Mol Gen Genet (1996) 251:599–608

ORIGINAL PAPER

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Phosphorylation/dephosphorylation steps are key events in the phytochrome-mediated enhancement of nitrate reductase mRNA levels and enzyme activity in maize

Received: 19 January 1996 / Accepted: 17 March 1996

Abstract We provide evidence to show that the increase in nitrate reductase (NR) transcript level stimulated by red light is mediated via a phosphorylation-dependent step. The light-stimulated enhancement of NR transcript level was significantly inhibited by H-7, a protein kinase inhibitor, whereas okadaic acid (OKA), a phosphatase inhibitor, had no effect. Phorbol myristate acetate (PMA), an activator of protein kinase C (PKC) enhanced the NR transcript level in dark-grown leaves. No correlation between changes in NR transcript level and NR activity (NRA) was observed. Inhibition of NRA by OKA and stimulation by H-7 indicated that NRA is increased by dephosphorylating the enzyme. We have identified a protein kinase (C type) that can phosphorylate the purified NR in vitro without the involvement of other accessory proteins. By in vivo labelling with ^{32}P and immunoprecipitation of NR with NR antibodies it was found that in the presence of OKA most NR protein (NRP) was present in phosphorylated state, while with H-7 the reverse was seen. The red (R) and far-red (FR) light reversible experiments suggested that phytochrome (Pfr, an active form) stimulation of NRA is mediated by dephosphorylation of the enzyme, suggesting that Pfr regulates both NR transcription and NRA via phosphorylation/dephosphorylation steps controlled by separate signal transduction pathways.

Key words Nitrate reductase · Phytochrome · Phosphorylation · Protein kinase C · *Zea mays*

Introduction

Nitrate reductase is one of the key enzymes involved in nitrogen assimilation in plants. Nitrate, the inducer, triggers the synthesis of this enzyme (Sommers et al. 1983), while light, via phytochrome, modifies the overall expression of the enzyme either at transcriptional (Rao et al. 1980; Gowri and Campbell 1989; Caboche and Rouze 1990; Melzer et al. 1989; Lillo 1991; Li and Oaks 1993; Sharma et al. 1994) or post-transcriptional levels (Remmler and Campbell 1986; Deng et al. 1990; Crawford et al. 1992; Pilgrim et al. 1993). Regulation of NR at the transcriptional level was demonstrated by inhibitor studies (Campbell and Smarrelli 1986) and by in vitro translation of mRNA (Cheng et al. 1986). Transcriptional regulation of NR by Pfr in maize was shown by Gowri and Campbell (1989).

The rapid response of nitrate reductase to alterations in environmental conditions, like changes in oxygen (Glaab and Kaiser 1993) or carbon dioxide concentration (Kaiser and Forster 1989; Kaiser and Brendle-Behnisch 1991) and light (Campbell 1988), led to the idea that, besides transcriptional and translational regulation, post-translational control may also regulate the activity of NR. Experiments with transgenic plants further indicated that post-translational regulation may be operative in NR regulation (Vincentz and Caboche 1991).

Huber et al. (1992) showed that phosphorylation and dephosphorylation are important post-translational modification steps by which NR activity (NRA) is regulated. They showed that NR in spinach leaves exists in a phosphorylated form in vivo and that the degree of phosphorylation of several tryptic fragments varied with dark and light conditions. Kaiser et al. (1993) provided indirect evidence for the involvement of protein phosphorylation/dephosphorylation in the rapid modulation of NR in leaves of spinach and roots of pea. They also reported that 67- and 100-kDa proteins are involved in an ATP-dependent inactivation of NR. The fraction containing the 67 kDa protein was found to have kinase activity (Spill and Kaiser 1994), while 100 kDa protein may be identical to nitrate inhibitor protein (NIP; Mackintosh et al. 1995). Recently it was

Communicated by R. G. Herrmann

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shown (Huber et al. 1994) that even in maize, a monocot, NR exists in a phosphorylated form in vivo.

We had shown earlier that in etiolated maize leaves light regulation of NR is mediated through phytochrome, a red/far red light photoreversible pigment (Rao et al. 1980). We have also shown that Pfr induces a transmitter which can interact with nitrate to induce NR (Sharma et al. 1994). The nature of the transmitter, the signal transduction chain and the site of action still remain to be elucidated. Since NR in maize is regulated by light both at the transcriptional level (Rajasekhar et al. 1988) and at the post-translational level via phosphorylation (Huber et al. 1994), we have tried to investigate how Pfr signalling operates at both levels.

Previously we had found that phorbol myristate acetate (PMA), an activator of protein kinase C, could enhance NR transcript levels and also increase NRA in the absence of light. This implicated a PKC-type kinase in the light-mediated expression of NR (Chandok and Sopory 1992; Sharma et al. 1994; Raghuram and Sopory 1995b). Since PMA is an analogue of diacylglycerol (DAG), which is one of the products of phosphatidylinositol-bis-phosphate (PIP_2) breakdown, it was suggested that Pfr-mediated stimulation of NR gene expression could occur via polyphosphoinositide (PPI) turnover. We have earlier shown that Pfr affects the turnover rate of the PPI cycle (Guron et al. 1992) and 5-hydroxytryptamine (5-HT), an activator of the PPI cycle, mimics the light effect in stimulating NRA and gene expression in the dark (Chandok and Sopory 1994; Raghuram and Sopory 1995b). In the present study we provide evidence that phosphorylation via a PKC-type enzyme may be a key event in Pfr-mediated signal transduction for the regulation of NR transcript level, and that Pfr may also affect the level/activity of phosphatases of type 1 or 2A to maintain the level of active NR. As the regulation by Pfr seems to involve independent steps, our results indicate that Pfr may initiate more than one signalling event, which affect mRNA levels and NR activity.

Materials and methods

Plant material and light source

Seed of *Zea mays* var. Ganga-5 were obtained from the National Seed Corporation, New Delhi, India. Red light (R) was obtained from 100 W tungsten lamps using CBS 650 (Carolina Biological Supply Co.) filter (emission λ_{max} 650 nm, intensity 1.47 $\mu W/cm^2$) and far red (FR) was obtained using a CBS 730 filter (λ_{max} 730 nm, 1.5 $\mu W/cm^2$) as reported earlier (Sharma and Sopory 1984). All the manipulations were done under green safe light ($0.01 W/m^2$), which was obtained by filtering the light from a cool flourescent tube through several layers of green cellophane paper (emission λ_{max} 500 nm).

Growth conditions

Seeds were washed thoroughly, soaked in water overnight and again washed in deionized or distilled water. Seeds of uniform size were grown in petri dishes on moist paper in total darkness in an incubator for 8–9 days at 27 + 1° C. Seedlings were watered daily. Petri dishes were screened regularly for ungerminated or infected seeds. Fully opened primary leaves of uniform size were excised from the seedlings of similar morphology. All the manipulations during growth and treatment were done under green safe light. Okadaic acid and H-7 treatments were given to cut leaves that were 8–9 days old. Earlier it was found that in leaves irradiated with red light for 5 min and kept in darkness (dt) for 2 h before nitrate addition, nitrate reductase activity was stimulated more than in those treated without a dark period (Chandok 1993). Therefore, in the present study, a dark period was used even in H-7 and OKA treatments.

Extraction and assay of nitrate reductase

Etiolated leaves of 8 to 9 day-old seedlings were excised and floated on nitrate solution (60 mM) for induction of NR. Irradiation with red light and PMA treatment were carried out on excised, etiolated leaves as described earlier (Chandok and Sopory 1992). Extraction of NR was done as reported (Sharma and Sopory 1984) and NR was assayed according to the procedure of Hageman and Hucklesby (1971). The assay mixture contained the following components: 1.5 ml 0.1 M phosphate buffer (pH 7.5), 0.2 ml 0.1 M KNO_3, 0.2 ml NADH (1 mg/ml). Nitrate reductase activity is expressed as nmol nitrite produced per h per mg protein. Nitrate content of leaves was estimated by the zinc dust method (Wooley et al. 1960).

Protein estimation

Protein determinations on extracts, column fractions and purified enzyme were done according to Bradford's (1976) method.

Purification and in in vitro phosphorylation of nitrate reductase

The NR was purified according to the procedure of Nakagawa et al. (1984). The extract was prepared from 8-day-old, dark-grown leaves exposed to light for 18 h in the presence of 60 mM KNO_3. The leaves were ground in extraction buffer (1 : 4), containing 250 mM TRIS-HCl (pH 8.5), 10 mM cysteine, 20 µM FAD, 1 mM EDTA and 10% glycerol (v/v). The homogenate was centrifuged at 16000 rpm in a Hitachi refrigerated centrifuge using an RPR 20-2 rotor for 30 min. Solid ammonium sulphate was added to the crude extract to 40% saturation. The enzyme extract was stirred for 1–2 h and then centrifuged to collect the precipitate, which was resuspended in a minimum amount of 10 mM potassium phosphate buffer pH 7.5, containing 1 mM 2-mercaptoethanol and 10% glycerol (v/v). The solution was dialysed against 2 l of 10 mM potassium phosphate buffer (pH 7.5) for 4 h. The dialysed sample was applied to a pre-equilibrated blue dextran affinity column. The column was washed with 100 mM TRIS-HCl buffer containing 1 mM EDTA, 1 mM 2-mercaptoethanol, 20 µM FAD and 10% glycerol. The flow rate was maintained at 30 ml/h. Washing was done until the A_{280} was less than 0.02. The enzyme was eluted by using washing buffer containing 10 µM NADH and 5-ml fractions were collected in tubes containing 0.1 ml of 1M KNO_3. The second elution step was done with 3 M KNO_3. Fractions were assayed for NR activity and protein concentration.

In vitro phosphorylation was carried out in a reaction mixture containing 30 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 400 ng purified NR, 5 µg phosphatidylserine, 100 µM $CaCl_2$, 1 µg oleyl acetyl glycerol and 50 µl of purified kinase. The total reaction volume was 100 µl. The reaction was started by adding 100 µM of [γ-^{32}P]ATP (200000 cpm, with a specific activity of 3000 Ci/mmol). The reaction was carried out at 30° C for 5 min, stopped by the addition of SDS. sample buffer and boiled for 2–3 min. For assay of radioactivity

Table 1 Effects of the various compounds used to study signal transduction elements involved in phytochrome-mediated stimulation of nitrate reductase gene expression and activity

Compound	Effect	Reference
H-7	Inhibitor of protein kinases	Hidaka et al. (1991) Lee and Assmann (1991)
Staurosporine	Inhibitor of protein kinases with greater specificity for protein kinase C	Tamaoki (1991) Longeran (1990)
Okadaic acid (OKA)	Inhibitor of protein phosphatases 1 and 2A	Hardie et al. (1991) Sheen (1993)
Phorbol myristate acetate (PMA)	Stimulator of protein kinase C	Castagna et al. (1982) Olah and Kiss (1986) Nanmori et al. (1994)

incorporated, the reaction was stopped using 10% ice-cold TCA. The precipitates were collected on Whatman filter discs and washed 4–5 times with cold 10% TCA. The filters were dried and counted in scintillation fluid in an LKB scintillation counter. Phospho-amino acid analysis of phosphorylated NR was done according to Neufeld et al. (1989).

Immunoprecipitation and Western blotting of nitrate reductase

In vivo phosphorylation with and without nitrate (as control) was performed and leaves were washed thoroughly with distilled water and treated with OKA, H-7 or PMA as described above. In case of the in vivo phosphorylation experiments on NR, 30 mM HEPES (pH 7.5) was used instead of phosphate buffer. Protein was extracted and precipitated (at 1 mg/ml) with NH_4SO_4 and dialysed as described above. Immunoprecipitation of NRP was done using NR antibodies (1:1000 dilution), according to the procedure of Huber et al. (1992), using immunoprecipitin as a precipitating agent. Immunoprecipitated NR was washed thoroughly and either checked for incorporation of ^{32}P into NRP or subjected to SDS-PAGE and transferred to nitrocellulose paper according to the procedure of Towbin et al. (1979). The blot was probed with NR antibodies (1:500) followed by incubation with anti-rabbit IgG conjugated to horseradish peroxidase. The protein-antibody complex was detected by using 4-chloronapthol as a substrate. The blot was autoradiographed for the detection of phosphorylated NRP. Dot blotting of immunoprecipitated NR was done on nitrocellulose paper using a commercial dot blot apparatus (Schleicher and Schuell).

Northern blot analysis

RNA was isolated according to method of Logemann et al. (1987). Aliquots (20 or 40 µg) of the RNA samples were denatured in the presence of 6% formaldehyde and 50% formamide at 50° C for 1 h and blotted onto a GSP membrane (DuPont NEN). Northern blot analysis was performed as described by Sambrook et al. (1989). The RNA was subsequently crosslinked to the membrane under UV using Stratalinker (Stratagene), and the filter baked at 80° C and used for hybridization. A ^{32}P-labelled probe was generated by the random primer extension method using a purified insert from a NR cDNA clone and a commercial labeling kit (USB) as per manufacturer's instructions. The specific activity of the probe was over 5×10^8 dpm/µg. Pre-hybridization was performed for 15 min at 65° C in 0.5 M NaCl, 0.1 M NaH_2PO_4, 0.1 M TRIS base, 2 mM EDTA, 1% SDS and 100 µg/ml denatured salmon sperm DNA. The denatured probe (1×10^6 dpm/ml) was then added and hybridization was allowed to continue for about 24 h at 65° C. Pre-hybridization, hybridization and washings were performed in a hybridization incubator. Washing was performed using 10 mM sodium phosphate buffer (pH 7.0), 2 mM EDTA and 1% SDS, initially at room temperature and subsequently at hybridization temperature. The washed filter were exposed to Kodak x-AR films using two intensifying screens for appropriate periods of time.

For the chicken actin probe, the specific activity obtained was 7×10^9 dpm/µg. The hybridization was carried out at 55° C in 6 × SSC, 1% SDS, 100 µg/ml denatured salmon sperm DNA and 1×10^7 dpm/ml denatured probe. Washing was done twice in 2 × SSC and 1% SDS at room temperature followed by washing twice with 1 × SSPE (Sambrook et al. 1989) and 1% SDS at hybridization temperature followed by 0.5 × SSPE at room temperature. Washed filters were exposed for autoradiography.

Chemicals

[γ-^{32}P]ATP and carrier-free ^{32}P were obtained from BARC, India, PMA, 4α-PMA and all other chemicals were purchased from Sigma. Okadaic acid and alkaline phosphatase were purchased from Boehringer Mannheim and H-7 and precipitin were obtained from Gibco BRL.

Statistical analysis

Experiments were repeated three times, using triplicate samples each time. The Northern and Western blots shown are from representative experiments.

Results

Phosphorylation as a key event in the regulation of NR transcript level

From our previous work on PMA stimulation of NR transcript level (Sharma et al. 1994; Raghuram and Sopory 1995b) it appeared likely that phosphorylation/dephosphorylation events are involved in light-mediated regulation of NR transcription, and this effect might be regulated by PKC-type enzymes. We have identified a PMA-stimulated kinase (70 kDa) which also binds to ^3H-PMA (Chandok and Sopory, in preparation). To check for the involvement of phosphorylation/dephosphorylation in Pfr-mediated stimulation of NR gene expression we have used various compounds (Table 1). Leaves from dark-grown plants were treated with PMA and R light-irradiated leaves were incubated with H-7 and OKA. Total RNA was isolated after 2 h

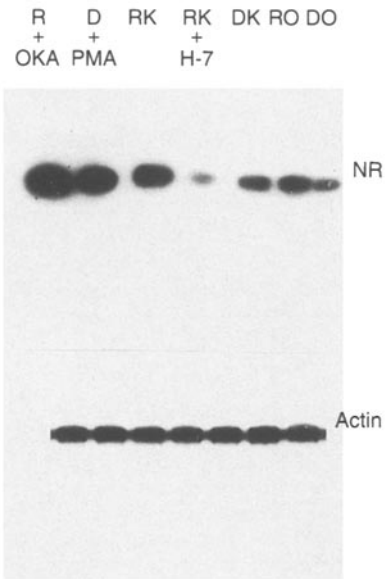

Fig. 1 Northern blotting analysis showing effects of PMA, OKA and H-7 on the red light-stimulated increase in nitrate reductase (NR) transcript levels. Etiolated leaves, 8–9 days old, were floated on water in the presence of H-7 and Okadaic acid (OKA) and irradiated with red light for 5 min. Phorbol myristate acetate (PMA) treatment and red light irradiation of etiolated excised leaves was carried out for 5 min. After treatment, leaves were kept for 2 h in darkness in water and then floated on nitrate solution (60 mM); RNA was isolated 4 h later. Hybridization with NR cDNA probe was done as described in experimental procedures. The Northern blot was boiled to remove the first probe and rehybridized with actin cDNA. Lanes: D. dark + water; R. red light (5 min) + water; DK, dark + KNO_3; RK, red light (5 min) + KNO_3; RK + H-7, red light (5 min) + H-7 + KNO_3; D + PMA, dark + PMA (5 min) + KNO_3; R + OKA, red light (5 min) + OKA + KNO_3

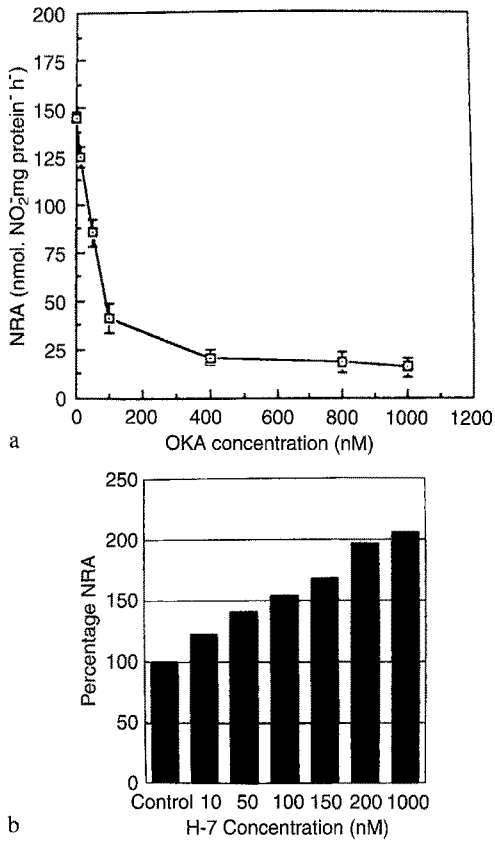

Fig. 2a, b Effect of okadaic acid and H-7 on nitrate reductase activity. Excised etiolated maize leaves (8–9 days old) were floated on different concentrations of OKA (**a**) or H-7 (**b**) and irradiated with R light for 5 min. Incubation in OKA was continued for another 2 h in the dark. Leaves were washed and then incubated in KNO_3 (60 mM) in the dark and NRA was measured after 12 h

of nitrate treatment and analyzed by Northern blotting. The data showed that there was very low level of NR mRNA in leaves that were kept in water instead of nitrate. In samples irradiated with R light an increase in NR was seen. This was also observed in the case of dark-grown leaves incubated with PMA (Fig. 1). Leaves treated with an inactive analogue of PMA, 4α-PMA, did not show any increase in NR mRNA (data not shown). Interestingly, in RNA samples isolated from R + H-7 treated leaves, very low levels of NR mRNA were seen, whereas in R + OKA treated leaves the stimulatory effect of R light was not inhibited and in fact a slight increase was noticed. There was no change in the level of actin mRNA in any of the samples analyzed.

This data shows that phosphorylation is involved in regulation of NR transcript level.

The NR transcript level does not reflect the level of activity of NR

In view of the above results, attempts were made to check whether the increase and decrease in the transcript level brought about by OKA and H-7, respectively, had any bearing on NR activity status. In contrast to their effect at the transcript level (shown in Fig. 1) OKA inhibited NRA, while H-7 showed stimulation of NRA (Fig. 2a, b), indicating that the NR transcript level does not reflect the level of activity of the NR and showing that NRA is regulated independently by phosphorylation and dephosphorylation mechanisms.

Phosphorylation of purified NR in in vitro

Huber et al. (1994) showed that NR exists in a phosphorylated form in vivo in maize and that the phosphorylation occurs at serine residues. Our data obtained by in vivo labelling of proteins with ^{32}P also showed that NR is phosphorylated in vivo in our system (data not shown). In view of our earlier work (Chandok and Sopory 1992) we tested whether NR could act as a substrate for a PKC-type enzyme. We have recently purified a kinase of 55 kDa ($PKC_{55}M$) from maize, whose activity is stimulated by

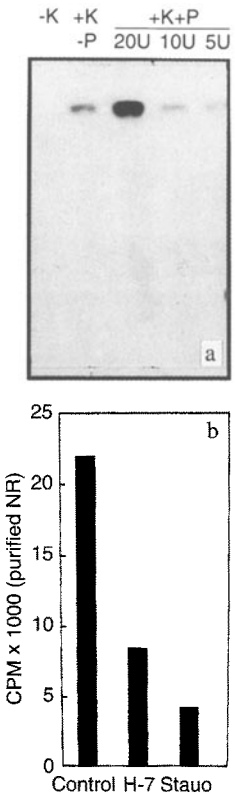

Fig. 3a, b In vitro phosphorylation of purified nitrate reductase. **a** Purified NR was phosphorylated either in the absence of purified kinase from maize (−K) or with $PKC_{55}M$ kinase in the presence of phosphatidyl serine, oleyl acetyl glycerol and calcium (+K−P). In some cases (+K+P) purified NR was first incubated with alkaline phosphatase (5, 10 and 20 units) for 20 min at 30° C and then the protein was precipitated with $(NH_4)_2SO_4$ (40%), dialysed and used for in vitro phosphorylation studies. In vitro phosphorylation was done as described in experimental procedures and phosphorylated product was separated on SDS-PAGE and autoradiographed. **b** In vitro phosphorylation of NR was done as in (**a**) but in presence of H-7 and stauorosporine and the counts incorporated in NR were measured. The enzyme preparation was treated with alkaline phosphatase prior to the kinase reaction

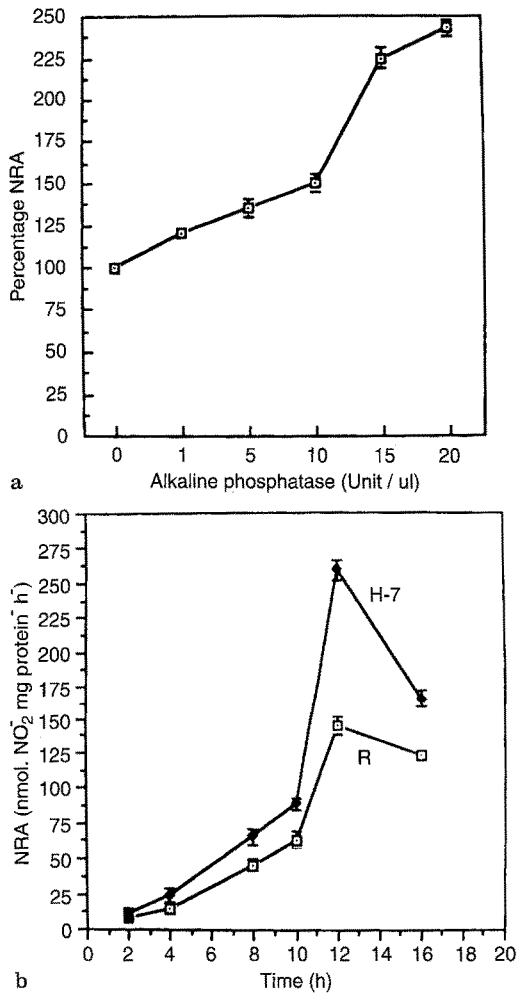

Fig. 4a, b Effects of alkaline phosphatase on in vitro NR activity and of H-7 on NR activity in vivo. **a** NR protein (100 µg) was incubated with different amounts of alkaline phosphates for 20 min at 30° C. After the treatment the protein was precipitated either with $(NH_4)_2SO_4$ and dialysed or precipitated with polyethylene glycol PEG8000 and the pellet was dissolved in buffer containing cysteine and glycerol before monitoring NRA. **b** Etiolated maize leaves were floated either in the presence (*filled squares*) or absence (*dotted squares*) of H-7 (200 nM) and irradiated with red light (R) for 5 min. Experimental conditions were as given in Fig. 5. NRA was analysed at different times after treatment

phosphatidylserine, oleyl acetyl glycerol and Ca^{2+} (Chandok and Sopory, in preparation). We purified NR to homogeneity by using the protocol given in Materials and methods. A 365-fold purification of NR was achieved. The peak fraction containing NRA obtained after affinity chromatography showed a single band at 115 kDa after silver staining (data not shown). It was found that $PKC_{55}M$ phosphorylated purified NR in vitro (Fig. 3a). The extent of phosphorylation increased when the purified NR was treated with alkaline phosphatase prior to the kinase reaction. Autophosphorylation of NR was not observed in the present case (Fig. 3a, lane-K). The sites of in vitro phosphorylation by $PKC_{55}M$ were serine residues (data not shown). One important property of $PKC_{55}M$ is that its activity is strongly inhibited by H-7 and staurosporine (data not shown). Both these inhibitors also blocked the in vitro phosphorylation of NR by $PKC_{55}M$ (Fig. 3b).

The dephosphorylated form is the active form of NR

The results in Fig. 2a, b indicated that the dephosphorylated form of NR may be the active enzyme. This was confirmed by treating the partially purified NR with increasing concentrations of alkaline phosphatase, which leads to an increase in NRA (Fig. 4a). A kinetic study following H-7 treatment showed that the NRA was not inhibited (Fig. 4b), thus confirming that the dephosphorylation leads to an increase in NRA. It was found that changes in NRA brought about by H-7 and OKA treatments were not due to a change in nitrate level (data not shown).

Changes in NRA are not due to change in NRP concentration

In order to find out if the changes in NRA brought about by the treatments described above were due to changes in the level of NR protein (NRP), NR antibodies were used to check NRP levels. The data

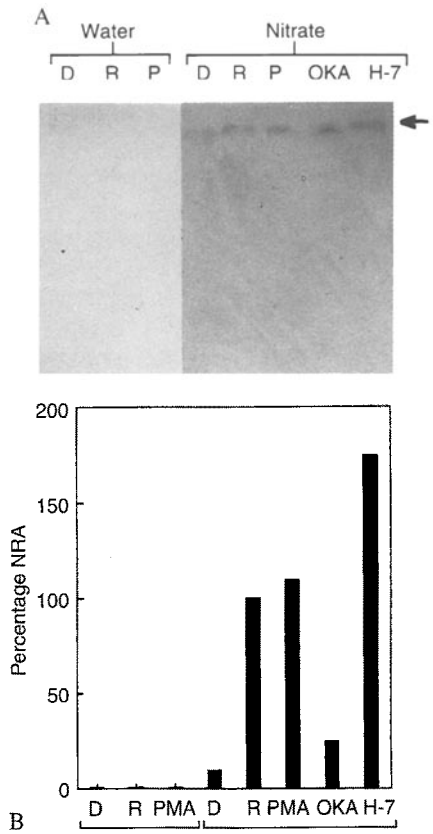

Fig. 5A, B Effect of H-7 and OKA on nitrate reductase protein and nitrate reductase activity. Etiolated maize leaves were floated on nitrate (for 8 h) or on water after the indicated treatments and changes in NRP were measured by Western blot analysis using NR antibodies (**A**) and NRA was monitored (**B**). D, dark; R, red light (5 min); P, PMA (5 min)

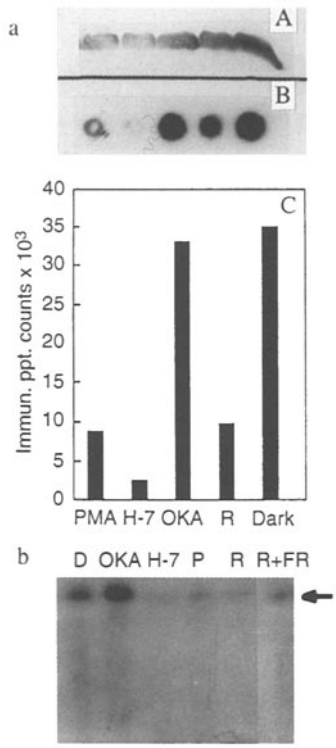

Fig. 6a, b Changes in the phosphorylation state of nitrate reductase protein under various treatment regimes. **a** Etiolated maize leaves were labelled in vivo with ^{32}P and treated with H-7, OKA or PMA for 5 mins; NRP was extracted after 8 h of induction. **A** Western blot showing amounts of NRP in the various samples treatments. **B** Autoradiograph of dot blot of immunoprecipitated NRP. **C** Amounts of ^{32}P present in immunoprecipited NRP after the various treatments. **b** Gel electrophoresis of immunoprecipitated NR. The ^{32}P-labelled protein was visualized by autoradiography

given in Fig. 5a and b clearly shows that there is no NRP, and consequently no NRA, in leaves incubated in water. In nitrate-treated samples, in R light-irradiated and PMA-incubated leaves, the level of NRP and NRA was more. Interestingly, there was no major difference in the levels of NRP detected in OKA and H-7 treated samples (Fig. 5b), whereas there was very significant difference in the NRA induced by these two treatments (Fig. 5b). This suggests that the data given on NRA in Figs. 3a and 4a is not due to changes in NRP level, and probably reflects the ratio of NR existing in phosphorylated and dephosphorylated forms.

To check whether the treatments in Fig. 5 reflect the phosphorylation/dephosphorylation status of NRP, the experiments were repeated, except that in this case the proteins were simultaneously labelled with ^{32}P. NR was isolated and immunoblotted. Based on this quantitative data, NR protein amounts were equalized and again checked with NR antibodies (Fig. 6A). Equal amounts of immunoprecipitable protein from each set was either dot blotted and autoradiographed (Fig. 6B), or counted (Fig. 6C). As is clear, after the dark and R+OKA treatments, more NRP was present in the phosphorylated form compared to either R light-irradiated leaves or leaves treated with PMA or R+H-7.

Phytochrome regulation of the phosphorylation state of NRP

From the experiments given in Figs. 5 and 6, it follows that R light triggers the dephosphorylation of NRP, and under such conditions higher NRA levels were seen than in unirradiated samples. To check if this is a phytochrome-mediated response, the experiment shown in Fig. 6 was repeated. In one case the leaves were irradiated with 5 min of R light and in the other R light irradiation was followed by FR light irradiation for 5 min. As can be seen in Fig. 7, in dark-incubated leaves more NRP is present in phosphorylated form, whereas with R light irradiation there was a significant decrease in the concentration of the phosphorylated form of NR; and this effect was reversed by FR light. Reversibility was almost total. This data suggests that Pfr may activate phosphatases to dephosphorylate NRP and thereby increase NRA. Our earlier data on a PMA-mediated increase in NRA in the absence of light (Chandok and Sopory 1992) and our present data (Figs. 5, 6) showing that after immunoprecipitation fewer counts were found in PMA-treated leaves than in those kept in darkness suggest that PMA may also act at the post-translational level via dephosphorylation

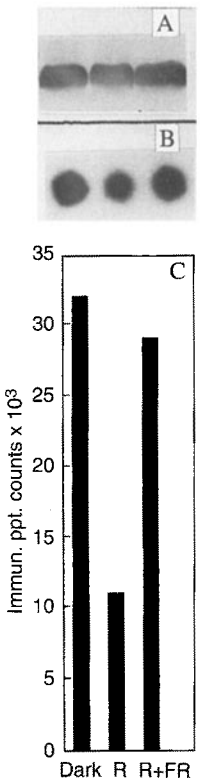

Fig. 7A–C Phytochrome-mediated changes in the phosphorylation state of nitrate reductase protein. After labelling with ^{32}P, etiolated maize leaves were either irradiated with R light for 5 min or R light was followed by far red (FR) light for 5 min and NRP was extracted after 8 h. **A** Western blot showing that equal amounts of NRP were used for the different treatments. **B** Nitrate reductase protein was immunoprecipitated and blotted onto nitrocellulose paper and autoradiographed. **C** Amounts of radioactive phosphate incorporated into NRP after immunoprecipitation

events. This was also confirmed by comparison, on gels, of the levels of phosphorylation of NRP following the different treatments (Fig. 6b).

Discussion

Phytochromes are the best characterized photoreceptors known to mediate light-dependent responses in higher plants (Furuya 1993), and regulate the transcription of specific genes (Silverthrone and Tobin 1984; Kuhlemeir et al. 1988; Terzaghi and Cashmore 1995). Although a lot of work has been done on the analysis of promoters of various families of nuclear genes, and various sequence elements that may be involved in Pfr regulation have been defined (Terzaghi and Cashmore 1995), the components of the signal transduction pathways have remained unidentified. Recent studies have, however, shown that Ca^{2+} and cGMP (Bowler et al. 1994; Neuhaus et al. 1993) and protein phosphorylation/dephosphorylation steps (Singh and Song 1990; Quail 1991; Dooshi et al. 1992) are involved in initiating signal transduction. It is assumed that phosphorylation events affect the binding of many transcription factors to cis-acting elements in light-regulated genes (Datta and Cashmore 1989; Klimczak et al. 1992; Sarokin and Chua 1992). The report by Sheen (1993) on the role of protein phosphatases in light-inducible gene expression in maize is one piece of direct evidence for the importance of phosphorylation status of proteins in Pfr signal transduction. However, Romero and Lam (1993) did not find any effect of protein kinase inhibitors on Pfr-mediated transcriptional stimulation of the *Lhcp* gene. Therefore at present the role of phosphorylation/dephosphorylation in Pfr-mediated responses is not fully established.

Nitrate reductase is a substrate-inducible enzyme and light influences the accumulation of NR mRNA, appearance of NR protein and NR activity (Caboche and Rouze 1987; Sopory and Sharma 1990; Raghuram and Sopory 1995a); the last is regulated by phosphorylation/dephosphorylation event with the dephosphorylated form of NR being the active enzyme (Kaiser et al. 1993; Huber et al. 1992). In the present study we have checked (i) whether a phosphorylation/dephosphorylation event is involved in Pfr-mediated, nitrate-dependent enhancement of NR transcript level and (ii) whether Pfr affects the phosphorylation status of NR protein in vivo, independently of its effect on the transcript level.

Recent studies in our laboratory have indicated that the products of the PPI cycle and protein kinase (s) may be involved in Pfr regulation of NR transcript level (Sharma et al. 1994; Raghuram and Sopory 1995b). As was shown earlier (Raghuram and Sopory 1995b) we found that addition of PMA, an activator of PKC, can enhance NR transcript levels, thus suggesting the involvement of a PKC-type enzyme in Pfr signal-response coupling. Such kinases have been reported in other systems (Schafer et al. 1985; Elliot and Skinner 1986; Nanmori et al. 1994) and we have also identified and characterized a PMA-stimulated protein kinase of 70 kDa (Chandok and Sopory, in preparation). An inhibitor of such kinases, H-7, significantly blocked the R light-dependent enhancement of NR transcript level (Fig. 1); however, OKA, an inhibitor of phosphatase type 2A had no effect, suggesting that Pfr stimulation of NR mRNA involves a protein phosphorylation event, which might be controlled by a PKC-type kinase. However, it needs to be established whether NR gene regulation is achieved via direct phosphorylation of trans-acting factors, although binding of some of them to different cis elements has been shown to be dependent on their phosphorylation status (Datta and Cashmore 1989; Sarokin and Chua 1992; Klimczak et al. 1992, 1995). In the same system, a role for protein phosphatases in chlorophyll accumulation and photosynthetic gene activation has been demonstrated (Sheen 1993), suggesting that light could transduce signals by activating either kinases or phosphatases or both. We have shown earlier that 5-HT, a stimulator of the PPI cycle in maize (Chandok and Sopory 1994), could mimic the light effect (Raghuram and Sopory 1995b); thus a role for the PPI cycle is also indicated. In fact, turnover of PPI is affected by Pfr, has been shown in maize (Guron et al. 1992).

The mechanism by which PMA mimics red light in increasing NR transcript level is not known. It could be via the involvement of a PKC-type enzyme (Raghuram

and Sopory 1995b). In other systems, regulation of cis elements by trans-acting factors like AP-1 (Angel et al. 1987; Chiu et al. 1987; Imbra and Karin 1987; Lee et al. 1987) or other transcription factors (Imagawa et al. 1987; Mitchell et al. 1987; Lenardo et al. 1987; Sen and Baltimore 1986) may be PMA dependent.

We have previously demonstrated that NRA in maize is also regulated by light via phytochrome (Rao et al. 1980; Sharma and Sopory 1984). Our present data shows that the level of NR transcript cannot in all cases be correlated with NRA (Figs. 1, 2). To understand the mechanism of Pfr-mediated stimulation of NRA, the possibility of post-translational modification via phosphorylation/dephosphorylation was explored. Our present results with H-7 and OKA confirm that NR is active in dephosphorylated form and our in vitro phosphorylation data suggests that, in contrast to the report by Spill and Kaiser (1994), only one protein, a PKC-type enzyme, has potential to phosphorylate NR. This conclusion is further supported by the finding that H-7 and staurosporine inhibit NR phosphorylation.

To answer the question whether Pfr affects the phosphorylation/dephosphorylation state of NR in vivo, we checked in vivo phosphorylation of NR following various treatments. In darkness less protein was synthesized than in leaves that were irradiated with red light. Although H-7 inhibited, any increase in transcript level, the protein level was not affected because the H-7 effect is masked following the transfer of leaves to nitrate solution (data not shown). Even though NRP levels were the same in H-7 and OKA treated samples, NRA was different, suggesting that dephosphorylation of NR is required to obtain active NR. Our data clearly shows that regulation of the phosphorylation state of NRP is also light mediated and is altered by Pfr. Huber et al. (1994) have previously shown a decrease in phosphorylation in response to light by peptide analysis; however, they did not identify the photoreceptor involved. It is possible that, at the post-translational level, Pfr may be inhibit kinases or activate phosphatases in order to stimulate NRA.

The data on PMA-mediated increase in NRA (Chandok and Sopory 1992) and the decrease in the phosphorylation of NRP, shown in this paper, is difficult to explain at present. The effect could be mediated via suppression of another set of kinases or by activating phosphatases as well. In fact PMA has been shown to stimulate PP 1-type phosphatases in other systems (Srinivasan and Begum 1994).

In conclusion, it follows that Pfr affects both the level of NR transcript and the phosphorylation state of NR protein (see Fig. 8). These two switches seem to be regulated independently, since in R light-irradiated leaves in the presence of OKA NR transcript level was not affected, whereas the enzyme activity was inhibited. This shows that a phosphorylation event leads to an increase in NR transcript level, whereas a dephosphorylation event at the post-translational level can lead to an increase in the NRA. There thus seems to be no direct correlation between the amount of mRNA and NRA. An increase in the NR transcript level need not always result in an increase in the NRA.

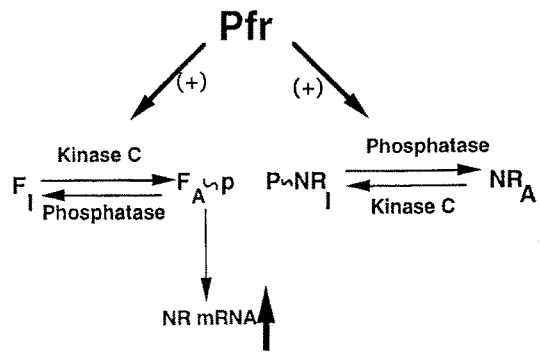

Fig. 8 A proposed model for the role of phosphorylation and dephosphorylation in Pfr-mediated enhancement of nitrate reductase transcript levels and NRA. It is proposed that phosphorylation of a transcription factor by a PKC-type enzyme (either directly or via a cascade of phosphorylation steps) leads to an increase in NR transcript levels. NR in the dephosphorylated form is active and Pfr mediates either inactivation of protein kinases or activation of phosphatases to stimulate NR activity. Regulation of Pfr at the transcriptional and post-translational levels may occur independently. F_I, inactive transcription factor; F_A, active transcription factor; NR_I, inactive NR; NR_A active NR

Acknowledgements The research was supported by a grant from Department of Biotechnology, Govt. of India. NR cDNA and NR antibodies were kindly provided by Prof. W. H. Campbell. We are thankful for BIC, JNU for their help in printing the manuscript.

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