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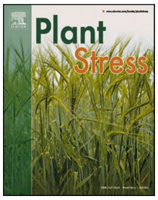

Pearl millet WRKY transcription factor PgWRKY52 positively regulates salt stress tolerance through ABA-MeJA mediated transcriptional regulation

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ABSTRACT

Environmental stresses adversely affect plant growth and development by disturbing physiological and metabolic equilibrium. Plants counteract these stresses through intricate genetic and biochemical pathways, which are largely mediated by signalling networks that involve key transcription factors (TFs). Among these, WRKY TFs are crucial in modulating plant responses to various stresses. In previous studies, WRKY TFs have been identified in millets. However, their functional characterization in millets remains vastly unexplored. Therefore, we have isolated and characterized *PgWRKY52*, a Group IIc WRKY TFs from pearl millet, along with its upstream promoter region to understand its functional regulatory role. Ectopic expression of *PgWRKY52* in transgenic Arabidopsis improved seed germination under salt stress and phytohormonal treatments of abscisic acid (ABA) and methyl jasmonate (MeJA). Improved stress tolerance was linked to reduced reactive oxygen species (ROS) accumulation and upregulation of stress-responsive genes, indicating an enhanced defense system. Promoter analysis unveiled that the *PgWRKY52* promoter was constitutively active across vegetative and reproductive tissues, with strong stress-inducible activity under salt, heat, and ABA treatments. Cis-regulatory element (CRE) analysis identified key stress-responsive elements, including ABRE, MYB, W-box and MYC, which were validated through mutational studies as essential for promoter activity. Additionally, *PgWRKY52* exhibited W-box-dependent DNA-binding capability, a characteristic feature of WRKY TFs. These findings emphasize the important function of the *PgWRKY52* promoter in driving stress-responsive transcription. Altogether, these findings establish *PgWRKY52* as a stress-responsive TF that enhances salt stress tolerance through crosstalk of ABA-MeJA signalling pathways and the regulatory role of its promoter, presenting a promising tool for developing climate-resilient crops.

Introduction

Unpredictable climate variables and associated environmental problems, such as drought, flood, heat stress and soil salination, present significant challenges to agricultural production (Rezaei et al., 2023, Mittler, 2006). These pressures negatively impact plant growth and severely decrease crop yields, posing a risk to global food security. As climate change continues to intensify, the frequency and severity of these stresses are predicted to increase, further exacerbating the pressure on agricultural systems. Despite these challenges, plants have evolved intricate genetic and biochemical strategies to effectively counter these stresses. (Nguyen et al., 2016). Their complex signalling networks efficiently sense and adapt to unfavourable environmental conditions. (Gill and Tuteja, 2010, Singh et al., 2002).

Central to the regulation of these adaptive mechanisms are transcription factors (TFs), which act as molecular units to control gene expression (Porto et al., 2014). These TFs are crucial components in the regulatory network and function as molecular switches by binding to

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their cognate site (cis-elements) present in the promoter region (Liu et al., 2014). Through "cis-trans" interactions, TFs regulate target genes that control key cellular processes involved in stress response. Among these, WRKY TFs which are known for their pivotal role in numerous biological processes linked to plant development and responses to stress. (Jiang et al., 2017, Banerjee and Roychoudhury, 2015). WRKY TFs are defined by a conserved "WRKYGQK" motif and a zinc finger structure. WRKY TFs binds to the W-box [(T)TGAC(C/T)] and transcriptionally modulate the downstream stress-responsive genes (Banerjee and Roychoudhury, 2015, Chen et al., 2019, Eulgem and Somssich, 2007).

WRKY TFs are broadly known for their participation in mediating stress responses, with various studies demonstrating their functional involvement through genetic and molecular approaches (Eulgem and Somssich, 2007). For example, *ZmWRKY65*, improves stress tolerance in transgenic plants (Y Yu et al., 2023), while *SlWRKY36* and *SlWRKY51* positively regulate salt stress response by controlling ion equilibrium and proline biosynthesis (Mehboob et al., 2024). WRKY TFs often work in coordination with other genes, forming regulatory modules to regulate stress responses. In rice, the OsWRKY72-OsAAT30/OsGSTU26 complex facilitates ROS scavenging, contributing to salinity stress tolerance (Liu et al., 2024). *GhWRKY17* regulate abiotic stress responses through ABA signalling and ROS modulation in transgenic *N. benthamiana* (Yan et al., 2014). Additionally, *WRKY39* coordinates the SA and JAactivated pathways during thermal stress (Li et al., 2010), while *WRKY75* is essential for regulating defense responses in Arabidopsis through the JA signalling pathway (Chen et al., 2021). The overexpression of stress-related TFs has been demonstrated to enhance crop tolerance by regulating key signalling pathways. However, research exploring the functional role of WRKY TFs in millets remains scarce. This gap emphasizes the importance of investigating regulatory mechanisms in pearl millet, particularly how WRKY TFs and their regulatory regions mediate stress responses.

Pearl millet (*Pennisetum glaucum*) is an extensively cultivated C4 crop in subtropical zones of the world with challenging environmental conditions. (Satyavathi et al., 2021). It is mostly grown as a primary food source and is furthermore utilized for fodder and fuel in semi-arid regions (Rhoné et al., 2020). The crop is recognized for its resistance, thriving in adverse conditions such as elevated temperatures, dryness, and low soil fertility (Satyavathi et al., 2021). The C4 photosynthetic pathway enhances its carbon fixation efficiency and minimizes water loss, enabling it to thrive efficiently in hot and arid environments (Shrestha et al., 2023). It also contain essential nutrients, including carbohydrates, proteins, lipids, vitamins, and dietary fibre and micronutrients, rendering it a promising crop for enhancing nutritional security (Satyavathi et al., 2021, J Chanwala et al., 2024). Due to these attributes, pearl millet is gaining global attention as a potential solution for food security and climate adaptation. However, achieving sustainable pearl millet production remains challenging due to inadequate advancements in genetic tools to unfold its genomic architecture to improve crop productivity.

The recent release of pearl millet genome datasets by Yan et al., 2023 and Varshney et al., 2017, (Yan et al., 2023, Varshney et al., 2017) provides invaluable resources on genome composition and architecture for addressing this research gap. These resources facilitate for understanding molecular mechanism of pearl millet, specifically how regulatory elements and associated TFs are involved in transcriptional regulation, which can be useful to enhance crop yield and resilience. Also, our earlier studies have identified 97 PgWRKYs and shown the participation of several *PgWRKY*s in abiotic stress responses of pearl millet (Chanwala et al., 2020). Additionally, their genomic structure, enriched with stress responsiveness motifs, indicated their potential role in regulating stress-related pathways. Despite these attributes the specific functional roles of individual *PgWRKYs* in stress responses remain largely unexplored. Given this gap in knowledge, PgWRKY52 was selected for functional characterization due to its significant differential expression under drought, salinity, and heat stress, as revealed by transcriptome analysis. Also, its promoter region harbours multiple stress-related cis-regulatory elements, indicating a key role in stress regulation. Previous studies have also highlighted the role of *PgWRKY74* and *PgWRKY44* in abiotic stress responses (J Chanwala et al., 2024, Qazi et al., 2024). These findings suggest that different PgWRKYs contribute to distinct stress pathways, emphasizing the importance of functional validation.

Building on this, the current study focuses on isolating and characterizing *PgWRKY52*, along with its upstream promoter sequence, to explore its regulatory role in stress responses. This research enhances our comprehension of the regulatory components involved in stress responses in pearl millet, with potential applications in improving crop performance in marginal regions and developing stress-resilient crops through targeted genetic modifications.

Materials and Methods

Plant materials and growth conditions

Seeds of *Pennisetum glaucum* (PRLT 2/89–33) and *Oryza sativa* cv. (IR64) were acquired from ICRISAT, Hyderabad and ICAR-NRRI, Cuttack, respectively. The *Nicotiana tabacum* Samsun NN and *Nicotiana benthamiana* seeds were available at Institute of Life Sciences, Bhubaneswar. The seeds were germinated in an equal mixture of black and red soil and cultivated in a controlled greenhouse at 28 °C (± 2) with a 16-hour light period and an 8-hour dark period.

Gene isolation and in-silico prediction of functional features

The *PgWRKY52* was isolated from pearl millet cDNA using Ex Taq polymerase (Takara, Japan) and inserted into the pGEM-T Easy vector (Promega, USA). Positive constructs were confirmed through Sanger sequencing. Gene structure and domain integrity were analyzed using the GSDS server and SMART tools, respectively (Bailey et al., 2009, Hu et al., 2014). Multiple sequence alignment was conducted through PRALINE. (Bawono and Heringa, 2014). A phylogenetic tree with the neighbour-joining method was generated using MEGA software (Kumar et al., 2016).

Relative expression profiling

The expression of *PgWRKY52* was checked under salt, drought and heat conditions and phytohormonal treatments as described in Chanwala et al., 2024 (J Chanwala et al., 2024). Briefly, salt stress was induced by subjecting four-week-old pearl millet seedlings to a 250 mM NaCl solution for 24 hours, while drought stress was applied through 9 days of water withholding, followed by a three-day recovery period. For phytohormones treatment, seedlings were exposed to 100 μM of ABA, SA and MeJA for 24 hours (Jha et al., 2021). Datasets were normalized using GAPDH and Actin.

Subcellular localization and transactivation assay

The CDS (without stop codon) of *PgWRKY52* was inserted upstream of GFP to create the 35S: *PgWRKY52*-GFP-pCAMBIA2300 construct. This construct, along with vector control (35S: GFP-pCAMBIA2300), was individually bombarded into onion cells via particle bombardment, as described in Chanwala et al., 2024 (J Chanwala et al., 2024). DAPI (358/461 nm) and GFP (488/509 nm) fluorescence were examined using a confocal microscope. For transactivation assay, full-length (1–648 bp), N-terminal (1–324 bp), and C-terminal (325–648 bp) regions were subcloned into pGBKT7. These constructs were then transformed into the Y2H Gold yeast strain and streaked on SD/-Trp, SD/-Trp/-His, and SD/-Trp/-His + X-α-gal media using Clontech (USA) protocol. Colony PCR was employed to confirm the insert presence in positive colonies, followed by plasmid extraction and verification via sequencing. An empty vector (pGBKT7) was used as a negative control to avoid false positives. The transactivation ability was assessed by monitoring growth and colouration of transformed yeast cells on various selection media.

Y1H assay

To investigate the DNA binding ability of *PgWRKY52*, the CDS was inserted into the pGADT7 vector. Additionally, oligonucleotides containing three W-box and mutated versions were independently cloned into pABAi plasmid to generate the pABAi-Wbox and pABAi-mWbox constructs. These constructs were individually co-transformed with pGADT7-*PgWRKY52* in yeast cells using Clontech (USA) Matchmaker Gold Yeast One-Hybrid System. Transformed colonies were streaked on synthetic dropout (SD) medium lacking leucine (SDO), double dropout (DDO) medium lacking uracil and leucine, and DDO supplemented with Aureobasidin A.

Development of transgenic Arabidopsis

The CDS of *PgWRKY52* was inserted into modified pCAMBIA2300. The agrobacterium culture harbouring CaMV35S: *PgWRKY52*-pCAMBIA2300 was used for floral dipping of wild-type (WT) Arabidopsis (Col-0) as described earlier (Zhang et al., 2006). T_0 seeds were screened on ½ MS medium containing 50 μg/mL Kanamycin. The healthier green seedlings were then transferred to pots and reconfirmed by analyzing gene integrity analysis.

Stress response evaluation

For seed germination rate, transgenic and WT seeds were germinated on ½ strength MS medium supplemented with 200 mM NaCl, 20μM MeJa and 1 μM ABA individually. Germination rates were recorded over 10 days and photographed. For phenotypic analysis, transgenic and WT seedlings were grown for four weeks and then treated with 250 mM NaCl for two weeks to induce salt stress, after which phenotypic images were captured.

Measurement of antioxidant and peroxidase activities

The levels of antioxidant enzymes, such as MDA, SOD, POD, and catalase, were measured as described previously (Schmedes and Hølmer, 1989, Aebi, 1984, Zhang, 1992, Khan et al., 2021, Jambunathan, 2010, Chen and Zhang, 2016). Furthermore, DAB and NBT staining were employed to quantify H_2O_2 and O_2^- accumulation (Jambunathan, 2010, Kumar et al., 2014). Additionally, expression analysis was carried out to assess transcript levels of genes related to stress responses.

Genetic transformation of pearl millet

The agrobacterium cells carrying CaMV35S: *PgWRKY52*-pCAMBIA2300 were used to transform two-week-old wild-type pearl millet calli, as mentioned earlier (J Chanwala et al., 2024, J Chanwala et al., 2024). For salt stress treatment, transgenic calli were placed on a CIM medium supplemented with 250 mM NaCl, while drought stress was simulated using 10 % PEG. qRT-PCR was employed to measure the transcript levels of stress-related genes and *PgWRKY52*.

Promoter isolation and cis-distribution analysis

A 1000 bp upstream sequence (P1) was amplified from pearl millet and cloned into the pGEM-T Easy vector. Further, two deletion fragments, P2 (676 bp) and P3 (500 bp) were derived from P1 fragment. These three fragments subcloned upstream of the GUS reporter gene into modified pCAMBIA2300 plasmid, generating P1:GUS, P2:GUS and P3:GUS clones. The upstream promoter sequence of *PgWRKY52* was examined for potential cis-regulatory elements (CREs) through Plant-Care database (Lescot et al., 2002).

Transient assay of promoter fragments

For transient assay of the promoter fragments P1, P2, and P3 in tobacco, rice and pearl millet seedlings, agroinfiltration-mediated expression analysis was done. The promoter constructs cloned in modified pCAMBIA2300-GUS were transformed into *Agrobacterium tumefaciens* strain GV3101 using freeze-thaw method (Chen et al., 1994). Briefly, in regards to tobacco, the positive agrobacterium colony having respective promoter construct was grown overnight and resuspended in agroinfiltration buffer comprising 50 mM MES, 20 mM Na_3PO_4, 27 mM D-glucose and 0.1 mM acetosyringone. A needleless syringe was used to infiltrate the agrobacterium suspension into the abaxial side of fresh young tobacco leaves. (Yang et al., 2000, Sherpa et al., 2023).

For the infiltration of rice and pearl millet seedlings, positive Agrobacterium colonies were resuspended in liquid infiltration media (LIM), which contains 4g/L MS basal salt, 200 mM sucrose, 200 mM glucose, 42 mM $MgCl_2$, 40 mM KCl, 150 μM acetosyringone, and 0.01% Silwet. The seedlings were submerged in this resuspension and then infiltrated under a vacuum at a pressure of 0.933 bar for 10 minutes. Afterwards, they were dried on sterile blotting paper to remove excess Agrobacterium. Finally, the seedlings were kept in a solution of 150 μM acetosyringone mixed with sterile water for 48 hours in complete darkness. (Sherpa et al., 2023, Sethi et al., 2022).

For *GFP* analysis, P1:GFP was transformed in *A. tumefaciens* GV3101 and, following the above-described protocol, agroinfiltrated into young *Nicotiana benthamiana* leaves. The leaves were kept at 25 °C in complete darkness. After five days, the agroinfiltrated leaves were detached, and images were taken using Gel Doc XR + System (Bio-Rad). ImageJ software was used to measure the GFP intensity. (Schneider et al., 2012; Kumari et al., 2024).

Development of transgenic tobacco plants

All three promoter constructs P1:GUS, P2:GUS, and P3:GUS were used to raise stable transgenic tobacco plants employing leaf disk method (Dey and Maiti, 1999). T_O seeds were screened on ½ MS medium containing 300 mg/L Kanamycin. Segregation analysis was performed to calculate the ratio of Kan^R (resistant) and Kan^S (sensitive) seedlings for chi-square analysis.

Tissue-specific promoter activity

The expression of GUS in different cross-sections of tissue samples and plant parts of transgenic tobacco plants was checked by dipping it in X-gluc solution (5-Bromo-4-chloro-3-indolyl-β-D-glucuronide) consisting of 0.3% X-gluc, 50 mM $NaPO_4$, 10 mM EDTA and 0.01% Tween-20 for 12 hr at 37 °C. After chlorophyll removal and blue colouration was captured using an Olympus SZ Stereo microscope (Acharya et al., 2014).

Fluorometric analysis

The GUS activity in both transgenic tobacco plants and those that were agroinfiltrated was quantitatively assessed using a fluorometric GUS assay (Jefferson et al., 1987). Total protein from agroinfiltrated plants was extracted 48 hours post infiltration or from transgenic plants by crushing the plant in acidified sand mixed with GUS extraction buffer and 0.1% β-Mercaptoethanol. After centrifugation, the supernatant was incubated with MUG (4-Methylumbelliferyl beta-D-glucuronide), which is a fluorogenic substrate for β-glucuronidase (GUS) at 37 °C. Interactions between total protein and MUG were monitored at three different time points: 0 min, 10 min, and 20 min. At each interval, the mixture was transferred to a stopping solution containing 0.2 M Na_2CO_3, and fluorescence was measured using the GloMax®20/20 Fluorescent

Module (excitation at 365–395 nm and emission at 440–470 nm). GUS activity was quantified as nmol MU/min/mg protein, with three replicates for each sample. The concentration of total protein in the extract was determined through the Bradford method (Bradford, 1976).

Stress treatment

To check the promoter's stress inducibility, transgenic tobacco line of P1 construct was germinated on half strength of MS medium with 300 mg/L kanamycin sulfate. After 21 days, the germinated seedlings were exposed to abiotic and phytohormonal stress conditions. To simulate salt stress, seedlings were subjected to 250 mM NaCl for 12 hrs and for heat treatment, the seedlings were kept at 45 °C for 2 hrs. Simultaneously, for phytohormonal stress treatment, the seedlings were immersed in 150 µM Salicyclic acid (SA), 150 µM MeJa and 100 µM ABA for 12 hrs following the previously described protocol (Deb and Dey, 2019). Total protein was isolated after stress treatment, and fluorometric GUS analysis was performed, as mentioned earlier.

Mutational analysis

To check the effect of different cis-elements on promoter strength, site-directed mutation of key cis-elements was done using PCR-based site-directed mutagenesis as described by Reikofski and Tao 1992 (Reikofski and Tao, 1992). The P1 promoter has two ABRE, two MYB, two W-Box and four MYC cis-element sites widely distributed throughout the promoter length as found through PlantCare database. The first ABRE (starting from 5′) cis-element mutation has been named 'M1', the second mutation named 'M2', while complete deprivation of ABRE cis-elements from P1 promoter has been named 'M3'. Similarly, two MYB site mutations have been labelled 'M4' and 'M5' while 'M6' for mutation of both MYB sites in P1 fragment. Single W-box mutations were labelled as 'M7' and 'M8', and both W-box site mutations as 'M9'. There are four MYC sites, and individual mutations were named 'M10', 'M11', 'M12', and 'M13', while the complete MYC mutation is named 'M14'. P1 promoter with complete mutation of ABRE, MYB, W-Box and MYC in a single sequence was synthesised in pUC57 and named 'M15'. All the mutation fragments were cloned in pCAMBIA-2300, expressing the GUS reporter gene. Next, to analyse the effect of cis-element mutations, the positive GV3101 agrobacterium colonies of respective constructs were grown overnight and then used for agroinfiltration in *N. benthamiana* young leaves. After 48 hrs, fluorometric GUS analysis was done. Similarly, the above-mutated clones were also transiently expressed in pearl millet following the previously described protocol.

Transcription factor binding sites prediction

The upstream sequence (1 Kb) of *PgWRKY52* was analyzed for putative TF binding sites using the PlantRegMap database (Tian et al., 2019).

Statistical analysis

Experiments were performed in replicates. Their mean values, along with their standard deviations, were calculated. Statistical analysis was performed using Student's *t*-test to evaluate the significance of the differences between mean values ($^*p \leq 0.05$, $^{**}p \leq 0.01$).

Results

Isolation and characterization of PgWRKY52: structure, homology, and stress response

A cDNA fragment corresponding to *PgWRKY52* was successfully PCR amplified from pearl millet cDNA library (*Pgl_GLEAN_10026906*). The coding sequence of *PgWRKY52* is 648 base pairs long, encoding a protein of 216 amino acids. Gene structure analysis depicted two exons and one intron in *PgWRKY52* (Fig. 1A). The PgWRKY52 amino acid sequence possesses a signature "WRKYGQK" motif and a C_2H_2 zinc-finger-like structure, as shown in Fig. 1B. Phylogenetic analysis revealed close identity of PgWRKY52 with Group IIc members from other plants, including OsWRKY72 (87.6%), AtWRKY45 (79.8%), and AtWRKY75 (83.5%) (Fig. 1C).

The functional role of *PgWRKY52* in abiotic stress responses was validated using RT-qPCR, which demonstrated its upregulation during salt and drought stress (Figure S1A-B), consistent with RNA-seq data (Figure S1C). Phytohormone treatments revealed that *PgWRKY52* expression was significantly upregulated at 3 hours following 100 µM ABA and MeJA treatments. However, SA treatment led to a reduction in expression levels at the 24-hour time point (Figure S1D), highlighting its dynamic regulation in stress and hormonal signalling pathways.

Subcellular localization and transactivation activity analysis of PgWRKY52

Confocal microscopy showed that the onion cells transformed with the control construct exhibited GFP fluorescence in both the nucleus and cytoplasm. In contrast, GFP signals were exclusively detected in nucleus for cells transformed with 35S: *PgWRKY52*-GFP fusion construct. These findings confirmed that PgWRKY52 is a nuclear-localized protein (Fig. 2A).

Further, the transactivation potential of PgWRKY52 was evaluated by transforming yeast cells with the full-length *PgWRKY52* gene, along with its N-terminal and C-terminal regions, as mentioned in Material and Methods. As depicted in Fig. 2C, transformed yeast cells harbouring full-length PgWRKY52 and C-terminal region grew well and showed blue colouration on SD/-His/-Trp+X-α-gal plates. These findings confirmed that PgWRKY52 possesses self-transactivation activity.

PgWRKY52 binds to W-box

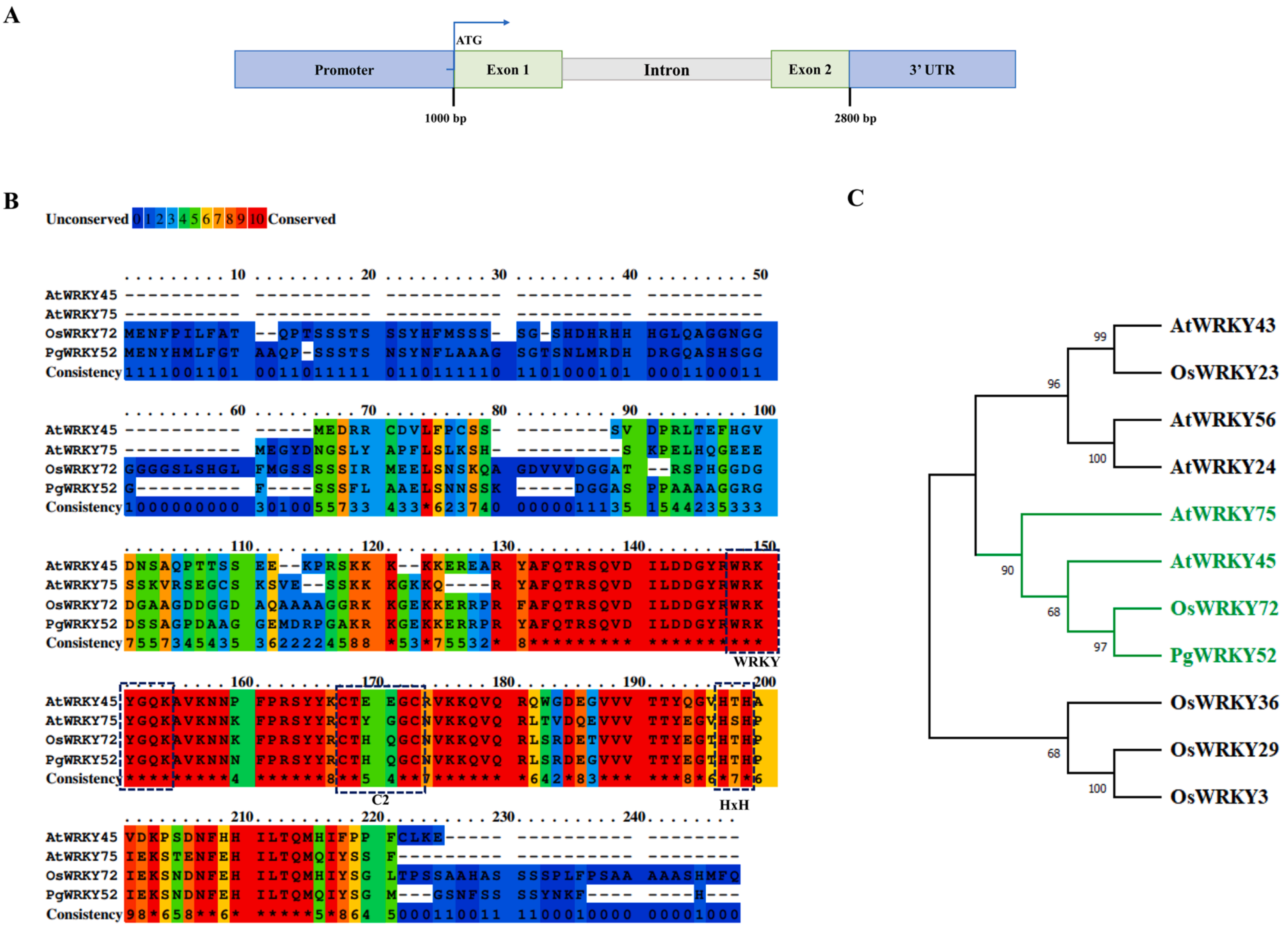
Yeast one-hybrid assay result demonstrated that cells carrying both pGADT7- PgWRKY52 and pABAi-W-box constructs grew successfully on SD/-Ura/-Leu (DDO) media containing 400 ng/ml Aureobasidin A. In contrast, yeast cells with mutated W-box construct failed to grow. These findings confirm that PgWRKY52 binds to the W-box and activates the reporter gene in yeast (Figure S2B).

PgWRKY52 mediates stress response in transgenic Arabidopsis

To explore the functional role of *PgWRKY52*, transgenic Arabidopsis lines overexpressing *PgWRKY52* were generated. Two homozygous T_3 lines, L2 and L11, were chosen for further analysis based on their segregation patterns and expression levels. The seed germination efficiency of these T_3 lines and wild type (WT) was evaluated under various stress treatments. Under normal conditions, the germination percentage of the T_3 lines was comparable to WT, showing no significant differences. However, under stress conditions such as ½ MS medium containing 200 mM NaCl, 1.0 µM ABA, or 20.0 µM MeJA, the germination percentage of transgenic seeds (L2, L11) was 15–30% greater than WT (Fig. 3A-H). Further, a pot experiment was conducted to explore the influence of *PgWRKY52* on salt stress tolerance in transgenic lines. After four weeks of growth, the plants were subjected to salt stress. We observed that in the absence of stress, both the transgenic and WT plants showed similar growth. However, after exposure to salt stress, the transgenic lines were healthy and green than the WT plants (Fig. 3I). These findings imply that overexpression of *PgWRKY52* improves salt stress tolerance in Arabidopsis plants.

PgWRKY52 modulated the efficiency of ROS scavenging during salt stress

Abiotic stress often leads to lipid peroxidation and excessive ROS



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            .........10 .........20 .........30 .........40 .........50
AtWRKY45    ---------- ---------- ---------- ---------- ----------
AtWRKY75    ---------- ---------- ---------- ---------- ----------
OsWRKY72    MENFPILFAT --QPTSSSTS SSYHFMSSS- SG-SHDHRHH HGLQAGGNGG
PgWRKY52    MENYHMLFGT AAQP-SSSTS NSYNFLAAAG SGTSNLMRDH DRGQASHSGG
Consistency 1111001101 0011011111 0110111110 1101000101 0001100011

            .........60 .........70 .........80 .........90 ........100
AtWRKY45    ---------- -----MEDRR CDVLFPCSS- --------SV DPRLTEFHGV
AtWRKY75    ---------- MEGYDNGSLY APFLSLKSH- ---------S KPELHQGEEE
OsWRKY72    GGGGSLSHGL FMGSSSSSIR MEELSNSKQA GDVVVDGGAT --RSPHGGDG
PgWRKY52    G--------- F----SSSFL AAELSNNSSK -----DGGAS PPAAAAGGRG
Consistency 1000000000 3010055733 433*623740 0000011135 1544235333

            .........110.........120.........130.........140.........150
AtWRKY45    DNSAQPTTSS EE--KPRSKK K--KKEREAR YAFQTRSQVD ILDDGYRWRK
AtWRKY75    SSKVRSEGCS KSVE--SSKK KGKKQ----R YAFQTRSQVD ILDDGYRWRK
OsWRKY72    DGAAGDDGGD AQAAAAGGRK KGEKKERRPR FAFQTRSQVD ILDDGYRWRK
PgWRKY52    DSSAGPDAAG GEMDRPGAKR KGEKKERRPR YAFQTRSQVD ILDDGYRWRK
Consistency 7557345435 3622224588 *53*75532* 8********* **********

            .........160.........170.........180.........190.........200
AtWRKY45    YGQKAVKNNP FPRSYYKCTE EGCRVKKQVQ RQWGDEGVVV TTYQGVHTHA
AtWRKY75    YGQKAVKNNK FPRSYYRCTY GGCNVKKQVQ RLTVDQEVVV TTYEGVHSHP
OsWRKY72    YGQKAVKNNK FPRSYYRCTH QGCNVKKQVQ RLSRDETVVV TTYEGTHTHP
PgWRKY52    YGQKAVKNNN FPRSYYRCTH QGCNVKKQVQ RLSRDEGVVV TTYEGTHTHP
Consistency *********4 ******8**5 4**7****** *642*83*** ***8*6*7*6

            .........210.........220.........230.........240.......
AtWRKY45    VDKPSDNFHH ILTQMHIFPP FCLKE----- ---------- --------
AtWRKY75    IEKSTENFEH ILTQMQIYSS F--------- ---------- --------
OsWRKY72    IEKSNDNFEH ILTQMHIYSG LTPSSAAHAS SSSPLFPSAA AAASHMFQ
PgWRKY52    IEKSNDNFEH ILTQMQIYSG M---GSNFSS SSYNKF---- ----H---
Consistency 98*658**6* *****5*864 5000110011 1100010000 00001000
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Fig. 1. ***In-silico* Features of *PgWRKY52*.** (A) The gene structure of *PgWRKY52* displaying its exons, introns, and UTR regions.(B) Multiple sequence alignment of the *PgWRKY52* with homologous WRKY members. (C) Phylogenetic analysis depicting the homologous relationship between *PgWRKY52* and its homologous WRKY proteins.

production, causing oxidative stress. Therefore, we analyzed antioxidant enzyme activities in transgenic and WT plants under salt stress. NBT and DAB staining showed significantly lower levels of O_2^- and H_2O_2 in transgenic plants compared to WT under salt stress (Fig. 4A-B), suggesting enhanced ROS scavenging in the OE lines. Biochemical analysis of transgenic and WT plants revealed that under salt stress, the transgenic lines exhibited higher levels of antioxidant enzymes, particularly SOD, CAT, and POD, compared to the WT plants (Fig. 4C). Additionally, the transgenic lines showed notably lower levels of MDA, a marker of lipid peroxidation, suggesting reduced oxidative damage. Moreover, the expression levels of stress-responsive genes (*AtRD22, AtRD29, AtNCED, AtCAT, AtSOD,* and *AtPOD*) were increased over time under salt stress, with higher levels observed in the transgenic lines compared to WT (Fig. 4D). These findings highlight that *PgWRKY52* improves salt stress tolerance by enhancing ROS scavenging and minimizing oxidative stress-induced damage.

Overexpression of PgWRKY52 modulates stress responses in pearl millet

Transformed transgenic calli overexpressing *PgWRKY52* were exposed to drought and salinity stress, as described in Material and Methods. Under these stress conditions, *PgWRKY52* and other stress marker genes (*OST1, NCED*) were upregulated and higher than vector control. These results confirm that the stress-inducible *PgWRKY52* is important in positively regulating stress responses (Fig. 5A-C).

Isolation and genetic architecture analysis of the PgWRKY52's promoter

The upstream 1000 bp promoter of *PgWRKY52* gene (*P1*) was amplified from pearl millet genomic DNA. The full-length (P1) and 5′ truncated (P2 and P3) promoter fragments with sizes of 1000 bp, 676 bp, and 500 bp were PCR amplified (Figure S3). Also, the promoter cis-element analysis showed the presence of numerous critical motifs linked to various biological processes (Figure S4), such as stress-responsive elements (ARE, MYB, STRE, W-box, WRE3, WUN-motif, MYC), metabolism responsive (O2-site), plant growth and development (RY-element, dOCT), light responsive (Box 4, G-box, TCCC-motif, TCT-motif) and hormonal responsive (ABRE, CGTCA-motif, TGACG-motif). Other important elements, including TATA boxes and CAAT boxes, essential for basal transcription machinery, were also present. These findings indicate that *PgWRKY52* might be regulated by a complex network of cis-elements responding to environmental stimuli, metabolic processes, and hormonal signals.

Promoter activity analysis

Transient gene expression of all three promoter fragments (P1, P2, and P3) fused with the uidA gene in tobacco leaves, as well as rice and pearl millet seedlings confirmed that all three fragments were active and could drive reporter gene expression in all three species. In rice and millet seedlings (Fig. 6A-B), both P1 and P2 constructs showed similar GUS activity, which was ~2–4-fold higher than the P3 construct, suggesting that critical regulatory elements might be missing or less functional in this truncated version. A similar trend was observed in tobacco

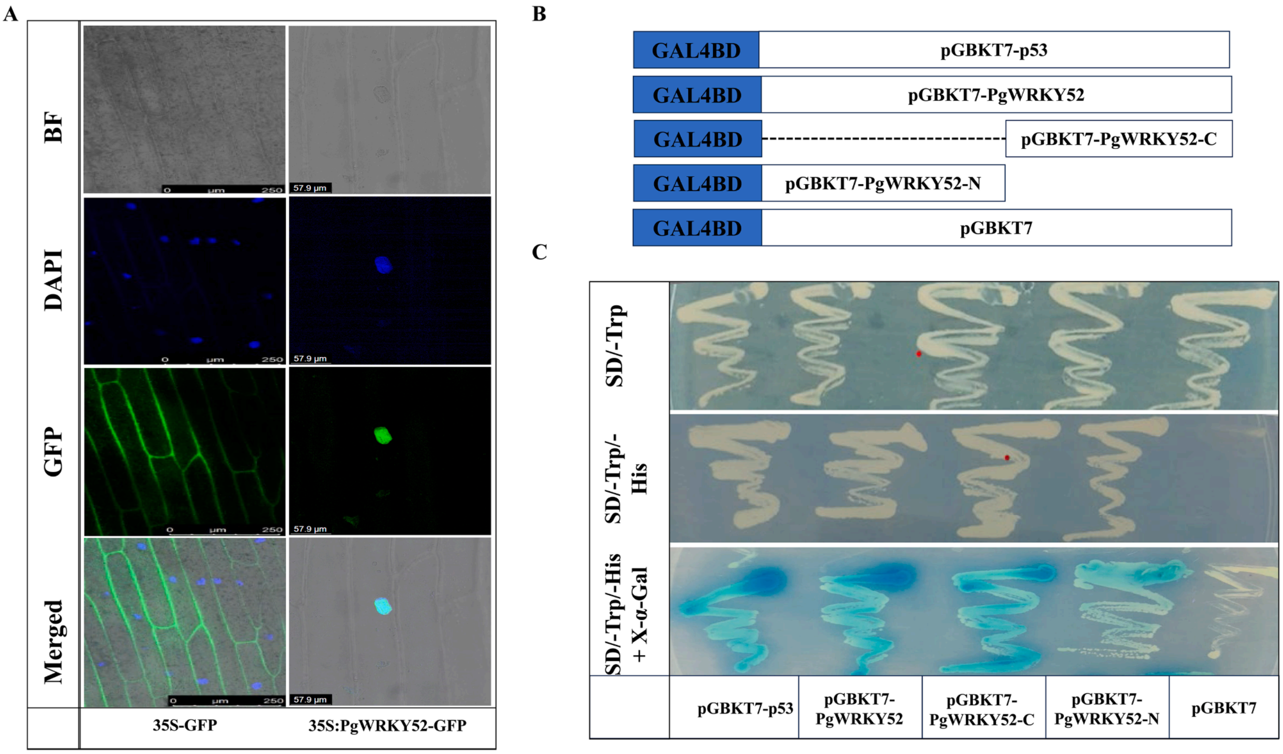

Fig. 2. Subcellular Localization and Transactivation Assay of PgWRKY52. (A) Confocal microscopy depicting the subcellular localization. BF: Bright field, DAPI: Nuclear staining, GFP: Green fluorescence protein, Merged: Combined image. (B) A diagram depicting full-length and truncated *PgWRKY52* fragments fused to the GAL4 DNA-binding domain. (C) Yeast growth on SD/-Trp, SD/-Trp/-His, and SD/-Trp/-His + X-α-gal media.

plants, with P1 and P2 constructs demonstrating higher GUS activity than P3 (Fig. 6C). Furthermore, GFP fluorescence for the P1 promoter fragment fused with a GFP was observed across the leaf tissues, with a strong and widespread signal compared to control plants, which exhibited no detectable GFP expression (Fig. 6D-E).

For stable expression analysis, transgenic tobacco plants were raised for all three promoter fragments (P1, P2, P3) constructs as described in Materials and Methods. Further, 8–9 lines were advanced to T_2 generation based on gene integrity analysis (Figure S5). Based on GUS activity in 21-day-old T_2 seedlings, P1 (L5), P2 (L4), and P3 (L6) were selected for subsequent experiments. A comparative assessment revealed that P1 and P2 constructs exhibited significantly higher activity, approximately 1.5–2.0 times stronger than that of the P3 fragment, indicating greater transcriptional activity in the transgenic tobacco plants (Fig. 7A). These findings were further corroborated by qRT-PCR analysis (Fig. 7B).

Tissue-specific activity of PgWRKY52's promoter

To study the spatiotemporal *uid*A gene expression in transgenic plants, GUS expression was quantified in different plant tissues under normal conditions. The data revealed that the transgenic lines carrying the uidA gene under the control of promoter fragments (P1, P2, and P3) exhibited higher GUS expression in leaves and stem tissues while showing lower activity in root tissues (Fig. 7C). These findings were further supported by X-Gluc staining, the formation of intense blue colouration in seedlings, leaves, stems, and root tissues (Fig. 7D). Furthermore, X-Gluc staining of different reproductive and vegetative parts (petiole, ovary, anther, style, and filament) of T_2 generation tobacco plants of P1 construct confirmed constitutive nature of the *PgWRKY52* promoter (Fig. 7E).

Stress inducibility of PgWRKY52 promoter

To examine the stress-inducible nature of the *PgWRKY52* promoter, we analyzed GUS activity in T_2 transgenic tobacco plants carrying the P1 construct under various abiotic stress treatments. Specifically, salt stress led to a ~1.2 times higher GUS activity than control seedlings (Fig. 8A), while GUS activity was ~1.3-fold higher under heat stress than controls (Fig. 8B). These findings highlight the crucial role of the *PgWRKY52* promoter in mediating plant responses to heat and salinity stress.

Phytohormones are crucial in regulating plant stress responses. Enriching several hormone-responsive CREs, like ABRE and TGACG-motif in *PgWRKY52* promoter, leads to checking its inducibility under phytohormone treatments. For that, twenty-one-day-old transgenic tobacco seedlings were exposed to SA, MeJA, and ABA hormones, followed by GUS activity measurement as described in Materials and Method. Our findings revealed that the *PgWRKY52* promoter was strongly induced by ABA and MeJA, with a ~1.15 to ~2.5-fold increase in GUS activity over 48 hours, respectively (Fig. 8C). These findings indicate that the *PgWRKY52* promoter was inducible by ABA and MeJA.

Cis-regulatory elements (CREs) role in PgWRKY52's promoter activity

CREs are important in controlling promoter activity to mediate the plant's response to environmental stimuli. Therefore, a targeted mutation experiment was performed to evaluate the involvement of key CREs in *PgWRKY52* promoter's regulation. Based on their known roles in stress signalling, several important CREs, including ABRE (2 sites), MYB (2 sites), W-box (2 sites), and MYC (4 sites), were selected for mutation. A total of 15 mutation constructs were generated (Fig. 8D), with single, double, quadra, and full mutations of these CREs to dissect their individual and combined contributions to promoter strength.

These mutated constructs were transiently expressed in tobacco and pearl millet, and GUS activity was measured to determine the impact of specific mutations as mentioned in Material and Methods. The cis-element mutation analysis revealed significant reductions in GUS activity (Fig. 8D, Figure S6), underscoring the critical roles of these CREs in promoter activity. Single mutations in the ABRE (M1, M2) element

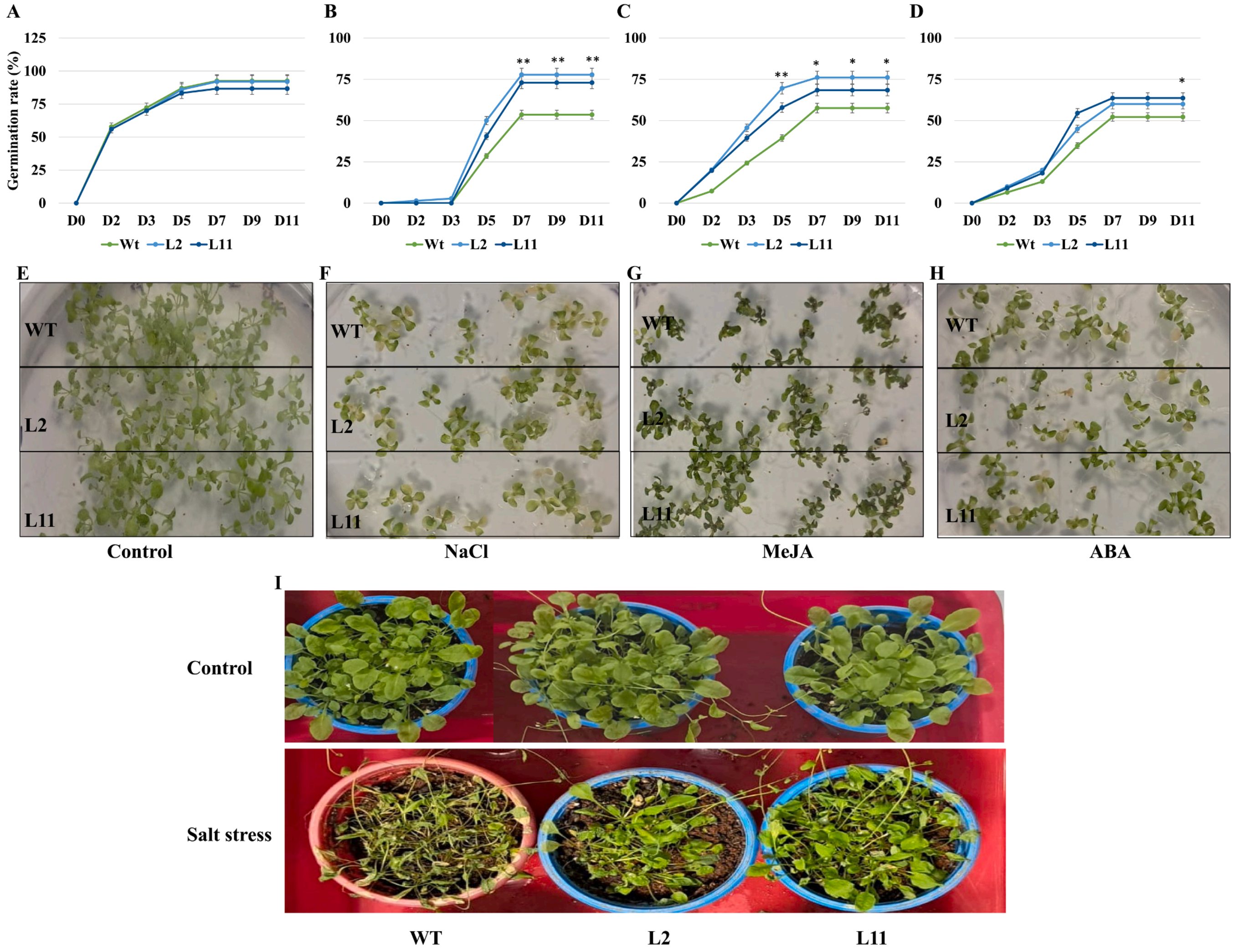

Fig. 3. Stress Tolerance Evaluation. (A-D) The germination percentage of wild-type (WT) and transgenic seeds after 7 days of growth on ½ MS agar medium supplemented with varying levels of NaCl, MeJA, and ABA. (E-H) Representative images displaying seed germination performance under stress conditions. (I) Morphological comparison of WT and transgenic plants grown under salt stress.

resulted in reductions of ~30–50% in GUS activity, with a double ABRE mutant (M3) showing a similar ~40–50% decrease. Mutations in the MYB element (M4, M5) led to consistent reductions of ~45%, with the double MYB mutant (M6) showing an even greater reduction of ~60%. For W-box mutations (M7, M8), single mutants caused substantial declines of ~70–80%, and the double W-box mutant (M9) resulted in a significant ~90% reduction in GUS activity. The MYC mutations (M10-M13) displayed varying effects, with 30–70% reductions in single mutants, while the quadra mutant (M14) exhibited a ~75% decrease in GUS activity. Finally, a complete mutation across all 10 CREs (M15) resulted in a drastic ~95% reduction in GUS activity. These results clearly illustrate the vital role of individual and combined CREs in regulating the *PgWRKY52* promoter's regulation. The substantial reductions in GUS activity, especially in the double and quadra mutants, indicate that these CREs work synergistically to drive promoter function in an integrated network. Also, the near-complete loss of activity in the full mutant highlights the complex regulatory network controlling *PgWRKY52* expression.

Putative TFBs in PgWRKY52's promoter

The putative transcription factor binding sites (TFBs) were screened for *PgWRKY52* promoter, and findings revealed the presence of multiple TFBs associated with important regulatory families such as AP2, bHLH, bZIP, ERF, MYB, NAC, WRKY, etc. These TFBs were distributed across the promoter region, with notable clusters between positions 373–856 bp (Table S2). This distribution suggests the complexity of transcriptional regulation within the *PgWRKY52* promoter and the ability to integrate multiple stress and developmental signals.

Discussion

Pearl millet is mostly cultivated in semiarid regions and often encounters various abiotic stresses that significantly affect its growth and productivity (Satyavathi et al., 2021, Rhoné et al., 2020). To thrive in these conditions, the plant has developed complex gene regulation systems that allow it to adjust effectively to environmental changes (Satyavathi et al., 2021). Transcription factors (TFs) are essential in these processes, as they modulate target gene expression that governs stress responses, signal processing, and physiological changes. The TFs, including WRKY, MYB, NAC, bZIP, AP2/ERF have been implicated in stress responses in pearl millet (Chanwala et al., 2020, 2023; Jha et al., 2024, 2025; Xu et al., 2024). Among these, WRKY TFs are known to play a key role in regulating plant stress responses and associated signalling pathways. Studies have reported that overexpressing WRKYs can enhance plant's resistance against various environmental stresses (Singh et al., 2002, Jiang et al., 2017, Banerjee and Roychoudhury, 2015). However, specific functions and molecular mechanisms of WRKY in

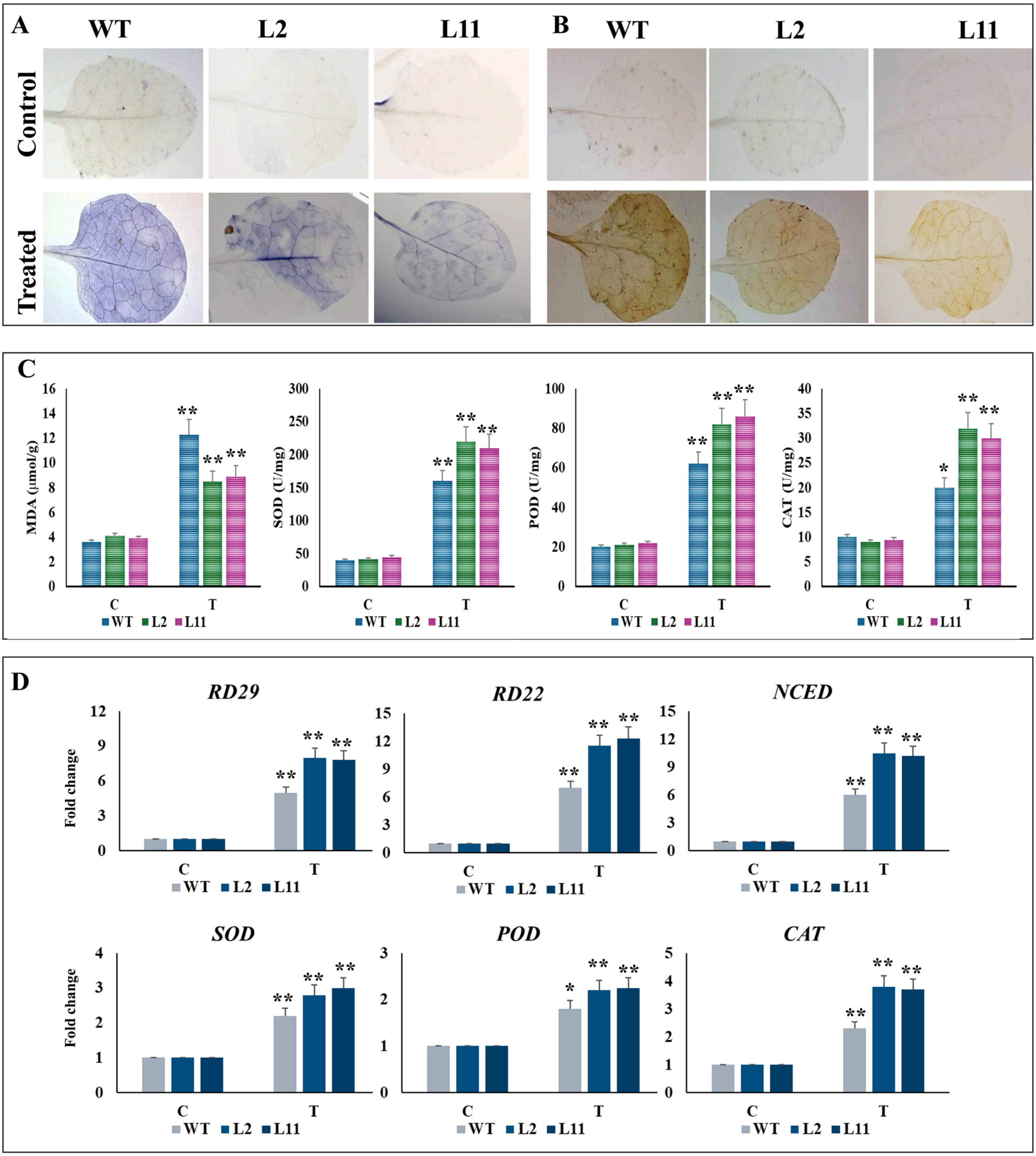

Fig. 4. Biochemical and Physiological Assessments Under Salt Stress. (A-B) NBT and DAB-stained leaves showing H_2O_2 and O_2^- levels. (C) Biochemical and antioxidant enzyme levels. (D) Expression analysis of stress-related marker genes. The Y-axis shows fold changes in transcript levels, while the X-axis represents the plant samples analyzed. "C" indicates control, and "T" indicates treated samples.

pearl millet remain poorly understood. Therefore, understanding the functional dynamics of WRKY TFs and their regulatory architecture is essential for developing strategies to enhance stress resilience through genetic engineering.

In this investigation, we have isolated and characterized *PgWRKY52*, a WRKY TF from pearl millet involved in stress responses. Phylogenetic analysis demonstrated that *PgWRKY52* shares a close relationship with Group IIc WRKY genes, such as *OsWRKY72, AtWRKY75* and *AtWRKY45*, which are reported to be involved in regulating stress responses (Liu et al., 2024, Lu et al., 2023, Zhou et al., 2025). These findings imply that *PgWRKY52* could have a similar role in regulating stress-related pathways in pearl millet. Expression analysis revealed that *PgWRKY52* is induced by salt and drought stress. Furthermore, ABA and MeJA treatments significantly upregulated its expression. Similar WRKY TFs, such

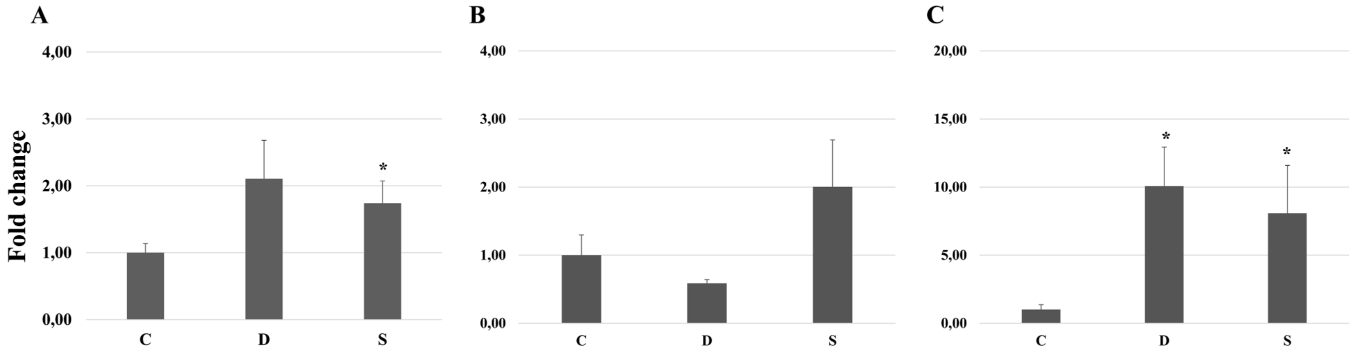

Fig. 5. Relative Expression Analysis of *PgWRKY52* (A), NCED (B), and OST1 (C) in transformed pearl millet calli overexpressing *PgWRKY52* under drought (D) and salt stress (S) conditions.

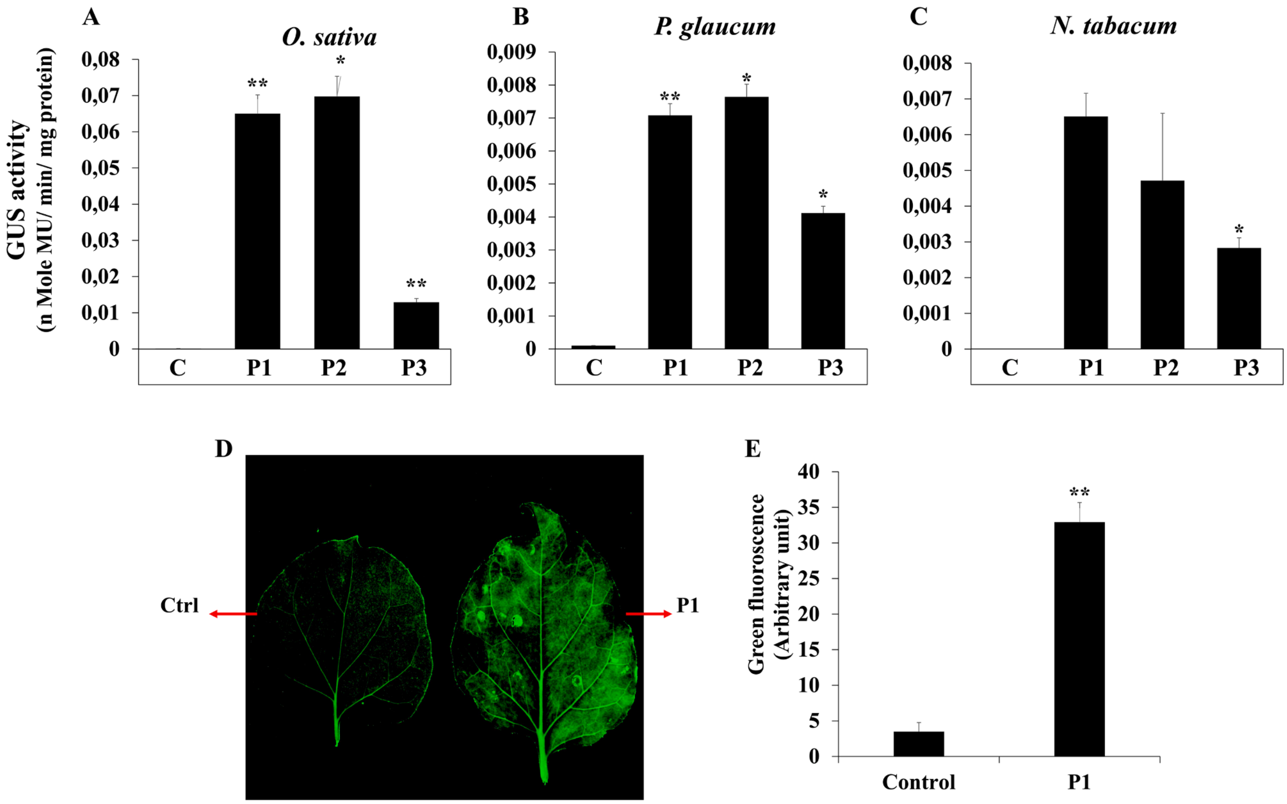

Fig. 6. Transient Promoter Activity Analysis. (A-C) Transient GUS expression analysis of P1, P2 and P3 promoter constructs in *O. sativa, P. glaucum* and *N. tabacum.* (D-E) Transient GFP expression analysis in *N. benthamiana* leaves infiltrated with the P1 promoter construct.

as *TaWRKY24, GhWRKY16* and *GbWRKY1*, have been shown to enhance stress tolerance through ABA-mediated pathways (Y Yu et al., 2023, Ma et al., 2019, Luo et al., 2020). These findings suggest that *PgWRKY52* may function in a similar manner, highlighting its potential role in stress adaptation.

Abiotic stresses pose major challenges to plant growth, often hindering vital processes like seed germination and viability. In this study, overexpressing *PgWRKY52* in Arabidopsis significantly enhanced seed germination and better growth under salt stress, ABA and MeJA treatment compared to WT plants. Furthermore, abiotic stress, particularly salt stress, induces oxidative stress through excessive ROS production, leading to lipid peroxidation and cellular damage (Gill and Tuteja, 2010, Peleg and Blumwald, 2011). Here also, transgenic Arabidopsis lines overexpressing *PgWRKY52* showed significantly lower levels of superoxide and hydrogen peroxide than WT plants under salt stress, indicating enhanced ROS scavenging. Biochemical assays revealed that the transgenic lines exhibited significantly higher activities of antioxidant enzymes (SOD, CAT, and POD) and lower MDA levels, suggesting reduced oxidative damage. This aligns with previous findings on *SiWRKY36* and *SiWRKY51*, enhancing ROS scavenging and activating stress-responsive genes to mitigate salt stress effects (Mehboob et al., 2024). Additionally, the expression of stress-responsive genes, including *AtRD22, AtRD29, AtNCED, AtSOD, AtPOD*, and *AtCAT*, was markedly higher in transgenic plants compared to WT under salt stress, indicating *PgWRKY52*'s role in promoting stress tolerance. Overall, these findings highlight *PgWRKY52*'s potential as a key regulator in improving salt stress tolerance through enhanced ROS scavenging and activation of stress-responsive genes (Gill and Tuteja, 2010, Ahmad et al., 2010).

Similar findings were observed in transgenic pearl millet calli overexpressing *PgWRKY52*. Upon drought and salt stress exposure, these transgenic calli showed significantly higher expression levels of *PgWRKY52* and stress-related marker genes such as *OST1* and *NCED*

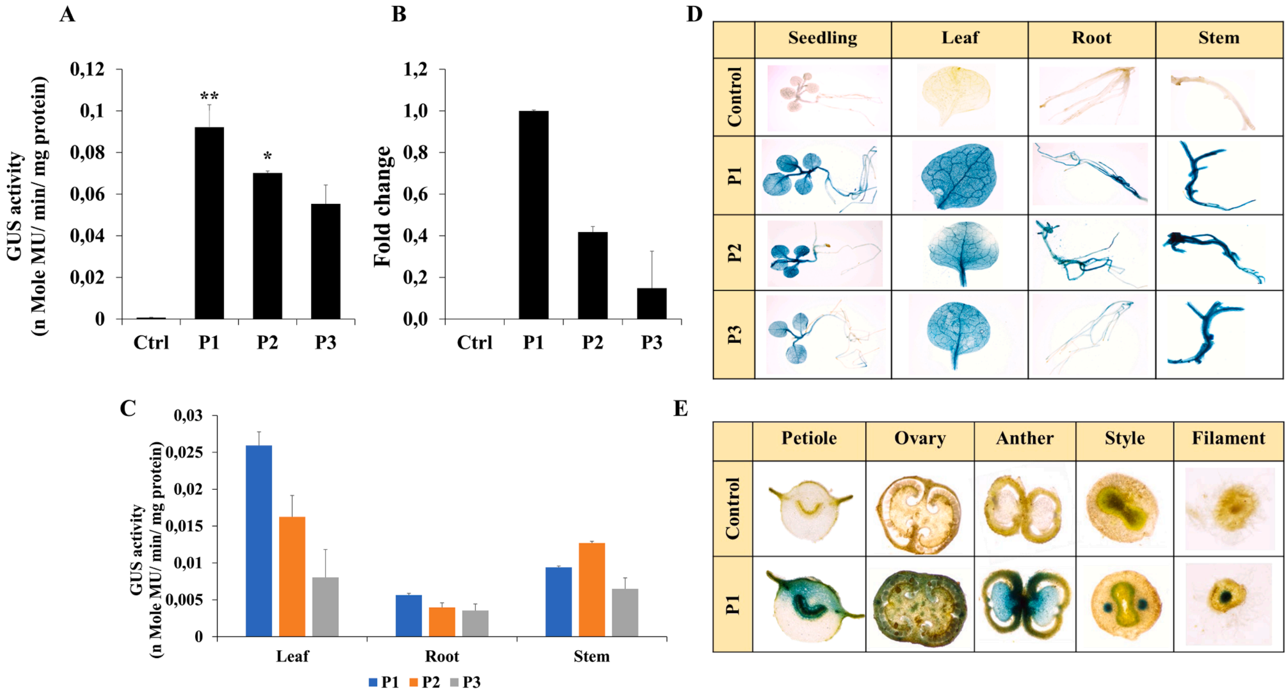

Fig. 7. Functional Analysis of *PgWRKY52* Promoter Fragments (P1, P2, and P3) in Transgenic *N. tabacum* Seedlings. (A) Fluorometric GUS activity and (B) Expression analysis of uidA (GUS) in 21-day-old T_2 transgenic seedlings. (C) Fluorometric quantification of GUS activity in different tissues and (D) Histochemical X-gluc staining to visualize GUS expression in seedlings, as well as leaf, stem, and root tissues of transgenic plants. (E) Histochemical localization of GUS activity in various tissues of P1 transgenic plants, including petiole, ovary, anther, style, and filament.

compared to vector control. These results confirm that *PgWRKY52* is stress-inducible and important for enhancing the stress tolerance of pearl millet, resembling its function observed in Arabidopsis.

These findings align with the function of other WRKY TFs, such as *AtWRKY45*, which positively regulates salinity and osmotic stress responses via ABA signalling and enhanced antioxidant capacity (Zhou et al., 2025). Similarly, *WRKY75* is involved in jasmonate-mediated plant defense (Chen et al., 2021). *WRKY75* improves salt stress tolerance by increasing *SOS1* expression (Lu et al., 2023). Also, methyl jasmonate (MeJA) has been reported to improve salt tolerance by altering ABA signalling, osmotic adjustment, photosynthesis, and ROS metabolism (Delgado et al., 2021, Munemasa et al., 2011). Together, these findings suggest that *PgWRKY52* may operate through similar phytohormonal signalling pathways, including ABA and MeJA, to improve salt stress tolerance. However, further research is needed to elucidate its interactions with other transcriptional regulators, downstream target genes, and overall contribution to stress adaptation mechanisms.

To further examine the role of *PgWRKY52*'s transcriptional alteration in abiotic stress responses, we characterized its promoter sequence. *In-silico* analysis revealed the presence of multiple stress-responsive cis-regulatory elements (CREs) in the *PgWRKY52* promoter, such as the ARE, MYB, STRE, W-box, WRE3, WUN, MYC, ABRE, and TGACG-motif. These CREs play critical roles in controlling gene expression under stress conditions by responding to various external stimuli. The presence of W-box suggests that *PgWRKY52* may not only autoregulate its expression but also cooperate with other WRKY family members to coordinate stress responses, ensuring efficient adaptation to stress conditions (Chen et al., 2012).

Promoter activity analysis of *PgWRKY52* across different plant species, including rice, tobacco, and millet, confirmed its broad functionality and stress-inducible nature. The promoter was active even in truncated forms, indicating the conservation of core regulatory elements essential for stress responsiveness. Specifically, GUS activity assays in transgenic plants subjected to heat and salinity stress revealed significant promoter activation, further supporting its role in mediating stress responses. Notably, *PgWRKY52*'s promoter demonstrated strong phytohormone-inducible nature, responding to ABA and MeJA. Similar promoter activities were also reported in other species, such as Arabidopsis (*AtMYB44*-involved in ABA-mediated drought signalling) (Jung et al., 2008), wheat (*TaWRKY10*-involved in drought tolerance) (Wang et al., 2013), rice (*OsWRKY45*-induced by both JA and ABA) (Qiu and Yu, 2009), and *OsbZIP72* (regulating ABA signalling and drought tolerance) (Lu et al., 2009), etc. Comparatively, *PgWRKY52's* promoter exhibits similar stress-responsive characteristics, emphasizing its potential as a universal stress-inducible regulatory element. The ability of its minimal promoter to retain significant activity suggests that key cis-regulatory elements are present within its core region. Understanding these regulatory sequences further could facilitate the development of synthetic promoters for stress-tolerant crops, providing a valuable tool for biotechnological applications in agriculture.

Mutation analysis of specific cis-elements revealed that WRKY, MYC, MYB, and ABRE elements significantly contribute to promoter strength. The cumulative deletion of these motifs led to a marked reduction (up to 90%) in GUS activity, emphasizing their central role in maintaining high transcriptional activity. Sherpa et al. also reported the importance of W-box and as-1 (TGAC motif) elements in maintaining promoter activity (Sherpa et al., 2023). By leveraging these cis-elements, synthetic promoters could be designed to optimize stress-responsive gene expression, improving crop resilience and stability under adverse conditions (Liu and Stewart, 2016, Yang et al., 2021). Furthermore, the presence of multiple TFBs, including WRKY, MYB, and AP2/ERF, implies that the *PgWRKY52's* promoter is a key component of complex regulatory pathways involving different TFs.

This ability of *PgWRKY52*'s promoter to integrate multiple stress signals, including those from key phytohormones such as ABA and MeJA, highlights its regulatory complexity and suggests a robust network of transcriptional regulation in response to stress conditions. This integration underscores *PgWRKY52*'s potential as a crucial player in stress adaptation and makes it a promising target for improving multi-

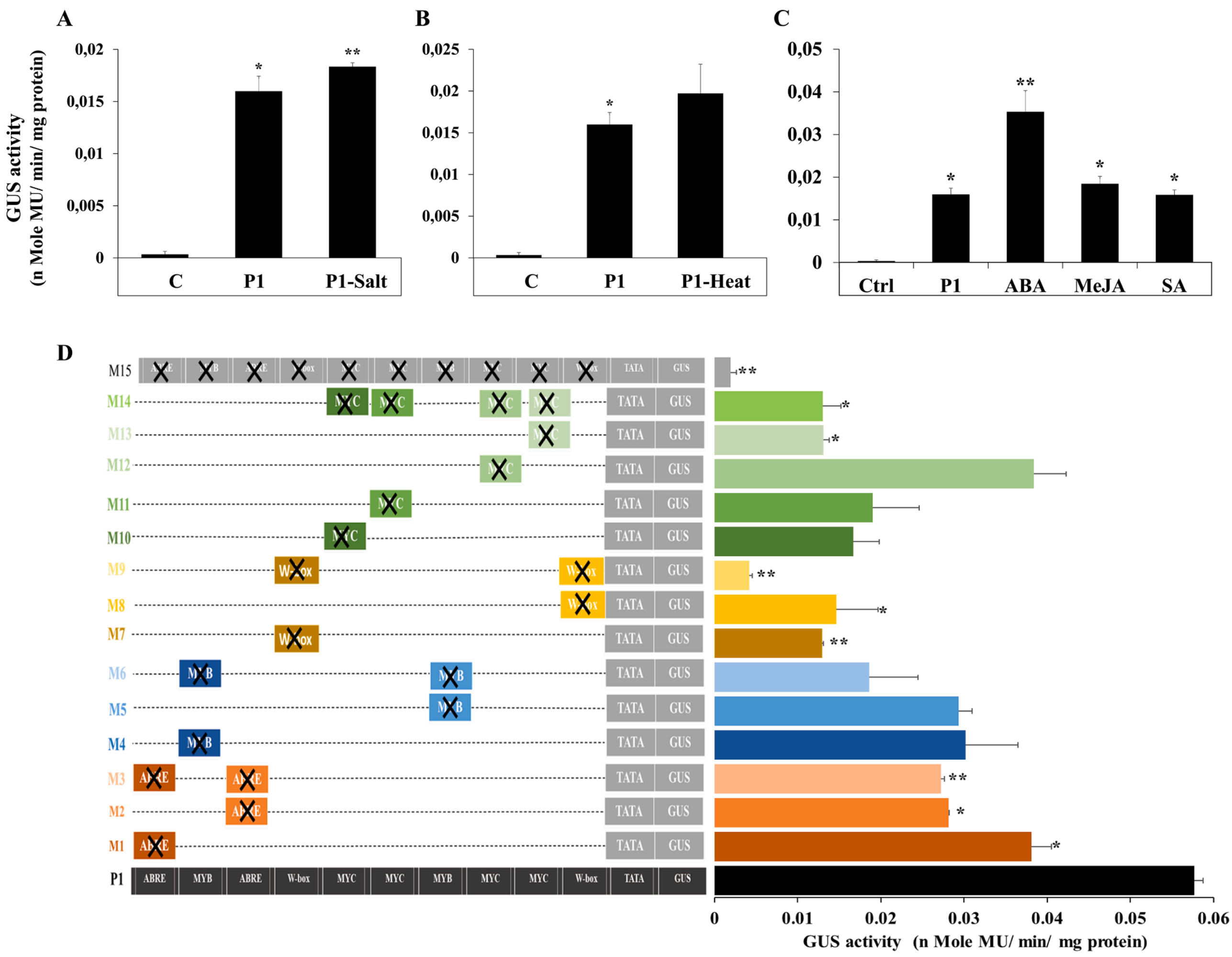

Fig. 8. Analysis of Stress-Inducible Nature. (A-C) Fluorometric GUS activity of transgenic plants harbouring the P1 construct under salt stress, heat stress and phytohormones (ABA, MeJA, and SA) treatments. (D) Transient GUS expression analysis to assess the impact of mutations in stress-responsive ABRE, MYB, W-Box, and MYC cis-elements (M1-M15 sequences) compared to the native P1 promoter.

stress resistance in pearl millet through transgenic approaches. By modulating endogenous hormonal pathways and expression of stress-responsive genes, *PgWRKY52* could play a pivotal role in developing crops with improved resilience and productivity under diverse environmental challenges. These findings position *PgWRKY52* as a key TF for genetic engineering approaches to boost stress resilience, particularly millets.

Conclusion

In conclusion, the comprehensive functional characterization of the pearl millet *PgWRKY52* gene and its promoter highlights their pivotal roles in salt stress tolerance. Ectopic expression of *PgWRKY52* in Arabidopsis significantly increases resistance to salt stress by activating stress signalling pathways and improving enzymatic antioxidant defense systems. Furthermore, the promoter architecture of *PgWRKY52*, enriched with a diverse array of stress-responsive hormone-regulated cis-elements, along with constitutive expression and inducibility under stress conditions, underscores its regulatory versatility and adaptability. These findings validate *PgWRKY52* as a key regulator of salt stress tolerance and position it as a promising candidate for engineering crops, with considerable potential to improve agricultural productivity in challenging, stress-affected regions.

CRediT authorship contribution statement

Jeky Chanwala: Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Khushbu Kumari:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Deepak Kumar Jha:** Writing – review & editing, Validation, Methodology, Formal analysis. **Mrunmay Kumar Giri:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Investigation, Conceptualization. **Nrisingha Dey:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, 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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NA

Data availability

All data relevant to the findings of this study are incorporated in the manuscript.

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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.2025.100814.

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