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## Mutation of the regulatory phosphorylation site of tobacco nitrate reductase results in high nitrite excretion and NO emission from leaf and root tissue

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**Abstract** In wild-type *Nicotiana plumbaginifolia* Viv. and other higher plants, nitrate reductase (NR) is regulated at the post-translational level and is rapidly inactivated in response to, for example, a light-to-dark transition. This inactivation is caused by phosphorylation of a conserved regulatory serine residue, Ser 521 in tobacco, and interaction with divalent cations or polyamines, and 14-3-3 proteins. The physiological importance of the post-translational NR modulation is presently under investigation using a transgenic *N. plumbaginifolia* line. This line expresses a mutated tobacco NR where Ser 521 has been changed into aspartic acid (Asp) by site-directed mutagenesis, resulting in a permanently active NR enzyme [C. Lillo et al. (2003) *Plant J* 35:566–573]. When cut leaves or roots of this line (S<sub>521</sub>) were placed in darkness in a buffer containing 50 mM KNO<sub>3</sub>, nitrite was excreted from the tissue at rates of 0.08–0.2 μmol (g FW)<sup>-1</sup> h<sup>-1</sup> for at least 5 h. For the control transgenic plant (C1), which had the regulatory serine of NR intact, nitrite excretion was low and halted completely after 1–3 h. Without nitrate in the buffer in which the tissue was immersed, nitrite excretion was also low for S<sub>521</sub>, although 20–40 μmol (g FW)<sup>-1</sup> nitrate was present inside the tissue. Apparently, stored nitrate was not readily available for reduction in darkness. Leaf tissue and root segments of S<sub>521</sub> also emitted much more

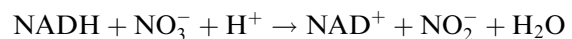
nitric oxide (NO) than the control. Importantly, NO emission from leaf tissue of S<sub>521</sub> was higher in the dark than in the light, opposite to what was usually observed when post-translational NR modulation was operating.

**Keywords** *Nicotiana* · Nitrate reductase · Nitric oxide · Nitrite excretion · Phosphorylation

**Abbreviations** NR: Nitrate reductase · NO: Nitric oxide · Ser: Serine · WT: Wild type

### Introduction

Nitrate reductase (NR, EC 1.6.6.1) catalyses a key step in nitrogen assimilation:



NR is known to be regulated post-translationally by phosphorylation of a conserved serine residue, Ser 543 in spinach (Douglas et al. 1995; Bachmann et al. 1996) and Ser 534 in *Arabidopsis* (Su et al. 1996), corresponding to Ser 521 in *Nicotiana tabacum*. In the presence of Mg<sup>2+</sup> or spermidine the phosphorylated form of NR interacts with 14-3-3 proteins, and NR activity is then inhibited (Provan et al. 2000; Athwal and Huber 2002). This regulatory mechanism assures rapid inactivation of NR under certain conditions, like a sudden decrease in light intensity. The nitrite formed in the reaction step catalysed by NR is further converted into ammonium by nitrite reductase (NiR) and then incorporated into organic compounds by glutamine synthetase and glutamate synthase (GOGAT). NiR and GOGAT are closely coupled to photosynthesis because, in leaves, reduced ferredoxin generated in photosynthesis is used as the electron donor for these enzymes. In darkened leaves, or root tissue, reducing power for NiR and GOGAT must be generated by other means than photosynthesis, but still involves ferredoxin or a ferredoxin-like protein (Meyer and Stitt 2001). Rapid down-regulation of NR due to, for

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instance, a sudden decrease in light intensity has been claimed to be necessary to avoid accumulation of nitrite, which is toxic to the plant (Riens and Heldt 1992; Huber et al. 2002; Lillo 2004), and the work presented here indeed demonstrates that the Ser 521 can be important for cessation of nitrite production.

In roots from many plant species, anaerobic conditions result in nitrite excretion (Botrel et al. 1996; Allègre 2003). This efflux of nitrite has been ascribed to an activation of NR by dephosphorylation, probably triggered in part by a decrease in cytosolic pH (Botrel and Kaiser 1997; Allègre 2003). In barley roots, nitrite assimilation was also impaired in anaerobic conditions (Botrel et al. 1996). It has been known for a long time that supplementary nitrate increases a plant's resistance to hypoxia and anoxia, and recently it has been shown that an impairment of NR activity reduced the resistance of tomato plants to anaerobiosis (Allègre 2003). Also, under anoxia, tobacco mutants deficient in root NR showed much higher ethanol and lactate fermentation than wild-type (WT) roots (Stoimenova et al. 2003a, 2003b). These observations are in agreement with nitrate reductase taking part in regeneration of  $\text{NAD}^+$  for the glycolytic pathway since respiration cannot recycle the  $\text{NAD}^+$  under anoxic conditions.

NR also catalyses the conversion of nitrite into nitric oxide (NO), a gaseous free radical exerting numerous physiological effects in plants (Kaiser et al. 2004). NO can further react with superoxide, formed by NR or other enzymes in the plant, to form peroxynitrite, a highly toxic and reactive compound (Ruoff and Lillo 1990; Yamasaki and Sakihama 2000; Rockel et al. 2002; Kaiser et al. 2004).

We have previously produced several *N. plumbaginifolia* transgenic lines ( $S_{521}$ ) expressing a tobacco NR with the regulatory Ser 521 changed to aspartic acid (Asp 521). Under optimal growth conditions nitrite did not accumulate in response to a light–dark transition in  $S_{521}$  leaves although NR was always in its active form (Lillo et al. 2003). The reason for this could be that NR is restrained in situ also by other mechanisms than phosphorylation and association with 14-3-3 proteins. Restriction in substrate availability can indeed be important. Although the  $K_m$  value for NADH is very low for spinach and tobacco NR, i.e. 1–2  $\mu\text{M}$  (Sanchez and Heldt 1990; Lillo et al. 1997), it has been suggested that NADH may decrease in darkness and be limiting for nitrate reduction (Kaiser et al. 2000). The present work shows that although NR in  $S_{521}$  is in its active form in darkness, formation of nitrite is still restricted, but will increase to higher levels when external nitrate is provided. The compartmentation of nitrate into a metabolic and a storage pool appears to be important for regulation of nitrogen assimilation, and may prevent conversion of nitrate to nitrite under conditions that cannot support adequate further metabolism of nitrite. This complex regulation of nitrate compartmentation and reduction is apparently aimed at avoiding accumulation of toxic compounds.

## Materials and methods

### Plant material

After germination in sand or agar, plants were grown in perlite overlaid by a thin layer of soil for about 6 weeks. The plants were in the rosette stage and had approximately seven leaves when leaf samples were harvested. Seeds were provided by Unité de Nutrition Azotée des Plantes INRA, Versailles, France. Plants tested were: WT *Nicotiana plumbaginifolia* Viv., transgenic *N. plumbaginifolia* (var. *viviani*) with the full length tobacco NR (C1) (Vincenz and Caboche 1991), and  $S_{521-7}$  where tobacco NR Ser 521 was mutated into Asp; otherwise, the  $S_{521-7}$  line resembles the C1 line (Lillo et al. 2003). In C1 and  $S_{521}$ , 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). Plants were grown at 20°C with a 12-h photoperiod at 80  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , and were irrigated with Hoagland solution containing 15 mM  $\text{KNO}_3$  three times a week, and always the day before harvesting.

### Nitrite excretion

Leaves were cut into pieces of approximately 0.50  $\text{cm}^2$ . Roots and leaves were washed in deionised water and cut into 0.7-cm-long pieces. The tissue, 5 $\times$ 1 g, was placed in a 100-ml Erlenmeyer flask with 20 ml of 50 mM Mes buffer pH 6.5 (Hepes pH 7.0 was also used, and gave the same results) and various concentrations of  $\text{KNO}_3$ . The Erlenmeyer flasks were kept in darkness under slow agitation. Samples of the buffer were taken every hour for measurement of nitrite content, and the tissue was used for assay of NR activity.

### Nitrate measurements

Crude extracts were diluted 100 times, and all the nitrate was converted into nitrite enzymatically by adding NR purified from *N. plumbaginifolia*. Nitrite was then determined as described below. Alternatively, aliquots of 20–25 mg of freeze-dried powder were extracted successively with 1 ml of 80% (v/v in water) ethanol, 1 ml of 60% ethanol and finally with 1 ml of water (1 h of extraction for each step) at 4°C. The three successive extractions were pooled and homogenised. An aliquot (2 $\times$ 1 ml) of the supernatant was then evaporated in a Speed-Vac and resuspended in 1 ml of water for nitrate determination. Nitrate concentrations were determined by HPLC (Dionex analyser, DX-120; HPLC AS14 column; 3.5 mM  $\text{Na}_2\text{CO}_3$  and 1 mM  $\text{NaHCO}_3$  as eluant). Peaks were identified and quantified in an integrator (Peak-net station) by comparison with standard nitrate concentrations (10  $\mu\text{M}$  to 1 mM).

### Extraction and assay of NR

Leaves, 1 g, were homogenised with 4 ml of 0.1 M Hepes–KOH (pH 7.5), 3% (w/v) polyvinylpyrrolidone, 1 mM EDTA and 7 mM cysteine. The assay mixture contained 50 mM Hepes–KOH (pH 7.5), 100  $\mu\text{M}$  NADH, 5 mM  $\text{KNO}_3$  with 2 mM EDTA or 6 mM  $\text{MgCl}_2$ . The assay volume was 0.70 ml. Activity was measured in crude extracts by determining  $\text{NO}_2^-$  formation by the addition of 1% sulphanilamide and 0.02% N-(1-naphthyl)-ethylene-diamine dihydrochloride in 1.5 M HCl (Lillo and Henriksen 1984). The activity state is defined as NR assayed in the presence of  $\text{Mg}^{2+}$  (and 14-3-3 proteins) as a 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.

## Gas-phase NO measurements

*N. plumbaginifolia* plants were cultivated on vermiculite under a 16 h light/8 h dark regime at 25°C day/22°C night temperature. Three leaves were harvested for NO emission and weighed. The leaves with freshly cut petioles were placed in a beaker with Hoagland nutrient solution (15 mM nitrate) and mounted in a chamber with a transparent lid and 4 l air volume. A constant flow of measuring gas (1.5 l min<sup>-1</sup>) was pulled through the chamber and subsequently through the chemiluminescence detector (CLD 770 AL ppt; Eco-Physics, Dürnten, Switzerland; detection limit 20 ppt; 1 min time resolution) by a vacuum pump connected to an ozone destroyer. The ozone generator of the chemiluminescence detector was supplied with dry oxygen (99%). The measuring gas (air or nitrogen) was made NO-free by conducting it through a custom-made charcoal column (1 m long, 3 cm internal diameter, particle size 2 mm). Calibration was routinely carried out with NO-free air (0 ppt NO) and with various concentrations of NO (1–35 ppb) adjusted by mixing the calibration gas (500 ppb NO in nitrogen; Messer Griesheim, Darmstadt, Germany) with NO-free air. Flow controllers (FC-260; Tylan General, Eching, Germany) were used to adjust all gas flows. Light was provided by a 400-W HQI-lamp (Schreder, Winterbach, Germany) above the cuvette. Quantum flux density was 200 μmol m<sup>-2</sup> s<sup>-1</sup> PAR). Air temperature in the cuvette was continuously monitored, and was usually about 20°C in the dark and 23–25°C in the light.

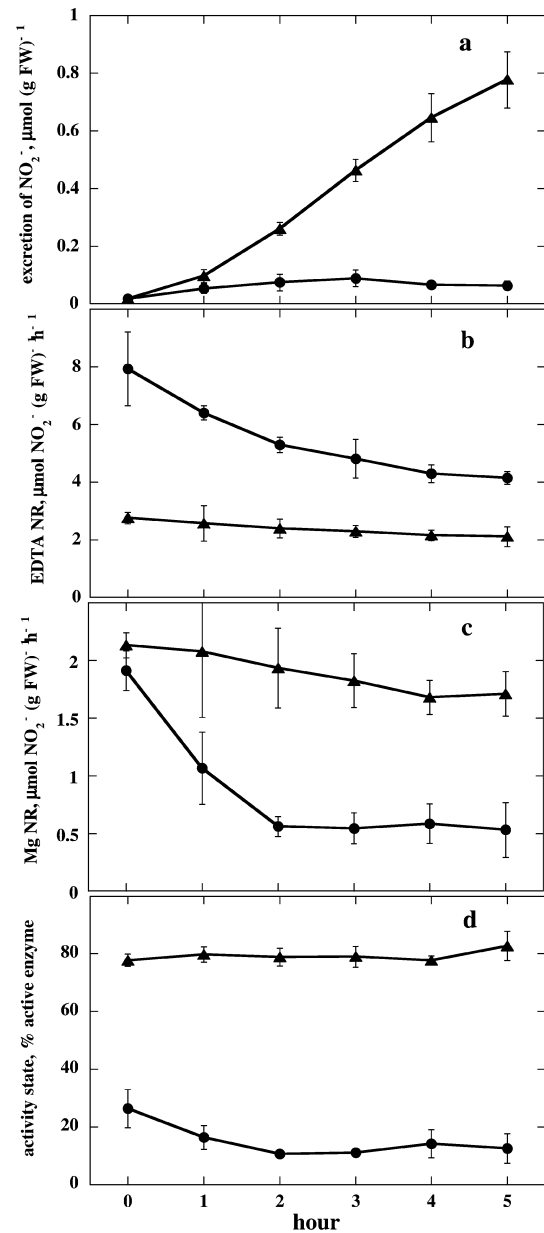
## NO emission from roots

Roots were harvested from hydroponically grown *N. plumbaginifolia* plants, washed briefly and cut into segments of approx. 0.5 cm. 2 g of root segments were immersed in a beaker with 20 ml Hoagland nutrient solution and this beaker was placed in the measuring cuvette. NO was measured under constant shaking (150 rpm), as described.

## Results

### Nitrite excretion from leaf tissue

Leaves of *N. plumbaginifolia* were harvested in the morning after 3 h of light, cut into narrow pieces, and placed in Erlenmeyer flasks in a buffer with various concentrations of nitrate. The flasks were kept in darkness under slow agitation. Figure 1a shows nitrite excretion from leaf tissue with 50 mM nitrate in the buffer. For S<sub>521</sub> tissue, nitrite was excreted almost linearly with time during the 5 h of the experiment, whereas nitrite excretion from the control plant expressing a WT NR with the Ser 521 intact (C1) was very low, and stopped completely after 3 h. Total NR activity (assayed in the presence of EDTA) showed a 45% decrease during the first 4 h for C1 and 22% for S<sub>521</sub> (Fig. 1b). NR activity tested in the presence of Mg<sup>2+</sup> (actual NR activity) is believed to reflect the in situ NR activity more adequately than NR assayed in the presence of EDTA (Kaiser et al. 2000). The actual NR activity shown in Fig. 1c was similar at time zero for C1 and S<sub>521</sub>, but NR activity in C1 then decreased sharply, whereas actual activity in S<sub>521</sub> was almost constant. This is correlated with the data showing nitrite excretion (Fig. 1a). Mean values for nitrite excretion during the first hour were 0.04 and 0.08 μmol (g FW)<sup>-1</sup> h<sup>-1</sup> for C1 and S<sub>521</sub>, respectively. During the next 2 h nitrite excretion from S<sub>521</sub> was 10 times higher than for C1. Thereafter



**Fig. 1a–d** Nitrite excretion and NR activity in cut leaf tissue during 5 h darkness in the presence of 50 mM exogenous nitrate for S<sub>521</sub> (▲) and C1 (●) *Nicotiana plumbaginifolia* plants. **a** Nitrite excretion. **b** Total NR activity, measured in the presence of EDTA. **c** Actual NR activity, measured in the presence of Mg<sup>2+</sup>. **d** Activity state of NR. Means ± SE, n = 3

nitrite excretion remained high for S<sub>521</sub>, but stopped completely for C1. As expected, the NR activity state was always high for S<sub>521</sub>. For C1, the activity state was relatively low at the start of the experiments and decreased further during the first 2 h (Fig. 1d). Post-translational inactivation of NR therefore appears to be important for cessation of nitrite formation under these conditions.

Figure 2 shows the effect of different exogenous concentrations of nitrate on nitrite excretion from S<sub>521</sub> and C1 leaf tissue. When no nitrate was added to the buffer wherein the tissue was immersed, nitrite excretion was low also in S<sub>521</sub> plants. After the first 2 h, during the 2- to

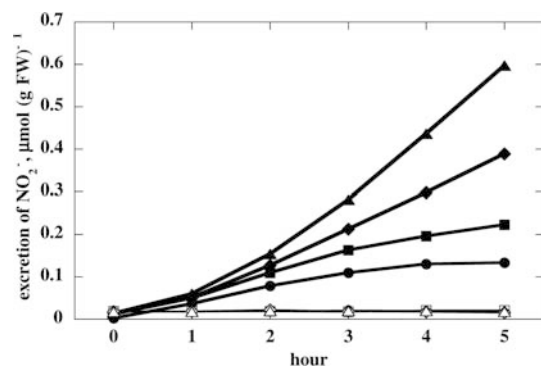
5-h time span, nitrite excretion from  $S_{521}$  tissue with no exogenous nitrate was only 12% of that from tissue exposed to 50 mM exogenous nitrate. For C1 tissue, nitrite excretion was close to zero regardless of the exogenous concentrations of nitrate. Different batches of plants varied with respect to nitrite excretion as well as NR activity. As shown in Fig. 1, some nitrite excretion could sometimes be observed from C1 plants during the first 3 h of incubation, although excretion from C1 plants was very low compared with the  $S_{521}$  mutant. To examine how different concentrations of nitrate affected nitrite excretion, samples were harvested from the same batch of plants and tested at various nitrate concentrations. Figure 2 represents one out of several experiments showing a strong effect of nitrate concentration on nitrite excretion for the  $S_{521}$  plants, and zero or very low nitrite excretion from C1 regardless of nitrate concentration.

Nitrite content was also measured after boiling the tissue in the incubation buffer, which releases nitrite from within the cells. These measurements did not show a different nitrite formation rate compared with the data presented in Figs. 1a or 2; hence, not much nitrite accumulated within the tissue of  $S_{521}$  or C1 plants (data not shown). This was also confirmed in the samples prepared for NR activity measurements because, although slightly increasing, these samples did not show high background values for nitrite at the start of the NR assay.

Nitrate content of leaves was determined, and showed that  $S_{521}$  leaves contained 20–40  $\mu\text{mol}$  nitrate  $(\text{g FW})^{-1}$ , i.e. 25–100 times more than the amount of nitrate that was actually reduced to nitrite in the experiments presented in Figs. 1 and 2. This indicates that the endogenous nitrate is not easily available for NR since high exogenous nitrate concentrations were necessary for sustained nitrite formation.

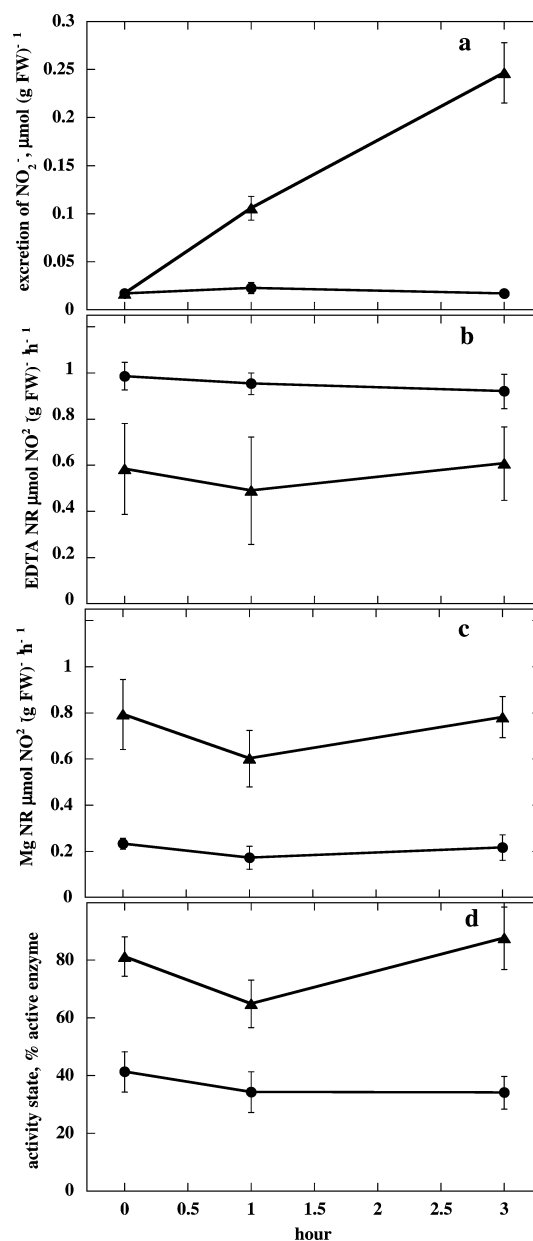
#### Nitrite excretion from root tissue

Nitrite excretion from root pieces was much higher in  $S_{521}$  plants compared with control (C1) plants (Fig. 3a).



**Fig. 2** Nitrite excretion from *N. plumbaginifolia* cut leaf tissue immersed in various concentrations of nitrate during 5 h of darkness. The data presented represent one out of three similar repeats. Filled symbols  $S_{521}$ , open symbols control plant C1. Nitrate concentrations tested were: 0 (●, ○), 1 (■, □), 10 (◆, ◇), and 50 mM (▲, △)

Actual NR activity was about 4 times higher in  $S_{521}$  than in control plants (Fig. 3c). The nitrate content of roots always exceeded 30  $\mu\text{mol}$   $(\text{g FW})^{-1}$ . When comparing roots submerged in buffers with different concentrations of nitrate it was found that nitrite excretion depended on high exogenous nitrate concentrations also for roots. Without exogenous nitrate, nitrite excretion stopped after 1 h (Fig. 4). Three different repeats were made of this experiment, showing basically the same results, but maximal excretion rates for  $S_{521}$  tissue (in 50 mM



**Fig. 3a–d** Nitrite excretion and NR activity in cut roots of *N. plumbaginifolia* during 3 h darkness in the presence of 50 mM exogenous nitrate for  $S_{521}$  (▲) and C1 (●) plants. **a** Nitrite excretion. **b** Total NR activity, measured in the presence of EDTA. **c** Actual NR activity, measured in the presence of  $\text{Mg}^{2+}$ . **d** Activity state of NR. Means  $\pm$  SE,  $n=3$

nitrate) varied from 0.65 to 1.0  $\mu\text{mol (g FW)}^{-1}$  during the 5-h time span. Nitrite excretion always stopped after 1 h when no exogenous nitrate was present. Nitrite excretion from C1 roots was also tested and showed close to zero excretion rates for all nitrate concentrations used (Figs. 3a, 4).

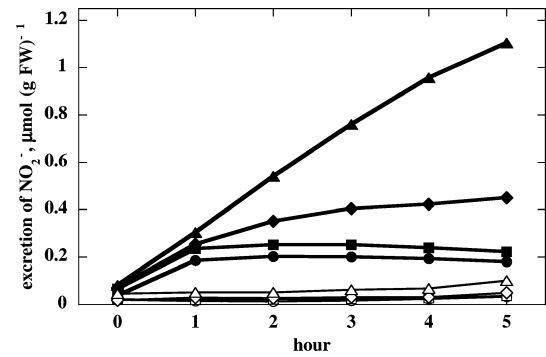
### NO formation

As a side reaction, NR can use nitrite as a substrate and form NO by a one-electron transfer reaction (Yamasaki and Sakihama 2000; Rockel et al. 2002; Kaiser et al. 2004). We therefore tested the formation of NO under conditions that could lead to (transient) accumulation of nitrite due to lack of post-translational regulation of NR in  $S_{521}$ . NO emission from C1 was low in the dark, increased during the light phase and remained high in the second dark phase (Fig. 5). Quite in contrast, in  $S_{521}$  leaves, NO emission was generally much higher in the dark than in the light. Significant differences in  $\text{NO}_2^-$  concentrations in situ were not found between  $S_{521}$  and C1 under these conditions (data not shown).

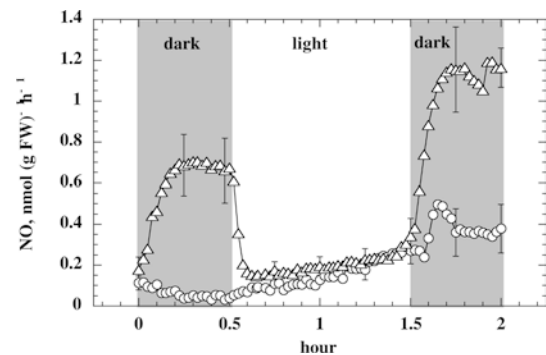
Higher NO emission from  $S_{521}$  than for C1 was also found for root pieces floating in 15 mM nitrate solution (Fig. 6). Generally, NO emission from roots exposed to normal air was not detectable, but under anaerobic conditions 8 and 1  $\text{nmol (g FW)}^{-1} \text{h}^{-1}$  were emitted from  $S_{521}$  and C1 roots, respectively. Nitrite concentrations in the roots increased during 2 h of anaerobiosis from about 1 to 40  $\text{nmol (g FW)}^{-1}$  in  $S_{521}$  and from 0.02 to 27  $\text{nmol (g FW)}^{-1}$  in C1 plants. It seems that in the roots of the  $S_{521}$  line a significant proportion of the stored nitrite is reduced to NO (compare the level of nitrite to the maximal emission rate in Fig. 6). After returning to aerobic conditions, nitrite concentrations decreased only slowly (not shown), whereas NO emission ceased almost immediately (Fig. 6). Thus, the difference in anoxic NO emission between  $S_{521}$  and C1 roots was much higher than would be expected from the differences in the nitrite concentration.

### Discussion

Previous work had shown that transforming the E23 *N. plumbaginifolia* NR-deficient mutant with a reporter gene or structural NR gene linked to the NR promoter often led to no or very low expression (Meyer and Stitt 2001), and to assure proper expression the mutated NR gene was placed under control of the 35S promoter. Mutated NR is therefore expressed constitutively in all kinds of cells (ectopic expression), even in cells that might have little or no NR activity. The results for the  $S_{521}$  plants were always compared with the C1 plants in which the non-mutated NR gene was also linked to the 35S promoter. Generally, we found WT and C1 plants to give the same results (data not shown), with the exception of NO emission under anoxia (Fig. 6). Under



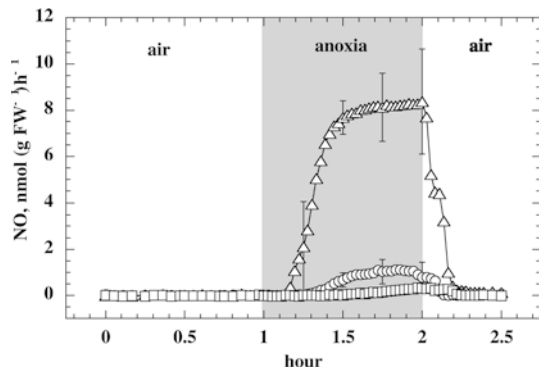
**Fig. 4** Nitrite excretion from *N. plumbaginifolia* cut root tissue immersed in various concentrations of nitrate during 5 h of darkness. The data presented represent one out of three similar experiments. Filled symbols  $S_{521}$ , open symbols the control plant C1. Nitrate concentrations tested were: 0 ( $\bullet$ ,  $\circ$ ), 1 ( $\blacksquare$ ,  $\square$ ), 10 ( $\blacklozenge$ ,  $\lozenge$ ), and 50 mM ( $\blacktriangle$ ,  $\triangle$ )



**Fig. 5** NO flux density from leaves during dark–light–dark transitions. Detached leaves of *N. plumbaginifolia* were placed with their petioles in a nutrient solution containing 15 mM nitrate.  $S_{521}$  ( $\Delta$ ) and C1 ( $\circ$ ). Means  $\pm$  SE,  $n=5$

anoxia, 12% of the NO emission could have been caused by ectopic expression of NR.

Excretion of nitrite by anaerobic roots or by roots treated with inhibitors of respiration has been often reported (Lee 1979; Mann et al. 1979; Dry et al. 1981; Botrel et al. 1996). Similarly, tissue incubated in darkness and anoxia has been used for the so-called “in vivo NR activity test” where nitrite accumulation in the medium is also detected (Mann et al. 1979; Lillo and Henriksen 1984). This increase in nitrite accumulation in both roots and shoots sometimes has been linked to a decrease in ATP and/or glucose 6-phosphate concentration which was then supposed to restrict nitrite reduction in plastids, and to an acidification of the cytosol which is known to activate NR. Indeed, only a partial reduction in nitrite assimilation was observed in anoxic barley roots (Botrel et al. 1996). We show here that nitrite excretion from detached leaves and roots of *N. plumbaginifolia* is also linked to the loss of post-translational regulation of NR resulting from mutation of the regulatory Ser residue. Nitrite excretion, although low, was also detected at the beginning of the experiment



**Fig. 6** NO flux density from root segments in air and anoxia. Cut roots of *N. plumbaginifolia* were floated on nutrient solution with 15 mM nitrate. S<sub>521</sub> (Δ), C1 (○) and WT (□). Means ± SE (C1 *n*=3, S<sub>521</sub> *n*=3, WT *n*=2)

when using leaf tissue of the control transgenic *N. plumbaginifolia* line, but this nitrite excretion stopped after 3 h of incubation in the dark. A concomitant decrease in NR activation state was also observed. It thus seems that the major factor controlling nitrite production is the modulation of NR activity by phosphorylation and 14-3-3 binding. Interestingly, even in the deregulated transgenic plants (S<sub>521</sub>), nitrite was not found to accumulate in leaves under optimal growth conditions (Lillo et al. 2003). In mature plant cells the total cell volume of the cytosol will make up only 5–10% of the cell, and the concentrations of nitrate or nitrite in different compartments can be very different from the values obtained by averaging the total content of the whole tissue. Local accumulation of nitrite may therefore occur, and be, for instance, 20 times higher than the averaged values obtained. Because of this dilution effect by the whole cell, increase of cytosolic nitrite can be hard to determine, and accumulation of nitrite is only seen under more extreme conditions.

The present work shows that when fresh nitrate is provided externally to cut leaf or root tissue nitrite formation is stimulated in the S<sub>521</sub> plants (Figs. 2, 4). Even though high nitrate concentrations were present in both root and leaf tissues a source of external nitrate was necessary for sustained nitrite excretion. This indicates that compartmentation of nitrate into a metabolic pool (cytosolic) and a storage pool (vacuole) is important for regulation of nitrate reduction. The concept of two nitrate pools, a large storage pool in the vacuole and a small metabolically available pool in the cytosol, was launched a long time ago (Ferrari et al. 1973; Martinoia et al. 1981). Although a substantial mobilisation of vacuolar stored nitrate has been shown to occur in N-starved barley roots (van der Leij et al. 1998), it seems that in our conditions this remobilisation did not occur, or at least not at a rate sufficient for sustaining maximal nitrite production. Alternatively, the reduction of exogenous nitrate into nitrite by NR could be restricted to cells of the epidermal layer, which implies that the available vacuolar nitrate pool is much lower than when calculated from the total organ.

In leaves of the S<sub>521</sub> line, NO emission was generally higher than in the control C1 transgenic line, both in the light and in the dark. Importantly, the dark/light pattern of NO emission was also changed by the mutation of Ser 521. In leaves of WT plants, NO emission was usually higher in the light than in the dark (Fig. 5, also compare Rockel et al. 2002), whereas in S<sub>521</sub>, the pattern was opposite (higher in dark than in light). No accumulation of nitrite was seen, but the higher rate of NO emission in the S<sub>521</sub> line could be due to a slight increase in nitrite formation and to a higher rate of NO formation from nitrite by the constitutively active NR in S<sub>521</sub> plants. It has previously been shown for other species that production of NO by NR occurs in tissue where nitrite concentration is high (Morot-Gaudry-Talamain et al. 2002; Rockel et al. 2002). However, in agreement with previous results (Lillo et al. 2003), accumulation of nitrite was not observed in leaves during 30 min of darkness in the WT, or in transgenic *N. plumbaginifolia* unless extra nitrate was added. Apparently, the assay for nitrite is not sufficiently sensitive to detect any increase during the time span (30 min) generally used to see optimal inactivation of NR, although nitrite may increase locally. Measurements of NO were, however, very sensitive and showed increasing emission of NO during 30 min of darkness.

A much higher NO emission than in C1 was also detected in anaerobic S<sub>521</sub> roots, together with a small nitrite accumulation. Interestingly, an increased NO production has been detected also in anoxic alfalfa root cultures (Dordas et al. 2003) and it has been suggested that NO may be involved in the regulation of anoxic metabolism (Stoimenova et al. 2003a, 2003b). The present work shows a clear correlation between *in vivo* formation of NO and regulation of NR. However, direct evidence that NO emitted is the product of nitrate reduction remains to be obtained, and to fully evaluate the effects of abolishing phosphorylation of the regulatory Ser 521, <sup>15</sup>N labelling in the S<sub>521</sub> line will be done. This will allow us to quantify the rate of nitrate reduction (to nitrite and also NO) in both the C1 and S<sub>521</sub> lines, and to follow the metabolic perturbations resulting from the NR deregulation.

Plants have evolved various complementary mechanisms to avoid the potential problem of nitrite accumulation under certain conditions when further assimilation of nitrite is slow. As shown in the experiments presented here, mutation of the regulatory serine in NR and thereby constitutively high NR activity can lead to formation and excretion of nitrite. It is also apparent that the post-translational regulation of NR not only regulates NR with respect to nitrite production but also functions to regulate NO emission.

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## References

- Allège A (2003) Approche physiologique et biomoléculaire du rôle de la nitrate réductase dans la résistance à l'asphyxie racinaire de la tomate. Thesis, Institut National Polytechnique de Toulouse, France
- Athwal GS, Huber SC (2002) Divalent cations and polyamines bind to loop 8 of 14-3-3 proteins, modulating their interaction with phosphorylated nitrate reductase. *Plant J* 29:119–129
- Bachmann M, Shiraishi N, Campbell WH, Yoo B-C, Harmon AC, Huber SC (1996) Identification of Ser-543 as the major regulatory phosphorylation site in spinach leaf nitrate reductase. *Plant Cell* 8:505–517
- Botrel A, Kaiser WM (1997) Nitrate reductase activation state in barley roots in relation to the energy and carbohydrate status. *Planta* 201:496–501
- Botrel A, Magné C, Kaiser WM (1996) Nitrate reduction, nitrite reduction and ammonia assimilation in barley roots in response to anoxia. *Plant Physiol Biochem* 34:645–652
- Dordas C, Hasinoff BB, Igamberdiev AU, Manac'h N, Rivoal J, Hill RD (2003) Expression of a stress-induced hemoglobin affects NO levels produced by alfalfa root cultures under hypoxic stress. *Plant J* 35:763–770
- Douglas P, Morrice N, MacKintosh C (1995) Identification of a regulatory phosphorylation site in the hinge 1 region of nitrate reductase from spinach (*Spinacea oleracea*) leaves. *FEBS Lett* 377:113–117
- Dry I, Wallace W, Nicholas DJD (1981) Role of ATP in nitrite reduction in roots of wheat and pea. *Planta* 152:234–238
- Ferrari TE, Yoder OC, Filner P (1973) Anaerobic nitrite production by plant cells and tissues: evidence for two nitrate pools. *Plant Physiol* 51:423–431
- Huber SC, MacKintosh C, Kaiser WM (2002) Metabolic enzymes as targets for 14-3-3 proteins. *Plant Mol Biol* 50:1053–1063
- Kaiser WM, Kandlbinder A, Stoimenova M, Glaab J (2000) Discrepancy between nitrate reduction rates in intact leaves and nitrate reductase in leaf extracts: What limits nitrate reduction in situ? *Planta* 210:801–807
- Kaiser WM, Planchet E, Stoimenova M, Sonoda M (2004) Modulation of nitrate reductase activity and (eco)physiological implications. In: Stulen I, Amancio Z (eds) N metabolism and plant adaption to the environment, chapter 7. Kluwer, Dordrecht
- Lee RB (1979) The release of nitrite from barley roots in response to metabolic inhibitors, uncoupling agents and anoxia. *J Exp Bot* 30:119–133
- Leprince A-S, Grandbastien M-A, Meyer C (2001) Retrotransposons of the Tnt1B family are mobile in *Nicotiana plumbaginifolia* and can induce alternative splicing of the host gene upon insertion. *Plant Mol Biol* 47:533–541
- Lillo C (2004) Light regulation of nitrate uptake, assimilation and metabolism. In: Stulen I, Amancio Z (eds) N metabolism and plant adaption to the environment, chapter 6. Kluwer, Dordrecht
- Lillo C, Henriksen A (1984) Comparative studies of diurnal variations of nitrate reductase activity in wheat, oat and barley. *Physiol Plant* 62:89–94
- Lillo C, Kazaic S, Ruoff P, Meyer C (1997) Characterization of nitrate reductase from light- and dark-exposed leaves. *Plant Physiol* 114:1377–1383
- Lillo C, Lea US, Leydecker M-T, Meyer C (2003) Mutation of the regulatory phosphorylation site of tobacco nitrate reductase results in constitutive activation of the enzyme in vivo and nitrite accumulation. *Plant J* 35:566–573
- Mann AF, Hucklesby DP, Hewitt EJ (1979) Effect of aerobic and anaerobic conditions on the in vivo nitrate reductase assay in spinach leaves. *Planta* 146:83–89
- Martinoia E, Heck U, Wiemken A (1981) Vacuoles as storage compartment for nitrate in barley leaves. *Nature* 289:292–294
- Meyer C, Stitt M (2001) Nitrate reduction and signalling. In: Lea PJ, Morot-Gaudry JF (eds) Plant nitrogen. Springer, Berlin Heidelberg New York, pp 37–59
- Morot-Gaudry-Talamain Y, Rockel P, Moureaux T, Quilleré I, Leydecker M-T, Kaiser WM, Morot-Gaudry JF (2002) Nitrite accumulation and NO emission in relation to cellular signaling in NiR antisense tobacco. *Planta* 215:708–715
- Provan F, Aksland L-M, Meyer C, Lillo C (2000) Deletion of the nitrate reductase N-terminal domain still allows binding of 14-3-3 proteins but affects their inhibitory properties. *Plant Physiol* 123:757–764
- Riens B, Heldt HW (1992) Decrease of nitrate reductase activity in spinach leaves during a light-dark transition. *Plant Physiol* 98:573–577
- Rockel P, Strube F, Rockel A, Wildt J, Kaiser WM (2002) Regulation of nitric oxide (NO) production by plant nitrate reductase in vivo and in vitro. *J Exp Bot* 53:103–110
- Ruoff P, Lillo C (1990) Molecular oxygen as electron acceptor in the NADH-nitrate reductase system. *Biochem Biophys Res Commun* 172:1000–1005
- Sanchez J, Heldt HW (1990) On the regulation of spinach nitrate reductase. *Plant Physiol* 92:684–689
- Stoimenova M, Hänsch R, Mendel R, Gimmler H, Kaiser WM (2003a) The role of nitrate reduction in the anoxic metabolism of roots. I. Characterization of root morphology and normoxic metabolism of wild type tobacco and a transformant lacking root nitrate reductase. *Plant Soil* 253:145–153
- Stoimenova M, Liboureligl, Ratcliffe RG, Kaiser WM (2003b) The role of nitrate reduction in the anoxic metabolism of roots. II. Anoxic metabolism of tobacco roots with or without nitrate reductase activity. *Plant Soil* 253:155–167
- Su W, Huber SC, Crawford NM (1996) Identification in vitro of a post-translational regulatory site in the hinge 1 region of *Arabidopsis* nitrate reductase. *Plant Cell* 8:519–527
- van der Leij M, Smith SJ, Miller AJ (1998) Remobilisation of vacuolar stored nitrate in barley root cells. *Planta* 205:64–72
- Vincentz M, Caboche M (1991) Constitutive expression of nitrate reductase allows normal growth and development of *Nicotiana plumbaginifolia* plants *EMBO J* 10:1027–1035
- Yamasaki H, Sakihama Y (2000) Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species. *FEBS Lett* 468:89–92