Indian Journal of Biochemistry & Biophysics
Vol. 62, November 2025, pp. 1187-1197
DOI: 10.56042/ijbb.v62i11.16688

Comprehensive *in silico* analysis of upstream sequences of *TaNRT2* gene family of bread wheat: Unveiling effects of hormonal cross-talk on differential homeolog expressions of *TaNRT2.1-6* under low nitrogen conditions

Anuj Kumar[1,2], Muhammed Shamnas V[1,2], Amit Kumar Singh[3], Gyan Prakash Mishra[4] & Subodh Kumar Sinha[1]*

[1]ICAR-National Institute for Plant Biotechnology; [2]Division of Molecular Biology & Biotechnology, ICAR- Indian Agricultural Research Institute; [3]Division of Genomic Resources, ICAR-National Bureau of Plant Genetic Resources; & [4]Division of Genetics, ICAR-Indian Agricultural Research Institute, Pusa Campus, New Delhi-110 012, India

Received 26 January 2025; *revised 09 June 2025*

Nitrate is one of the major nitrogen forms available in soil that are crucial for plant growth and development. This study employed multiple bioinformatics tools for a comprehensive *in silico* analysis of approximately 3 kb upstream regions of all *TaNRT2* family genes, which encode members of high-affinity nitrate transporters (HATS) in wheat, retrieved from the Chinese Spring reference genome sequence. The analysis concentrated on identifying Cis-Regulatory Elements (CREs), Transcription Factor Binding Sites (TFBS), evolutionary relationships, tandem repeats, and CpG/CpNpG islands. Furthermore, transcript expression patterns were studied based on CREs of three homeologs of *TaNRT2.1-6* in root and shoot tissues at the seedling stage under low nitrate conditions along with Gibberellic acid (GA_3) and Salicylic acid (SA). The sequence analysis of 3 kb upstream sequence revealed varied types and occurrences of Cis-Regulatory Elements and Transcription Factor Binding Sites, predicting variations in nitrate-dependent gene expression. Key TFBS (LBD, GATA, NIN-like, Dof, MYB, NAC, MIKC_MADS, and bZIP), involved in growth regulation under low nitrogen conditions, were identified in all *TaNRT2* promoters. The present study provides a detailed analysis of the *TaNRT2* gene promoters in wheat, offering insights for designing future studies to understand regulatory mechanisms underlying wheat's N-responsive growth responses.

Keywords: Cis-regulatory elements, Gibberellic acid (GA_3), High-affinity nitrate transporter (NRT2), Salicylic acid (SA), Wheat

Nitrate, a common form of nitrogen in soil, is absorbed by roots and translocated to above-ground parts of the plant. Nitrate molecules also trigger signal transduction that affects the plant's molecular, metabolic, and developmental processes. Nitrate transporters are essential for the absorption and distribution of nitrate (NO_3^-) from soil medium and within plants, respectively. In wheat (*Triticum aestivum* L.), high-affinity nitrate transporters (*HATS*) encoded by *NRT2* (Nitrate Transporter2) family genes are essential for nitrate uptake under low nitrate conditions ranging from 10 to 250 µM[1]. A comprehensive genome-wide analysis identified 46 *NRT2* genes in wheat's genome, many of which are located on the sixth chromosome. Their expression patterns under nitrate deprivation conditions exhibit distinct expression patterns. For instance, *TaNRT2.1* is predominantly expressed in roots and is significantly upregulated under nitrogen-limiting conditions, highlighting their possible role in nitrogen acquisition[2-4]. Interestingly, a few of the *NRT2* genes exhibit reduced expression under similar conditions, suggesting functional divergence within the family. Understanding the functional diversity and regulatory mechanisms of *NRT2* genes is crucial for improving wheat's nitrogen use efficiency.

Previously, a few important CREs were identified in N-responsive genes of Arabidopsis and rice. The nitrate-specific regulation of a 150 bp segment from the promoter of the nitrate transporter gene, *AtNRT2.1*, was demonstrated in Arabidopsis[5]. Major cis-acting elements identified in the promoter of *bHLH001* of *Chenopodium glaucum* L. revealed hormone-associated signalling, enabling it to trigger a response to abiotic stress and light-induced mechanisms[6]. *GhNAC2* promoter analysis has shown increased expression in root tissues under the influence of GA, ethylene, auxin, ABA, mannitol, and NaCl, suggesting that *GhNAC2* promoter elements

*Correspondence:
E-mail: subsinha@gmail.com
Suppl. data available on respective page of NOPR

independently govern *GhNAC2* expression, influencing root growth and stress-related functions. *TaNAC2-5A* is a nitrate-inducible NAC transcription factor in wheat, and *TaNAC2* binds to promoters of *TaNRT2.5-3B* nitrate transporter genes[7]. Overexpression of *OsSNAC1* with the promoters of *OsNRT2.1/2.2* increased nitrate in roots and shoots, leading to higher N uptake and plant biomass[8,29]. Conversely, mutations in *OsSNAC1* reduced N uptake and NUE, inhibiting plant growth[8]. In *Arabidopsis thaliana*, a 1.8 kb promoter fragment of the *NIA1* gene enhances nitrate response and shows enhancer activity, which is repressed by ammonium[9]. An *in silico* analysis of 40 *OsGLP* gene promoters in rice revealed that genes with promoters from within the same clade exhibited identical expression patterns of genes at different binding sites for transcription factors, enabling them to respond effectively to diverse biotic and abiotic stresses[10].

Plant hormones like salicylic acid, ABA, GA_3, and auxin play key roles in coordinating local and long-distance signalling pathways[11]. The transcript levels of *ZmNRT2.1* and *ZmNRT2.2* were inhibited in plants with *zmga3ox* mutations, whereas GA_3 treatment increased their expression[12]. SA also plays a role in controlling N assimilation by influencing NR function in *Citrullus lanatus*[13]. As SA concentration rises, total N contents first rise and subsequently fall in tandem with changes in NR activity, particularly during germination and seedling growth[14,15]. The present study aims to identify the CREs from the promoter region of 46 *NRT2* genes in the Chinese Spring reference genome sequence of bread wheat to understand the functional diversity among members of the *NRT2* family genes. Furthermore, we also identified transcription factor binding sites, tandem repeats, and other features in these regions. We also tested the effects of GA, SA, and their combination on seedling morphology and expression patterns of *TaNRT2.1,* one of the main genes for the high-affinity nitrate transporter.

Materials and Methods

Sequence retrieval and phylogenetic relationship in 3 kb upstream sequence of 46 *TaNRT2* genes

The 3 kb promoter sequences and protein sequences of all 46 *TaNRT2* genes were retrieved from the Plant Ensemble database (https://plants.ensembl.org/Triticum_aestivum/Info/Index)[16]. The accuracy of these promoter sequences was reconfirmed using Phytozome v13 (https://phytozome-next.jgi.doe.gov/)[17]. For phylogenetic analysis, these 46 promoter sequences were examined utilizing the MEGA7 software's p-distance model and the Neighbor-Joining method[18]. The percentage of parallel trees where related taxa clustered together was determined using the bootstrap test (1000 repetitions). We followed the nomenclature of genes as described by Kumar *et al*[2], considering phylogenetic relationships, sub-genomic locations (A, B, D), and in-paralog relationships. Each nitrate transporter gene (*TaNRT2*) was assigned a unique identifier for analysis and interpretation[2] (Suppl. Table S1).

Examination of 3 kb upstream promoter sequences for Transcription Factor Binding Sites, CpG/CpNpG islands, tandem repeats, and Cis-Regulatory Elements

To examine Cis-Regulatory Elements in the upstream sequence of 46 *TaNRT2* genes, the 3 kb upstream sequences from the translation start site (ATG) were obtained from the Plant Ensemble database (https://plants.ensembl.org/Triticum aestivum/Info/Index)[2], following that, the promoter sequences were uploaded to the PLANTCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/)[19] and verified in NEW PLACE (https://www.dna.affrc.go.jp/PLACE/?action=newplace) to identify various Cis-Regulatory Elements of the promoters of *TaNRT2* genes of wheat (Suppl. Table S2). Additionally, to comprehend the regulatory pathways of *TaNRT2* gene expression patterns using PlantPAN 3.0 software, Transcription Factor Binding Sites were identified in the promoter regions (http://plantpan.itps.ncku. edu.tw/promoter.php). The MPAP (Multiple Promoter Analysis Program) was also used to identify common TFBS among promoters (Suppl. Table S3).

Additionally, the tandem repeats (TRs) of these genes, and CpG/CpNpG islands within the 3 kb upstream promoter sequences were analysed using PlantPAN 3.0[20]. Three factors were taken into consideration to classify a DNA sequence as a CpG island: (i) content of GC exceeding 50%, (ii) a CpG between observed and expected dinucleotide proportion should be above 0.6, and (iii) length greater than 200 base pairs.

Seedling growth, biomass measurement, RNA extraction, and qRT-PCR analysis

The wheat genotype K9107 (K9), an efficient nitrogen uptake genotype[4], was utilized to investigate the expression of *TaNRT2.1A/B/D* genes. Wheat seedlings were grown in a hydroponic setup using MS nutrient medium as previously described[2], under two

nitrate concentrations: 8 mM (N-plus; N+) and 0.08 mM (N-minus; N-). Initially, the seedlings were grown in the N-plus condition for ten days, followed by a transfer to media with or without nitrate, along with gibberellic acid (100 nM GA_3; GA) and salicylic acid (0.5 mM SA; SA) and their combination, such as GA_3+SA(GS), in both conditions for an additional four days[21-23]. All experiments were conducted under controlled, sterile conditions at 25 ± 1°C, with a light intensity of 150-200 µmoles photon/m²/s, with a 10/14 h dark/light cycle and 70% relative humidity. Total RNA was extracted from the frozen root and shoot tissues of 14-day-old K9107 seedlings. cDNA was synthesized following the method outlined[2].To ensure specificity, sub-genome sequence-specific primers were designed by aligning the homeologous sequences to identify SNPs at the 3' end, facilitating precise primer binding and amplification. The pairs of primers were confirmed for their specific binding in the wheat cDNA at the Plant Ensemble database. The expression patterns of *TaNRT2.1A/B/D* gene in root and shoot tissues were studied under different conditions described above. The fold change expression of each homeolog's expression under N- (N-minus) conditions was compared to N-plus conditions.

Results

Phylogenetic examination of promoter sequences of 46 *TaNRT2* genes

To investigate the phylogenetic relationship among the 3 kb upstream sequence of *TaNRT2*s, a phylogenetic tree based on neighbour-joining method consisting of 46 *TaNRT2* was generated after the multiple-alignment of the upstream sequences. The 46 upstream sequences are separated into three distinct clades with bootstrap values ranging from 0 to 1000. Of these, clade I was divided into two clusters as well. Cluster I included five promoter sequences from the *TaNRT2.4* class (Fig. 1). Clade II consisted of 5 clusters, with 19 promoter sequences from *TaNRT2.1*, along with three promoter sequences from the *TaNRT2.5* class. Cluster I of clade II contained three gene promoters (*TaNRT2.5*-A1, *TaNRT2.5*-B1, and *TaNRT2.5*-D1), while Cluster II consisted of 3 promoters (*TaNRT2.1*-A6, *TaNRT2.1*-D6, and *TaNRT2.1*-B6). Cluster III contained five promoters of genes, *viz*., *TaNRT2.1*-A4, *TaNRT2.1*-D3, *TaNRT2.1*-B3, and *TaNRT2.1*-A2), while Cluster IV contained three promoters of genes, viz., *TaNRT2.1*-A5, *TaNRT2.1*-D5, and *TaNRT2.1*-B5). Lastly, Cluster V had five promoter sequences (*TaNRT2.1*-A1, *TaNRT2.1*-D1, *TaNRT2.1*-B1, *TaNRT2.1*-B2, and *TaNRT2.1*-A2). In Clade III, a total of 22 promoter sequences of the *TaNRT2.2* class were observed, with two promoter sequences associated with *TaNRT2.3-A1* and *TaNRT2.3-D1* (Fig. 1). This detailed mapping and phylogenetic analysis provide insights into the genetic organization and evolutionary relationships of the *TaNRT2* gene promoters.

Identification and frequency of transcription factor binding sites in the upstream sequence of the nitrate transporter (*NRT2*) genes

The promoter sequences of 46 *TaNRT2* genes were analysed using PlantPAN 3.0 to identify common Transcription Factor Binding Sites. The database provided 11,447 TFBS based on the input queries. Out of the total TFBS, we identified a total of eight types of TFBS with a total of 3322 binding sites, which are known for their response in the regulation of development and growth under low nitrogen conditions and response to both biotic and abiotic stresses. These TFBS have been shown to have some association with nitrate transporter (LBD, GATA, NIN-like, Dof, MYB, NAC, MIKC_MADS, and bZIP)[24-33]. In this study, the most prevalent (found in every input query gene) TFBS were MIKC_MADS (812) followed by MYB (693), sharing 24.44% and 20.86% binding sites of a total of 3322 repeats in all

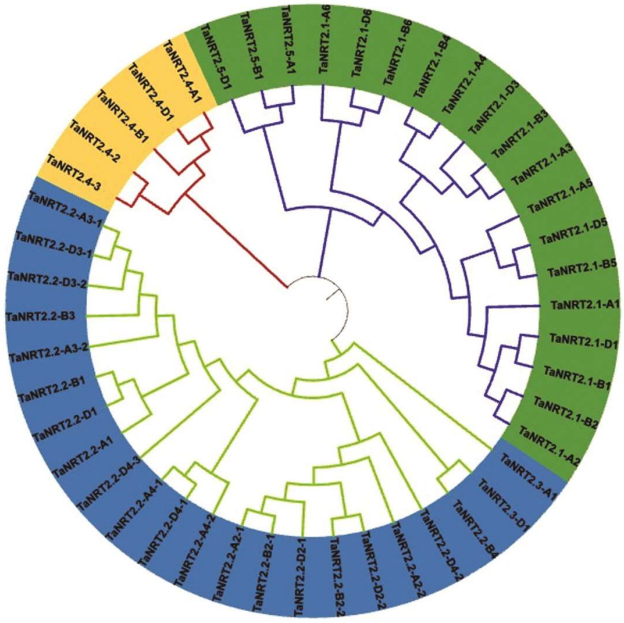

Fig. 1 — Phylogenetic relationship among 3 kb promoter sequences of the 46 *TaNRT2* genes of wheat. MUSCLE was used to align the sequences, and MEGA 7 was to build tree following the neighbor-joining approach

46 promoter sequences. The highest numbers of TFBS were found in *TaNRT2.4-2*, followed by *TaNRT2.1-B6*, with 121 and 119 repeats, respectively. The other TFBSs, including LBD, GATA, NIN-like, Dof, NAC, and bZIP, were also present, albeit with relatively lower frequency with 202, 505, 218, 461, 431, and 328 binding sites, respectively. The *TaNRT2.4-3* promoter region exhibited the highest number of LBD (27), while the *TaNRT2.1-D6* promoter had the most GATA (28), NIN-like (16) in the *TaNRT2.4-B1*, Dof (37) in the *TaNRT2.4-A1,* MYB (40) in the *TaNRT2.1-D,* NAC (27) in the *TaNRT2.1-B6*, MIKC_MADS (42) and bZIP (28) *TaNRT2.2-B4* in the *TaNRT2.1-D5* gene promoter. Our findings on the frequency of TFBS indicate the expression pattern of several *TaNRT2* genes (*TaNRT2.4-3, TaNRT2.1-D6, TaNRT2.4-B1, TaNRT2.4-A1, TaNRT2.1-D, TaNRT2.1-B6, and TaNRT2.1-D5*) is controlled by various transcription factor families that respond to low nitrogen conditions (Fig.2, and Suppl. Table S3).

CpG/CpNpG analysis and tandem repeats in targeted gene promoters

Tandem repeats and CpG/CpNpG analysis in targeted promoter sequences are important because they can help elucidate epigenetic regulation. Our investigation found CpG/CpNpG islands along with tandem repeats in a 3 kb upstream sequence of the 46 *TaNRT2* genes. Furthermore, out of the 46 promoter regions, 34 contained CpG/CpNpG islands, aside from *TaNRT2.1-A3, TaNRT2.1-B3, TaNRT2.1-D3, TaNRT2.1-A4, TaNRT2.2-B1, TaNRT2.2-D1, TaNRT2.2-A2-2, TaNRT2.2-A3-1, TaNRT2.2-D3-1, TaNRT2.2-D3-2, TaNRT2.2-A4-1, TaNRT2.2-B4, TaNRT2.2-D4-1, TaNRT2.2-D4-2,* and *TaNRT2.5-B1* (Table 1A). These genes encode proteins involved in nitrate transport at different stages. Transcriptional divergence rates are higher in genes that contain tandem repeats (TRs) in their promoters. TRs, often referred to as satellite DNA, are sequences that indicate a higher mutation rate[44]. TRs fall into three categories based on repeat length: microsatellites (1-6 nucleotides), mini satellites (6–100 bp), and mega satellites (>135 nucleotides). This study found TRs in 18 out of 46 promoter sequences, indicating a higher mutation potential due to polymerase slippage. The promoters of five genes (*TaNRT2.1-A4, TaNRT2.2-A1, TaNRT2.2-A3-1, TaNRT2.2-D3-1,* and *TaNRT2.2-D3-2*) contained repeats of less than six nucleotides, classifying them as microsatellites. Thirteen gene promoters (*TaNRT2.1-A3, TaNRT2.1-B3, TaNRT2.1-A4, TaNRT2.2-B1, TaNRT2.2-A2-2, TaNRT2.2-B2-2, TaNRT2.2-D2-1, TaNRT2.2-D2-2, TaNRT2.2-A4-2, TaNRT2.3-A1, TaNRT2.4-2, and TaNRT2.4-3*) contained mini satellites, and only the promoters of *TaNRT2.2-D4-2* and *TaNRT2.4-B1* contained mega satellites (Table 1B).

Cis-Regulatory Element analysis of 46 *TaNRT2* gene promoters

The upstream region of 46 *TaNRT2* genes was analysed using PLANTCARE software to identify possible Cis-Regulatory Elements. Seventy-seven elements were identified and categorized into six groups: light-responsive, core promoter-related,

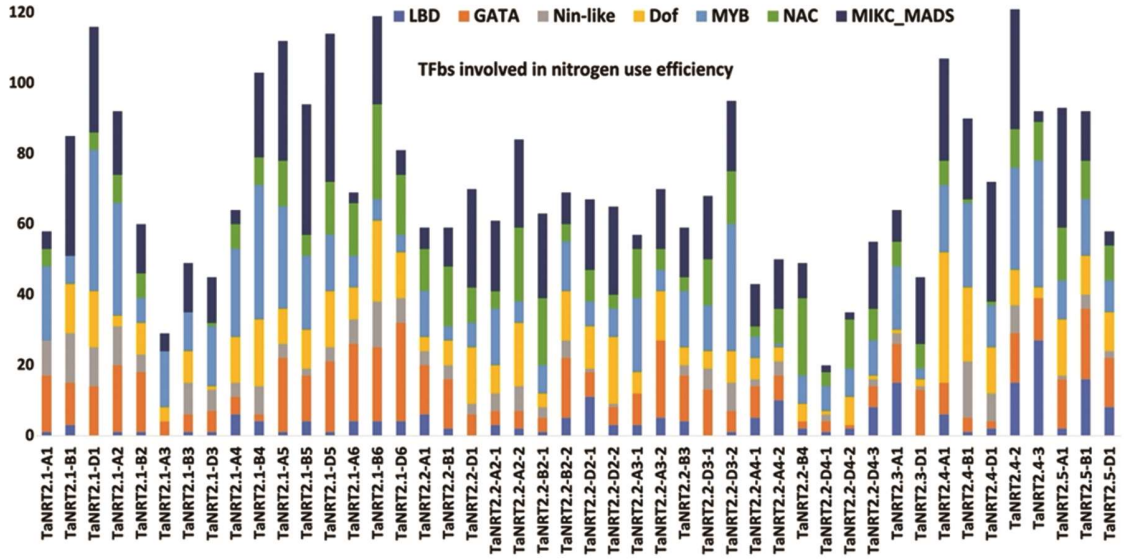

Fig. 2 — Transcription Factor Binding Sites (TFBS) in each promoter sequence of *TaNRT2* are represented by different colours

Table 1 — The details of CpG/ CpNpG motifs (A) or tandem repeats (B) identified in upstream regions.

A. CpG Islands						B. Tandem Repeats								
Gene name	Begin site	End site	Length	G+C frequency	CpG o/e ratio	Gene name	Location	Period size	Copy number	Consensus size	Percent matches	Percent indels	Score	Entropy (0-2)
TaNRT2.1-A1	16	536	513	0.5	0.6	TaNRT2.1-A3	891-938	23	2.1	23	88	3	71	1.91
TaNRT2.1-B1	1763	2989	1206	0.5	0.84	TaNRT2.1-B3	865-899	18	1.9	18	94	0	61	1.92
TaNRT2.1-D1	2025	2989	949	0.51	0.8		1049-1096	23	2.1	23	84	3	62	1.85
TaNRT2.1-A2	945	1619	664	0.48	0.6	TaNRT2.1-A4	62-95	15	2.3	15	90	5	52	1.66
TaNRT2.1-B2	1073	1675	593	0.48	0.73		1093-1154	2	31	2	100	0	124	1
TaNRT2.1-B4	1780	2340	552	0.5	0.85		1757-1802	18	2.6	18	96	0	83	1.87
TaNRT2.1-A5	2181	2989	795	0.5	0.87	TaNRT2.2-A1	1627-1665	2	19.5	2	100	0	78	1
TaNRT2.1-B5	2018	2853	823	0.5	0.87	TaNRT2.2-B1	538-573	17	2.1	17	94	0	63	1.8
TaNRT2.1-D5	1998	3008	975	0.5	0.79	TaNRT2.2-A2-2	569-652	43	2	43	90	0	132	1.85
TaNRT2.1-A6	2184	3008	792	0.49	0.83		1732-1782	24	2.1	24	92	0	84	1.91
TaNRT2.1-B6	2205	2913	698	0.5	0.73		2592-2635	22	2	22	90	0	70	1.95
TaNRT2.1-D6	1	604	595	0.52	1.01	TaNRT2.2-B2-2	1409-1511	37	2.8	37	91	2	145	1.71
TaNRT2.2-A1	1	1349	1327	0.57	1		1446-1509	21	3.3	21	64	28	59	1.62
TaNRT2.2-A2-1	966	1960	978	0.5	0.9		2583-2628	23	2	23	95	0	83	1.88
TaNRT2.2-B2-1	795	1716	897	0.49	0.84	TaNRT2.2-D2-1	233-269	17	2.1	18	90	5	58	1.94
TaNRT2.2-B2-2	1006	1999	978	0.5	0.96	TaNRT2.2-D2-2	2591-2634	22	2	22	95	0	79	1.93
TaNRT2.2-D2-1	963	1838	861	0.48	0.86	TaNRT2.2-A3-1	2319-2355	2	18.5	2	100	0	74	1
TaNRT2.2-D2-2	1124	1737	604	0.47	1.07	TaNRT2.2-D3-1	2328-2364	2	18.5	2	100	0	74	1
TaNRT2.2-A3-2	855	2284	1265	0.6	0.83	TaNRT2.2-D3-2	2303-2353	2	25.5	2	100	0	102	1
TaNRT2.2-B3	15	1176	1143	0.53	0.64		2353-2380	2	14	2	100	0	56	1
TaNRT2.2-A4-2	638	2389	1723	0.56	0.89	TaNRT2.2-A4-2	913-1014	38	2.7	38	92	0	168	1.96
TaNRT2.2-D4-3	774	2457	1684	0.49	0.89	TaNRT2.2-D4-2	580-880	160	1.9	160	90	2	480	1.89
TaNRT2.3-A1	1	1875	1845	0.56	0.89	TaNRT2.3-A1	1363-1442	35	2.3	33	79	12	88	1.99
TaNRT2.3-D1	1	1628	1602	0.52	0.83		1380-1448	35	2	35	94	0	120	1.99
	286	927	631	0.49	0.98	TaNRT2.4-2	1648-1907	130	2	130	84	4	337	1.97
TaNRT2.4-D1	2455	3001	526	0.5	1.04	TaNRT2.4-B1	1160-1207	19	2.5	19	100	0	96	1.74
TaNRT2.4-2	2342	3000	637	0.52	0.92		2248-2377	28	4.6	28	94	1	208	1.62
TaNRT2.4-A1	2229	3001	748	0.54	1.07		2269-2377	16	7.6	16	60	22	53	1.6
TaNRT2.4-B1	1750	3003	1219	0.55	0.95	TaNRT2.4-3	375-570	93	2.1	92	98	0	374	1.95
TaNRT2.4-3	554	3003	2450	0.57	1.02		1298-1403	52	2	53	87	1	151	1.56
TaNRT2.5-B1	54	3004	2951	0.55	0.99									
TaNRT2.5-D1	265	1114	694	0.52	1.06									

abiotic and biotic stress-responsive, development related, hormone-related, and circadian cis-regulatory elements (Fig.3 & Suppl. Table S2). The *TaNRT2.1-A4* gene had the highest number of elements, followed by *TaNRT2.2-D3-2* and *TaNRT2.2-D4-2*. Promoter-related cis-elements comprised the most significant proportion (38.90% in *TaNRT2.1-A4*), followed by abiotic and biotic stress-responsive elements (28.51% in *TaNRT2.2-D2-1*), hormone-related elements (13.56% in *TaNRT2.2-D4-3*), light-responsive elements (11.01% in *TaNRT2.2-D4-3*), and development-related elements (7.84% in *TaNRT2.2-D3-2, TaNRT2.3-A1, TaNRT2.4-B1*) (Suppl. Table S3). More than 85% of CREs belong to CAAT-box (97.82%), TATA-box (91.30%), CGTCA-motif (93.47%), ABRE (82.60%), G-box (82.04%), and ARE (63.04%) (Fig. 4). These elements are involved in various regulatory functions, including hormones regulation, light responses, stress responses, growth stages, and anaerobic induction. Analysis of *TaNRT2* genes showed an abundant presence of core promoter elements like TATA-box, CAAT-box, AT~TATA box, and A-box elements in all 46 *TaNRT2* genes. *TaNRT2.1-A4* and *TaNRT2.2-D3-2* promoter had the highest number of TATA-box (87), and CAAT-box (23), respectively. The above elements serve as transcription factor binding sites (Fig. 4). Box 4, G-Box, GA-motif, GATA-motif, GT1-motif, I-box, TCT-motif, ACE, CAG-motif, chs-CMA2a, Sp1, TCCC-motif, AE-box, ATCT-motif, GTGGC-motif, ATC-motif, MRE, Box II, 3-AF1 binding site, chs-CMA2c, chs-Unit 1 m1, LS7, and C-box were all included in the light-responsive category. The transcription rates of light-controlled genes were maintained by the abundance of Box 4 and G-box motifs, particularly in the promoters of *TaNRT2.2-D4-3* (14), *TaNRT2.2-D4-1, TaNRT2.2-D4-2,* and *TaNRT2.4-3* (each with 13 light-responsive motifs) (Fig. 4). All *TaNRT2* gene promoters had stress-responsive Cis-elements, which could be divided into biotic and abiotic stress-responsive elements. ARE, CCAAT-box, DRE core, DRE 1, GC-motif, LTR,

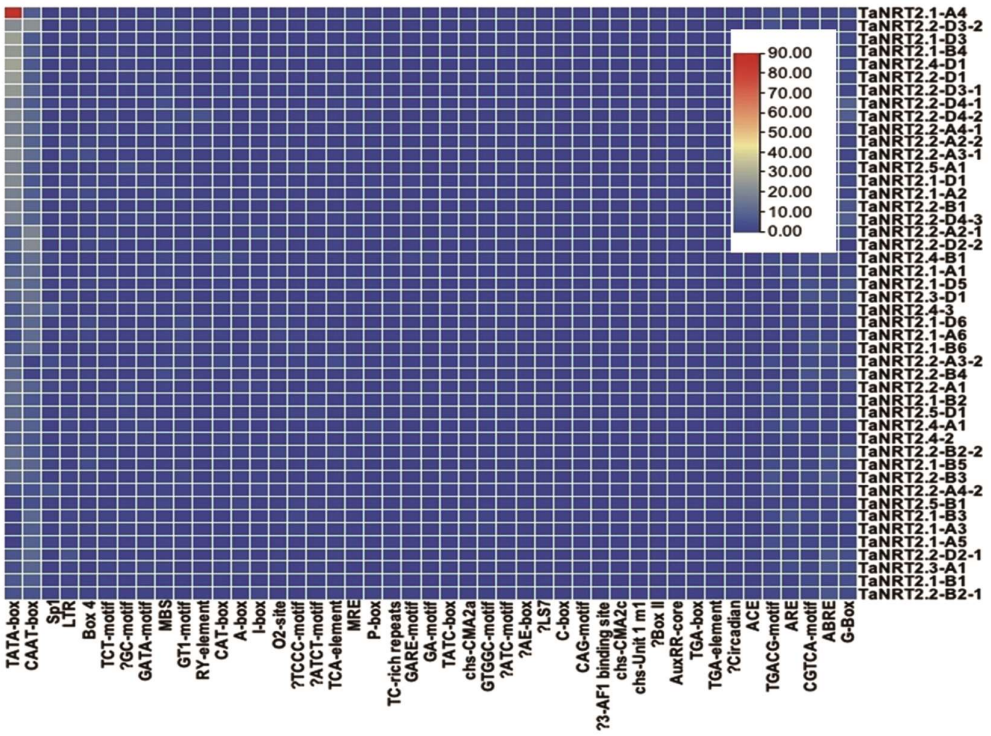

Fig. 3 — A graphical depiction of common Cis-Regulatory Elements in the promoter sequences of the 46 *TaNRT2* genes in wheat

MBS, MBS 1, STRE, TC rich repeats, MYB, MYC, MYB recognition site, MYB binding site, MYB-like site, and AT-rich element were among the elements linked to abiotic stress. The components associated with biotic stress included box S, WRE3, W-box, and WUN-motif. *TaNRT2.2-D2-1* had the highest frequency of stress-responsive elements (29), followed by *TaNRT2.1-A1* and *TaNRT2.4-B1* (28 each) (Fig. 4). The wound-responsive elements, Box S, W-box, WRE 3, and WUN-motif elements interact with WRKY transcription factors to protect against biotic stresses[46].

Hormone-related CREs present in promoter sequences include ABRE, TCA-element, TGACG-motif, TGA-element, ABRE4, ABRE2, ABRE3a, ERE, GARE-motif, P-Box, TATC-box, AuxRR-Core, and CGTCA-motif. Promoters of *TaNRT2.2-D4-3*, *TaNRT2.2-D2-1*, and *TaNRT2.2-D4-1*contain 18, 16, and 15 hormone-related motifs, respectively. ABRE and CGTCA-motif, classified as methyl jasmonate-responsive and abscisic acid-responsive elements, were common in response to hormone-related changes linked to nitrogen use efficiency[34-36]. ABRE4, ABRE2, and ABRE3a acted as binding sites for the abscisic acid hormone-related transcription factors[46].Whereas CGTCA-motif and TGACG-motif reacted to methyl jasmonate, AuxRE, TGA-element, and AuxRR-Core reacted to auxin[40]. The salicylic reaction engaged the TCA element, while gibberellin elicited responses from P-box, TATC-box, and GARE-motif (Fig. 4).

Development-related CREs includes CAT-box, O2-site, RY-element, AAGAA-motif, AACA-motif, CCGTCC-box, CCGTCC motif, as-1, E2Fb, CARE, dOCT, F-box, GCN4-motif, MSA-like, and NON-motif. Among these, as-1 and CAT-box elements were abundantly present, 97 and 28, respectively, in all promoter sequences, functioning as meristem-specific stimulation promoters[46]. The genes with the highest number of growth and development-related motifs are *TaNRT2.2-D3-2, TaNRT2.3-A1,* and *TaNRT2.4-B1*, each with 11 motifs in their promoter sequences. Out of 46, only five genes (*TaNRT2.2-B2-1, TaNRT2.2-B2-2, TaNRT2.2-D2-, TaNRT2.2-B4 and TaNRT2.2-D4-3*), has circadian control element each with single motif in their upstream sequence. Different development-related cis-elements play roles in pathogenesis, vascular growth, endosperm expression, zein metabolism regulation, cell cycle regulation, mitosis-specific activation, and production and breakdown of biological macromolecules, as demonstrated in (Fig. 4).

Effects of gibberellic and salicylic acid hormones on *TaNRT2.1* gene expression

The high-affinity nitrate transporter, which is

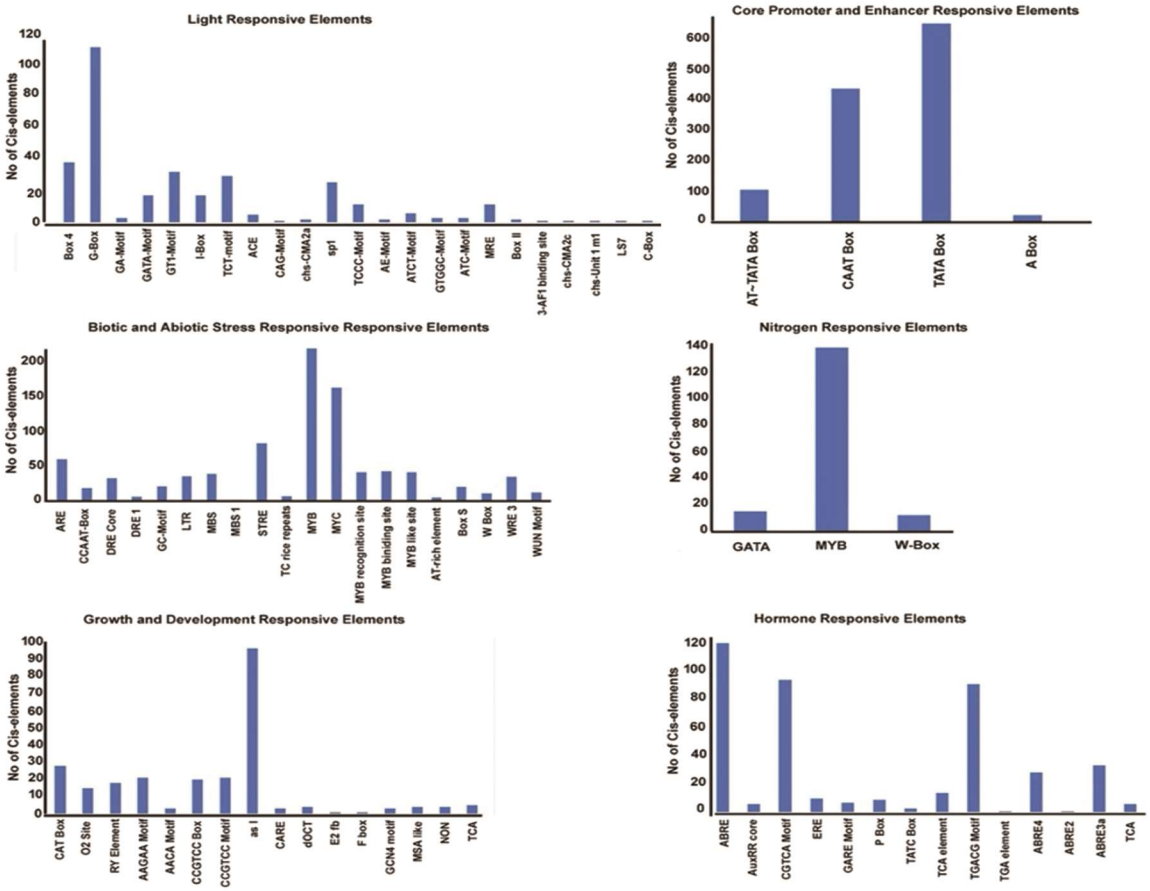

Fig. 4 — Cis-Regulatory Elements distribution in the upstream sequences of 46 *TaNRT2* genes of wheat. CREs are categorized into six groups: core-promoter, light, hormone, growth and development, abiotic and biotic stress, development-related and nitrogen-responsible elements. Notable motifs include TATA, CAAT, ARE, DRE, W-box, ABRE, G-box, CAT-box, RY-element, and GC-motif, highlighting their diverse transcriptional regulation under diverse conditions and stress responses

expressed in the phloem roots[1], epidermis, seeds, leaves, and other plant organs, is crucial for nitrate uptake and transport in plants[38]. The *TaNRT2* genes are widely expressed in all major organs, with the highest expression of *TaNRT2.1* in root tissues, particularly in 14-day-old seedlings[4]. In this study, we assessed the *TaNRT2.1-6A/B/D* genes responses of 14-day-old wheat seedlings grown under hydroponic conditions with varying nitrate levels in the presence of gibberellic acid (GA) and salicylic acid (SA). The qRT-PCR results demonstrate that *TaNRT2.1-6D* and *TaNRT2.1-6B* genes are highly responsive to low nitrogen conditions, followed by *TaNRT2.1-6D* and *TaNRT2.1-6B* in the root (Fig. 5A & B). *TaNRT2.1-6A* has a minor response in the root or shoot, particularly when treated with salicylic acid than that of B and D derived transcripts. When both GA and SA were applied under low nitrogen conditions, only *TaNRT2.1-6D* showed moderate expression in both roots and shoots. This indicates that *TaNRT2.1-6D* might be essential to the mutual reaction of wheat seedlings to the combined hormonal treatment under nitrogen stress (Fig. 5C). The application of salicylic acid and gibberellic acid, both individually and in combination, under low nitrogen conditions significantly encouraged wheat growth seedlings. The increased shoot and root lengths, along with higher fresh and dry biomass, demonstrate the potential of these hormones to enhance plant growth and stress resilience (Fig. 5D-G). These findings suggest that SA and GA treatments might have some cross-talks with nitrate sensing and acquisition in wheat seedlings in nitrogen-limited environments.

Discussion

Wheat has a relatively large number of *NRT2* genes compared to rice, Arabidopsis, and other species due to its large and complex genomes[4]. Uncovering the CRE that dictates the timing and level of gene transcription at tissue and cellular level is a central

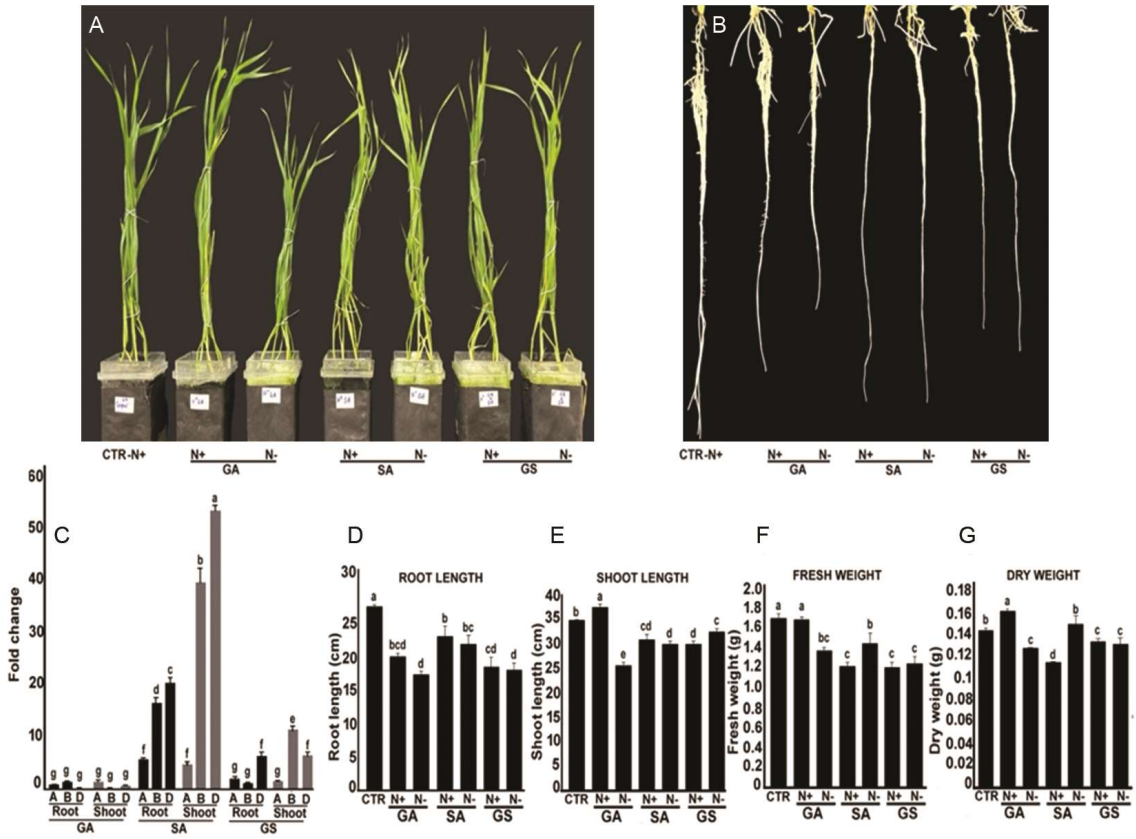

Fig. 5 — GA3 and SA responses on wheat seedlings under different external nitrate conditions. (A) Wheat seedlings grown under different N and hormones treatment in hydroponic condition; (B) Root length under different treatments; (C) Expression pattern of the *TaNRT2.1-6A/B/D* genes in response to low nitrogen conditions along with GA_3 and SA hormones. Biomass; (D) Root length; (E) Shoot length; (F) Fresh weight; and (G) Dry weight) of wheat seedlings under different treatments

goal of biology. In this analysis, we employed web-based bioinformatics resources to analyse the promoter sequences of 46 *TaNRT2* genes in wheat. We identified transcription factor binding sites, cis-regulatory elements, and motifs. Our results revealed key cis-elements and their transcription factors, which play roles in nitrate transportation during growth, development, and stress response. This information can help understand nitrate pathways in wheat and other crops.

The promoter sequences of *TaNRT2* genes can be grouped into three main clades. Clade I contain five *TaNRT2.4* promoter sequences. Clade II has 19 *TaNRT2.1* and three *TaNRT2.5* promoter sequences. Clade III includes 22 *TaNRT2.2* and two *TaNRT2.3* promoter sequences. Two *TaNRT2.4* promoter sequences on different chromosomes are highly similar. This analysis helps us understand the genetic and evolutionary relationships among *TaNRT2* genes and their structural conservation. The TF families (TCP, WRKY, bHLH, NAC, BES1, bZIP, MYB, GATA, and AP2/ERF) in wheat and other crop species are categorised based on their pathways[39]. We found out multiple TFBS (LBD, GATA, NIN-like, Dof, MYB, NAC, MIKC_MADS and bZIP) having roles in controlling growth and development, low-nitrate response in *TaNRT2* genes. The OsMADS57 has been previously shown to interact with promoter sequence of *OsNRT2.3a,* involved in nitrate translocation[6]. The Dof binding site has been observed in different *NRT2* gene promoters, which has been implicated in several agronomic traits, such as taller height, longer spikes, more spikes per plant, more grains perspike, and heavier grains, in wheat when *TaDof1* was overexpressed[25]. On contrary, the overexpression of *TabZIP60* have shown negative effect on N uptake, lateral root branching, spike number *etc*, in wheat[40]. Under low-nitrogen conditions, *OsMYB305* overexpression increased tiller number, shoot dry weight, and total N concentration and promoted rice growth[27]. LBD37, a novel repressor, found to repress many N-responsive genes

involved in N uptake and assimilation in Arabidopsis[28]. In addition to its involvement in nitrate signalling, overexpression of *TaNAC2-5A* improved root growth, nitrate influx traits and its ability to efficient N uptake[29].

We also examined the 3 kb upstream promoter sequences of 46 nitrate transporter genes to identify tandem repeats (TRs). In this analysis, the promoters of five genes (*TaNRT2.1-A4, TaNRT2.2-A1, TaNRT2.2-A3-1, TaNRT2.2-D3-1,* and *TaNRT2.2-D3-2*) contained repeats of less than six nucleotides, classifying them as microsatellites. Thirteen gene promoters (*TaNRT2.1-A3, TaNRT2.1-B3, TaNRT2.1-A4, TaNRT2.2-B1, TaNRT2.2-A2-2, TaNRT2.2-B2-2, TaNRT2.2-D2-1, TaNRT2.2-D2-2, TaNRT2.2-A4-2, TaNRT2.3-A1, TaNRT2.4-2,* and *TaNRT2.4-3*) contained mini satellites, and only the promoters of *TaNRT2.2-D4-2* and *TaNRT2.4-B1* contained mega satellites. A general relationship between variations in gene expression and variations in Short Tandem Repeats (STR) length in *A. thaliana* was supported by the STR length, which showed a correlation between gene expression and STR length variation[44].Being dynamic in nature in the promoter, tandem repeats can alter local chromatin structure, and may consequently help with the evolutionary tuning of gene expression[44-45].

The presence of CpG/CpNpG islands in 34*TaNRT2* gene promoters suggests potential methylation-induced transcriptional repression that affects gene expression. However, the absence of these islands in some *TaNRT2* genes indicates that their expression may not be repressed by cytosine methylation and might be controlled by some other epigenetic processes, like post-translational changes of histones[45]. The longest island of CpG was spotted in the *TaNRT2.5-B1* promoter sequence (2951 bp), closely matching the targeted query length (3000 bp). Many protein-coding genes also showed promoter DNA methylation, which can be used to silence their expression[42].

Seventy-seven motifs were identified and categorized into six groups: the *TaNRT2.1-A4* gene had the highest number of motifs, followed by *TaNRT2.2-D3-2* and *TaNRT2.2-D4-2*. Promoter-related cis-elements comprised the largest proportion (38.90% in *TaNRT2.1-A4*), followed by abiotic and biotic stress-responsive elements (28.51% in *TaNRT2.2-D2-1*), hormone-related elements (13.56% in *TaNRT2.2-D4-3*), light-responsive elements (11.01% in *TaNRT2.2-D4-3*), and development-related elements (7.84% in *TaNRT2.2-D3-2, TaNRT2.3-A1, TaNRT2.4-B1*). An enhancer-like component involved in anoxic-specific inducibility is the GC-motif, while elicitor-mediated activation during stress reactions is linked to the AT-rich region[46]. Hormone signal transduction, disease resistance, abiotic stress tolerance, secondary metabolism, and growth in plants all depend on MYB transcription factors[31]. The DRE, STRE, LTR, and MBS motifs are among the Cis-Regulatory Elements that affect the regulation of abiotic responsive genes in plants[41]. WRKY TFs interact with the fungal elicitor-responsive transcription factor W-box[42]. The upstream sequence of the genes linked to abiotic stresses has also been shown to contain a number of cis-elements, such as G-Box, ACE, Sp1, TCT-motif, Box-4, and GATA motif[46]. The G-box motif has been linked to stress reactions, hormone signalling (ABA and ethylene), and photosynthesis[43]. The ABRE core primarily binds the bZIP TFs, and a number of bZIP TFs are involved in controlling the ABA-dependent stress response[46].

Hormone-related components, such as ABA, auxin, salicylic, and jasmonic acid elements, were present in every promoter sequence[42]. It showed that a wide range of hormones control the expression of the nitrate-responsive genes, including *TaNRT2s* of wheat, which highlights cross-talks of different hormones in nitrate mediated coordination of physiological and developmental processes of wheat. Nitrogen uptake and transport in plants depends on the activity and efficiency of nitrate transporters, which are genetically governed by physiological processes in the presence of different external cues. However, *TaNRT2* family members in wheat have not been systematically identified. Our study shows that *TaNRT2.1-6D* and *TaNRT2.1-6B* are highly responsive to low nitrogen, especially in roots. *TaNRT2.1-6A* shows minimal response, particularly under salicylic acid treatment. *TaNRT2.1-6B* gene is predominantly expressed in root, and found to recover[15] N uptake of *atnrt2.1* mutant[2]. The hormones like, abscisic acid (ABA) and salicylic acid have been found to cause alteration on expression of *MsNRT2.1-2.3*[34]. A hormone profile analysis suggesting SA might be crucial for in preventing the overgrowth of root system under low N conditions and deregulation of FIP1 encoding factor interacting with poly(A) polymerase 1[13]. Wheat seedlings treated with 0.5 mM salicylic acid (SA) showed a significant reduction in drought-induced growth suppression, *e.g.,* decreased fresh mass, dry mass, root length, plant height, and increased lipid

peroxidation by significantly increasing ascorbate and glutathione content[36]. When plants are subjected to a variety of abiotic stresses, including heat, cold, heavy metals, osmotic stress, and salt, SA triggers the stress-induced antioxidant system[14].

Conclusion

We focused on understanding the mechanisms regulating gene expression for nitrate transport in wheat, a crucial process for growth traits such as yield, tillering, height, and grain filling. Using online bioinformatics tools, we analysed the promoter sequences of 46 *TaNRT2* genes in wheat, identifying key Cis-Regulatory Elements, motifs, and transcription factor binding sites. We identified several transcription factors involved in growth and stress responses that bind to these promoters. Our phylogenetic analysis grouped the promoter sequence of *TaNRT2* genes into three main clades, revealing their genetic and evolutionary relationships. We also examined variable tandem repeats and CpG islands in upstream promoter sequences of these genes, which suggest potential regulatory mechanisms such as transcriptional regulation due to methylation and post-translational histone modifications. Overexpression studies indicated that certain *NRT2* genes significantly improve agronomic traits and nitrate uptake, which highlights the complex regulation of nitrate-responsive genes in wheat through various cis-elements and hormone-related elements, permitting the inter-hormonal synchronization of physiological and developmental processes. Furthermore, our research provides insightful information about the various regulatory aspects of nitrate uptake and transport pathways of wheat. These insights might be crucial in the development of wheat varieties that are more efficient in nitrate uptake and transport under different unfavourable conditions.

Acknowledgement

AK acknowledges Indian Council of Agricultural Research, New Delhi, for the Senior Research Fellowship for his doctoral studies. ICAR-NIPB's institutional funds financially supported the present study.

Conflict of interests

All authors declare no conflict of interest.

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