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Research Article

Effect of Nitrate, Nitrite, Ammonium, Glutamate, Glutamine and 2-oxoglutarate on the RNA Levels and Enzyme Activities of Nitrate Reductase and Nitrite Reductase in Rice

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In this study the induction and regulation of NR and NiR by various N metabolites in excised leaves of rice (*Oryza sativa* ssp. indica var. Panvel I) seedlings grown hydroponically (nutrient starved) for 10-12 days and adapted for 2 days in darkness was examined. Nitrate induced the activity of both the enzymes reaching an optimum at 40 mM in 6 hrs. Nitrite and ammonium inhibited NR in light in a concentration-dependent manner. Glutamine, which had little effect of its own on NR in light and no effect on NiR in both light and dark, strongly inhibited Nitrate-induced NR in the dark. When the activities of these enzymes were measured from leaves treated with glutamate and 2-oxoglutarate, a similar pattern of induction was observed for NR and NiR. The transcript levels of NR and NiR increased to a similar extent in the presence of nitrate. However light did not cause any significant change in transcript levels. These results indicate that both the enzymes are under tight regulation by nitrogen metabolites and light and are co-regulated under certain conditions.

INTRODUCTION

The assimilatory nitrate reduction pathway is a vital biological process in higher plants, algae, and fungi, as it is the principal route by which inorganic nitrogen is incorporated into organic compounds. It involves energy-dependent uptake of nitrate through the roots and its reduction to nitrite and further to ammonium in a two-step process catalysed by the highly regulated enzymes, nitrate reductase (NR) and nitrite reductase (NiR) in cytosol and chloroplast respectively. The reduced nitrogen, ammonium, is converted into organic-N forms, glutamine (Gln) and glutamate (Glu) by combined action of GS and GOGAT in a cyclic manner (Sivasankar and Oaks, 1996). These amino acids subsequently serve as the N donor for all other amino acids.

Nitrate assimilation is a highly regulated process because of its dependence on photosynthesis for energy and reductants as well as the toxicity of the metabolites of this pathway, nitrite and ammonium. Accordingly, 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 (Aslam *et al.*, 1997). However, the strongest responses are induction by nitrate and repression by ammonium or its derivatives, in a combined action of feedforward and feedback regulation mechanism (Crawford, 1995). The activity of nitrate reductase is controlled according to the nitrogen status of plants by a hierarchy of transcriptional, post-transcriptional and post-translational regulation (Crawford, 1995; Daniel-Vedele *et al.*, 1998). Recently, Lea and co-workers (2006) have shown that the posttranslational regulation of NR is apparently much more important than the transcriptional regulation for setting the levels of amino acids, ammonium, and nitrate in *N. plumbaginifolia*. Control of NR gene transcription facilitates long-term responses to the nitrate signal (hrs to days), whereas post-translational regulation allows rapid changes in NR activity (mins to hrs). Nitrite is never allowed to accumulate in the cells because of its toxicity. In maize, it has been shown to inhibit NR transcript level and activity (Raghuram and Sopory, 1999). Ammonium and glutamine have been shown to have varied responses (no effect, inhibition or stimulation) depending on the species, genotype, tissue and experimental conditions (Aslam *et al.*, 1979; Shankar and Srivastava, 1998). On the other hand, the roles of glutamate and 2-oxoglutarate in regulating NR and NiR are not

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available in literature except a few reports suggesting their importance in tobacco plants (Stitt *et al.*, 2002). Besides, the effect of all these metabolites have not been studied systematically for a single plant and most of the papers are confined to a single enzyme.

In the present study, attempts were made to examine the induction of nitrate assimilation enzymes by nitrate and light as well as their regulation by downstream metabolites of nitrogen metabolism in rice. As light/dark transition causes changes in the expression of NR and NiR as well as nitrogen status, the effect of these metabolites on NR and NiR enzyme activities were examined under both light and dark conditions. Even though rice is a major staple food of the world and its genome has been sequenced, nitrate metabolism has not been studied in great detail. Another factor which makes this study important is the low nitrogen use efficiency of rice (~25 %), poorest among cereals. For this purpose *Oryza sativa* ssp. indica var. Panvel I was selected. Results presented in this paper indicate a tight and co-ordinated regulation between nitrate and nitrite reduction. Both these processes were co-regulated by nitrate in the presence of light as well as by nitrite, glutamate and 2-oxoglutarate in dark. However, ammonium and glutamine differentially regulated the activities of NR and NiR. The transcript levels of NR and NiR were also measured using RT-PCR and it was found that nitrate, not light, plays a more significant role in the regulation of transcript levels in rice. It can be concluded, therefore, that nitrate assimilation system of rice is under stringent control of nitrogen metabolites.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of hybrid *Oryza sativa* var. Panvel I, obtained from the Kharland Research Station, Panvel (Maharashtra, India) were washed thoroughly, surface sterilized for 10 minutes in 5 % v/v sodium hypochlorite, washed several times with tap water and soaked in distilled water and kept in dark for two days. Imbibed seeds were plated on wet germination paper in a plastic tray and incubated at 25+2 °C under white light derived from 4 Osram 36 W fluorescent lamps. The light intensity was 7 Klux at the plant level. The seedlings were watered daily with distilled water for 10-12 days.

Treatments

To study the involvement of the nitrogen metabolites in the regulation of enzymes, excised leaves from 10-12 days old seedlings were treated with nitrate (0-100 mM), nitrite (0.1-1.0 mM), ammonium (10-40 mM), glutamine (10-40 mM), glutamate (20 mM), 2-oxoglutarate (10 mM), each of these with and without nitrate (40 mM). At the end of 4 hrs, the leaves were washed, blotted on tissue paper, wrapped in foil, frozen in liquid N_2 and used immediately or stored at –70° C to determine enzyme activities and protein content.

Dark adaptation

For studies in the dark, light grown seedlings were transferred to complete darkness 2 days before treatment since minimal nitrate induced NR activity was observed at this time point. This was carried out in order to make sure that light signal, stored in any form, is completely exhausted in the system. Initial standardisation was carried out by assaying NR activity at 6-hour intervals after transferring the leaves to dark. At the end of 24 hours, no NR activity could be detected. Similar conditions were used for studies on NiR.

Enzyme assays

The buffer used for preparation of crude extracts contained potassium phosphate buffer (100 mM, pH 7.5), magnesium acetate (5 mM), glycerol (10 % v/v), polyvinylpyrollidone (10 % w/v), Triton X-100 (0.1 % v/v), EDTA (1 mM), DTT (1 mM), PMSF (1 mM), benzamidine (1 mM) (prepared fresh) and 6-aminocaproic acid (1 mM). The leaf tissue (0.25 g) was ground into a fine powder in liquid N_2 using a mortar and pestle. The extraction buffer was added soon after liquid nitrogen evaporated, but before thawing set in. The tissue to buffer ratio was 1:3 (w/v) and the mixture was homogenised thoroughly. The extract was filtered through nylon net (80 mm) and centrifuged at 14,000 rpm for 15 minutes. The clear supernatant was used immediately for the measurement of enzyme activities and protein content.

Nitrate reductase assay

The assay was performed as described by Hageman (1979) and the nitrite formed was estimated by Snell & Snell method (1949). The assay mixture consisted of 50 mM potassium phosphate buffer (pH 7.5), 5 mM EDTA, 5 mM KNO_3 and an appropriate amount of crude extract in a total volume of 0.4 ml. The blanks contained all the assay components except NADH. The reactions were set up in triplicates and carried out at RT (25° C) and stopped after 20 minutes by adding 0.6 ml of 1:1 (v/v) mixture of sulphanilamide (1 % w/v in 3 N HCl) and NED (0.1 % w/v). The reaction was incubated for a further 15 min at RT and the pink colour developed was measured at 540 nm. The amount of nitrite formed was calculated from a standard curve plotted using the A_{540} values obtained from known amounts of nitrite. NR activity was defined as nmoles of nitrite produced per ml extract per hour and the specific activity as enzyme activity/mg protein. Each such experiment was repeated thrice and the mean data

was plotted as relative specific activity (%) along with standard errors.

Nitrite reductase assay

Nitrite reductase (NiR) activity was assayed as described by Wray and Fido (1990) by using dithionite-reduced methyl viologen as an artificial electron donor. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.5), 2.5 mM KNO_2, 3 mM methyl viologen, 20 mM sodium dithionite (prepared freshly in 290 mM sodium bicarbonate) and 10 µl of enzyme extract in a final volume of 0.1 ml. The reaction was started by the addition of sodium dithionite. The blanks contained all the assay components except methyl viologen. The reactions were set up in triplicate and carried out at RT (25° C) and stopped after 10 minutes by the addition of 1.9 ml of reaction stopping and colour developing reagent which consisted of water (0.7 ml), sulphanilamide (1 % w/v in 3 N HCl; 0.6 ml) and NED (0.1 % w/v; 0.6 ml). The reaction was incubated for a further 15 min at RT and the pink colour developed was measured at 540 nm. The amount of nitrite consumed was calculated from a standard curve plotted using the A_{540} values obtained from known amounts of nitrite. NiR activity was defined as nmoles of nitrite consumed/ml extract/hour and the specific activity as enzyme activity/mg protein. Each such experiment was repeated thrice and the mean data was plotted as relative specific activity (%) along with standard errors.

Protein estimation

Protein content was estimated according to the Bradford's method using BSA as standard (Bradford, 1976).

RNA isolation

RNA was isolated according to the method of Chomczynski and Sachhi (1987) using commercial TRIZOL from GIBCO. Excised leaves were treated with or without KNO_3 (40 mM) upto two hours and RNA isolated according to the manufacturer's instruction. The quantity of RNA was measured by absorbance at 260 nm and integrity checked by agarose gel electrophoresis.

RT-PCR and gel analysis

RT-PCR was performed in a Techne Progene (UK) thermal cycler fitted with a heated lid. Gene specific primers were used for NR, NiR and Tubulin. The sense and antisense primer sequences of NR were AGGGGATGATGAACAACTGC and GAGTTGTCGGAGCTGTACCC. NiR sense and antisense primer sequences were TGCGAAGTACATTGCCGTAG and CTGCTCTTCAAGGAACGTCC respectively.

Tubulin sense and antisense primer sequences were TGAGGTTTGATGGTGCTCTG and GTAGTTGATGCCGCACTTGA. The target gene transcript was amplified using one-step RT-PCR kit supplied by QIAGEN (Germany), according to the supplier's instructions. Cycling conditions were optimised to give a linear relationship between the template used and product formed. Reverse transcription and amplification of the genes were done simultaneously as follows: 1. RT step (50 °C, 30 min, 1 cycle) 2. PCR Activation step (95 °C, 15 min, 1 cycle) 3. Three-step PCR cycling for 30 cycles involving (a) denaturation at 94 °C, 30 sec (b) annealing at 58 °C, 30 sec (c) extension at 72 °C, 60 sec and 4. Final Extension (72 °C, 10 min, 1 cycle).

PCR products were run on 2 % agarose gel and visualised under UV light after staining with ethidium bromide.

Image analysis of RT-PCR products

The RT-PCR gels were photographed with a Canon G2 Digital Camera (Japan) using a yellow filter and the intensity of the bands was quantified using the image analysis software of Scion Corp, USA (www.scioncorp.com). A 100-bp ladder was used to identify the band size of the products. The numerical values obtained for different treatments were plotted in the form of histogram.

RESULTS

Effect of nitrate and light

Light and nitrate are known to modulate the expression of the components of nitrate assimilation at both transcriptional and posttranscriptional levels, although the exact mechanism is not known. Results presented

Table I : Effect of Nitrate on NR (A) and NiR (B) Activity in Light and Dark (Concentration of Nitrate – 40 mM).

	Treatment	Relative Specific Activity (%)
(A)	Water (Light)	5.93 ± 0.92
	Water (Dark)	5.23 ± 3.65
	Nitrate (Light)	100
	Nitrate (Dark)	15.51 ± 2.07
(B)	Water (Light)	33.16 ± 3.16
	Water (Dark)	36.15 ± 3.04
	Nitrate (Light)	100
	Nitrate (Dark)	73.19 ± 2.45

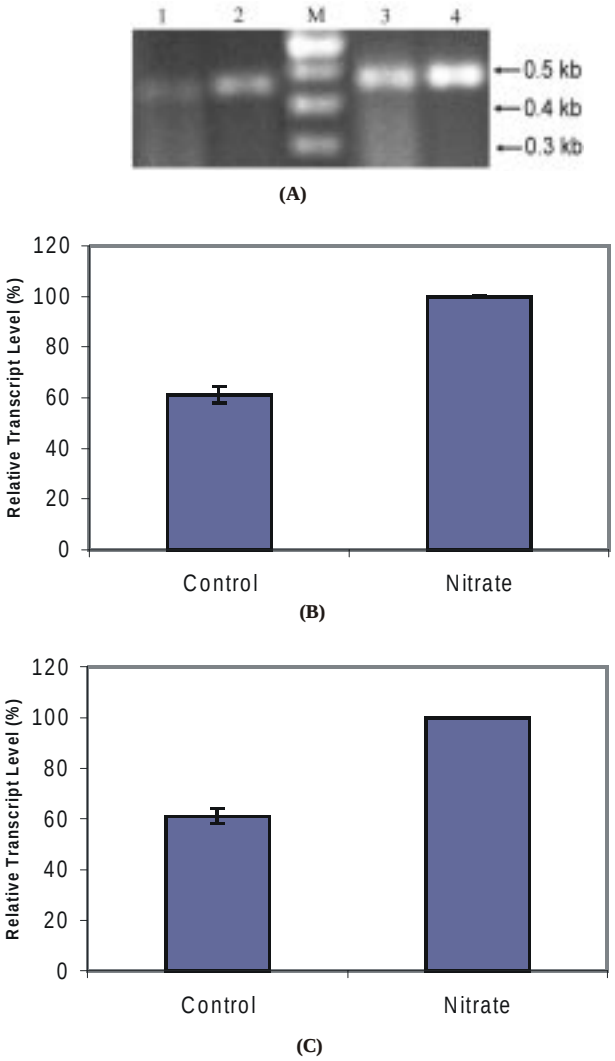

Figure 1 : Effect of Nitrate on NR mRNA in Light and Dark. (A) Agarose Gel Electrophoresis of RT-PCR products from total RNA isolated using TRIZOL. The gels were stained with Ethidium Bromide and photographed using Canon Camera. Lane 1, 2 & Fig. B – Light, Lane 3, 4 & Fig. C – Dark, Lane 1. Control, Lane 2. KNO_3 (40 mM), Lane M. Marker (B) & (C) Image Analysis using Scion Image. Transcript level in the presence of 40 mM KNO_3 was considered as 100 % control.

here show that NR activity increased almost 20-fold in the presence of nitrate and light while NiR activity increased 3-fold only under similar conditions (Table IA). In the absence of light there was a drastic decrease in NR activity. On the other hand, NiR activity did not show a significant decrease (Table IB). When effects of nitrate and light were checked on NR and NiR mRNA levels, a similar kind of pattern was observed for both the transcripts (Fig. 1 & 2). Light did not change mRNA levels like nitrate, which increased the transcript levels by 45 %.

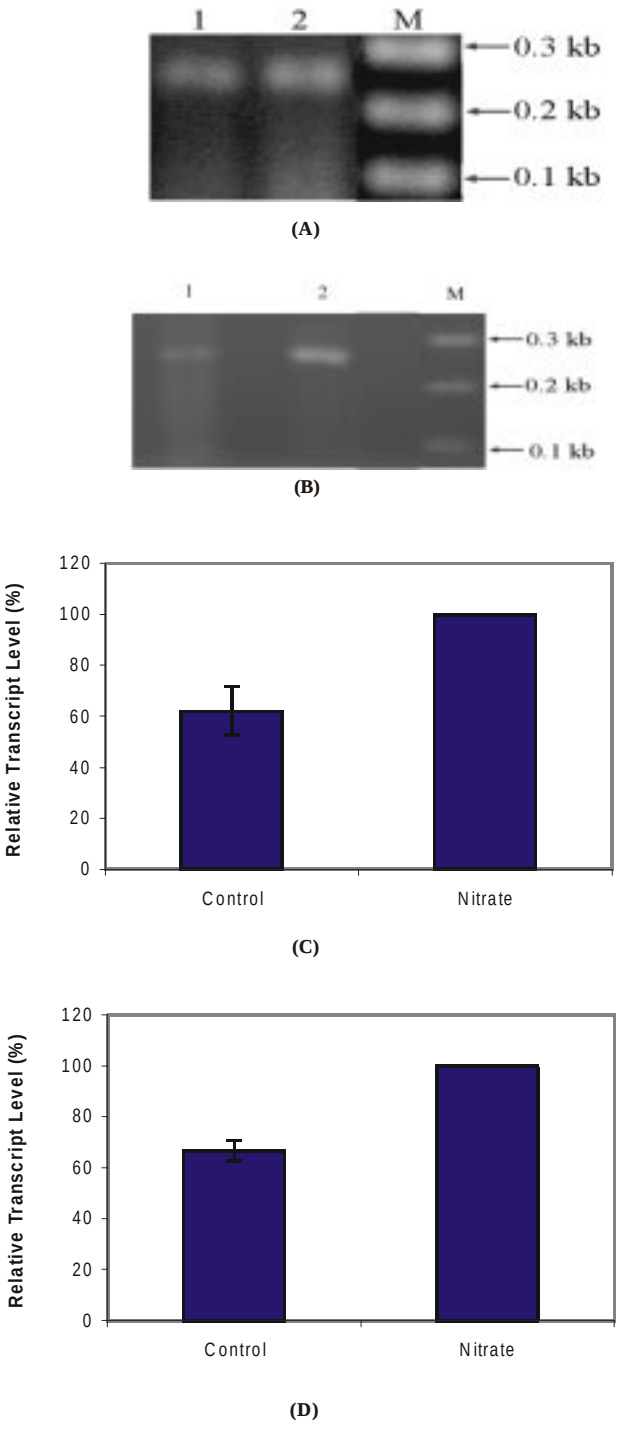

Figure 2 : Effect of Nitrate on NiR mRNA in Light (A & C) and Dark (B & D). (A) & (B) Agarose Gel Electrophoresis of RT-PCR products from total RNA isolated using TRIZOL. The gels were stained with Ethidium Bromide and photographed using Canon Camera. Lane 1. Control, Lane 2. KNO_3 (40 mM), Lane M. Marker (C) & (D) Image Analysis using Scion Image. Transcript level in the presence of 40 mM KNO_3 was considered as 100 % control.

Effect of nitrite

To check the effect of nitrite on the enzymes of nitrate assimilation excised leaves were floated on different concentrations of potassium nitrite (0.1 to 1 mM) and combinations of nitrite and nitrate (40 mM KNO_3). The data presented in Table II shows that nitrite alone had no effect on NR activity but it was inhibitory to nitrate-induced NR activity, and the level of inhibition increased with increasing concentration of nitrite, from 24 % in 0.1 mM KNO_2 to 54 % in 1 mM KNO_2. In the dark, when nitrite (1.0 mM) was provided alongwith nitrate to the excised leaves, it was found that inhibition was more significant (75 %) (Fig. 4A).

Similar experiments were carried out to check the effect of nitrite on the activity of nitrite reductase. In light, nitrite partially stimulated nitrate-induced NiR activity (Fig. 3B) whereas in dark it clearly inhibited this activity (Fig. 4B).

Effect of ammonium

We tested whether ammonium plays a significant role in the regulation of nitrate assimilation in rice. Leaves were floated on different concentrations of ammonium chloride (10-40 mM), and combinations of ammonium chloride and potassium nitrate (40 mM). The data presented in Table II shows that NH_4 did not have any effect on NR activity in the absence of nitrate, but it was inhibitory to nitrate-induced NR activity, and the level of inhibition increased with increasing concentration of ammonium, from 9 % to 62 % (10 mM to 40 mM NH_4Cl). In dark, ammonium stimulated nitrate-induced NR by almost 50 % (Fig. 4A) showing a pattern opposite to that in light. Fig. 4B shows that while ammonium had a partial inhibitory effect on nitrate-induced NiR in light, there was no effect of ammonium on NiR activity in the dark.

Effect of glutamine

Glutamine has been considered as a suitable candidate for feedback inhibition of N assimilation. Excised leaves were floated on different concentrations of glutamine (10-40 mM) and combinations of glutamine and potassium nitrate (40 mM). The data presented in Fig. 3A shows that glutamine alone did not have any effect on NR activity in the absence of nitrate but partially inhibited (~ 20 %) nitrate-induced NR activity. This inhibition was observed to increase to almost 40 % in the dark (Fig. 4A). On the other hand, there was no effect of glutamine on NiR activity in both light and dark (Fig. 3B and 4B).

Effect of glutamate

Excised leaves were floated on 20 mM glutamate and combinations of glutamate and potassium nitrate (40 mM) with controls using distilled water and 40 mM KNO_3. The data presented in Fig. 3A show that nitrate-induced NR activity increased to 180 % in light and 250 % in the dark (Fig. 4A). There was no effect of glutamate on NiR activity in light (Fig. 3B) while in the dark it increased to 160 % (Fig. 4B).

Table II : Effect of Range of Concentration of Nitrite, Ammonium and Glutamine on NR activity in Light.

Treatments	NR Relative Specific Activity (%)	Treatments	NR Relative Specific Activity (%)
W	5.93 ± 0.74	10 mM AC	5.17 ± 0.85
PN	100	20 mM AC	6.87 ± 1.41
0.1 mM NI	7.48 ± 1.23	40 mM AC	6.28 ± 1.22
0.2 mM NI	6.66 ± 1.11	PN + 10 mM AC	90.94 ± 5.54
0.5 mM NI	6.76 ± 2.01	PN + 20 mM AC	78.05 ± 4.32
1.0 mM NI	5.96 ± 1.23	PN + 40 mM AC	37.77 ± 1.97
PN + 0.1 mM NI	76.16 ± 2.23	10 mM G	3.37 ± 0.65
PN + 0.2 mM NI	73.85 ± 3.94	20 mM G	4.23 ± 0.82
PN + 0.5 mM NI	64.30 ± 4.32	40 mM G	7.88 ± 1.24
PN + 1.0 mM NI	45.57 ± 0.46	PN + 10 mM G	86.63 ± 4.56
		PN + 20 mM G	91.06 ± 5.65
		PN + 40 mM G	73.75 ± 5.43

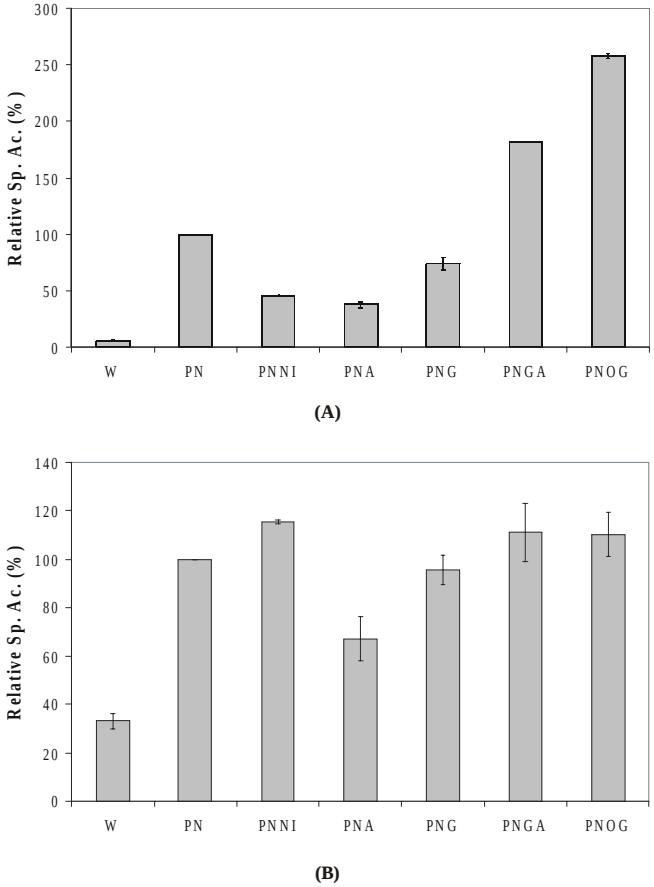

Figure 3 : Effect of Downstream Metabolites on NR (A) and NiR (B) Activity in Light. Excised leaves from 11-day old rice seedlings were floated on downstream metabolites of nitrogen metabolism in combination with 40 mM KNO_3 (PN) in the light. Activity in the presence of 40 mM KNO_3 was considered as 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), PNNI – Potassium Nitrite (1 mM), PNA – Ammonium Chloride (40 mM), PNG – Glutamine (40 mM), PNGA – Glutamate (40 mM), PNOG – 2-Oxoglutarate (10 mM)

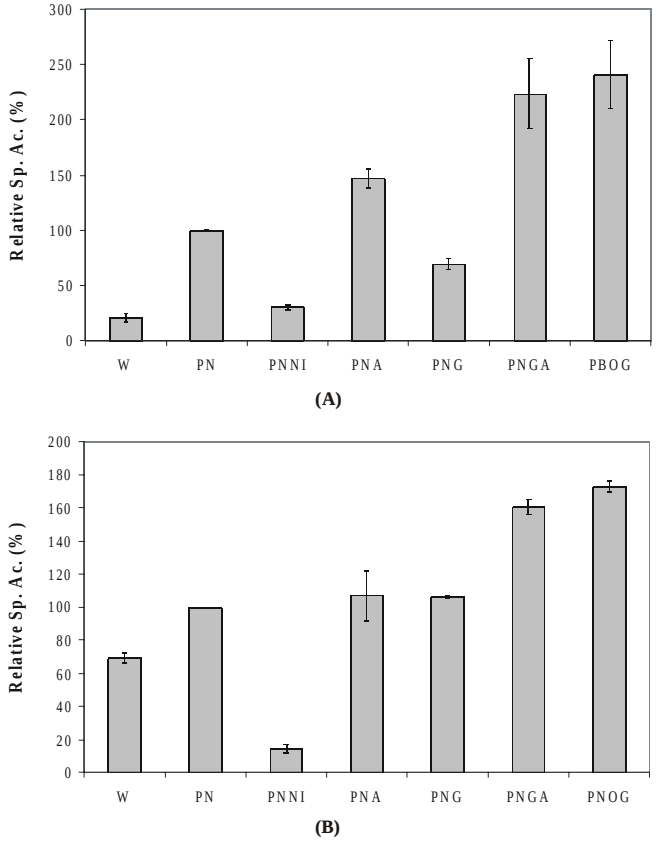

Figure 4 : Effect of Downstream Metabolites on NR (A) and NiR (B) Activity in Dark. Excised leaves from 11-day old rice seedlings were floated on downstream metabolites of nitrogen metabolism in combination with 40 mM KNO_3 (PN) in the dark. Activity in the presence of 40 mM KNO_3 was considered as 100 % 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), PNNI – Potassium Nitrite (1 mM), PNA – Ammonium Chloride (40 mM), PNG – Glutamine (40 mM), PNGA – Glutamate (40 mM), PNOG – 2-Oxoglutarate (10 mM)

Effect of 2-oxoglutarate

2-oxoglutarate is the form in which carbon skeleton is provided by the TCA cycle to accept reduced nitrogen (ammonium). Although it is an important metabolite occupying central position in carbon and nitrogen metabolism there have not been many reports in the literature regarding its role in the regulation of nitrate assimilation. Excised leaves were floated on 2-oxoglutarate (10 mM) and combinations of 2-oxoglutarate and potassium nitrate (40 mM). The data presented in Fig. 3A and 4A show that 2-oxoglutarate stimulated NR activity by 250 % in both light and dark only in the presence of nitrate. No change was observed in NiR activity in light (Fig. 3B) but it increased to 180 % in the dark (Fig. 4B).

DISCUSSION

In this study the roles of nitrogen metabolites in the regulation of nitrate assimilation have been characterised in rice. As there are very few reports in the literature regarding induction and regulation of nitrate assimilation pathway in rice, this study started with basic characterisation like nitrate concentration and time required for maximum induction of activities of NR and NiR. It was found that an incubation with 40 mM nitrate for 4-6 hrs gave maximum activities in leaves. NR activity was optimum when nitrate was supplied to leaves in light and it started decreasing

after 6 hrs of dark, reaching basal level in 24 hrs (data not shown). Several mechanisms may contribute to this decline. These include a dramatic decrease of the transcript level that commences soon after illumination (Scheible *et al*., 1997, 2000; Matt *et al*., 2001) and post-translational inactivation of NR in the dark (Kaiser and Huber, 1994; Scheible *et al*., 1997; MacKintosh, 1998). Changes of the cytosolic NADH concentration might also affect *in vivo* NR activity (Kaiser *et al*., 2000). When 2 days dark adapted leaves were resupplied with nitrate in light, NR activity again reached its optimum value in 6 hrs. A similar pattern was observed for NiR activity with 40 mM nitrate and 6 hrs being optimum. However it did not decrease drastically even when light was withdrawn for 2 days. On the other hand, NR and NiR mRNA levels were very much similar in the presence of nitrate and light.

All these results show that the expression of nitrate reductase and nitrite reductase are under co-regulation of nitrate and light albeit different amplitudes. Although the relative importance of light and nitrate varies with different plant species and tissues as well as experimental conditions, earlier studies have also suggested a similar mechanism for the regulation of nitrate assimilation in maize, cultured spinach cells, *N. plumbaginifolia* (Faure *et al*., 1991; Ogawa *et al*., 2000; Sivasankar and Oaks, 1996). Faure *et al*. (2001) have shown that NR and NiR mRNA and activity decreases after 2 days of darkness and are reinduced to their maximum after 4 to 6 hrs in light.

Nitrite is a particularly interesting and strong candidate for feedback regulation because it does not accumulate in plant cells due to its toxicity and the rate-limiting nature of NR (Oaks, 1994). Coordinated regulation of NR and NiR also ensures that nitrite is not accumulated in the cell (Ogawa *et al*., 2000). Nitrite has been shown to inhibit NR activity and mRNA levels in maize seedlings without affecting uptake or nitrate availability (Ivashikina and Sokolov, 1997; Raghuram and Sopory, 1999). In this study, nitrate-induced NR activity was significantly inhibited in the presence of nitrite in light and this inhibition increased in the dark. Although nitrite had no effect on NiR activity in light, a complete inhibition of NiR was observed in the dark. In fact NiR activity reached below the basal level. The severe loss of enzyme activities by nitrite in the dark reflects the tight control of expression of these enzymes in rice. This can be explained by the fact that the reduction of nitrite to ammonium requires reduced ferredoxin (a product of photosynthesis). An abrupt decrease in photosynthesis (absence of light) would, therefore, limit further assimilation, and could lead to the accumulation of nitrite unless NR activity was down regulated (Lillo *et al*., 2004). This also serves to save the plant from nitrite and ammonium toxicity.

Although the role of ammonium in the regulation of nitrate assimilation has been studied in many plants, the exact mechanism by which it modulates this pathway and its components are still far from clear. Differential effects of ammonium on NR have been shown to vary from plant to plant and growth conditions (green/etiolated plants). Solomonson and Barber (1990) have shown that when it was given alone to plants, there was an undetectable level of NR mRNA, protein or its activity. However, along with nitrate the effect of ammonium varied between no effect in *Arabidopsis* (Crawford *et al*., 1988) and maize (Oaks *et al*., 1988), to inhibitory effect in wheat (Botella *et al*., 1993) and stimulatory effect in etiolated wheat (Datta *et al*., 1981) and maize (Raghuram and Sopory, 1999). The results presented in this study show both NR and NiR activities were significantly inhibited in ammonium treated leaves in the light showing a similar pattern of regulation for both. In the dark, however, ammonium had differential effects on NR and NiR activities. While NR activity was induced significantly, there was no effect on NiR activity. These results suggest that nitrite and ammonium play critical roles in regulation of these activities.

Glutamine, a product of nitrate assimilation and an intermediate for the synthesis of other amino acids and nitrogen-containing compounds, has been shown to have diverse effects on the activity of NR. The effects varied from species to species and from tissue to tissue (Shankar and Srivastava, 1998). The results in the present study show that glutamine in light showed a partial inhibition of NR in leaves. Several other workers have also reported a similar kind of inhibition in tobacco and maize leaves (Sivasankar *et al*., 1997; Vincentz *et al*., 1993). However, glutamine was found to severely inhibit NR activity in roots (Deng *et al*., 1991; Li *et al*., 1995). Sivasankar *et al*. (1997) proposed three different possibilities which could contribute to the differential effects of glutamine on the induction of NR activity in shoot and root: (a) differences in the sensitivity of the isozymes to glutamine, (b) differential compartmentation of exogenously fed glutamine and (c) differences in the turnover rates of the enzymes. Shankar and Srivastava (1998) have also shown that NADPH:NR, a root specific isoform, is more sensitive to glutamine than the NADH:NR, present in shoot.

Under similar conditions, no effect of glutamine was observed on NiR activity in the leaves treated with glutamine in light. This result is in agreement with the report of Sivasankar *et al*. (1997) where it was shown that NiR activity is not inhibited by glutamine in maize shoot. However the same group of workers has reported that NiR mRNA in maize seedlings is regulated similar to NR mRNA. It was suggested by these workers that the differences in regulation at mRNA and enzyme activity levels by glutamine are

probably due to the slower turnover rate of the NiR protein (Li *et al.,* 1995; Sivasankar *et al.,* 1997).

Glutamine caused a significant decrease in NR activity in the dark. However, no significant change was observed in the NiR activity under same conditions. The results presented here show that glutamine plays an important role in the regulation of nitrate reductase in leaves in the dark. It has been shown earlier that glutamine downregulates NR by promoting post-translational inactivation (Klein *et al.,* 2000; Scheible *et al.,* 1997). It is now an established fact that NR is phosphorylated in the dark and 14-3-3 binds to the phosphorylated form leading to inactivation and degradation of the enzyme (Mackintosh, 1998).

When glutamate was provided to tobacco leaves, a decrease in both NR mRNA and activity was observed. However, in our studies on rice, study glutamate was found to have stimulatory effects on NR activity both in the presence and absence of light. Glutamate had no effect on NiR activity in light but a significant increase in NiR was observed in the dark. The differences in results between this study and tobacco may be explained by differences in route of supply of glutamate (through petiole in tobacco) as well as treatment time (start of the light in case of tobacco).

2-oxoglutarate is the acceptor of the amino group donated by glutamine and glutamate. Although it is supposed to play a crucial role in the regulation of nitrate assimilation, there have not been many studies in higher plants, especially rice. In fungi and bacteria 2-oxoglutarate has been suggested to act antagonistically to glutamine (Stitt and Krapp, 1999). Müller and co-workers (2001) have shown an increase in NR activity when tobacco leaves were supplied with 2-oxoglutarate. In our study also an increase was observed in NR activity in both light and dark. However, an increase in NiR activity was observed only in the dark.

All these results indicate towards a co-regulation of NR and NiR by light and nitrate. However, NR activity is more responsive to circadian rhythm. For instance, the activity of nitrate reductase decreases to a great extent when light is removed and is not recovered by supplying nitrate. On the other hand, NiR activity responds slowly to light removal. NR is also under a tighter regulation than NiR by most of the metabolites. It can be concluded from all the results presented here that not all the downstream metabolites are feedback inhibitors of nitrate assimilation and whenever a carbon skeleton, 2-oxoglutarate, is available nitrate assimilation proceeds towards the formation of amino acids. However, it is difficult to distinguish between transcriptional or posttranscriptional regulation at this stage.

It is interesting to note that none of these metabolites had any effect on the activities of NR and NiR on their own, i.e., in the absence of nitrate. Effect of these metabolites was also measured on nitrate uptake in leaves. It was observed that none of these agents had any effect on nitrate uptake process (data not shown). From these results it can be concluded that the observed effects of these metabolites were directly on nitrate-induced responses of NR & NiR.

To our knowledge this is first study in which the effect of all the downstream metabolites of nitrate assimilation has been examined simultaneously on both nitrate reductase and nitrite reductase, especially in rice. The further characterisation of this regulation and their interaction with C-metabolism at the whole plant level may help us in increasing the nitrogen use efficiency (NUE) of rice.

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