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Nitrogen Stress Leads to Induce Change in Expression of Genes for Nitrate Transporter in Wheat Genotypes

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A B S T R A C T

Nitrogen use efficiency (NUE) is very important for reducing the cost of production, sustainable agriculture and mitigates the environment pollution. It is more so in case of major cereals like wheat, where the NUE is approximately 40%. NUE comprises of N-uptake by the root and then their assimilation, utilization, remobilization by the shoot. However, utilization primarily dependent on available resources, i.e. amount of N-uptaken by the root system. Root system architecture (RSA) and the transporters are key factors which determine the amount of nitrogen forage could be possible by a genotypes at different level of soil nitrogen. Here in this study we are reporting N stress induced changes in gene expression of different high and low affinity nitrate transporters among eight diverse wheat genotypes with respect to NUE at seedling stage. This seems to be one of first reports of nitrate transporters gene expression under N-deprived condition in different NUE genotypes of wheat. Kharchia, showed minimum change in expression, whereas VL-401 and Kalyansona were distinctly different from the rest of the genotypes for LATS and Kharchia also showed its distinct character by significantly down regulating for HAT under N-stress condition.

Introduction

Nitrogen is one of the most critical limiting element for plant growth, primarily constituent of the nucleotides and proteins that make up the building blocks essential for life (Xu *et al.,* 2012), therefore quantitatively most important nutrient and limiting factor for growth and development of plants (Kraiser *et al.,* 2009). Inadequate nitrogen seriously affects yields of crops while excess has no significant effect on yield, but contributes N pollution by means of leaching, surface runoff, denitrification, and emission of greenhouse gas like nitrous oxide, etc. (Liao *et al.,* 2012). Serious health hazards are of great concern due to intake of nitrate-contaminated water (Abrol *et al.,* 1999). However, low recovery rate and high loss of fertilizer N would increase the cost of food production and the eutrophication of many natural aquatic and terrestrial ecosystems (An *et al.,* 2006). Rational application of N to avoid excessive fertilization together with use of cultivars which efficiently use N sources

have been proposed as prime factor for improvement of NUE (Noulas *et al.,* 2002). These desirable cultivars with greater NUE are thought to produce higher yields even at low N supply and have been called as efficient germplasms (Haefele *et al.,* 2008). It is reported that increased N fertilization in combination with shorter varieties are important factor in increasing wheat yield during 20th century (Khush, 1999). The long-time objectives for a sustainable agriculture can be met not only by using efficient farming techniques (e.g., decrease of N fertilizer supply, distribution in several split applications, use of coated forms of nitrogen fertilizer) but also by using varieties which absorb N from soil and metabolize them better i.e. by using varieties that have a better NUE (Gallais *et al.,* 2005). The NUE reported in case of cereals including wheat is only about 40%, which means 60% of the applied fertilizer is lost to the environment polluting it one or the other way (Raghuram *et al.,* 2007). Therefore, increasing emphasis in growing wheat cultivars with improved NUE for reducing excessive input of fertilizers along with maintaining an acceptable yield is a global requirement. (Foulkes *et al.,* 2009). NUE is a function of multiple interacting genetic and environmental factors and is therefore an inherently complex character. NUE includes N-uptake, N-assimilation, N-utilization or N-remobilisation efficiency, expressed as a ratio of output (total plant N, grain N, biomass yield, grain yield) and input N in the form of fertilizers (Pathak *et al.,* 2008). That is why it is necessary to identify contrasting wheat genotypes for NUE for further study them to understand the mechanism of NUE and the key molecular regulatory factor(s) in wheat. There have been several reports suggesting genetic variability in NUE pertaining to genetic differences in N uptake and utilization efficiency in different crops including wheat (Namai *et al.,* 2009). Controlled environmental condition could be used to know the inherent mechanism and regulation for imparting efficiency in both terms of N uptake and utilization. Nitrogen uptake up by plant mainly depends upon the nature of root system along with N transporter system present in root. To acquire sufficient amounts of nitrogen needed to maintain optimal growth, higher plants have to couple with marked spatial and temporal changes in the availability of nitrogen sources (mainly NO_3^- and NH_4^+) in the soil (Robinson *et al.,* 1994) and for this constraint, plants have evolved adaptive mechanisms such as High Affinity Transporter System (HATS) and Low Affinity Transporter System (LATS) allowing them to enhance their nitrogen capture efficiency in situations of nitrogen limitation (Clarkson *et al.,* 1985). Physiological investigations of NO_3^- uptake by the roots of many different types of plants have led to the conclusion that plants have developed three types of transport system such as Constitutive HATS (CHATS), Inducible HATS (IHATS) and LATS, to cope with the variations in NO_3^- concentrations in cultivated soils (Crawford and Glass, 1998). The low affinity transport system (LATS) is used preferentially at high external nitrate concentrations above 1 mM, LATS is constitutive in nature and possibly has a signaling role to induce the expression of HATS and nitrate assimilatory genes, presumably playing a nutritional role only above a certain threshold (Pathak *et al.,* 2008). It is generally assumed that the Nitrate Transporter 1 (NRT1) gene family mediates the root Low-Affinity Transport System (LATS), with the exception of the *AtNRT1.1*, which is both a dual affinity transporter (Wang *et al.,* 1998; Liu *et al.,* 1999) and a nitrate sensor (Ho *et al.,* 2009). The high affinity transport system (HATS) works at low concentrations (1 μM–1 mM) (Pathak *et al.,* 2008), relies on the activity of the so-called NRT2 family genes (reviewed in Williams and Miller, 2001). The current study started with field evaluation of several wheat genotypes,

and eight highly N-responsive genotypes were selected based on the field observation. Eight diverse genotypes for NUE were studied at their seedling stage under NO_3^- -optimum and NO_3^- -stress conditions after growing them in mixture of perlite and vermiculite (complete nutrient free medium). Candidate nitrate transporters gene expression were studied under both NO_3^--optimum as well as NO_3^--stress conditions to decipher the N-responsive behavior of wheat genotypes at seedling stage.

Materials and Methods

Selection of genotypes

Based on evaluation of field data at ICAR-IIWBR, Karnal, eight wheat genotypes having diverse features for NUE have been selected for the study (Table 1).

Growing condition for seedling

Briefly, the healthy seeds of all the selected genotypes were first rinsed with 70 % ethanol for 3 min and then surface sterilized using 0.5 % Sodium hypochlorite for 3 min. After several washes with ddH2O, the seeds were kept for germination in incubator at 25 ± 1 °C in the dark. Three days old uniformly germinated seeds having the primary roots length of approximately 1cm were carefully transplanted in4 inch pots containing 2:1 mixture of vermiculite and perliter after moisturizing with distilled water. The culture room growth condition was as mentioned by Sinha *et al.*, 2015. Murashige and Skoog medium (MS) (minus N) was used as nutrient media in which 8.00mM and 0.4mM nitrogen was added from $Ca(NO_3)_2.4H_2O$ and $NH_4^+NO3^-$ respectively for N controlled condition while, for N- stress condition 0.08mM and 0.004mMnitrogen was added from $Ca(NO_3)_2.4H_2O$ and $NH_4^+NO3^-$ respectively. Freshly prepared nutrient solution was applied as per the requirement during its growth phases for the entire 15 days period at three days interval.

Total RNA extraction and cDNA preparation

Root tissue (100 mg) of 15 days old seedling were harvested for total RNA extraction using pure link® RNA Mini Kit. RNA yield and quality was determined by spectrophotometry using Nanodrop (Thermo Scientific, USA). RNA sample was treated with DNase from Thermo Scientific kit to remove traces of genomic DNA. Their integrity was checked on 1.2% formaldehyde agarose gel. First strand cDNA was synthesized using SuperScriptIII® first strand cDNA synthesis system (Invitrogen, USA) and synthesized cDNA was stored at -20ºC for further use.

Gene expression study through semi quantitative PCR and qPCR

For expression study the primers, were designed for total 16 candidate genes (Table 2)by using IDT software from the EST/ gene sequences of both high and low affinity nitrate trasporter genes (available in public domain). Semi quantitative PCRs were carried out for all the primers for selection of primers, those were giving differential expression under control and stress condition only those primers were selected for qPCR. Semi quantitative PCR carried out for 30 cycle. Reaction volume contain following components: 2µL of 10X buffer, 0.4 µL of 10mM dNTP mix, 0.4 µL of 50mM $MgCl_2$, 1U *Taq* polymerase, 1µL each forward and reverse primer of 10µM, 0.5 µL cDNA of 5µg/µL, and remain 14.2µL milliQ water added in each PCR tube. The PCR programme was set as: 95˚C for 4 min., 95˚C for 30 sec., 55˚C for 30 seconds, 72ºC for 30 seconds for 30 cycles and final extension at 72ºC for 5 minutes. After completion of semi quantitative PCR the expression pattern was checked by gel electrophoresis.

For gene expression studies, qPCR was carried out by using fluorescence detection using fluorescent ds DNA binding dye *Power*® SYBR Green PCR Master Mix Reference No. 4367659 using the protocol of Sinha *et al.,* 2015. Actin was taken as the reference gene for all the reactions. MicroAmp®fast 96- well reaction plate used. The reaction plate then covered with an adhesive sealing sheet and were run on Step One^{TM} Plus ABI (USA) Real Time PCR. The PCR programme was set for 40 cycles consisting of 95˚C for 10 minutes, 95˚C for 15 seconds and 60˚C for 1 minute. Following this, a melting curve analysis step was also carried out and the result was calculated in the form of fold change in gene expression calculated using $2^{-\Delta\Delta Ct}$ (Livak *et al.,* 2001) in stressed samples with respect to optimal and data was normalized taking Actin as the normalizer in the experiment.

Statistical analysis of data

In case of gene expression study, standard error of means were calculated and presented as error bars.

Results and Discussion

Gene expression of various nitrate transporters

Based on field evaluation at ICAR-IIWBR, Karnal, eight genotypes (Table1) were used in the present study. In order to understand the effect of Nitrogen stress on gene expression of candidate genes of nitrate transporter. We have grown these selected eight wheat genotypes under complete controlled condition as mentioned in materials and methods to note the response of the nitrate transporter genes under nitrogen stress.

There are total 16 genes related to nitrate transporter were studied (Table 2). Expression study was carried out by using semi quantitative PCR. Out of the 16genes were analysed, most of them did not shown differential expression. Differential expressions were observed among the genotypes as well as between the N-optimum and N-stress condition only in case of five transporters genes i.e. *TaNRT2* (high affinity), and rest *TaNPF6.6, TaNPF6.2, TaNPF6.7* and *TaNPF6.1* are low affinity (Fig.1). In case of high affinity nitrate transporter *TaNRT2,* WH-542 did not show the expression under control condition whereas WH-147 and Sujata shown negligible expression under N- stress.

Differential expression of *TaNRT2* even observed under N-stress in comparison to control in case of WH-542, Sujata, VL-401 and Kalyansona. In case of low affinity transporter gene *TaNPF6.6* maximum expression observed in HS-277 under N-optimum condition, and rest of the genotypes exhibited higher expression under N- stress. Gene *TaNPF6.2* shown higher expression in case of four genotypes namely HS-277, Sujata, VL-401 and Kalyansona under N-stress condition; whereas in GW-322, WH-542 shown relatively higher expression under N-optimum condition. Genotype WH-147, and Kharchia did not show any variation under N-stress. Under N-optimum condition, the expression of *TaNPF6.7* was higher that than of under stress in WH-542, WH-147, Kharchia and Sujata genotypes. In case of gene *TaNPF6.1* higher expression observed under N-stress condition in Sujata, VL-401and Kalyansona as compare to N- optimum. To see fold change in gene expression q-PCR was carried out for transporter genes those five, which showed differential expression in semi quantitative PCR.

Expression study include both high and low affinity nitrate transporter system genes. Among them *TaNRT2* is high affinity nitrate transporter system gene. All genotypes were

shown upregulation ranging from 1.16 fold in WH-542 to 4.31 fold in VL-401 under N stress sample, except Kharchia and GW-322 both these genotypes were showing down regulation of *TaNRT2* in range of 10 fold to 1.20 fold respectively (Fig.2).

Genotypes such as HS-277, WH-542, Sujata, VL-401 and Kalyansona were showing down regulation for *TaNPF6.6* gene under N stress. Maximum down regulation was observed in WH-542 which is around 7.1 fold whereas maximum up regulation was observed in GW-322, around 2.21 fold. The expression was almost unchanged in case of Kharchia and WH-147 (Fig.3a).All genotypes were showing down regulation of *TaNPF6.2* gene except genotype Kalyansona (with 6.4 fold change). GW-322 showed maximum level of down regulation with 20 fold change. Gene expression in case of V-L401, Sujata and WH-147 were unchanged under N-stress condition (Fig.3b).Genotypes HS-277, Sujata, VL401, were showing up regulation of *TaNPF6.7* and genotypes GW-322, WH-542, Kharchia, and Kalyansona were observed with down regulation of *TaNPF6.7*. Up regulation in HS-277 was 1.90 fold, in Sujata and VL-401 were 2.74 and 2.42 fold respectively and same down regulated in GW-322 was 3.5 fold, in WH-542 and Kharchia were 3.7 and 3.9 fold respectively. WH-147 did not change the expression of the gene under N-stress (Fig.3c). Four genotypes among eight such as HS-277, GW-322, WH-147 and VL-401 were showing upregulation for *TaNPF6*.1 gene expression under N-stress condition with highest (7.43 fold) change in GW-322. WH-542 showed 3.8 fold and Sujata 3.21 fold down regulation. Minimum changes in gene expression was in case of Kharchia and Kalyansona (Fig.3d).

Transporters are mainly responsible for the uptake of nutrient of which nitrate transporter are responsible for uptake of N-nutrition in case of wheat. Studying these transporters, which mainly belongs to the roots, is very important to understand the nature of individual genotypes for their N-uptake. Many of the transporters are known for dual-affinity such as NRT1.1 (Sun *et al.*, 2014). The regulation of these transporters is known by their gene expression and hence the gene expression of the transporters under N-stress condition was taken up for the study. Present study have been started with the downloading of available nitrate transporter from public domain, of which, 16 could be amplified by semi quantitative PCR, followed by qPCR for those which showed differences under N-stress in any of the eight genotypes. Since the result of semi quantitive PCR is not conclusive, but give an indication that in a set of diverse of genotypes, the expression pattern under N stress is different, further carrying out qPCR analysis was important. Five transporter genes, one of them HAT and fours LATS were finally studied through qPCR.

The high affinity transport system (HATS) works at low concentrations (1 μM–1 mM) (Pathak *et al.*, 2008), relies on the activity of the so-called NRT2 family genes (Williams and Miller, 2001). NRT2 genes in *Arabidopsis* showed that NRT2 involves in nitrate transportation. (Cerezo *et al.*, 2001; Wang *et al.*, 2012).Out of the two important HATs studied under this experiment, one of them (*TaNRT2*) showed differential expression at 30 cycle of amplification, which was studied further by qPCR. In wheat, complete CDS of this gene (*TaNRT2*) was reported during 2005 by Tong *et al.*, (NCBI GenBank: AF288688.1). Known plant *NRT2* genes occur within a single monophylic group (Yin *et al.*, 2007). Several genes from these family are being reported based on the sequence similarity. It is also known that Nitrate availability and other factors regulate the gene expression of many NRT2 genes (Zhuo *et al.*, 1999; Orsel *et al.*, 2002 and 2006).

Fig.1 Expression pattern of NO_3^- transporter genes by Semi-quantitative PCR of 15 days old seedlings of diverse wheat genotypes under N- optimum and N- stress condition

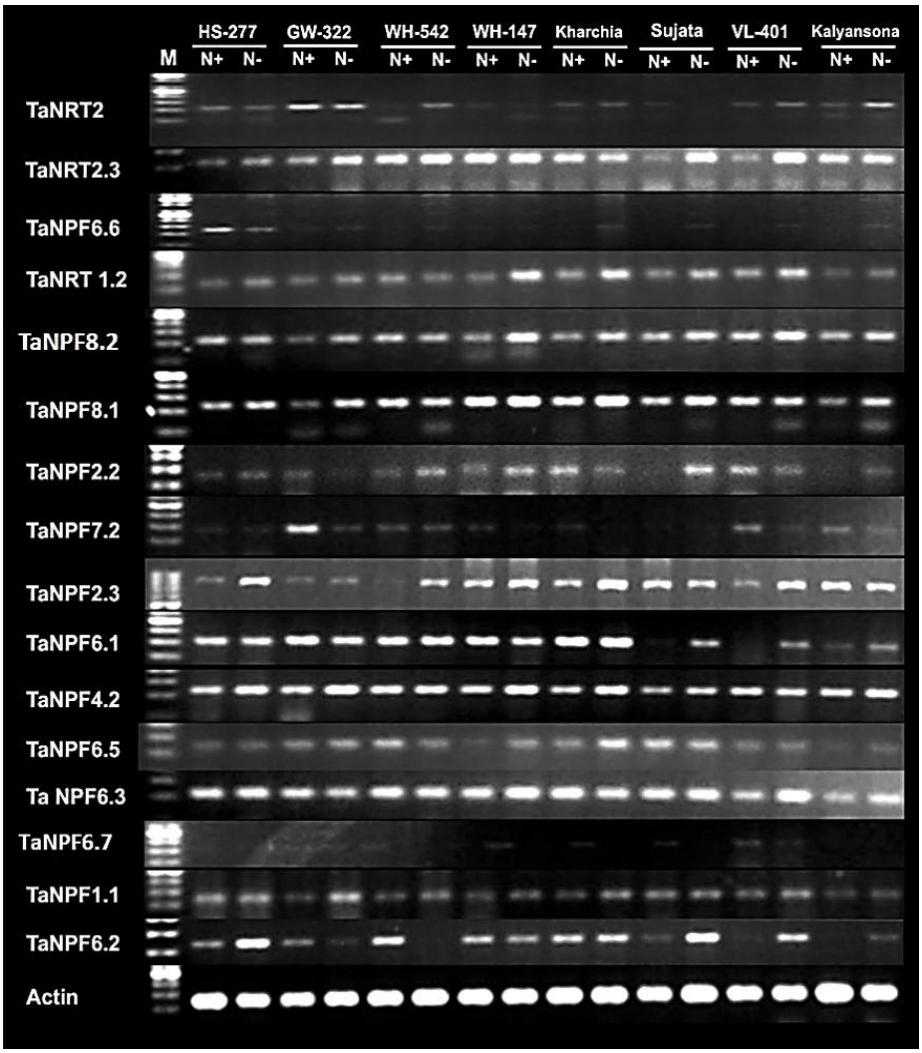

Fig.2 Expression profile of *TaNRT2* gene by qPCR

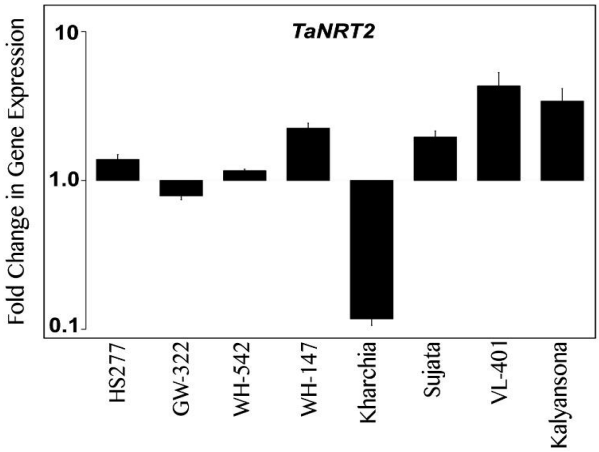

Fig.3 Expression profile of genes by qPCR (a) *TaNPF6.6*, (b) *TaNPF6.2*, (c) *TaNPF6.7* and (d) *TaNPF6.1* genes by qPCR

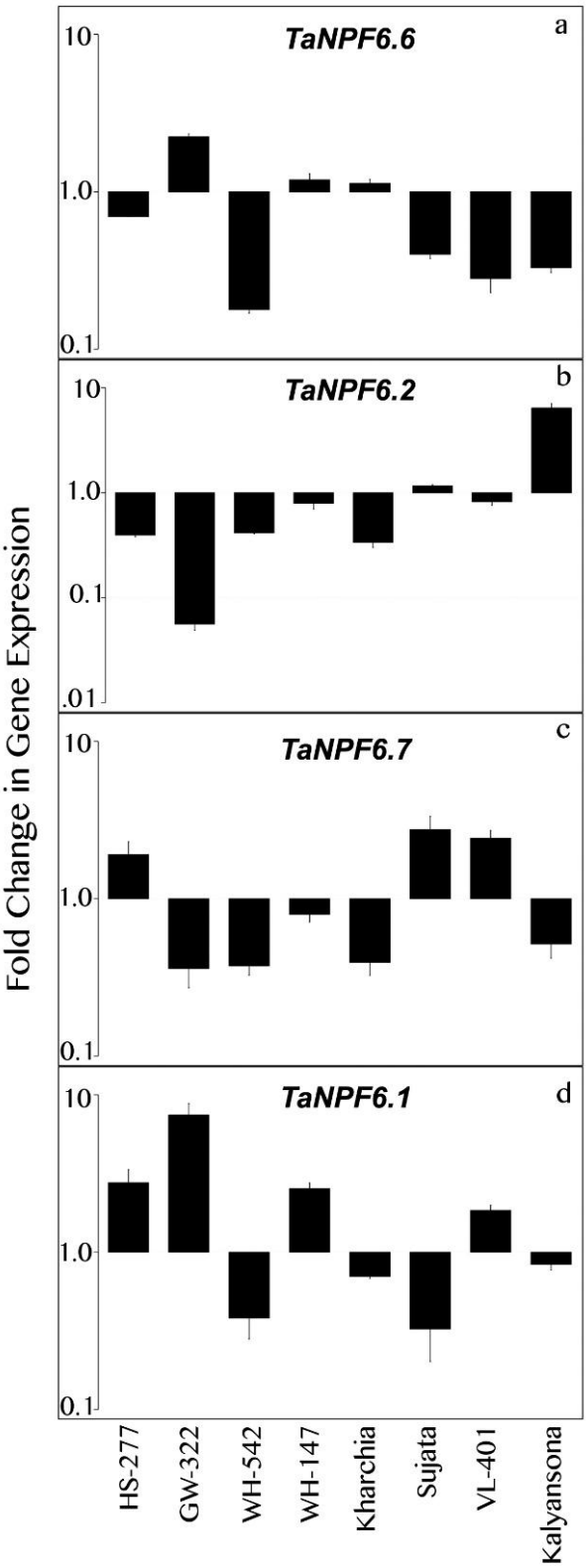

Table.1 Wheat genotypes and their features used for the experiment

Genotypes	Features related to Nitrogen Use Efficiency(NUE)
WH-542,GW-322	High Nitrogen responsive genotypes
HS-277,WH-147	High Nitrogen use efficient
Sujata,VL-401	Poor Nitrogen use efficient
Kharchia	Least Nitrogen uptake and utilization ability
Kalyansona	The most popular varieties during 1980s

Table.2 Candidate genes used in study

S. N.	Acc. number	Transporterid	Forward Primer	Reverse Primer
1	AF288688.1	TaNRT2	*GGGCTAACGCAACTTCTT*	*AGAGCGACGGGTAATGT*
2	AY053452.1	TaNRT2.3	*GGCTCACACAACTTCTCTTC*	*GCACCAGAGTGATAGGTAATG*
3	HF544990.1	TaNPF6.6	*CCTCTTCACCTCCCTCAA*	*CACGGTTGCGAAGACAA*
4	AY587264.1	TaNRT1.2	*GACGCCATGAGAAGTTTAGG*	*CCTCCTCTGGCTGTGAATA*
5	HF544997.1	TaNPF8.2	*CTTTCCCTTGTGCCAGTATT*	*CAGCCATCATCAGGTAGAATC*
6	HF544996.1	TaNPF8.1	*CTCGGCTGGAAATTACCTTAG*	*GTCCAGATGCCCTTCATTC*
7	HF545000.1	TaNPF2.2	*TACGCGAGCGGTCTAAT*	*GGCTGTGACAAGGTAGAATAG*
8	HF544993.1	TaNPF7.2	*TCACGGGACTTGTGATACT*	*TACGTCGACAGGTAGAAGAG*
9	HF545001.1	TaNPF2.3	*CCGTCAACCTCATCTACTTTG*	*GCAGTCGCAGCTTTCTT*
10	HF544988.1	TaNPF6.1	*CTATGCGCAGATGACCAC*	*AATGAGGCGGTCGTAGATA*
11	HF545003.1	TaNPF4.2	*GGTGGCACTCATCAACTATG*	*CTCGCTATGCTTGGCTATTT*
12	HF544991.1	TaNPF6.5	*ATCAACCTGGCCGCTTAC*	*TGCACCAGCTAGCATTTCT*
13	HF544987.1	TaNPF6.3	*AGGCTCGACTACTTCTACTG*	*CCATGCGTTTCTCCTTGT*
14	HF545004.1	TaNPF6.7	*CCGGCACCAGTACAAAC*	*CCTATTCGATCCACCCTACA*
15	HF545002.1	TaNPF1.1	*AGACGGAAATTGGAGCATAC*	*CTGAGTGACAGTGCAAATCT*
16	HF544986.1	TaNPF6.2	*ACTTCTTCCTGCGAGAGT*	*CTTGTGCACGATGGTTACT*

Mostly the expression of HATS gets induced by N-starvation and was evident in case all the genotypes except Kharchia and GW-322. qPCR result sowed that Kharchia had a complete contrast in comparison to all other genotypes for the HAT *TaNRT2* gene expression under N-stress. With all the earlier observation in mind, this data points out towards uniqueness about the genotype, and it also shows the lower N-foraging capability from the beginning of the growth period, i.e. at its seedling stage.

As discussed earlier, NRT1s are known as low affinity transporters, and active when the nitrate concentration in the soil is high. Later these transporters are named as NPF, and all the transporters, with ID as NTR1 or NPF, are low affinity ones. In the present study, there was no correlation of the expression of these four LATS were found among the genotypes, which indicated the variability of the LATS and need for many LATS as they must be working in a different manner and might not be possible to replace one with the other one. Though the genotypic variation was evident for all the four LATS, different genotypes showed different level of expression for different LATS. This indicates the role of each LATS are different. However, some of the LATS showed genotype specific higher expression under N-stressed condition. Individual LATS under the present study are discussed below.

TaNPF6.6 expression also indicates that Kharchia is different by its static expression under N-stress condition, where most of the genotypes showed a lower level expression under N-stress condition. The trend was similar in case of *TaNPF6.2*also. Only Kalyansona had a higher expression, Kharchia and VL-401 did not alter their expression under N-stress. Kalyansona and Kharchia showed minimal change in expression for *TaNPF6.1* too. It is reported that *TaNPF6.1* and *TaNPF6.2* transcripts were present with high abundance in the roots and very low abundance in the shoots (Buchner *et al.,* 2014), but their expression under low nitrogen is not much elaborated. The regulation of wheat NFP genes by plant N-status indicated involvement of these transporters in substrate transport in relation to N-metabolism (Buchner *et al.,* 2014).

WH-147 changes its gene expression insignificantly for *TaNPF6.7*. This study reveals different LATS are regulated and expressed in a genotype specific manner and all LATS together decide the uptake capability of the genotype. However, some of the contrasting genotypes like Kharchia, VL-401, Kalyansona, which are not known for their N-use capability, showed the different gene expression pattern in most of the LATS.

Under N-stress condition, the expression of LATS are not be reported in wheat, but over expression of some of the LATS have been reported in rice, which increased the plant growth, not the nitrogen use efficiency (Fan *et al.,* 2014). Some of the LATS are required for redistribution of nitrate and there by promoting growth, mainly NRT1.11 and NRT1.12, which are xylem borne (Hsu and Tsay, 2013). Similarly Arabidopsis Nitrate Transporter NRT1.9 is important in Phloem Nitrate Transport (Wang and Tsay, 2011). With respect to the transporters investigated presently – NPF6.1, NPF6.2, NPF6.6 and NPF6.7, none of them are characterized so far with N-stressed condition. Neither functions of them are well established, except they are categorised as NRT1/NPF family (LATS) based on the sequence information (http://www.uniprot.org/uniprot/). Present study depicted some genotype specific information on their expression, but functional genomics studies for these genes will make the clear about their exact function.

Gene expression of the LATS was first-hand information on this area and no reports on the functional properties of these transporters (NPF6.1, NPF6.2, NPF6.6 and NPF6.7) are not known. Hence, this may be possibly the first report and genotypic variation were evident from this study. Kharchia, showed minimum change in expression, whereas VL-401 and Kalyansona were distinctly different from the study under N-stress condition. One HAT gene *TaNRT2* expression was as expected and induced under low N, though the experiment was with chronic stress. However, Kharchia showed its distinct character by significantly down regulating.

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Author's contribution

CKN has actually done the most part of the work, G and AB has carried out the standardization and designing some of the primers, SKS has suggested for detail designing the experiment, KV has grown the materials in field from where the genotypes were selected, PKM has over all idea of the research experiment and guidance as group leader.

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