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Cloning, *in silico* analysis of upstream sequences and tissue and different external conditions mediated expression studies of three homeologs of *TaNPF7.2* of wheat (*Triticum aestivum*)

MUHAMMED SHAMNAS V[1], AKANKSHA SINGH[1], ANUJ KUMAR[1] and SUBODH KUMAR SINHA[1]*

ICAR-National Institute for Plant Biotechnology, New Delhi 110 012, India

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ABSTRACT

The present study was carried out during 2020–2021 at ICAR-National Institute for Plant Biotechnology, New Delhi during 2020–2021 to investigate the cis-regulatory elements, transcription factor (TF) binding sites and stress- and development-responsive expression dynamics of *TaNPF7.2*, a wheat (*Triticum aestivum* L.) homolog of the *AtNRT1.5/NPF7.3* responsible for root-to-shoot nitrate transport. Promoter regions (~2 kb) of *TaNPF7.2*-A, -B, and –D were analyzed to identify cis-regulatory elements and TF-binding sites. A homeolog-specific expression study was conducted at early and later developmental stages (GS19, GS39, GS65) under different treatments in the nitrogen-uptake-efficient genotype, i.e., K9107. Promoter analysis revealed strong homeolog divergenc with *TaNPF7.2*-A enriched in general stress-responsive STRE motifs, *TaNPF7.2*-B harbouring higher MYB and DRE content and *TaNPF7.2*-D uniquely lacking ABREs but exhibiting dense WRKY-, HD-ZIP– and ZF-HD–binding sites. Expression profiling showed that under optimal nitrate, *TaNPF7.2*-A was the most strongly induced in seminal roots. Salt stress caused widespread transcriptional suppression whereas drought triggered a delayed but strong induction of *TaNPF7.2*-B. Cadmium stress induced rapid activation of *TaNPF7.2*-D, consistent with its WRKY-rich promoter element. During later developmental stages, expression remained predominantly root-centric, with shifting homeolog dominance from 7.2-D (GS19) to 7.2-A (GS65). These findings provide insights into understanding of nitrate translocation in wheat and identify promising regulatory nodes for enhancing NUE under both favourable and stress conditions.

Keywords: NUE, Promoter, Stress response, *TaNPF7.2*, Wheat

Wheat (*Triticum aestivum* L.), the most widely cultivated cereal, depends heavily on nitrogen fertilizers yet it's nitrogen use efficiency remains low, with less than one-third of applied nitrogen utilized by wheat (Hawkesford 2014). Nitrate transport in plants is controlled by transporter families such as NRT1 (NPF), which function under high nitrate availability (Tsay *et al.* 2007). In wheat, 331 *NPF* genes have been identified (Tsay *et al.* 2007, Leran *et al.* 2014, Wang *et al.* 2020). In *Arabidopsis*, NRT1.5 (NPF7.3) expressed in root pericycle cells mediates xylem loading of nitrate and participates in abiotic stress responses (Lin *et al.* 2008, Chen *et al.* 2012).

Gene transcription is regulated by complex networks of transcription factors (TFs) and cis-regulatory elements (CREs). Key CREs such as abscisic acid-responsive (ABREs) and dehydration-responsive (DREs) elements mediate ABA-dependent and -independent pathways during drought, salinity and cold stress (Zhou *et al.* 2010, Dar *et al.* 2017). MYB-binding sites and stress-responsive elements (STRE) further contribute to transcriptional reprogramming under osmotic, oxidative and nutrient stress (Ambawat *et al.* 2013, Sheshadri *et al.* 2016). Multiple TF families–including C2H2, WRKY, TCP (e.g., *ZmTCP14*), HD-ZIP, RAV, SBP, WOX and ZF-HD–play crucial roles in regulating genes and hormone signaling to enhance plant stress tolerance and adaptation (Jiang *et al.* 2015, Zhang *et al.* 2020, Liu *et al.* 2021, Karami *et al.* 2022, Li *et al.* 2022, Liu *et al.* 2022, Jiao *et al.* 2023, Wang *et al.* 2024).

Regulatory understanding of wheat nitrate transporters is very limited. Therefore, in the present study we analysed the promoter regions and stress-responsive expression patterns of *TaNPF7.2* homeologs, given their homology to a nitrate transporter in Arabidopsis involved in root-to-shoot nitrate translocation, revealing distinct regulatory signatures and environmental responsiveness and providing insights for improving nitrate transport and nitrogen use efficiency under optimal and stress conditions.

MATERIALS AND METHODS

The experiment was conducted during 2020–21 (19 November 2020 to April 5, 2021) at ICAR-National Institute

[1]ICAR-National Institute for Plant Biotechnology, New Delhi.
*Corresponding author email: subsinha@gmail.com

for Plant Biotechnology, New Delhi.

Sequence retrieval of 2 kb upstream sequence TaNPF7.2 gene: The 2 kb upstream promoter regions of *TaNPF7.2* homeologs were retrieved from the Plant Ensembl database (https://plants.ensembl.org/index.html).

Plant materials and growth conditions: Uniform K9107 seeds, a nitrogen/nitrate uptake efficient wheat genotype (Kumar *et al.* 2023) were surface sterilized following the procedure of Sinha *et al.* (2015) and germinated in aerated, deionized distilled water at 25 ± 1°C. Seedlings were grown in a modified hydroponic setup as previously described by Sinha *et al.* (2019). For later growth stages, K9107 seeds were grown in natural conditions in net-house in pots containing vermiculite and perlite in (2:1). For promoter amplification, genomic DNA was extracted from leaves of two-week-old seedlings using the CTAB protocol (Doyle and Doyle 1987).

Cloning and in silico *analysis of upstream sequences of tanpf7.2 homeologs for cis-regulatory elements and transcription factor binding sites*: Approximately 2 kb upstream promoter region of *TaNPF7.2* gene was amplified using homeolog specific primers (Table 1). The amplicons were cloned in pJET1.2 cloning vector followed by Sanger sequencing. The promoter sequences were then analyzed using PLANTCARE (https://bioinformatics.psb.ugent.be/webtools/ plantcare/html/) (Rombauts *et al.* 1999) to identify stress-responsive cis-regulatory elements. To further elucidate the regulatory framework, transcription factor binding sites (TFBSs) in the promoter regions were predicted using PlantRegMap (https://plantregmap.gao-lab.org/binding_site_prediction.php) (Tian *et al.* 2020).

RNA extraction, and qRT-PCR analysis: Seeds were grown hydroponically as described previously (Sinha *et al.* 2015). For expression studies in the K9107 genotype, optimum nitrogen (ON; 8.0 mM nitrate) and low nitrogen (LN; 0.08 mM nitrate) conditions were established using nitrate-free 1× MS medium (HiMedia, Cat. No. PT093) supplemented with ON and LN. The nutrient medium was replaced every third day. For salt stress treatments, seedlings initially grown under ON and LN conditions for 11 and 18 days respectively, were subjected to 100 mM NaCl stress (Elhabashy *et al.* 2025). Similarly for 200 µM cadmium chloride treatment (Ozygit *et al.* 2021) and 10% PEG for drought induction (Robin *et al.* 2021). Seminal and lateral roots were harvested separately at 14 and 21 days.

For later developmental stages, K9107 plants were grown under ON and LN conditions and sampled at GS19 (three-leaf stage), GS39 (flag leaf) and GS65 (half flowering). Root, stem, leaf and ear tissues were collected and stored.

Total RNA was extracted and cDNA was synthesized using Oligo dT primers. To ensure amplification specificity, sub-genome-specific primers were designed by aligning homeologous sequences and selecting SNPs at the 3′ end to enable precise primer binding (Table 1). The expression patterns of *TaNPF7*.2-A/-B/-D in root and shoot tissues (3 biological replicates) were analyzed under the different experimental conditions described above. *Actin* was used as the internal reference gene for normalization. The ΔCt value was determined as $Ct_{treatment} - Ct_{reference}$ and the ΔΔCt value was calculated as $\Delta Ct(_{N^+}) - \Delta Ct(_{N^-})$ to derive the relative expression levels. The fold-change in gene expression was determined using the $2^{-}\Delta\Delta Ct$ method (Livak and Schmittgen 2001) where values greater than one indicate upregulation and values less than one indicate downregulation under N^+ conditions relative to N^-.

RESULTS AND DISCUSSION

Cloning of upstream sequence of TaNPF7.2-A, -B, -D in pJET1.2 vector: PCR amplification of upstream promoter region of *TaNPF7*.2-A, *TaNPF7*.1-B, *TaNPF7*.2-D was performed using homeolog specific primers (Fig. 1A) and cloned into pJET1.2 cloning vector and the positive clones were identified by restriction digestion (Fig. 1B). Sanger sequencing results demonstrated the cloning of 1976, 1995 and 1984 bp of *pTaNPF7.2-A*, *pTaNPF7.2-B* and *pTaNPF7*.2-D upstream sequences respectively (Supplementary Data).

Table 1 Details of homeolog-specific primer used for cloning in pJET1.2 vector and expression analysis.

Primer Name	Primer sequence (5’ to 3’)	Remarks
pTaNPF7.2_AF	CTCAACACCCAGGACCTAGG	Forward primer for cloning of *TaNPF7.2A* promoter
pTaNPF7.2_AR	GAGGTGAGCCGGGGGCG	Reverse primer for cloning of *TaNPF7.2A* promoter
pTaNPF7.2_BF	GTGCTCGGATGCTTGCCTGA	Forward primer for cloning of *TaNPF7.2B* promoter
pTaNPF7.2_BR	GAGGTGAGCCGGGGGTGG	Reverse primer for cloning of *TaNPF7.2B* promoter
pTaNPF7.2_DF	AGCAACTCATGTAGTATTGGC	Forward primer for cloning of *TaNPF7.2D* promoter
pTaNPF7.2_DR	GAGGTGAGCCGGGGGCG	Reverse primer for cloning of *TaNPF7.2D* promoter
TaNPF7.2_AF	GCTCCATCTTCTCCAACACGA	Forward primer for qRT-PCR of *TaNPF7.2A* gene
TaNPF7.2_AR	GTAGTTGGGGGTGCCGAG	Reverse primer for qRT-PCR of *TaNPF7.2A* gene
TaNPF7.2_BF	CTCCATCTTCTCCAACACGG	Forward primer for qRT-PCR of *TaNPF7.2B* gene
TaNPF7.2_BR	GTAGTTGGGGGTGCCGAG	Reverse primer for qRT-PCR of *TaNPF7.2B* gene
TaNPF7.2_DF	CTCCATCTTCTCCAACACCG	Forward primer for qRT-PCR of *TaNPF7.2D* gene
TaNPF7.2_DR	GTAGTTGGGGGTGCCGAG	Reverse primer for qRT-PCR of *TaNPF7.2D* gene

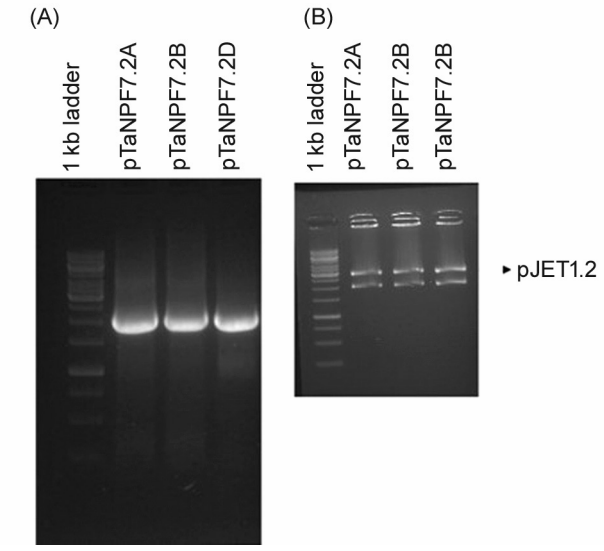

Fig. 1 Agarose gel showing amplification of upstream region of (A) *TaNPF7.2*-A, -B, -D genes; and (B) agarose gel with restriction digestion of upstream sequences in pJET1.2 vector.

Homeolog-specific distribution of stress-responsive cis-regulatory elements: The genome-wide analysis of the cloned promoter regions revealed distinct homeolog-dependent differences in the abundance and distribution of stress-associated cis-regulatory motifs. ABA-responsive elements (ABREs) were present exclusively in the A and B homeologs with the A genome harboring ABRE4-1, ABRE-2 and ABRE3a-1 numbers while the B genome contained a higher number with 6 ABREs and 1 ABRE3a (Fig. 2A). Notably, no ABRE motifs were identified in the D homeolog. The dehydration-responsive DRE core motif exhibited a broader distribution across all homeologs. The A, B and D genomes contained 3, 4 and 1 DRE motifs respectively, suggesting differential potential for ABA-independent drought-responsive transcriptional regulation (Fig 2A). Motifs recognized by MYB transcription factors were also unequally distributed, with the B homeolog exhibiting the highest number (A=3, B=4, D=2) (Fig. 2A). Elements associated with anaerobic or hypoxic signaling, specifically the ARE motif, were identified across all sub-genomes (A=3, B=2, D=3) (Fig. 2A), indicating no marked sub-genomic bias in anaerobic response ability at the promoter level. The general stress-responsive STRE element showed significant variation in abundance among the homeologs. The A homeolog contained 12 STRE motifs whereas the B and D homeologs carried only 2 each (Fig. 2A). The GC-motif, another stress and anaerobic-associated cis-element, was detected at unequal frequencies across homeologs (A=4, B=3, D=2) (Fig. 2A).

These differences in cis-regulatory elements may arise from sub-functionalization during wheat polyploidization enabling the A, B and D sub-genomes to partition regulatory functions across tissues and environmental contexts, as supported by recent evidence linking chromatin accessibility, DNA methylation and sequence variation in CREs to homoeolog-biased gene expression in wheat (Zheng *et al.* 2024). *TaNPF2.12*, has been shown to exhibit this kind of promoter-based divergence, which is widely acknowledged as a major factor in the phenotypic diversity associated with NUE (Siddiqui *et al.* 2023). The enrichment of stress-related motifs in *TaNPF7.*2-A and *TaNPF7.*2-B suggests their potential dominance in mediating nitrate transport and signaling under stress conditions (Jia *et al.* 2023). Together, these findings highlight how promoter-level evolution can fine-tune gene responsiveness and contribute to the regulatory plasticity essential for wheat's adaptation to fluctuating environments (Chen *et al.* 2023).

Homeolog-specific distribution of stress-responsive transcription factor binding sites: A comparative assessment of transcription factor (TF) families revealed marked differences in the abundance and composition of stress-responsive regulators across the three homeologs. The C2H2 zinc-finger family was consistently represented in all genomes with the A and D homeologs each containing 14 each whereas the B genome carried only 9 (Fig. 2B). This pattern indicates stronger C2H2-mediated stress regulation in the A and D sub-genomes relative to B (Fig. 2B, Table 2). In contrast, the WRKY family displayed a striking homeolog-specific enrichment in the D genome, where 17 members were identified (Fig. 2B). WRKY TFs were absent or minimally represented in the A and B homologs, (Fig. 2B, Table 2). The TCP family was more prominent in the B genome, each with 7, compared to only a single representative in the A homeolog and 5 representatives in the D homeolog (Fig. 2B). For HD-ZIP significant variation is observed with 23 in D homeolog followed by 7 in *TaNPF7.2B* and only 1 in case of A homeolog (Fig. 2B). Smaller stress-associated TF families such as RAV, SBP, WOX and ZF-HD (Table 2) were present at lower abundance across the genomes, but exhibited homeolog-specific signatures. The A and D sub-genomes each contained 1 RAV member, whereas the B genome lacked this family (Fig. 2B). SBP and WOX TFs were represented in both the B and D genomes but were absent from the A homeolog (Fig. 2B). Conversely, the ZF-HD family was specific to the D genome with 4 members, re-emphasizing the stress-oriented complexity of this sub-genome (Fig. 2B).

TFBS predictions further supported the presence of strong homeolog-specific regulatory divergence reflecting differential cis-regulatory architectures that are likely to underlie variation in gene expression and functional specialization among wheat homologs, consistent with genome-wide TF binding variation reported in other cereals crops like maize (Galli *et al.* 2025) *TaNPF7.*2-D appears to be the main stress-integrative homeolog, especially for drought, salinity and pathogen-associated signals based on the significant enrichment of WRKY TFBSs in the D genome (Khoso *et al.* 2022). The enrichment of WRKY, HD-ZIP and ZF-HD TFs in D sub-genome indicates its potential role as a key stress-response integrator consistent with prior reports highlighting D-sub-genome dominance in stress adaptation (Gudi *et al.* 2024). On the other hand,

Table 2 Functions of different stress and development associated transcription factors present in upstream region of TaNPF7.2 gene

Transcription factor	Functions
C2H2	Response to oxidative stress, Response to cold, Response to water deprivation, Response to wounding, Response to high light intensity, Response to salt stress, Response to abscisic acid
WRKY	Responses to environmental stresses like drought, salt, temperature and pathogen-associated stress responses as well as growth and development
TCP	Cell growth and proliferation, Plant reproductive development, Plant immunity, translate environmental signals into growth regulation
HD-ZIP	Key role in plant growth, development, and stress responses
RAV	Response to development and to environmental stresses
SBP	Crucial role in various developmental processes, including flower development, fruit development, and stress responses
WOX	Crucial role in regulating development and responding to stress
ZF-HD	Regulate growth, development, and responses to stress by binding to DNA
GATA	Involved in seed germination, plant growth and development, chlorophyll synthesis, and nitrogen metabolism

C2H2-binding sites were more in the A and D homeologs, suggesting that these sub-genomes may facilitate early stress adaption (Han *et al.* 2020).

Comparative analysis of development-related transcription factors across TaNPF7.2 homeologs: Several transcription factor (TF) families associated primarily with plant growth and developmental processes were differentially distributed across the three wheat homeologs (Table 2). The GATA transcription factor family showed the highest number of predicted binding sites in the A homeolog with nine sites followed by five in the B homeolog while no GATA-associated sites were detected in the D genome (Fig. 2B, Table 2) suggesting a greater regulatory contribution of the A genome to nutrient assimilation and early growth. In contrast, TCP binding sites were most abundant in the B homeolog with seven sites compared with one in the A genome and five in the D genome. Similarly, HD-ZIP binding sites were strongly enriched in the D genome with twenty-three sites, whereas only one site was present in the A homeolog and seven in the B genome. SBP (SQUAMOSA promoter-binding protein) family binding sites were more frequent in the B homeolog with four sites compared with three in the D genome and were absent from the A genome indicating shared regulatory control between the B and D genomes. Similarly, WOX (WUSCHEL-related homeobox) binding sites were detected mainly in the B and D sub-genomes with three and one sites respectively while absent from the A homeolog. Finally, ZF-HD binding sites were exclusively detected in the D homeolog with four sites indicating clear sub-genome–specific enrichment.

The observed uneven distribution of developmental TFs across wheat homeologs highlights the asymmetric contribution of each sub-genome to plant growth regulation. The predominance of GATA and TCP families in the A and B genomes suggests that these sub-genomes are central to early vegetative growth and organogenesis, whereas the D genome, enriched with HD-ZIP and ZF-HD members likely supports morphological robustness and structural adaptation (Li 2015, Li *et al.* 2017, Liu *et al.* 2021, Feng *et al.* 2022). Co-occurrence of SBP and WOX families in the B and D sub-genomes implies coordinated regulation of reproductive development and meristem activity consistent with reports linking homeolog-specific TF expression to floral determinacy and tiller formation (Tregear *et*

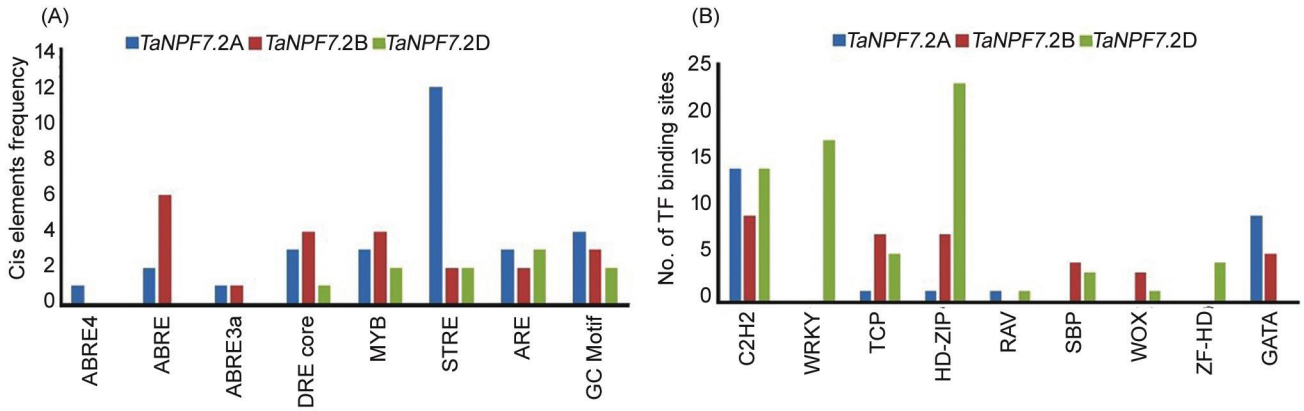

Fig. 2 Different stress related (A) Cis-Regulatory Elements, and (B) transcription factor binding sites, present in upstream region of three homeologs of *TaNPF7.2* gene of a wheat genotype, K9107.

al. 2022, Schoen *et al.* 2025). Together, these findings suggest that transcriptional partitioning among wheat sub-genomes contributes not only to stress tolerance but also to developmental flexibility and adaptability across environments consistent with recent evidence showing extensive homeolog divergence driven by sub-genome–biased transposable element distributions in wheat (Jia *et al.* 2025).

Differential expression of TaNPF7.2 under nitrate and abiotic stress conditions: Under optimal nitrate supply (8.0 mM), *TaNPF7.2* exhibited maximal transcript accumulation in seminal roots at 14 days post-germination (Fig. 3A). Among the homeologs, *TaNPF7.2*-A displayed the highest induction, followed sequentially by *TaNPF7.2*-B and *TaNPF7.2*-D (Fig. 3A). Notably, *TaNPF7.2* maintained measurable expression in lateral roots at both 14 and 21 days (Fig. 3A). Under salt stress (Fig. 3B), *TaNPF7.2* retained its temporal expression peak at 14 days in seminal roots; however, the induction was markedly reduced relative to nitrate conditions. Lateral roots showed only low transcript levels and all homeologs declined sharply by 21 days indicating a short-lived and relatively weak response to salt stress (Fig. 2B). These observations indicate that *TaNPF7.2* plays a secondary role in salinity adaptation. Under drought (PEG-induced) stress (Fig. 3C), *TaNPF7.2* exhibited negligible transcriptional activation at 14 days. A distinct shift occurred at 21st day, where *TaNPF7.2*-B showed upregulation in seminal roots (Fig. 3C). This delayed induction suggests a specialized function of the *TaNPF7.2*-B homeolog in prolonged drought adaptation. Cadmium exposure elicited a distinct transcriptional response with *TaNPF7.2*-D emerging as the predominant early-induced homeolog in both seminal and lateral roots at 14th day (Fig. 3D). *TaNPF7.2*-A and *7.2*-B displayed moderate but consistent induction. By 21 days, expression of all homeologs declined sharply (Fig. 3D), reflecting a rapid yet short-lived responsiveness to cadmium toxicity. This pattern emphasizes a homeolog-specific contribution, with *TaNPF7.2*-D serving as the principal cadmium-responsive homeolog (Fig. 3D).

The expression analyses highlight a dynamic and stress-dependent hierarchy among the *TaNPF7.2* homeologs consistent with recent findings that homoeolog expression bias in hexaploid wheat is dynamic, reprogrammed across conditions and contributes to regulatory variation in response to environmental and genetic contexts (Kumar *et al.* 2023, Glombik *et al.* 2025). Under optimal nitrate conditions, *TaNPF7.2*A exhibited the strongest induction in seminal roots at early seedling stages. The sustained basal expression in root tissues supports the role for *TaNPF7.2* in maintaining nitrate loading into the xylem during early growth stages. Under salt stress, the transcription of all homeologs were significantly reduced, indicating that *TaNPF7.2* is not a major contributor to salinity adaptation. On the other hand, at 14th day cadmium exposure strongly activated *TaNPF7.2*D, which is consistent with the promoter's exceptionally high WRKY enrichment (Wu *et al.* 2022) which suggests

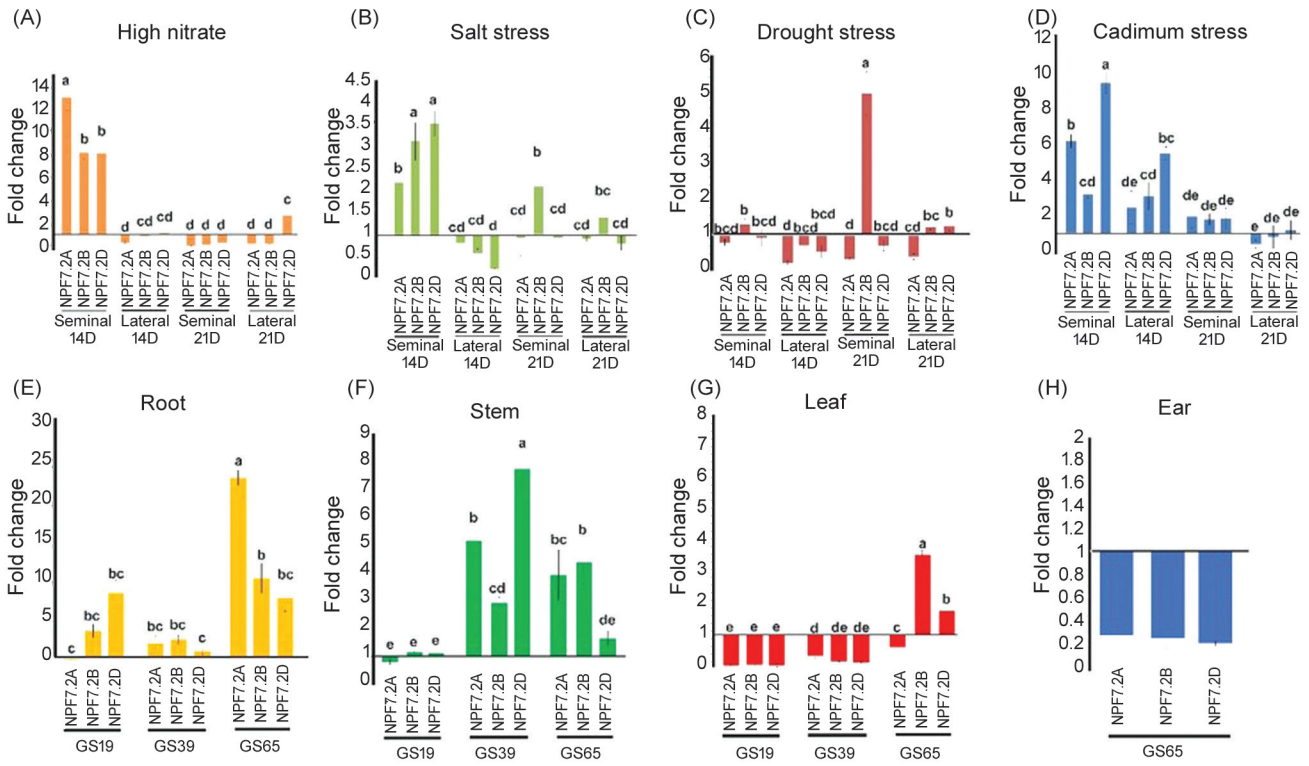

Fig. 3 Relative expression of *TaNPF7.2* homeologs under different treatments, nitrate (8.0 mM), NaCl (100 mM, $CdCl_2$(100 um) and PEG (10) % (A-D) and later growth stages (E-H). Values are mean ± SE of three biological replicates. Different letters indicate statistically significant differences at $p<0.05$.

that *TaNPF7*.2-D may help modulate nitrate transport or root homeostasis under heavy metal-induced oxidative stress. Drought stress induced a delayed activation with *TaNPF7*.2-B showing significant induction only at 21 days. This temporal shift suggests that *TaNPF7*.2-B may function in sustained drought adaptation rather than immediate stress responses. This kind of stress-induced modulation of nitrate transporter activity has been widely reported across multiple nitrate transporter families under diverse abiotic stress conditions (Vashi *et al.* 2025).

Expression dynamics of TaNPF7.2 *during later developmental stages of wheat*: The expression profiling of *TaNPF7*.2 across later growth stages revealed a distinct pattern of homeolog- and tissue-specific regulation (Fig. 3 E-H). In root tissue, the *TaNPF7*.2D homeolog was most strongly expressed at GS19 whereas GS39 showed no substantial homeolog-specific variation (Fig. 3E). By GS65, a shift occurred with *TaNPF7*.2-A exhibiting the highest transcript abundance followed by *7*.2-B and *7*.2-D (Fig. 3E) indicating developmental reprogramming of homeolog dominance as plants progressed toward reproductive stages. In stem tissue, *TaNPF7*.2-D showed consistent and detectable expression at both GS39 and GS65 (Fig. 3F). Leaf tissues displayed overall minimal responsiveness with only mild expression of B and D homeologs at GS65 (Fig. 3G). In ear tissue, expression of all *TaNPF7*.2 homeologs remained negligible across stages (Fig. 3H). The developmental reprogramming observed in this study, with a transition from D-genome dominance in early root stages to A-genome activation during reproductive growth, reflects a dynamic redistribution of nitrate transport capacity in response to metabolic demand (Wang *et al.* 2020).

The present study demonstrates diverse cis-regulatory motifs, transcription factor-binding site connections, and differential expression across stress and developmental phases, generating homeolog-specific regulatory diversity in *TaNPF7*.2. Under nitrate, salt, drought, and cadmium conditions, *TaNPF7*.2-A, *7*.2-B, and *7*.2-D, showed distinct activation patterns, indicating sub-functionalization of the transporter in wheat. Stress treatments significantly reduced total nitrogen accumulation and translocation efficiency, aligning with the transcriptional suppression or delayed activation of *TaNPF7*.2 homeologs. Together, these results enhance our knowledge of how wheat regulates nitrate transport and point to *TaNPF7*.2 as a potential target for increasing nitrogen utilization efficiency in both ideal and stressful growth conditions.

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