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FOCUS PAPER

Mechanism and importance of post-translational regulation of nitrate reductase

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Abstract

In higher plants, nitrate reductase (NR) is inactivated by the phosphorylation of a conserved Ser residue and binding of 14-3-3 proteins in the presence of divalent cations or polyamines. A transgenic *Nicotiana plumbaginifolia* line (S_{521}) has been constructed where the regulatory, conserved Ser 521 of tobacco NR (corresponding to Ser 534 in *Arabidopsis*) was mutated into Asp. This mutation resulted in the complete abolition of activation/inactivation in response to light/dark transitions or other treatments known to regulate the activation state of NR. Analysis of the transgenic plants showed that, under certain conditions, when whole plants or cut tissues are exposed to high nitrate supply, post-translational regulation is necessary to avoid nitrite accumulation. Abolition of the post-translational regulation of NR also results in an increased flux of nitric oxide from the leaves and roots. In view of the results obtained from examining the different transgenic *N. plumbaginifolia* lines, compartmentation of nitrate into an active metabolic pool and a large storage pool appears to be an important factor for regulating nitrate reduction. The complex regulation of nitrate reduction is likely to have evolved not only to optimize nitrogen assimilation, but also to prevent and control the formation of toxic, and possibly regulatory, products of NR activities. Phosphorylation of NR has previously been found to influence the degradation of NR in spinach leaves and *Arabidopsis* cell cultures. However, experiments with whole plants of *N. plumbaginifolia*, *Arabidopsis*, or squash are in favour of NR degradation being the same in light and darkness and independent of phosphorylation at the regulatory Ser.

Key words: Degradation, nitrate reductase, phosphorylation, post-translational regulation.

Introduction

In higher plants, NR is rapidly inactivated/activated by phosphorylation/dephosphorylation (Fig. 1) in response to environmental stimuli and various treatments. The adjustment of NR activity by a post-translational mechanism takes place in only 5–20 min, depending on the species (Riens and Heldt, 1992; Provan and Lillo, 1999; Lillo *et al*., 2003). Sugars, cytosolic acidification, and anaerobiosis are factors all known to activate NR in both leaves and roots (Provan and Lillo, 1999; Kaiser and Huber, 2001). In leaves, the regulation of NR is closely coupled to photosynthesis, and post-translational inactivation of NR takes place when light intensity is suddenly decreased or the leaves are deprived of CO_2 (Kaiser and Brendle-Behnisch, 1991; Provan and Lillo, 1999). The close coupling of NR regulation to photosynthesis may be important in order to avoid the accumulation of the product of the NR reaction, NO_2^-. The reduction of NO_2^- to ammonium, the next step in the process of incorporating inorganic nitrogen into organic compounds, needs reduced ferredoxin, a product of photosynthesis. An abrupt stop or decrease in photosynthesis would, therefore, limit further assimilation, and could lead to the accumulation of NO_2^- unless NR activity was rapidly down-regulated. Indeed, accumulation of NO_2^- is observed in transgenic *Nicotiana plumbaginifolia* where post-translational regulation is abolished by site-directed mutagenesis, and plants are kept in darkness with a high nitrate supply (Lillo *et al*., 2003; Lea *et al*., 2004). For roots it is less obvious what physiological events should lead to changes of NR activity.

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It has been shown that under sudden anoxia NR is activated, and that NO_2^- accumulates in root tissue and/or is excreted (Kaiser and Huber, 2001). Interestingly, tobacco plants devoid of root NR activity showed a higher sensitivity to anoxic conditions associated with a higher rate of ethanol and lactate formation in the roots (Stoimenova *et al*., 2003). This suggests that the reduction of nitrate into nitrite, linked to the oxidation of NADH to NAD, may be an important reaction in anoxic roots under conditions when respiration cannot recycle NAD^+, although the precise role of NR in these conditions remains largely to be determined.

Serine phosphorylation, 14-3-3 proteins

More than ten years ago it was shown for higher plants that NR is inactivated by phosphorylation, and activated by dephosphorylation (Kaiser and Spill, 1991; MacKintosh, 1992). The phosphorylation site is located to a special motif (R/K-S/T-X-pS-X-P) in the hinge 1 between the Mo-cofactor binding domain and the haem binding domain. The regulatory Ser is Ser 534 in *Arabidopis*, Ser 543 in spinach, and Ser 521 in tobacco (Douglas *et al*., 1995; Bachmann *et al*., 1996*b*; Su *et al*., 1996; Lillo *et al*., 2003). However, the system is more complex than simply phosphorylation and dephosphorylation because members of the 14-3-3 protein family also bind to phosphorylated NR (reviewed by Kaiser and Huber, 2001; MacKintosh and Meek, 2001; Huber *et al*., 2002). 14-3-3 proteins belong to a highly conserved protein family with regulatory roles in plant, fungal, and mammalian cells (MacKintosh and Meek, 2001; Huber *et al*., 2002). It is after the binding of these 14-3-3 proteins to the phosphorylated Ser that NR is actually inhibited, and inhibition is only observed in the presence of cations. The most important cations for NR inhibition *in situ* are likely to be Mg^{2+} and polyamines (Provan *et al*., 2000).

Ion-exchange chromatography of spinach leaf extracts revealed three peaks of kinase activity using a peptide derived from the sucrose-phosphate synthase (SPS) phosphorylation site as the substrate (McMichael *et al*., 1995; Douglas *et al*., 1997). The third kinase peak was sometimes found only to be specific for SPS (McMichael *et al*., 1995), while other studies showed that all three kinase peaks were able to inactivate NR (Douglas *et al*., 1997). The first and second kinase activity peaks are modulated by calcium and the latter may correspond to a CDPK (Douglas *et al*., 1997). The third kinase peak is calcium independent and is probably a member of the plant family of yeast SNF1 kinase homologues (Douglas *et al*., 1997; Sugden *et al*., 1999). Phosphorylated NR is reactivated by a PP2A protein phosphatase (MacKintosh and Meek, 2001). Photosynthesis clearly induces activation of NR, but the signal transduction chain(s) linking processes in the chloroplasts to activities of kinases and phosphatases acting on NR in the cytosol is still unknown. Although depriving leaf tissue of CO_2 in the light will inactivate NR, the regenerative part of the Calvin cycle is apparently not necessary for light-activation of NR as shown using inhibitors of photosynthesis with cut barley leaves or protoplasts (Provan and Lillo, 1999). Rather, the reductive part of the Calvin cycle by which glycerate-3-P is converted to triose-P appears to be important; however, this needs further investigation.

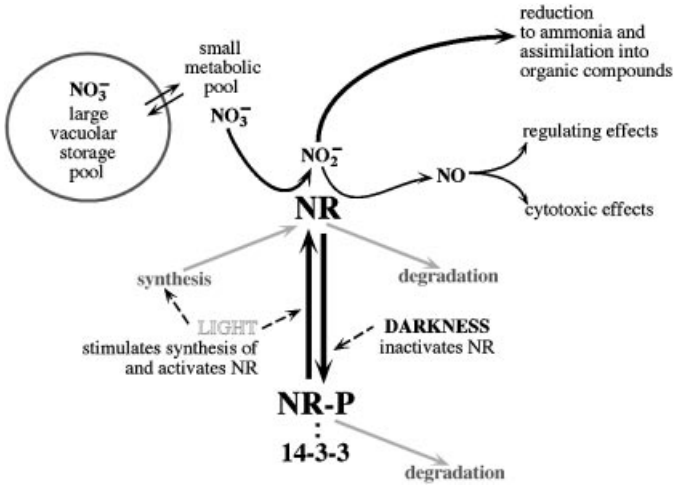

Fig. 1. Simplified scheme for regulation of nitrate reductase activity. Nitrate reductase (NR) is generally active in the light, and the main reaction catalysed is the reduction of NO_3^- into NO_2^-. Nitrite is then further reduced and assimilated in the chloroplasts. Nitrate is partioned between an active metabolic pool in the cytosol and a storage pool in the vacuole. This compartmentation can be important for the regulation of nitrate reduction since the vacuolar nitrate is not readily available for reduction, and therefore restricts substrate availability for the NR enzyme (US Lea *et al*., unpublished data). In darkness NR is phosphorylated on a regulatory Ser residue in the hinge 1, and then binds 14-3-3 proteins and is inactive. Light induces dephosphorylation and thereby activation of NR. The balance between activities of kinases and phosphatases acting on NR and NR-P determines the phosphorylation status of the enzyme. NR(-P) is degraded at a constant rate in light as well as darkness, independently of post-translational phosphorylation of the regulatory Ser. The higher level of NR protein in the light appears to be caused by the positive effects of light on NR synthesis, transcription, and translation. NR also catalyses a side-reaction which converts nitrite into nitric oxide (NO). Nitric oxide has regulatory as well as toxic effects in plants. The main reaction of NR (nitrite formation) as well as the side reaction (nitric oxide formation) are regulated by phosphorylation of the regulatory Ser in hinge 1 of NR.

Why post-translational regulation? Nitrite toxicity

Although the effects of nitrite on the formation of mutagenic (and carcinogenic) compounds have been much studied in mammals (Bartsch *et al*., 1992; Sugimura, 2000), the influence of such nitrite-derived compounds in plants has not been investigated as much. Certainly, similar reactions between nitrite and the large diversity of organic compounds present in plants are as

likely to result in mutagenic compounds as in mammals. If the various steps of nitrate assimilation are not properly co-regulated this could result in the temporary accumulation of nitrite and a higher mutation frequency than desirable. In the evolution of plants, nitrite accumulation is likely to have been eliminated by introducing proper regulation of the pathway; post-translational inactivation of NR being an important mechanism. A short-term increase of nitrite in the plant will not visibly influence the plant, however, repeated exposure to nitrite could lead to an unfavourably high frequency of mutations. Nitrite ions also present a more acute toxicity toward the photosynthetic apparatus especially in their acid form, nitrous acid, which can diffuse freely across membranes (Sinclair, 1987). Nitrite can also lead to the formation of NO (Fig. 1) which can further react with active oxygen species to produce peroxynitrite; a strong oxidant able to nitrate tyrosine residues and thus to modify protein activities (Morot-Gaudry-Talamain *et al*., 2002). Recently, it was shown that post-translational regulation of NR is important for avoiding the accumulation of nitrite under certain growth conditions (Lillo *et al*., 2003). In the transgenic *N. plumbaginifolia* S_{521} line the regulatory serine in the hinge 1 of NR was changed by site-directed mutagenesis. When these plants were irrigated with a high concentration (150 mM) of KNO_3 for about 1 week, nitrite accumulated during the night, but disappeared again after about 1 h of high light intensity in the morning. This disappearance of nitrite in the light is linked with the onset of photosynthesis, which generates reduced ferredoxin used by nitrite reductase, and therefore stimulates further metabolism of nitrite. Chlorosis of young leaves developed in the S_{521} mutant after about 3 weeks. The importance of the regulatory serine for the avoidance of nitrite accumulation is clearly demonstrated when cut roots or leaves of *N. plumbaginifolia* are placed in a buffer with 50 mM nitrate and kept in the dark. Only roots from the transgenic S_{521} continue to excrete nitrite during the 5 h incubation time (Fig. 2), and only in S_{521} plants was the activity state (percentage of active enzyme) high during the whole period (Lea *et al*., 2004). In leaves this was even more clear; nitrite excretion was negligible after 1 h in all lines tested except for S_{521} for which nitrite excretion continued at a high rate for at least 5 h (Lea *et al*., 2004).

A special enzyme, nitric oxide synthase, is responsible for NO formation in mammals. In plants, NO also appears to be synthesized from NO_2^- with the help of NR. In agreement with NR being involved in NO formation in plants, post-translational modification of NR modulates NO production (Rockel *et al*., 2002). NO production depends also on the level of nitrite (Morot-Gaudry-Talamain *et al*., 2002). NO is known to be mutagenic in *Salmonella* and assumed to be mutagenic in mammals and leads to the production of peroxynitrite (Wink *et al*., 1991; Grisham *et al*., 2000). In addition to harmful effects, NO

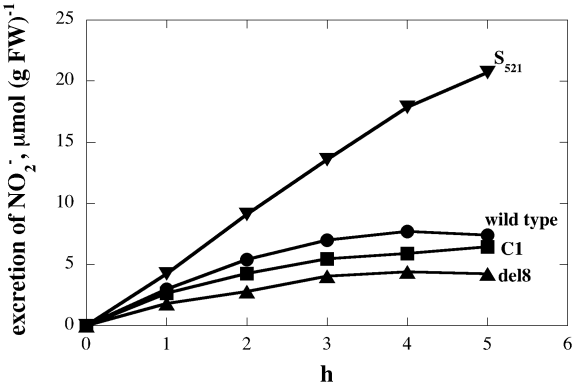

Fig. 2. The regulatory serine is necessary for cessation in nitrite excretion. *Nicotiana plumbaginifolia* was grown in perlite overlaid with a thin layer of soil and supplied with Hoagland solution containing 15 mM KNO_3 at sowing (Lillo *et al*., 2003). Roots (0.2 g) were submerged in 2 ml 50 mM KNO_3. Samples were removed from the liquid surrounding the roots and tested for nitrite every hour. The S_{521} line which is not post-translationally regulated with respect to NR showed a much higher rate of nitrite excretion than the other lines. The data are means from five different experiments and SE was less than 15%.

also acts as a signal molecule, influencing growth and development, and promoting stomatal closing (Beligni and Lamattina, 2001; Garcia-Mata and Lamattina, 2003). The accumulation of nitrite may, therefore, not only result in cytotoxic effects, but also influence growth and development. NO emission from leaves and roots of *N. plumbaginifolia* was higher in plants when the post-translational regulation of NR was abolished (Lea *et al*., 2004). NR is not the only source of NO synthesis in plants. It has been shown very recently that *Arabidopsis* possesses a nitric oxide synthase gene, which was discovered through its homology to a snail enzyme, implicated in NO production (Guo *et al*., 2003). Before that, another plant NO synthase gene was identified as a variant of the P protein of the glycine decarboxylase complex (Chandok *et al*., 2003). The question which remains open is, is the NO produced by NR actually biologically active and if not, is there any mechanism which could possibly allow the plants to discriminate between the different sources of NO production?

N-terminal end

Although most of the NR amino acid sequence is well conserved among eukaryotes this is not the case for the N-terminal end. In fungi the N-terminal end varies in length from 7 (*Ustilago maydis*) to 121 amino acids (*Neurospora crassa*), and comprises no common feature (Nussaume *et al*., 1995). In higher plants the N-terminal end varies from 60 (bean) to 99 (spinach) amino acids, and the sequences of this region are very variable. However, they are largely hydrophilic and all have a region with

acidic amino acids (Nussaume *et al*., 1995). Since this region shows great variation among species it is unlikely to be essential for the function of the enzyme, but is likely to have a role in regulation. This region was deleted from the tobacco NR gene (*Nia2*) and the resulting truncated coding sequence was expressed in the NR-deficient E23 mutant of *N. plumbaginifolia*. It was then found that the dark-inactivation of NR in these transgenic plants (del8) was reduced, compared with wild-type plants when NR activity was measured *in vitro* (Nussaume *et al*., 1995). However, when partially purified NR was examined, the truncated enzyme could still bind 14-3-3 proteins, and was inactivated *in vitro* to the same extent as the full-length enzyme (Provan *et al*., 2000). The truncated enzyme showed some differences from the full-length enzyme, like poorer stability and a different pH optimum *in vitro*. All these properties could be ascribed to the conserved acidic domain present in the NR N-terminal region since a deletion of this acidic motif resulted in a truncated NR enzyme with the same properties as the NR deleted of the complete N-terminal domain (Pigaglio *et al*., 1999). Interestingly, the del8 plants behaved like wild type and C1 plants when assaying nitrite excretion from cut roots and leaves, indicating that post-translational regulation is functioning in these plants *in situ* (Fig. 2; Lea *et al*., 2004). However, in the del plants, the rate of nitrate reduction was less affected by CO_2 removal than in the wild-type plants (Lejay *et al*., 1997). This suggests that different mechanisms could be involved in NR inactivation in the dark as opposed to illuminated plants exposed to low CO_2 concentrations where there is an excess of reducing power but less sugars. Indeed, in the former, NR is clearly inactivated by phosphorylation of the Ser 521 residue since, when this residue is mutated, NR activity is no longer inhibited. In the latter case, one could envisage another mechanism of inactivation in the presence of light and the absence of sugars in which the N-terminal domain would have an important role. It is known that several kinases phosphorylate NR and these could have specific roles for the inactivation of NR in response to different factors such as the absence of reducing power and sugars. For instance the plants Snf1 homologues, of which the third NR kinase peak is a member, may respond more specifically to variations in sugar levels. The fact that fungal NRs are devoid of the N-terminal extension supports the hypothesis that this region may participate in the inactivation of NR when CO_2 fixation, which is lacking in these organisms, is non-functional.

Diurnal variations

Nitrate reductase expression varies during the day and night (Lillo *et al*., 2001). The highest activity of NR is generally observed during the first part of the photoperiod, NR activity then declines during the latter part of the photoperiod and dark period. NR mRNA starts to increase towards the end of the night in many plants, and this indicates that NR expression is controlled by a circadian clock, and that the up-regulation of NR expression is preparing the plant for efficient nitrogen metabolism the next day. Work with *N. tabacum* has previously shown that the number of *Nia* genes per plant will influence expression of the gene, NR activity per gene is higher in plants with one or two *Nia* genes compared with wild-type plants which has four genes (Scheible *et al*., 1997). This difference is probably caused by weakened negative feedback from N-assimilation products in the plants having only one NR gene. Scheible *et al*. (1997) also showed that diurnal post-translational regulation was adjusted so that plants with fewer NR genes compensated for this by keeping more of the NR enzyme in its active form during the dark period.

When the NR gene is linked to the cauliflower mosaic virus 35S promoter the NR gene is constitutively expressed, and NR mRNA is high during both the day and night (Vincentz and Caboche, 1991). Diurnal variations of NR activity are still clearly pronounced in the plants (C1) although the gene is constitutively transcribed (US Lea *et al*., unpublished results). Diurnal variations are most pronounced in the presence of Mg^{2+} which gives a measure for the NR activity *in situ* (only the non-phosphorylated NR is active in the $+Mg^{2+}$ assay). Post-translational phosphorylation contributed strongly to the diurnal variations of NR activity in wild-type as well as C1 plants. When the NR gene was mutated either by a deletion in the N-terminal end (Nussaume *et al*., 1995) or by mutating the Ser 521 (Lillo *et al*., 2003), and the gene was linked to the 35S promoter, diurnal variations in NR activity were severely dampened (US Lea *et al*., unpublished results). The maximal variations in actual NR activity during a day and a night was 400% in the wild type, 300% in C1, 67% in del8, and 51% in S_{521} (US Lea *et al*., unpublished results).

Mg^{2+}, Ca^{2+}, polyamines

Divalent cations can influence NR activity by different means. For example, in spinach, three different kinases phosphorylate NR which leads to the inactivation of the enzyme. Two of these kinases depend on Ca^{2+} for activity (Douglas *et al*., 1997; MacKintosh and Meek, 2001; Kaiser and Huber, 2001). Calcium fluxes in the cell may, therefore, result in changes in NR kinase activity and thereby influence the phosphorylation state of NR. Cations are also important in the activation of the already phosphorylated NR. In addition to 14-3-3 proteins, Mg^{2+}, Ca^{2+}, or polyamines have repeatedly been shown to be necessary for the inactivation of NR, and the effects of these ions on NR inactivation are complex (Provan *et al*., 2000; Kaiser and Huber, 2001).

A subset of the 14-3-3 isoforms in *Arabidopsis* have been found to contain an EF hand-like cation-binding domain, close to the C-terminal end (Lu *et al*., 1994; DeLille *et al*., 2001). When specific amino acids of the GF14ω isoform, which belongs to this subset, were mutated, it was found that the inhibition of NR activity was decreased (Athwal and Huber, 2002). The binding constant for Ca^{2+} is 5.5×10^4 M^{-1} for the GF14ω isoform (Lu *et al*., 1994), and this implies that the expected I_{50} (concentration for half-maximal effect) for Ca^{2+} would be in the micromolar range (18 μM). The I_{50} values reported for Mg^{2+} and Ca^{2+} inhibition *in vitro* are in the mM range (Provan *et al*., 2000; Athwal and Huber, 2002), and this indicates that the effect of Mg^{2+} and Ca^{2+} on the inhibition of NR is not explained by binding of these ions to 14-3-3 only. It is interesting to note that the GF14psi and GF14phi isoforms contain the EF-hand like cation domain, yet apparently show no affinity towards NR (Bachmann *et al*., 1996*a*; DeLille *et al*., 2001).

It is probable that an interacting site for divalent cations is also present on the NR enzyme itself. Squash NR is phosphorylated and inhibited by 14-3-3 proteins and divalent cations, equivalent with other plant NR (Lillo *et al*., 1997). In addition, for purified squash NR, strong inhibitory effects of Mg^{2+} can also be demonstrated in the absence of 14-3-3 proteins. Squash NR was inhibited 70–90% by 5 mM Mg^{2+} or Ca^{2+} (and no 14-3-3s), but this kind of inhibition was eliminated when the enzyme was preincubated with the substrates, NADH and NO_3^- or by the addition of thiol compounds (Lillo, 1993). This probable redox control by reduced molecules (NADH or thiol compounds) could provide a link between nitrate reduction and photosynthesis. Effects of Mg^{2+} independently of phosphorylation and 14-3-3 proteins were also found for *Ricinus communis* (Tsai *et al*., 2003). For the inactivation of *R. communis* NR, the inhibition depended on the addition of desalted crude extract from the same plant. Altogether, results with these various plants strongly suggest that Mg^{2+} and Ca^{2+} interact directly with NR, although the mechanism, as well as the physiological implications, are obscure. The conserved and negatively charged N-terminal acidic domain may be involved in cation binding and subsequent NR modulation.

Phosphorylation and importance for degradation of NR

Protease activity, inactivating or degrading NR *in vitro* has been frequently reported, but the exact way that initiation of NR degradation takes place *in vivo* is still unknown (Callis, 1995; MacKintosh and Meek, 2001). Proteolysis is also likely to be influenced by the metabolic and developmental status of the plant, and influence of 14-3-3 proteins and phosphorylation status can only be fully understood when the proteases acting on NR are known. Attempts to test for the possible involvement of ubiquitination and the proteasome in the degradation of *Arabidopsis* and spinach NR were negative (MacKintosh and Meek, 2001). In prolonged darkness or when nitrate is withdrawn from plants, NR protein and activity decline in plants. This decline could be caused by increased degradation of NR and/or decreased synthesis. Generally, NR is more phosphorylated in darkness than in the light and this has been suggested to be a signal for the degradation of the enzyme. However, work with different plant species has given contradictory results concerning the effects of phosphorylation on the degradation of NR. Kaiser and Huber (2001) showed in their experiments with spinach whole leaves and leaf discs that there was a correlation between low activity state (high degree of phosphorylation) and rapid degradation of NR. Another work has suggested that 14-3-3 proteins may promote NR degradation (Weiner and Kaiser, 1999). Conversely, in sugar-fed cell cultures of *Arabidopsis* high binding ability for 14-3-3 proteins protected many targets, including NR, against degradation (Cotelle *et al*., 2000), indicating that the phosphorylated form of NR is protected from degradation. Work with transgenic *N. plumbaginifolia* in which the NR gene was under the control of the 35S promoter, and hence constitutively transcribed in darkness as well as light, did not reveal any differences in NR degradation related to light/darkness (Lillo *et al*., 2003). In two transgenic lines which had very different activity states in darkness; i.e. high activity state (80%) for S_{521} (mutated at the regulatory serine), and low activity state (20%) for the control line C1, the decline in NR was the same in darkness (Lillo *et al*., 2003). Insignificance of NR activity state in relation to degradation of NR in *N. plumbaginifolia* was also confirmed by comparing NR degradation rates in the presence of tungstate in light and darkness. NR is a molybdenum-containing enzyme and tungstate, a molybdate analogue, has been known to inhibit the formation of active NR enzyme *in vivo* by preventing the incorporation of molybdenum (Deng *et al*., 1989; Wray and Filner, 1970). In the presence of tungstate, NR protein is still synthesized, but the NADH-nitrate reducing activity is defective. Since treatment of plants with tungstate inhibits the formation of new active NR, the decrease in NR activity in tungstate-treated plants reflects the actual rate of NR degradation. This is a method of studying NR degradation per se with no, or little, interference from *de novo* synthesis of the enzyme. Although activity state was high for wild-type as well as C1 plants in the light, and low in darkness, the decline in NR activity was the same in the light and darkness for these lines when fed with tungstate (Lillo *et al*., 2003). Similar experiments were performed with wild-type *Arabidopsis*.

Although NR is under the control of the light-inducible NR promoter in *Arabidopsis* which complicates the experiments, a rapid degradation rate of NR in light as

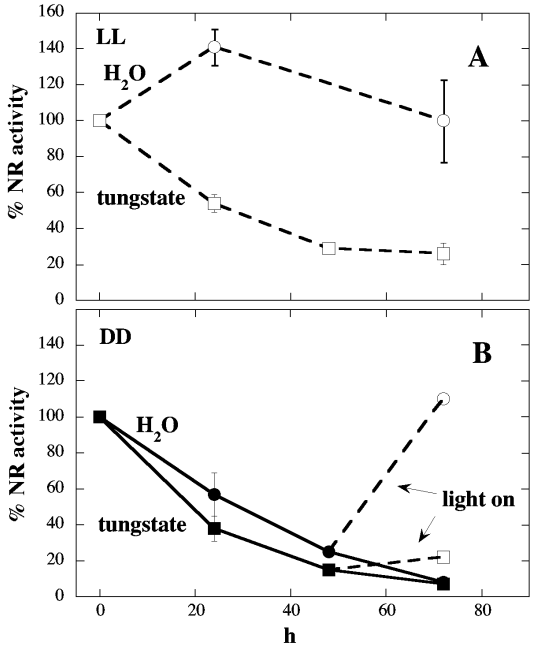

Fig. 3. NR is degraded in light and darkness. *Arabidopis thaliana* was grown in perlite overlaid with a thin layer of soil and supplied with Hoagland solution containing 15 mM KNO_3 at sowing (Lillo *et al*., 2003). After 3 weeks plants were irrigated with excess water or 1 mM tungstate (time zero) and kept in constant light (A) or darkness (B). NR activity (assayed in the presence of EDTA) declined rapidly in darkness in the presence or absence of tungstate. When kept in the light and given tungstate, NR activity also declined rapidly. After 48 h in darkness plants were transferred to light to test how efficiently tungstate inhibited the formation of new NR (Fig. 2B, broken lines). A small increase in NR activity was observed even when tungstate had been added. Hence tungstate addition did not completely block new synthesis of NR in *Arabidopsis*. New synthesis of NR therefore accounts for the slightly higher values of NR activity found in light/tungstate-treated plants compared with darkness/tungstate. Hence degradation rates were very similar in light and darkness for *Arabidopsis* as well as for *N. plubaginifolia* as shown previously (Lillo *et al*., 2003). At 48 h n=2, otherwise n=4 and SE is indicated with vertical bars when exceeding the size of the symbols.

well as darkness was confirmed (Fig. 3). As expected, NR activity in *Arabidopsis* was high in continuous light, and decreased rapidly in plants transferred to darkness. When plants were treated with tungstate, NR activity decreased almost as rapidly in light as in darkness. Activity state was 44% in plants treated with tungstate and kept in the light, but only 2% when kept in the dark (Oltedal, 2003). However, in *Arabidopsis*, the formation of new NR was not completely inhibited even in tungstate-treated plants because when plants were taken into light after 48 h of darkness, tungstate-treated plants showed a slight increase in NR activity, although very modest compared with plants treated with water only (Fig. 3B, broken lines). This modest formation of new NR activity accounts for the slightly higher NR activity in light compared with darkness. Similar experiments were performed with squash (F Provan, unpublished results), and when analysing a large set of data for squash no correlation between degradation rate and activity state was found.

The NR level is a result of synthesis and degradation, and light is well known to act positively on NR synthesis through increased transcription (Lillo, 1994). Light probably also acts positively on NR synthesis at the translational level. When such a positive factor is eliminated this can account for the declining NR protein levels even though NR degradation is the same as under NR-inducing conditions. In conclusion, it appears that in leaves of *N. plumbaginifolia, Arabidopis*, and squash the degradation rate of NR is not correlated with phosphorylation of the regulatory serine residue in hinge 1, and degradation rates are the same in light and darkness.

Perspectives

The post-translational control of NR by phosphorylation is important for controlling nitrite accumulation and subsequent deleterious effects in conditions where nitrite reduction is limited. The measured activation state of NR, which is supposed to reflect phosphorylation of the enzyme, is clearly not linked to the degradation rate which seems the same in all conditions. But there are still many open questions concerning NR inactivation by phosphorylation. What is the role of the N-terminal domain and of cations in NR inactivation? How is NR inactivated by 14-3-3 binding and what regulates this protein interaction? How are processes in the chloroplasts linked to activities of the kinases and phosphatases acting on NR? What is the role, if any, of NR in producing biologically active NO?

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