



External Nitrogen and Carbon Source-Mediated Response on Modulation of Root System Architecture and Nitrate Uptake in Wheat Seedlings

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Abstract

Nitrogen uptake efficiency is an important component trait that could be targeted for improving nitrogen use efficiency of crop plants. To understand the responses of different nitrate transport systems and the influence of root system architecture on nitrate uptake under limited nitrate conditions in wheat (*Triticum aestivum* L.) at the seedling stage, we studied nitrate uptake, root system architecture, and expression of different nitrate transporter genes in induced and non-induced wheat seedlings. Further, effects of inclusion of sucrose and two amino acids (glutamine and asparagine) in induction medium on these parameters were also studied. We observed that the induced wheat root system took up more nitrate as compared to non-induced root system in a dose-dependent manner. Gene expression of both high- and low-affinity nitrate transporter gene showed differential expression in the induced root tissues, as compared to non-induced tissues, depending on the concentration of nitrate present in induction medium. External nutrient media containing sucrose, glutamine, and asparagine reduce nitrate concentration in both root and shoot tissues and also influence the gene expression of these transporters. Our observations indicate that upon induction with milder external nitrate concentrations, the root architecture is modulated by changing overall lateral root size and 1st order lateral root numbers along with activation of nitrate transporters which acquire and transport nitrate in roots and shoots, respectively, depending on the carbon and nitrogen source available to seedlings.

Keywords Nitrate transporter · Nitrate starvation · Root system architecture · Nitrate uptake efficiency · Wheat

Introduction

Nitrogen (N) is a key essential mineral element that is required for various life processes of plants such as biosynthesis of amino acids, nucleic acids, chlorophylls, cofactors, several secondary metabolites, and so on (Crawford 1995; Scheible et al. 1997; Stitt 1999). Plants are able to acquire both inorganic as well as organic N from the soil in the form of nitrate (NO_3^-), ammonium (NH_4^+), urea, amino acids, and so on depending on their species and the physico-chemical properties of soil (Masclaux-Daubresse et al. 2010;

Miller et al. 2007). Among the different forms of nitrogen, nitrate is the predominant form in aerobic soil (Forde 2000) and hence the most preferred form of nitrogen for cultivation of wheat. Owing to the mobile nature of nitrate in the soil, the percentage of the applied nitrogenous fertilizer actually used by the wheat crop is very low and consequently a majority of it is lost to the environment by processes such as leaching, denitrification, volatilization, and so on (Good et al. 2004). The N losses lead to environmental pollution globally (Zhang et al. 2015). Further, because the lost N is not available to plants for growth, development, or yield, it causes economical losses for farmers. Being one of the staple foods of the major part of the world, enhancing nitrogen use efficiency (NUE) of wheat has now become a major concern for wheat improvement programs. Nitrate uptake efficiency (NUpE), that is, the ability of a genotype to take up nitrate from the soil, is probably the most important component trait toward improving NUE in wheat.

The first step of nitrate metabolism in plants is its uptake from the external environment into the cell. Nitrate (NO_3^-)

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molecules are actively transported by nitrate transporters located across plasma membrane of epidermal and cortical cells of the root tissues belonging to mainly two types of nitrate uptake systems, that is, high-affinity transport system (HATS) and low-affinity transport system (LATS) (Fan et al. 2017). These transport systems are further classified based on their transport kinetics into mainly three distinct nitrate transport categories (Miller et al. 2007; Tsay et al. 2007; Wang et al. 2012): (i) Constitutive high-affinity nitrate transport systems (CHATS), which are characterized by their lower values of both K_m and V_{max} ; (ii) inducible high-affinity nitrate transport systems, which show higher values of K_m and V_{max} and are induced by exposure of NO_3^- within hours to days; (iii) constitutive low-affinity transporters that take up nitrate at higher concentrations and in contrast to HATS fail to saturate at NO_3^- concentration as high as 50 mM (Crawford and Glass 1998). In *Arabidopsis*, constitutive LATS (CLATS) and inducible LATS (ILATS) have also been reported (Tsay et al. 1993; Huang et al. 1999). LATS and HATS are encoded by the *NRT1* and *NRT2* gene families, which have 53 and 7 known members, respectively, in *Arabidopsis thaliana* (Wang et al. 2012 and references therein). The various aspects of nitrate transport, sensing, and signaling have been reviewed in recent publications (O'Brien et al. 2016; Kiba and Krapp 2016; Noguero and Lacombe 2016; Fan et al. 2017). The expression study of some of the putative low- and high-affinity nitrate transporter genes of wheat has been studied under different growth stages and external concentrations of nitrogen (Buchner and Hawkesford 2014; Guo et al. 2014; Melino et al. 2015; Taulemesse et al. 2015), however, only *TaNRT2.1* was isolated and characterized in detail (Yin et al. 2007). The N status and growth stage at the time of N treatment in these studies are entirely different than the present study.

Nitrate acquisition by plants depends on several internal and external conditions such as fluctuating nitrate availability in the soil and physiological conditions of plants (for example, NO_3^- assimilation, acquisition of other nutrients, growth condition, etc.) (Ruffel et al. 2014). To compensate for fluctuations of external NO_3^- concentrations and the actual N demand of plants for normal growth and development, the plant root system undergoes continuous modulation for its NO_3^- uptake kinetics (Alvarez et al. 2012; Krouk et al. 2010; Vidal and Gutierrez 2008). The monocot cereals including wheat have complex root structures composed of several root types (Klepper 1992). Apart from an embryonic primary root, wheat seedlings also form variable numbers of seminal roots that emerge from the scutellar node which are also embryonic. All of these embryonic roots can form first- and second-order lateral roots. Later, they also form several crown roots which are non-embryonic in nature. All of these root systems play significant roles in nutrient and water acquisition. The effects of different nutrients on growth and

development of roots in model plants have been widely studied. However, detailed root system architecture studies under the influence of external nutrients are by and large more restricted to the model plant, that is, *Arabidopsis* (Gruber et al. 2013; Kellermeier et al. 2014). However, the influence of nitrate on overall growth of barley root systems including lateral roots has been reported (Drew et al. 1973). Knowledge of the best root system architecture for efficient nitrate uptake will play an important role in developing strategies for root breeding for efficient nitrate uptake in its limited and heterogeneous availability condition in the soil. Nitrate uptake by roots is under the control of complex regulation by at least two different N-signaling pathways. NO_3^- itself locally induces the expression of several genes encoding its own transport systems, as well as the main enzymes involved in its assimilation (Wang et al. 2004; Gojon et al. 2009). The root NO_3^- uptake system is also under systemic feedback repression by whole-plant signals associated with high N status of the organism (Tabata et al. 2014; Ohkubo et al. 2017).

After the entry of NO_3^- into the root tissues, it is metabolized to ammonium by two enzymes (nitrate reductase and nitrite reductase) (Meyer and Stitt 2001), and is further assimilated to glutamate by the GS-GOGAT pathway (Lea and Mifflin 1974; Lea and Forde 1994). The assimilation of ammonium requires a carbon (C) skeleton for the biosynthesis of important molecules such as amino acids, proteins, and nucleic acids. Therefore, NO_3^- uptake is not only determined by NO_3^- availability and N demands of the whole plant (Imsande and Touraine 1994; Forde 2002; Alvarez et al. 2012), but also by the C metabolites produced by photosynthesis (Delhon et al. 1996). It has been observed that plant root tissues are capable of taking up exogenously supplied carbon sources like sucrose and biochemically diverse types of amino acids (Malamy and Ryan 2001; Hirner et al. 2006; Svennerstam et al. 2007; Walch-Liu et al. 2006; Nasholm et al. 2009). Our current understanding about nitrate uptake and its regulation is more or less restricted to the model plant *Arabidopsis*, and not to field crops such as wheat. The number of genes that encode low- and high-affinity nitrate transport systems seems to be relatively high in wheat owing to its large genome size and the complex environment in which it grows as compared to *Arabidopsis*. Therefore, understanding the contribution of HATS and LATS towards overall N uptake and modulation of root system architecture under limited N conditions will be highly useful to mine the best allele among several nitrate uptake efficient wheat genotypes.

We studied nitrate uptake in seedlings of a high N-responsive wheat genotype that was subjected to induction with low to high nitrate concentrations (0.04–2 mM NO_3^-) and made comparisons to non-induced seedlings. We hypothesized that the presence of even a smaller amount of nitrate

in the induction system would modulate root system architecture and also the activities of different nitrate transporters, which consequently change the nitrate–N content in root and shoot tissues. The carbon and nitrogen status of the plants would most likely impact different nitrate transporter activities and hence, we included sucrose, asparagines, and glutamine (as C and N sources) in one of the induction systems (2 mM NO_3^-) to observe their effects on these parameters. The present study would give an insight towards the relation of nitrate transport system under external NO_3^- induction and RSA which might play a role in nitrogen use efficiency of wheat which is conferred by the complex root system.

The objectives of the present investigation were to study the gene expression of kinetically different nitrate transporters in root tissue under different induced and non-induced conditions, changes in root morphology, and root system architecture during the induction period. The effects of inclusion of sucrose and two amino acids (glutamine and asparagines; the end products of nitrate and ammonium assimilation) in the nutrient media on these parameters were also studied.

Materials and Methods

Plant Material and Growth Conditions

In the present study, a widely grown Indian wheat variety known as HD-2967 (a highly N-responsive variety released from ICAR-Indian Agricultural Research Institute, New Delhi, India in 2011 for North Western and North Eastern Plain Zone of wheat growing zones of India with yield potential 5.4 ton/ha in timely sown irrigated condition) was used in all experiments.

Wheat seedlings were hydroponically grown after surface sterilization of uniform size seeds following the methods described by Goyal and Huffaker (1986) and Sinha et al. (2015). The modified hydroponic system was used as described by Negi et al. (2016), except for using square containers (Phyta Jar (box) from HIMEDIA, India; width \times length \times height = 75 mm \times 74 mm \times 138 mm) for growing wheat seedlings. The Phyta Jar consists of a stand supported net having perforations through which the radicle emerges and grows in liquid nutrient media. The germinated seeds were kept on the net which supports seedling growth throughout the experiment. The number of seedlings grown was 4 seedlings/box and all the experiments were carried out in controlled and aseptic conditions. The seeds were germinated by imbibing aerated de-ionized distilled water at 25 ± 1 °C in the dark. Then seedlings were kept in 0.2 mM CaSO_4 solution for 10 days in the dark at 25 ± 1 °C as described previously (Aslam et al. 1992). After 10 days of growth, the 0.2 mM CaSO_4 solution was

replaced by 1 \times MS (Murashige and Skoog) media (Nitrogen minus) and grown further for 3 days at 25 ± 1 °C under 150–200 $\mu\text{mol photon/m}^2/\text{s}$ light intensity, 10/14 dark/light hours, 70% relative humidity. The MS media at this stage were devoid of nitrogen just to prevent any induction of the transporters during this period.

After 13 days of growth, seedlings (four uniformly grown seedlings/box) were subjected to various pre-treatments on the 14th day with the solution (hereafter induction and non-induction media) specified in Fig. 1 for 24 h under similar growth condition as described above (Aslam et al. 1992) to observe various physiological and molecular changes as discussed below. The source of NO_3^- in all the experiments was $\text{Ca}(\text{NO}_3)_2$. The calcium concentration in all media was kept constant by adjusting the amount of CaCl_2 . During the entire period of plant growth, continuous aeration was ensured to provide ample O_2 and to maintain the uniform solution concentration at the root surfaces. The complete experiment plan is explained with the help of a flow chart (Fig. 1).

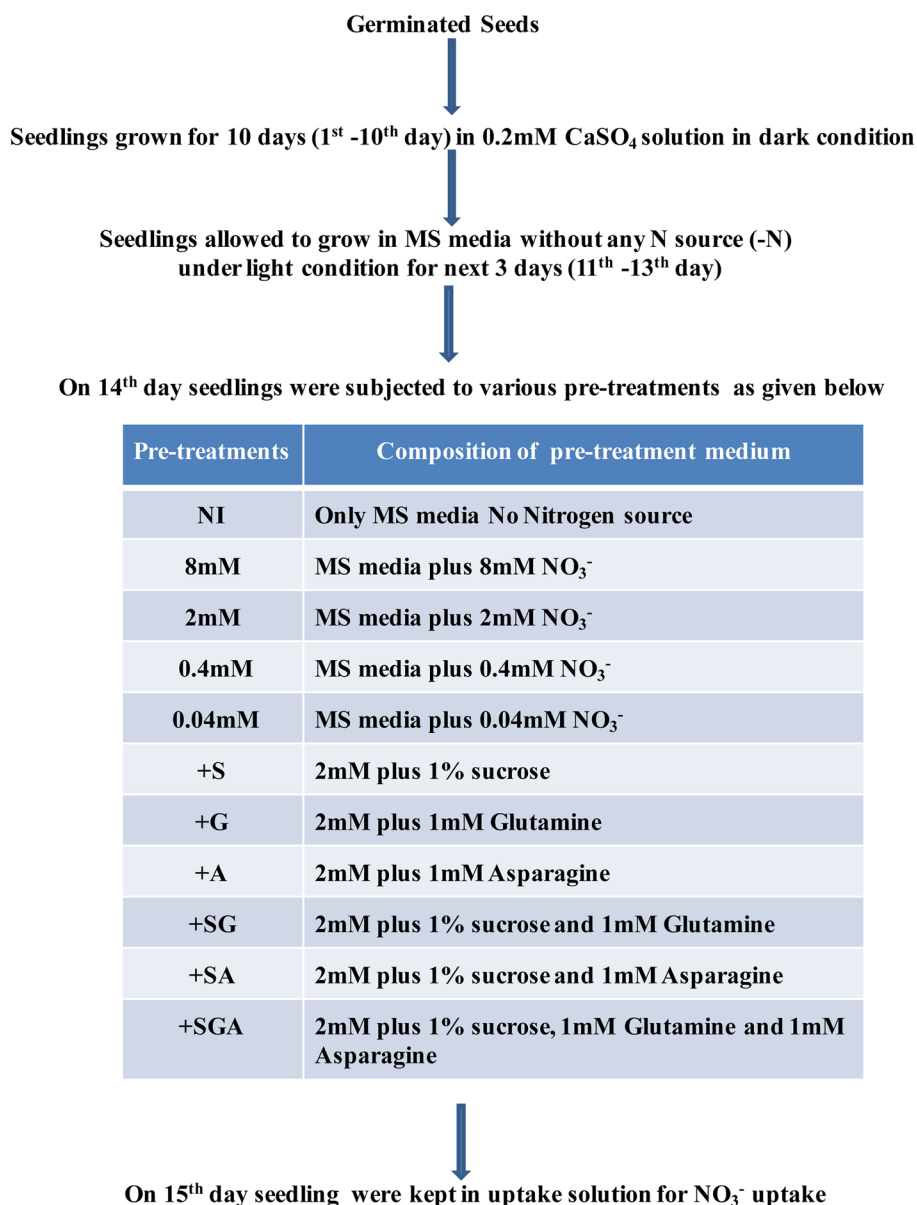
To observe the effect of chronic N starvation, seedlings were also grown in another set of experiments for 15 days; however, in this case, germinated seeds were directly transferred to a box (Phyta Jar) containing MS media consisting of either 8 mM NO_3^- (optimum) or 0.08 mM NO_3^- (stress). In this case, media were refreshed after every third day. Other growing conditions were the same as described above.

For biomass and morphological studies wherever mentioned, seedlings were divided into shoot and root and then the root and shoot length and fresh weight were measured separately. Dried weight of these tissues was measured after drying them at 50 °C temperature in hot air oven.

Nitrate–Nitrogen Estimation

After pre-treatment of seedlings as described (Fig. 1), the seedlings were transferred to uptake solution containing 8 mM NO_3^- (1 mM Mes buffer, pH 6.0; 0.2 mM CaSO_4 , and 4 mM calcium nitrate). NO_3^- –N content was measured in aliquots (20 μL) taken at the end of uptake and expressed as $\mu\text{g NO}_3^-$ –N/mL by following the method of Cataldo et al. (1975). The fine ground root and shoot tissues were suspended in de-ionized water and incubated at 45 °C for 1 h. Then samples were mixed and centrifuged at 5000 $\times g$ for 15 min and supernatants were used for analysis. Nitrate–nitrogen was also estimated in dried root and shoot tissues by the same method. In the case of dried tissue, 100 mg of dried root/shoot tissue was ground and mixed in 10 mL of de-ionized water for nitrate extraction and for further downstream process. Uptake solution was directly used for NO_3^- –N content analysis.

Fig. 1 Flow chart depicting the experimental plan



Root System Architecture Study

Images of properly washed and well spread roots were taken immediately after pre-treatment of seedlings using a flatbed scanner (Epson Perfection V700) at a resolution of 400 dpi. The Images thus generated were then analyzed with WinRhizo software which analyses the morphology of roots in the form of link analysis and can be converted into different root classes (Regent Instruments Canada Inc., Arsenault et al. 1995). WinRhizo analyses morphology (length, area, volume etc.), topology, architecture, and color analysis of roots. The data are stored in excel files and images in tiff, jpeg, or bmp files. All roots emerging from either the radicle or the scutellar node are here referred to as seminal roots; however, among all such seminal roots the one that emerges

from the radicle is referred to as the main/primary seminal root, whereas those emerging from scutellar nodes are referred to as lateral seminal roots. The post-embryonically developed lateral roots that emerged from these seminal roots are classified as 1st order lateral roots (LRs), whereas those developed from 1st order LRs are referred to as 2nd order LRs (Kellermeier et al. 2014). Out of the huge amount of data generated, some of them were processed for interpretation of five root parameters as per the given definition such as total root size (TRS: sum of path length of all types of roots described here), main root path (MRP length; primary root (seminal) that emerges from the radical, here the longest seminal root considered to be emerged from the radicle, and hence called a primary root), LRS (lateral root size; sum of path length of lateral roots (post-embryonic;

1st and 2nd order LRs) as a fraction of TRS), 1st order LR number (number of LRs emerging from primary and lateral seminal roots), and 2nd order LR number (number of LRs emerging from first-order LRs) were studied as described by Kellermeier et al. (2014) in the case of *Arabidopsis* roots with minor modification in Sinha et al. (2015) in case of wheat roots.

Nitrate Transporter Genes Expression Profile

Root tissues of induced (0.04 mM, 0.4 mM, 2 mM, and 8 mM NO_3^-) and non-induced seedlings were harvested and immediately snap-frozen in liquid nitrogen. Total RNA was extracted from three biological replicates following the Trizol method and treated with DNaseI as per the manufacturer's instructions. The integrity of RNA was confirmed by agarose (1.2%) gel electrophoresis. Complementary DNA was synthesized from the DNase-treated RNA using Superscript III Reverse Transcriptase (Invitrogen) as per the manufacturer's instructions. Results were normalized to the expression of the actin gene whose expression stability had already been validated in our several such experiments. Primers were synthesized to quantify the expression level of four nitrate transporter genes *TaNRT2.1* (AF332214.1), *TaNRT2.3* (AY053452.1), *TaNRT2* (AF288688.1), and *TaNRT1.1* (AY587265.1) (Suppl. Table 1). The quantitative real-time PCR experiments (qPCR) were conducted on an Eppendorf realplex with the SYBR Green Master Kit (Applied Biosystems). The qPCR program involved pre-incubation for 10 min at 95 °C, followed by 40 cycles of amplification, each consisting of denaturation for 15 s at 95 °C followed by annealing for 1 min at 60 °C. To ensure that single products were amplified, a melting curve was analyzed at the end of each assay. cDNA templates were diluted to 100 ng/ μL and qPCR was carried out using 10 μL reaction volume containing 1 μL diluted cDNA, 5 μL SYBR green mix, 1 μL each of 10 μM forward and reverse primers, and 2 μL nuclease-free water of three replicates of each samples. Relative expression was determined by the $\Delta\Delta C_T$ method corrected for primer efficiency (Livak and Schmittgen 2001) and compared against the non-induced treatment. In case of inclusion of sucrose, Gln, and Asn (+S, +G, and +A, respectively), the gene expressions were compared with a 2 mM induction system.

Statistical Analysis

All experiments and/or measurements reported in this study were done with five biological and three technical replicates except for the gene expression study wherein three biological replicates were taken. Mean values were presented for all the parameters with error bars (standard error of means). Least significant difference (LSD) at 5% was calculated for

significance of treatment effect and further a range test was performed to show the level of significance between and among the treatments in each experiment. The statistical analysis was performed using Statistical Package for the Social Sciences (SPSS, 2003) and MSTATC V2.1 (MSU, USA) (MSTATC 1990) software at alpha level 0.05. For comparing the means of optimum (8 mM NO_3^-) versus N stress (0.08 mM NO_3^-), we conducted a *t* test and wherever significant results were obtained results were presented with '*.' For any given two samples, initially an *F*-test at alpha level 0.05 was performed to see if the variances are equal. Based on the result of *F*-test, an appropriate *t* test was performed to see if the means are equal. For all other experiments in this article, means were compared and significant differences were determined, followed by ranking according to their significant differences. Factorial analysis (one factor) of variance (ANOVA) has been performed with a complete random design using MSTAT C version 2.1. Each experiment was performed with three biological replicates and four plants in each biological replicate. Ranking has been obtained as ascending order by 'range test' using the same software. Error mean square, degree of freedom, number of means to be separated, and number of observations per means were obtained from the ANOVA table. The least significant different difference test was performed as a sub-program under the range test to finally obtain the range with distinct letters.

Results

Chronic N starvation (0.08 mM NO_3^-) to wheat seedlings (HD-2967) grown for 15 days showed a significant increase in root/shoot ratios for their length, fresh weight, and dry weight (Fig. 2a). However, the NO_3^- -N content in both the tissues was drastically under stress conditions reflecting the imposition of N stress conditions (Fig. 2b).

In a further study with a different induction system, we observed that 0.4 mM NO_3^- induced maximum uptake of NO_3^- (Fig. 3a). Therefore, we were interested to know what happened immediately after induction to roots with respect to their morphology, architecture, and expression of genes encoding high- and low-affinity nitrate transporters.

Effect of Different Nitrate Induction Systems on Nitrate Uptake and Its Allocation in Roots and Shoots

We first measured depletion of nitrate by quantifying NO_3^- -N in the uptake solution containing induced and non-induced (NI) wheat seedlings (14 days old; 10 days in dark, 3 days in light without N, and 1 day in induced and non-induced condition as described in M and M) and observed

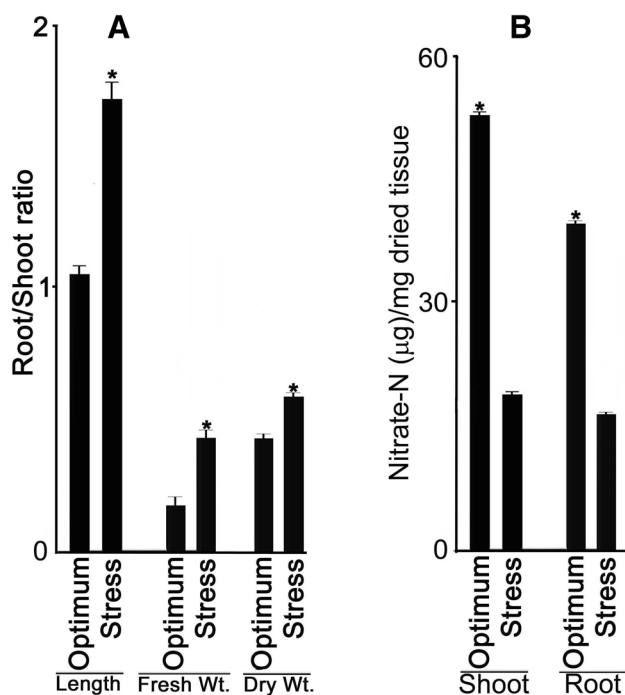


Fig. 2 Wheat seedlings grown under optimum (8 mM) and stress (0.08 mM) NO_3^- conditions for 15 days. Root and shoot ratio in terms of length, fresh weight, and dry weight basis under optimum and stress condition (a). Nitrate-N content in shoot and root tissues under optimum and stress condition (b). *Above bars indicate significant differences ($p < 0.05$) between the treatments

the lowest amount of NO_3^- -N (463 $\mu\text{g NO}_3^-$ -N/mL) in the uptake solution containing wheat seedlings induced by 0.4 mM NO_3^- as compared to non-induced, and the other two inducible systems (0.04 mM and 2.0 mM) (Fig. 3a). The uptake solution containing NI wheat seedlings contains the maximum amount of NO_3^- -N, that is, 873 $\mu\text{g NO}_3^-$ -N/mL. The other two systems have intermediate amounts ranging from NI to 0.04 mM. Our results in this case demonstrated that these inducible systems induce the nitrate uptake process to different degrees and among them the 0.4 mM inducible system caused maximum nitrate uptake by wheat seedlings.

NO_3^- -N was measured in both root and shoot tissues of wheat seedlings after 24 h of induction compared to NI systems. We detected very low levels of NO_3^- -N in shoot and root tissues of NI seedlings. However, exposure of various amounts of nitrate present in the induction solution for 24 h influences NO_3^- -N content in shoot and root tissues. The maximum amount was found in seedlings induced with 2 mM nitrate solution (8.83 and 14.5 $\mu\text{g NO}_3^-$ -N/mg dry tissues in both in shoots and roots, respectively). In the case of other NO_3^- induction systems, NO_3^- accumulation in shoots was on par with NI shoots in 0.04 and 0.4 mM induction, but NO_3^- accumulation was significantly higher in the 0.4 mM

induction system but not in the case of the 0.04 mM system. Interestingly after a further increase beyond 2–8 mM NO_3^- , the induction system reduced NO_3^- accumulation significantly, however, it was still significantly higher than that of the NI system in both shoot and root tissues (Fig. 3b and c). As the 2 mM NO_3^- induction system caused the maximum amount of NO_3^- -N, we further included different C and N sources in the 2 mM NO_3^- induction system to observe their effect on NO_3^- -N content (or nitrate uptake) in shoot and root tissue. All of them influenced the allocation of NO_3^- in root and shoot tissues differently. The major observation was that the C and N sources were significantly inhibitory in NO_3^- -N accumulation/uptake in shoot and root tissues as compared to the 2 mM induction solution (8.83 and 14.5 $\mu\text{g NO}_3^{2-}$ -N/mg in shoot and root, respectively). Addition of 1% sucrose (w/v) and 1 mM asparagine (1.0 $\mu\text{g NO}_3^-$ -N/mg in both cases) severely inhibits NO_3^- -N accumulation in shoot tissues. Similar inhibition of NO_3^- accumulation was observed in the case of root tissues as well. Interestingly, glutamine was less inhibitory compared to asparagines and sucrose and other combinations (4.17 $\mu\text{g NO}_3^{2-}$ -N/mg) in both shoot and root tissues except the +SA condition in shoots (Fig. 3d and e).

Effect of Different Induction Systems on Morphological Attributes

As far as morphological attributes of induced and non-induced wheat seedlings are concerned, we did not observe significant differences in a few parameters such as shoot length and root dry weight during both induction as well as C and N interaction; shoot dry weight was affected during the induction experiment only (Fig. 4a, c, i, j and l). However, root length showed significant changes wherein 2 mM induction system caused a maximum increase in root length, that is, 29.26 cm as compared to NI (13.33 cm) and the other induction systems. The 8 mM condition, however, had less impacts on root length than 2 mM, that is, 21.9 cm, but more impact compared to the NI, 0.04 and 0.4 mM conditions which have statistically similar effects (Fig. 4b). Sucrose, Gln, and Asn were inhibitory as far as total root length was concerned in comparison to the 2 mM condition (Fig. 4d). Fresh shoot weight was at a maximum in the case of 2 mM induction system (Fig. 4e). The other induction systems had less influence than NI. Root fresh weight in the NI condition at a maximum, that is, 41.07 mg, among all treatments followed by the 2 mM condition wherein the fresh weight was 29.33 mg (Fig. 4f). However, sucrose induction showed increased root fresh weight, that is, 36.33 mg, as compared to 2 mM, yet less than the NI condition (41.07 mg) (Fig. 4h). In the case of shoot fresh weight among all combinations, only the +SA system had a similar effect as the 2 mM system, whereas the other systems had inhibitory effects (Fig. 3g). In

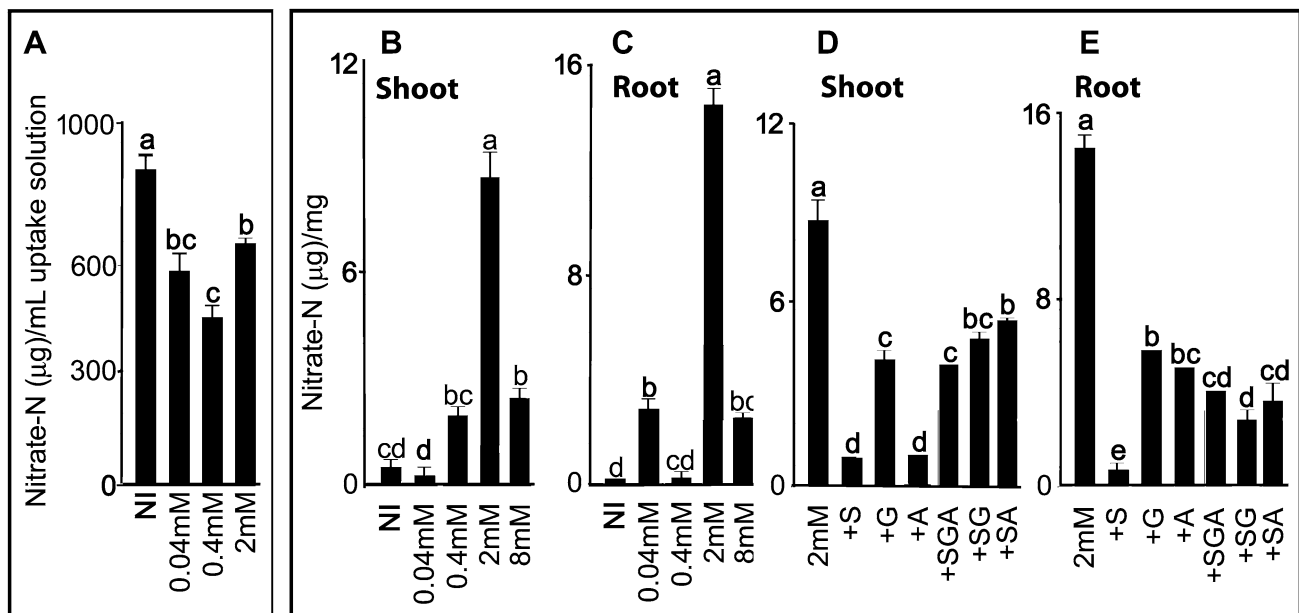


Fig. 3 Nitrate-N estimation in uptake solution and shoot and root tissues: amount of nitrate-N present in nitrate depleted uptake solution (8 mM NO_3^-) estimated after 24 h containing pre-treated (induced/non-induced (NI) wheat seedlings with different NO_3^- concentrations (0.04 mM, 0.4 mM, and 2.0 mM) (a). NO_3^- -N accumulation in shoot tissues (dried) immediately after induction with different NO_3^- concentrations (0.04 mM, 0.4 mM, 2.0 mM, and 8.0 mM) or

non-induced (NI) wheat (b) and root tissue (c). NO_3^- -N accumulation in shoot tissues (dried) induced with 2.0 mM NO_3^- along with sucrose (+S), glutamine (+G), asparagines (+A), sucrose + glutamine + asparagines (+SGA), sucrose + glutamine (+SG), sucrose + asparagines (+SA) (d), and root tissues (e). Letters above bars indicate significant differences ($p < 0.05$) between the treatments

the case of shoot dry weight, +SA conditions showed little increase as compared to all other systems (Fig. 4k).

Effect of Different Induction Systems on Root System Architecture

As far as TRS is concerned, the induction system containing 8 mM nitrate was stimulatory, that is, (220.45 cm) (Fig. 5IIe), as compared to NI which was just 101.29 cm (Fig. 5IIa), whereas the other induction systems were either on par or less than the NI condition (Fig. 5Ia). Among all C and N sources, the +S and +SGA induction systems (236.44 cm, 251.97 cm, respectively) and +A (210.51 cm) had interestingly stimulatory effects on TRS when incorporated in the 2 mM (108.87 cm) induction system (Fig. 5If). Although sucrose and asparagine were stimulatory in TRS when used separately (Fig. 5IIh and h), glutamine and its combination, that is, +SG (40.81 cm) were inhibitory (47.73 cm) (Fig. 5Ij and j). In this case, the stimulatory effect of asparagines was reduced when combined with sucrose (+SA-39.15 cm) (Fig. 5If and IIIk). In the case of the main root path length (MRP) that represents the length of the root that emerges from the radicle or primary root, it does not get induced by any of the induction systems except 2 mM (46.84 cm) as compared to NI (42.47 cm) (Fig. 5Ib and IIId). Both C and N sources and their combinations were inhibitory compared to 2 mM (Fig. 5Ij and IIIf-k). Another RSA

parameter, that is LRS, which reflects the fraction of lateral root length to TRS, in the cases of 0.04 mM (0.58), 0.4 mM (0.52), and 2 mM (0.53) were interestingly on par with NI (0.54) except the 8 mM (0.19) induction system which was inhibitory in nature (Fig. 5Ic and IIe). However, in this case, +G (0.619) imparted a significant increase as compared to 2 mM (0.53), whereas other incorporations were inhibitory in nature (Fig. 5Ih and IIg). As far as lateral root numbers are concerned, the 1st order lateral root numbers increased in 0.4 mM (18.5) and 8 mM (23.66) conditions as compared to NI (12.33) (Fig. 5Id and IIc and e). However, the 2nd order lateral root numbers in the NI and 2 mM conditions were on par, whereas both lower concentration (0.04 mM and 0.4 mM) and higher concentration (8.0 mM) conditions had lower numbers of second-order lateral roots (Fig. 5Ie). Incorporation of sucrose in the 2 mM induction system, that is +S, induced the number of first-order lateral root (Fig. 5II). As far as 2nd order lateral root numbers were concerned, neither C or N metabolites had any significant impact on this parameter (Fig. 5Ij).

Effect of Different Induction Systems on Expression of Nitrate Transporter Genes

All high-affinity nitrate transporter genes (*TaNRT2.1*, *TaNRT2.3*, and *TaNRT2*) were up-regulated in root tissues ranging from 4 to 40-fold, which were exposed with lower

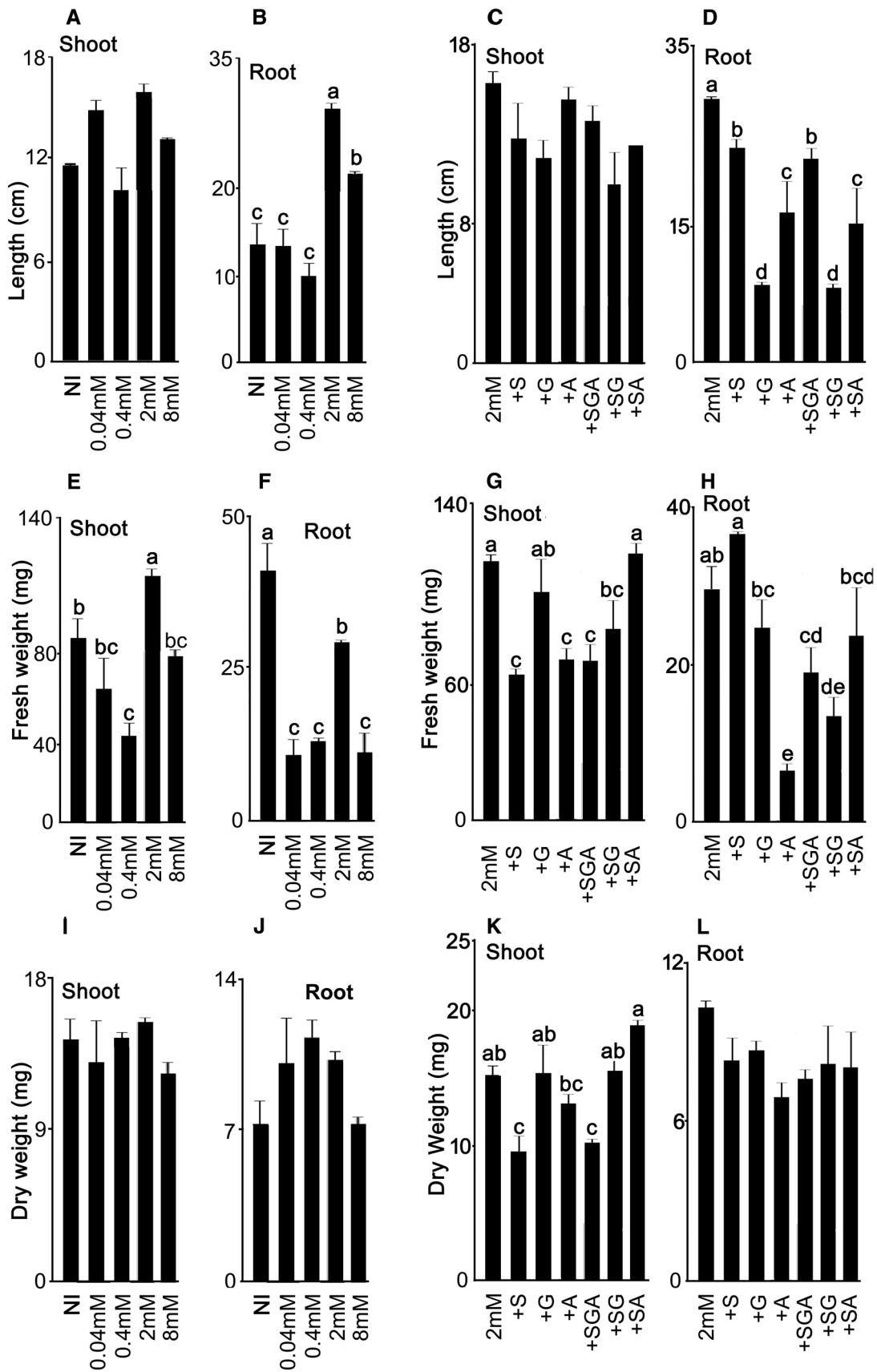


Fig. 4 Variations in different morphological parameters under induced, non-induced, and different combinations of C and N metabolites in 2 mM induction conditions: length (NI and induced systems: **a, b**); C and N combination systems: **c, d** fresh weight (NI and induced systems: **e, f**); C and N combination systems: **g, h**); dry weight (NI and induced systems: **i, j**); C and N combination systems: **k, l**). Letters above bars indicate significant differences ($p < 0.05$) between the treatments, whereas no letters above bar shows no significant difference between treatments

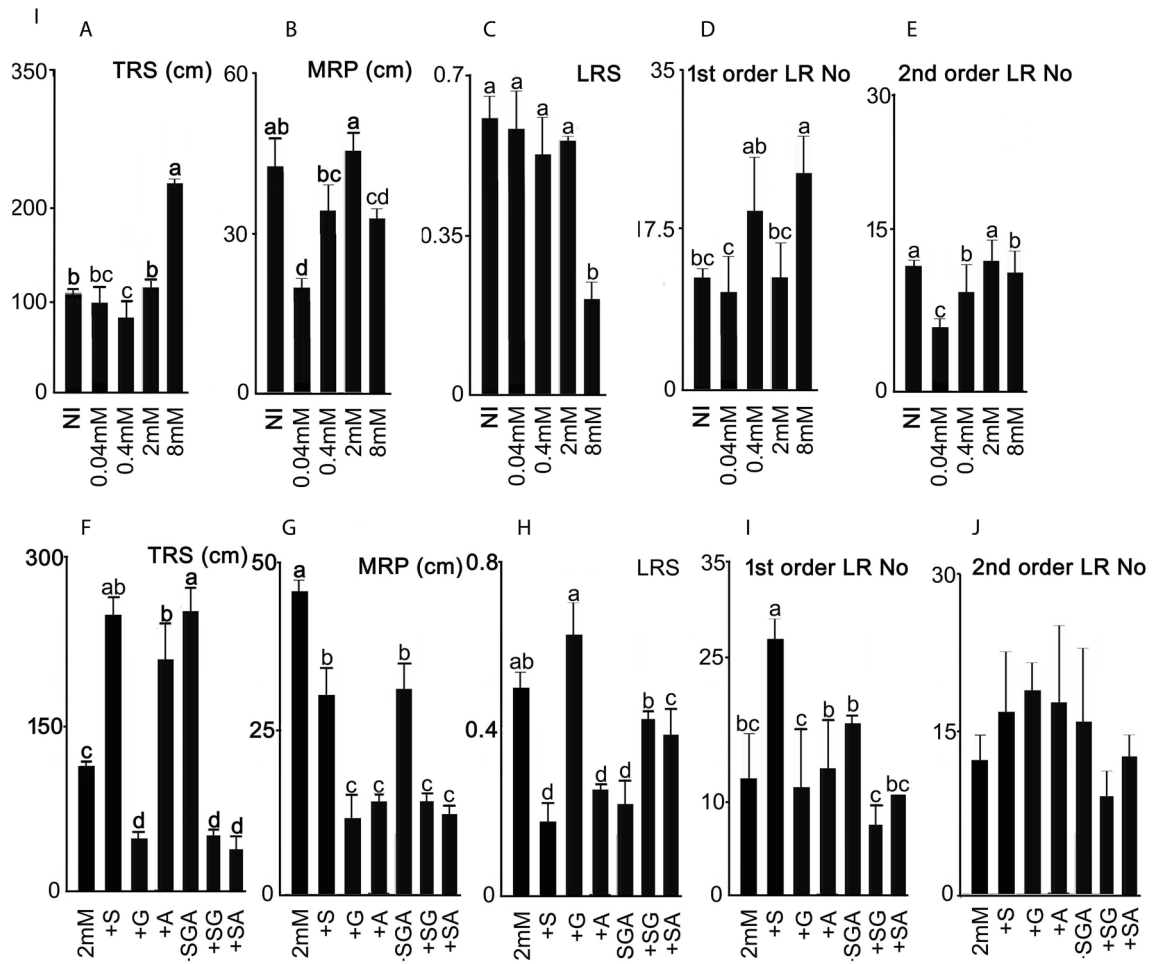
external nitrate concentrations (0.04 mM and 0.4 mM) compared to NI root tissues. Except in the case of *TaNRT2.3* (down-regulated by almost eight times), the 2 mM condition induced the expression of two other high-affinity nitrate transporter genes as compared to NI root tissues. The 8 mM induction system always down-regulated gene expression of all transporters except *TaNRT2.1*. As far as C and N metabolite inclusion in the 2 mM induction system is concerned, +S and +SGA caused up-regulation of *TaNRT2.3* as compared to 2 mM root tissues. +G, +A and their combinations with sucrose down-regulated the expression of *TaNRT2.3*. In the case of *TaNRT2.1*, all N and C combinations cause down-regulation of the gene when included in the 2 mM induction system but independently these sources have positive regulations. These systems, however, down-regulated *TaNRT2*, except +G and +S, when included in the 2 mM induction system. The low-affinity nitrate transporter *TaNRT1.1* was down-regulated in 0.04 mM, 2 mM, and 8 mM root tissues, but interestingly in the case of the 0.4 mM induction system it was up-regulated. In this case except +A and +SG, other C and N systems caused up-regulation of *TaNRT1.1* expression when included in the 2 mM induction system (Fig. 6).

Discussion

In this study, we observed reduction in biomass in terms of the shoot and root length, fresh, and dry weight in N stressed wheat seedlings undergoing 15 days of chronic N starvation. At the whole-plant level, the ratios of root versus shoot in terms of length, fresh weight, and dry weight significantly increased under N stress conditions indicating partitioning of sources towards roots to support their growth so that they can forage for N sources from the external media under N-limited conditions. Previous studies also suggest that mild nitrogen unavailability to the plant suppresses shoot growth and stimulates root growth; however, extreme conditions lead to an overall inhibition of plant growth, but still the inhibition is more prominent in shoots than root growth (Agren and Ingestad 1987; Fichtner and Schulze 1992).

Although NO_3^- uptake capacity varies with the nitrate concentration in the induction media it is always more than that of the condition wherein roots were never exposed to external nitrogen. Because nitrate uptake is absolutely a root

physiological phenomenon, it seems quite likely that nitrate available in the induction system could have modulated the root systems vis-a-vis nitrate uptake system activity which enables them to take up considerably higher amounts of nitrate from uptake solution than the NI system. Considering these assumptions, we focus on the physiological and molecular changes that have been induced in wheat seedlings during the nitrate induction period. We observed varied levels of nitrate-N both in root and shoot tissues irrespective of the initial concentration of N in the induction systems indicating tight regulation and dynamic allocation of nitrate in both roots and shoots for its further metabolism depending on C and N demand (Fig. 3b and c). The lower amount of NO_3^- -N in the 8 mM condition in both roots and shoots as compared to the 2 mM condition may be because of further metabolism of nitrate by nitrate reductase (NR) and/or nitrite reductase (NiR) in these tissues. At low external nitrate concentrations, NR is found primarily in epidermal cells and cortical cells of the root, whereas at higher external nitrate concentrations, activity is also detected in cells of the cortex and vascular system. We speculated that at higher nitrate concentrations, that is 8 mM, both NR concentration and activity might be more as compared to the 2 mM condition, therefore it is metabolized faster in this case and consequently nitrate was found in lesser amounts. NR activity has also been found to be inducible by nitrate itself (Kronzucker et al. 1995). We also observed that both external C and N sources inhibit nitrate uptake, however, surprisingly the degree of inhibition of nitrate uptake differs with the kind of N source, for example, Gln and Asn, which suggests their distinct direct or indirect regulatory roles (Fig. 3d and e). It has been observed in several studies that the downstream metabolites of ammonium assimilation such as amino acids translocated from the shoot to the roots via the phloem may act as signal molecules which mediate the down-regulation of root N uptake (Cooper and Clarkson 1989; Muller and Touraine 1992). Inclusion of sucrose, Gln, and Asn in the 2 mM induction system reduced the nitrate content in both root and shoot tissues. This might have happened either because of further assimilation of nitrate to ammonium during this period or the absence of accelerating activity of sucrose as a signal molecule for the nitrate uptake process because of a longer lag time in this case (Zhou et al. 2009). However, the degree of decreased concentration varies with the C and N sources used indicating their diverse role apart from acting just as a C or N source. The negative feedback relationship of external amino acids or tissue concentration of amino acids on nitrate uptake by plants has been observed in crops as well as model plants (Lee et al. 1992; Muller et al. 1995) with some degree of deviation (for example, *Brassica napus*; Laine et al. 1995). Biomass results indicate that under such circumstances roots are more plastic in nature than shoots to adjust the N requirement of wheat



5 cm

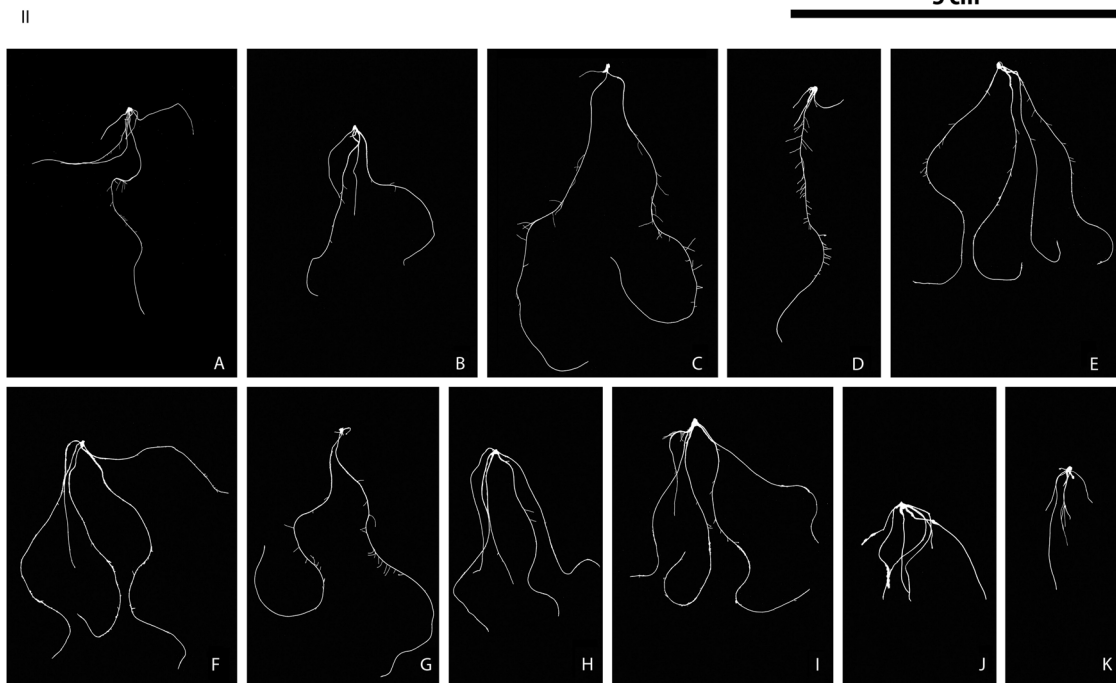


Fig. 5 Variations in root system architecture parameters under induced, non-induced (upper panel I: **a–e**), and different combinations of C and N metabolites in 2 mM induction conditions (lower panel I: **f–j**): TRS (total root size), MRP (main root size) LRS (lateral root size), and 1°—LRN and 2°—LRN (1st and 2nd lateral root number). Root morphology of non-induced (II:**a**), 0.04 mM (II:**b**), 0.4 mM (II:**c**), 2.0 mM (II:**d**), 8.0 mM (II:**e**), +S(II:**f**), +G(II:**g**), +A(II:**h**), +SGA(II:**i**), +SG(j), and +SA(i) studied using flatbed scanner (Epson Perfection V700) at a resolution of 400 dpi

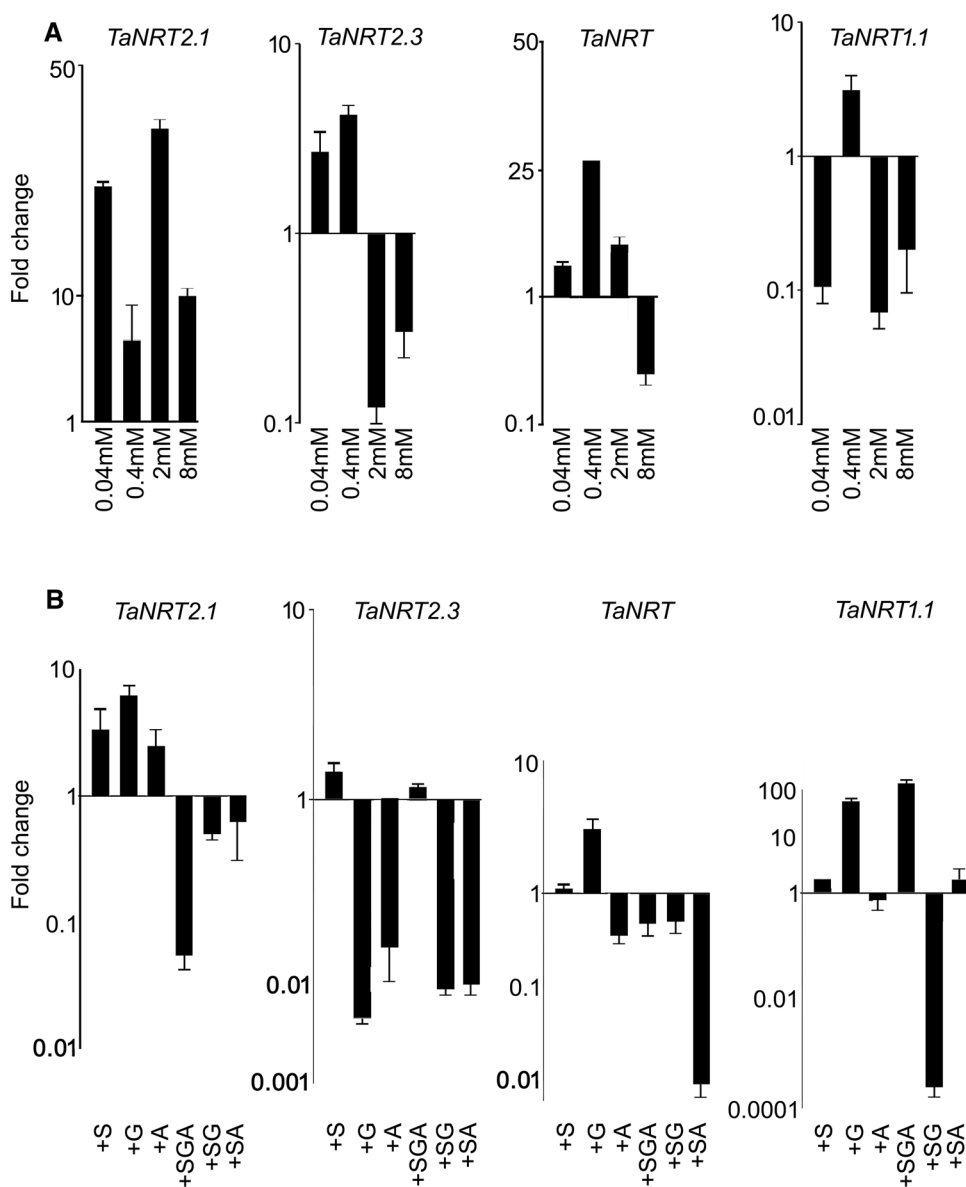
seedlings by changing length and fresh weight without any fluctuations in dry weight (Fig. 4).

To understand the possible role of different components of the root system in nitrate uptake in a changing external N concentration, we measured the major five root system architecture (RSA) parameters of wheat seedlings immediately after the induction period together with effects of external C and N sources. We observed variation in TRS under induced and non-induced conditions which has been resolved further into its component parameters (Fig. 5I and II). Our results show the significant influence of either lower or complete absence of external nitrate on lateral root parameters (LRS and 1st order LR numbers) than to main roots (MR) with some degree of variation. Within the LR parameters, the number (1st order LR numbers) and length might have played some role in nitrate sensing and acquisition. The variation in RSA parameters under different conditions indicates their probable role in nitrate sensing and acquisition under different external N status. Both the nutritional status of the plant and the external nutrient availability can induce changes in the overall root morphology (Giehl et al. 2014). Severe nitrogen starvation in the external media decreases total root length, whereas its availability even in a milder concentration induces its increase (Gruber et al. 2013). Such RSA alterations may reflect different strategies used by plants in response to the amount of available nutrient (Giehl and Wiren 2014). We observed that mild external NO_3^- concentrations in the induction media inhibit MRP significantly as compared to complete N starvation. It has been observed that supply of nitrate to nitrogen-deficient plants inhibits primary root elongation and stimulates LR growth by regulating auxin activity by a mechanism that involves the auxin receptor AUXIN SIGNALING F-BOX3 (AFB3; Vidal et al. 2010). However, the TRS in the case of NI is considerably less than that of 8.0 mM. We observed that the addition of C and N sources separately or in combinations in the induction media causes a specific impact on these RSA parameters. These observations indicate the possibility of their roles in either cell division or elongation of root cells, however, this needs further study.

As far as wheat is concerned, except *TaNRT2.1*, no other nitrate transporters are characterized in detail. Based on sequence homology these genes have been categorized in respective groups. Sequences available in the public domain

were used in this study for gene expression. To understand the possible reasons for variation in nitrate concentrations in roots and shoots under inducible and non-inducible conditions, we examined the expression pattern of the three high- and one low-affinity putative nitrate transporter genes in root tissues. We observed that all three high-affinity nitrate transporter genes were up-regulated in root tissues, which were induced with nitrate present at low concentrations (0.04 mM and 0.4 mM) in the induction media as compared to non-induced root tissue (Fig. 6). Their degree of up-regulation differs with the external nitrate concentration indicating the possibility of their activation at specific external N concentrations. However, the pattern of expression of these transporters varies at two concentration levels, that is 2 mM and 8 mM. Although *TaNRT2.3* and *TaNRT2.1* were down- and up-regulated, respectively, at these concentrations, *TaNRT2* was up-regulated at the 2 mM condition but down-regulated at the 8 mM condition. Under different growth conditions, *TaNRT2.3* has been found to be up-regulated in root tissues after exposure with different concentrations of nitrate (Yin et al. 2007; Guo et al. 2014). Melino et al. (2015) have also found the up-regulation of high-affinity nitrate transporter genes by lower concentrations of nitrate, but did not observe any changes in low-affinity nitrate transporter gene expression (*TaNRT1.1* in this case), though the N status of wheat plants at the time of gene expression was different than in the present study. Because in this study seedlings exhausted almost all stored food during first 10 days of growth in the dark, exposure to different concentrations of nitrate during 24 h of induction causes changes in expression of different high- and low-affinity nitrate transporters as compared to non-induced tissues. Our results corroborate the existence of kinetically different nitrate transport systems within each category of HATS and LATS in wheat too. The down-regulation of *TaNRT2.1* and *TaNRT2.3* gene expression under the +G condition together with the up-regulation of *TaNRT1.1* as compared to 2 mM wheat seedlings which suggests that *TaNRT1.1* probably suppresses IHATS (*TaNRT2.1* and *TaNRT2.3* in this case) activity when high concentrations of alternative N sources (Gln in this case) are present (Munos et al. 2004; Krouk et al. 2006). Several groups (Quesada et al. 1997; Krapp et al. 1998; Vidmar et al. 2000) also demonstrated a role for Gln in inhibiting the expression of inducible high-affinity nitrate transporters. Both nitrate-induced influx and transporter transcript abundance decrease simultaneously in root tissue when treated with exogenously applied amino acids (Vidmar et al. 2000). *NRT1.1* has been shown to act as a NO_3^- transceptor (transporter as well as receptor) (Gojon et al. 2011), which controls the expression of the high-affinity NO_3^- transporter gene *NRT2.1*, and the development of lateral roots (Krouk et al. 2006; Remans et al. 2006; Ho et al. 2009; Wang et al. 2009). In our *TaNRT1.1* expression study, we observed the

Fig. 6 Real-time gene expression profile under induced, non-induced (a) and different combinations of C and N metabolites in 2 mM induction conditions. **b** Genes are shown by their names, for example, *TaNRT2.1*, *TaNRT2.3*, *TaNRT2*, and *TaNRT1.1*



up-regulation of *TaNRT1.1* in the 0.4 mM induction system and down-regulation at the other two lower concentrations. However, even at the higher concentrations, that is 8 mM and 2 mM, there was lower expression as compared to the non-induced. Under high nitrate concentration, *NRT1.1* switches to a low-affinity function and *NRT2.1* gene expression is strongly induced which is evident in our *TaNRT2.1* expression. In one of the cases (*TaNRT2.3*), we found that the +S condition induced gene expression which is similar to the condition in which *NRT2.4* expression was found to be induced by N starvation and C (Kiba et al. 2012). Further, all levels of *NRT2.1* expression (promoter activity, transport level, transport activity) have been shown to be repressed by downstream N metabolites and induced by NO_3^- (Ruffel et al. 2014).

Overall in the present studies, we observed that wheat seedlings modulate their root system architecture more specifically its lateral root traits (LRS and 1st order LR numbers), under either complete absence of nitrate or presence of smaller amounts of it (Fig. 5c and d). Further, these smaller amounts of nitrate (0.04, 0.4, and 2 mM nitrate) in the external media cause higher amounts of nitrate uptake as compared to the non-induced root (Fig. 3a) probably because of activation of different high-affinity nitrate transporters which result in acquisition and translocation of nitrate into roots and shoots, respectively (Fig. 3b and c). The presence of smaller amounts of nitrate probably in this case passes a sense of availability of nitrogen in the external media to the plant which allows specific root growth to forage for nitrogen sources and concomitantly

activates the nitrate uptake system. On the other hand, the presence of externally available sucrose has been found to induce TRS and 1st order LR No. of root systems (Fig. 5f and i) and probably thereby influencing higher activity of HATS and LATS (Fig. 6b) in root tissue. However, the reduced nitrogenous end product of nitrate and ammonium assimilation, that is Gln and Asn, have been found to be inhibitory in nitrate–N content in both roots and shoots (Fig. 3d and e), whereas they have been found to both up- and down-regulate the HATS and LATS genes. The result indicates the involvement of potential post-translational regulation of nitrate transporters and feedback control by reduced N compounds in triggering nitrate uptake. Amino acids including Gln and Asn have been considered as feedback inhibitors of nitrate uptake (Muller and Touraine 1992). However, studies in this direction are needed to find explanations for these complex regulative parameters.

Conclusion

The results show that inducible nitrate transporters in roots contribute more in higher amounts of nitrate uptake from the external media specifically under nitrogen-limited conditions in wheat seedlings than that of non-inducible conditions. The lower external nitrate concentrations modulate the root system architecture, more precisely the lateral root parameters, both in terms of their number as well as length which probably contributes in nitrate uptake by increasing the expression of all high-affinity nitrate transporters gene in coordination with LATS thereby causing more uptake of NO_3^- -N from the uptake solution. The data also suggest that root tissues have different types of high-affinity nitrate transporters which seem to be working at different concentrations of external nitrate. Interestingly, C and N sources impact wheat seedlings both positively and negatively for its morphological as well as RSA attributes indicating C and N crosstalk in N sensing and signaling which ultimately impacts the overall growth and development, RSA, and also activation of nitrate transporter genes. Therefore, from the present studies, we can conclude that wheat seedlings utilize a specific and selective nitrate transport system which works in coordination with a network of regulatory steps of the nitrate assimilation pathway together with carbon assimilation to maintain a nutritional balance for optimal growth of shoots and roots including root architecture. Therefore, RSA traits, that is LRS and LR numbers, together with identification of efficient allele(s) for inducible high-affinity nitrate transporters will pave the way towards improving nitrogen use efficiency in general or nitrate uptake efficiency in particular in wheat.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflicts of interest.

References

- Agren GI, Ingestad T (1987) Root: shoot ratio as a balance between nitrogen productivity and photosynthesis. *Plant Cell Environ* 10:579–586
- Alvarez JM, Vidal EA, Gutierrez RA (2012) Integration of local and systemic signaling pathways for plant N responses. *Curr Opin Plant Biol* 15:185–191
- Arsenault JL, Pouleur S, Messier C, Guay R (1995) WinRHIZO™, a root-measuring system with a unique overlap correction method. *HortScience* 30:906
- Aslam M, Travis RL, Huffaker RC (1992) Comparative kinetics and reciprocal inhibition of nitrate and nitrite uptake in roots of uninduced and induced barley (*Hordeum vulgare* L.) seedlings. *Plant Physiol* 99:1124–1133
- Buchner P, Hawkesford MJ (2014) Complex phylogeny and gene expression patterns of members of the NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER family (NPF) in wheat. *J Expt Bot* 65:5697–5710
- Cataldo DA, Haroon M, Schrader LE, Youngs VL (1975) Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Commun Soil Sci Plant Anal* 6:71–80
- Cooper HD, Clarkson DT (1989) Cycling of amino-nitrogen and other nutrient between shoots and roots in cereals: a possible mechanism integrating shoot and root in the regulation of nutrient uptake. *J Expt Bot* 40:753–762
- Crawford NM (1995) Nitrate: nutrient and signal for plant growth. *Plant Cell* 7:859–868
- Crawford NM, Glass ADM (1998) Molecular and physiological aspects of nitrate uptake in plants. *Trends Plant Sci* 3:395–398
- Delhon P, Gojon A, Tillard P, Passama L (1996) Diurnal regulation of NO_3^- uptake in soybean plants IV. Dependence on current photosynthesis and sugar availability to the roots. *J Expt Bot* 47:893–900
- Drew MC, Saker LR, Ashley TW (1973) Nutrient supply and growth of seminal root system in barley. *J Expt Bot* 24:1189–1202
- Fan X, Naz M, Fan X, Wei X, Miller AJ, Xu G (2017) Plant nitrate transporters: from gene function to application. *J Expt Bot* 68:2463–2475
- Fichtner K, Schulze ED (1992) The effect of nitrogen nutrition on growth and biomass partitioning of annual plants originating from habitats of different nitrogen availability. *Oecologia* 92: 236–341
- Forde BG (2000) Nitrate transporters in plants: structure, function and regulation. *Biochem Biophys Acta* 1465:219–235
- Forde BG (2002) Local and long-range signaling pathways regulating plant responses to nitrate. *Ann Rev Plant Biol* 53:203–224
- Giehl RFH, Wirén N (2014) Root nutrient foraging. *Plant Physiol* 166:509–517
- Giehl RFH, Gruber BD, vonWirén N (2014) It's time to make changes: modulation of root system architecture by nutrient signals. *J Expt Bot* 65:769–778

- Gojon A, Nacry P, Davidian JC (2009) Root uptake regulation: a central process for NPS homeostasis in plants. *Curr Opin Plant Biol* 12:328–338
- Gojon A, Krouk G, Perrine-Walker F, Laugier E (2011) Nitrate transporter(s) in plants. *J Expt Bot* 62:2299–2308
- Good AG, Shrawat AK, Muench DG (2004) Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? *Trends Plant Sci* 9:597–605
- Goyal SS, Huffaker RC (1986) A novel approach and a fully automated microcomputer-based system to study kinetics of NO_3^- ; NO_2^- and NW transport simultaneously by intact wheat seedlings. *Plant Cell Environ* 9:209–215
- Gruber BD, Giehl RFH, Friedel S, Wiren N (2013) Plasticity of the Arabidopsis root system under nutrient deficiencies. *Plant Physiol* 168:161–179
- Guo T, Xuan H, Yang Y, Wang L, Wei L, Wang Y, Kang G (2014) Transcription analysis of genes encoding the wheat root transporter NRT1 and NRT2 families during nitrogen starvation. *J Plant Growth Regul* 33:837–848
- Hirner A, Ladwig F, Stransky H et al (2006) Arabidopsis LHT1 is a high affinity transporter for cellular amino acid uptake in both root epidermis and leaf mesophyll. *Plant Cell* 18:1931–1946
- Ho CH, Lin SH, Hu HC, Tsay YF (2009) CHL1 functions as a nitrate sensor in plants. *Cell* 138:1184–1194
- Huang NC, Liu KH, Lo HJ, Tsay YF (1999) Cloning and functional characterization of an Arabidopsis nitrate transporter gene that encodes a constitutive component of low-affinity uptake. *Plant Cell* 11:1381–1392
- Imsande J, Touraine B (1994) N demand and the regulation of nitrate uptake. *Plant Physiol* 105:3–7
- Kellermeier F, Armengaud P, Seditas TJ, Danku J, Salt DE, Amtmann A (2014) Analysis of the root system architecture of Arabidopsis provides a quantitative readout of crosstalk between nutritional signals. *Plant Cell* 26:1480–1496
- Kiba T, Krapp A (2016) Plant nitrogen acquisition under low availability: regulation of uptake and root architecture. *Plant Cell Physiol* 57:707–714
- Kiba T, Feria-Bourrellier AB, Lafouge F, Lezhneva L, Boutet-Mercey S, Orsel M (2012) The Arabidopsis nitrate transporter NRT2.4 plays a double role in roots and shoots of nitrogen-starved plants. *Plant Cell* 24:245–258
- Klepper B (1992) Development and growth of crop root system. In: Hatfield JA, Stewart BA (eds) *Advances in soil science, limitations to plant root growth*, vol 19. Springer-Verlag, New York, pp 1–25
- Krapp A, Fraissier V, Scheible WR, Quesada A, Gojon A, Stitt M (1998) Expression studies of *Nrt2:INp*, a putative high-affinity nitrate transporter: evidence for its role in nitrate uptake. *Plant J* 14:723–731
- Kronzucker H, Glass ADM, Siddiqi MY (1995) Nitrate induction in spruce: an approach using compartmental analysis. *Planta* 196:683–690
- Krouk G, Tillard P, Gojon A (2006) Regulation of the high-affinity NO_3^{2-} uptake system by NRT1.1-mediated NO_3^{2-} demand signaling in Arabidopsis. *Plant Physiol* 142:1075–1086
- Krouk G, Crawford NM, Coruzzi GM, Tsay YF (2010) Nitrate signaling: adaptation to fluctuating environments. *Curr Opin Plant Biol* 13:265–272
- Laine P, Ourry A, Boucaud J (1995) Shoot control of nitrate uptake rates by roots of *Brassica napus* L.: effects of localized nitrate supply. *Planta* 196:77–83
- Lea PJ, Forde BG (1994) The use of mutants and transgenic plants to study amino acid metabolism. *Plant Cell Environ* 17:541–556
- Lea P, Mifflin B (1974) Alternative route for nitrogen assimilation in higher plants. *Nature* 251:614–616
- Lee RB, Purves JV, Ratcliffe RG, Saker LR (1992) Nitrogen assimilation and the control of ammonium and nitrate absorption by maize roots. *J Expt Bot* 43:1385–1396
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using realtime quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25:402–408
- Malamy J, Ryan K (2001) Environmental regulation of lateral root initiation in Arabidopsis. *Plant Physiol* 127:899–909
- Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gaufichon L, Suzuki A (2010) Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. *AnnBot* 105:1141–1157
- Melino VJ, Fiene G, Enju A, Cai J, Buchner P, Heuer S (2015) Genetic diversity for root plasticity and nitrogen uptake in wheat seedling. *Funct Plant Biol* 42:942–956
- Meyer C, Stitt M (2001) Nitrate reductase and signallin. In: Lea PJ, Morot-Gaudry JF (Eds) *Plant nitrogen*. Springer, New York
- Miller AJ, Fan X, Orsel M, Smith SJ, Wells DM (2007) Nitrate transport and signalling. *J Exp Bot* 58:2297–2306
- MSTATC (1990) A microcomputer program for the design, management, and analysis of research agronomic experiments. Michigan State University, East Lansing
- Muller B, Tilliard P, Touraine B (1995) Nitrate fluxes in soybean seedling roots and their response to amino acids: an approach using ^{15}N . *Plant Cell Environ* 18:1267–1279
- Müller B, Touraine B (1992) Inhibition of NO_3^- —uptake by various phloem translocated amino acids in soybean seedlings. *J Expt Bot* 43:617–623
- Muños S, Cazettes C, Fizames C, Gaymard F, Tillard P, Lepetit M et al (2004) Transcript profiling in the *chl1-5* mutant of Arabidopsis reveals a role of the nitrate transporter NRT1.1 in the regulation of another nitrate transporter, NRT2.1. *Plant Cell* 16:2433–2447
- Näsholm T, Kielland K, Ganeteg U (2009) Uptake of organic nitrogen by plants. *New Phytol* 182:31–48
- Negi M, Sanagala R, Rai V, Jain A (2016) Deciphering phosphate deficiency-mediated temporal effects on different root traits in rice grown in a modified hydroponic system. *Front Plant Sci* 7:550
- Noguero M, Lacombe B (2016) Transporters involved in root nitrate uptake and sensing by Arabidopsis. *Front Plant Sci* 7:1391
- O'Brien JA, Vega A, Bouguyon E, Krouk G, Gojon A, Coruzzi G et al (2016) Nitrate transport, sensing, and responses in plants. *Mol Plant* 9:837–856
- Ohkubo Y, Tanaka M, Tabata R, Ogawa-Ohnishi M, Matsubayashi Y (2017) Shoot-to-root mobile polypeptide involved in systemic regulation of nitrogen acquisition. *Nat Plants*. <https://doi.org/10.1038/nplants.2017>
- Quesada A, Krapp A, Trueman LJ, Daniel-Vedele F, Ferná'ndez E, Forde BG et al (1997) PCR-identification of a *Nicotiana plumbaginifolia* cDNA homologous to the high affinity nitrate transporters of the crnA family. *Plant Mol Biol* 34:265–274
- Remans T, Nacry P, Pervent M, Filleur S, Diatloff E, Mounier E et al (2006) The Arabidopsis NRT1.1 transporter participates in the signaling pathway triggering root colonization of nitrate-rich patches. *Proc Natl Acad Sci USA* 103:19206–19211
- Ruffel S, Gojon A, Lejay L (2014) Signal interactions in the regulation of root nitrate uptake. *J Expt Bot* 65:5509–5517
- Scheible WR, Gonzalez-Fontes A, Lauerer M, Muller-Rober B, Caboche M, Stitt M (1997) Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. *Plant Cell* 9:783–798
- Sinha SK, Rani M, Bansal N, Venkatesh K, Mandal PK (2015) Nitrate starvation induced changes in root system architecture, carbon-nitrogen metabolism, and miRNAs expression in nitrogen-responsive wheat genotypes. *Appl Biochem Biotechnol* 177:1299–1312
- Stitt M (1999) Nitrate regulation of metabolism and growth. *Curr Opin Plant Biol* 2:178–186

- Svennerstam H, Ganeteg U, Bellini C, Nasholm T (2007) Comprehensive screening of Arabidopsis mutants suggests the lysine histidine transporter 1 to be involved in plant uptake of amino acids. *Plant Physiol* 143:1853–1860
- Tabata R, Sumida K, Yoshii T, Ohyama K, Shinohara H, Matsubayashi Y (2014) Perception of root-derived peptides by shoot LRR-RKs mediates systemic N-demand signaling. *Science* 346:343–346
- Taulemesse F, Le Gouis J, Gouache D, Gibon Y, Allard V (2015) Post-Flowering nitrate uptake in wheat is controlled by N status at flowering, with a putative major role of root nitrate transporter NRT2.1. *PLoS ONE* 10(3):e0120291
- Tsay YF, Schroeder JI, Feldmann KA, Crawford NM (1993) The herbicide sensitivity gene CHL1 of Arabidopsis encodes a nitrate-inducible nitrate transporter. *Cell* 72:705–713
- Tsay FY, Chiu CC, Tsai CB, Ho CH, Hsu PK (2007) Nitrate transporters and peptide transporters. *FEBS Lett* 581:2290–2300
- Vidal EA, Gutierrez RA (2008) A systems view of nitrogen nutrient and metabolite responses in Arabidopsis. *Curr Opin Plant Biol* 11:521–529
- Vidal EA, Araus V, Lu C, Parry G, Green PJ, Coruzzi GM, Gutierrez RA (2010) Nitrate-responsive miR393/AFB3 regulatory module controls root system architecture in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 107:4477–4482
- Vidmar JJ, Zhuo D, Siddiqi MY, Schoerring JK, Touraine B, Glass ADM (2000) Regulation of high affinity nitrate transporter genes and high affinity nitrate influx by nitrogen pools in plant roots. *Plant Physiol* 123:307–318
- Walch-Liu P, Liu LH, Remans T, Tester M, Forde BG (2006) Evidence that L-glutamate can act as an exogenous signal to modulate root growth and branching in *Arabidopsis thaliana*. *Plant Cell Physiol* 47:1045–1057
- Wang R, Tischner R, Gutierrez RA, Hoffman M, Xing X, Chen M et al (2004) Genomic analysis of the nitrate response using a nitrate reductase-null mutant of Arabidopsis. *Plant Physiol* 136:2512–2522
- Wang R, Xing X, Wang Y, Tran A, Crawford NM (2009) A genetic screen for nitrate regulatory mutants captures the nitrate transporter gene *NRT1.1*. *Plant Physiol* 151:472–478
- Wang YY, Hsu PK, Tsay YF (2012) Uptake, allocation and signaling of nitrate. *Trends Plant Sci* 17:458–467
- Yin LP, Li P, Wen B, Taylor D, Berry JO (2007) Characterization and expression of a high-affinity nitrate system transporter gene (*TaNRT2.1*) from wheat root, and its evolutionary relationship to another *NRT2* genes. *Plant Sci* 171:621–631
- Zhang X, Davidson EA, Mauzerall DL, Searchinger TD, Dumas P, Shen Y (2015) Managing nitrogen for sustainable development. *Nature*. <https://doi.org/10.1038/nature15743>
- Zhou S, Gao X, Wang C, Yang G, Cram WJ, He G (2009) Identification of sugar signals controlling the nitrate uptake by rice roots using a noninvasive technique. *Z Naturforsch C* 64:697–703