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Mutation of the regulatory phosphorylation site of tobacco nitrate reductase results in constitutive activation of the enzyme *in vivo* and nitrite accumulation

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Summary

In wild-type *Nicotiana plumbaginifolia* and other higher plants, nitrate reductase (NR) is rapidly inactivated/activated in response to dark/light transitions. Inactivation of NR is believed to be caused by phosphorylation at a special conserved regulatory Ser residue, Ser 521, and interactions with divalent cations and inhibitory 14-3-3 proteins. A transgenic *N. plumbaginifolia* line (S_{521}) was constructed where the Ser 521 had been changed by site-directed mutagenesis into Asp. This mutation resulted in complete abolishment of inactivation in response to light/dark transitions or other treatments known to inactivate NR. During prolonged darkness, NR in wild-type plants is in the inactivated form, whereas NR in the S_{521} line is always in the active form. Differences in degradation rate between NR from S_{521} and lines with non-mutated NR were not found. Kinetic constants like K_m values for NADH and NO_3^- were not changed, but a slightly different pH profile was observed for mutated NR as opposed to non-mutated NR. Under optimal growth conditions, the phenotype of the S_{521} plants was not different from the wild type (WT). However, when plants were irrigated with high nitrate concentration, 150 mM, the transgenic plants accumulated nitrite in darkness, and young leaves showed chlorosis.

Keywords: nitrate reductase, *Nicotiana*, phosphorylation, protein degradation, transgenic plant.

Introduction

Nitrate reductase (NR; EC 1.6.6.1) catalyses the rate-limiting, key step in nitrogen assimilation:

$$NADH + NO_3^- + H^+ \rightarrow NAD^+ + NO_2^- + H_2O.$$

Nitrate reductase is regulated at the transcriptional, translational and post-translational levels. Transcription of NR is enhanced by nitrate availability and light in higher plants (Lillo, 1994; Meyer and Stitt, 2001; Tischner, 2000). NR is known to be regulated post-translationally by phosphorylation on a conserved Ser residue; Ser 543 in spinach (Bachmann *et al.*, 1996; Douglas *et al.*, 1995) and Ser 534 in *Arabidopsis* (Su *et al.*, 1996), corresponding to Ser 521 in *Nicotiana tabacum*. In the presence of Mg^{2+} or spermidine, the phosphorylated form of NR interacts with 14-3-3 proteins, and NR activity is then inhibited (Athwal and Huber, 2002; Provan *et al.*, 2000). Activity state is a frequently used term for describing post-translational effects on NR, and is defined as NR assayed in the presence of Mg^{2+} and 14-3-3 proteins, as percentage of NR activity measured in the presence of EDTA (total activity). Activity state reflects how much of the enzyme is in the non-phosphorylated active form. Regulation of NR also involves subtle aspects. Although NR mRNA levels were not influenced by light/darkness or nitrogen source in transgenic plants expressing a 35S-NR construct (C1 plants), a three- to fourfold drop in NR protein and activity was seen after 60 h in the dark (Vincentz and Caboche, 1991). Light therefore appears to influence mRNA translation, or stability of the protein.

In the work presented here, three different lines of *Nicotiana plumbaginifolia* were tested: wild type (WT) with the original NR structural gene and inducible NR promoter; C1 with the original NR coding sequence linked to the constitutive cauliflower mosaic virus 35S promoter; and S_{521} with a mutated NR coding sequence linked to the 35S promoter. In S_{521}, the regulatory Ser was substituted by Asp. In the transgenic plants, regulation of NR on the transcriptional level was eliminated as previously shown (Vincentz and Caboche, 1991). Normal growth and development of C1

plants was demonstrated before (Vincentz and Caboche, 1991). Here we compare S_{521} with C1 and the WT.

Generally, NR is phosphorylated more in darkness than in the light, and this has been suggested to be important for the degradation rate of the enzyme; however, work on different plant species has given contradictory results concerning effects of phosphorylation. Kaiser and Huber (1997) showed in their experiments with spinach whole leaves and leaf discs that there was a correlation between low activity state and rapid degradation of NR. To obtain different activity states, the tissue was treated with either a 5′-AMP analogue, a respiration uncoupler, a weak acid or glucose, all compounds known to increase the activity state of NR. In conclusion, their work supported the assumption that the inactive phosphorylated form of NR was more rapidly degraded than the non-phosphorylated form. This work was further confirmed by using [^{35}S]methionine labelling of proteins and following degradation of the labelled NR during 2.5 h in light and darkness (Weiner and Kaiser, 1999). Furthermore, *in vitro* experiments indicated that binding of 14-3-3 proteins was important for degradation of NR. When 14-3-3 proteins were removed from the extract, NR was stable (Weiner and Kaiser, 1999). Conversely, in barley, extracts from plants in the light had a much more labile NR than extracts from plants in the dark had (Lillo and Henriksen, 1984). In cell cultures of *Arabidopsis*, 14-3-3 proteins protected many targets, including NR, against degradation (Cotelle *et al.*, 2000). It is therefore far from clear what effect phosphorylation may have on NR stability in different plants, or if there is a general relationship between phosphorylation state and degradation rate in all species. Tungstate, a molybdate analogue, has been known to inhibit formation of active NR enzyme *in vivo* by preventing incorporation of molybdenum (Deng *et al.*, 1989; Wray and Filner, 1970). In the presence of tungstate, NR protein is still synthesised, but the NADH-nitrate-reducing activity is defective. As treatment of plants with tungstate inhibits formation of new active NR, the decrease in NR activity reflects the actual rate of NR degradation. This is a method of studying NR degradation *per se* with no interference from the synthesis rate of the enzyme. Also, as transcription of genes linked to the 35S promoter is not influenced by light, the transgenic plants harbouring the 35S-NR constructs provide excellent tools to test for the effect of light on other steps in NR regulation, like degradation. In this work, we examine degradation of NR in S_{521}, C1, as well as in WT, to test the influence of activity state on NR stability in darkness and light. Although activity state was very different in S_{521} compared to the two other lines, we did not find significant differences in degradation rate.

Evidence for the involvement of the conserved Ser 521 residue in NR inactivation by phosphorylation is hitherto mainly based on *in vitro* experiments (Bachmann *et al.*, 1996; Douglas *et al.*, 1995), where phosphorylation of this residue was correlated with inactivation of the enzyme. But the real mechanism of NR inactivation and the actual role of Ser 521 *in situ* are not established so far. Moreover, the impact of the loss of NR inactivation on physiology and metabolism is also not known. We had previously shown that plants expressing an N-terminal-deleted NR protein did not present any differences in terms of nitrate assimilation rate in the dark, but that they had a higher nitrate assimilation rate when grown in the light in the absence of CO_2 (Lejay *et al.*, 1997). However, as we showed later, this deleted protein can still be phosphorylated and inactivated *in vitro* (Provan *et al.*, 2000). In this work, we have addressed the role of the conserved Ser 521 residue in NR regulation by changing it into Asp and expressing the mutated enzyme in plants. As the effect of de-regulation of NR on both the transcriptional and post-translational levels has not been tested previously in any plant, we wished to study how this de-regulation of NR would influence growth, development and biochemical parameters in the plants.

Results

NR activity during rapid light/dark transitions

Figure 1 shows changes in NR activity state and NR activity during short-term light-to-dark and dark-to-light transitions. In both WT (*Nia* promoter) and C1 (35S promoter), NR activity state fell from approximately 65 to 30% during 15 min of darkness. When the light was switched on again (after 45 min in darkness), NR activity state increased. For the S_{521} mutant, no significant variations in NR activity state was observed during light/dark/light transitions (Figure 1a). A small decrease in NR activity + EDTA was seen in response to light-to-dark transitions for all varieties (Figure 1b). NR activity + Mg^{2+} decreased by more than 70% for WT and C1, during 45 min of darkness, and increased again, although to a lesser extent, when the light was switched on. For the S_{521} mutant, decrease in darkness was only 15%, and no clear increase was observed after the light was switched on (Figure 1c).

Degradation of NR activity during prolonged darkness or light

Figure 2 shows the decrease in NR + EDTA activity during 48 h of darkness in the presence (a) or absence (c) of 0.5 mM tungstate in the irrigation water. The first sample was harvested 2.5 h into the light period (0 h darkness), then the light was switched off, and samples were taken after 24 and 48 h of darkness. In the presence of tungstate, NR activity decreased at the same rate in WT, C1 and S_{521} plants (Figure 2a), and reflects degradation of enzyme already present at the start of the experiment, as no new (active)

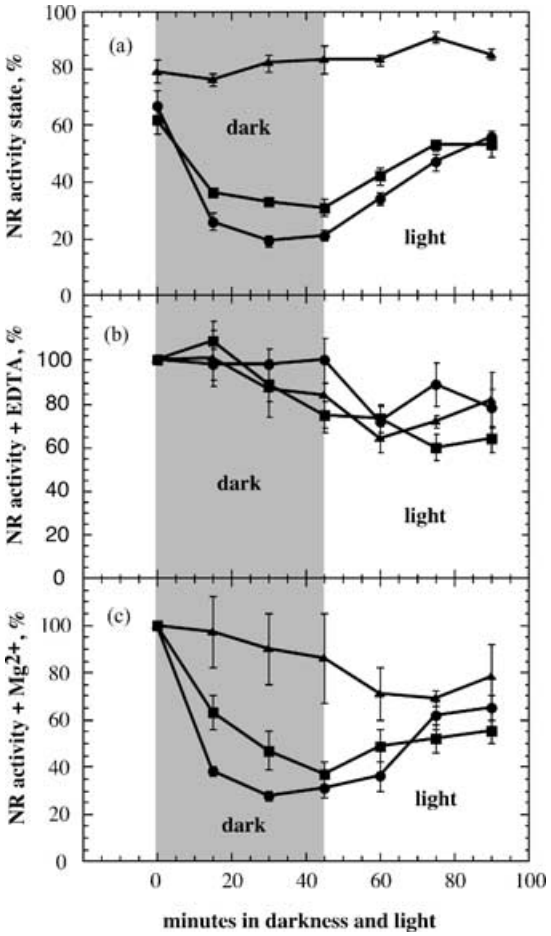

Figure 1. Changes in NR activity state and NR activity during light/dark transitions.
(a) NR activity state.
(b) NR activity assayed in the presence of EDTA.
(c) NR activity assayed in the presence of Mg^{2+}. Samples were harvested 2.5 h into the light period (time 0), then the light was turned off, and samples were harvested after 15, 30 and 45 min of darkness. The light was switched on again, and samples were taken after another 15, 30 and 45 min of light. Plants tested were WT (dots), C1 (squares) and S_{521} (triangles), respectively. The mean initial NR activity (+ EDTA assay) was for WT: 3.6, C1: 2.8 and S_{521}: 2.2 μmol NO_2^- g^{-1} FW h^{-1}. Because of variations among batches of plants, the experiments were repeated at least five times, and the data given are mean values. SE values are given by vertical bars. The results presented were obtained using the heterozygous S_{521}-1 line, but the results were confirmed with the two homozygous lines.

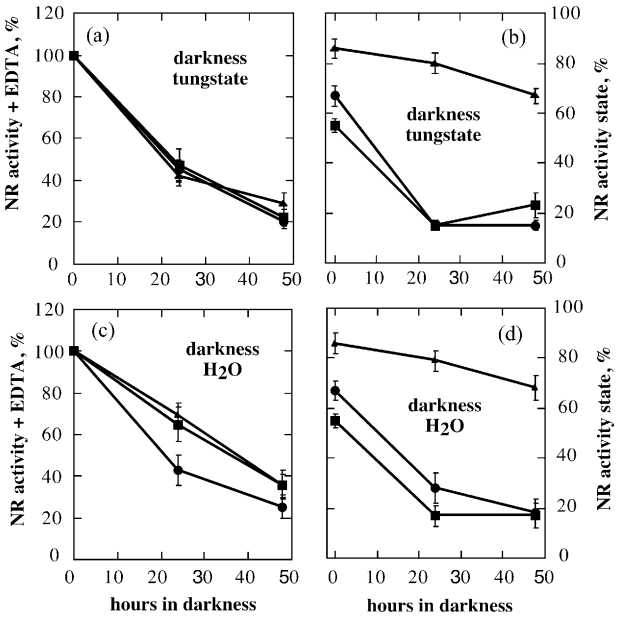

Figure 2. Degradation of total NR activity (EDTA NR) during 48 h of darkness, in the presence or absence of 0.5 mM tungstate.
The first sample was harvested 2.5 h into the light period (0 h darkness), then the light was turned off, and samples were taken after 24 and 48 h of darkness. Plants were irrigated with water or 0.5 mM tungstate before the light was turned off. The mean initial NR activity was set to 100%, and was for WT: 4.3, C1: 4.7 and S_{521}: 3.3 μmol NO_2^- g^{-1} FW h^{-1}. Symbols are as in Figure 1.
(a) EDTA NR activity in plants irrigated with 0.5 mM tungstate and transferred to darkness.
(b) NR activity state in plants irrigated with 0.5 mM tungstate and transferred to darkness.
(c) EDTA NR activity in plants irrigated with water and transferred to darkness.
(d) NR activity state in plants irrigated with water and transferred to darkness.
The data are the means of eight independent experiments. For S_{521}, four experiments represent data from the heterozygous S_{521}-1 and two each from the S_{521}-7 and S_{521}-5 lines. SE values are given by vertical bars.

enzyme is prepared (Deng *et al.*, 1989). Without tungstate, the observed decline in NR activity reflects the balance between synthesis and degradation of NR. In wild-type plants, the *Nia* structural gene is under control of the light-stimulated *Nia* promoter, and transcription is therefore restricted in darkness. On the other hand, in C1 and S_{521} lines, the NR gene is under control of the 35S promoter, hence constitutively transcribed. As expected, the NR activity level declined more slowly in C1 and S_{521} plants compared with that in the WT (Figure 2c). No difference was found between C1 and S_{521} plants regarding decline in NR activity. Activity state of NR in C1 plants in darkness was between 15 and 30% (Figures 1a and 2b,d), reflecting that most of the enzyme was phosphorylated and binding 14-3-3 proteins. On the other hand, activity state of NR in S_{521} was always high, i.e. approximately 80% (Figures 1a and 2b,d). As degradation rates of NR activity in C1 and S_{521} were very similar, activity state did not appear to influence degradation. The NR activity at the start of the experiment (time zero in Figure 2) varied among batches of plants from 2.6 to 6.1 μmol NO_2^- g^{-1} FW h^{-1}. The experiment was therefore repeated eight times. On a percentage basis, there were no indications that variations in the initial NR activity within the range mentioned above influenced degradation rates in these experiments, and the data are presented as mean values. These experiments also gave an estimation of NR activity in the S_{521} compared with C1 and WT. When harvested 2.5 h into the light period, maximal (EDTA) NR activity in S_{521} was 23% lower than that in WT and 30% lower than that in C1

plants, on an average. The amount of NR protein was measured by Western blots, and examples are presented in Figure 4. In this experiment, NR activity of S_{521}-7 plants decreased in water-treated plants from 100 to 52 and 21% after 24 and 48 h in darkness, and NR protein decreased to 42 and 29% after 24 and 48 h in darkness. For C1 plants, NR activity decreased from 100 to 64 and 36% in water-treated plants after 24 and 48 h of darkness, and NR protein decreased to 55 and 31%. It has previously been shown for WT and C1 plants that maximal (EDTA) NR activity was closely correlated with the level of NR protein under different growth conditions, including prolonged darkness (Vincentz and Caboche, 1991). As expected, such a correlation was confirmed here also for S_{521} plants. The same trend between the level of NR activity and the amount of NR protein was found when plants were watered with tungstate (Figure 4), which suggests that little new NR protein is produced in the dark. In the case of tungstate-treated plants, the total amount of NR protein is the sum of the newly synthesised NR containing tungstate (inactive), and of the molybdo-NR (active) already present at the start of the experiment. The decrease in the amount of molybdo-NR can thus be accurately measured by following the decrease in NR activity without interference of newly synthesised NR protein.

The insignificance of activity state in relation to degradation of NR was further confirmed when plants were kept in continuous light and given tungstate (Figure 3a,b). In continuous light, activity state was higher for WT and C1 as compared with darkness. However, degradation rates in light was not significantly different from those in continuous darkness (compare Figures 2a and 3a) showing that light/darkness or activity state had little influence on degradation of NR activity.

Kinetics and in vitro *stability of NR activity*

The degradation rate of NR activity in crude extracts was tested at 25 and 4°C at pH 7.5 and 8.5, but no differences

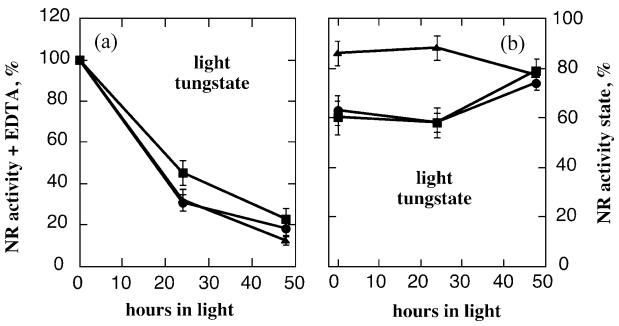

Figure 3. Degradation of total NR activity (EDTA NR) during 48 h of light in the presence of 0.5 mM tungstate.
(a) EDTA NR activity in plants irrigated with 0.5 mM tungstate.
(b) NR activity state in plants irrigated with 0.5 mM tungstate. Otherwise, as given in Figure 2.

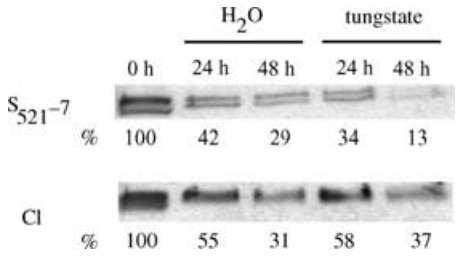

Figure 4. NR protein measured by Western blotting in S_{521}-7 and C1 during 48 h of darkness in the presence or absence of tungstate.
Plants were treated as described in Figure 2. Relative results of densitometer analysis are given below each blot, and the signal at the start of the experiments (0 h darkness) is set to 100%.

were found between mutated and wild-type NRs. Furthermore, no differences between the WT and the transgenic lines were found for K_m values of the substrates NADH or KNO_3. The pH optimum was also the same for mutated and wild-type NR, but the pH curves were slightly different. In the higher pH range, wild-type NR showed a sharper decrease. This was found when using crude extracts as well as Blue Sepharose purified enzyme.

Nitrite accumulation

As stated above, under normal growth conditions, S_{521} plants grew and developed as the WT or C1, and no special phenotype was apparent. However, when plants were irrigated with high concentrations of KNO_3, 100–150 mM, young S_{521} leaves turned yellow 3–4 weeks after onset of the new irrigation regimen, whereas this was not the case for C1 or WT plants (Figure 5). When irrigated with 150 mM KNO_3, the nitrite content in the S_{521} leaves started to increase slowly. After about 1 week, nitrite concentration was always high, about 100 nmol NO_2^- g^{-1} FW, as found repeatedly in four independent batches of plants (Figure 6). NR activity in these extracts was approximately 2 μmol NO_2^- g^{-1} FW h^{-1}, and did not change significantly throughout the experiment. WT and C1 plants did not accumulate nitrite. S_{521} plants given 150 mM KCl did not accumulate nitrite nor develop chlorosis. The results shown in Figure 6 refer to samples harvested in the morning, at the end of a 12-h dark period. When S_{521} leaves were harvested

Figure 5. Development of chlorotic leaves in transgenic *N. plumbaginifolia*. Wild type (right) and S_{521}-5 (left) were irrigated with 150 mM KNO_3 during 4 weeks.

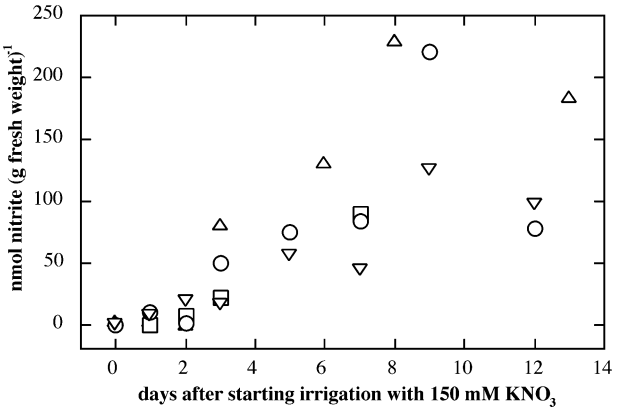

Figure 6. Nitrite accumulation in leaves of S_{521}.
After starting irrigation with 150 mM KNO_3, nitrite was measured in the leaf samples harvested in the morning at the end of a 12-h dark period during 12 days. Four independent batches of plants were followed: two for S_{521}-7 and two for S_{521}-5.

later in the day, after 3 h of light exposure, no nitrite accumulation was detected (results not shown). Indeed, disappearance of the nitrite occurred rather rapidly during about half an hour after onset of illumination (Figure 7). When light was turned off in the middle of the day, nitrite started to accumulate and reached high levels, like those found in the morning, after 1 h (Figure 7).

Discussion

Mutations of the Ser within the binding motif of 14-3-3 in hinge 1 of NR have not been tested *in planta* previously. In the *N. plumbaginifolia* S_{521} line, Ser 521 was changed into Asp. Although another mutated form of NR, i.e. Ser substituted by Ala, was also planned to be introduced into the NR-deficient mutant, this was not successful because of unknown reasons. However, Su *et al.* (1996) had previously observed that the NR with Ser to Ala mutation was not stable when expressed in the yeast *Pichia pastoris*. In fact, the NR containing the Ser to Ala mutation was only expressed in the protease-deficient mutant of *Pichia*. When Ser is replaced by Ala, a new cutting site for proteases may be introduced, thus rendering NR unstable.

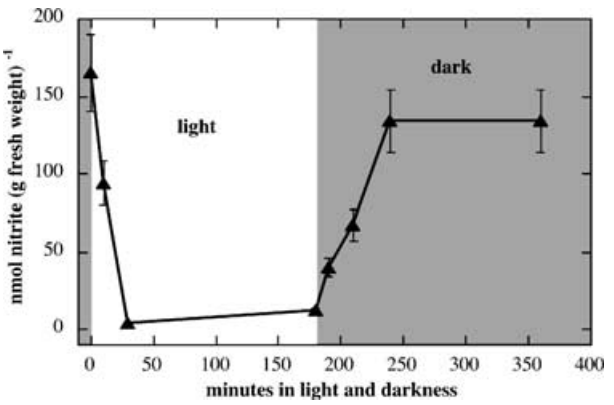

Figure 7. Changes in nitrite concentration in leaves in response to light on and off.
S_{521}-7 leaves were harvested 10 days after the start of irrigation with 150 mM KNO_3. Leaves were harvested in the morning after 12 h of darkness, and at different time points during the first 3 h of the light period. The light was then turned off, and samples were taken during a 3-h dark period ($n = 3$; SE values are indicated by vertical bars).

In some systems, Asp has been shown to mimic a phosphorylated Ser residue (Wang *et al.*, 1992). If this would be the case concerning NR, Mg^{2+} should be inhibitory; however, this was not found for NR from S_{521} plants. This is in agreement with the results for mutated *Arabidopsis* NR expressed in the *Pichia* system, which was not inactivated by Mg^{2+} and 14-3-3 proteins after changing the regulatory Ser into Asp (Su *et al.*, 1996). Clearly, Asp does not mimic phosphorylated Ser at the regulatory site in NR. As expected from *in vitro* experiments, NR activity in the S_{521} mutant did not respond to changes in light/darkness. This *in vivo* proof of the involvement of Ser 521 in NR inactivation in the dark also validates the model of NR regulation by phosphorylation of this residue. Activity state remained high, about 80%, during short-term dark treatments (Figure 1a) as well as prolonged darkness (Figure 2), in contrast to NR activity state in WT or C1 plants, which was low in darkness. The post-translational control of NR in S_{521} was, indeed, abolished when Ser 521 was changed into Asp.

During the day, the S_{521} line accumulated high levels of Gln, and during the night it accumulated high levels of Asn, the end-products of nitrate assimilation (data not shown). This shows that inactivation of NR is an important mechanism for control of the metabolite flux in this pathway. These differences between plants with de-regulated NR and other plants will be further investigated.

Degradation of NR was tested under continuous light and darkness (Figures 2 and 3). No difference was found in degradation rates of NR in C1 and S_{521} lines, both of which have the structural/mutated-structural NR linked to the 35S promoter. It has been suggested that activity state will influence degradation rate of NR (Cotelle *et al.*, 2000; Kaiser and Huber, 1997; Weiner and Kaiser, 1999). For spinach NR, it was shown that phosphorylated NR in the presence of 14-3-3 proteins was more rapidly degraded than was the non-phosphorylated NR, and increased degradation of NR was correlated with low activity state *in vivo* (Kaiser and Huber, 1997; Weiner and Kaiser, 1999). On the other hand, in *Arabidopsis* cell cultures, phosphorylated proteins, including NR, appeared to be protected from degradation *in vivo* (Cotelle *et al.*, 2000). The experiments presented here for *Nicotiana* NR did not indicate any connection between activity state and degradation rate because activity state in darkness was very different in S_{521} and C1 plants without resulting in different degradation rates.

Insignificance of NR activity state in relation to degradation of NR was also confirmed by comparing degradation of NR in the presence of tungstate in light and darkness. Although the different activity states in light and darkness for NR in the WT and C1 were striking, degradation of NR was almost the same (Figures 2a and 3a).

Nitride reductase expression underlies important regulation mechanisms related to the transcriptional as well as post-translational levels. However, the control mechanisms of NR expression may be abolished, and apparently the plant still thrives well as observed for the S_{521} plants. Regulation at the transcriptional level was already abolished previously (C1 plants) by using the 35S promoter and introducing constitutively transcribed NR to NR-deficient plants (Vincentz and Caboche, 1991). From studies of different *Nicotiana* mutants possessing one, two or four functional copies of the NR gene, it became clear that the plant compensates for a decreased number in functional *Nia* genes by keeping more of NR in the non-phosphorylated active form. Regulation on different levels thus appears to assure balance in the metabolism (Scheible *et al.*, 1997). Under optimal growth conditions, de-regulation of NR on the transcriptional as well as post-translational levels still allows the S_{521} plants to grow normally. However, under certain conditions, the lack of NR regulation was harmful to the plant, and young leaves became chlorotic (Figure 5). When plants were irrigated with high concentrations of nitrate during several days, nitrite was found to accumulate in the leaves when tested at the end of the night (Figure 6). The accumulated nitrite disappeared quickly when light was turned on. These variations in nitrite levels are probably because of, and linked to, variations in nitrite reductase activity *in situ*. Indeed, nitrite reductase is a chloroplastic enzyme which depends on reduced ferredoxin for activity. In the leaves, ferredoxin is directly reduced by the photosystems, and thus, the availability of this reducing power is dependent on light. Accumulation of nitrite was in fact a rather rapid process, detectable already after 10 min of darkness, and levelling off after 1 h in darkness (Figure 7). The levelling off in nitrite accumulation might be caused by lack of available nitrate for the NR enzyme in the cytosol, or by lack of reducing power. The former hypothesis will be further investigated and appears attractive because in other experiments (unpublished), nitrite accumulation was observed only when cut leaves were fed with exogenous nitrate. There is considerable evidence for the existence of two nitrate pools: a small metabolic pool in the cytosol and a large storage pool in the vacuole (Ferrari *et al.*, 1973; Van der Leij *et al.*, 1998); only the small metabolic pool is assumed to be available for the NR enzyme.

The reason for the complex regulation of NR may partly be that plants need to decrease nitrite formation rapidly under conditions where the metabolism cannot assure efficient further assimilation into organic compounds. Accumulation of nitrite during the night, or in response to changes in cloudiness, could be harmful to the plant because of toxic effects of nitrite. Post-translational regulation of NR may also be desirable to avoid formation of byproducts of the NR reactions like activated oxygen and peroxynitrite (Yamasaki, 2000), compounds that may disturb the metabolism of the cell. This work shows that mutation of Ser 521 eliminates the post-translational regulation of NR, which leads to accumulation of nitrite and causes damage (chlorosis) of plant leaves under certain growth conditions. The chlorosis observed in S_{521} plants irrigated with high concentrations of nitrate provides the first evidence *in planta* for a physiological role of NR phosphorylation to control nitrite production in the dark.

Experimental procedures

NR mutagenesis

The tobacco NR-coding sequence was mutated by PCR by using a primer carrying the Ser 521 → Asp 521 (TCA → GAT) change and a primer upstream of the mutation site. The resulting PCR product was used as a 'megaprimer' for another round of PCR with primer OI2 as described previously by Pigaglio *et al.* (1999). Part of the original NR-coding sequence cloned in an yeast expression vector (Truong *et al.*, 1991) was then replaced by the final PCR product. The functionality of the mutated NR protein was verified by expression in yeast (data not shown). This new NR-coding sequence was subsequently cloned in a plant expression vector under the control of the 35S promoter, and transferred to the *N. plumbaginifolia* E23 *nia* mutants as previously described (Pigaglio *et al.*, 1999). Primary transgenic plants were selected by kanamycin resistance and restoration of NR activity. This line was called S_{521}. Three independent S_{521} lines carrying a single active transgene were selected for further analysis (S_{521}-1, S_{521}-5 and S_{521}-7) as previously described (Pigaglio *et al.*, 1999), and homozygous lines were obtained in their progeny.

Plant material

After germination in sand or agar, plants were grown in perlite overlaid by a thin layer of soil for about 6 weeks (or sometimes longer when mentioned in results). The plants were in the rosette stage and had approximately seven leaves when leaf samples were harvested. The plants tested were: wild type *N. plumbaginifolia* (WT) and transgenic *N. plumbaginifolia* (var. Viviani) with the full length tobacco NR constitutively expressed using the cauliflower mosaic virus 35S promoter (C1) (Vincentz and Caboche, 1991). In C1, NR was constitutively expressed using the cauliflower mosaic virus 35S promoter. All the NR activity detected in these transgenic plants is derived from the transgene expression, as the endogenous NR gene is inactivated by a retrotransposon insertion (Leprince *et al.*, 2001). As described above, another transgenic *N. plumbaginifolia* line was constructed where the *Nia* gene was under the control of the constitutive 35S promoter, and the Ser in hinge 1 known as the regulatory phosphorylation target was mutated into Asp. Otherwise, the S_{521} line resembles the C1 line. Plants were grown at 20°C with a 12-h photoperiod at 80 μmol m^{-2} sec^{-1}, and were irrigated with Hoagland solution

containing 15 mM KNO_3, three times a week and always on the day before harvesting (Lillo and Henriksen, 1984; Provan *et al.*, 2000).

Extraction and assay of NR

Leaves, 2 g, were homogenised with 8 ml of 0.1 M Hepes-KOH (pH 7.5), 3% (w/v) polyvinylpolypyrrolidone, 1 mM EDTA and 7 mM cysteine. The assay mixture contained 50 mM Hepes-KOH (pH 7.5), 100 μM NADH, 5 mM KNO_3, with 2 mM EDTA or 6 mM $MgCl_2$. The assay volume was 0.75 ml. Activity was measured in crude extracts by determining NO_2^- formation, and also in (partially) purified NR preparation by continuously following the decrease of NADH at 340 nm (Lillo and Henriksen, 1984; Lillo and Ruoff, 1992). NADH oxidation in the absence of KNO_3 was negligible. Activity state is defined as NR assayed in the presence of Mg^{2+} (and 14-3-3 proteins) as percentage of NR activity measured in the presence of EDTA, and reflects how much of the enzyme is in the non-phosphorylated active form. Assays were run at 25°C.

Partial purification of NR

Leaves, 10 g, were homogenised with 30 ml of 0.1 M K-phosphate buffer (pH 7.5), 3% (w/v) polyvinylpolypyrrolidone, 1 mM EDTA, 7 mM cysteine, 7.5 μM leupeptin, 0.1 mM phenyl methanesulfonyl flouride, 1 μM FAD, 1 mM Na_2MoO_4, and purified with Blue Sepharose, as previously described by Provan *et al.* (2000).

Immunoblot analysis

Proteins were separated using a discontinuous buffer system SDS–PAGE (12% gels) electrophoresis (Provan *et al.*, 2000). Protein preparations, 12 μg, were applied to each well. Proteins were transferred to a nitrocellulose membrane (0.45 μm) using a mini trans-blot electrophoretic cell (Bio-Rad, Hercules, CA, USA). The transfer buffer used was 25 mM Tris, 192 mM glycine, 20% methanol, with pH 8.3. The membrane was incubated with rabbit polyclonal antibodies raised against purified squash NR. The immunocomplexes were recognised using protein G conjugated to horseradish peroxidase. The membranes were developed using a chemiluminescent detection method (Amersham Pharmacia, Uppsala, Sweden). Because the antibodies were not strictly specific when used with crude *N. plumbaginifolia* extracts, extracts were first treated with Blue Sepharose. All the affinity-purified NR activity eluted from the Blue Sepharose was pooled, and activity could be related quantitatively to gram FW. Western blots were analysed densitometrically by NIH Image 1.61.

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