequences employing MEGA V10.0 software. The SMART server was used to predict the conserved domain in PgNAC103. The exon-intron arrangement was analyzed with GSDS and tertiary structure was predicted with HHpred (Zimmermann, et al. 2018). String database was used to predict the protein-protein interaction of PgNAC103 (https://string-db.org/) (Szklarczyk, et al. 2023).

The CTAB (cetyltrimethylammonium bromide) method was employed to extract the genomic DNA of pearl millet (Porebski, et al. 1997). From DNA, the upstream region (1000 bp) of the *PgNAC103* gene was isolated with a specific pair of primers through PCR and then cloned into pGEM®-T Easy Vector. The *cis*-element analysis of the upstream region of *PgNAC103* was done and visualized through TBtools (Chen, et al. 2020).

The coding region of *PgNAC103* was inserted into pCAMBIA-2300-GFP (modified) vector without stop codon to perform subcellular localization and transgenic development. Also, the full-length *PgNAC103* and two truncated genes, namely *PgNAC103*-N and *PgNAC103-C* were cloned in the yeast vector pGBKT7 using gene-specific primer pairs (**Supplementary Table S1)**. The full-length putative promoter region PgNAC103-F1 (−1 to −1000) of 1000 bp and one 5′ deletion fragment PgNAC103-F2 (−1 to −500) of 500 bp were inserted into pCAMBIA2300-GUS (modified).

2.7. *Transactivation assay and subcellular localization*

The WoLFPSORT server predicted nuclear localization of PgNAC103. To further confirm the sub-cellular localization of PgNAC103, the 35S: PgNAC103-GFP-pCAMBIA2300 construct and vector control (pCAMBIA-2300-GFP) was bombarded into epidermal cells of onion through gene gun (Bio-Rad Helios). Then, transformed onions were transferred in dark condition for 36–48 h; the fluorescence signals were visualized through CLSM (Leica, STED microscope).

For yeast transformation, the Y2H Gold yeast strain was used (Clontech, Palo Alto, CA, USA). To check the transactivation ability of the PgNAC103, three constructs, namely pGBKT-PgNAC103(1–1350 bp), PgNAC103-N (1–675 bp) and PgNAC103-C (676–1350 bp) with negative control (empty pGBKT) and positive control (pGBKT-p53) were separately transformed into the yeast. On three different selection media, namely SD/-Trp, SD/-Trp/-His, SD/-Trp/-His/-Ade and SD/-Trp/-His/-Ade +*X*-α-Gal, transformed yeast cells were spotted and

kept at 30 °C for 3–5 days.

2.8. Generation of transgenic Arabidopsis and pearl millet calli

The *Agrobacterium* strain GV3101 transformed with the constructs 35S:PgNAC103-pCAMBIA2300-GFP, PgNAC103-F1-pCAMBIA2300-GUS and PgNAC103-F2-pCAMBIA2300-GUS were used for infecting wild-type Arabidopsis (Col-0) by employing floral dip method (Zhang, et al. 2006). For screening the transgenic plants, the T_0 seeds were germinated on ½ MS media plates having 50ug/ml Kanamycin. The healthy transgenic Arabidopsis seedlings were transferred to pots under greenhouse condition and further confirmed by gene integrity assay. T_3 seeds were harvested from independent T2 lines for further analysis.

The well-developed H77/833–2 calli were transformed with *Agrobacterium* strain GV3101 harboring 35S:PgNAC103:GFP construct to develop transgenic pearl millet calli; similarly, H77/833–2 seedlings were also infected with the same construct following the procedures described earlier (Chanwala, et al. 2024; Jha, et al. 2024).

2.9. Stress tolerance assays

The seeds of transgenic lines of PgNAC103-overexpressing Arabidopsis with wild type were sown on half MS medium with 200 mM mannitol, 1uM ABA and only ½ MS separately. After vernalization, the seeds were placed at 22–24 °C in a growth chamber. The rate of seed germination noted till 11 days and a photograph was taken. For root length assay, 4-days old seedlings were transferred on stress media (½ MS plates having 200 mM Mannitol and 1 μM ABA) and control media (without mannitol and ABA) and then allowed to grow further for two weeks. After that, root length was measured, and a photograph was taken.

NBT (nitroblue tetrazolium) and DAB (3,3′ -diaminobenzidine) staining was done on two weeks old transgenic and wild-type seedlings under normal and drought conditions. The abundance of superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) was evaluated (Jambunathan 2010; Kumar, et al. 2014).

Also, the wild-type and *PgNAC103*-overexpressing transgenic Arabidopsis seedlings were placed in pots for growing at optimum greenhouse conditions for 4-weeks followed by drought stress induction (watering was stopped for 3-weeks), whereas for control plants, water was provided regularly. Photographs were taken, and fresh weight and chlorophyll content were calculated.

Samples from drought-induced *PgNAC103*-overexpressing transgenic Arabidopsis plants and wild-type were used for expression analysis of ROS-scavenging genes and stress genes through q-RTPCR. Biochemical assays of antioxidants and peroxidase activity were performed. The content of malondialdehyde (MDA) in wild-type and transgenic plants under drought and normal conditions was calculated using thiobarbituric acid (Schmedes and Hølmer 1989). For determining the SOD, CAT and POD activity, the protocol described by Chen *et al.*, 2016 was followed (Chen and Zhang 2016).

The transgenic pearl millet H77/833–2 calli were transferred to co-cultivation media containing 20 % 4000 PEG for the determination of expression pattern of stress genes, namely *OST2, ERD,* and *CAT* (Jha, et al. 2024). The RNA was isolated and expression analysis done through qRT-PCR. Similarly, dehydration stress was imposed on the transformed H77/833–2 seedlings and expression levels of *OST2, ERD,* and *CAT* were checked.

2.10. *Promoter activity assay of putative PgNAC103 promoters*

Initially, to check the promoter activity, a transient assay of PgNAC103-F1:GUS and PgNAC103-F2::GUS was performed in N. *benthamiana* and pearl millet. In healthy leaves of N. *benthamiana, Agrobacterium* strain GV3101 harboring the above two constructs were infiltrated using a needle-less syringe following standard protocol (Sherpa, et al. 2023). In the case of pearl millet, one week old seedlings dipped into *Agrobacterium* culture and vacuum infiltrated for 10 min at 800 mm of Hg. GUS assay was performed after 48–72 h of incubation following the standard protocols (Jefferson 1987).

As mentioned above, Arabidopsis transgenic lines of two fragments of PgNAC103 promoter were generated. The T_3 transgenic seeds of PgNAC103-F1: GUS and PgNAC103-F2:GUS were germinated on ½ MS media containing kanamycin (50 mg/liter). GUS assay of individual lines was done. To check the inducibility of PgNAC103 promoter fragments, seedlings of both promoter fragments were dipped into 200 mM mannitol for 24 h GUS assay of treated and non-treated samples was performed. Also, histochemical GUS staining of seedlings, leaves, and flowers of selected lines of each promoter construct was done following the method mentioned by Chanwala et al. (Chanwala, et al. 2024)

2.11. Statistical analysis

For the analysis of all data sets, Student's *t*-tests were performed. The mean values and standard deviation of three biological and two technical of each experiment were used for data presentation; the * $p < 0.05$ and * * $p < 0.01$ showed statistically significant differences among the mean values.

3. Results

3.1. Identification of NAC gene family in pearl millet

For the identification of the NAC TFs in pearl millet, HMM search was performed by using NAC protein sequences from *A. thaliana, O. sativa* and *S. italica* as queries . HMM scan analysis was done with the help of HMM profiles of both NAC (PF01849) and NAM (PF02365) domains. After confirmation of NAC and NAM domains, 155 NACs were confirmed and named as PgNAC1 to PgNAC155 on the basis of their location on pearl millet chromosomes. Interestingly, we have found 4 new PgNACs [PgNAC11 (Pgl_GLEAN_10,009,387), PgNAC85 (Pgl_GLEAN_10,033,240), PgNAC117 (Pgl_GLEAN_10,022,065) and PgNAC139 (Pgl_GLEAN_10,001,756) that were not reported earlier. The relative molecular weight among these 155 PgNAC members varied from 6.06991 kDa (PgNAC12) to 81.75198 kDa (PgNAC137), while pIs ranged from 4.2 (PgNAC145) to 10.8 (PgNAC69) with 95 members having pI<7 while others with pI>7 (**Supplementary Table S2)**. The instability index of most PgNAC proteins (124 out of 155) was higher than 40 and thus was unstable. PgNAC110 was the most stable protein with a stability index of 30.91. Most of the PgNAC proteins were predicted to have subcellular localization in the nucleus (**Supplementary Table S2)**.

3.2. Phylogenetic and evolutionary analysis of NAC members in pearl millet

For exploring the evolutionary relationship between NAC proteins of *P. glaucum, A. thaliana, O. sativa & S. italica*, a neighbor-joining phylogenetic tree was generated by using MEGA V10.0 software. A total of 573 sequences comprising of 155 sequences from pearl millet, 131 from Arabidopsis, 142 from rice and 145 from foxtail millet were used for analysis. The phylogenetic tree (Fig. 1**)** divided all 155 NAC proteins into 15 groups (I-XV) and they were distributed in all 15 groups.

To identify the duplication events of *PgNACs* in pearl millet, we have performed collinearity analysis. Twenty-nine segmental duplicated genes and eighteen tandem duplicated genes were found **(**Fig. 2**, Supplementary Table S3).** Interestingly, the tandem pairs were identified on majority of pearl millet chromosomes except for Chr 1 and Chr 5. Furthermore, it was noticed that few *PgNAC* genes were involved in more than one segmental duplication events (*PgNAC114* with *PgNAC1* and *PgNAC95, PgNAC112* with *PgNAC3* and *PgNAC94, PgNAC11* with *PgNAC13* and *PgNAC92, PgNAC106* with *PgNAC16* and *PgNAC124,*

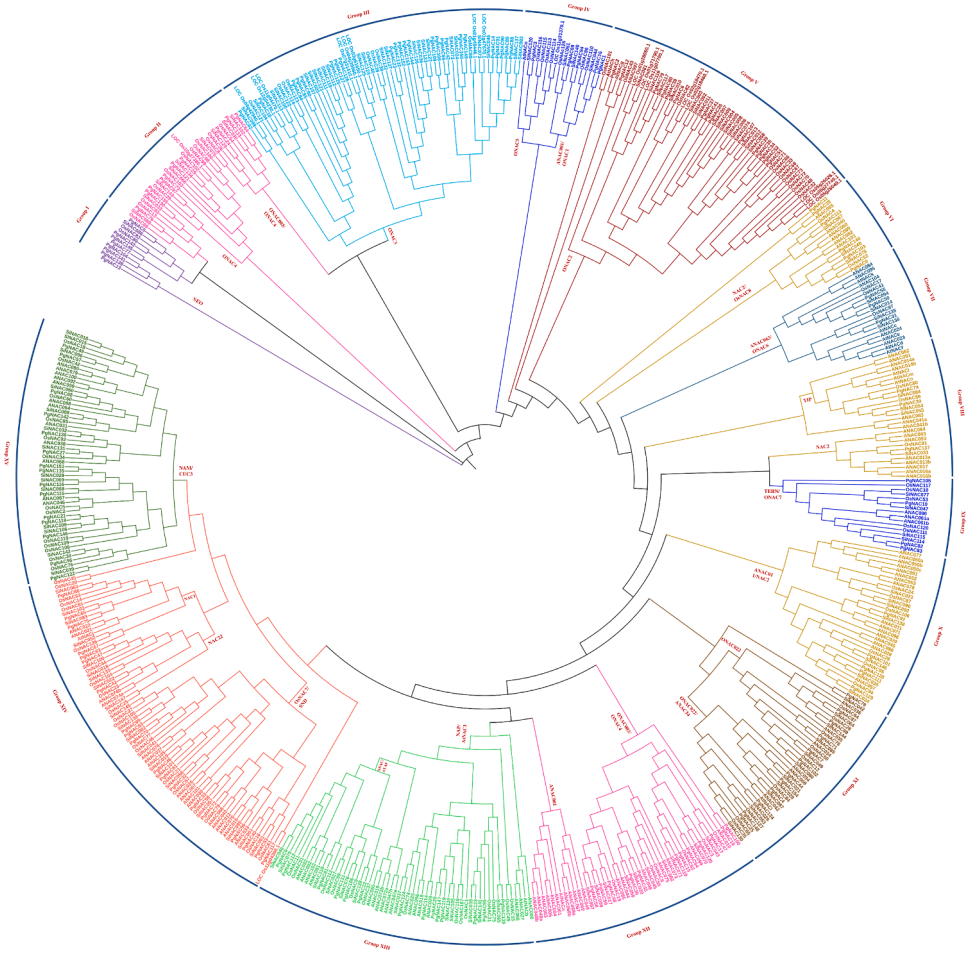

Fig. 1. Phylogenetic representation of NAC proteins of *P. glaucum* with *A. thaliana, O. sativa & S. italica*: A total of 573 NAC proteins were aligned by MUSCLE for generating phylogenetic tree employing neighbor-joining method with 1000 bootstrap replication using MEGA v10.0. The sub-groups are numbered from I-XV.

PgNAC63 with *PgNAC29* and *PgNAC43*).

Additionally, we attempted to explore the orthologous pairs between 573 NACs of *P. glaucum, A. thaliana, O. sativa & S. italica* through synteny. The results showed 138 orthologous pairs were identified that includes 49 pairs between *AtNAC* and *PgNAC* genes, 28 pairs between *OsNAC* and *PgNAC* genes and 61 pairs between Si*NAC* and *PgNAC* genes **(Supplementary Table S3).** Further, to understand the evolutionary pressure on the *PgNAC* family, the Ks and Ka values have also been calculated **(Supplementary Table S4).** In total, the values for all the identified pairs were less than one (<1), which indicated a purifying selection of *PgNAC* genes in the course of evolution.

A total of 66 *cis*-elements were identified in the 2000 bp upstream region of identified NAC genes. *Cis*-elements like TATA Box, CAAT Box, STRE, MYB, G Box, ABRE, AS1, CGTCA Motif, TGACG Motif, ARE and WRE3 were most common, whereas TGA BOX, Plant AP2 like, HD-ZIP3, JERE, AT-rich sequence and SARE were less in number **(**Fig. 3**).**

3.3. *Spatial expression profiles of PgNACs*

We have selected 10 genes for the expression analysis study based on phylogenetic, synteny analysis and genome wide chromosomal distribution (**Supplementary Table S1**). Of the selected genes, *PgNAC20* and *PgNAC21* were located on the Chr1; *PgNAC22* on Chr2; *PgNAC81* on Chr4; *PgNAC98, PgNAC103, PgNAC106, PgNAC114, PgNAC119* belongs to Chr6 and PgNAC155 located on Chr7. The majority of the selected genes belonged to NAP/ATAF of the NAC gene family, members of these groups have been well characterised to play crucial role in stress response. RNA samples from root, stem and leaf were harvested to explore the expression patterns of selected *PgNAC* genes in the above tissues. The expression analysis of these *PgNACs* revealed that the majority of them showed higher expression in root. The expression of *PgNAC21, PgNAC81, PgNAC106* and *PgNAC114* was significantly high in root tissues, indicating a possible role in root development **(**Fig. 4**)**. However, the expression of *PgNAC22* and *PgNAC98* was highest in leaf tissues. On the contrary, few genes showed differential expression patterns**.**

3.4. *Expression analysis of PgNACs under abiotic and hormonal treatments*

The above mentioned *PgNAC* genes were explored for their expression analysis in response to phytohormonal treatments (ABA, MeJA and SA) under abiotic stimuli (drought, heat and salinity). The majority of the *PgNAC* genes showed the upregulation pattern in drought conditions (Fig. 5a). However, the expression of *PgNAC22* and *PgNAC106* was downregulated on 9th day post-treatment. Interestingly, the expression of *PgNAC114* was recovered after re-watering (at the 9th day), suggesting its possible involvement in drought stress response. Similarly, under salinity stress, majority of the *PgNAC* genes showed differential expression patterns (Fig. 5b). *PgNAC20, PgNAC21, PgNAC22,* and *PgNAC114* were significantly upregulated at both 3 hr and 24 hr time points. On the contrary, expression of *PgNAC81, PgNAC103* and *PgNAC106* significantly decreased at both the time points. In response to heat stress, 6 *PgNAC* genes were upregulated whereas 3 *PgNAC* genes were downregulated **(**Fig. 5c**)**. *PgNAC20, PgNAC22, PgNAC106* and *PgNAC114* were highly upregulated under heat stress. In contrast,

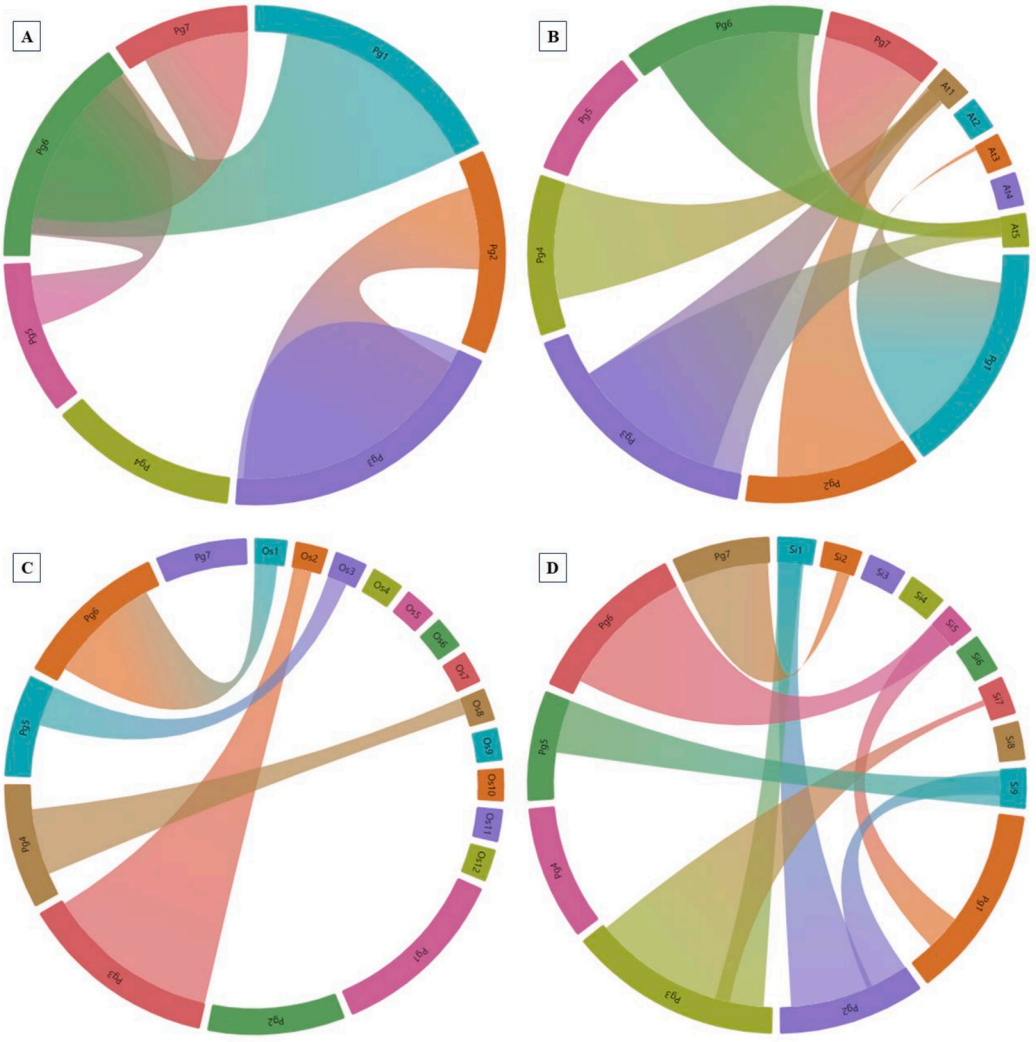

Fig. 2. Synteny relationship between NAC genes of *P. glaucum, A. thaliana, O. sativa* and *S. italica*: Orthologous and paralogous pairs were shown through connected colored line shows among the species. **A)** Synteny connections in *P. glaucum* **B)** Synteny connections between *P. glaucum* and *A. thaliana* **C)** Synteny connections between *P. glaucum* and *O. sativa* **D)** Synteny connections between *P. glaucum* and *S. italica.*

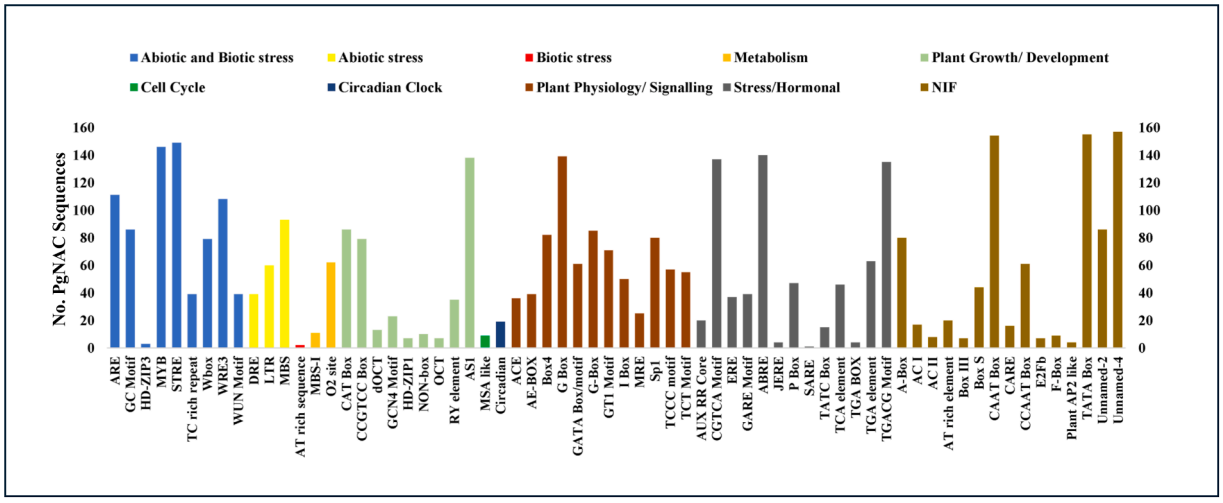

Fig. 3. *Cis*-element analysis on a 2000 bp upstream region of *PgNAC* genes. Color bars indicate the group to which *cis* elements belongs. The Y axis represents number of *PgNAC* upstream region where specific *cis*-element present, and the X axis denotes different *cis* elements found on upstream of *PgNAC* genes.

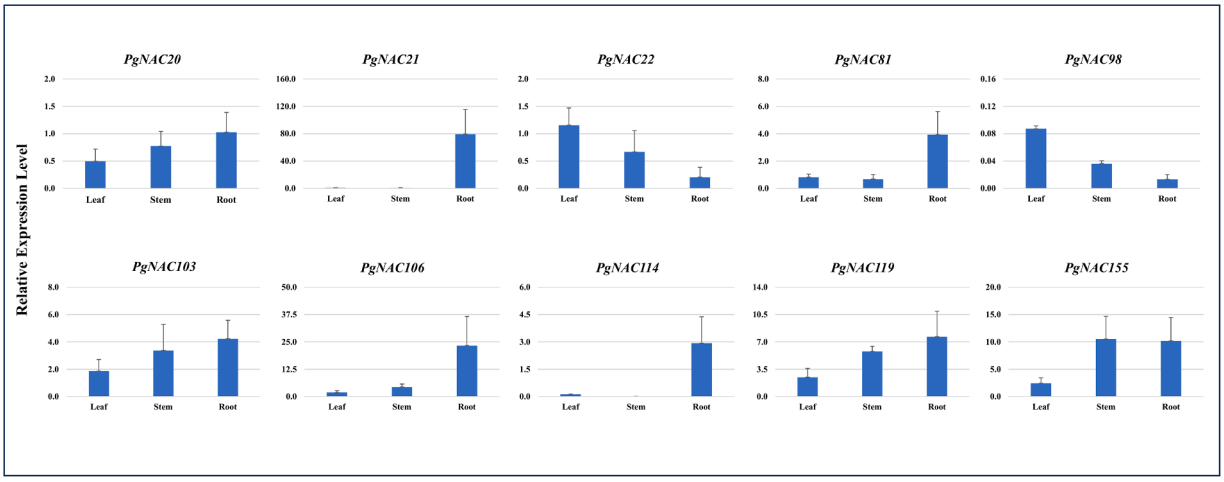

Fig. 4. Expression analysis of *PgNAC* genes in root, stem, and leaf tissues by qRTPCR analysis. The Y axis represents relative expression, and the X axis denotes different plant tissues.

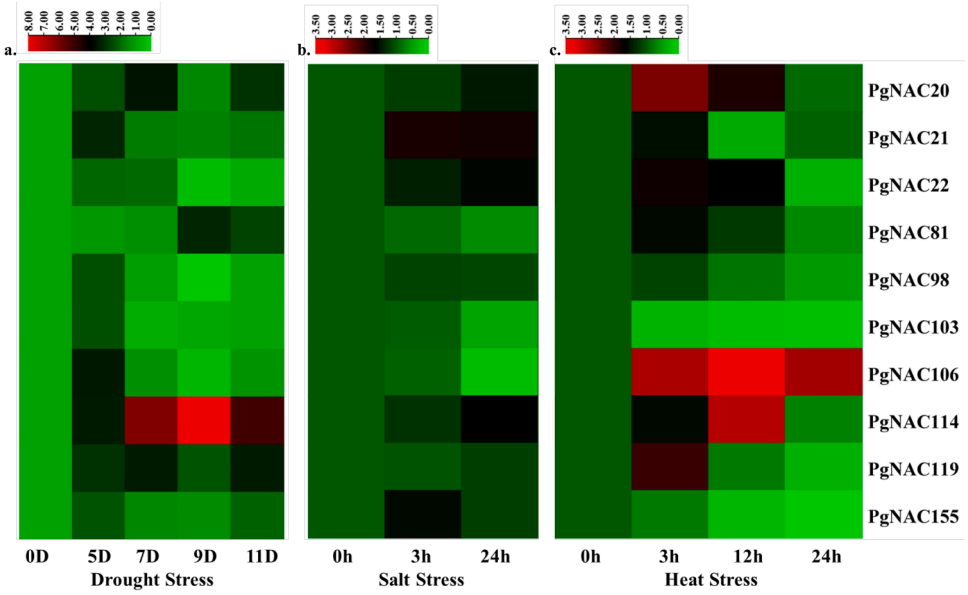

Fig. 5. Heat map showing the expression pattern of *PgNAC* genes under (5a) Drought stress (at 0th day, 5th day, 7th day, 9th day and 11th); (5b) Salt stress (at 0 h, 3 h and 24 h); and (5c) Heat stress (at 0 h, 3 h, and 12 h and 24 h). The transcript abundance level has been normalized and hierarchically clustered. The heat map was generated using TBtools v0.66831.

PgNAC103 and *PgNAC155* expression was downregulated.

Phytohormones are known to activate specific signal cascades in response to various external stimuli (Verma, et al. 2016). The involvement of NAC proteins with various phytohormones under abiotic stress has been reported in different species. Under ABA treatment, *PgNAC22, PgNAC81 PgNAC95* and *PgNAC114* at 3 hr and 24 hr time points were upregulated (Fig. 6a). Meanwhile, 4 genes (*PgNAC21, PgNAC98, PgNAC103* and *PgNAC106*) followed a downregulation pattern upon ABA treatment. *PgNAC155* did not show a noticeable change in response to ABA.

Under MeJA treatment, *PgNAC114* was significantly upregulated at both time points. We also observed early transcript accumulation (at 3 h time point) of *PgNAC21, PgNAC103, PgNAC106* and *PgNAC155* during MeJA treatment. In contrast, low transcript levels were also observed for *PgNAC20, PgNAC22, PgNAC81,* and *PgNAC119* (Fig. 6b).

Upon exogenous SA treatment, transcript levels of *PgNAC22, PgNAC106* were significantly upregulated, whereas *PgNAC20, PgNAC103, PgNAC114* and *PgNAC119* were significantly downregulated (Fig. 6c). However, *PgNAC155* did not show a noticeable trend in response to SA.

3.5. *Sequence analysis of PgNAC103*

We have isolated a 1353 bp long *PgNAC103* from the cDNA of pearl millet with a start and stop codon. The sequence analysis of PgNAC103 showed that it has typical NAC protein features (Fig. 7a) and contains the conserved NAM domain in the N-terminal region with 5 conserved sub-domains, namely (A-E) (Fig. 7b). The evolutionary relationship of PgNAC103 with NAC proteins of other species was analyzed through a phylogenetic tree; AtNAC103 was most closely related to PgNAC103 (Fig. 7c). Presence of 4 exons and 3 introns was predicted by GSDS server (Fig. 7d). The predicted structure of PgNAC103 revealed that it mainly has disordered C-terminal region while the N-terminal region was comparatively structured (Fig. 7e).

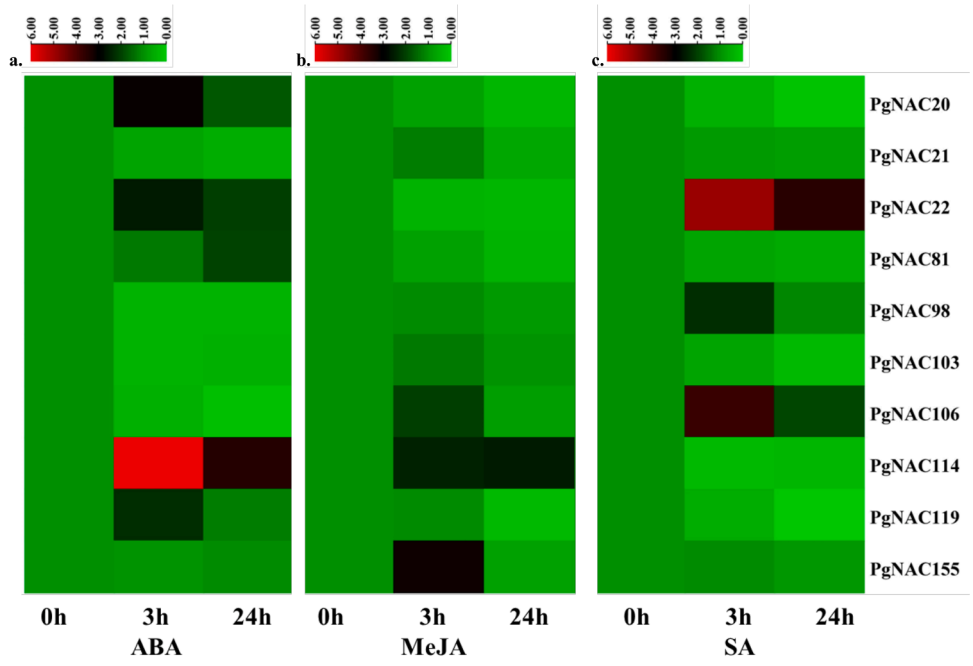

Fig. 6. Heat map showing expression analysis of *PgNAC* genes under ABA (6a), MeJA (6b), and SA (6c) treatment. The transcript abundance level has been normalized and hierarchically clustered. The heat map was generated using TBtools v0.66831.

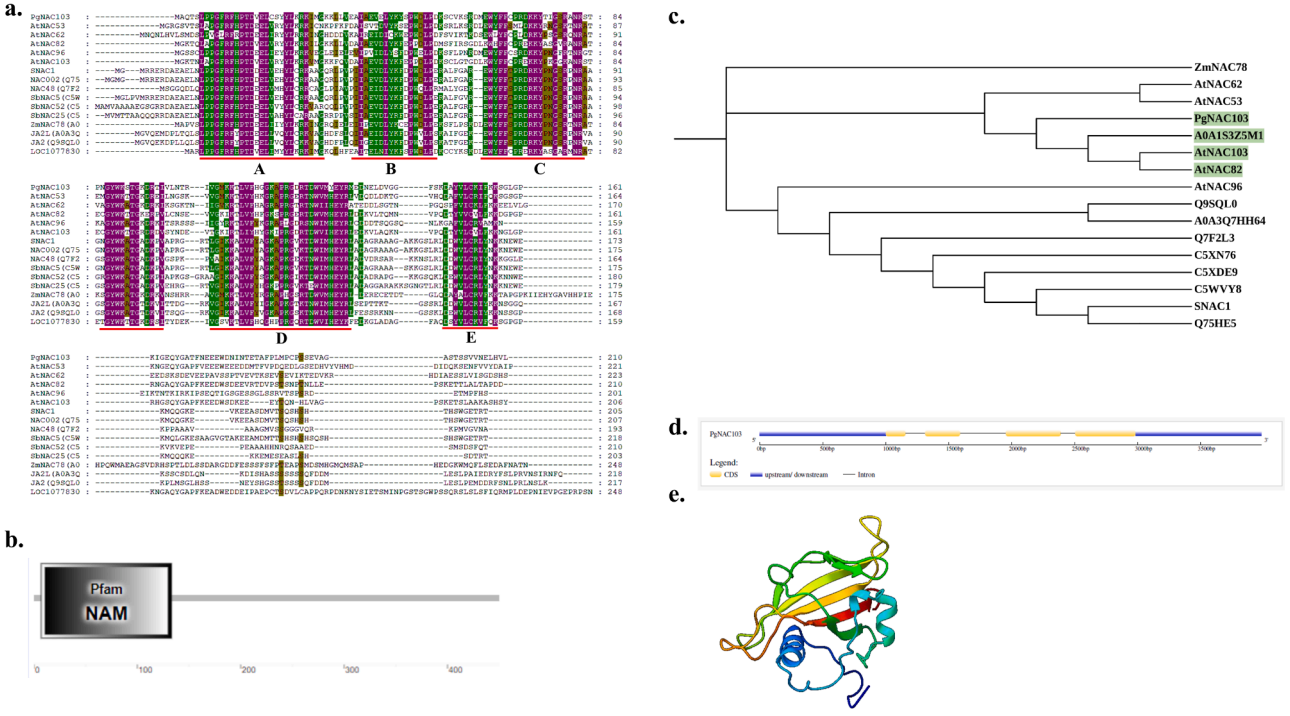

Fig. 7. (a) Protein sequence alignment of PgNAC103 along with NAC proteins of other species showed the conserved NAM domain; (b) NAC domain confirmation with SMART server; (c) Phylogenetic analysis of PgNAC103; (d) Exon-intron organization done with GSDS server and (e) PgNAC103 structure prediction through HHpred server.

3.6. Transactivation activity and subcellular localization of PgNAC103

The PgNAC103 was predicted to localize in the nucleus which was further verified by expressing GFP fused PgNAC103 and control vector transiently in the onion epidermal cell as described in Method Section 2.8 (Fig. 8a). Under confocal microscope, a sharp green fluorescence was obtained from nucleus bombarded with 35S:PgNAC103-GFP construct, while in vector control (35:GFP-pCAMBIA2300) diffused GFP signal was visualized from both nucleus and cytoplasm (Fig. 8b). These findings confirmed that PgNAC103 was localized in the nucleus.

The transactivation feature of PgNAC103 was analyzed in the yeast cell. The GAL4 DNA-binding domain fused with 3 constructs of PgNAC103 (full-length PgNAC103, PgNAC103-N and PgNAC103-C)

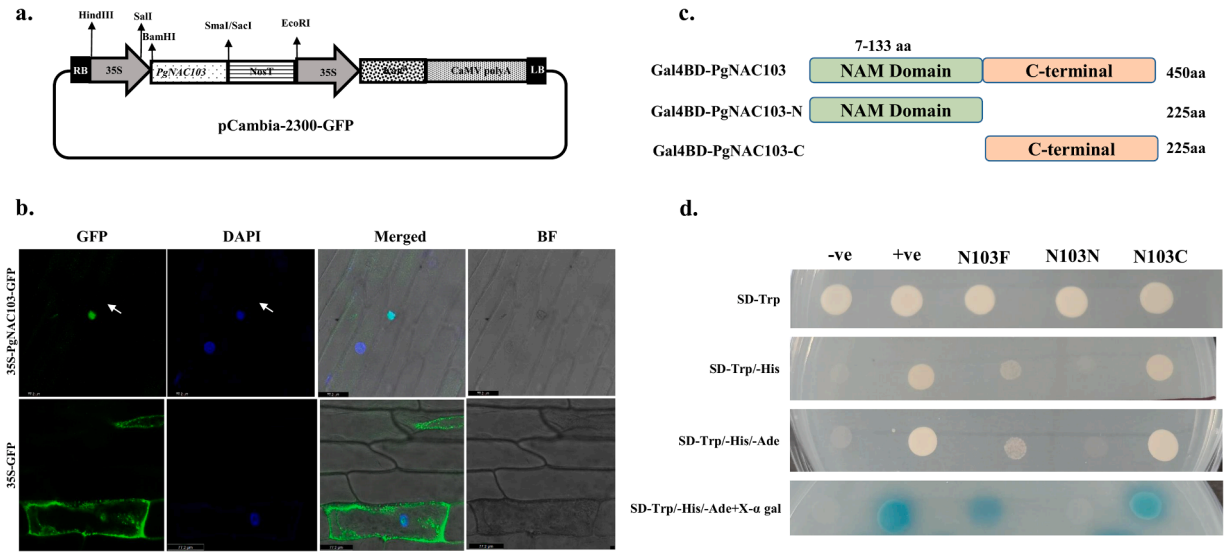

Fig. 8. (a) Schematic representation of 35S:PgNAC103-pCAMBIA2300-GFP construct; (b) Subcellular location of *PgNAC103* gene along with vector control. (DAPI: 4′,6-diamidino-2-phenylindole; GFP: Green fluorescence; Merged: Merged images of GFP, DAPI and BF: Bright field); (c) Schematic representation of constructs for transcriptional activation assay of PgNAC103; (d) Transactivation assays of PgNAC103 in a yeast expression system.

were transformed into the yeast (Fig. 8c). As shown in Fig. 8d the pGBKT7-PgNAC103-N and negative control (pGBKT7) were not able to grow on SD/-Trp/-His/-Ade plates whereas the positive control, pGBKT7-PgNAC103, and pGBKT7-PgNAC103-C grew on SD/-Trp/-His/-Ade plates. They also turned SD/-Trp/-His/-Ade+*X*-α-gal plates in blue coloration. These observations suggest that PgNAC103 is a transcriptional activator, and its C-terminal region has an activator domain.

3.7. *Growth analysis of Arabidopsis overexpressing PgNAC103*

Transgenic lines of Arabidopsis overexpressing *PgNAC103* were developed and confirmed through gene integration assays (**Supplementary Fig. S1**). The seed germination rate of wild-type and transgenic lines overexpressing *PgNAC103* (OE1, OE7 and OE12) on ½MS medium under control conditions was almost identical. Under osmotic stress, the germination rate of wild-type Arabidopsis was observed to reduce by around 50 %, whereas the germination rate of transgenic lines reduced by 13–22 % (Fig. 9a,b, d and e). Also, upon exposure to osmotic stress at post germination stage, the primary root length of the transgenic lines overexpressing *PgNAC103* was comparatively higher than wild-type seedlings (Fig. 9g **and** h). Under ABA treatment, the transgenic lines exhibited a significantly higher germination rate than the wild-type plants, which experienced a drastic reduction of up to 70 % (Fig. 9c **and** f). Additionally, the root length of the transgenic lines was longer than that of the wild-type plants (Fig. 9i).

Further, to check the level of O_2^- (superoxide radicals) and H_2O_2, the

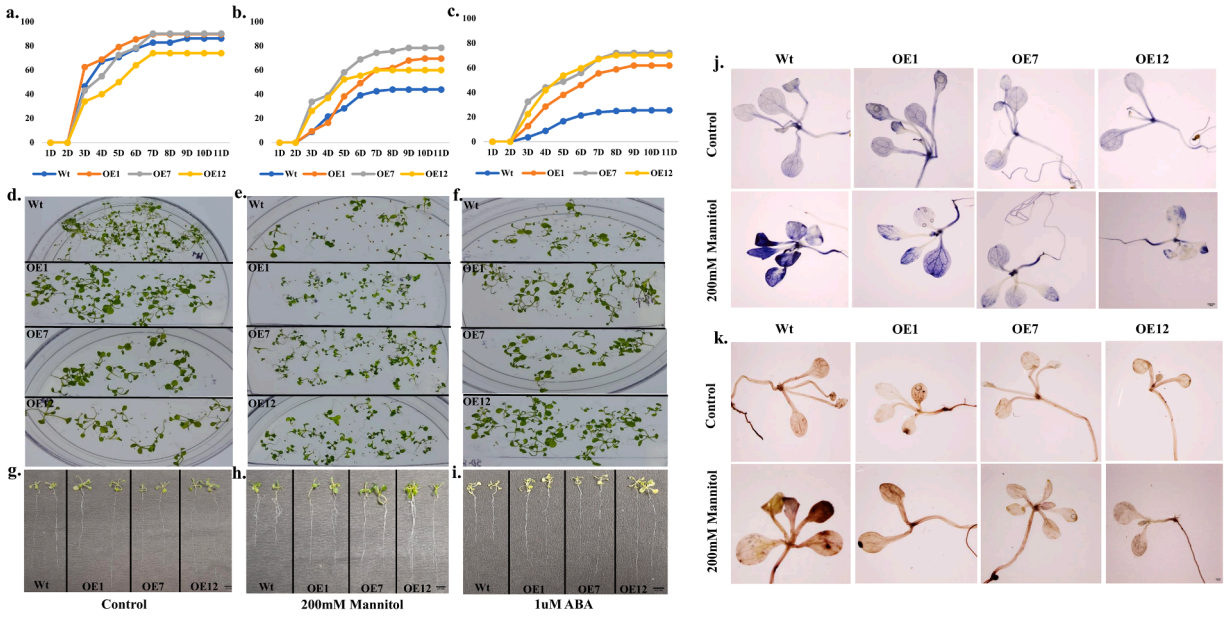

Fig. 9. Effect of Osmotic stress and ABA on seedlings of the *PgNAC103* transgenic Arabidopsis lines (OE1, OE7 and OE12). (a-c) Rate of germination of transgenic and WT seeds on ½ MS medium, ½ MS medium with 200 mM mannitol and ½ MS medium with 1 uM ABA; (d-f) Photographs showing seed germination of WT and transgenic lines on ½ MS medium, ½ MS medium with 200 mM mannitol and ½ MS medium with 1 uM ABA; (g-i) Root length of wild-type and transgenic Arabidopsis. Four-day-old seedlings were transferred to ½ MS medium, ½ MS medium with 200 mM mannitol and ½ MS medium with 1 uM ABA. After 14 days, representative images were taken; (j-k) O_2^- and H_2O_2 content indicated by NBT and DAB staining in transgenic lines and WT under normal and stress conditions.

wild-type and transgenic seedlings were treated with NBT and DAB solutions as described earlier. Under control conditions the O_2^- and H_2O_2 content appeared to be the same in all the samples. Whereas under osmotic stress the O_2^- and H_2O_2 content in wild-type was much higher than the transgenic lines (Fig. 9i–k). The expression profiling of stress marker genes (*AtRD22, AtCOR15* and *AtKIN*) and ROS scavenging effector genes (*AtSOD, AtPOD, AtCAT*) was done for understanding the molecular mechanism of stress response in Arabidopsis overexpressing *PgNAC103*. The transcript level of most of these genes were increased upon osmotic stress (**Supplementary Fig. S2**). Specifically, *AtPOD, AtRD22, AtCOR15* and *AtKIN* genes were highly upregulated in transgenic lines upon osmotic stress.

3.8. Stress tolerance analysis of Arabidopsis transgenic plants under drought

For further understanding the role of PgNAC103 in drought response at higher developmental stages, the 4-week-old Arabidopsis transgenic lines (T3) and wild-type plants were employed for the evaluation of stress responses. The phenotypic analysis of 4-week-old *PgNAC103*-overexpressing Arabidopsis lines and wild type was done. We observed that under normal conditions, they grew well, and no significant morphological differences were found. However, under drought stress, the transgenic lines were comparatively greener and healthier than wild-type plants (Fig. 10a). As shown in Fig. 10b, the fresh weight of both transgenic lines and wild-type plants were nearly similar without stress, whereas under drought stress, the fresh weight of transgenic lines was higher. Also, under drought, the chlorophyll content was comparatively higher in transgenic lines than the wild type plants (Fig. 10c). We observed that the MDA content of the wild type was higher than transgenic lines under drought stress (Fig. 10d). SOD, CAT and POD activities of transgenic lines were comparatively higher than the wild-type plants upon induction of drought stress whereas under normal condition antioxidant enzymatic activity was very much similar in both wild-type and transgenic Arabidopsis plants (Fig. 10d). Under drought stress, the NBT and DAB staining suggested a higher amount of O_2^- and H_2O_2 in the leaves of wild-type plants compared to transgenic lines (Fig. 11a). As shown in Fig. 11b, ROS-scavenging genes and stress-responsive genes were highly expressed in the *PgNAC103* overexpressing Arabidopsis lines under stress.

3.9. Genetic transformation of *PgNAC103* in pearl millet

Gene integration confirmed the successful transformation of pearl millet calli and seedlings with 35S:PgNAC103-pCAMBIA2300GFP and vector control (35-pCAMBIA2300GFP) construct (**Supplementary Fig. 3a**). Transformed transgenic calli were treated with 20 % PEG for 7 days, whereas seedlings were treated for 24 h. We found that the expression level of the *PgNAC103* was significantly enhanced upon drought treatment. Also, some known stress genes like *OST2, ERD* and *CAT* were upregulated in *PgNAC103*-overexpressing samples compared to vector control in both transformed calli and seedlings. The data obtained was represented in **Supplementary Fig. 3b-c** with respective standard deviations. This suggested the overexpression of *PgNAC103* might activate downstream stress genes in pearl millet.

3.10. Characterization of *PgNAC103* promoter

The upstream sequence of *PgNAC103* was analysed using the PLANTCARE database. It revealed the presence of several *cis*-elements associated with various biological processes. It was noticed that multiple copies of MYB and MYB-related *cis*-elements along with core cis-elements were present (Fig. 12a). To assess the promoter activity of upstream region of *PgNAC103*, two constructs (PgNAC103-F1:GUS and PgNAC103-F2::GUS) were prepared (**Supplementary Fig. 4a**). PgNAC103-F1 found to have higher GUS activity compared to that of PgNAC103 F2 upon transient expression in the pearl millet seedlings and in the leaves of N. *benthamiana* (**Supplementary Fig 4b-c**). Transgenic Arabidopsis lines expressing GUS under the control of PgNAC103-F1 and PgNAC103-F2 were developed and confirmed through gene integration assay **(Supplementary Fig. 4d)**. Higher GUS activity in transgenic lines of PgNAC103 F1 was found compared to PgNAC103 F2 transgenic lines which was in accordance with the transient GUS assay in tobacco and pearl millet. (Fig. 12b and c). We also found the GUS expression under

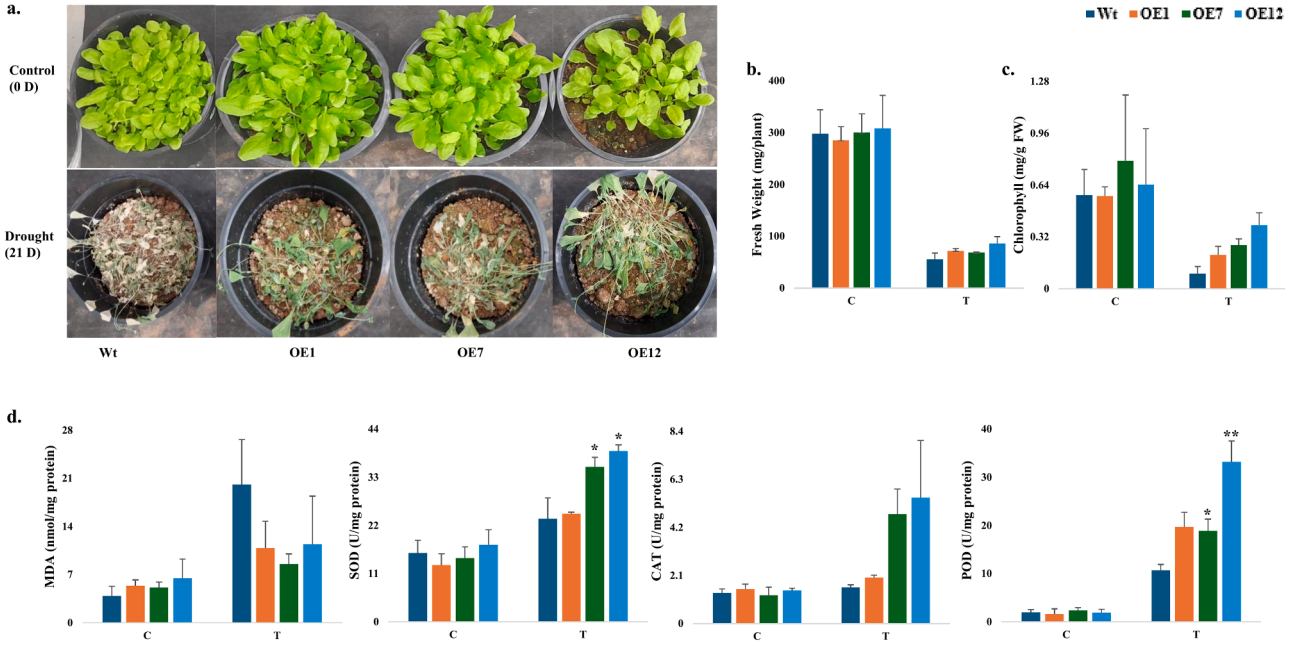

Fig. 10. (a) Phenotypic analysis of 4-weeks old *PgNAC103*-overexpressing transgenic lines and wild-type under normal conditions (OD) and drought stress (3-weeks). (b) Fresh weight and (c) and chlorophyll content of transgenic and wild-type plants under normal and drought conditions. The Y axis represents fresh activity and chlorophyll content respectively, and the X axis denotes different plant lines. (c) MDA content and antioxidant enzyme activity (SOD, CAT and POD) of transgenic and wild-type plants under normal and drought stress. C: Control, T: Treated. The Y axis represents enzymatic activity, and the X axis denotes different plant lines.

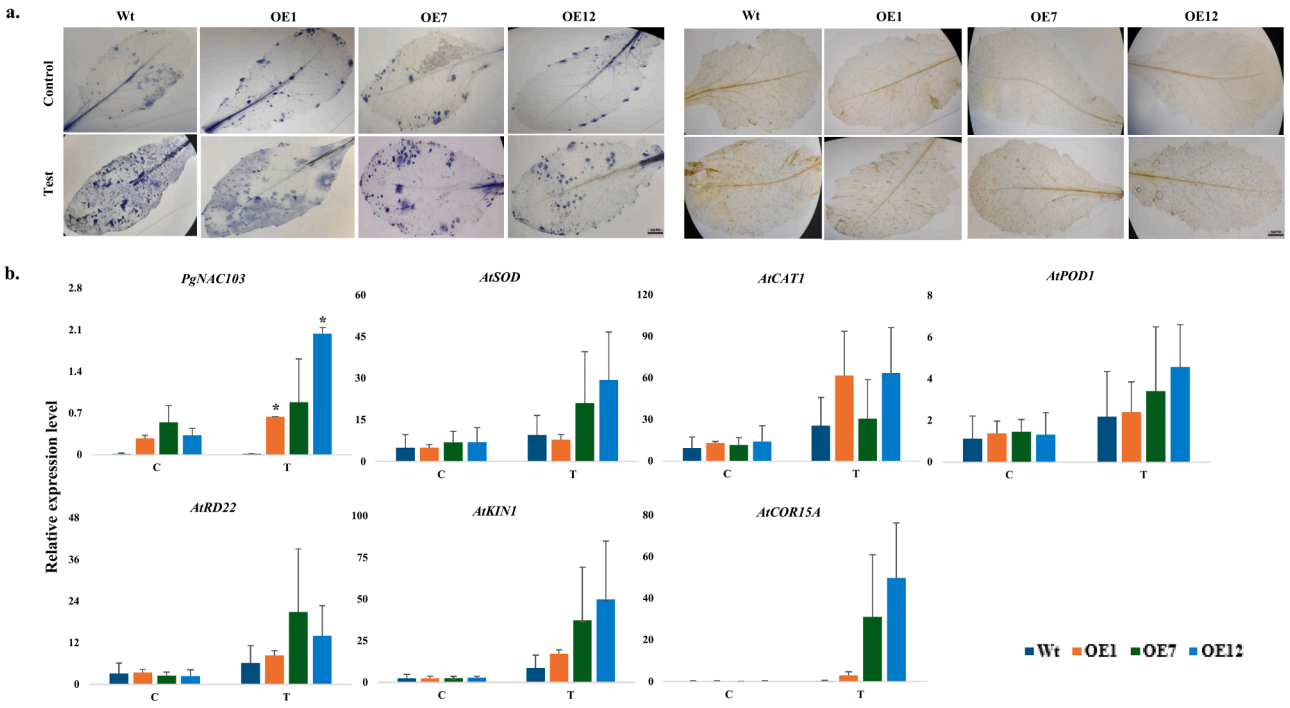

Fig. 11. Stress tolerance evaluation of 4-week-old *PgNAC103*-overexpressing transgenic lines (OE1, OE7 and OE12) and wild-types (Wt) under drought conditions. (a) Superoxide (O_2^-) and hydrogen peroxide (H_2O_2) content under normal and drought conditions; (b) Analysis of expression levels of stress-related genes under drought in transgenic Arabidopsis and WT plants. The Y axis represents relative expression, and the X axis denotes different plant lines. C: Control, T: Treated. The significant difference in mean between the samples for a given set is indicated by $*p < 0.05$, as obtained by Student's *t*-test, which employs the observation is significant.

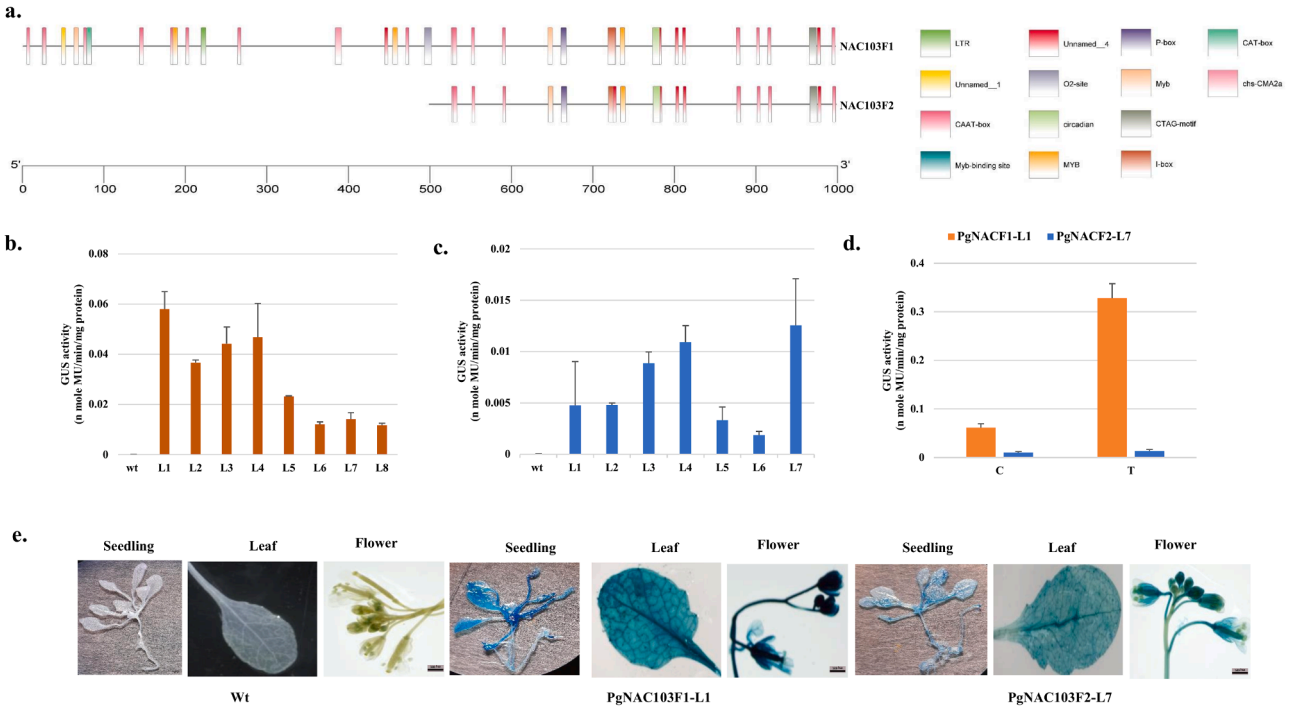

Fig. 12. (a) Putative *cis*-element analysis of PgNAC103-F1 (1000 bp) and PgNAC103-F2 (500 bp) promoter; GUS activity analysis for Arabidopsis transgenic lines of F1 (b), and F2 (c) and under drought stress (d). Histochemical staining of transgenic seedling, leaf and flower showing GUS activity under *PgNAC103* promoter (F1, and F2) (e).

PgNAC103 promoter fragments was induced under drought stress, particularly GUS activity under the full-length fragment was higher (Fig. 12d). The histochemical GUS staining of seedlings, leaves, and flowers further confirmed the higher GUS activity of full-length PgNAC103 promoter. Also the promoter activity of full-length PgNAC103 promoter was higher than that of PgNAC103-F2 in mature transgenic Arabidopsis plants' seedlings, leaves, and flowers (Fig. 12e).

4. Discussion

In pearl millet, the significant role of TF members belonging to WRKY (Chanwala, et al. 2020), GRAS (Jha, et al. 2021), MYB (Chanwala et al., 2023), bZIP (Jha, et al. 2024), Trihelix (Chanwala et al., 2024) and AP2/ERF (Xu, et al. 2024) families has been established under diverse stresses. In our independent study , we have identified 155 NAC members in pearl millet which is very close to that of *O. sativa* (140) (Nuruzzaman, et al. 2010) , soybean (152) (Le, et al. 2011), *S. italica* (147) (Puranik, et al. 2013).

We performed a phylogeny analysis that classified 155 NAC pearl millet in 15 groups which were consistent with previously reported NAC groupings in other species (Nuruzzaman, et al. 2010; Ooka, et al. 2003); Group 13 consisted of subgroups ATAF and NAP, whose members are reported to be involved in stress responses (Christianson, et al. 2010; Tran, et al. 2004). A total of 14 PgNAC members belong to this group, which suggests their functional similarity with reported ATAF and NAP members of other specie (Arabidopsis). Phylogenetic analysis also revealed the presence of orthologous pairs between pearl millet and rice [PgNAC112 and OsNAC11 (LOC_Os01g66120); PgNAC98 and OsNAC28 (LOC_Os03g04070)], indicating the existence of an ancestral set of genes/proteins among rice and pearl millet before divergence. Transgenic rice plants overexpressing *OsNAC11* (LOC_Os01g66120) were tolerant to drought stress with higher grain yield, suggesting a similar type of function of *PgNAC112* (Chung, et al. 2018). PgNAC98 has high similarity with OsNAC28 (LOC_Os03g04070) and transgenic rice overexpressing *OsNAC28* showed enhanced ABA-mediated abiotic stress tolerance (Hong, et al. 2016). The collinearity analysis showed that the *PgNAC*s were involved in both paralogous and orthologous duplication. Some were associated with more than one segmental duplication like *PgNAC63, PgNAC106, PgNAC111, PgNAC112* and *PgNAC114*. The involvement of *NAC* genes in more than one duplication event has also been observed in bananas (Sun, et al. 2020). These results suggest the prominent role of segmental duplication in *PgNAC* gene evolution. Also, *PgNAC* genes showed the highest synteny with Si*NAC*s followed by *AtNAC*s and *OsNAC*s. Additionally, 6 *PgNAC*s showed collinearity with NAC members of *A. thaliana, O. sativa* and *S. italica* that suggested their existence prior to the divergence of dicot and monocot plants. Similarly, 12 *PgNAC*s showed collinearity with NAC members of *O. sativa* and *S. italica* not with *A. thaliana*, so these pairs might be evolved after divergence of dicot and monocot plants. The Ks/Ka values for all the identified pairs were less than 1, indicating a purifying selection of *PgNAC* genes in the course of evolution. Studies on NAC gene family in different plants also showed purifying selection as a driving force in NAC gene evolution (Zong, et al. 2020; Gong, et al. 2019). As purifying selection limits the divergence of duplicated genes, the duplicated *PgNAC* genes may not have a divergent function during subsequent evolution (Sun, et al. 2020). The presence of wide range of *cis* elements (specific to growth, development, abiotic stress, biotic stress, and hormonal signaling,) in *PgNAC* genes indicates their diverse functionality in the context to different stress and developmental factors.

Earlier studies have confirmed the importance of *NAC* genes in various developmental stages and tissue-specific expression in planta (Breeze, et al. 2011; Singh, et al. 2021; Yang, et al. 2011). Most *PgNAC* genes were expressed in root followed by leaf as observed in other species (Fang, et al. 2008; Min, et al. 2019). Moreover, overexpression of tissue-specific *NAC* gene (*AtNAC2*) facilitated lateral root development in Arabidopsis (He, et al. 2005). Similarly, overexpression of *OsNAC5* resulted in enlarged root diameter, leading to improved drought tolerance in rice (Jeong, et al. 2013). Based on these observations, we may assume that *PgNAC20, PgNAC21, PgNAC81, PgNAC103, PgNAC106, PgNAC114* and *PgNAC119* having higher expression in root, could have the potential for enhanced root development in pearl millet, leading to drought tolerance in planta.

Upon drought stress most of the selected *PgNAC* genes were upregulated and few of them like *PgNAC22* and *PgNAC106* were downregulated. Earlier studies suggest that *NAC* genes function as both positive and negative regulators of drought stress. For instance, overexpression of *OsNAC6* and *OsNAC10* increased drought tolerance ability in transgenic rice (Jeong, et al. 2010; Nakashima, et al. 2007). In contrast, *ANAC016* and *ANAC053* were reported to be negative regulators of drought stress (Sakuraba, et al. 2015). A similar expression pattern was observed under salinity and heat stresses. Further, these observations are in accordance with the previous reports on functions of NAC family in other species that have suggested their role as both positive and negative regulators of salt and heat stress responses (Diao, et al. 2018; He, et al. 2005; Park, et al. 2011). In a study, Dudhate et al. showed *PgNAC113* (named *PgNAC119* in present study) acted as positive regulator in salinity stress in pearl millet (Dudhate, et al. 2021); a similar result was observed in our study. Notably, *PgNAC20* and *PgNAC114* were upregulated under salt, drought, and heat stress, which suggested their important role in pearl millet abiotic stress responses. Moreover, many studies have established the importance of NAC TFs under different stresses (Shao, et al. 2015); as Ta*NAC67* overexpression provided multiple stress tolerance in Arabidopsis (Mao, et al. 2014). In another study, significant induction of *SlNAC11* was seen under heat, dehydration and cold stress (Wang, et al. 2017).

The selected *PgNAC*s in this study were found to have differential expression patterns upon three phytohormonal (ABA, MeJA and SA) treatments. In Arabidopsis, overexpression of Ta*NAC29* showed ABA-mediated improved salt and drought stress tolerance (Huang, et al. 2015). In pearl millet, *PgNAC81*, and *PgNAC114* showed upregulation patterns under both drought and ABA stresses; accordingly, we speculate that these genes might be associated with ABA signaling pathways for drought tolerance. Arabidopsis overexpressing of *ZmNAC55* enhanced drought resistance through ABA-dependent pathway (Mao, et al. 2016). *PgNAC114* was upregulated under drought stress, ABA and MeJA treatment which suggests a positive role of *PgNAC114* in drought stress response via ABA and MeJA signaling pathways in pearl millet. A recent study also reported positive role of *VaNAC17* in conferring drought tolerance to transgenic Arabidopsis by modulating endogenous JA biosynthesis (Su, et al. 2020). Taken together, expression analysis of *PgNAC* genes in response to phytohormones clearly indicated their involvement in different signaling responses. It was also seen that few *PgNAC*s were upregulated/downregulated under more than one phytohormone treatment. In this study, *PgNAC22* was upregulated upon ABA and SA treatment. *PgNAC20* and *PgNAC119* were downregulated under MeJA and SA application. *PgNAC22*, which shares a close phylogenetic relationship with Si*NAC128*, was found to be upregulated under all phytohormone treatments except MeJA. Also, in this context *PgNAC114* was upregulated under all the applied stresses (phytohormones) except SA. This finding was supported by an earlier study where Si*NAC128* was upregulated in foxtail millet responding to ABA and SA (Puranik, et al. 2013). From this, we hypothesize that *PgNAC*s could be involved in protecting pearl millet via activating hormone (ABA, MeJA and SA) signaling pathways from multiple environmental stresses.

Many NAC TFs have been demonstrated to play an important role in imparting drought stress. Our initial expression analysis revealed that *PgNAC103* expression was influenced by drought, heat, salinity, and phytohormones, with a five-fold upregulation observed at an early time point during drought stress. Therefore, *PgNAC103* was selected as a candidate gene for further functional analysis under drought stress conditions. Sequence analysis showed that it had more than 50 % similarity with its Arabidopsis counterpart NAC protein *ANAC103* and had a larger C-terminal protein stretch, which might contribute to its functional diversity in pearl millet. PgNAC103 was found to be a nuclear protein; it had highly conserved N-terminal region (NAM domain) and variable C-terminal regions, characteristic features of NAC TF (He, et al. 2019). The transactivation activity of transcription factors represents a fundamental mechanism by which the expression of genes under their control can be modulated, with far-reaching implications for plant development, physiology, and adaptation PgNAC103, with its

impressively high transcriptional activity, has the potential to modulate the downstream genes for various biological functions.

Under stress, plants undergo a multitude of physiological and morphological changes to protect themselves. Recent studies have shed light on the physiological and biochemical mechanisms by which drought influences fundamental biological processes like seed germination and root length. For instance, a water deficit can result in a decline in photosynthetic rate and carbon assimilation, leading to reduced energy and resources available for seed germination and root elongation leading to their inhibition (Dalal and Tripathy 2018). In our study, we observed that transgenic Arabidopsis plants overexpressing *PgNAC103* exhibited enhanced seed germination and root growth, potentially serving as a stress response. Similar findings were found in earlier studies also (He et al., 2019a,b).

Plants detect and react to signs of water deficiency through their roots then the stress signal is transmitted to aerial portions, such as the leaves, where it causes them to shut their stomata and synthesize ABA to conserve water (Soma, et al. 2021). Then, ABA induces the other stress genes to mitigate the drought effect. Before the accumulation of ABA upon drought, some genes activate all the response mechanisms against dehydration. They are associated with the ABA-independent pathway and induce early stress responses which is crucial for plants to avoid early oxidative damage. The expression pattern of *PgNAC103* under drought and ABA treatment was corelated with this early stress response mechanism. Our study found that the transcript level of *PgNAC103* was highly increased in the early stages of drought, but in later stages where ABA is synthesized, its expression level came to normal. Also, upon ABA treatment, the expression level of *PgNAC103* was reduced. From these findings we hypothesize that *PgNAC103* is an early responsive gene and might impart drought tolerance by ABA-independent pathway. To further confirm this, we exposed the *PgNAC103* overexpressing Arabidopsis to ABA treatment and found that the transgenic lines were less sensitive to ABA than the wild type. The NAC family of transcription factors has been shown to be an early responder to drought stress, quickly altering the expression of target genes to initiate adaptive responses. Previous studies suggested early responsive genes are crucial for imparting stress responses (Harb, et al. 2010; He, et al. 2020).

It is well-studied that in plants overexpression of reactive oxygen species (ROS) might cause damaged cellular proteins, lipids, and DNA (Carillo, et al. 2011; Nxele, et al. 2017). To overcome these oxidative damages, plants scavenge the overproduction of ROS and keep their level low. In our study, the level of common ROS molecules like superoxide (O^{2-}) and hydrogen peroxide (H_2O_2) was comparatively less in the transgenic lines under drought stress. A similar response was observed upon overexpression of *SlNAC6* under drought stress (Jian, et al. 2021). Upon stress, ROS accumulation depends on their generation and subsequent scavenging. In plants, several enzymes are associated with ROS scavenging system; specifically, three antioxidant enzymes, namely SOD, CAT and POD play a crucial role (Miller, et al. 2010). The lower level of ROS in transgenic plants indicated an enhanced scavenging system, which was positively correlated with higher enzymatic activity of SOD, CAT and POD in the transgenic lines. These findings showed that overexpression lines have a stronger scavenging system to remove the excess ROS produced during stress than wild-type ones. Under stress, reactive oxygen species cause cell injury, leading to increase MDA levels, a marker of lipid peroxidation (Chanwala, et al. 2024). In this study, we found that the transgenic lines had less MDA content under drought stress than wild-type plants indicating transgenic lines were less prone to injury under stress.

We observed that in the transgenic Arabidopsis lines overexpressing *PgNAC103*, the expression level of three ROS scavenger genes like, *AtSOD, AtCAT* and *AtPOD* were much higher relative to wild type under drought, which was in accordance with their higher enzymatic activities. Plants regulate the expression of several stress-responsive genes to adapt the adverse environmental conditions; these genes form a crucial molecular basis for the response and adaptation of plants to stresses (Umezawa, et al. 2006). To understand the molecular basis of *PgNAC103*-mediated stress responses, we analyzed the transcript level of stress-responsive marker genes like *AtCOR15A, AtKIN1, AtRD22,* etc. These genes were highly upregulated both at the early seedling stage and later developmental stage; these observations were supported by the study of He et al., where it was reported that overexpression of *ThNAC7* showed upregulation of similar genes (He, et al. 2019). We observed similar findings in transformed pearl millet calli and seedlings expressing the *PgNAC103* under drought stress. The stress-responsive genes *ERD, OST2*, and CAT were highly upregulated in *PgNAC103* transgenic pearl millet calli and seedlings under drought stress compared to vector control. This finding suggests that *PgNAC103* plays a significant role in enhancing the plant's ability to survive under drought conditions.

The regulation of gene function is a complex and intricate process crucial for the proper functioning of living organisms. The promoter region of a gene is important for the regulation, and it initiates the transcription process. The interplay between promoters and transcription factors is particularly important in plant responses to biotic and abiotic stresses. This intrigued us to investigate the promoter region of *PgNAC103* gene. Our analysis of the upstream region of PgNAC103 revealed the presence of *cis*-elements associated with drought induction (MYB and MYB-binding sites), cold-responsive (LTR) and other core promoter *cis*-elements (CAAT, TATA etc.) were present. Notably, multiple copies of MYB binding and MYB-related *cis*-elements were present. This analysis indicated that the PgNAC103 promoter might regulate the gene expression in drought and temperature stress responses. We found that the upstream region of *PgNAC103* showed transcriptional activity in Arabidopsis, tobacco, and pearl millet. Upon deletion of the 5′ region (−1000 to −501), the promoter activity drastically decreased, as evidenced truncated fragment (PgNAC103-F2) showed much lower activity than the full-length promoter (PgNAC103-F1); suggesting the fragment between coordinates (−1000 to −501) is highly essential for the PgNAC103 promoter activity. Also, upon drought induction, we observed that the full-length PgNAC103 promoter was highly induced. From these findings, we assume that the PgNAC103 promoter could have direct involvement for imparting *PgNAC103*-mediated stress tolerance in pearl millet.

Protein-protein interaction prediction suggested potential interactions of PgNAC103 with stress-responsive transcription factors such as WRKY, MYB, bZIP, and DREB (**Supplementary Fig. 5**). This raises the possibility of PgNAC103 forming complexes or interacting independently with the promoter regions of stress-related genes like CAT, SOD, and ERD. Promoter analysis further suggested potential regulation of PgNAC103 expression by MYB genes, hinting at a transcriptional network mediating drought stress response. Based on our findings, *PgNAC103* appears to enhance drought tolerance in transgenic plants by modulating stress gene expression and antioxidant enzyme activity. However, the precise mechanism underlying PgNAC103′s role in drought stress response remains unclear and warrants further investigation.

5. Conclusion

In this independent study, we identified 155 PgNACs in the pearl millet genome. Our phylogenetic and synteny analysis provided insights into the evolutionary progression and the functional diversity of PgNACs. Furthermore, our expression analysis of selected *PgNACs* under various stress treatments and in different tissues revealed their potential involvement in stress resistance and development of pearl millet. The majority of the *PgNACs* showed positive expression under hormonal treatments, indicating their significant role in phytohormonal signaling pathways for stress responses. We also functionally characterized *PgNAC103*, a NAC transcription factor that demonstrated tolerance against drought stress in the transgenic Arabidopsis through an ABA-independent pathway. The increased seed germination rate and reduced superoxide radicals' accumulation in transgenic lines confirmed

PgNAC103 as a positive regulator of drought responses. These findings on pearl millet NAC are a significant step towards understanding the functional diversity and downstream signal pathways of candidate *PgNAC*s under various abiotic stresses and propose *PgNAC103* as a potential candidate to develop climate-resilient crop plants to support sustainable agriculture.

CRediT authorship contribution statement

Deepak Kumar Jha: Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Jeky Chanwala:** Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **I. Sriram Sandeep:** Writing – original draft, Validation, Software, Methodology, Formal analysis, Data curation. **Preeti Barla:** Writing – review & editing, Methodology, Investigation, Data curation. **Nrisingha Dey:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The authors declare no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2024.100728.

Data availability

Data will be made available on request.

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