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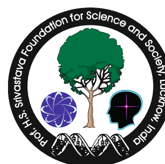

RESEARCH ARTICLE

Comparative study of the genetic basis of nitrogen use efficiency in wild and cultivated barley

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Abstract To curb the increasing demand for nitrogenous fertilizers, it is imperative to develop new cultivars with comparatively greater nitrogen use efficiency (NUE). Nonetheless, so far very meager information is available concerning the variances among barley (*Hordeum vulgare* L.) varieties for their response to nitrogen deprivation. The current study was carried out to explore the potential of barley genotypes for higher NUE. A hydroponic experiment was conducted at seedling stage to compare the performance of four barley genotypes, *ZD9* and *XZ149* (with higher NUE) and *HXRL* and *XZ56* (with lower NUE) in response to low (0.1 mM) and normal nitrogen (2 mM) levels. Under low N, all the genotypes expressed less number of tillers, decreased soluble proteins, chlorophyll and N concentrations in both roots and shoots, in comparison with normal N supply. However, significant differences were found among the genotypes. The genotypes with high NUE (*ZD9* and *XZ149*) showed higher N concentration, increased number of tillers, improved chlorophyll and soluble proteins in both roots and shoots as compared to the inefficient ones (*HXRL* and *XZ56*). Furthermore, nitrate transporter gene (NRT2.1) showed higher expression under low N, both in roots and leaves of N efficient genotypes, as compared to the N inefficient ones. However, N assimilatory genes (GS1 and GS2) showed higher expression under normal and low N level, in leaves and roots respectively. The outcome of the study revealed that genotypes with higher NUE (*ZD9* and *XZ149*) performed better under reduced N supply, and may require relatively less N fertilizer for normal growth and development, as compared to those with lower NUE. The study also revealed a time-specific expression pattern of studied

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genes, indicating the duration of low N stress. The current study suggested that future work must involve the time course as a key factor while studying expression patterns of these genes to better understand the genetic basis of low-N tolerance.

Keywords Barley · Nitrogen metabolism · Gene expression

Introduction

Nitrogen (N) is a vital element in plant nutrition and is known to be a key factor in limiting crop productivity (Kraiser et al. 2011). Being a major component of RNA, DNA, chlorophyll, ATPs, cytokinins, auxin and enzymes, N plays a pivotal role in growth and development of plants (Raven et al. 2004; Hawkesford et al. 2012). In 2016, the world total use of nitrogenous fertilizer was 110 million tones, representing a 34% increase with respect to 2002 (FAO 2018). However, it has substantial impacts on the quality of environment throughout the globe. Only 30–50% of the applied N is taken up by crops, depending upon the crop species and cultivars, as well as management practices, with the rest fertilizer amount being lost and ultimately polluting the local agro-ecology (Garnett et al. 2009).

Within soil, most of the nitrogen is absorbed by plants as nitrate (NO_3^-) (Crawford and Forde 2002), which is reduced to ammonium by nitrate reductase (NR) and nitrite reductase (NiR), followed by amino acid and protein biosynthesis. Little et al. (2005) has narrated an important function of NRT2.1, a high-affinity nitrate transporter, in controlling branching pattern of roots, which implies that the NUE may directly be influenced by genes involved in nitrogen absorption (Lea and Azevedo 2007). Owing to its pivotal role in assimilation of inorganic nitrogen, glutamine synthetase (GS; EC 6.3.1.2) fascinated the researchers for several decades (Mc Nally et al. 1983; Edwards et al. 1990). The studies regarding expression and knockout behavior of individual GS isogenes revealed their cell and tissue specific localization and their pivotal role in growth and development of cereals. The expression pattern of NRT2.1 gene has thoroughly been investigated at mRNA level. Its expression is stimulated by low NO_3^- availability which controls its uptake by roots in response to plant's nitrogen status (Okamoto et al. 2003; Filleur and Daniel-Vedele 1999). The expression of NRT2.1 is suppressed by NO_3^- itself, through a mechanism independent of the feedback repression, employed by nitrogen metabolites, but specifically prompted by the dual-affinity NRT1.1 NO_3^- transporter (Munos et al. 2004; Krouk et al. 2006). The NO_3^- high affinity nitrate transporters (HATS) are regulated by the same factors, showing a robust correlations of changes in NRT2.1 transcript level and the activity of HATS, indicating a central role of NRT2.1 transcriptional regulation in NO_3^- absorption.

The genetic diversity of cultivated barley becomes narrower due to intensive breeding and cultivation, posing a bottleneck for genetic improvement. Contrarily, wild barley has a highly rich source of genetic variability, and can serve as a valuable gene reservoir for future breeding programs (Dai et al. 2012). Due to immense genetic diversity, it is hypothesized that wild barley can perform better under low N availability. To curb the ever rising global N demands, it is imperative to identify the barley genotypes showing tolerance to N starvation, which could certainly serve as a genetic resource for developing high yielding crops, with comparatively low N requirement, ultimately giving a healthy environment to the future generations.

Genetic differences in physiological responses to applied N have already been pointed out in various species of plants like wheat, maize, barley, rice and sorghum (Ortiz-Monasterio et al. 1997; Le Gouis et al. 2000; Muchow 1998; Presterl et al. 2003; Namai et al. 2009; Anbessa et al. 2009). However, the information regarding intra-specific genetic diversity is very limited. A hydroponic experiment was designed to investigate the genotypic differences between Tibetan wild and cultivated barley under reduced N availability.

Materials and methods

Plant materials, growth conditions and N doses

This experiment was performed under a controlled environment at Zijingang Campus of Zhejiang University, Hangzhou (30.29°N and 120.16°E), P.R. China. On the basis of previous investigation (Shah et al. 2017a), two accessions (XZ56 and XZ149) of Tibetan annual wild barley, and two cultivars (HXRL and ZD9), varying in NUE, were chosen for the experiment. Seeds of barley cultivars were obtained from key Laboratory of Crop Germplasm Resource of Zhejiang Province, Department of Agronomy, Zhejiang University, Hangzhou, China. Two Tibetan annual wild barley accessions (XZ56 and XZ149) were obtained from Huazhong Agricultural University, China. Seeds were treated with 2% H_2O_2 for 30 min, for surface sterilization (Quan et al. 2016). After washing, the seeds were germinated on moist Whatman grade-1 filter papers. 12 days after germination (second leaf stage), uniformly healthy and vigorous seedlings were transplanted to containers filled with 5 L basic nutrition solution. The concentration of different nutrients in the solution

was: 2 mmol $NaNO_3^-$, 0.63 mmol $MgSO_4$, 0.18 mmol K_2SO_4, 0.18 mmol KH_2PO_4, 0.36 mmol $CaCl_2$, 20.9 µmol Fe-citrate, 4.5 µmol $MnCl_2$, 0.38 µmol $ZnSO_4$, 0.16 µmol $CuSO_4$, 46.9 µmol H_3BO_3 and 0.062 µmol H_2MoO_4. The solution pH was managed at 5.8 ± 0.1, by using HCl or NaOH. Prior to treatment, the plants were grown normally for 7 days for acclimatization in basic nutrient solution. Based on the previous studies (Quan et al. 2016), two N concentrations, i.e. 0.1 (low) and 2 mM (normal), were maintained in the basic nutrition solution for the study. Completely randomized block design was employed with four independent replicates. The containers were continuously supplied with air through pumps and the nutrition solution was replaced after every 5 days. After 22 days exposure to treatments, the plants were harvested for measuring morphological, physiological and biochemical characteristics of roots and shoots. However, the samples for molecular study were harvested after 24 h, 10 days and 15 days exposure to N treatments and immediately stored at − 80 °C.

Measurement of N concentration

Total N concentration in plant tissues was measured by the Kjeldahl method (Jones 1991). After drying, the material was ground into fine powder. Around 0.2 g sample powder was weighed, digested in sulfuric acid, and analyzed for N content according to Li et al. (2006).

Measurement of soluble protein

Bradford (1976) method was employed to determine the soluble protein contents. Fresh leaf samples (2 g each) were homogenized with 0.05 M Tris buffer (1 mL pH 8.5) using pestle and mortar, followed by centrifugation at 9000 for 10 min. The supernatant (100 µL) was mixed with 3 mL Bradford reagent (Sigma, prepared using 10 mL reagent + 50 mL distilled water), followed by incubation for 5 min. The absorbance was recorded at 595 nm using spectrophotometer. Standard calibration curve was generated by using bovine serum (Sigma). The concentration of protein in leaf samples was demonstrated in terms of mg/g fresh weight.

Chlorophyll content

On fresh weight basis, total chlorophyll content was determined as mentioned by Farida et al. (2017). The leaf disks, excised from the youngest fully expanded leaves, were embedded in acetone (20 mL) under dark conditions for approximately 24 h at 4 °C till the complete disappearance of green color. The resultant solution was used to measure the absorbance at 664 and 647 nm using a spectrometer (UV-3101P, Labomed Inc., USA).

qRT-PCR and gene expression analysis

By using the TRIzol reagent, total leaf and root RNA was extracted according to manufacturers' protocols (Invitrogen, Karlsruhe, Germany). cDNA was synthesized using 1 µg of each RNA sample with 0.5 of oligo (dT) 12–18 and 200 units of Superscript II (Invitrogen, Karlsruhe, Germany). cDNA samples were analyzed by quantitative real time PCR (qRT-PCR) in the iCycler iQTM Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the SYBR Green PCR Master Mix (Applied Biosystems). The PCR outlines were as follows: pre-denaturation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 5 s and annealing at 60 °C for 30 s, followed by steps for Melt-Curve analysis (60–95 °C, 0.5 °C increment for 5 s per step). Each sample was replicated three times. Primer express soft-ware (Applied Biosystems) was used to design gene-specific primers. The primer used for *Hv*GS1; (accession no JX878489 forward-5′-GAGCGTCACCATT GCTCTCT-3′, reverse-5′-CTGCCTTCCTTCTCGGTGT C-3′) for *Hv*GS2; (accession no AK360336 forward-5′-GA CACCTACACACCACAGGG-3′, reverse -5′-GCTATGT GCAAGTCATGGCG-3′) for *Hv*NRT2.1; (accession no U34198 forward-5′-CTGACCTTGGTGCCCGTTAT-3′, reverse-5′-GGAGACCCTTGGCTTTCTCC-3′). Barley *GAPDH* (glyceralde-hyde-3-phosphate dehydrogenase) gene (accession no. M36650, forward-5′-AAGCATGAA GATACAGGGAGTGTG-3′, reverse-5′- AATTTATTCT CGGAAGAGGTTGTACA-3′) was used as control. The relative expression level was determined by using $2^{-\Delta\Delta CT}$ method, described by Livak and Schmittgen (2001).

Statistical analysis

The data are the means of four independent replicates. The significance of the differences between two barley genotypes, under varying N supply, was evaluated by two-way analysis of variance (ANOVA), followed by the LSD (least significant difference) multiple range test ($P < 0.05$), employing DPS 9.50 (Data Processing System) (Bukhari et al. 2016).

Results

N concentration in shoots and roots

Shoot N concentration (SNC) was significantly reduced in low N treatment relative to control in all barley genotypes (Table 1). However, a considerable variation among the four genotypes was observed, with ZD9 (3.74 and 4.95% at 0.1 and 2.0 mM N respectively) and XZ149 (3.76 and 5.79% at 0.1 and 2.0 mM N respectively) showing

Table 1 Effect of N levels on chlorophyll content, N concentration (roots and shoots) and number of tillers per plant of four barley genotypes

N level (mM)	Genotype	Chl a mg g^{-1} FW	Chl b mg g^{-1} FW	Chl a + b	SNC (%)	RNC (%)	Tillers per plant
0.1	ZD9	38.86 ab	60.44 c	99.83 b	3.74 c	1.44 d	2.39 cd
	XZ149	38.26 ab	64.81 abc	103.19 ab	3.76 c	1.66 d	2.53 c
	HXRL	38.38 ab	50.54 d	89.82 c	2.95 de	1.12 e	1.41 e
	XZ56	38.75 ab	61.56 bc	100.31 b	2.81 e	1.43 d	2.21 d
2.00	ZD9	39.39 a	70.25 a	109.11 a	4.95 b	3.10 a	3.25 a
	XZ149	39.28 a	67.95 ab	106.20 ab	5.79 a	2.87 ab	3.30 a
	HXRL	38.08 b	67.43 abc	105.51 ab	4.78 b	2.51 c	2.17 d
	XZ56	38.95 ab	62.23 bc	101.18 b	3.60 cd	2.74 bc	2.95 b
	Interaction (G × N)	ns	**	*	*	ns	**

Means sharing the common letter(s) are statistically at par with each other

ns non-significant, *RNC* root nitrogen concentration, *SNC* shoot nitrogen concentration

*Significant at $P < 0.05$, **significant at $P < 0.01$

significantly higher SNC at both N levels than other two genotypes. Similarly root N concentration (RNC) was also significantly reduced in low N level relative to control (Table 1). Again ZD9 (1.44 and 3.10% at 0.1 and 2.0 mM N respectively) and XZ149 (1.66 and 2.87% at 0.1 and 2.0 mM N respectively) depicted the higher RNC than HXRL (1.12 and 2.51% at 0.1 and 2.0 mM N respectively) and XZ56 (1.43 and 2.73% at 0.1 and 2.0 mM N respectively). Moreover, wild genotype XZ149 contained the highest SNC and RNC at both N levels, indicating its high capability of N uptake and transportation.

Chlorophyll content and soluble proteins

As compared with the control, a declining trend was observed for chlorophyll content in the low N treatment. Chlorophyll a + b significantly differed among all genotypes at both N levels. However, chl a showed variation at higher N dose only. ZD9 (38.86, 39.39 and 99.83, 109.11 mg g^{-1} FW Chl a and Chl a + b at 0.1 and 2.0 mM N respectively) and XZ149 (64.81, 67.95 and 103.19, 106.20 mg g^{-1} FW Cha a and Chl a + b at 0.1 and 2.0 mM N respectively) had higher chlorophyll a and a + b contents at both N treatments (Table 1).

Like N concentration, the soluble protein contents were also significantly reduced in low N treatment relative to control (Fig. 1) in both shoots and roots of all four barley genotypes. Meanwhile, ZD9 and XZ149 had significantly higher leaf soluble protein contents than other two genotypes. However for root soluble protein contents, no considerable variation was noticed among the genotypes at low nitrogen level, except XZ56 which showed significantly lower root soluble protein content than other three genotypes. At normal nitrogen level, ZD9 had the highest soluble protein content than other three genotypes while HXRL exhibited the lowest value.

Number of tillers per plant

The tillering capacity of plants was significantly reduced under low N availability, as compared with the control. However, considerable differences were found among different barley genotypes for this trait, in response to low N supply. Both ZD9 (2.39 and 3.25 tillers at 0.1 and 2.0 mM N respectively) and XZ149 (2.53 and 3.30 tillers at 0.1 and 2.0 mM N respectively) produced more tillers per plant than other two genotypes at both N levels (Table 1).

GS1, GS2 and NRT2.1 expression

The results indicated a significant influence of time interval on the expression pattern of genes in both leaves and roots. The interaction between genotype and time interval was also significant for all three genes (Figs. 2, 3, 4). The expression of GS1 and GS2 in leaves was dramatically reduced in low N treatment relative to control for all the genotypes except GS2 at 15 days after N treatment, where its expression was increased at low N in all four genotypes, while in roots, it was up-regulated under low N at all three sampling times. In contrast to N assimilatory genes, an increasing trend in expression of NRT2.1 (nitrate transporter) gene was observed, both in roots and leaves of the four genotypes under low N level during the whole stress treatment, with a marked genotypic difference. The expression of GS1 in ZD9 leaves was significantly increased from 24 h to 10 days, and then decreased at 15 days. In HXRL and XZ149, the expression of GS1 in leaves was increased at 10 days and 15 days, respectively (Fig. 2a–c). The expression level of GS1 in ZD9 was not different from that of HXRL at 10 days, but became significantly lower than other genotypes at 15 days (Fig. 2b, c). In roots, significant difference was found for expression

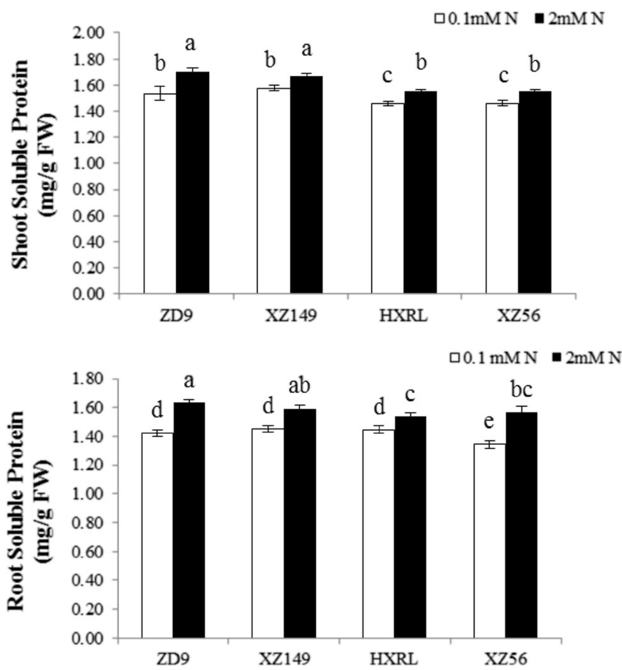

Fig. 1 Effect of N levels on soluble proteins in shoot and root of four barley genotypes. Different letters indicate significant difference among the genotypes at $P < 0.05$

level of GS1 among genotypes at 10 days, with HXRL being considerably lower at all sampling times than other genotypes. In addition, a considerable difference in GS1 expression was found between the two efficient genotypes at 10 and 15 days, with the wild barley (XZ149) exhibiting higher GS1 expression level than the cultivar ZD9.

The GS2, a main isoform of GS in leaves, depicted a similarly clear reduction in its expression under low N availability relative to control. For GS2 expression level in leaves, only XZ56 was significantly higher at 24 h while no significant difference was found among the genotypes at 10 days after N treatment (Fig. 3a, b). At 15 days, GS2 expression reversed where HXRL showed increase in leaves (Fig. 3c). In roots, all the genotypes showed the similar expression. ZD9, XZ149 and XZ56 showed higher NRT2.1 expression in leaves at 24 h, 10 and 15 days after N treatment, respectively (Fig. 4a–c). XZ149 showed significantly higher expression level than ZD9 and HXRL, but lower than XZ56 at 15 days. No significant difference was found for root NRT2.1 expression level at 24 h and 15 days. However at 10 days, ZD9 (high N efficient genotype) showed significantly higher expression than the two genotypes with low NUE.

Low N decreased and increased the overall expression of GS1 and GS2 in leaves and roots, respectively, as compared to normal N supply. However slight difference was observed among the genotypes as well as sampling times. For GS1 expression, XZ149 and ZD9 showed the lowest value at 24 h, while HXRL exhibited the highest figure at 10 days treatment at low N (Fig. 2a–c). In roots, N efficient genotypes showed enhanced GS1 expression at low N as compared to the inefficient genotypes (Fig. 2d–f). Interestingly, the two N efficient genotypes differed in root GS1 expression at 10 and 15 days, XZ149 with higher expression than ZD9 (Fig. 2e, f). Similar trend was observed for GS2 in leaves where its expression was reduced under low N supply, but increased when the stress prolonged, i.e. at 15 days. Contrasting results were obtained at 15 days in low N level and genotypes differed significantly, where HXRL and XZ56 showed higher values than ZD9 and XZ149 in GS2 expression (Fig. 3c). In roots, N efficient genotypes XZ149 and *ZD9* showed significant increase in GS2 expression at 24 h and 10 days, while XZ149 showed marked decrease at 15 days. NRT2.1 was highly expressed in ZD9 and XZ149 at 24 h and 10 days, respectively, in leaves at low N, while at 15 days, XZ56 had higher expression.

Discussion

The N concentrations often fluctuate under field condition, thus affecting the normal growth and development of plants. Therefore, adaption to low N availability is an important mechanism for the existence of plants in changing environments, and in crops, it is vital to maximize the yields (Kant et al. 2011). Plants' adaptation to N deficiency consists of composite morphological, physiological, and developmental responses (Yang et al. 2011). Wide-range of alterations in primary and secondary metabolism, protein synthesis and cellular growth processes can be observed in plants under low N environments (Peng et al. 2008). Various crops, including maize, rice, oilseed rapes have been extensively investigated for their response to varying N doses (Kessel et al. 2012; Ikram et al. 2012; Wei et al. 2012; Abdel-Ghani et al. 2013). However, comparatively less information is available regarding the physiological and molecular variations in barley under reduced N availability. The traits that contribute to NUE comprise of a wider range of genetic variations (Kessel et al. 2012; Dawson et al. 2008). The emphasis of this experiment was to observe the expression of nitrogen transporter and assimilation genes in roots and leaves under different N supply. The plants growing under high nitrate supply showed down-regulation of leading GS1 and GS2 genes in the roots and a relative up-regulation in leaves, particularly *HvGS1* and *HvGS2* (Fig. 4). This response shows that *HvGS1* gene responds to the shift from roots to shoot nitrate assimilation, owing to enhanced movement of nitrate to leaves as indicated by previous researchers (Lewis et al. 1982; Goodall et al. 2013). *HvGS1,* therefore

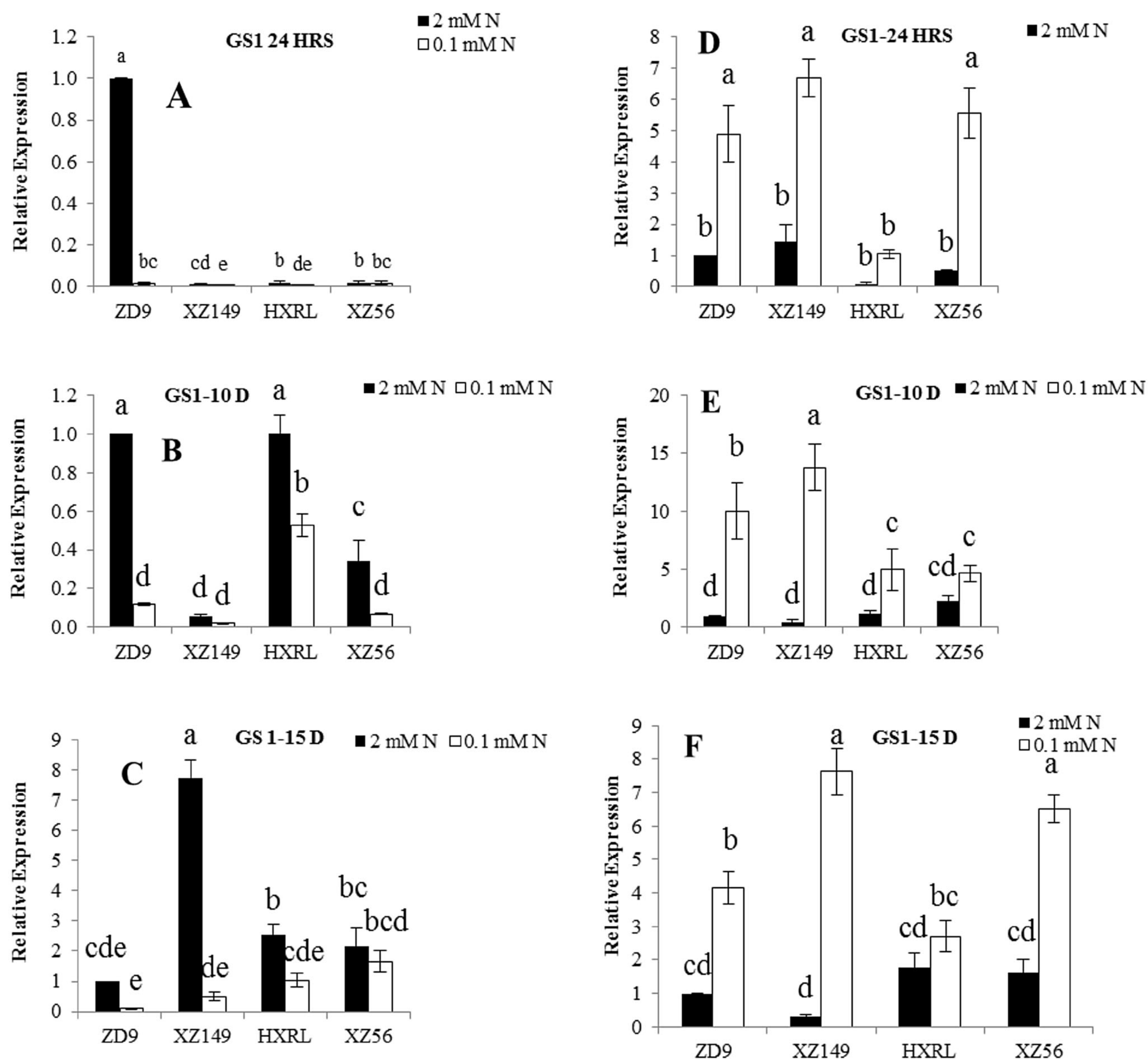

Fig. 2 Expression patterns of GS1 gene in four barley genotypes at different time intervals. **a**, **b** and **c** in leaves; **d**, **e** and **f** in roots at 24 h, 10 days and 15 days after N treatment, respectively. Different letters indicate significant difference among the genotypes at $P < 0.05$

has an important role to assimilate ammonia generated as a result of nitrate reduction in leaves. Similarly improved GS expression in leaves enhanced GS2 protein in the mesophyll and GS1 protein in vascular tissues (bundle sheath and vascular bundle) of plants growing under increased level of ammonium or nitrate (Tobin and Yamaya 2001; Goodall et al. 2013). The expression of GS2 under low nitrogen in leaves was higher at 15 days after N treatment (Fig. 3c). It has also been reported that in leaves, GS2 activity is usually greater, but it decreases with progressing senescence as chloroplasts are degraded (Mc Nally et al. 1983; Bernard et al. 2008). Thus, obvious increase was observed in N inefficient genotypes (Shah et al. 2017a, b).

The up-regulation of *HvGS1* and *HvGS2* in plant roots, grown under reduced N supply is interesting (Figs. 2, 3). The higher expression of these genes under N scarcity might be the consequence of general stress response. Previous studies reported the highest expression of *HvGS1* at 0.1 mM NH_4NO_3, which endorses this possibility, as ammonia is known to limit nitrate absorption and as a result aggravates the N deficiency in barley (Kronzucker et al. 1999; Lewis et al. 1982; Goodall et al. 2013). In contrast to that, increasing N level decreases expression of *HvGS1* isoforms and *HvGS2* in all tissues, further showing its role in re-assimilation of ammonia, synthesized during N remobilization. Likewise, in rice, Zhao and Shi (2006)

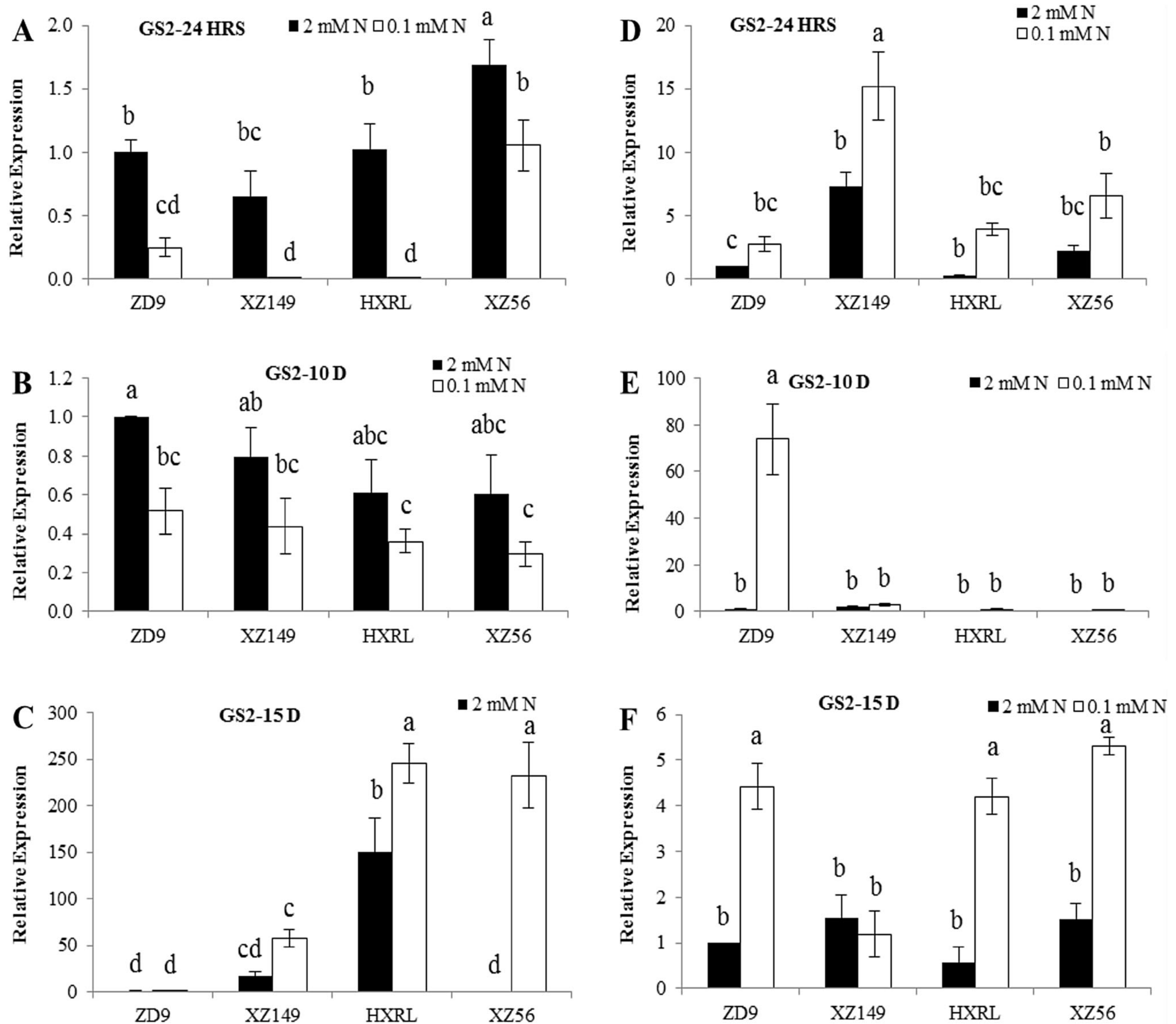

Fig. 3 Expression patterns of GS2 gene in four barley genotypes at different time intervals. **a**, **b** and **c** in leaves; **d**, **e** and **f** in roots at 24 h, 10 days and 15 days, respectively. Different letters indicate significant difference among the genotypes at $P < 0.05$

observed an up-regulation of root GS isoforms under high N tension, while under normal N, GS1 had high expression.

The expression of the N assimilation genes *HvGS1* and *HvGS2* in the roots of N-efficient barley genotype ZD9 and XZ149 were greater than that in HXRL and XZ56 (inefficient) genotypes at early stage of N stress, i.e. 24 h and 10 days, suggesting that these genes may play important role in manipulating the NUEs of crops by controlling N uptake during N stress. The higher activity of GS and protein synthesis, in response to reduced N supply and stressful environment, is in agreement with the up-regulation of *HvGS* (Mack 1995; Peat and Tobin 1996; Goodall et al. 2013). In this study, the increased expression of GS in barley leaves at normal N condition in N efficient genotypes resulted in increased synthesis of soluble proteins (Fig. 1), higher N concentration and chlorophyll contents which improved the vegetative growth of plants, as reflected by number of tillers per plant (Table 1). Previous reports indicated that soluble protein synthesis and GS activity were directly linked to GS expression and that root GS expression increased under low N environment and other abiotic stresses, when rapid re-assimilation of ammonia, produced by protein degradation, was required (Mack 1995; Peat and Tobin 1996; Goodall et al. 2013). Furthermore, in view of the low N availability for protein synthesis, two scenarios might also be advocated for explaining the lower protein concentration in N-starved plants: (1) NO_3^-–N at comparatively high concentration

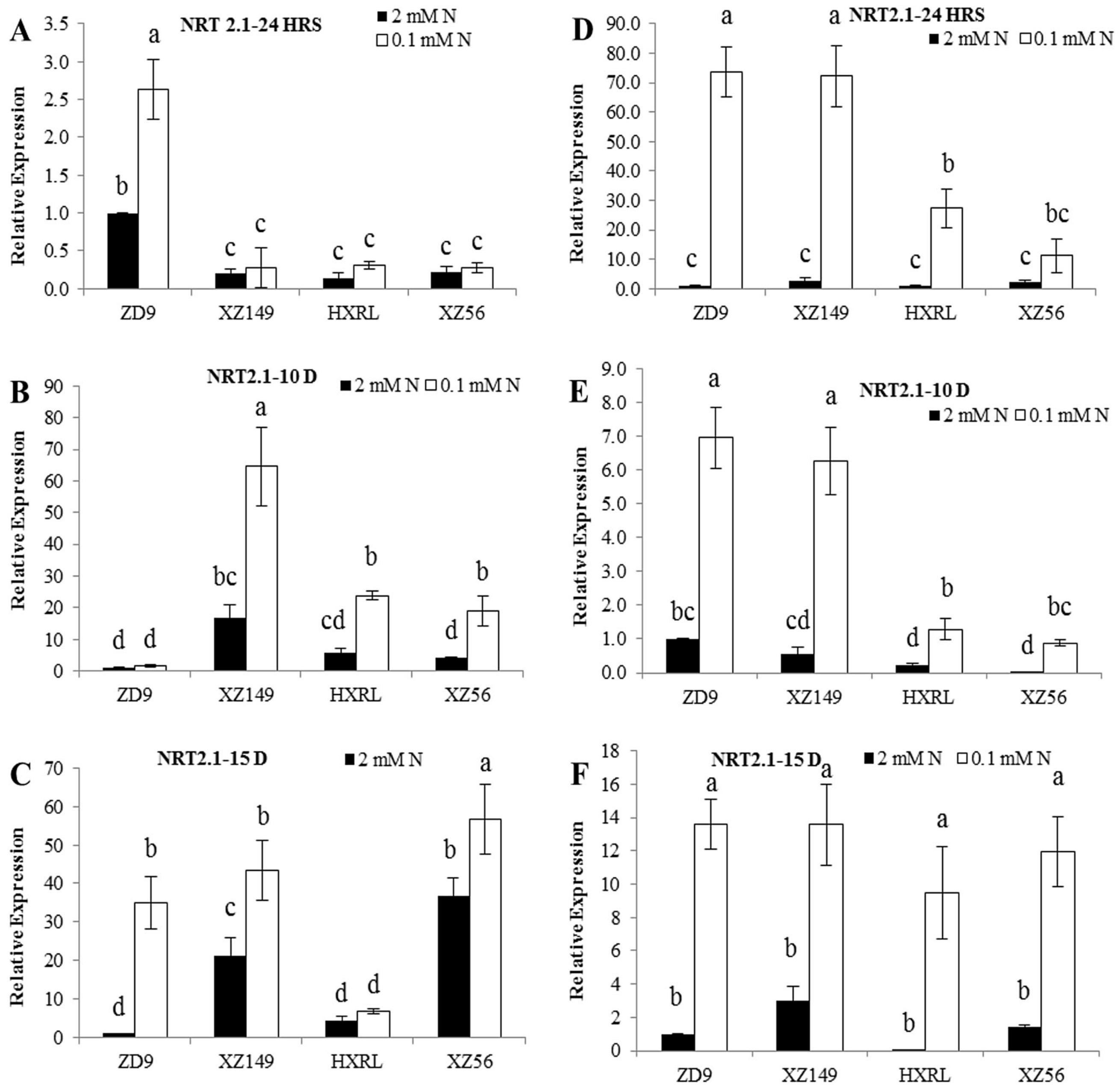

Fig. 4 Expression patterns of NRT2.1 gene in four barley genotypes at different time intervals. **a**, **b** and **c** in leaves; **d**, **e** and **f** in roots at 24 h, 10 days and 15 days, respectively. Different letters indicate significant difference among the genotypes at $P < 0.05$

would lead to an obvious increase in transcripts encoding various proteins (Stitt 1999); and (2) greater activity of proteases in N-starved plants which would lead to a rise in protein degradation (Galangau et al. 1988). Whatever the case, the capacity of maintaining a large proportion of N in a soluble form and divert it to the formation of soluble proteins might help plants to tolerate low N stress. Similar results were also observed previously in barley (Robredo et al. 2011) and in cassava (Gleadow et al. 2009).

Plants absorb N from the soil by nitrate transporters. The studies revealed a preferential expression of NRT2 genes in roots. However, no clear information is reported regarding the organ specificity in a wide range of plant species. In general, the expression of high affinity nitrate transport system increases under N starvation (Crawford and Glass 1998). It was also reported earlier that high affinity nitrate transporter *AtNRT*2.1 was induced in N-deprived *Arabidopsis* roots (Kiba et al. 2012; Lezhneva et al. 2014). In this study, nitrate transporter NRT2.1 was up-regulated at low N only in the roots and leaves of N efficient genotypes XZ149 and ZD9 (Fig. 4), suggesting enhanced uptake and translocation of NO_3^- from roots to leaves. While in

inefficient genotypes, it showed a slower response. This distinctive up-regulation of nitrate transporters in XZ149 and ZD9 may contribute to enhanced NO_3^- absorption, generating higher amount of N-containing metabolites essential for their endurance under N scarcity. Thus, it could be deduced that better performance of ZD9 and XZ149 genotypes under low N availability is attributed to higher N absorption and accumulation.

Conclusion

By improving NUE of the crops, application of fertilizer and pollution in the environment could be decreased. The present study illustrated a huge variation among 4 barley genotypes in terms of NUE, providing an opportunity in developing new cultivars with high NUE, which will ultimately reduce the fertilizer application and its loss into environment. The plants, supplied with limited N revealed a negative correlation with total chlorophyll content, soluble proteins and tissue N concentration. The studied genes showed time specific expression patterns which indicates that the time of the stress is an important factor while manipulating variances among genotypes. So the present study suggests that the future work must involve the time course as a key factor while studying expression patterns of these genes which would enable us to better understand the genetic basis of low-N tolerance. The present study also supports the genes HvNRT2.1, *Hv*GS1 and *Hv*GS2 as possible targets to improve N uptake and assimilation in barley that could improve NUE.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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