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Effects of Limiting Nitrate Concentration on Morphological and Differential Expression of High- and Low-affinity Nitrate Transporter Genes of Diverse Wheat Genotypes

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ABSTRACT

Nitrate uptake in wheat is an essential and complex process that involves multiple proteins. Roots are the major plant organs that take nitrate molecules from the soil and transport them to the above-ground parts. They involve several high- and low-affinity nitrate transporters located in the plasma membrane of different root and shoot tissues. In the present study, we investigated the responses of different selected wheat genotypes, released in India for different agroclimatic regions in a different year, for their biomass, root traits, and expression of high- and low-affinity nitrate transporters genes under optimal and limiting nitrate conditions at the 14 days seedling stage. The maximum number of genotypes showed increased biomass and total root size (TRS) under nitrate starvation, which indicates that the variation of TRS traits in different genotypes responds differently to nitrate starvation. Gene expression of *TaNRT2.1-4* showed up-regulated expression under low external N-concentration in all genotypes. Among the four Ta*NRT1.1* orthologs, *TaNPF6.1* and *TaNPF6.4* showed up-regulated expression, whereas *TaNPF6.2* and *TaNPF6.3* expressed down-regulated in root at higher nitrate concentrations. *TaNRT1.1* (*TaNPF7.1* and *TaNPF7.2*) showed up-regulated in root at optimum nitrate concentrations. *TaNPF6.1*, *TaNPF6.4*, *TaNPF7.1,* and *TaNPF7.2* showed a typical expression of low-affinity nitrate transporter genes under optimum external N-concentrations. Our findings indicate that HD2967, NP890, and VL804 showed the highest expression of *TaNRT2.1-4, TaNRT1.1,* and *TaNRT1.5,* respectively, possibly involved in nitrate uptake and translocation (from root to shoot).

Key words: Nitrogen use efficiency, Nitrogen uptake efficiency, RSA, Nitrate-flux, Nitrate-uptake

INTRODUCTION

Wheat is one of the major cereal crops that provides a primary staple food and occupies the maximum land area under cultivation worldwide. The world production of wheat (*Triticum aestivum* L.) is 777 million tonnes, which is the second most-produced cereal after maize (Anonymous 2023a). Due to the growing population, global demand for wheat will reach 1.6% per year by 2050; however, the current production rate of wheat increases only by 1.1% annually, causing a massive gap between demand and supply (Hall and Richards 2013). To bridge this gap, there is an inclination to increase the application of nitrogenous fertilizers to enhance wheat productivity. Wheat can utilize only 30-35% of the available nitrogen in the soil, while the remaining 65-70% is lost to the environment, which is equivalent to nearly one-third of crop fertilizer consumption globally (Tyagi et al. 2020, Kumari et al. 2022). The unused nitrogenous fertilizer increases environmental pollution due to various physical-biochemical processes, e.g., denitrification and eutrophication etc. Therefore, novel approaches to increase crop nitrogen use efficiency (NUE) need to be identified and developed to reduce this loss and trim-down overdependency on nitrogenous fertilizers. The NUE trait comprises two major component traits. One of the component traits is Nitrogen Uptake Efficiency (NUpE), which refers to the amount of available nitrate taken from soil to the root of the plant, and the second component trait is N-Utilization Efficiency (NUtE), which corresponds to grain dry matter yield per unit N uptake (Sinha et al. 2020). It would be essential to improve the NUE of wheat under restricted N-supply (low N). In this condition, N-uptake and its subsequent transport to different plant parts would

possibly be controlled mainly by high-affinity nitrate transporters (NRT2) family genes and possibly by regulatory proteins/RNAs (Kumar et al. 2023).

Nitrogen is the essential nutrient required for the growth and development of wheat, a cereal crop grown aerobically that favours nitrate over any other nitrogen source. Nitrate is actively taken up by the root epidermal layer using different nitrate transporters. The nitrate uptake by plant roots is a crucial trait that can be utilized to enhance the NUE. The nitrate transporters of Arabidopsis have been thoroughly studied and characterized compared to other plant species. The movement of nitrate from the soil to the root and its transportation from the root to other plant parts requires the membrane transport proteins. It is believed that due to the varying availability of nitrate in the soil, four distinct gene families have been identified that encode nitrate transporters and channels (Krapp et al. 2014, Crawford and Glass 1998). Among them, NPF and NRT2 are the major contributors to nitrate uptake and translocation of nitrate throughout the plant system. HATS works when nitrate is present in low concentrations, while LATS works when nitrate is present in high concentrations and transports them across the plasma membrane of different tissues, e.g., root epidermal and cortical cells (Sinha et al. 2020, Kumar et al. 2022). Arabidopsis is the only plant species extensively studied for its various nitrate transporters. Its genome has 53 LATS and 7 HATS genes, belonging to the NPF (Léran et al. 2014) and NRT2 gene family (Orsel et al. 2002), respectively. AtNRT2 genes, encoding high-affinity nitrate transporters, are induced in low nitrate substrate conditions. Arabidopsis has 7 NRT2 genes, but only *NRT2.1, 2.2, 2.4*, and *2.5* are expressed in N-deprived root conditions, and these four NRT2 transporters showed that they cause 95% and alone AtNRT2.1 cause 75% of HATS influx under N-limited conditions (Lezhneva et al. 2014, O'Brien et al. 2016). Recently, *TaNPF* and *TaNRT2* family genes have been identified in wheat genomes, and there are 46 *TaNRT2* (HATS) genes were predicted in bread wheat (Wang et al. 2020, Li et al. 2021, Kumar et al. 2023). The complementation of *atnrt2.1* mutant with one high-affinity nitrate transporter gene of bread wheat, i.e., *TaNRT2.1-B6*, caused recovery of about 1.5 times 15N influx in Arabidopsis mutant than wild type, confirming the function of one of the high-affinity nitrate transporters (Kumar et al. 2023). *AtNRT1.1* (*NPF6.3*) was first identified as a low-affinity transporter involved in nitrate uptake from soil (Tsay et al. 1993). However, it was later demonstrated to be a dual-affinity nitrate transporter, capable of acting as a high- and low-affinity nitrate transporter. *AtNRT1.5* (*NPF7.3*), located in the plasma membrane of root pericycle cells near the xylem, transports nitrate as a low-affinity system. Root distribution also depends on the concentration and distribution of external nitrogen in the soil, which modulates the root system architecture. The overall structure of the root system is influenced by the interaction between the internal genetic developmental plan and the external environment, i.e., changes in the root system architecture are triggered by the plant's nitrogen status and local signals within the root (Hodge 2004, Forde 2002, Kumar et al. 2024).

The present study investigates the genetic diversity within eight wheat genotypes of India released in India between 1960 and 2012 (Anonymous 2021). We investigate the potential relationship between these genotypes and various physio-molecular traits, e.g., biomass, RSA, and expression of high- and low-affinity nitrate transporter systems. We also investigated the expression pattern of three major nitrate transporter genes, *TaNRT2.1* (HATS, transport nitrate from soil to root) and *TaNRT1.1* (LATS, transport nitrate from soil to root) and *TaNRT1.5* (LATS, transport nitrate from root to shoot of plant) through qRT-PCR. Based on our findings, an external N-concentration can affect the RSA and biomass distribution in both the shoot and root. HD2967 had a significantly higher expression in low nitrate availability. NP890 and VL804 had significantly high expression in *TaNRT1.1* and *TaNRT1.5*, respectively, in high nitrate availability in soil. These genotypes can be used for the NUE program to develop high nitrate uptake and use efficient genotypes.

MATERIALS AND METHODS

Plant material and seedling growth conditions

Eight wheat (*Triticum aestivum* L.) cultivars were selected from the genotypes released in India for

Table 1. Wheat genotypes used in present study

S. No.	Genotypes	S. No.	Genotypes
1	HD2967	5	VL804
2	DBW71	6	MACS6222
3	UP262	7	AKAW4731
4	HD3086	8	NP890

different agroclimatic regions in different years (Table 1). The lines of origin selected for the study included the varieties released in India by the All India Coordinated Research Project on Wheat and Barley between 1960 and 2012 (Anonymous 2021). All eight genotypes were taken and surface sterilized with 0.1% mercuric chloride and 70% ethanol, the seeds were germinated at 25±1°C in the dark. After germination, five plantlets were transferred in a 4-inch pot in N-free medium, i.e., perlite and vermiculite mixture (perlite: vermiculite, 1:2 (v/v)) under two N-concentrations (8.0 and 0.08 mM) of nitrate for 14 days. A liquid MS medium without any nitrogen source was supplemented with 4.0 and 0.04 mM $Ca(NO_3)_2$ in N+ and N- media at uniform intervals for the plant's growth period. The calcium concentration in all growth medium was kept constant and balanced with $CaCl_2$ (Sinha et al. 2018). All experiments were conducted in controlled and aseptic conditions at 25 ± 1°C under 150-200 µmol photon/m^2/s light intensity, 10/14 dark/light hours, and 70% relative humidity.

Biomass and root system architecture

Wheat seedlings were separated into roots and shoots to study the biomass and RSA of 14-day seedlings. Root and shoot lengths and fresh weights were measured separately for each seedling. Following drying at 50°C in a hot air oven, the dry weight of root and shoot was measured. The images of the properly cleaned roots were taken using a flatbed scanner (Epson Perfection V700) at a resolution of 400 dpi. The root images were analyzed with WinRhizo software (Regent Instruments Canada Inc., 2013). Five root parameters, i.e., TRS (total root size), MRP (main root path length), LRS (lateral root size), FOLRN (First Order LR number), and SOLRN (2nd Order LR number), were measured as described by Sinha et al. (2018).

***In-silico* expression of NRT2 and NPF genes analysis**

The differential gene expression analysis of *TaNRT2* and *TaNPF* genes was estimated based on publically available RNA-seq data for root tissues, available in expVIP Wheat Expression Browser (http://www.wheat-expression.com/), in terms of log2 transcript per million (TPM) (Borrill et al. 2016, Ramrez-Gonzalez et al. 2018).

Gene expression studies of high and low-affinity nitrate transporters

RNA isolation and cDNA synthesis

Total RNA was isolated from root tissue using the plant RNA isolation Kit (Pure link™ RNA mini kit, Ambion, Life technologies). RNase-free DNase I (New England BioLabs) was used to remove genomic DNA contamination. The RNA quality and concentration were determined using agarose gel electrophoresis and NanoDrop spectrophotometer ND1000, respectively. cDNA was synthesized from isolated total RNA using reverse transcriptase to generate first-strand cDNAs. 2 µg total RNA was used to synthesize cDNA using a mixture of OligodT and hexamer as a reverse primer. A SuperScript III First-Strand Synthesis System kit (Invitrogen, Life Technologies Corporation) was used per manufacturer instructions for cDNA synthesis. qRT-PCR was performed using the Eppendorf MasterCycler RealPlex4 Real-Time PCR system and PowerUp™ SYBR Green Master Mix (Applied Biosystems, CA, USA). The reaction mixture contained 5 ìl SYBR Green Master Mix (2X), 5 µM (0.5 ìl) each of gene-specific forward and reverse primers, and 100 ng (1 µl) of cDNA of total reaction volume was 10 µl. The PCR reaction conditions were as follows: 2 min at 50°C, 2 min at 95°C for one cycle, and 15 s at 95°C and 60 s at 60°C for 40 cycles.

Primer designing

Gene-specific primer pairs for the selected transcripts were designed by aligning the nucleotide sequence of all homeologs to search one/two SNPs at 3'end for either one or both primer pairs to ensure homeolog-specific primer binding and amplification (Kumar et al. 2022) (Table 2). The resulting primer pairs were cross-checked in the cDNA database of Ensembl Plants (http://plants.ensembl.org/Triticum_aestivum) for their unique template

Table 2. Primer sequences used for real-time PCR

S. No.	Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')
1	*TaNRT2.1-4*	CACATCGTGATGGGCTTGCT	CCATGTCCGGTAGTTGGTGA
2	*TaNPF6.1*	AGATGACCACCTTCTCGGTGT	GGAGGATGGAGCCGACGAA
3	*TaNPF6.2*	CAGGGGAGGCTCGACTACTT	GCCAAGCGCTTCTCCTTGTA
4	*TaNPF6.3*	AGCAAGGAGTGCCGATTCC	ACCTGCTTCACCTCCTCCA
5	*TaNPF6.4*	TCCCTATCTACGACCGCCTC	ATGGCGAGGATGGAGAGGAA
6	*TaNPF7.1*	TACAAGACCTGCGCCATCTT	GAAGATCCCCATCTCCGTGC
7	*TaNPF7.2*	ACATGATCGCGTTCGGCAA	AAGTAGCTGAAGAAGGCCACC
8	Actin	GACCGTATGAGCAAGGAGATC	GTACTAAGGGAGGCAAGAATCG

binding, which was later confirmed by cDNA sequencing of respective clones. The following stringent criteria, e.g., melting temperature (Tm), GC content, hairpin loop formation, base number, and self-complementation, were considered to analyse primers using the OligoAnalyzer™ Tool.

qRT PCR

The gene expression profiles of selected nitrate transporters in root tissue under nitrate-limiting and optimum conditions of 14-day-old seedlings were analysed by quantitative PCR. Eight genotypes were taken for nitrate transporters gene expression analysis. Gene expression of *TaNRT2.1*-4 (TraesCS6A 02G031100 and TraesCS6B02G044100), *TaNPF6.1* (TraesCS7A02G301700, TraesCS7B 02G201900, and TraesCS7D02G297000), *TaNPF6.2* (TraesCS1A02G210900, TraesCS1B 02G224900, and TraesCS1D02G214200), *TaNPF6.3* (TraesCS1A02G211000, TraesCS1B 02G225000, and TraesCS1D02G214300), *TaNPF6.4* (TraesCS5A02G537100, TraesCS4B 02G371000, TraesCS4B02G375800, and Traes CS4D02G361500), *TaNPF7.1* (TraesCS6A 02G280200, TraesCS6B02G309200, and Traes CS6D02G260500) and *TaNPF7.2* (TraesCS6A 02G263500, TraesCS6B02G290500, and Traes CS6D02G251500) (Table 3), was studied in N+ and N- nitrate conditions. The nomenclature of *TaNRT2.1-4* was followed as described by Kumar et al. (2023). Relative expression was determined by the ΔΔCT method corrected for the primer efficiency (Livak and Schmittgen 2001), and the N+ and N– treatments are considered as control (expression is normalized to fold change 1) for high-affinity (*TaNRT2.1-4*) and low affinity (*TaNPF6.1, TaNPF6.2, TaNPF6.3, TaNPF6.4 TaNPF7.1* and *TaNPF7.2*) nitrate transporter genes, respectively, for calculating fold change in the gene expression.

Statistical analysis

All data reported in this study were conducted with five replicates, and gene expression was conducted with three biological and three technical replicates. Mean values were computed for all parameters, with

Table 3. List of HATS and LATS genes and their homologous pair in bread wheat

Gene name	Wheat gene ID (Genome A)	Wheat gene ID (Genome B)	Wheat gene ID (Genome D)
TaNRT2.1-4	TraesCS6A02G031100	TraesCS6B02G044100	
TaNPF6.1	TraesCS7A02G301700	TraesCS7B02G201900	TraesCS7D02G297000
TaNPF6.2	TraesCS1A02G210900	TraesCS1B02G224900	TraesCS1D02G214200
TaN3F6.3	TraesCS1A02G211000	TraesCS1B02G225000	TraesCS1D02G214300
TaNPF6.4	TraesCS5A02G537100	TraesCS4B02G371000	TraesCS4B02G375800
		TraesCS4D02G361500	
TaNPF7.1	TraesCS6A02G280200	TraesCS6B02G309200	TraesCS6D02G260500
TaNPF7.2	TraesCS6A02G263500	TraesCS6B02G290500	TraesCS6D02G251500

error bars representing the standard error of the means. A least significant difference (LSD) at a significance level of 5% was calculated for treatment × genotype interactions. The statistical analysis was performed using the Statistical Package for the Social Sciences (Anonymous 2003b) software at $\alpha = 0.05$.

RESULTS

Effect of nitrate-limiting conditions on biomass

The maximum shoot length was observed in HD2967 in optimum (N+) nitrate conditions and HD2967 and DBW71 under nitrate (N-) limiting conditions (Fig. 1A). Root length was highest in UP262 and MACS6222 in nitrate optimum conditions and MACS6222 in nitrate limiting conditions, whereas HD3086 showed the lowest root length in N+ medium than N- medium (Fig. 1B). The higher shoot length was observed in the N+ medium than the N- medium in all genotypes, but interestingly, most of the genotypes showed higher root length in the N- medium than the N+ medium except UP262 and NP890 (Fig. 1A, B). Across all genotypes, shoot fresh weight was higher in N+ medium than N- medium, whereas root fresh weight was not uniform among genotypes (Fig. 1C, D). Shoot dry weight was maximum in DBW71 in both N+ and N- conditions, whereas UP262 and VL804 showed a minimum dry weight in N+ and N- conditions, respectively. The root dry weights of NP890 and HD2967 were maximum and minimum under nitrate-optimum conditions, respectively, and NP890 and VL804 were maximum and minimum under nitrate-limited conditions, respectively (Fig. 1E, F). This data suggested that high nitrate availability promotes increased biomass and plant length, whereas low nitrate decreases it.

Effect on root system architecture under limited nitrate

Under nitrate-limiting (N-) conditions, most genotypes showed significantly increased TRS. However, DBW71 and HD3086 showed maximum and minimum TRS under N- conditions (Fig. 2A). Most genotypes also showed a significantly higher root length that emerges from the radicle (Main Root

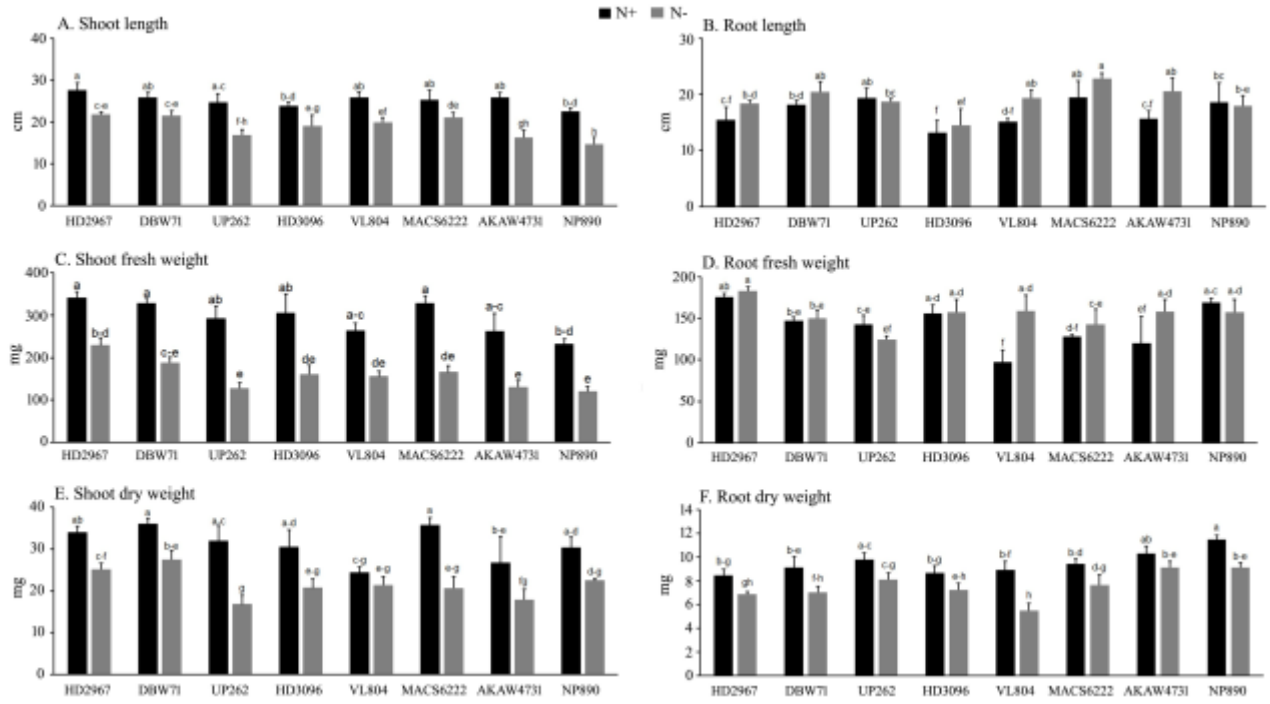

Figure 1. Growth of wheat seedlings in response to two external N+ and N- concentrations in eight genotypes. Shoot length (A), root length (B), shoot fresh weight (C), root fresh weight (D), shoot dry weight (E) and root dry weight (F), in N+ and N- external conditions for each genotype. The significant difference ($p < 0.05$) among N concentration (N+ vs. N-) and genotypes is indicated in the letters above the error bars. Values are means ± SEM (n=5).

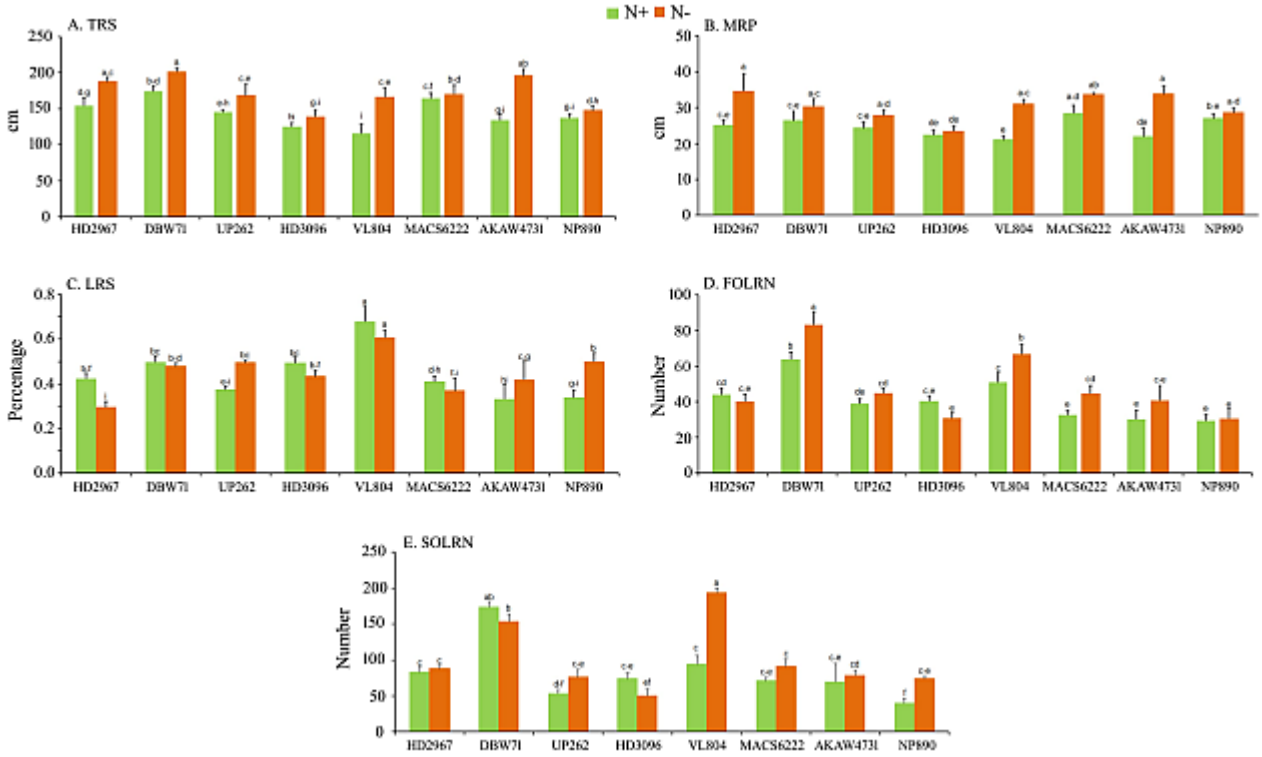

Figure 2. Root system architecture traits of wheat seedlings in response to two external N+ and N- concentration. (A) Total Root Size (TRS; cm), (B) Main Root Pathlength (MRP; cm), (C) Lateral Root Size (LRS), (D) First Order Lateral Root Number (FOLRN), (E) Second-Order Lateral Root Number (SOLRN). The axes denote the name of the genotypes studied and are represented by two colours denoting external N+ and N- conditions, for each genotype. Values are means ± SEM (n=5).

Path length, MRP) in nitrate-limiting conditions. HD3086 and NP890 significantly did not show nitrate starvation response on MRP. In N-growth condition, the MRP was significantly higher in HD2967 and AKAW4731 and lower in HD3086 (Fig. 2B). LRS is determined by the proportion of lateral root length that contributes to TRS. The majority of genotypes showed higher LRS in nitrate-optimum than nitrate-limiting conditions. UP262, NP890, and AKAW4731 genotypes significantly demonstrated higher LRS in the N- condition than in the nitrate optimum condition. However, interestingly, VL804 exhibited the highest LRS in both nitrate-limiting and optimum conditions (Fig. 2C). In most genotypes, the First Order Lateral Root Number (FOLRN) was significantly higher under nitrate-limited conditions than under nitrate-optimum conditions. Interestingly, DBW71 has the highest number of FOLRN in both N+ and N- conditions (Fig. 2D). Most genotypes exhibited more Second Order Lateral Roots (SOLRN) under N- than N+ conditions. VL804 and DBW71 have the highest SOLRN among all the genotypes in N- and N+ conditions, respectively. It was observed that all genotypes had a higher number of SOLRN than FOLRN. Most genotypes showed a higher degree of modulation in RSA parameters, i.e., TRS, MRP, LRS, FOLRN, and SOLRN under N- growth conditions (Fig. 3).

In-silico and qRT-PCR Expression patterns of selected HATS and LATS gene

Using RNASeq data (Borrill et al. 2016, Ramrez-Gonzalez et al. 2018), we investigated the expression pattern of *TaNRT2* and *TaNPF* genes in root tissues at different stages of growth. The transcript (in tpm) analysis showed that *TaNRT2* and *TaNPF* genes expressed in root at different growth stages, but *TaNPF6.4* and *TaNPF7.1* showed the lowest expression (Fig. 4). When exposed to low nitrate

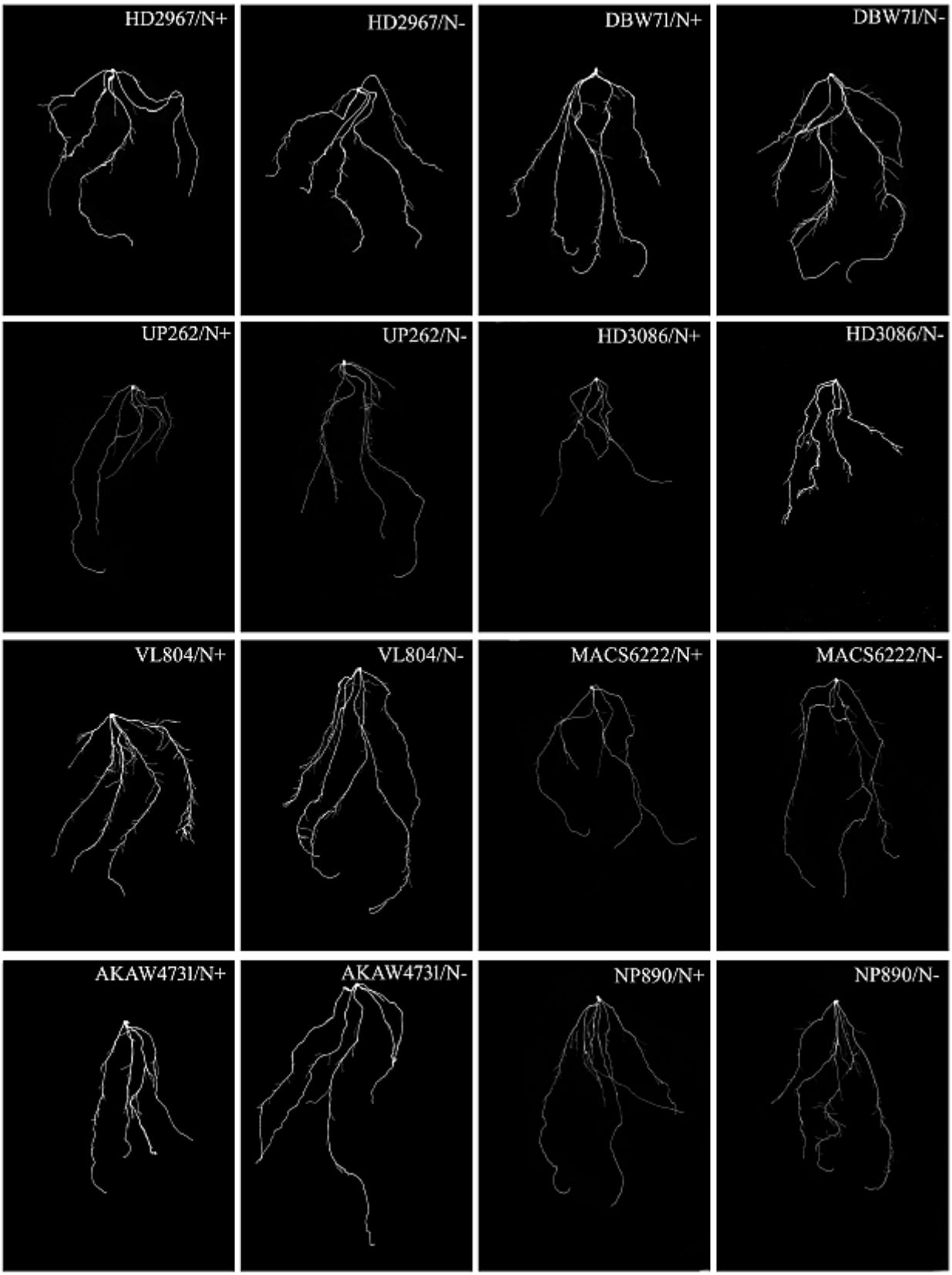

Figure 3. 2D root images of wheat seedling genotypes under two external N+ and N- conditions. The genotype name and N conditions are given in each image. The images were taken using a flatbed scanner (Epson Perfection V700) at a resolution of 400 dpi

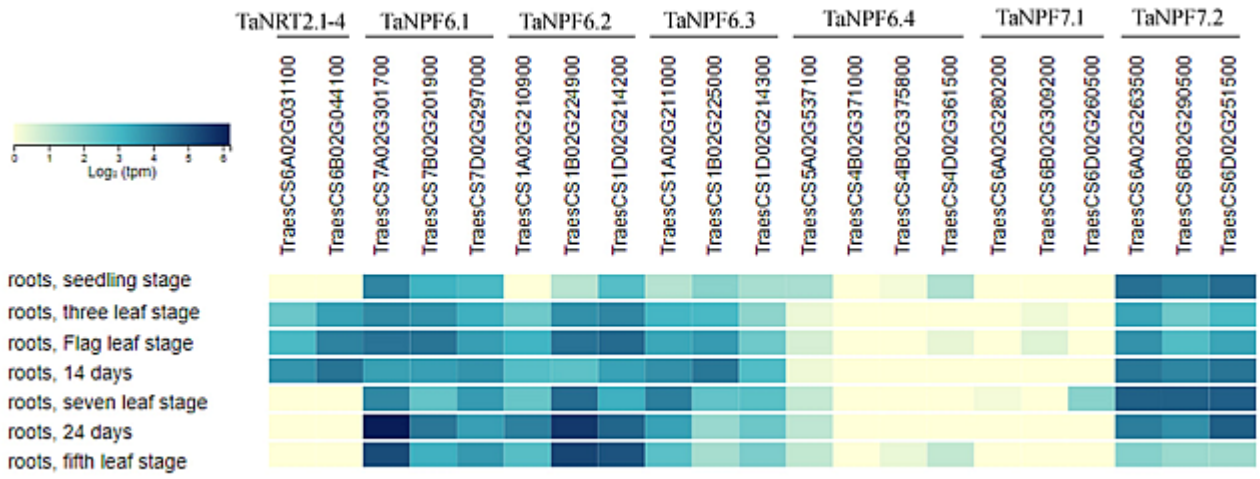

Figure 4. Heatmap showing the expression pattern of *TaNRT2* and *TaNPF* genes in root at different growth stages, using RNASeq data (Ramírez-Gonz´alez et al. 2018). The expression level of all genes is represented in terms of log2 tpm values

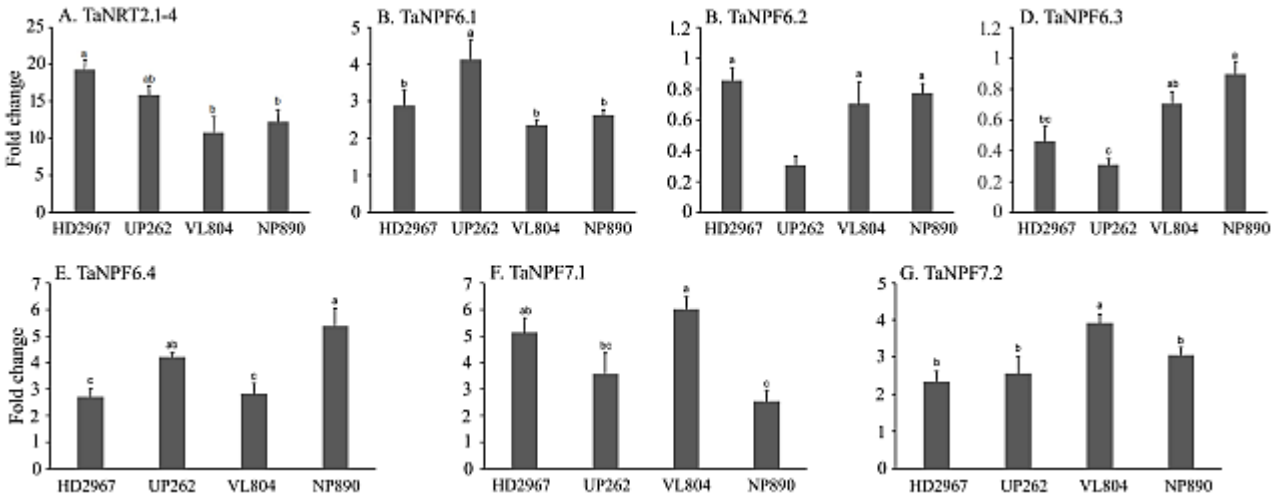

Figure 5. qPCR expression analysis of high and low affinity nitrate transporter genes in wheat genotypes grown under two different external N levels (N+ and N-). Gene expression of high affinity nitrate transporter, *TaNRT2.1-4* (A) and low affinity nitrate transporter, *TaNPF6.1* (B), *TaNPF6.2* (C), *TaNPF6.3* (D), *TaNPF6.4* (E) *TaNPF7.1* (F) and *TaNPF7.2* (G) was studied in response to two external nitrate conditions (N+ and N-) in root tissue. TaNRT2.1-4 gene expression is shown relative to N+ condition. *TaNPF6.1, TaNPF6.2, TaNPF6.3, TaNPF6.4, TaNPF7.1* and *TaNPF7.2* gene expression is shown relative to N- condition. The significant difference ($p < 0.05$) among genotypes is indicated above the error bars. Values are means of three technical and biological replicates ± SEM.

concentrations, *TaNRT2.1-4*, a high-affinity nitrate transporter (HATS) gene, is consistently up-regulated in genotypes. HD-2967 (19.24 fold) and VL804 and NP890 (10.75 and 12.18 fold) showed the significant highest and lowest expression among these four genotypes, respectively (Fig. 5A). *TaNPF6.1* and *TaNPF6.4* showed higher gene expression in N+ comparison to N- conditions. Expression of *TaNPF6.1* showed significantly higher expression in UP262 (4.12 fold) than in other genotypes (Fig. 5B). *TaNPF6.2* and *TaNPF6.3* showed a down-regulated expression in N+ than N- conditions. HD2967 (0.85 fold), VL804 (0.70), and NP890 (0.77) had the highest expressions but there was no significant

difference in the *TaNPF6.2* gene (Fig. 5C). *TaNPF6.3* (Fig. 5D) gene expression was highest in NP890 (0.89 fold), and lowest in UP262 (0.30 fold) genotype. NP890 (5.37 fold) showed the highest and HD2697 (2.71 fold) and VL890 (2.80 fold) showed significantly lowest gene expression in N+ in comparison to N- conditions in *TaNPF6.3* (Fig. 5E). Expression of the *TaNPF7.1* gene was highest in VL804 (6.01 fold) and lowest in NP890 (2.52 fold) (Fig. 5F) whereas VL804 (3.91 fold) had the highest expression and except VL804 all three showed significantly lowest *TaNPF7.2* gene expression (Fig. 5G).

DISCUSSION

The roots absorb water and nutrients from the soil and transport them to the upper part of the plant, i.e., shoots. Our findings indicate that nitrate-limiting conditions consistently cause an increase in root length for all genotypes. It is likely associated with a signaling cascade induced by the lack of nitrogen and the ability of the genotypes to forage for nitrogen (Gruber et al. 2013) and due to the amount of biomass that is transported to the root for further extension (Garnett et al. 2009). All genotypes exhibited similar nitrate responsiveness traits, with exposure to nitrate starvation resulting in longer roots, indicating that nitrogen sensing and acquisition mechanisms are operated uniformly. However, the root length was more responsive to N-starvation compared to other genotypes. As root length increased, shoot length decreased in all genotypes, indicating that biomass was translocated from the shoot to the root to maximize their foraging potential under N-starvation. When nitrate is lacking in the external media, it causes imbalances in plant biomass. To cope with this, plants allocate reserved biomass to different plant parts, with roots playing a crucial role in scavenging deficient resources for survival (Sinha et al. 2015, 2020). The RSA images represent the change in different root system components under nitrogen starvation. We analysed 2D images of the complete root system of all genotypes grown under nitrogen-optimal and starved conditions to estimate the nitrogen-mediated root plasticity. Plant root growth can be reprogrammed from shallow to deep or vice versa to maximize the uptake of nutrients (Kumari et al. 2023). TRS of wheat seedlings was measured as the total length of embryonic and non-embryonic roots, and it was found to be increased upon N-starvation across all genotypes. However, the LRS of most of the genotypes was lower, and the MRP of the most genotypes was higher in nitrogen-starved conditions. This is evident in their higher seminal root (embryonic root) length and lower lateral root (non-embryonic root) length. Increased TRS has been reported previously and implicated in enhancing root foraging capacity in N-starvation conditions (Sinha et al. 2018, Melino et al. 2015). This ability allows the plants to access nutrients from the soil, helping to increase their overall growth and productivity. DBW71 was seen as having the highest TRS under N-starvation conditions with a lower value of LRS, which indicates that seminal roots are more likely than lateral roots to forage for N sources. Wheat has a fixed number of seminal roots that is generally 5 in number. In contrast, the number of first-order lateral roots (FOLRN) and second-order lateral roots (SOLRN) depends on the external environment. It is more likely that the biogenesis of newer roots is restricted in favour of elongating already existing lateral roots for foraging under nitrogen-limited conditions (Giehl and von Wiren 2014). We observed that all genotypes recognized the nitrogen starvation signal, but their responses differed.

Wheat uptakes nitrate as a nitrogen source from the soil, which is then transported to various plant parts through two major nitrate transporters, i.e., HATS and LATS (Sinha et al. 2020). The complexity of the wheat genome results in a greater number of genes encoding both high-affinity transport systems (HATS) and low-affinity transport systems (LATS) when compared to Arabidopsis. Most of the HATS and LATS genes have been well characterized in Arabidopsis, and recently 331 *TaNPF* genes and 46 *TaNRT2* genes have been identified in wheat (Wang et al. 2020, Kumar et al. 2023). To gain insight, an expression study of one HATS (*TaNRT2.1*) and two LATS (*TaNRT1.1* and *TaNRT1.5*) and their homeologs in the bread wheat under two nitrate growing conditions. *TaNRT2.1-4* typically shows a higher expression pattern in root tissue in all genotypes grown under low N-concentration than optimum N-concentration, confirming its HATS

activity. Previously, many groups also studied and reported the *TaNRT2.1* expression in the root tissue under nitrogen-limiting conditions, in which a lower nitrate concentration induced wheat seedlings (Guo et al. 2014, Melino et al. 2015, Sinha et al. 2020, Kumar et al. 2022). We observed significantly low expression of *TaNRT2.1-4* in NP890 and VL804 among the four genotypes, which indicates genotypic variation, suggesting that might be responsible for the different NUE in these genotypes. The wheat genome comprises four genes, namely *NPF6.1, NPF6.2, NPF6.3*, and *NPF6.4*, located on chromosomes no. 1, 4, 5 and 7 orthologous to *AtNRT1.1* (*NPF6.3*) (Buchner and Hawkesford 2014). Among the four TaNPF genes, *TaNPF6.1* and *TaNPF6.4* demonstrate an expression pattern typical of low-affinity transport systems (LATS), as they are highly expressed under nitrogen-optimum conditions. In contrast, *TaNPF6.2* and *TaNPF6.3* gene expression display a pattern somewhat of high-affinity transport systems (HATS), as they are more expressed under nitrogen-limited conditions. Downregulation of *TaNPF6.2* and *TaNPF6.3* genes in N-optimum conditions (Sinha et al. 2020) corroborated with our results and indicated their role in the uptake of nitrate only when starvation of nitrate in the soil. NRT1.1 functions as a dual-affinity transporter, i.e., functional in sufficient and starved nitrate conditions. *TaNPF7.1* and *TaNPF7.2* are located on chromosome number 6 of all three genomes in wheat, orthologous to *AtNRT1.5* (Buchner and Hawkesford 2014), and play an important role in the translocation of nitrate from root to shoots. Both *TaNPF7.1* and *TaNPF7.2* exhibit an expression level as they are expressed more under N-optimum conditions than N-limited conditions. *NRT1.5* is a LATS but not a dual-affinity transporter and transports nitrate in both directions (influx and efflux) in highly available nitrate, probably via a proton-coupled mechanism (Lin et al. 2008). The involvement of LATS has been demonstrated in both the uptake of nitrate from the soil and its subsequent translocation to the shoot in Arabidopsis (Liu and Tsay 2003, Lin et al. 2008). These RSA and gene expression data suggest that NRT2.1, NRT1.1, and NRT1.5 are involved in nitrate uptake from soil and its translocation to the upper parts of plants through HATS and LATS.

CONCLUSION

N-starvation modulated the root length of all wheat genotypes studied and increased root dry weight. RSA and biomass of plants are affected by external available N-concentration irrespective of the genetic background of the genotype. NRT2.1 and NRT1.1 are high and low-affinity nitrate transporter systems, respectively, showing a dual-affinity transporter. There was a higher level of HATS activity in N-responsive genotypes, indicating that the nitrate transporter gene that encodes for HATS might have undergone a favourable allelic variation. *TaNRT1.1* and *TaNRT1.5* showed differential gene expression among genotypes under high external N-concentration, and these genes might have different root-specific expressions. The present study demonstrates that LATS and HATS play specific roles in nitrate uptake and translocation, depending on the N status of wheat seedlings and the external nitrate concentration. Identifying the key genes in wheat genotypes superior in N-uptake and/or translocation using molecular breeding helps to develop higher yields and better-quality wheat varieties. In addition, it can help reduce the use of nitrogen fertilizers and protect the environment.

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