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Short-term modulation of nitrate reductase activity by exogenous nitrate in *Nicotiana plumbaginifolia* and *Zea mays* leaves

Sylvie Ferrario[1], **Marie-Hélène Valadier**[1], **Christine H. Foyer**[2]

[1] Laboratoire du Metabolisme, INRA, Route de Saint-Cyr F-78026 Versailles cedex, France
[2] Environmental Biology Department, IGER, Plas Gogerddan, Aberystwyth, Dyfed SY23 3EB, UK

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Abstract. Maize (*Zea mays* L.) grown on low (0.8 mM) NO_3^-, as well as untransformed and transformed *Nicotiana plumbaginifolia* constitutively expressing nitrate reductase (NR), was used to study the effects of NO_3^- on the NR activation state. The NR activation state was determined from the relationship of total activity extracted in the presence of ethylenediaminetetracetic acid to that extracted in the presence of Mg^{2+}. Light activation was observed in both maize and tobacco leaves. In the tobacco lines, NO_3^- did not influence the NR activation state. In excised maize leaves, no correlation was found between the foliar NO_3^- content and the NR activation state. Similarly, the NR activation state did not respond to NO_3^-. Since the NR activation state determined from the degree of Mg^{2+}-induced inhibition of NR activity is considered to reflect the phosphorylation state of the NR protein, the protein phosphatase inhibitor microcystin LR was used to test the importance of protein phosphorylation in the NO_3^--induced changes in NR activity. In-vivo inhibition of endogenous protein phosphatase activity by microcystin-LR decreased the level of NR activation in the light. This occurred to the same extent in the presence or absence of exogenous NO_3^-. We conclude that NO_3^- does not effect the NR activation state, as modulated by protein phosphorylation in either tobacco (a C_3 species) or maize (a C_4 species). The short-term regulation of NR therefore differs from the NO_3^--mediated responses observed for phospho*enol*pyruvate carboxylase and sucrose phosphate synthase.

Key words: *Nicotiana* (nitrate reductase) – Nitrate – Nitrate reductase (activation state) – Protein phosphorylation – Transgenic plants – *Zea* (nitrate reductase)

Abbreviations: Chl = chlorophyll; MC = microcystin-LR; PEPCase = phospho*enol*pyruvate carboxylase; SPS = sucrose-phosphate synthase

Correspondence to: C.H. Foyer; FAX: 44 (1970) 828357; E-mail: foyer@bbsrc.ac.uk

Introduction

The activity of an enzyme may be controlled at the transcriptional level or by post-transcriptional modulation, or both. Generally, this first type of regulation allows adaptive long-term adjustments of enzyme activity to prevailing conditions while the second is more important in short-term acclimation. Both types of regulation allow the co-ordination of metabolic pathways in response to fluctuations in supply and demand. The co-ordination of carbon (C) and nitrogen (N) metabolism in plants requires rapid short-term acclimatory responses as well as adaptive changes to accommodate variations in environmental conditions (irradiance, water availability and mineral supply).

Nitrate reductase (NR), the first enzyme of N assimilation is induced at the transcriptional level by NO_3^-, light and carbohydrates (Cheng et al. 1986, 1992; Vincentz et al. 1993). It is inhibited by glutamine (Deng et al. 1991). The activity of the NR protein is modulated via a protein phosphorylation/dephosphorylation process in response to changes in the availability of light and CO_2 (Kaiser et al. 1992; Kaiser and Brendle-Behnish 1991). The precise nature of the signal inducing the modulation of the NR activation state in such situations is unresolved. A decrease in photosynthesis, caused by a decrease in light or CO_2, leads to a rapid decrease of the NR activation state both in shoots and roots. In consequence, the phosphorylation state of the enzyme responds to the level of a photosynthetic intermediate or product.

Nitrate has been shown to be a powerful modulator of phospho*enol*pyruvate carboxylase (PEPCase) and sucrose phosphate synthase (SPS) activities. Nitrate-induced increases in the phosphorylation states of these enzymes favour the re-allocation of carbon skeletons towards amino acid synthesis and away from sucrose synthesis (Van Quy et al. 1991a; Foyer et al. 1994). Nitrate-induced regulation of PEPCase gene transcription is an essential adaptive mechanism (Sugiharto and Sugiyama 1992) but the NO_3^--mediated activation of the PEPCase protein kinase and hence the phosphorylation status of PEPCase is an important rapid short-term response (Van Quy et al.

1991b; Duff and Chollet 1995). The NO_3^- effect on SPS also involves the modulation of SPS phosphorylation status as suggested by experiments using mannose and okadaic acid (inhibitors of protein kinase and protein phosphatase activities; Champigny and Foyer 1992).

The NO_3^--induced effects on NR activation state have not been studied in detail (Huber et al. 1992; de Cires et al. 1993). Recently, short-term effects of NO_3^- on the NR activation state have been suggested (M. Stitt, Botanisches Institut, Universität Heidelberg, Germany; personal communication). It was, therefore, pertinent to determine the influence of NO_3^- on the activation state of NR in detail. The NR activation state was determined via a comparison of the activity in the presence of Mg^{2+} and the total extractable NR activity obtained in the presence of EDTA. The activity of the phosphorylated form of NR is inhibited in the presence of Mg^{2+} by the interaction between the phosphorylated protein and an inhibitory protein (NIP; MacKintosh et al. 1995). We were able to exploit a transformed tobacco line expressing only a constitutive NR gene (Vincentz and Caboche 1991) and compare the responses of NR activity in this transformant with those of the untransformed control in different environmental conditions. This has allowed us to distinguish transcriptional modulation from the post-transcriptional modulation of the NR protein. In the untransformed plants the modulation of NR activity results from NO_3^--mediated effects on transcription and on post-transcriptional regulation. In the transformant, only NO_3^--mediated post-transcriptional events can occur. In addition, we have compared NO_3^--induced modulation of NR activity in a C_4 plant, maize, with that observed in the C_3 tobacco lines.

Materials and methods

Plant material. Transformed (line C) and untransformed (WT) *Nicotiana plumbaginifolia* Viv. seedlings were grown in sand and watered with a complete nutrient solution containing 10 mM nitrate and 2 mM ammonium (Coïc and Lesaint 1975). These are designated as high-N plants. The plants were grown in a growth chamber with a 16-h photoperiod, a 23 °C day/18 °C night regime and an irradiance of 170 (low-light plants) or 600 (high-light plants) µmol photons · $m^{-2} \cdot s^{-1}$. When the plants were five weeks old, leaf disks were taken from half of the plants and used for the experimental procedures. Only mature leaves were chosen for disks; these were harvested after 2 h of illumination. The remaining plants were then watered with a complete nutrient solution containing 0.05 mM NO_3^- for 5 d (see Ferrario et al. 1995 for details), the time necessary for the plants to develop two new leaves depleted of nitrate. These are therefore designated as low-N plants, as opposed to the high-N plants described above. Leaf disks were then taken from these new leaves and used in the following experimental procedures.

Maize *Zea mays* L. (cv. Brulouis) plants were grown in the same conditions as described for tobacco (above) except that the irradiance level was 400 mol photons · $m^{-1} \cdot s^{-1}$ and the nutrient solution contained 0.8 mM NO_3^-. When the plants were two weeks old, the third expanded leaves were excised and used for the experimental procedure. Each complete experiment was repeated three times. The trends were entirely consistent between experiments and the degree of variation between the values obtained was less than 20%.

Experimental procedure. Tobacco leaf disks were floated on a complete nutrient solution containing 0.05 mM KNO_3 at an irradiance of 450 µmol photons · $m^{-2} \cdot s^{-1}$. After 1 h of light the nutrient solution was adjusted to either 30 mM KNO_3, 30 mM KCl, or 30 mM KCl plus 100 mM glutamine. Disks were sampled at the times indicated in the text and immediately frozen and stored at − 80 °C until use. In the case of maize leaves, the same procedure was used as for tobacco leaf disks except that the cut end of each leaf was submerged in the nutrient solution containing 0.05 mM KNO_3 for 1 h in the light (600 mol photons · $m^{-2} \cdot s^{-1}$) and then adjusted to either 40 mM KNO_3 or KCl. The excised leaves were allowed to take up the nutrient solution for up to 7 h and were harvested at the times indicated in the text. When the protein phosphatase inhibitor microcystin-LR (MC) was used, the excised leaves were allowed to take up 200 µl of 10 µM MC 1 h before or after the transfer to 40 mM KNO_3.

Nitrate reductase assay. Nitrate reductase was extracted from leaf tissue which had been reduced to a fine powder with liquid nitrogen in a mortar. The extraction buffer [50 mM 3-(N-morpholino)propanesulfonic acid (Mops) KOH, pH 7.8; 1 mM NaF; 1 µM Na_2MoO_4; 10 µM FAD; 1 µM leupeptin; 0.2 g/g FW polyvinylpyrrolidone (PVP); 2 mM β-mercaptoethanol for tobacco leaves or 5 mM cysteine for maize leaves] was then added to the leaf tissue powder (1 ml/100 mg FW) and the extract immediately divided into two fractions and adjusted to either 5 mM $MgCl_2$ or 5 mM EDTA before centrifugation 5 min at 12000·*g*. Nitrate reductase activity was measured immediately in the supernatants. The reaction mixtures consisted of buffer containing 50 mM Mops-KOH, pH 7.5; 1 mM NaF; 10 mM KNO_3; 0.17 mM NADH; and 5 mM $MgCl_2$ or 5 mM EDTA for the $MgCl_2$ or EDTA extracts respectively. The reaction was stopped after 8 or 16 min by the addition of an equal volume of sulphanilamide (1% in 3N HCl) to the mixture N-naphthylethyl-diamine dichlorohydrate (NNED) (0.02%) was then added and the absorbance measured at 540 nm. The activation state of NR is defined as the activity measured in the presence of 5 mM $MgCl_2$ divided by the activity measured in the presence of 5 mM EDTA (expressed as a percentage).

Nitrate and chlorophyll estimations. Chlorophyll (Chl) was estimated in the crude leaf extracts by the method of Arnon (1949). Nitrate was estimated using the same leaf extracts as for NR activity, by the method of Cataldo et al. (1975).

Results

Modulation of NR activity by nitrate and light. Feeding untransformed tobacco leaf disks with NO_3^- for 4–6 h resulted in an increase in the total extractable NR activity in the light (Fig. 1D, E, G, H) but the NR activation state was constant over the whole period of the experiments. The extent of the increase in NR activity induced by NO_3^- depended largely on the initial N status of the leaves. The basal level of NR activity (before the addition of NO_3^-) was lowest in the low-N tobacco (Fig. 1G, H) and highest for the high-N plants (Fig. 1A, B, D, E). The effect of NO_3^--feeding was most rapid and pronounced in the leaf disks from the low-N plants. This is consistent with an initial lower level of NR and hence a greater capacity to increase NR activity. Moreover, the nitrate effect appeared to be closely linked to the light conditions under which the plants were grown. No effect of NO_3^- on NR activity was observed when the plants were grown in high light and high N conditions (Fig. 1A, B), but a rapid increase in NR activity was detected in plants grown at low light. An increase in irradiance alone was not sufficient to increase NR activity since activity in disks from high-N leaves was not increased as shown by the KCl

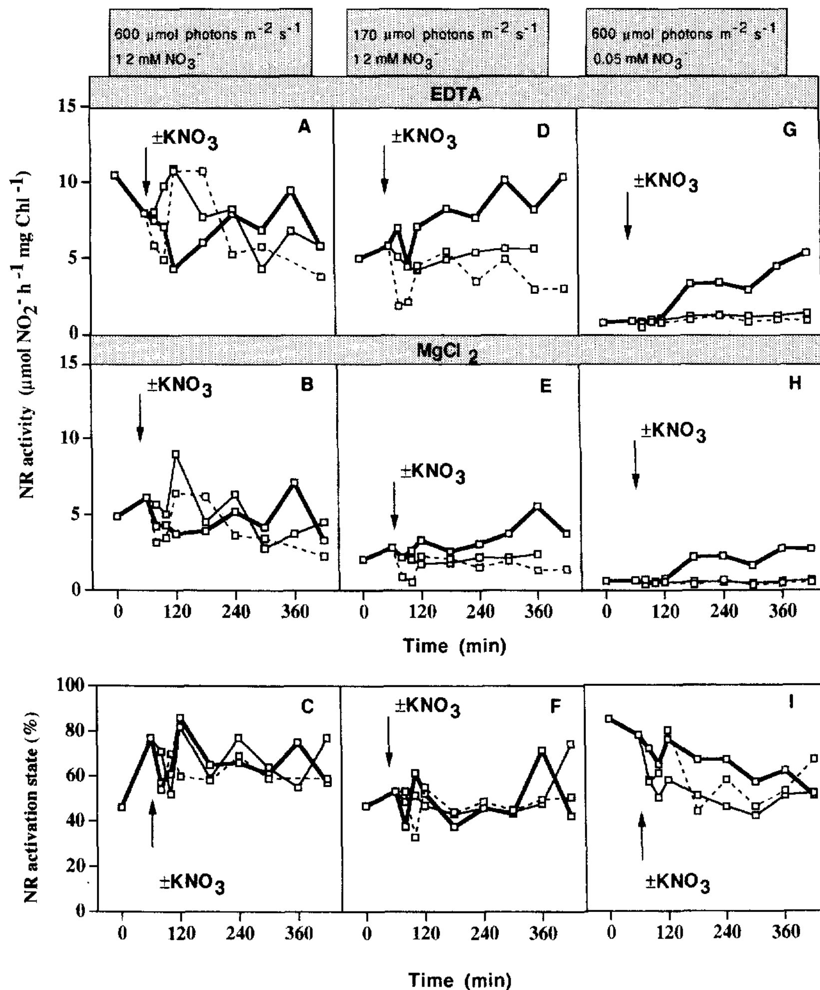

Fig. 1A–I. The effect of 30 mM KNO_3 (*bold line*), 30 mM KCl (*dashd line*) and 30 mM KCl plus 100 mM glutamine (*thin line*) on the extractable activities of NR in leaf disks of untransformed *Nicotiana plumbaginifolia* grown with different levels of NO_3^- and light as indicated. The NR activity was measured in the presence of either 5 mM EDTA or 5 mM Mg^{2+} and the activation state of NR was estimated by the ratio of NR activity measured in the presence of Mg^{2+} to that in the presence of EDTA, and expressed as a percentage. The *arrow* indicates the point of addition of KNO_3, KCl or KCl plus glutamine. The irradiance was 450 µmol photons·m^{-2}·s^1. Values are the average of three independent measurements.

control treatments (Fig. 1). All these effects on NR activity were observed in both the EDTA and $MgCl_2$ assays in the untransformed plants (Fig. 1) but were never observed in the transformed line (Fig. 2), suggesting that the change in activity resulted from effects at the transcriptional level. The NR activity was not modified by the supply of glutamine and was not distinct from the basal level of the KCl treatment (Figs. 1, 2).

The modulation of NR activity by NO_3^- was also observed in maize plants (Fig. 3A–D). Feeding excised low-N-grown maize leaves with NO_3^- (before or after illumination) resulted in an increase in the total extractable NR activity compared to the KCl control. This increase of NR activity was clearly evident 2–3 h after the addition of NO_3^- in the light (Fig. 3).

Modulation of NR activation state by nitrate and light. Nitrate increased the total NR activity of untransformed tobacco leaf discs but did not change the NR activation state in either the untransformed or transformed plants (Figs. 1C, F, I; 2C, F). Similarly, glutamine had no effect on the NR activation state (Figs. 1, 2). Illumination caused an increase of the NR activation state in both tobacco (Fig. 7) and maize leaves (Fig. 3E, F) but the subsequent addition of NO_3^- did not modify the activation state attained in the light. The response to light was always the same regardless of whether NO_3^- was supplied before or after illumination (Fig. 3E, F). The modulation of NR activation state was a rapid process detected soon after the onset of illumination.

Effect of phosphatase inhibitor on the modulation of NR activation state by light and nitrate. Protein phosphatase inhibitors such as microcystin-LR (MC) can be used to modify the phosphorylation state of NR in situ since they disrupt the balance between protein phosphatase and protein kinase activities. Microcystin-LR causes an increase of phosphorylation state of phosphorylated proteins such as NR. In the following experiments the application of MC to excised leaves allowed an indirect estimation of the NO_3^- effect on NR protein phosphorylation and indicated the response of the NR kinase to the effector NO_3^-. When MC was fed to excised maize leaves 1 h after the light was switched on (Fig. 4A–C) the NR activation state fell very quickly to the same level as was found in the dark (Fig. 4C). A subsequent addition of NO_3^- did not modify the effect of MC (Fig. 4C). The pretreatment with NO_3^- before the addition of MC (Fig. 4D–F) shows the absence of a stimulatory effect of NO_3^- on NR protein kinase more clearly. No differences were observed between the KNO_3 and KCl treatments (Fig. 4F). The addition of MC decreased not only

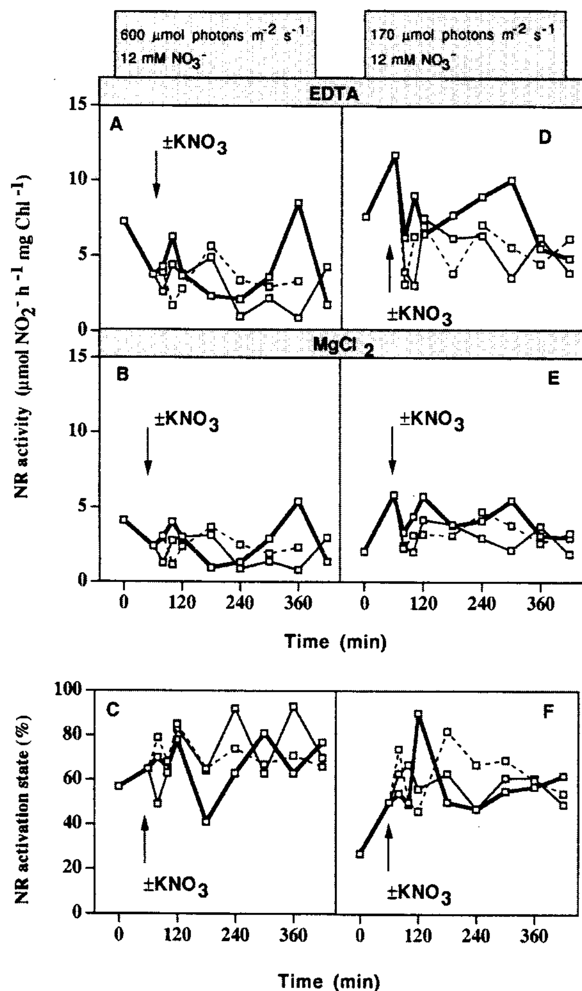

Fig. 2A–F. The effect of 30 mM KNO_3 (*bold line*), 30 mM KCl (*dashed line*) and 30 mM KCl plus 100 mM glutamine (*thin line*) on the extractable activities of NR in leaf disks of the transformed *Nicotiana plumbaginifolia* expressing a constitutive NR and grown with different levels of light as indicated. All other details were as in Fig. 1

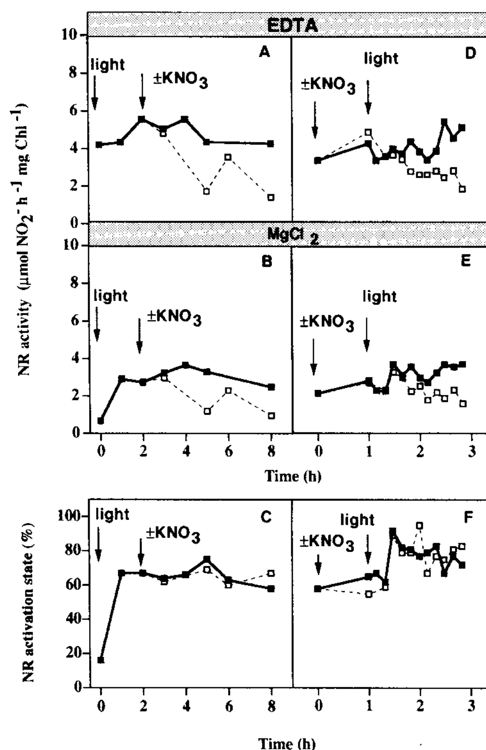

Fig. 3A–F. The effect of 30 mM KNO_3 (*bold line*) and 30 mM KCl (*dashed line*) on the extractable activities of NR in excised maize leaves growth with a low level of NO_3^- (0.8 mM NO_3^-). All other details were as in Fig. 1 except that the irradiance was 600 µmol photons$\cdot m^2 \cdot s^1$ and KNO_3 was supplied before (A–C) or after (D–F) the light, as indicated by the *arrows*. Values are the average of three independent measurements

the NR activation state but also the total extractable NR activity (Fig. 4 compared with Fig. 3).

Relationship between the foliar NO_3^- content and the NR activation state. Maize leaves fed with NO_3^- had increased NO_3^- levels (Fig. 5A). The addition of MC decreased NO_3^- uptake from the nutrient solution (Fig. 5A, B). There was no correlation between foliar NO_3^- content and NR activation state (Fig. 6).

Diurnal rhythm in the NR activation state. The NR activation state of attached leaves varied during the photoperiod as well as following the transition to darkness (Fig. 7). Maximum NR activation was observed soon after the onset of illumination (after 2 h of illumination). It decreased thereafter until the end of the photoperiod. The NR activation state decreased during the dark period with a minimal value at the end of the night (Fig. 7). The NR activation state was always slightly less in the transformed plants than in the untransformed controls throughout the day/night cycle.

Discussion

Nitrate-induced regulation of NR transcription is well characterised (Cheng et al. 1986, 1992; Vincentz et al. 1993) but the NO_3^--induced modulation of the NR activation state has not been demonstrated (Huber et al. 1992; de Cires et al. 1993). Nitrate enhances the light-modulation of enzymes of C metabolism such as PEPCase and SPS but the data presented here clearly show that the NR activation state is not influenced in maize or tobacco by NO_3^-. The only mechanism of NO_3^--mediated induction of NR activity occurs via changes in turnover. Hence, any increase in the NR activity induced by NO_3^- is due to changes in NR mRNA and protein levels or both. Our results support previous observations which suggest that the short-term light modulation of NR is not influenced by exogenous nitrate (Huber et al. 1992a; de Cires et al. 1993; Foyer et al. 1996). Nitrate reductase protein and NR activity have been shown to decrease more quickly than the NR mRNA when plants are deprived of NO_3^- (Galangau et al. 1988). This post-transcriptional effect of NO_3^- on NR protein turnover probably occurs simultaneously with transcriptional control since N-deprivation conditions caused marked decreases in NR

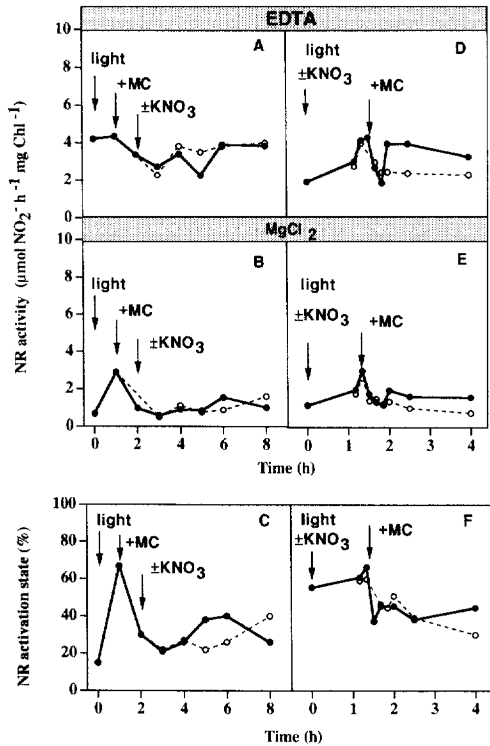

Fig. 4. The effect of 30 mM KNO_3 (*bold line*) and 30 mM KCl (*dashed line*) in the presence of 10 µM microcystin-LR (*MC*) on the extractable activities of NR in excised maize leaves grown with a low level of NO_3^- (0.8 mM NO_3^-). All other details were as in Fig. 1 except that the irradiance was 600 mol · photons · $m^{-2} \cdot s^1$ and MC was supplied before (Fig. 4A–C) or after (Fig. 4D–F) KNO_3 as indicated by the *arrows*. Values are the average of three independent measurements

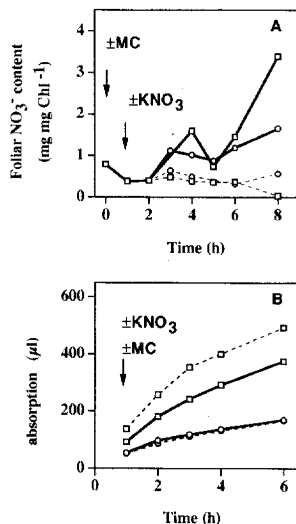

Fig. 5A, B. The kinetic of NO_3^- accumulation (**A**) and the volume uptake (**B**) by excised maize leaves in the presence (□) or absence (○) of microcystin-MR (*MC*) and in the KCl control (*dashed line*)

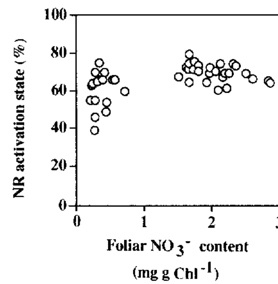

Fig. 6. The influence of foliar NO_3^- content on the NR activation state (estimated by the ratio of NR activity measured in the presence of 50 mM Mg^{2+} to that in the presence of 4 mM EDTA and expressed as a percentage) in excised maize leaves

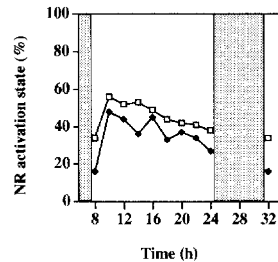

Fig. 7. Diurnal variation of the NR activation state (estimated by the ratio of NR activity measured in the presence of 5 mM Mg^{2+} to that in the presence of 5 mM EDTA and expressed as a percentage) in attached leaves of the untransformed (□) and transformed (◆) *Nicotiana plumbaginifolia* expressing a constitutive NR. The shaded areas on the figures represent the dark period

activity in both the 35S-NR expressors and in untransformed controls (Ferrario et al. 1995).

The apparent insensitivity of the NR activation state to exogenous NO_3^- in the presence of microcystin-LR, a phosphatase inhibitor, suggests that the NR-protein kinase is not regulated by NO_3^-. Hence, the regulation of the NR activation differs markedly from that of PEPCase and SPS. Phosphorylation of NR does not, by itself, cause inhibition of NR activity. Inhibition requires the binding of a second protein, NIP. Hence, the kinase may be activated by NO_3^- but if NIP is not present or does not bind to the protein then no loss of activity will occur. Light modulation of NR and SPS activities in spinach differ in terms of their relative sensitivity to the products of photosynthesis or to salts (Huber et al. 1992b, 1994). Despite other similarities in the post-transcriptional regulation of NR and SPS the possibility of one (or more) common protein kinase(s) remains open (McMichael et al. 1995). Perhaps other regulatory factors act in synergy with protein phosphorylation to allow precise regulatory responses.

Glutamine has been shown to increase PEPCase activity (Thi Manh et al. 1993; Foyer et al. 1996), indicating that the NO_3^- effect may be mediated by a product of N assimilation. Glutamine had no effect on the activation state of NR in the light in our experiments. Glutamine is known to repress the transcription of the NR gene but it had no observable effect on the total extractable NR activity within the timescale of our experiments. The light activation of NR cannot be related to a product of N assimilation such as glutamine.

This agrees with the hypothesis that the NR protein kinase may more likely be modulated by a product of C metabolism than by one derived from N metabolism (Huber et al. 1993; Kaiser and Huber 1994). The conditions necessary to promote the maximum activation/inactivation changes in NR activity are light/dark transitions (Huber et al. 1992a), CO_2 deprivation (Kaiser and Brendle Behnish 1991) and anoxia in roots (Glaab and Kaiser 1993). All these conditions induce a break in photosynthesis and the depletion of C assimilates. Moreover, an exogenous supply of hexoses to excised barley leaves increases the activation state of NR (de Cires et al. 1993). In attached tobacco leaves the NR activation state decreased from the beginning to the end of the photoperiod. A diurnal rhythm in the NR activation state may be superimposed on the circadian rhythm of gene expression as previously suggested for spinach (Huber et al. 1992b). The diurnal modulation of the NR activation state appears to be more related to the carbohydrate status of leaves than to the amino acid status. It is not possible, however, to correlate the changes in the NR activation state during the light/dark cycle with the observed changes in N compounds or C compounds or the N/C ratio.

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