

# Regulation of activity and transcript levels of NR in rice (*Oryza sativa*): Roles of protein kinase and G-proteins

Ahmad Ali<sup>a</sup>, Subramanian Sivakami<sup>a,\*</sup>, Nandula Raghuram<sup>b</sup>

<sup>a</sup>Department of Life Sciences, University of Mumbai, Vidyanaigari, Santacruz (E), Mumbai 400098, India

<sup>b</sup>School of Biotechnology, G.G.S. Indraprastha University, Kashmere Gate, Delhi 110006, India

Received 21 June 2006; received in revised form 3 October 2006; accepted 5 October 2006

Available online 10 November 2006

## Abstract

The aim of the present study was to determine the effects of activators and inhibitors of signaling pathways on nitrate-induced NR activity and transcript levels in rice leaves. Rice plants (*Oryza sativa* indica var. Panvel I) were grown hydroponically (without nutrients) on germination paper under continuous white light for 10–12 days. Leaves, adapted for darkness for 2 days, were treated with signaling agents both in light and dark. The results presented in this work show that okadaic acid (protein phosphatases 1 and 2A inhibitor) and lithium (inhibitor of IP<sub>3</sub> pathway) specifically inhibit activity and mRNA levels of NR under light conditions with no effect on NiR activity. Both NR and NiR activities are inhibited and stimulated in the dark by PMA and Bisindolylmaleimide (BIM) (PKC activator and inhibitor), respectively. Cholera toxin specifically enhances NR activity and steady state levels of mRNA in the dark showing the involvement of G-proteins. Calcium has a stimulatory effect on both the enzymes, with an increase in NR mRNA levels albeit to a lesser extent. These results suggest that the activities of nitrate-assimilating enzymes in rice are regulated independently by G-protein and IP<sub>3</sub> mediated pathways and co-regulated by PKC and calcium.

© 2006 Elsevier Ireland Ltd. All rights reserved.

**Keywords:** Nitrate assimilation; Signaling agonists and antagonists; PKC and phosphatase; PMA; Cholera toxin; *Oryza sativa*

## 1. Introduction

Nitrate, a major source of nitrogen for higher plants, is taken up from the soil by energy dependent nitrate transporters present in roots where it may be reduced to ammonium, stored in the vacuole or transported to leaf. The major site of nitrate assimilation is leaf for most of the crop plants and root for woody plants. Once inside the cell, nitrate is converted to ammonium (reduced nitrogen) in two successive steps catalysed by nitrate reductase (NR) and nitrite reductase (NiR) in cytosol and chloroplast, respectively. The first step is assumed to be the rate-limiting step. The enzymes of nitrate

assimilation are regulated at both the transcriptional and post-transcriptional levels by various endogenous and exogenous factors like nitrate, CO<sub>2</sub>, light, hormones, temperature, carbon and nitrogen metabolites [1]. Nitrate and light play major roles in the regulation of nitrate assimilation pathway. Although the exact mechanism is not known, the involvements of several signaling intermediates have been shown to mediate this regulation. For instance, the involvement of a G-protein in the regulation of NR has been reported in maize leaves [2]. Calcium is an important second messenger, which mediates the responses of various external and internal signals [3]. Presence of calcium-dependent protein kinases and their roles in regulating NR have been identified from various plants. Similarly phosphoinositides have been shown to play a role in NR regulation [4]. In spite of all these developments there have been very few reports on the regulation of these enzymes in rice, which is an ammonium utilising crop plant. Even though NiR is an important enzyme of nitrate assimilation pathway, its regulation has received less attention.

Several approaches have been used to study the target enzymes proteins/transcription factors of different signaling intermediates. In this study, a set of commercially available

**Abbreviations:** BIM, Bisindolylmaleimide; CDPK, calmodulin-domain (calcium dependent) protein kinase; DAG, diacylglycerol; IMPase, inositol monophosphatase; NED, naphthyl ethylene diamine dihydrochloride; NR, nitrate reductase; NiR, nitrite reductase; NUE, nitrogen use efficiency; PMA, phorbol myristate acetate; PKC, protein kinase C; PP 1 and 2A, protein phosphatase 1 and 2A; RT-PCR, reverse transcription-polymerase chain reaction

\* Corresponding author. Tel.: +91 22 26528847.

E-mail address: [sivakami\\_s2000@yahoo.com](mailto:sivakami_s2000@yahoo.com) (S. Sivakami).

signaling agonists and antagonists, highly specific to their targets, has been employed to determine their effects on NR and NiR activities. As light/dark transition causes changes in the expression of NR and NiR as well as protein kinases/phosphatases, the effect of these signaling agents were examined both under light and dark conditions. In the case of NR, wherever significant alterations in enzyme levels were observed, the steady state mRNA levels were measured by RT-PCR in order to check for a possible correlation between activity and transcript levels. Phorbol myristate acetate (PMA), a phorbol ester, is an artificial analogue of diacylglycerol (DAG) and a potent activator of PKC [5]. Bisindolylmaleimide (BIM), on the other hand, specifically inhibits PKC. In this study, both these protein kinase C modulators have been used to study the involvement of PKC in the regulation of nitrate assimilation. Okadaic acid, an inhibitor of protein phosphatase 1 and 2A, was used to investigate the involvement of protein phosphatase. Cholera toxin and lithium were also used to check the roles of G-proteins and phosphoinositides in the regulation of enzyme activities and NR mRNA.

It was found that NR activity is more responsive than NiR to the treatment of all these drugs. NiR activity followed a pattern similar to NR with agents like  $\text{Ca}^{2+}$  and PKC but in others it did not show any change. Although alterations in NR transcript levels were observed with the various treatments, these changes were not as pronounced as those of activity. It can be concluded from this study that NR regulation involves major signaling pathways and is at transcriptional as well as post-transcriptional levels in a coordinated manner. To the best of our knowledge this is the first report where a range of specific signaling agents have been used to study the regulation of nitrate-assimilating enzymes in rice.

## 2. Methods

### 2.1. 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 with tap and single distilled water (sd/w), soaked for 10 min in 5% (v/v) sodium hypochlorite (NaOCl) and washed several times with tap water. The seeds were again washed and soaked in sd/w and kept in dark for 2 days. Imbibed seeds were plated on wet germination paper in a plastic tray and incubated at  $25 \pm 2$  °C under white light derived from 4 Osram 36 W fluorescent lamps. The light intensity at the plant level was 7 Klux. The seedlings were watered daily with sd/w for 10–12 days.

### 2.2. Treatments

To study the involvement of the signaling pathways in the regulation of enzymes, excised leaves from 10–12 days old seedlings were treated with signaling agonists and antagonists like PMA (20 ng/ml), Bisindolylmaleimide I (10 nM), okadaic acid (0.5 nM), cholera toxin (1.0  $\mu\text{g}/\text{ml}$ ), calcium (5 mM), lithium (2 mM), with and without nitrate (40 mM). After 4 h

leaves were washed, blotted on tissue paper, wrapped in foil, frozen in liquid  $\text{N}_2$  and used immediately or stored at  $-70$  °C to determine enzyme activities, nitrate content and protein estimation. The concentrations of the signaling agents were chosen on the basis of preliminary experiments where a range of concentrations was tested.

### 2.3. 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 h intervals after transferring the leaves to dark. At the end of 24 h, no NR activity could be detected. Similar conditions were used for studies on NiR.

### 2.4. 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), Polyvinylpyrrolidone (10%, w/v), Triton X-100 (0.1% v/v), EDTA (1 mM), DTT (1 mM), PMSF (1 mM), Benzamidine (prepared fresh) (1 mM) and 6-Aminocaproic acid (1 mM). The leaf tissue (0.25 g) was ground into a fine powder using a mortar and pestle using liquid  $\text{N}_2$ . 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  $\mu\text{m}$ ) and centrifuged at 14,000 rpm for 15 min. The clear supernatant was used immediately for the measurement of enzyme activities and protein estimation.

### 2.5. Nitrate reductase assay

The assay was performed as described by Hageman [6] and the nitrite formed was estimated by Snell and Snell method [7]. The assay mixture consisted of 50 mM potassium phosphate buffer (pH 7.5), 5 mM EDTA, 5 mM  $\text{KNO}_3$  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 min 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 nmol of nitrite produced per ml extract per hour and the specific activity as enzyme activity per mg protein. Each such experiment was repeated thrice and the mean data was plotted as relative specific activity (%) along with standard errors.

## 2.6. Nitrite reductase assay

Nitrite reductase activity was assayed as described by Wray and Fido [8] 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<sub>2</sub>, 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 min by adding 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<sub>540</sub> values obtained from known amounts of nitrite. NiR activity was defined as nmol of nitrite consumed/ml extract/h 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.

## 2.7. Protein estimation

Protein content was estimated according to Bradford's method using BSA as standard [9].

## 2.8. RNA isolation

RNA was isolated according to the method of Chomczynski and Sacchi [10] using commercial TRIZOL from GIBCO. Excised leaves were treated with different metabolites and signaling agents either alone and/or with KNO<sub>3</sub> up to 2 h. Treated excised leaves were ground thoroughly to a powder using a mortar and pestle in the presence of liquid nitrogen. One millilitre TRIZOL was added immediately, homogenised thoroughly and left for thawing. The tissue to buffer ratio was 1:10 (w/v). The homogenate was left for 5 min at room temperature and centrifuged at 8500 rpm for 15 min at 4 °C. Chloroform (0.2 ml) was added in the supernatant and the tube was shaken vigorously for 15 s, incubated for 3–5 min at RT and centrifuged at 8500 rpm for 15 min at 4 °C. The upper aqueous phase containing RNA was transferred to another tube. A 0.5 ml isopropyl alcohol was added, left overnight at –20 °C and again centrifuged at 8500 rpm for 15 min at 4 °C. The pellet obtained was washed twice with 75% ethanol, dried and resuspended in 50 µl RNase-free (DEPC-treated) distilled water, aliquoted in several tubes and stored at –20 °C.

The quantity of RNA was measured by taking absorbance at 260 nm and purity checked by measuring the absorbance at 230, 260, 280 and 310 nm. Only that RNA sample was used as template for the RT-PCR which gave a 260/280 ratio of ~2. The integrity of RNA was checked, before performing RT-PCR, by agarose gel electrophoresis (0.8%). The gel was

stained with ethidium bromide and visualised on an UV transilluminator.

## 2.9. RT-PCR and gel analysis

RT-PCR was performed in a Techne Progene (UK) thermal cycler fitted with a heated lid. Gene specific primers for NR and tubulin were designed in-house and were also evaluated for various other parameters like melting temperature, presence of secondary structure like hairpin and dimer formation using software available on the internet ([www.premierbiosoft.com](http://www.premierbiosoft.com)). The sense and antisense primer sequences of NR: AGGGGATGATGAACAACACTGC and GAGTTGTCCGAGCTGTACCC. Tubulin sense and antisense primer sequences are TGAGGTTT-GATGGTGCTCTG and GTAGTTGATGCCGCACTTGA. The target gene transcript was amplified using one-step RT-PCR kit supplied by QIAGEN (Germany), according to the supplier's instructions. One microgram template and 0.6 µM each of both the primers (forward and reverse) were added into a 50 µl reaction mixture containing 5× RT-PCR buffer, 5× Q solution, dNTPs and enzyme mix. RNase inhibitor (4 units/reaction) was also added into the reaction mixture. Tubulin was used as a housekeeping control. 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 –94 °C, 30 s, (b) annealing –58 °C, 30 s, (c) extension –72 °C, 60 s 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.

## 2.10. Image analysis of RT-PCR products

The RT-PCR gels were photographed with a Canon G2 Digital Camera (Japan) using a yellow filter and gel images were downloaded on a computer. A 100-bp ladder was used to identify the band size of the products. The intensity of the bands was quantified using the image analysis software of Scion Corporation, USA ([www.scioncorp.com](http://www.scioncorp.com)). The numerical values obtained for different treatments were plotted in the form of Histogram.

## 3. Results

### 3.1. Effect of PMA and Bisindolylmaleimide

Phorbol myristate acetate [5,11] and Bisindolylmaleimide I [12] are specific activator and inhibitor of PKC, respectively, and have been used extensively to study the target enzymes or factors of PKC. In our study, these commercially available drugs were used to investigate the involvement of PKC-mediated pathways in the regulation of nitrate assimilation. When NR and NiR activities were measured from the leaves treated with PMA, it was found to have an inhibitory effect on both the enzymes in dark with no effect in light (Figs. 1 and 3

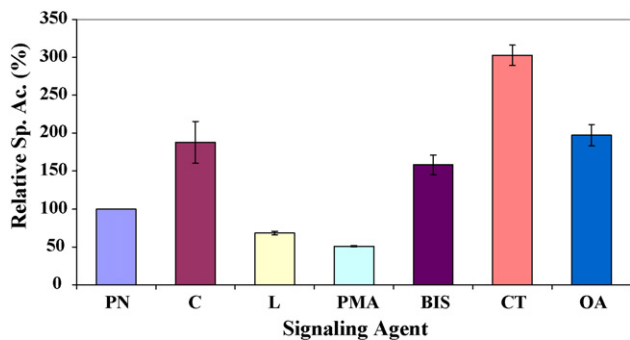


Fig. 1. Effect of signaling agents on nitrate reductase activity in the dark. Excised leaves from 11-days old rice seedlings (2-days dark adapted) were floated on signaling agents in combination with 40 mM KNO<sub>3</sub> in the dark for 4 h. Activity in the presence of 40 mM KNO<sub>3</sub> was considered as 100% control. The mean data from three different experiments are shown as relative specific activity (%) along with standard error bars. Specific Activity of NR after nitrate (PN) treatment: 8.07 ± 1.35 nmol nitrite produced/h/mg protein. PN, nitrate (40 mM); C, calcium (5 mM); L, lithium (2 mM); PMA, phorbol myristate acetate (20 ng/ml); BIS, Bisindolylmaleimide (10 nM); CT, cholera toxin (1 µg/ml); OA, okadaic acid (0.5 nM).

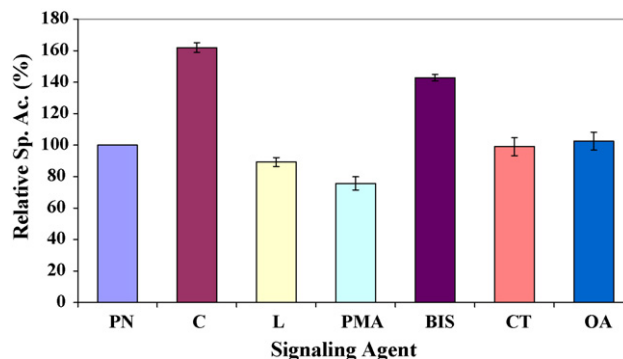


Fig. 3. Effect of signaling agents on nitrite reductase activity in the dark. Excised leaves from 11-day old rice (2-days dark adapted) seedlings were floated on signaling agents in combination with 40 mM KNO<sub>3</sub> in the dark for 4 h. Activity in the presence of 40 mM KNO<sub>3</sub> was considered as 100% control. The mean data from three different experiments are shown as relative specific activity (%) along with standard error bars. Specific Activity of NiR after nitrate (PN) treatment: 468.07 ± 15.35 nmol nitrite consumed/h/mg protein. PN, nitrate (40 mM); C, calcium (10 mM); L, lithium (2 mM); PMA, phorbol myristate acetate (20 ng/ml); BIS, Bisindolylmaleimide (10 nM); CT, cholera toxin (1 µg/ml); OA, okadaic acid (0.5 nM).

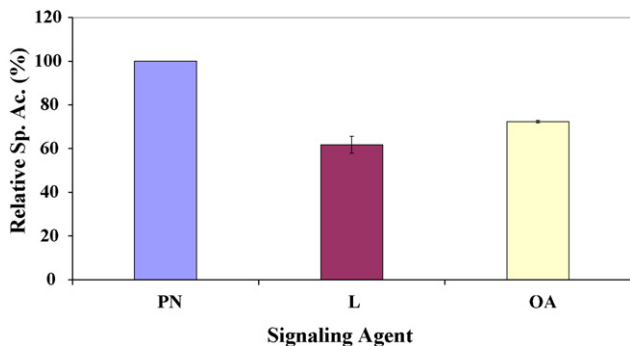


Fig. 2. Effect of lithium (L) and okadaic acid (OA) on NR activity in light. Excised leaves from 11-day old rice (2-days dark adapted) seedlings were floated on lithium (2 mM) and okadaic acid (0.5 nM) in combination with 40 mM KNO<sub>3</sub> in light for 4 h. Activity in the presence of 40 mM KNO<sub>3</sub> was considered as 100% control. The mean data from three different experiments are shown as relative specific activity (%) along with standard error bars. Specific Activity of NR after nitrate (PN) treatment: 52.04 ± 2.59 nmol nitrite produced/h/mg protein.

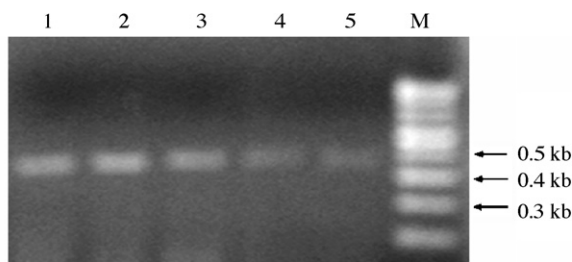
and Table 1). However, the effect was more significant on NR activity. On the other hand, Bisindolylmaleimide I stimulated the activity of these enzymes, almost an opposite effect of PMA (Figs. 1 and 3). In light, however, NR and NiR activities did not

Table 1  
Effect of signaling agents on NR and NiR activity in light

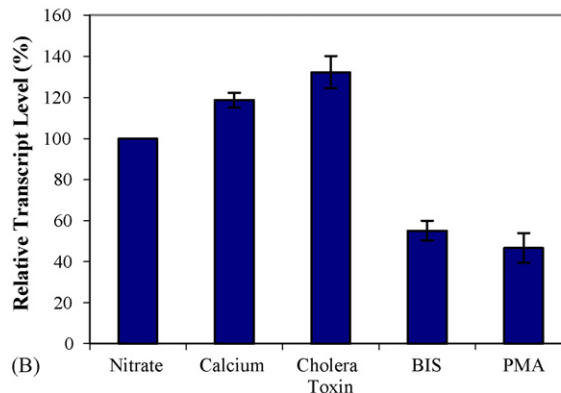
Signaling agent	NR	NiR
Nitrate (40 mM)	100	100
PMA (20 ng/ml)	83.56 ± 1.41	108.43 ± 1.32
Bisindolylmaleimide I (10 nM)	108.73 ± 4.15	116.98 ± 6.24
Okadaic acid (0.5 nM)	72.29 ± 0.56	103.16 ± 5.78
Lithium (2 mM)	61.75 ± 3.96	102.61 ± 13.09
Calcium (5 mM)	80.91 ± 14.85	95.85 ± 6.01
Cholera toxin (1 µg/ml)	96.83	117.98 ± 4.57

NR and NiR activities are expressed as relative specific activity (%) considering activity in the presence of nitrate as 100%.

change with Bisindolylmaleimide I. NR mRNA decreased to significant levels in the presence of both PMA (50%) and Bisindolylmaleimide I (56%) in the dark (Fig. 4). The effect of Bisindolylmaleimide I on NR mRNA is in contrast to its effect on activity.



(A)



(B)

Fig. 4. Effect of calcium, cholera toxin, Bisindolylmaleimide I and PMA on NR mRNA in the dark. (A) agarose gel electrophoresis of RT-PCR products from total RNA isolated using TRIZOL as mentioned in Section 2. The gels were stained with ethidium bromide and photographed using Canon Camera. Lane 1, KNO<sub>3</sub> (40 mM); Lane 2, calcium (5 mM); Lane 3, cholera toxin (1 µg/ml); Lane 4, Bisindolylmaleimide I (10 nM); Lane 5, PMA (20 ng/ml); Lane M, Marker. (B) Image analysis using Scion Image. Transcript level in the presence of 40 mM KNO<sub>3</sub> was considered as 100% control.



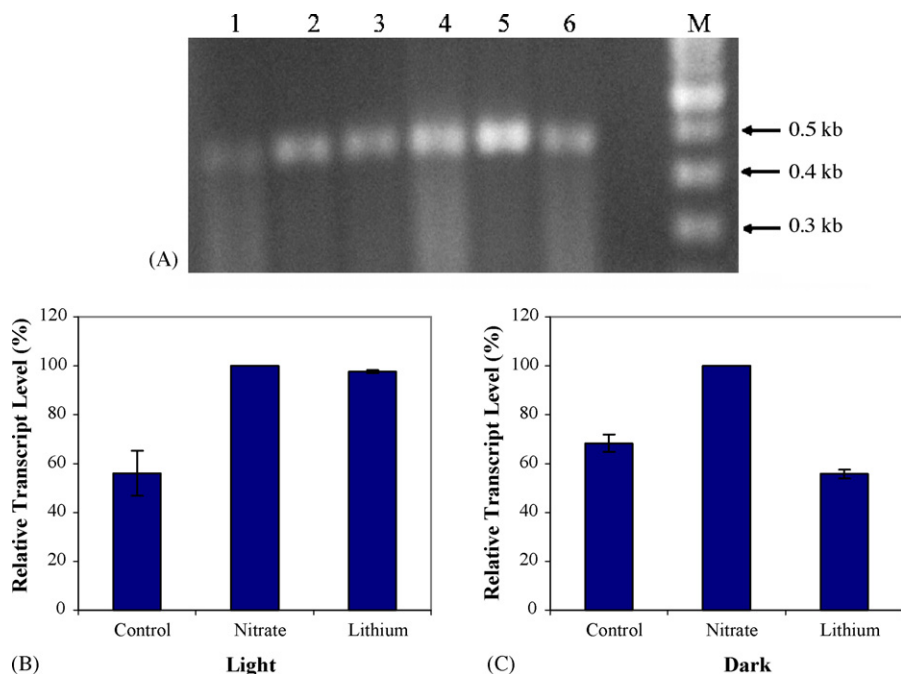


Fig. 5. Effect of lithium on NR mRNA in light and dark. (A) Agarose gel electrophoresis of RT-PCR products from total RNA isolated using TRIZOL as mentioned in Section 2. The gels were stained with ethidium bromide and photographed using Canon Camera. Lane 1–3: Light, Lanes 4–6: Dark. Lane 1, Control (Water); Lane 2,  $\text{KNO}_3$  (40 mM); Lane 3, LiCl (2 mM) +  $\text{KNO}_3$  (40 mM); Lane 4, Control (Water); Lane 5,  $\text{KNO}_3$  (40 mM); Lane 6, LiCl (2 mM) +  $\text{KNO}_3$  (40 mM); Lane M, Marker. (B and C) Image analysis using Scion Image. Transcript level in the presence of 40 mM  $\text{KNO}_3$  was considered as 100% control.

### 3.2. Effect of okadaic acid

Okadaic acid, an inhibitor of protein phosphatase 1 and 2A, was used to check the role of protein phosphatases in the regulation of nitrate assimilation. As can be seen from the Figs. 1 and 2, it caused an inhibition of NR activity in light conditions with a stimulation in the dark. On the other hand, okadaic acid caused no change in NiR activity either in light or dark (Table 1 and Fig. 3). NR mRNA also increased to a significant level (250%), similar to activity, in the dark (Fig. 6B). However, NR mRNA level did not change in presence of okadaic acid in light, in contrast to its effect on activity (Fig. 6A).

### 3.3. Effect of lithium

$\text{IP}_3$  and DAG are important second messengers of the cell and have been implicated in different cellular processes. They are generated as a result of breakdown of  $\text{PIP}_2$ . Inositol

monophosphatase (IMPase) is an important enzyme for the regeneration and formation of  $\text{PIP}_2$ . Lithium is an inhibitor of this enzyme and it has been shown earlier that plants IMPases are also sensitive to lithium inhibition like animal IMPases [13,14]. When lithium was given along with nitrate, NR activity decreased significantly in both light and the dark (Figs. 1 and 2). However, it did not have any effect on NiR activity either in the light or dark (Table 1 and Fig. 3). When NR transcript level was studied under similar conditions lithium showed a similar effect (44% inhibition) on the NR mRNA level in the dark (Fig. 5).

### 3.4. Effect of calcium

In the present study, calcium caused a significant stimulation of the activities of NR and NiR at 5 and 10 mM, respectively (Figs. 1 and 3). This effect was observed only in the dark with no effect in light (Table 1). However, calcium partially (120%) increased the NR transcript level that too in the dark specifically (Fig. 4). The requirement of different concentrations of calcium

Table 2  
Effect of signaling agents on tubulin mRNA in light (A) and dark (B)

Treatment	(A) Relative transcript level (%)	(B) Relative transcript level (%)
Water	100	100
Nitrate (40 mM)	103.93 ± 4.60	107.54 ± 5.65
PMA (20 ng/ml)	103.46 ± 7.14	107.83 ± 9.37
Bisindolylmaleimide (10 nM)	106.86 ± 4.65	102.65 ± 6.37
Okadaic acid (0.5 nM)	104.31 ± 9.43	105.14 ± 8.26
Cholera toxin (1 µg/ml)	106.67 ± 8.14	108.45 ± 6.38
Lithium (2 mM)	105.65 ± 5.75	109.43 ± 6.56
Calcium (5 mM)	104.33 ± 6.66	104.31 ± 7.76

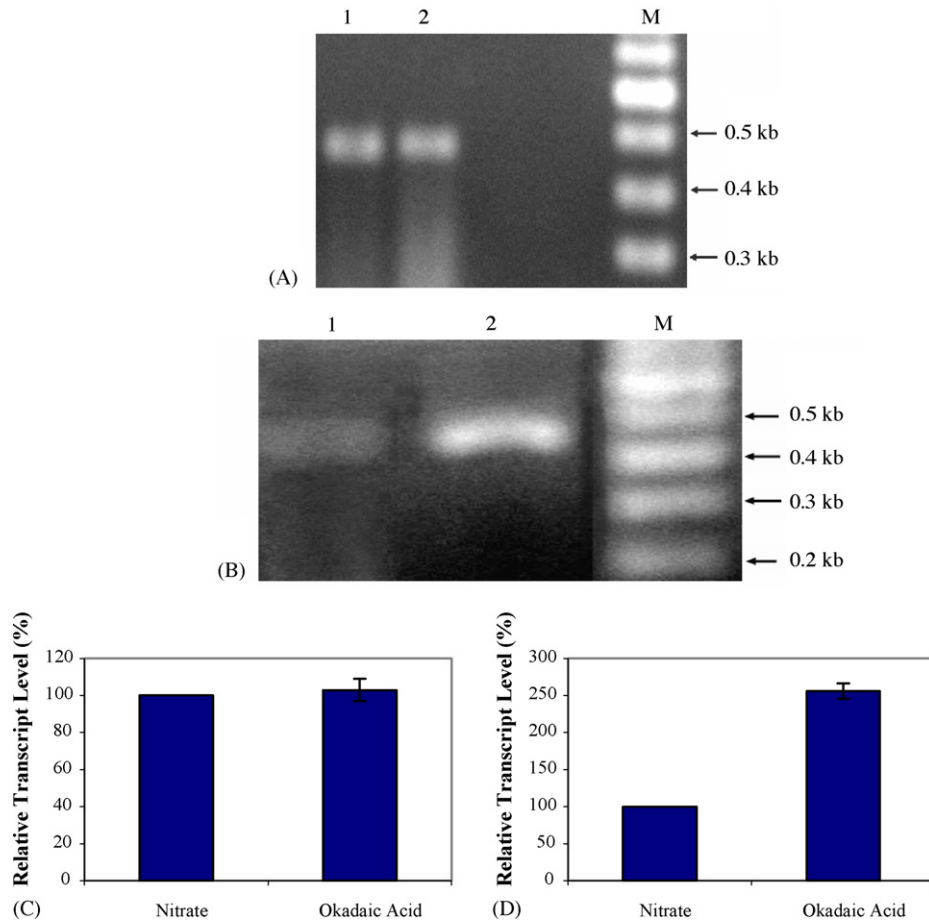


Fig. 6. Effect of okadaic acid on NR mRNA in light (A and C) and dark (B and D). (A and B) Agarose gel electrophoresis of RT-PCR products from total RNA isolated using TRIZOL as mentioned in Section 2. The gels were stained with ethidium bromide and photographed using Canon Camera. Lane 1,  $\text{KNO}_3$  (40 mM); Lane 2, okadaic acid (0.5 nM) +  $\text{KNO}_3$  (40 mM); Lane M, Marker. (C and D) Image analysis using Scion Image. Transcript level in the presence of 40 mM  $\text{KNO}_3$  was considered as 100% control.

for optimum NR and NiR activities may be due to fluctuations of its concentration caused by stimuli like light, hormones and environmental stresses [3].

#### 3.4.1. Effect of cholera toxin

Two bacterial toxins, cholera toxin and pertussis toxin, have been used to study the involvement of G-proteins in the metabolic pathways. Although the mechanism of action of these toxins is different, they have been shown to activate G-proteins. In the present study, cholera toxin, which activates G-protein by causing an ADP ribosylation of  $G\alpha$  subunit at its intrinsic GTPase site, was used. As can be seen from Fig. 1, cholera toxin brought about a three-fold stimulation of nitrate-induced NR activity in the dark with no effect on NiR activity in dark (Fig. 3). However, there were no significant changes in nitrate-induced NR and NiR activities in light (Table 1). When steady state level of NR mRNA of leaves treated with cholera toxin was measured, it showed an increase (~40%) (Fig. 4).

## 4. Discussion

Results presented in this study indicate to a complex and tight regulatory signaling network to control the reduction of

nitrate by NR and NiR. Significant effects of PMA and Bisindolylmaleimide I clearly suggest an involvement of a signaling pathway mediated by a protein kinase of PKC-type in the regulation of NR and NiR. PMA caused a marked decrease in activity of both the enzymes while Bisindolylmaleimide I had an effect opposite to that of PMA on NR and NiR (Fig. 1). The opposing effects of PMA and BIM are due to these drugs being agonist and antagonist of PKC, respectively. Inhibition of NR mRNA expression by PMA similar to its activity indicates the transcriptional regulation of NR by PKC-type protein kinase (Fig. 4). However, in experiments using etiolated seedlings of maize, an increase in transcript level was observed in the presence of PMA in the dark [15]. The causes for the differences in response to PMA may be due to differences in metabolic regulation between C3 and C4 plants, maize being a C4 plant. In addition, variations in growth conditions like the use of etiolated maize seedlings versus light grown dark adapted rice seedlings used in this study may have also contributed to the observed differences.

The use of PP1/PP2A inhibitors has led to identification of a role for reversible phosphorylation processes in plant systems. Okadaic acid, a PP1/PP2A inhibitor, caused a decrease in NR activity in light confirming the involvement of protein

phosphatases in the reactivation of NR in light [16]. This result is in accordance with earlier findings wherein it has been shown that NR dephosphorylation and consequent reactivation are blocked by protein phosphatase inhibitors like okadaic acid, calyculin and microcystin [17]. However, there was no change in NiR activity either in light or dark. An increase in NR activity and mRNA steady state level was also observed when leaves were treated with okadaic acid in the dark. The exact reason for this induction in NR needs to be further characterised. The results obtained using PKC modulators and protein phosphatase inhibitors show that rice NR is under the stringent control of protein phosphorylation and dephosphorylation. NiR was also found to be regulated by protein kinase C but not by protein phosphatases 1 and 2A. It is possible that other types of protein phosphatases are involved in the regulation of NiR.

Phosphoinositides as secondary messengers are also involved in many cellular processes [18]. Lithium, an inhibitor of inositol monophosphatases has been used by several workers to explore the involvement of IP<sub>3</sub> and DAG (products of PIP<sub>2</sub> breakdown) in metabolism of animals as well as plants [13,14]. In the present study, lithium decreased the activity of NR by 40% both in light and dark while the inhibitory effect of lithium on NR mRNA could be observed only in the dark. The precise mechanism of the differential effects of Li on NR mRNA levels in light and dark are not known, though phytochrome has been reported to influence the levels of phosphoinositides. The observed effect in this study may also be due to other factors like mRNA stability, post-translational regulation by light and nitrate availability. Raghuram and Sopory [15] have also reported a decrease in NR transcript level in etiolated maize leaves.

Calcium, a universal second messenger involved in mediating various signaling pathways was found to increase the activities of both NR and NiR in the dark. However, the induction in steady state level of NR mRNA in the dark was partial. Together, the effects of lithium and calcium on NR indicate its regulation by the IP<sub>3</sub> pathway. It can therefore be concluded that IP<sub>3</sub> mediated changes in calcium levels affect NR induction. The inhibition of NR activity and decrease in mRNA by lithium confirm this mechanism.

GTP analogues and bacterial toxins have been used in a number of studies to characterise the involvement of G-proteins in the light-stimulated signaling pathways in *Avena* seedlings, soybean suspension cultures and etiolated wheat protoplasts [2]. Both red/far red light and blue light photoreceptors, phytochrome and cryptochrome, have been shown to operate via G-proteins [19]. A significant increase in NR activity and mRNA levels in the dark brought about by cholera toxin implies an involvement of G-protein mediated signal transduction pathway. Although, the increase in NR activity was three-fold it could not mimic the effect of light in this system as reported for etiolated maize seedlings [2].

The induction of both activity as well as mRNA of NR by cholera toxin seems to suggest a role for phytochrome via G-proteins. The activation of G-proteins and changes in phosphoinositides levels are both known to be phytochrome mediated [20]. IP<sub>3</sub> and Ca<sup>2+</sup> may then be assumed to act

downstream of the phytochrome-activated-G-protein-mediated induction of NR mRNA and activity.

The role of phosphorylation and dephosphorylation as a mechanism of regulation of NR can be interpreted from the results obtained with PMA, BIM and okadaic acid. In our study, the inactivation of NR by PMA and activation by BIM suggests that a PKC-type protein kinase is involved in the phosphorylation-dependent inactivation of NR. Phorbol esters like PMA have also been shown to activate other type of protein kinases, known as PKDs, which are calcium-independent. In fact, only two subclasses of PKCs respond to calcium [5]. Further, inhibition of NR reactivation by okadaic acid in light suggests that protein phosphatases 1 and 2A are involved in the dephosphorylation mechanism. NR induction by calcium also indicates that this phosphorylation is not calcium-dependent in rice. Several studies have shown the involvement of CDPKs in the phosphorylation of NR [21].

It is interesting to note that none of these signaling agents had any effect on the activities of NR and NiR on their own, i.e., in the absence of nitrate as well as on nitrate uptake process in the leaf (data not shown). Therefore, it appears that the observed effects of the agents used in this study were directly on the activities and nitrate induction was also required. Tubulin was used as a control to study the steady state mRNA level of NR. Signaling agents did not change the expression of tubulin suggesting that changes observed in transcript level was specific to NR mRNA (Table 2).

Though nitrate reductase and nitrite reductase are important enzymes which catalyse successive steps in the nitrate

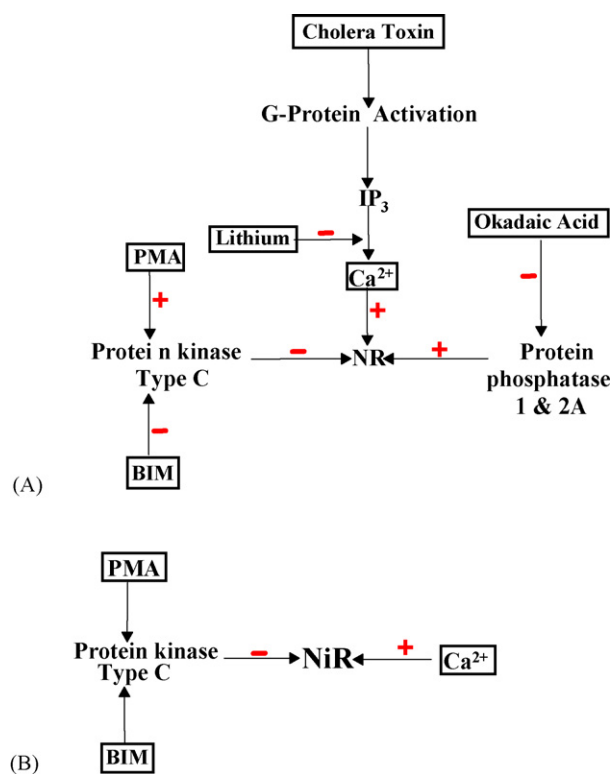


Fig. 7. A schematic model for regulation of NR (A) and NiR (B) by various signaling agents in rice.

assimilation pathway and have been shown to be co regulated by several factors [1], it was interesting to note that there were significant differences between the effects of the signaling molecules on NR and NiR activities. While PMA, BIM and calcium evoked very similar responses in NR and NiR activities, significant differences were observed with Li, okadaic acid and cholera toxin. The lack of effects of lithium, cholera toxin and okadaic acid on NiR activity either in the light or dark appear to indicate no involvement of phosphoinositides, G-protein and protein phosphatases in NiR regulation in rice (Fig. 7A and B). Taken together the effects of all the signaling agents used in the present study, regulation at post-transcriptional level appears to play a major role in rice.

The complete characterisation of the downstream steps of the signaling pathways and their interaction with C-metabolism will help us in increasing the nitrogen use efficiency (NUE) of rice, a crop known for its poor NUE (~25%). The results presented here point to a crucial role for NR, yet they pertain to the leaves of rice plant. Further attempts to increase NUE, by manipulating nitrate assimilation must keep in mind the metabolism of whole plant including nitrate uptake mechanism by the root.

## References

- [1] S. Sivasankar, A. Oaks, Nitrate assimilation in higher plants: the effect of metabolites and light, *Plant Physiol. Biochem.* 34 (1996) 609–620.
- [2] N. Raghuram, M.R. Chandok, S.K. Sopory, Light regulation of nitrate reductase gene expression in maize involves a G-protein, *Mol. Cell Biol. Res. Commun.* 2 (1999) 86–90.
- [3] P.K. Hepler, Calcium: a central regulator of plant growth and development, *Plant Cell* 17 (2005) 2142–2155.
- [4] M.R. Chandok, S.K. Sopory, 5-Hydroxytryptamine affects the turnover of polyphosphoinositides in maize and stimulates NR in the absence of light, *FEBS Lett.* 356 (1994) 39–42.
- [5] M.G. Kazanietz, Targeting protein kinase C and “non-kinase” phorbol ester reporters: emerging concepts and therapeutic implications, *Biochim. Biophys. Acta* 1754 (2005) 296–304.
- [6] R.H. Hageman, Integration of nitrogen assimilation in relation to yield, in: E.J. Hewitt, C.V. Cutting (Eds.), *Nitrogen Assimilation of Plants*, Academic Press, New York, 1979, pp. 591–611.
- [7] F.D. Snell, C.T. Snell, *Colorimetric methods of analysis*, vol. 3, D. Van Nostrand, New York, 1949, pp. 83–133.
- [8] J.L. Wray, R.L. Fido, Nitrate reductase and nitrite reductase, in: P.J. Lea (Ed.), *Enzymes of Primary Metabolism*, Academic Press, London, 1990, pp. 241–256.
- [9] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of proteins utilising the principle of protein dye binding, *Anal. Biochem.* 72 (1976) 248–254.
- [10] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction, *Anal. Biochem.* 9 (1987) 167–172.
- [11] M.R. Chandok, S.K. Sopory, ZmcPKC70, a protein kinase C-type enzyme from maize, *J. Biol. Chem.* 273 (1998) 19235–19242.
- [12] D. Toullec, P. Pianetti, H. Coste, P. Bellevergue, T. Grand-Perret, M. Ajakane, V. Baudet, P. Boissin, E. Boursier, F. Loriolle, L. Duhamel, D. Charon, J. Kirilobsky, The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C, *J. Biol. Chem.* 266 (1991) 15771–15781.
- [13] G.E. Gillaspay, J.S. Keddie, K. Oda, W. Gruissem, Plant inositol monophosphatase is a lithium-sensitive enzyme encoded by a multigene family, *Plant Cell* 7 (1995) 2175–2185.
- [14] M. Berridge, C.P. Downes, M.R. Hanley, Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands, *Biochem. J.* 206 (1982) 587–595.
- [15] N. Raghuram, S.K. Sopory, Roles of nitrate, nitrite and ammonium ions in phytochrome regulation of nitrate reductase gene expression in maize, *Biochem. Mol. Biol. Int.* 47 (1999) 239–249.
- [16] C. MacKintosh, Regulation of cytosolic enzymes in primary metabolism by reversible protein phosphorylation, *Curr. Opin. Plant Biol.* 1 (1998) 224–229.
- [17] M.G. Redinbaugh, S.C. Huber, J.L. Huber, K.W. Hendrix, W.H. Campbell, Nitrate reductase expression in maize leaves (*Zea mays*) during dark–light transitions. Complex effects of protein phosphatase inhibitors on enzyme activity, protein synthesis and transcript levels, *Physiol. Plant* 98 (1996) 67–76.
- [18] J.M. Stevenson, I.Y. Perera, I. Heilmann, S. Persson, W.F. Boss, Inositol signaling and plant growth, *Trends Plant Sci.* 5 (2000) 252–258.
- [19] H. Ma, GTP-binding proteins in plants: new members of an old family, *Plant Mol. Biol.* 26 (1994) 1611–1636.
- [20] K. Guron, M.R. Chandok, S.K. Sopory, Phytochrome-mediated changes in the level of phosphoinositides in etiolated leaves of *Zea mays*, *Photochem. Photobiol.* 56 (1992) 691–695.
- [21] P. Douglas, E. Pigaglio, A. Ferrer, N.G. Halford, C. MacKintosh, Three spinach leaf nitrate reductase-3-hydroxy-3-methylglutaryl-CoA reductase kinases that are regulated by reversible phosphorylation and/or Ca<sup>2+</sup> ions, *Biochem. J.* 325 (1997) 101–109.