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ORIGINAL PAPER

Enhancement of root architecture and nitrate transporter gene expression improves plant growth and nitrogen uptake under long-term low-nitrogen stress in barley (*Hordeum vulgare* L.) seedlings

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Abstract

Over application of nitrogen (N) fertilizers to crops ultimately causes N pollution in the ecosphere. Studying the response of plant growth and N uptake to low-N stress may aid in elucidating the mechanism of low N tolerance in plants and developing crop cultivars with high nitrogen use efficiency (NUE). In this study, a high-NUE mutant line A9-29 and the wild-type barley cultivar Hua30 were subjected to hydroponic culture with high and low N supply, and the dry weight, N accumulation, root morphology, and expression levels of the potential genes involved in nitrate uptake and assimilation were measured at seedling stage. The results showed that under low-N conditions, A9-29 had a higher dry weight, N content, N influx rate and larger root uptake area than did Hua30. Under long-term low-N stress, compared with Hua30, A9-29 demonstrated higher expression of the HvNRT2/3 genes, especially *HvNRT2.1*, *HvNRT2.5*, and *HvNRT3.3*. Similarly, the expression levels of N assimilation genes including *HvNIA1*, *HvNIR1*, *HvGS1_1*, *HvGS1_3*, and *HvGLU2* increased significantly in A9-29. Taken together, our results suggested that the larger root area and the upregulation of nitrate transporter and assimilation genes may contribute to stronger N uptake capacity for plant growth and N accumulation in responding to long-term low-N stress. These findings may aid in understanding the mechanism of low N tolerance and developing barley cultivars with high-NUE.

Keywords Barley (*Hordeum vulgare* L.) · Low-nitrogen stress · Root morphology · Nitrogen influx rate · Nitrate transporter · Nitrogen assimilation

Background

Nitrogen (N) is an essential element for plant growth and development, which is fixed into numerous enzymatic proteins and plays a vital role in various biological processes such as photosynthesis (Islam 2019). For agriculture, N fertilization can significantly increase crop production. Therefore, N fertilizer are applied in large amounts to achieve maximum crop productivity. However, only 33% of the total N applied is present in the harvested grain (Raun and Johnson 1999). The high level of N input in agriculture causes environmental problems and adds to the financial burden of farmers (Gojon 2017; Sinha et al. 2020). Improving nitrogen use efficiency (NUE) or increasing low N (LN) tolerance in crops to minimize adverse effects and reduce over-dependency on N fertilizers is important (Chen et al. 2018).

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NUE is a complex trait comprising two physiological components: N uptake efficiency (NUpE) and N utilization efficiency (NUtE) (Moll et al. 1982; Xu et al. 2012). The root is the main organ of N uptake, and root architecture often determines the plant ability to capture N (Iqbal et al. 2020; Jiang et al. 2017). N shows a remarkable regulatory effect on the development of the root system, and LN promotes root system growth (Duan 2019; Guo et al. 2017). Therefore, the investigation of root system development is vital for understanding plant responses to LN stress.

Nitrate is the predominant form of N available in cereal crops (Miller et al. 2007). The nitrate uptake system consists of the low-affinity transport system (LATS) and high-affinity transport system (HATS) (Fan et al. 2017). NRT2s and the partner proteins NRT3s—also named NAR2—belong to the HATS, which play important roles under limited N conditions (Fan et al. 2017; Li et al. 2017; O'Brien et al. 2016). The expression of some NRT2s increases with the reductions in N availability—consistent with the increases in nitrate uptake capacity (Plett et al. 2018). Moreover, the transcript levels of NRT2s showed distinct correlations with nitrate uptake capacity and N availability during maize's lifecycle (Garnett et al. 2013). Among seven NRT2 genes in *Arabidopsis*, *AtNRT2.1*, *AtNRT2.2*, *AtNRT2.4*, and *AtNRT2.5* are expressed in the roots of N-deprived plants (Kiba and Krapp 2016). In *Arabidopsis*, the interplay between these genes is required for the adaptation to N limitation (Lezhneva et al. 2014). In rice, three of the *OsNRT2* members (*OsNRT2.1*, *OsNRT2.2*, and *OsNRT2.3a*) with *OsNAR2.1* are involved in root acquisition of nitrate (Fan et al. 2017; Yan et al. 2011). Ten putative NRT2 genes and three putative NRT3 genes have been bioinformatically identified in barley (*Hordeum vulgare* L.), all of which are induced in the roots under different N levels (Guo et al. 2020). At present, three very similar barley NAR2 genes (*HvNAR2.1–HvNAR2.3*) (Tong et al. 2005), two *Arabidopsis* NAR2 genes (*AtNAR2.1* and *AtNAR2.2*) (Orsel et al. 2006), and two rice NAR2 genes (*OsNAR2.1* and *OsNAR2.2*) (Yan et al. 2011) have been characterized. However, only one of these is essential for nitrate HATS, such as *HvNAR2.3* in barley, *OsNAR2.1* in rice, and *AtNAR2.1* in *Arabidopsis* (Feng et al. 2011a; Orsel et al. 2006; Tong et al. 2005; Yan et al. 2011).

After absorption by plants, nitrate is first reduced to nitrite by nitrate reductase (NR) and then further reduced to ammonium by nitrite reductase (NiR). Subsequently, ammonium is assimilated into amino acids by the glutamine synthetase (GS)/glutamine-2-oxoglutarate aminotransferase (GOGAT) cycle (Xu et al. 2012; Criado et al. 2017). NR—the first enzyme involved in plant N assimilation—has a root architecture remodeling function (Fu et al. 2020). NiR—a central role in N metabolism—is involved in nitrite detoxification and NR activity and senescence regulation (Davenport et al. 2015). The expression of GS isogenes is affected by N form and availability (Goodall et al. 2013; Thomsen et al. 2014), and the increase in total GS activity under low nitrate is mainly due to GS1 activity (Avila-Ospina et al. 2015). The GS/GOGAT cycle is considered the major pathway for ammonium assimilation in plants. These N assimilation genes are crucial in plant N metabolism regulation.

Barley—the fourth major cereal crops worldwide—is used for malt and feedstock production and human consumption globally (Beatty et al. 2010). It has recently become an important model species for Triticeae genomics because it has a simple simple genome (diploid) and an integrated genome database (Han et al. 2016; International Barley Genome Sequencing et al. 2012). Extensive physiological information on N uptake and transport in barley has been acquired (Chen et al. 2018; Han et al. 2016; Quan et al. 2016). In our previous work, the barley double haploid lines with high-NUE were generated via microspore mutagenesis combined with field screening at anthesis and maturity under LN condition (Gao et al. 2018). These homogenous lines with the genetic background similar to the wild-type Hua30 represent excellent materials to investigate N uptake and utilization. In the present work, the high-NUE mutant line A9-29 and wild-type Hua30 at the seedling stage were studied, including shoot and root dry weight, N content, N influx rate, root morphology, high-affinity nitrate transporter and nitrogen assimilation genes expression.

Materials and methods

Plant materials and greenhouse experiment

Hua30 is one of the main barley varieties in Yangtze River Delta in China. A9-29, derived from Hua30 by microspore mutagenesis, has been characterized as a high-NUE line under LN treatment (Gao et al. 2018). Hua30 and A9-29 seeds were surface-sterilized in 75% ethanol for 1 min, rinsed several times, soaked in distilled water for 6–8 h, and then, placed on moist filter paper in culture dishes in a growth chamber. After 5 d, seedlings with uniform growth were removed seeds and cultured in water for 2d and then, transferred into a plastic container (52 × 35 × 15 cm in length × width × height) containing 10 L of nutrient solution—as described in Chen et al. (2018) with some modifications. NH_4NO_3 were supplied at two concentrations: 2.5 mM (HN) and 0.1 mM (LN).

All plants were cultivated in a glasshouse with a 16-h day at 20 °C ± 2 °C. The treatment was conducted at 10:00 am. The roots were collected at 1, 3, 7, 14 and 21 d of LN treatment, frozen in liquid N, and stored at − 80 °C for RNA extraction.

Measurement of dry weight and total N

The Hua30 and A9-29 seedlings were cultured in HN and LN nutrient solution for 21d. Samples were collected every 7d. The shoots and roots were separated and heated at 105 °C for 30 min for enzyme inactivation and dried to a constant weight at 75 °C for 3d. The resulting dry weights were recorded. Then, the dried plant tissue was ground and digested for total N determination by using the Kjeldahl method. A 5-mL aliquot from a total of 100 mL per digested sample was analyzed using a continuous-flow autoanalyzer (FlowSys; Systea, Anagni, Italy).

Measurement of root morphology

Fresh roots were sampled at 7d after HN and LN treatment and scanned on a flatbed scanner at 300 dpi. Root images were then analyzed on the LA-S Plant Root Analysis System (Wan Shen, Hangzhou, China). The root traits were described in terms of the total root length, main root length, root surface area, root volume, and root average diameter.

Determination of root ^{15}N influx and accumulation

The Hua30 and A9-29 seedlings were grown in a nutrient solution containing 1.43 mM NH_4NO_3 for 3 weeks and then deprived of N for 1 week. The plants were transferred first to 0.1 mM $CaSO_4$ for 1 min and then to a nutrient solution containing either 0.1 or 2.5 mM $^{15}NH_4{}^{15}NO_3$ (atom% ^{15}N: 99%) for 5 min for root ^{15}N influx and 7d for ^{15}N accumulation separately, and finally to 0.1 mM $CaSO_4$ for 1 min. Roots and shoots were separated immediately after the final transfer to $CaSO_4$ and then frozen in liquid N. Samples were ground to a powder and dried to a constant weight at 75 °C. Ten milligrams of the powder from each sample was analyzed on the MAT253-Flash EA1112-MS system (Thermo Fisher Scientific, USA). ^{15}N influx rate was then calculated using the method of Tang et al. (2012).

RNA extraction, cDNA synthesis, and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from roots by using TRIzol reagent (Invitrogen) and treated with DNase I (Promega), according to the manufacturer's protocol. The quality of the extracted RNA was confirmed using a NanoDrop ND-1000 spectrophotometer. The cDNA synthesis of all RNA samples was performed using a PrimeScript II 1st Strand cDNA Synthesis kit (TaKaRa, Dalian, China) with 1 μg of DNase-treated RNA, according to the manufacturer's protocol. The qRT-PCR reaction mix consisted of 10 μL of 2 × PowerUp SYBR Green Master Mix (Applied Biosystems, California, USA), 2.5 μL of 10 × diluted cDNA, and 1.6 μL of 10 mM stock solution of each primer to a final volume of 20 μL. Reactions were performed on 7500 Real-Time PCR System (Applied Biosystems) with the following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min to calculate cycle threshold (Ct) values. After cycles, the melting curves were generated to verify the primers specificity. *HvGAPDH* expression was used as a reference. The primers for *HvNRT2.1, HvNRT2.5, HvNRT2.6*, and *HvNIA1* were designed by primer 3 (http://primer3.ut.ee/). The other primers were derived from published articles. All the primers used in this study are listed in Supplementary Table S1. The qRT-PCR results were analyzed by the $2^{-\Delta\Delta Ct}$ comparative method according to Livak and Schmittgen (2001) by using the following equation:

$$2^{-\Delta\Delta Ct} = 2^{-[\Delta Ct\,(Treatment) - \Delta Ct\,(Ck)]} = 2^{-[Ct\,(Treatment) - Ct\,(HvGAPDH)] - [Ct\,(Ck) - Ct\,(HvGAPDH)]}$$

Three biological replicates were used to calculate the means and determine the statistically significant differences in each treatment.

Statistical analysis

Biomass, N content and concentration, and ^{15}N influx rate and accumulation were analyzed statistically using the t -test in MS Excel 2007. A two-way analysis of variance (ANOVA) with least significant difference (LSD) multiple comparison test was conducted in gene expression on DPS (version 7.05; China). Graphs were plotted using OriginPro (version 8.0; OriginLab Corporation, USA).

Results

Comparison of plant growth and N accumulation between Hua30 and A9-29 under HN and LN treatments

Compared with HN treatment, LN treatment led to severely decreased shoots growth and elongated root length in Hua30 and A9-29. Moreover, Hua30 was more seriously inhibited than was A9-29. Furthermore, the oldest leaf showed chlorosis and withering earlier in Hua30 than in A9-29 under LN treatment (Supplementary Fig S1).

The biomasses of shoots and roots in Hua30 and A9-29 were weighed separately at the different time points under HN and LN treatments. Under HN treatment, shoot and root dry weights did not show significant differences between Hua30 and A9-29 at different time points (Fig. 1a and b).

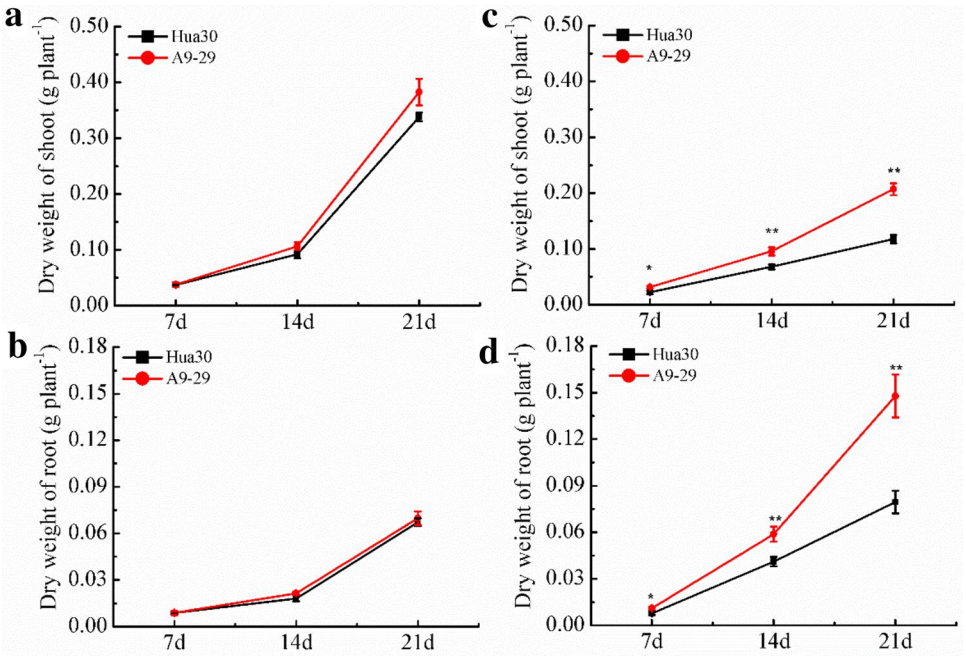

Fig. 1 Effect of high nitrogen (HN) (**a**, **b**) and low nitrogen (LN) (**c**, **d**) supply on dry weight of shoots (**a**, **c**) and roots (**b**, **d**) after 21d treatment. Data are means ± standard deviation (SD) of five biological replicates. * or ** indicated the significant difference ($P < 0.05$ or $P < 0.01$) between cultivars in same treatment

In contrast, under LN treatment, shoot and root dry weights increased more remarkably in A9-29 than in Hua30 at different time points (Fig. 1c and d).

The N contents of shoot and root in Hua30 and A9-29 were also measured. At 7 and 14d of HN treatment, the differences in shoot and root N contents between Hua30 and A9-29 were nonsignificant. At 21d of HN treatment, root N contents were significantly higher in A9-29 than in Hua30 (Fig. 2a and b). LN treatment led to lower N contents than did HN treatment. In Hua30 and A9-29 under LN treatment, shoot N contents showed different trends compared with those in roots. In both Hua30 and A9-29, shoot N contents increased very slightly from 7 to 21d, whereas those in roots demonstrated a slight decrease from 7 to 14d, followed by an increase from 14 to 21d. In particular, under LN treatment, A9-29 showed significantly higher shoot N contents at different time points and

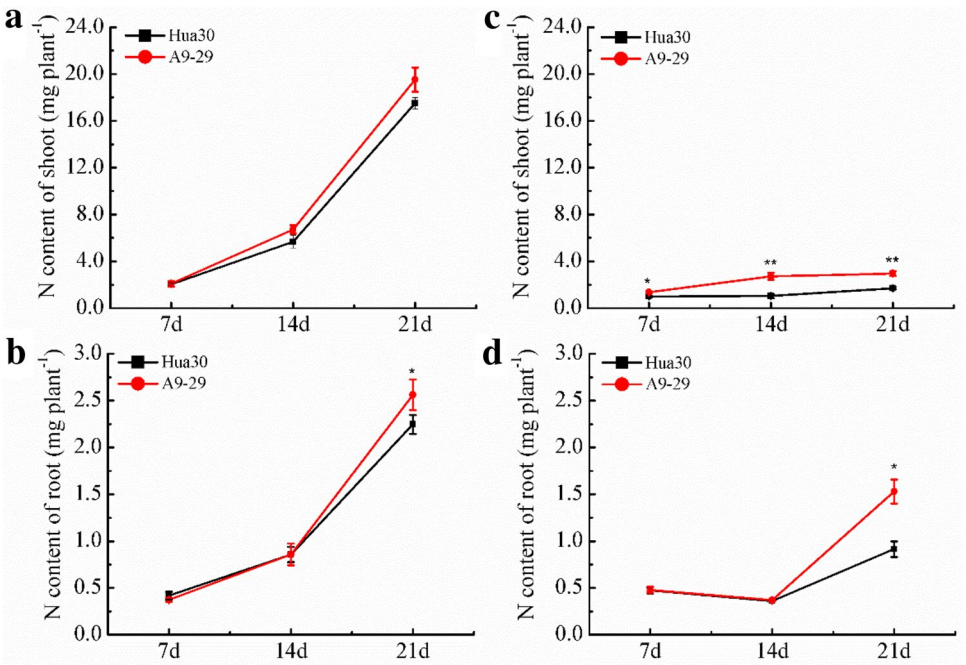

Fig. 2 Effect of HN (**a**, **b**) and LN (**c**, **d**) supply on nitrogen content of shoots (**a**, **c**) and roots (**b**, **d**) after 21d treatment. Data are means ± standard deviation (SD) of five biological replicates. * or ** indicated the significant difference ($P < 0.05$ or $P < 0.01$) between cultivars in same treatment

higher root N contents only at 21d than did Hua30 (Fig. 2c and d).

The N concentrations of shoot and root in Hua30 and A9-29 were analyzed further. The differences in shoot N concentrations at different time points or root N concentrations at 7d between Hua30 and A9-29 under HN treatment were nonsignificant. Under HN treatment, root N concentrations were significantly higher at 14d and then remarkably lower at 21d in Hua30 than in A9-29 (Supplementary Fig S2a and S2b). Under LN treatment, shoot N concentrations at 14d was remarkably higher in A9-29 than in Hua30. In contrast, root N concentrations at 7 and 14d were significantly higher in Hua30 than in A9-29 (Supplementary Fig S2c and S2d).

These results indicated that Hua30 and A9-29 had different responses to LN treatment. Under LN condition, A9-29 demonstrated better performance than did Hua30.

Table 1 Effect of HN and LN supply on root morphology between Hua30 and A9-29 on the 7th day of treatment

Root traits	Treatment	A9-29	Hua30
Root length (cm)	HN	238.24 ± 20.93a	170.44 ± 10.38b
	LN	360.70 ± 12.65a	240.17 ± 13.31b
Main root length (cm)	HN	20.25 ± 1.12a	14.69 ± 0.52b
	LN	29.43 ± 0.87a	18.30 ± 1.51b
Root surface area (cm^2)	HN	16.42 ± 1.02a	11.98 ± 0.48b
	LN	25.30 ± 1.52a	16.01 ± 1.01b
Root volume (cm^3)	HN	0.14 ± 0.01a	0.11 ± 0.01b
	LN	0.24 ± 0.04a	0.19 ± 0.04a
Root average diameter (cm)	HN	0.27 ± 0.01b	0.33 ± 0.01a
	LN	0.33 ± 0.04b	0.49 ± 0.05a
Root number	HN	7.4 ± 0.24a	7.8 ± 0.2a
	LN	7.6 ± 0.24a	7.8 ± 0.37a

Mean ± SD (n = 5) with the same line followed the different letters

Comparison of root morphology between Hua30 and A9-29 under HN and LN treatments

Root dry weights were significantly higher in A9-29 than in Hua30 at different time points under LN treatment and the root system became more complex with LN treatment time prolongation. Therefore, we analyzed root morphology after 7 d of HN and LN treatments by using a root system scanner (Table 1). Compared with HN treatment, LN treatment enhanced root growth and elongation in Hua30 and A9-29. Root length, main root length, and root surface area were approximately 39.78%, 37.85%, and 37.06% higher under HN treatment, respectively, and approximately 50.19%, 60.82%, and 58.03% higher under LN treatment, respectively, in A9-29 than in Hua30. Under HN and LN treatments, root volume was approximately 27 and 21% higher in A9-29 than in Hua30 respectively, with significant difference observed only under HN treatment. Moreover, root average diameter was approximately 22 and 48% larger in Hua30 than in A9-29 under HN and LN treatments, respectively. Root number did not show significant difference between Hua30 and A9-29 under the two N treatments. These findings demonstrated that A9-29 had a greater absorption area to capture N nutrients and thus adapt to N deficiency.

Comparison of N influx rate and accumulation between Hua30 and A9-29 on ^{15}N resupply after N starvation

To determine the root N influx and accumulation difference between Hua30 and A9-29, the short-term N uptake and long-term N accumulation were analyzed by exposing the N-starved roots to 2.5 and 0.1 mM $^{15}NH_4{}^{15}NO_3$ for 5 min and 7 d, respectively (Fig. 3). Compared with Hua30, A9-29 showed no differences in N influx rate at 2.5 mM NH_4NO_3, while a significantly higher N influx rate in roots was observed at 0.1 mM NH_4NO_3 (Fig. 3a). In the long-term treatments with both 2.5 mM and 0.1 mM

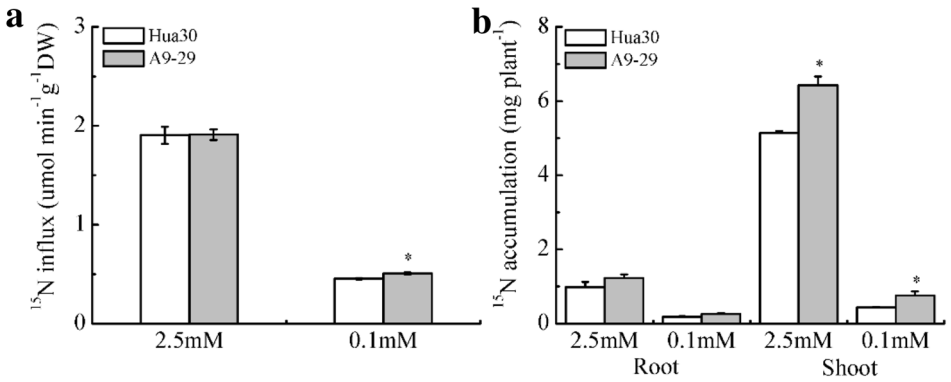

Fig. 3 N influx rate (**a**) and N accumulation (**b**) in Hua30 and A9-29 at different concentration of $^{15}NH_4{}^{15}NO_3$. Data are means ± standard deviation (SD) of three biological replicates. *indicated the significant difference ($P < 0.05$) between cultivars in same treatment

$^{15}NH_4{}^{15}NO_3$, ^{15}N accumulation in shoots was significantly higher in A9-29 than in Hua30 (Fig. 3b).

Comparison of root *HvNRT* expression levels between Hua30 and A9-29 under LN treatments

The relative expression of five *HvNRT2* and two *HvNRT3* genes in Hua30 and A9-29 roots were analyzed at LN treatment. Two-way ANOVA showed that the expression levels of all genes showed significant differences between Hua30 and A9-29 ($P < 0.05$). The expression levels of all genes showed significant differences between the different time points ($P < 0.05$) and a significant interaction between barley lines and time points ($P < 0.05$) (Supplementary Table S2).

The expression of each gene was then compared between Hua30 and A9-29 separately (Fig. 4). In Hua30 *HvNRT2.1* expression increased significantly only at 21d after LN treatment. Compared with Hua30, A9-29 showed significantly upregulated *HvNRT2.1* expression from 14 to 21d after LN treatment (Fig. 4a). *HvNRT2.2* and *HvNRT2.3* expression in the two materials demonstrated a similar pattern to that of *HvNRT2.1*. *HvNRT2.2* expression in Hua30 showed nonsignificant differences during the treatment time points, whereas *HvNRT2.2* expression in A9-29 was obviously upregulated from 14 to 21d and peaked at 14d after LN treatment (Fig. 4b). *HvNRT2.3* expression in Hua30 also showed nonsignificant difference during LN treatment, and *HvNRT2.3* expression in A9-29 was the identical to that of *HvNRT2.1* and *HvNRT2.2*, which increased significantly from 14 to 21d after LN treatment (Fig. 4c). *HvNRT2.5* expression in Hua30 and A9-29 sharply increased at 7d and then slightly decreased in Hua30 but persistently increased in A9-29 from 14 to 21d and peaked at 14d after LN treatment (Fig. 4d). The expression level of *HvNRT2.6* was upregulated in Hua30 at 21d and significantly enhanced in A9-29 from 14 to 21d (Fig. 4e). *HvNRT3.1* expression increased in two cultivars under LN treatment, with A9-29 showing a higher expression level from 14 to 21d (Fig. 4f). *HvNRT3.3* in both cultivars was upregulated from 3 to 21d, and in A9-29, it showed a higher expression from 14 to 21d than in Hua30 (Fig. 4g).

These results indicated that long-term LN treatment enhanced the expression of *HvNRTs*, and these *HvNRTs*—especially *HvNRT2.1*, *HvNRT2.5*, and *HvNRT3.3*—showed

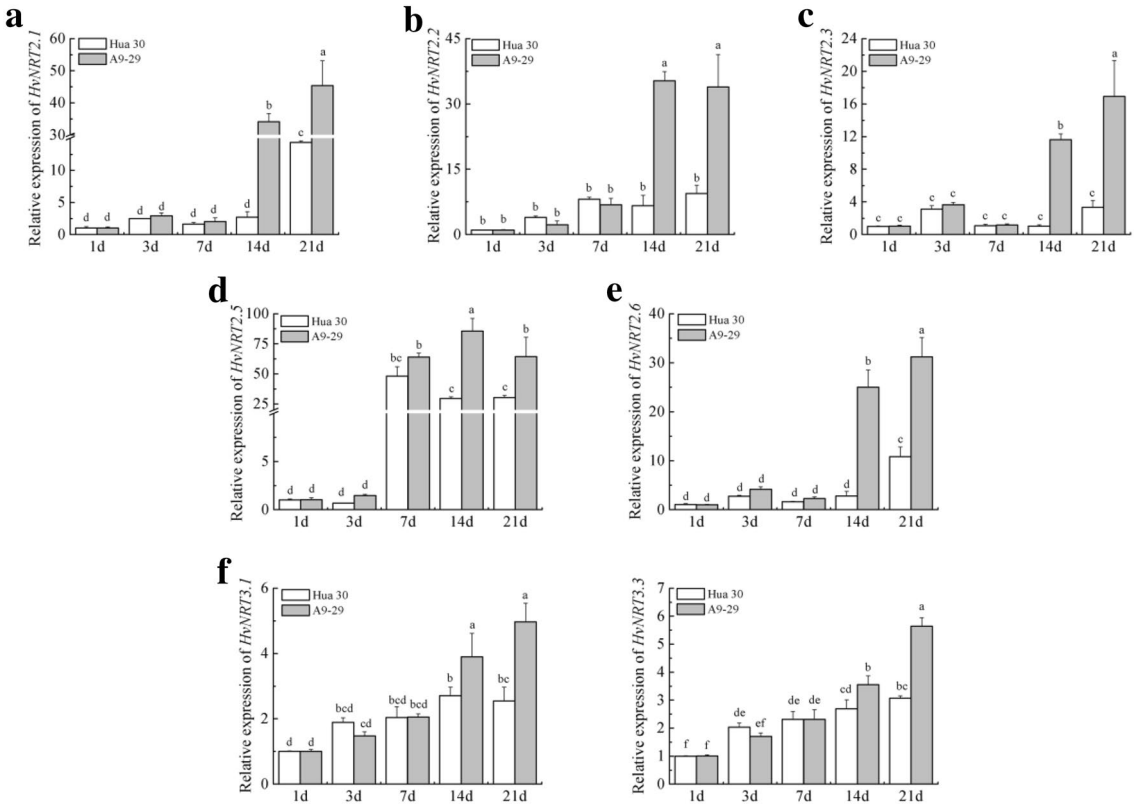

Fig. 4 Relative expressions of *HvNRTs* in roots between Hua30 and A9-29 under LN conditions. Data were means ± standard deviation (SD) of three biological replicates. Error bars labels with different letters indicate significant differences ($P < 0.05$) between time and cultivars according to ANOVA, the same as below

higher transcript levels in A9-29 than in Hua30 to improve N absorption.

Comparison of root N assimilation gene expression between Hua30 and A9-29 under LN treatments

The relative expression of eight genes involved in N assimilation were further investigated in Hua30 and A9-29 roots under LN treatment. Two-way ANOVA showed that the expression levels of all genes, except *HvGS1_2, HvGS1_4*, and *HvGS1_5*, showed significant differences between Hua30 and A9-29 ($P < 0.05$). The expression levels of all genes showed significant differences between the different time points ($P < 0.05$). The expression levels of all genes, except *HvGS1_2, HvGS1_4, HvGS1_5*, and *HvGLU2*, showed significant interactions between the different barley lines and time points ($P < 0.05$) (Supplementary Table S3).

Subsequently, the expression of these genes was compared between Hua30 and A9-29 (Fig. 5). In Hua30, *HvNIA1* expression only slightly increased at 14d after LN treatment, whereas that in A9-29 was significantly upregulated from 14 to 21d (Fig. 5a). *HvNIR1* expression in Hua30 and A9-29 was obviously downregulated at 7d after LN treatment and then upregulated significantly from 14 to 21 d in A9-29, peaking at 14d and was only upregulated at 21 d in Hua30 after LN treatment (Fig. 5b). *HvGS1_1* expression was upregulated in both the lines from 7 to 21d and peaked at 14 d after LN treatment. However, A9-29 had significantly higher *HvGS1_1* expression from 14 to 21d than did Hua30 (Fig. 5c). *HvGS1_3* expression was downregulated at 3d in both the lines and then sharply upregulated from 7 to 21 d in A9-29, which increased from 7 to 14d and then decreased at 21d in Hua30 (Fig. 5e). *HvGS1_2*, *HvGS1_4*, and *HvGS1_5* expression levels showed slight differences between Hua30 and A9-29, which only increased from 3 to 14d and then decreased at 21 d (Fig. 5d, f and g). *HvGLU2* expression was upregulated only in A9-29 from 14 to 21d (Fig. 5h). These results indicated that LN treatment enhanced N assimilation gene expression in A9-29. The higher transcript levels of *HvNIA1*, *HvNIR1, HvGS1_1*, *HvGS1_3*, and *HvGLU2*

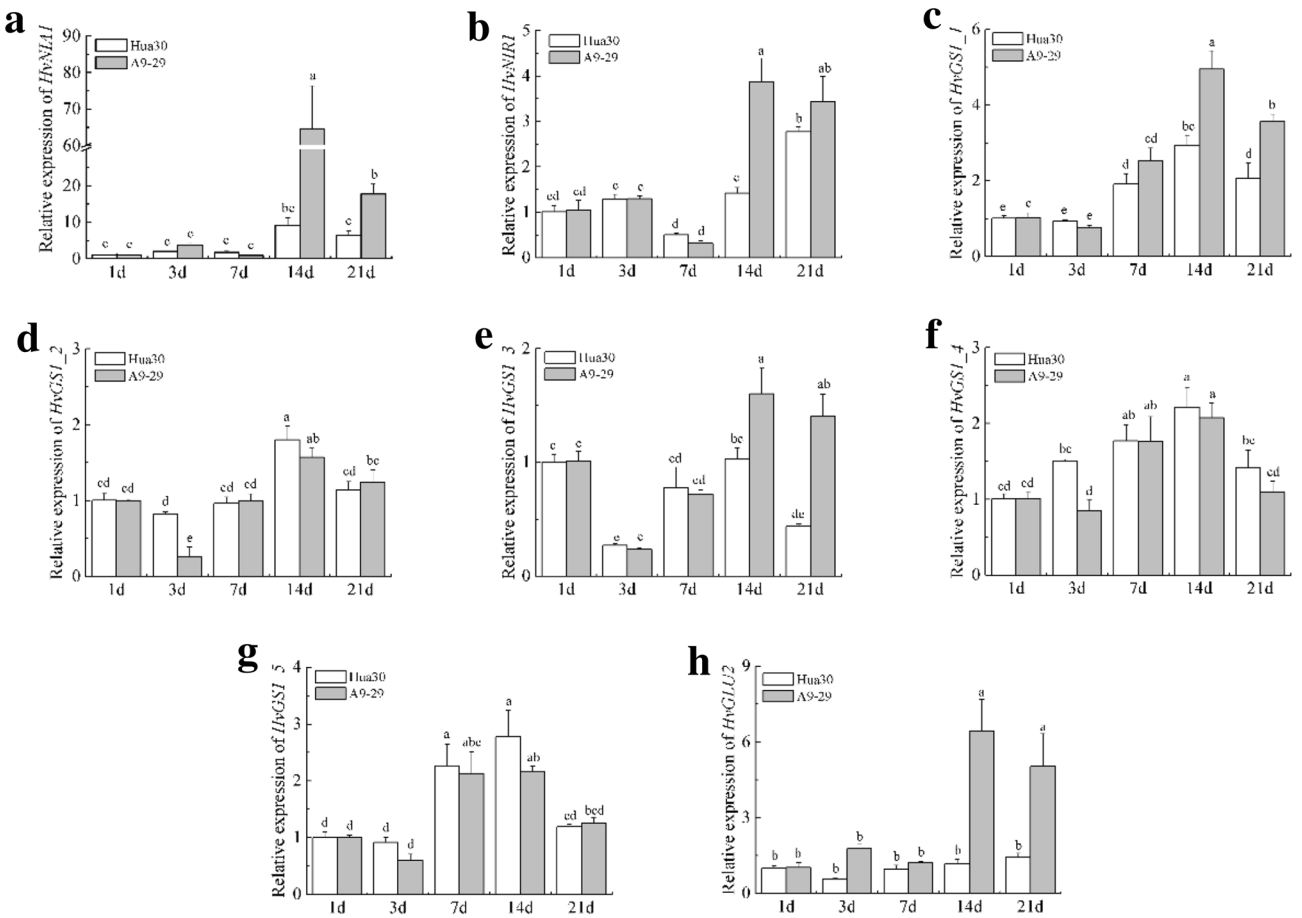

Fig. 5 Relative expressions of N assimilation genes in roots between Hua30 and A9-29 under LN conditions. Data were means ± standard deviation (SD) of three biological replicates

in A9-29 may play important roles in N assimilation in roots—which could further improve N absorption.

Discussion

Plant growth involves coordinated shoot and root development. A sufficient number of photosynthetic products generated in shoots is transferred to roots, which supports the development of root architecture and implementation of root function. Moreover, the larger the root biomass, the more is the biological yield (Duan 2019). N starvation severely affects plant growth, and high-NUE genotypes can generate more biomass and lead to higher N accumulation under LN stress (Liu et al. 2009; Xu et al. 2016). In this study, compared with the wild-type Hua30, A9-29 showed larger shoot and root biomass from 7 to 21 d under LN treatment (Fig. 1c and d), and higher N accumulation in both shoots and roots after long-term (21d) LN treatment (Fig. 2c and d). The lower root N concentration means more dry matter generated for A9-29 under LN treatment (Fig S2d). The difference in growth performance and N accumulation between the two barley genotypes indicated that A9-29 could maintain better growth than did Hua30 under LN treatment.

Nitrate is the most abundant N source (Miller et al. 2007). In addition to its role as a nutrient, nitrate can serve as a signaling molecule to modulate plant developmental processes, including root system architecture establishment (Krouk et al. 2010; O'Brien et al. 2016; Song et al. 2020). Under nitrate-limited conditions, plant roots employ an "active-foraging strategy" in response to nitrate deprivation by lateral root outgrowth and a shared pattern of transcriptome reprogramming (Ruffel et al. 2011). Root development is highly plastic and affected by various environmental factors (Forde 2014; Giehl et al. 2014). Nutrient availability has a widespread effect on the root system architecture by altering the numbers, length, angle and diameter of roots and root hairs (Gruber et al. 2013). An increase in root length under N deprivation might be related to the N deprivation–induced signaling cascade and the N foraging ability of the genotype (Gruber et al. 2013; Sinha et al. 2020). In this study, compared with Hua30, A9-29 had significantly increased main root length, total root length, and root surface area but decreased root average diameter under LN treatment. Moreover, the main root length only accounted for approximately 8% of the total root length (Table 1). These results indicated that larger root surface area in A9-29 was mainly attributable to higher lateral root length, thus resulting in an increased area for N absorption.

Plants have evolved two nitrate uptake systems, HATS and LATS, to cope with variable soil nitrate concentrations. NRT2.1 might be the main component of the HATS for root uptake under most conditions (Li et al. 2007; O'Brien et al. 2016). In *Arabidopsis*, NRT2.1, NRT2.2, NRT2.4, and NRT2.5 primarily mediates nitrate uptake, with NRT2.1 being the major contributor (Plett et al. 2018). NRT2.2 partially compensates for when NRT2.1 is lost (Li et al. 2007). *HvNRT2.1* in barley is vital in long-distance nitrate transport with low nitrate supply (Guo et al. 2020). In *Arabidopsis*, *AtNRT2.3* and *AtNRT2.6* both showed a constitutive expression pattern in the roots (Okamoto et al. 2003). *OsNRT2.3b* in rice is relatively stable in the roots and independent of the form and concentrations of the N supplied (Feng et al. 2011b). High *OsNRT2.3b* expression in rice enhances the pH-buffering capacity of the plant, thereby increasing N, Fe, and P uptake (Fan et al. 2016). *AtNRT2.5* expression in *Arabidopsis* is induced under N starvation, and among the seven members of NRT2 family, *AtNRT2.5* has the most abundant transcript levels in adult plants shoots and roots after long-term starvation (Lezhneva et al. 2014). *OsNRT2.5* in rice—also known as *OsNRT2.3a*—plays a role in the transport of nitrate from roots to shoots under low nitrate conditions (Tang et al. 2012). In wheat and maize, *NRT2.5* has been found to be induced under LN stress (Garnett et al. 2013; Guo et al. 2014). *HvNAR2.3* (*HvNRT3.3*)—the most abundantly expressed in roots among the three *NAR2* family members in barley—is upregulated by nitrate and N starvation (Tong et al. 2005). In the current study, the expression of *HvNRT2.1*, *HvNRT2.2*, *HvNRT2.3*, and *HvNRT2.6* in A9-29 was markedly enhanced at 14 and 21 d of LN stress, whereas only *HvNRT2.1* and *HvNRT2.6* expression in Hua30 significantly increased at 21 d of LN stress (Fig. 4a, b, c and e). Of the aforementioned four genes, *HvNRT2.1* was found to be expressed at the highest level, indicating that *HvNRT2.1* is predominant in nitrate uptake under LN conditions. *HvNRT2.5* expression was also induced under N starvation and showed the highest transcript level among the HvNRT2 genes detected after 7 d of LN treatment (Fig. 4d). Although *HvNRT3.1* and *HvNRT3.3* expression was upregulated under LN stress in Hua30 and A9-29, *HvNRT3.3* was more sensitive in response to LN stress (Fig. 4f, g). Krapp et al. (2011) considered that long-term N starvation increases the high-affinity nitrate uptake capacity, and their transcriptome analysis revealed *NRT2.4* and *NRT2.5* to be candidates implicated in the uptake process. In this study, *HvNRT2.4* expression levels were not measured because of unavailable specific primers to discriminate genes with high identity (95%) of CDS between *HvNRT2.4* (GenBank: AF091116.1) and *HvNRT2.6* (GenBank: DQ539043.1). The higher *HvNRT2.1*, *HvNRT2.5*, and *HvNRT3.3* expression levels in A9-29 on 14 and 21d of LN treatment suggested that they play vital roles in N uptake, which may

contribute to increased N uptake capacity in A9-29 in response to LN treatments.

In many plants, nitrate absorbed by roots is then assimilated in the roots and several enzymes, including NR, NiR, and GS, are involved in this process (Xu et al. 2012). NR is a highly regulated enzyme (Daniel-Vedele et al. 2010). In *Arabidopsis*, root NR activities might be well correlated with NIA1 transcript levels (Krapp et al. 2011). Compared with NR, only a single nitrite reductase, NiR1, was identified in barley (Ward et al. 1995). NiR is co-regulated with NR in response to N and C metabolites, at least at the transcriptional level (Meyer 2002). Moreover, NiR1 is a key target in the regulation of N assimilation and NO homeostasis and is relevant to the control of both plant growth and performance under stress conditions (Costa-Broseta et al. 2020). Cytosolic GS1 is important for primary $NH4^+$ assimilation in roots. GS1 isoforms are more abundant under LN treatment, and GS1 is more predominant than GS2 in barley (Avila-Ospina et al. 2015). GS1 also plays a key role in the maintenance of essential N flow and internal N sensing during the critical plant development stages (Thomsen et al. 2014). GLU2 encodes a ferredoxin-dependent glutamate synthase (Fd-GOGAT), which plays a major role in primary N assimilation in *Arabidopsis* roots (Coschigano et al. 1998). LN stress could result in increased N uptake rate and N accumulation in root cells, followed by the enhancement of the N assimilation ability. In contrast, increased N assimilation ability might be a feedback signal to promote N uptake under LN treatment (Jiang et al. 2017). In this study, large genotype-dependent differences were described in the expressional profile of the N assimilation genes that regulate N metabolism (Fig. 5). Compared with Hua30, A9-29 presented higher expression levels of N assimilation genes in roots, including *HvNIA1*, *HvNIR1*, *HvGS1_1*, *HvGS1_3*, and *HvGLU2*—which potentially contributed to the increased N uptake capacity in response to LN stress.

Conclusions

Plant growth, N accumulation, N influx rate, and potential gene expression were compared between the barley mutant line A9-29 and the barley wild-type Hua30 at the seedling stage. Compared with Hua30, A9-29 demonstrated increased N absorption area in root system architecture, higher root N influx and enhanced nitrate transport gene expression levels, all indicating a higher N uptake capacity. Moreover, increased N assimilation gene expression potentially promoted N absorption. Consequently, A9-29 demonstrated increased biomass and N accumulation in response to LN stress.

In conclusion, under long-term LN stress, the barley mutant line A9-29 absorbs N more efficiently than does its wild-type Hua30 at the seedling stage.

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Author Contributions All authors contributed to the study conception and design. Plant growth, treatment and sample preparation were performed by RG and GG. The RNA samples were prepared by HX and YL. The qPCR experiments were performed by RG and ZC. Data collection and analysis were performed by GR and RL. The first draft of the manuscript was prepared by RG and edited by CL and JC. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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