Nitrate assimilation in contrasting wheat genotypes

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Abstract The plants grown in natural conditions do not express their full potential of nitrogen (N) utilization due to limiting availability of N at later stages of growth. There is a likelihood of manifesting their full potential under non limiting nitrogen supply. In our earlier studies with 15 day old seedlings, it has been shown that high nitrate reductase (HNR) genotype with higher efficiency of the enzymes of the nitrate assimilatory pathway resulted in higher potential of this genotype for N utilization as compared to low nitrate reductase (LNR) genotype. In this study, the two wheat genotypes viz. HNR (HD 2285) and LNR (HD 1981) were grown in pots and were given N in three splits, so as to improve the availability of N at later stages of growth. In another experiment, 15 d old seedlings grown in hydroponics were used for nitrate uptake studies. Examination of the flag leaves at different growth stages revealed that except for nitrate levels which were higher in LNR genotype, the uptake of nitrate from the medium, the activity of the enzymes of the assimilatory pathway including total N were higher in HNR genotypes. In HNR genotype, higher amount of N was mobilized to the grains as compared to the LNR genotype. From our study, it is concluded that the higher N harvest of HNR genotype is due to the coordinated expression of all the enzymes of

the N metabolizing pathway and holistic approach for modifying the plant for better NUE will be beneficial.

Keywords Nitrate reductase · Nitrate uptake · Glutamate synthase · Glutamine synthetase · Wheat

Introduction

Nitrate is the major form of nitrogen (N) taken up and assimilated by most of the crop plants. Its low availability due to losses in various forms (Abrol et al. 1999), depending upon the soil types results in low utilization efficiency and limits the yield. Many management practices have been followed to enhance the nitrogen use efficiency (NUE) based on synchronization between supply and requirement (Pathak et al. 2008). In some studies the manipulation of the activities of the N assimilating enzymes in terms of over-expression in tobacco (Quillere et al. 1994; Kaiser et al. 2002), lotus (Suarez et al. 2002), rice (Hoshida et al. 2000) had limited effect on either NUE or grain filling and ultimately yield. Studies have also indicated that nitrate acts as a regulator of its own uptake, and identification of low and high affinity transporters have contributed towards unraveling the mechanisms of nitrate uptake in higher plants (Chopin et al. 2007). Katayama et al. (2009) studied the over-expression of the *OsNRT2.1* in rice with no effect on the stimulation of nitrate uptake or nitrate reductase (NR) activity. Transcriptional patterns of NR were similar to Nrt 1.5, which is involved in transporting nitrate from root to shoot (Lin et al. 2008). In *Arabidopsis* plants over-expressing Fd-GOGAT the changes in the amino acid pool were controlled by nitrate concentration (Ishizaki et al. 2010). Alteration of a single gene involved in N metabolism therefore, generally differed from the predicted results.

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Studies in field grown crops have depicted complex and dynamic changes in various physiological, biochemical markers representatives of N uptake, assimilation and recycling (Kichey et al. 2006).

In our laboratory, screening of a large number of wheat genotypes revealed 2–3 fold variation in NR activity (Nair and Abrol 1973; Abrol 1990). It was reported that high NR (HNR) genotypes invariably had higher reduced N and/or biomass production and sometimes higher grain yield. Our studies also showed that wheat cultivars with low NR activity had lower rate of nitrate uptake (Das et al. 2006). In other studies, however, efforts to relate NR activity to grain yield gave inconsistent results (Traore and Maranville 1999; Hirel et al. 2001). Analysis of biochemical factors contributing to variation in NR activity showed availability of low levels of reductant (NADH) and differences in transcript levels (Abdin et al. 1992; Jain and Abrol 2005).

In the present study nitrogen was made available throughout the growing period by split application so that the soil N was available to the upper leaf blades which contribute maximally to N at harvest (Nair and Abrol 1978; Abrol 1990). The N uptake and all the enzymes involved in the N assimilatory pathway were examined under ample N availability in the contrasting wheat genotypes.

Materials and methods

Experiment I: nitrate uptake by seedlings of HNR and LNR genotypes

Growth of seedlings The sterilized and germinated seeds of HNR and LNR wheat genotypes were inserted in thermocole sheets cut to fit 20 L hydroponic tanks. The roots were fully immersed in the nutrient solution. There were 40 seedlings per tray. When desired the entire sheet with all the plants could be transferred to the new solution with minimal disturbance. Seed germination, plant growth and all experiments were carried out in controlled environment in a growth chamber at 25 ± 2 °C and 70 % RH, with 16 h light provided by fluorescent tubes at the intensity of $500 \mu\text{E m}^{-2} \text{s}^{-1}$ for 15 days in aerated $\frac{1}{4}$ th strength (+N; NO_3^- concentration, 500 μM), Hoagland solution, pH 6.0 (induced seedlings). For the other set of the seedlings the N salts were omitted from the Hoagland medium (un-induced seedlings). To overcome the effect of the variations in biological material, three replicates per treatment were taken and 20 seedlings selected per replication for the uptake studies were of uniform size and the fresh weight (data not shown).

Nitrate uptake rate The 15 days old induced and un-induced seedlings of both the genotypes were incubated for 30 min in 10 mM nitrate solution (pH 5.5) and the uptake

rates were determined by measuring the depletion of nitrate from the uptake solution. The seedlings were separated into shoots and roots and dried till the constant dry weight was recorded. The roots and shoots were ground to a fine powder and 100 mg of samples from each were used for nitrate estimation by Downes (1978) method. The nitrate present in the solutions was also estimated by Downes method. The *in vivo* NR activity in the seedlings was estimated by the method of Jaworski (1971).

Experiment II

Two wheat genotypes viz. ‘HD 2285’ (HNR) and ‘HD 1981’ (LNR) were grown in the soil in pots (12 inch diameter containing 10 kg soil per pot). Two plants were maintained per pot. The fertilizers were applied at rates equivalent to 150 Kg N ha^{-1} (1.53 g urea pot^{-1} in three equal splits), 60 Kg P ha^{-1} (1.7 g single superphosphate pot^{-1}) and 40 Kg K ha^{-1} (0.45 g muriate of potash pot^{-1}), respectively. The phosphate and potash were applied as basal dose and N was given in three equal splits as basal dose, at crown root initiation and anthesis stages.

The activity of the enzymes of N assimilation pathway The activities of the various enzymes of the N assimilation pathway were estimated in the flag leaves from full expansion to senescence viz. 60 DAS to 110 DAS in both the wheat genotypes. The activity of the nitrate reductase (NR) was estimated *in vivo* (Jaworski 1971). Extracts for nitrite reductase (NiR) activity were prepared according to Gupta and Beevers (1984) and *in vitro* Nir activity was assayed by the method of Ida and Morita (1973). The extracts used for glutamine synthetase (GS) and glutamate synthase (GOGAT) were prepared by grinding 0.5 g of flag leaf tissue in 2.5 ml extraction buffer (Cooper and Beevers 1969). The homogenate was centrifuged at 500 g for 5 min to remove unbroken cells. The supernatant was centrifuged at 10,000 g for 15 min and supernatant was again centrifuged at 100,000 g for 30 min and resulting clear solution was used to assay GS and GOGAT. GS activity was determined according to Mohanty and Fletcher (1980) and Fd-GOGAT activity was determined using reduced methyl viologen as the electron donor (Lea et al. 1990). Fd-GOGAT activity was determined by the quantitative measurement of glutamate using High Performance Liquid Chromatography (HPLC) as described by (Esposito et al. 2003).

Estimation of nitrate, total N, nitrogen harvest and biomass Nitrate in the flag leaves was estimated with the improved hydrazine reduction method (Downes 1978). Nitrogen (N) concentration was determined as total reduced

N in 0.1 g samples of the various dried tissues subjected to digestion and distillation by using autoanalyzer (Gerhardt, Germany). The total N per plant and total grain N per plant was also calculated and harvested nitrogen index was calculated as: Grain N per plant/Total N per plant $\times 100$. The plants were harvested at various stages and dried at 70 °C till the constant dry weight and biomass was recorded.

Statistical analysis The data were analyzed statistically by analysis of variance (ANOVA) for each parameter separately using MSTAT program. Significance of differences among genotypes was determined by the least significance difference (LSD) calculated at $P < 0.05$

Results

Experiment I

Nitrate uptake by induced and un-induced seedlings

The 20 seedlings were incubated in 100 ml of 10 mM nitrate solution. The nitrate depleted from the medium after 30 min. was assessed and rate of nitrate uptake was calculated for the induced and un-induced seedlings of HNR and LNR genotypes for comparison. The induced seedlings of HNR genotype took up 29 % more nitrate from the incubation medium as compared to the LNR genotype. The un-induced seedlings of both the genotypes did not differ in the rate of nitrate uptake (Fig. 1).

Tissue nitrate content

The roots had lower nitrate content as compared to the shoots in both the genotypes (Fig. 2a,b). The nitrate

Fig. 1 Nitrate uptake by HNR and LNR genotype seedlings. The uninduced rate and rate for plants exposed to 10 mM nitrate for 30 min are shown for comparison. Each bar represents the mean $\pm$ SE of three replicates

Fig. 2 Nitrate content of the induced (a) and un-induced (b) seedlings of HNR and LNR genotypes. Each bar represents the mean $\pm$ SE of three replicates

content of the shoots of the un-induced seedlings of both the genotypes was lower than the tissue nitrate concentration of the induced seedlings (Fig. 2a,b). The nitrate content in the shoots of the induced seedlings of the LNR genotype were 2 fold higher as compared to the nitrate levels in the shoots of the HNR genotype. The nitrate content of the roots of the induced seedlings of both the genotypes was similar. The nitrate content in the shoots (49 % less) and roots (20 % less) of the un-induced seedlings of LNR genotype was lower as compared to the HNR genotype.

Activity of the enzyme nitrate reductase (NRA)

The NRA was high in both induced and un-induced seedlings of HNR genotype as compared to the activity of the enzyme in the leaves of LNR genotype. The NRA was 1.5 fold and 69 % more in the leaves of the induced and un-induced seedlings, respectively, of HNR genotype as compared the NRA in the leaves of LNR genotype (Fig. 3).

Experiment II

The activity of the enzymes of the N utilization pathway was assayed in the flag leaves (as the contribution of the flag leaves is maximum during grain development) of both the genotypes at various time intervals.

Fig. 3 In vivo activity of enzyme nitrate reductase of induced and un-induced seedlings of HNR and LNR genotypes. Each bar represents the mean $\pm$ SE of three replicates

Nitrate reductase, nitrite reductase and ammonia assimilating enzymes

The nitrate reductase activity (NRA) in the flag leaves of HNR genotype was higher than the NRA in the flag leaves of the LNR genotype (Fig. 4a). The difference in the NRA was more than 90 % at 60 DAS and at 110 DAS the flag leaves of HNR genotype had 1.7 fold higher activity of NR as compared to the activity in the flag leaves of the LNR genotype.

The flag leaves of the HNR genotype had higher activities of all the enzymes of the nitrate assimilation pathway as compared to their activities in the LNR genotype. The nitrite

reductase (NiR) activity was 45 % higher at 60 DAS in HNR genotype as compared to the level of the activity in the flag leaves of LNR genotype at the earlier stages of the measurement. At later stages, 110 DAS the HNR genotype had 2.4 fold higher NiR activity as compared to the activity LNR genotype (Fig. 4b).

The activities of the other enzymes of the pathway viz. GS/GOGAT were also high in the flag leaves of the HNR genotype as compared to their activity in the flag leaves of the LNR genotype (Fig. 4c,d). The GS activity was 41 %, 26 %, 42 % and 2 fold higher in HNR genotype at 60, 75, 90 and 110 DAS, respectively. Among the genotypes, the differences in the Fd-GOGAT activity were more than 1 fold and 3 fold at 60 and 110 DAS, respectively.

Total N content in different plant parts, nitrogen harvest and biomass

Amount of the total reduced N was estimated in the leaf laminae, leaf sheaths, chaff as well as the grains of both the genotypes (Fig. 5). The total reduced N in the leaves at the time of the harvest in LNR genotype (120 DAS) was 24 % higher in the flag leaves, 4 % more in leaf sheaths and 45 % higher in the chaff as compared to N in the respective tissues of the HNR genotype. The grains of the HNR genotype had almost 28 % higher N as compared to the grains of the LNR genotype. The total N per plant and total grain N per plant were 45 % and

Fig. 4 Changes in activities of nitrate reductase (a), nitrite reductase (b), glutamine synthetase (c) and glutamate synthase (d), respectively. The values are mean of $\pm$ SD of three replicates ($n=3$)

Fig. 5 Per cent total nitrogen in the various plant parts of the two wheat genotypes HD '2285' (HNR) and HD '1981' (LNR) measured at various growth stages. The values are mean of $\pm$ SD of ten replicates ($n=10$). * Significant at 5 % L.S.D

61 % more in HNR genotype (Table 1). The N harvest was therefore, higher in HNR genotype.

Plant biomass

The biomass of HNR genotype was significantly higher as compared to LNR genotype. The increase in the dry weight of the HNR genotype varied from 25 % to 30 % when compared with LNR genotype (Fig. 6).

Nitrate concentration of the flag leaves

The leaves of the LNR genotype accumulated much higher nitrate content as compared to the leaves of HNR genotype (Fig. 7). It was 94, 52, 26 and 36 % higher in the leaves of the LNR genotype at 60, 75, 90 and 110 DAS, respectively, as compared to the nitrate content in the flag leaves of the HNR genotype. The higher accumulation of nitrate in the leaves of LNR genotype was also observed in the seedlings in the experiment I.

Discussion

Earlier studies from our laboratory reported variation in the activity of enzyme nitrate reductase. Furthermore, it was observed that the genotypes with high NRA possessed

higher biomass, higher reduced N levels, grain protein. There may/may not be increase in grain yield (Abrol et al. 1999). In the recent study Anjana et al. (2011) in 15 days old seedlings grown under non limiting N supply, conclusively showed that it was the coordinated increase in the activity of all the enzymes in the N assimilatory pathway in HNR genotype.

The present studies were carried out at seedling stage as well as at various growth stages. Nitrate in the second experiment was given in splits so as to make N available at later stages. The enzyme activities were assayed in the flag leaves as they contribute towards the grain filling and grain N levels right up to maturity of the plants (Nair and Abrol 1978). The differences in the NR activity among the genotypes were also observed at the seedling stage. The study conclusively provided the evidence that high NR is associated with the enhanced activity of all the enzymes of the pathway. HNR genotypes differed in all aspects of N metabolism viz. amount of nitrate taken up, its rate of uptake, accumulation in the tissues, its assimilation and also N harvest. The nitrate content of the HNR genotype in both the experiments was lower than the nitrate content in the leaves of the LNR genotypes in spite of the higher rate of nitrate uptake from the medium. We presume that in LNR genotypes higher amount of nitrate is mobilized to storage pool, while in HNR genotypes assimilation is more. The nitrate levels in the shoots of the induced plants of the LNR genotypes were higher as compared to the HNR genotype but no difference was observed in the nitrate content of the roots of the induced seedlings of HNR and LNR genotype. This indicates the possibility of non inducing nature of NR enzyme in roots. Secondly, it may be possible that leaves being the major site of nitrate assimilation the major amount of nitrate is being transported to the shoots, but accumulates in LNR genotype due to lower NR activity.

Earlier, it was thought that NR is the first enzyme of the N assimilation pathway and was rate limiting and highly regulated. Beevers and Hageman (1969) suggested that the NR activity could be considered as an index of reduced nitrogen available in the plant and that plants with high NR activity possess a greater potential for accumulation of reduced nitrogen if soil nitrate is available throughout the growing period. Selection for high NRA was also associated with low leaf nitrate levels (Sherrard et al. 1986). A number of studies have been conducted in tobacco to relate

Table 1 Whole plant nitrogen (mg plant^{-1}) grain nitrogen (mg plant^{-1}) and harvested nitrogen index in two wheat genotypes

Cultivar	Whole plant nitrogen	Grain nitrogen	Harvested N index
HNR	166.2	141	85.0
LNR	107.8	87.3	81.0
L.S.D.(0.05)	14.5	12.5	1.69

Fig. 6 Changes in the dry weight of two wheat genotypes HD 2285 (HNR) and HD 1981 (LNR) at various days after sowing (DAS). L.S.D. (0.05) is 0.035, 0.087, 0.61 and 1.34 for 60, 75, 90 and 120 DAS, respectively. * Significant at least significant difference (L.S.D) at 5 % probability

over expression of NR to growth (Quillere et al. 1994; Kaiser et al. 2002). But, no relationship could be established between growth and NRA.

It was suggested by Stitt (1999) and Andrews et al. (2004) that GS and GOGAT are required in addition to NR for nitrate assimilation and one or both these enzymes may limit N assimilation and also growth and yield. While we reported higher activity of NR as a natural variation to enhance reduced N and biomass in different wheat genotypes (Abdin et al. 1992), the present study clarify that the HNR genotype exhibited higher activities of the nitrite reductase, GS and GOGAT leading to higher biomass and N harvest. Lower per cent N in the leaves of the HNR genotype at the harvest indicate that the mobilization of N was higher in this genotype as compared to LNR genotype. The results indicate that HNR genotype took up higher amount of nitrate, accumulated less and

Fig. 7 Nitrate content of the flag leaves of the two wheat genotypes HD '2285' (HNR) and HD '1981' (LNR). The values are mean of $\pm$ SD of ten replicates ($n=10$). * significant at 5 % L.S.D

assimilated more as compared to LNR genotype due the coordinated activity of all the enzymes of the pathway.

Lower nitrate levels in the tissues of HNR genotype also indicate better assimilation to ammonia. This is supported by the higher activities of all the N metabolizing enzymes till later stages of growth in HNR genotype. Higher shoot nitrate content has been positively correlated with higher uptake in some of the studies (Harrison et al. 2003a), but this was not observed in the present study as the nitrate concentration was lower in the vegetative tissues of the HNR genotype as compared to LNR genotype.

In the HNR genotype all the enzymes of the N assimilation pathway had the higher activity at all the stages of measurement. This may be one of the reasons that the studies in the transformants over-expressing single enzyme of the pathway have not given the desired results in terms of growth and yield (Fu et al. 2003; Harrison et al. 2000; Yamaya et al. 2002) Hence, overall there has been little indication that either increased NR, NiR or GS/GOGAT alone could be useful strategy to increase yield or NUE. In cereals there is a very strong evidence that GS1 is the key enzyme in the mobilization of N from the senescing leaves and its activity in leaves is positively related to grain N (Fei et al. 2003; Man et al. 2005). In HNR genotype higher overall activity of the GS (although not differentiated between GS1 and GS2) and also the higher activity of this enzyme in senescing leaves was responsible for translocation of the plant N to the seeds. In rice the NADH- GOGAT activity is important in utilization of N in grain filling and its activity in the developing grain is positively related to yield (Forde and Lea 2007).

Conclusions

Wheat genotypes with variation in the activity of nitrate reductase enzyme differ in the amount of N assimilated during the entire life span. The flag leaves of HNR genotype maintained higher activities of the N metabolizing enzymes till the later stages of growth. The seedlings of HNR genotype took up more nitrate from the medium as compared to LNR genotype. The higher grain N and higher N harvest was due the maintenance of the higher activity of all the N metabolizing enzymes. In the LNR genotypes a substantial portion of N is not re-translocated to the harvested structures as in this genotype the GS/GOGAT activity in the flag leaves especially during senescence declines significantly as compared to the activity in the leaves of the HNR genotype. Hence, the ability of the HNR genotypes to harvest more N and redistribute to grains is due to the well-coordinated system of N uptake and assimilation. The study can help us identify/select the cultivars that absorb and utilize N efficiently and such

cultivars can be utilized in the cisgenesis approach (Kichey et al. 2009), which is the main challenge for sustainable agriculture. The study also shows that why, in spite of the significant progress made on the regulation of N metabolism no significant breakthrough has been achieved, as it is difficult to rely on the results obtained by modifying the expression of a single gene as interactions among the pathways may be too complex. But, these data could be integrated at the whole plant level to find a pathway with universal switch controlling NUE.

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