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Differential regulation of nitrate assimilatory enzymes by methionine sulfoximine in rice

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Abstract
Nitrate assimilation is an important process for the growth and development of plants. It is regulated at both transcriptional and posttranscriptional level by various factors including nitrogen metabolites. In the present study the effect of nitrogen metabolites on the regulation of nitrate reductase (NR) and nitrite reductase (NiR) in the shoots of 11-days hydroponically grown rice seedlings were studied. Both ammonium and glutamine caused a decrease in the activity of nitrate-induced NR while these metabolites had only a partial inhibitory or no effect on NiR. These metabolites had no effect on the enzyme activities in the absence of nitrate and on the nitrate uptake. Methionine sulfoximine, (MSX) an inhibitor of glutamine synthetase, was used to check whether the observed inhibitory effect on NR and NiR is due to ammonium itself or some other downstream metabolites. The results obtained show that while MSX had a partial inhibitory effect on NR. However it had no effect on NiR activities. On the other hand it increased the inhibitory effect of ammonium on nitrate reductase activity. Increase in inhibition to such an extent was not observed with NiR activity. These results indicate that the inhibition of NR and NiR activities was a direct effect of ammonium accumulation rather than an effect of its assimilation products.

Keywords: Nitrate assimilation, Enzyme inhibitors, Methionine sulfoximine (MSX), Ammonium, Glutamine

Introduction

Nitrate is one of the most important sources of nitrogen for higher plants. The assimilation of nitrate begins with the uptake of nitrate from the soil by the root. Depending on the species nitrate can get stored or converted to ammonia in the root or is transported to leaves to be stored in the vacuoles or assimilated in the form of ammonium. The conversion of nitrate to ammonium is a two-step process catalysed by cytosolic nitrate reductase (NR; NADH:nitrate reductase; EC 1.6.6.1) and chloroplastic nitrite reductase (NiR; ferredoxin:nitrite oxidoreductase; EC 1.7.7.1). Ammonium is assimilated into the carbon skeleton by glutamate synthase (GOGAT) and glutamine synthetase (GS) in a cyclic manner[1]. Gene expression and enzyme activity of the various proteins involved in this pathway are regulated by both internal and external stimuli such as nitrate itself, carbon and nitrogen metabolites, growth regulators, light, temperature and carbon dioxide concentration [2,3,4]. However, the strongest responses are induction by nitrate and repression by ammonium or its derivatives[5]. NR activity is under the control of nitrogen status at all the levels of gene expression [5,6]. Nitrite, due to its toxicity, is never allowed to accumulate in the cells. On the other hand, ammonium and glutamine have been shown to have varied responses (no effect, inhibition or stimulation) depending on the species, genotype, tissue and experimental conditions [7].

In the present study the effect of various nitrogen metabolites were checked on the activities of nitrate assimilatory enzymes in rice (*Oryza sativa* var. Panvel I). This plant was selected as experimental system because of several reasons. There are a very few reports regarding the studies on the nitrate metabolism on this plant. Moreover, it is known for its poor nitrogen use effeiciency (NUE) which is ~ 25 %, poorest among

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cereals. Results presented in this study indicate that ammonium and glutamine differentially regulated NR and NiR. MSX (methionine sulfoximine) was also used to check whether the observed inhibitory effect of ammonium on NR and NiR is a direct effect of ammonium ions or due to some downstream product of ammonium assimilation like glutamine. It can be concluded from results presented in this paper that the observed inhibitory effect of ammonium on NR and NiR activities is a direct effect of ammonium accumulation rather than an effect of downstream metabolites like glutamine, glutamate or asparagine.

Materials and Methods

Rice crude extracts were prepared from excised leaves of 11-day old seedlings of *Oryza sativa* var. Panvel 1 and assayed for NR and NiR by using methods described by Hagemann (1979) and Wray and Fido (1990) respectively with slight modifications [8, 9, 10, 11]. NR and NiR specific activities were defined as nmoles of nitrite produced or consumed mg protein^{-1} min^{-1} respectively. The mean data from three different experiments have been represented as relative specific activity (%) along with standard error bars. The activities of NR and NiR in the presence of potassium nitrate (40 mM) have been considered as 100%. Total nitrate content was determined from leaves treated with various metabolites with or without nitrate following reduction to nitrite according to a method modified from Kamphake *et al.* (1967) [12]. Protein content was estimated according to the Bradford's method using BSA as standard [13].

Results

Effect of Ammonium

Ammonium and glutamine are the two major downstream metabolites of nitrate assimilation pathway. The effects of these two metabolites have been studied in many plant species. However the exact mechanism of regulation of NR and NiR by ammonium and glutamine is not known. Therefore attempts were made to analyze the role of these metabolites in the regulation of NR and NiR activities in rice. Excised leaves were floated on ammonium (40 mM), glutamine (40 mM) and MSX (1 mM) in the presence or absence of potassium nitrate (40 mM). The data presented in Tables 1 and 2 show that ammonium did not have any effect on NR and NiR activities in the absence of nitrate as well as on the nitrate uptake by leaves respectively. However it severely inhibited the nitrate-induced NR activity and the level of inhibition was 62% (Fig.1A). Figure 1B shows that ammonium has a partial inhibitory effect on nitrate-induced NiR in light.

Effect of Glutamine

Similarly the effect of glutamine was checked on the activities of NR and NiR by treating leaves with glutamine (40 mM) alone and in the presence of potassium nitrate (40 mM). The data presented in Tables 1 and 2 show that glutamine alone did not have any effect on NR and NiR activities in the absence of nitrate as well as on the nitrate uptake respectively but partially inhibited (~ 20%) nitrate-induced NR activity (Table 3). On the other hand, there was no effect of glutamine on NiR activity in light (Table 3).

Effect of MSX

Excised leaves were floated on 1 mM MSX either alone or in combination with nitrate (40 mM) and ammonium (40 mM) with distilled water and 40 mM KNO_3 as controls in light. The data presented in Fig. 1A show that MSX partially inhibited NR activity (20%) and this inhibition increased to 80% in the presence of ammonium. However, there was no effect of MSX on NiR activity (Fig 1B). It just enhanced the inhibitory effect of ammonium by about 10%. The data presented in the Tables 1 and 2 show that MSX did not have any effect on NR and NiR activities in the absence of nitrate as well as on the nitrate uptake process respectively.

Fig. 1: Effect of MSX on NR (A) and NiR (B) activity in Light

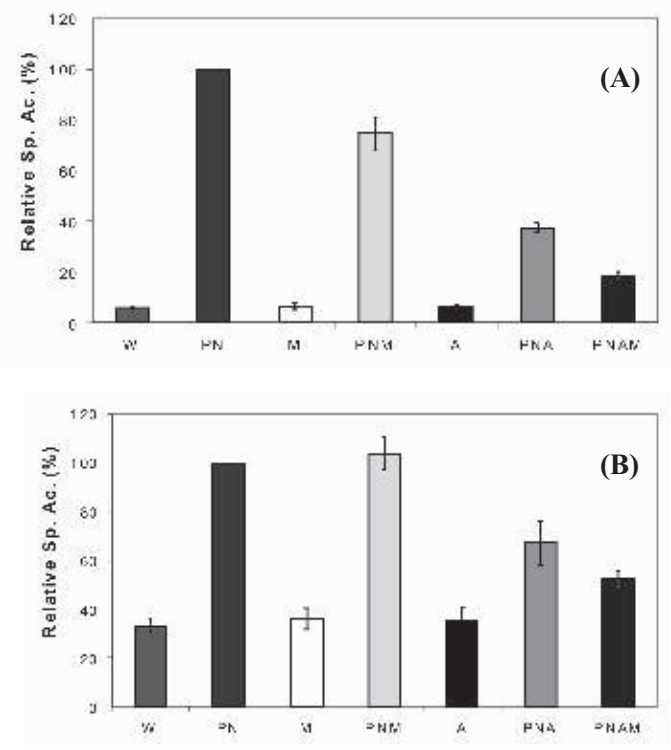

Fig. 1: (A) and (B)

Excised leaves from 11-day old rice seedlings were floated on MSX (1 mM) in combination with 40 mM KNO3 (PN) in the light. Activity in the presence of 40 mM KNO3 was considered as 100% control. The mean data from three different experiments are shown as relative specific activity (%) along with standard error bars.

W – Water
PN – Potassium nitrate (40 mM)
M – MSX (1 mM)
PNM – Potassium nitrate + MSX A – Ammonium chloride (40 mM)
PNA – Potassium nitrate + Ammonium chloride
PNAM – Potassium nitrate + Ammonium chloride + MSX

Table 1: Effect of Ammonium, Glutamine and MSX alone on NR and NiR Activity

Treatment	Relative Sp. Ac .(%)	
	Nitrate Reductase Activity	Nitrite Reductase Activity
Nitrate (40 mM)	100	100
Water	5.93 ± 1.60	33.16 ± 3.16
Ammonium (40 mM)	6.88 ± 2.33	31.58 ± 6.56
Glutamine (40 mM)	7.88 ± 0.43	32.54 ± 2.54
MSX (1 mM)	6.88 ± 1.35	36.22 ± 4.78

Table 2: Effect of Downstream Metabolites on Nitrate Uptake by Leaves

Treatment	Relative Nitrate Content (%)
Nitrate (40 mM)	100
Water	19.41 ± 2.38
Ammonium (40 mM)	110.54 ± 1.65
Glutamine (40 mM)	93.12 ± 5.13
MSX (1 mM)	102.34 ± 12.54

Table 3: Effect of Glutamine on NR and NiR activity

Treatment	Relative Sp. Ac. (%)	
	Nitrate Reductase Activity	Nitrite Reductase Activity
Nitrate (40 mM)	100	100
Water	5.93 ± 1.60	33.16 ± 3.16
Glutamine (40 mM)	73.75 ± 5.43	98.25 ± 4.54

Discussion

Nitrate assimilation is a highly regulated process because of its dependence on carbon metabolism for energy and reductants as well as the toxicity of the metabolites of this pathway, nitrite and ammonium. The activity of NR is controlled according to the status of nitrogen of plants at transcriptional, posttranscriptional and posttranslational levels [6]. Because of its central role in nitrogen metabolism in plants ammonium has been considered to be the most important metabolite for the regulation of nitrate and nitrite reductase and has been extensively reported in literature. However the exact mechanism by which it modulates the nitrate assimilation pathways is not clearly understood. It also has differential effect which depends on the plant type as well as growth conditions (etiolated/green plants). The effect varied between no effect in Arabidopsis to inhibitory effect in wheat and stimulatory effect in maize [14,15, 16]. The results presented in this study show that both NR and NiR activities were significantly inhibited in ammonium treated leaves showing a similar pattern of regulation for both.

The other metabolite of this pathway, glutamine, also has been shown to have diverse effects on the activity of NR and the effect varied from species to species and organ to organ [7]. The results presented in this study show that glutamine partially inhibited NR activity. A similar kind of effect has been reported in tobacco and maize leaves by several other groups [17, 18]. However in the roots the activity of NR has been shown to be severely inhibited by glutamine. Shankar and Srivastava (1998) have shown that NADPH:NR, a root specific isoform, is more sensitive to glutamine than NADH:NR, present in shoot [7]. Glutamine had no effect on the activity of NiR under similar conditions. Sivasankar et al. (1997) have reported that glutamine inhibited NiR mRNA and had no effect on

NiR protein [17]. This may be due to the slower turnover rate of NiR proteins [17,19].

Methionine sulfoximine (MSX) inhibits the assimilation of ammonium to amino-N by inhibiting GS. When it is supplied to many systems it gets phosphorylated and this phosphorylated form binds to the active site of GS resulting in a complete and irreversible inhibition[19]. This leads to the accumulation of ammonium in the system. MSX was used in the present study to check whether the observed inhibitory effect of ammonium on NR is on its own or due to some downstream product of ammonium assimilation like glutamine. The results obtained show that MSX had little effect on NR and NiR activities. However the extent of inhibition of NR activity increased in the presence of ammonium. Increase in inhibition to such an extent was not observed with NiR activity. These results indicate that the inhibition of NR and NiR activities was a direct effect of ammonium accumulation rather than an effect of its assimilation products. Several other studies have shown that MSX had no effect on NR and NiR activities [2, 20]. It can be concluded from above studies that NR is under tighter regulation than NiR by ammonium and glutamine. It can also be concluded that the observed effects of these metabolites were directly on the nitrate-induced NR and NiR.

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