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Effect of different oxygen availability on the nitrate reductase activity in *Cucumis sativus* roots

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Abstract

The effect of different oxygen availability on the nitrate reductase (NR, EC 1.6.6.1) activity in cucumber roots was studied. NR activity measured in the presence of Mg^{2+} (actual NR activity) as well as activity measured with EDTA (maximum NR activity) increased distinctly after 30 min of root incubation in a medium flushed with N_2 (anaerobic conditions). In contrast, aeration of roots (aerobic conditions) decreased both enzyme activities. Such inactivation of NR was rapidly reversed after transferring the roots to anaerobic conditions. An air-induced decrease of the actual enzyme activity was prevented by staurosporin, a protein kinase inhibitor; whereas microcistin LR, an inhibitor of protein phosphatases, completely eliminated the reactivation of NR actual activity under limited oxygen availability. An increase of the NR actual activity in roots incubated in a nitrogen-flushed buffer was correlated with a lower content of ATP in root tissues. These data suggest that reversible protein phosphorylation is involved in the regulation of NR activity under limited oxygen. On the other hand, feeding roots with inhibitors of protein kinases as well as phosphatases did not affect the maximal activity of NR indicating that other modification(s) of enzyme activity could also function in cucumber roots. Since the changes in the expression level of gene encoding nitrate reductase (*CsNR*) under different oxygen availability were not correlated with the enzyme activity, the transcription level of oxygen action was excluded. On the other hand, it was demonstrated that oxygen-induced alteration of NR was dependent on the ratio of oxidized/reduced pyridine nucleotides in tissues. In aerobic conditions, when maximal NR activity was inhibited, a drop of the NAD(P)H level was also observed. These data point to hysteretic modifications of NR protein induced by NAD(P)H as the target of reversible and rapid changes in maximal enzyme activity under different oxygen availability.

Additional key words: actual and maximum NR activities, cucumber, posttranslational regulation.

Introduction

Nitrate reductase (NR; EC 1.6.6.1.), the first and key enzyme of the nitrate assimilation pathway in higher plants, is subjected to a wide regulation of its catalytic activity in response to different environmental stimuli. Reactions of the enzyme to a number of external factors are very rapid and are due to the posttranslational modifications of nitrate reductase protein (Campbell 1999, Kaiser *et al.* 1999). The best known is reversible phosphorylation catalyzed by the specific protein kinases which phosphorylate the conserved serine residue in NR protein Ser-543 in spinach (Douglas *et al.* 1995, Bachmann *et al.* 1996a), Ser-534 in *Arabidopsis* (Su *et al.* 1996) and Ser-521 in *Nicotiana tabacum* (Lea *et al.* 2003). After phosphorylation, the enzyme associates with 14-3-3 protein in the presence of divalent cations (Mg^{2+}) and polyamines and forms an inactive complex (Athwal and Huber 2002). Reactivation of nitrate reductase occurs after dephosphorylation of the enzyme, catalyzed by protein phosphatases type 2A and subsequent dissociation of the inhibitor protein (reviewed by Kaiser and Huber 2001). These modifications of NR activity are well described for green tissue, as the response to changes in light conditions, carbon dioxide availability or low temperature (Kaiser *et al.* 1999).

Also in root tissue nitrate reductase activity is modified through reversible phosphorylation of enzyme protein due to the action of different external and internal stimuli. It was previously reported that NR activity in root extracts changed rapidly in the presence of adenine nucleotides and Mg^{2+} (Reda and Kłobus 2006). Different

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Abbreviations: DTT - dithiotreitol; EDTA - ethylenediaminetetraacetic acid; MTT - thiazolyl blue; NR - nitrate reductase; PES - phenazine ethosulphate; PMSF - phenylmethylsulphonyl fluoride; PVPP - polyvinylpolypyrrolidone.
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environmental conditions and uncoupler treatment of pea (Glaab and Kaiser 1993), barley (Bortel *et al.* 1997) and cucumber roots (De la Haba *et al.* 2001) caused posttranslational modifications of NR. Although modulation of the nitrate reductase activity resulted mostly from dephosphorylation of the enzyme protein, different mechanisms (reduction/oxidation or hysteresis) can also operate. In the present study we examined the changes of NR activity in cucumber roots growing under limited oxygen availability and, as we have shown, the anoxia action on the activity of nitrate reductase was very complex. We documented that, besides protein phosphorylation, other posttranslational enzyme modifications as well as changes in the expression of NR encoding genes may be involved.

Materials and methods

Plants: Cucumber (*Cucumis sativus* L.) plants were grown hydroponically, as described previously (Reda and Kłobus 2006). The roots of 7-d-old seedlings, excised from plants usually after 6-h illumination, were incubated up to 90 min in 10 mM Mes-KOH (pH 5.5) with 5 mM KNO_3, flushing with oxygen (aerobic conditions) or nitrogen (anoxia). After incubation the roots were blotted with paper, frozen in liquid nitrogen and used for the extraction of the enzyme.

Extraction and assay of nitrate reductase: 1 g of frozen roots was ground in a chilled mortar with 1.5 cm^3 of 50 mM Hepes-KOH (pH 7.5), 1 mM dithiotreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 % bovine serum albumin and 1 mM polyvinylpolypyrrolidone (PVPP). After filtration through 4 layers of cheesecloth and centrifugation at 15 000 *g* for 15 min at 4 °C, the supernatant was desalted on *Sephadex G-25 Fine*. Nitrate reductase activity was measured in the desalted supernatant in the presence and/or absence of $MgCl_2$ ($+Mg^{2+}$ and/or $-Mg^{2+}$) according to Kaiser and Huber (1997) with some modifications. The reaction medium contained 50 mM Hepes-KOH (pH 7.5), 5 mM $MgCl_2$ ($+Mg^{2+}$) or 5 mM ethylenediaminetetraacetic acid (EDTA; $-Mg^{2+}$), 10 mM KNO_3 and crude extract. The reaction was started by the addition of 0.2 mM NADH and, after 5 min of incubation at 27 °C was stopped with 0.066 cm^3 of 1 mM zinc acetate. The mixture was centrifuged, and the amount of nitrite was determined colorimetrically at 540 nm (Hageman and Reed 1980).

Extraction and determination of pyridine nucleotide content: Cucumber roots (1.5 g) incubated in different conditions were frozen in liquid nitrogen and then homogenized with 3 cm^3 ethanol-water (1:1; v:v) containing 0.1 M NaOH (alkaline extracts) or 0.1 M HCl (acid extracts) (Carrier and Neve 1979, modified by Juszczuk and Rychter 1997). Alkaline extracts were used to determine the reduced forms of pyridine nucleotides; whereas in acid extracts, their oxidized forms were measured. All extracts were stirred for 20 min at 0 °C and centrifuged for 10 min. (10 000 *g*) at 4 °C. The precipitates were then collected, resuspended in 1 cm^3 extraction solvent and centrifuged again. This step was repeated twice, and all supernatants were combined. Alkaline supernatants were heated for 5 min at 50 °C, and the pH was adjusted to 8.0 - 9.0 with 0.1 M HCl. The pH of the acid extracts was adjusted to 6.0 - 7.0 with 0.1 M NaOH, and all extracts were centrifuged again for 10 min (10 000 *g*) at 4 °C. Pyridine nucleotide levels were determined spectrophotometrically (*DU 640 Beckman*, Fullerton, USA) according to Lechevallier *et al*. (1977) with some modifications of Juszczuk and Rychter (1997). The reaction medium used to determine the phosphorylated forms of the pyridine nucleotides ($NADP^+$ and NADPH) contained in the final volume 1 cm^3: 30 mM Hepes-KOH pH 7.8, 0,5 mM EDTA, 5 mM glucose-6-phosphate, 0.1 mM thiazolyl blue (MTT), 2 mM phenazine etho-sulphate (PES), and acid or alkaline extract (for $NADP^+$ or NADPH measurement respectively). The reaction was started by adding 50 units of glucose-6-phosphate dehydrogenase. For determining the level of NAD^+ and NADH, 9.6 % ethanol was used instead of glucose-6-phosphate, and the reaction was started by the addition of alcohol dehydrogenase. The rate of MTT reduction at 570 nm was measured and changes in absorbance were compared with changes for each form of pyridine nucleotide standard solution. The total contents of pyridine nucleotides were calculated based on the fresh mass of the root tissue.

Extraction and determination of ATP: The determination of adenosine triphosphate (ATP) was made according to Glaab and Kaiser (1999) with some modifications. The root samples incubated in aerobic and anaerobic conditions were frozen (1 g) and ground in liquid nitrogen. Afterwards, 5 cm^3 of 4.5 % $HClO_4$ was added to the frozen powder and mixed until thawing. The mixture was then supplemented with 0.125 cm^3 of 2 mM Tris and centrifuged for 5 min at 5 000 *g* at 4 °C. The pH of the supernatant was adjusted to 7.4 with 5 mM K_2CO_3, and samples were centrifuged once again (5 min, 5 000 *g*, 4 °C). The ATP content was determined luminometrically using Firefly luciferin-luciferase in a *TD-20/20 Luminometer* (*Turner Designs*, Sunnyvale, USA).

Expression of nitrate reductase gene *CsNR*: Total RNA was isolated from 50 mg of roots with *Tri Reagent* (*Sigma*, St. Louis, USA). To evaluate the expression of the *CsNR* (accession number AY580989) and *CsEF1* (accession number EF446145) (internal standard) genes, the semi-quantitative RT-PCR (Titan one tube RT-PCR

system, *Roche*, Mannheim, Germany) with specific primers for each gene were performed. For the RT-PCR reaction, 150 ng of total RNA was used. CsNR specific primers were: 5`-AGCATCGGATTCAACTGGGG-3` (forward primer) and 5`-CGTTGATTATATGCTCG GGC-3` (reverse primer), and for the EF1α gene: 5`-GGAGGTA TTGACAAGCGTGTG-3` (forward primer) and 5`-GGAGAGTTGGTCCCTTGTACC-3` (reverse primer). The RT-PCR products were separated on a 1.5 % agarose gel stained with etidinum bromide. The gel images were digitally captured with a *Sony XC-ST50CE* camera and analyzed with the *Biocapt version 99* program. Results are presented as gel images and as the ratio of the signal for *CsNR* gene product selected line to the *EF1α* gene product signal.

Results

Effect of changes in oxygen availability on nitrate reductase activities: Cucumber roots were incubated in a buffer aerated or flushed with nitrogen for up to 90 min At the time indicated in the figures, the desalted extracts isolated from the roots were used to the determine the maximum (activity of both, phospho- and dephospho-NR determined with EDTA) and actual (only dephospho-NR activity assayed with Mg^{2+}) nitrate reductase activities. Aeration of the buffer with roots caused a decrease of both the maximum and actual NR activities (Fig 1*A*,*B*). The drops in activity were first observed after only 30 min of root aeration. When the incubation buffer was flushed with nitrogen, the maximum and actual NR activity in the roots increased rapidly after 30 min of treatment (Fig 1*A*,*B*). It should be noted that the changes observed in actual activity of NR were stronger than those which occurred in the maximum activity. Moreover, the activation state of NR (activity of dephospho-NR, measured in the presence of Mg^{2+} shown as a percentage of maximum activity) decreased during aeration of the roots and increased during the flushing with nitrogen (Table 1).

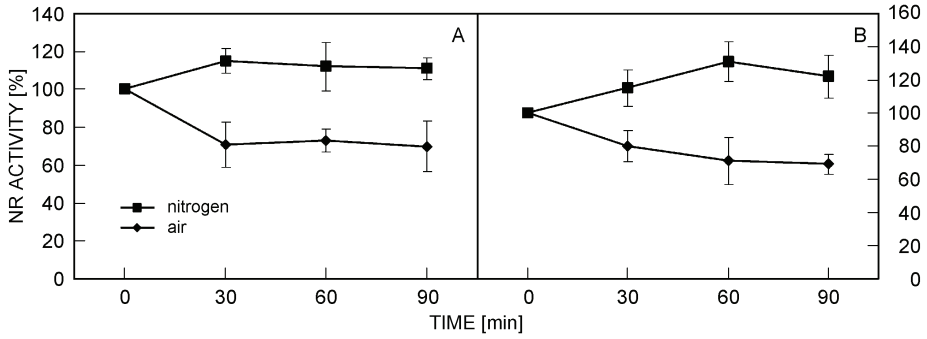

Fig. 1. Roots of 7-d-old cucumber seedlings were incubated in 10 mM Mes-KOH (pH 5.5) with 5 mM KNO_3. The incubation buffer was flushed with air or with N_2 for up to 90 min. In the time indicated in the figure the desalted extracts were obtained from roots and NR activity was measured in the presence of 5 mM EDTA (*A* - maximum activity) or in the presence of 5 mM $MgCl_2$ (*B* - actual activity) as described in Material and Methods. Initial NR activity measured in cucumber roots at the beginning of experiment (time 0) was used as 100 % (*A* - 0.56 ± 0.03 and *B* - 0.3 ± 0.015 μmol(NO_2^-) g^{-1}(f.m.) h^{-1}). Means of 15 replications of 5 independent experiments. *Error bars* represent SD.

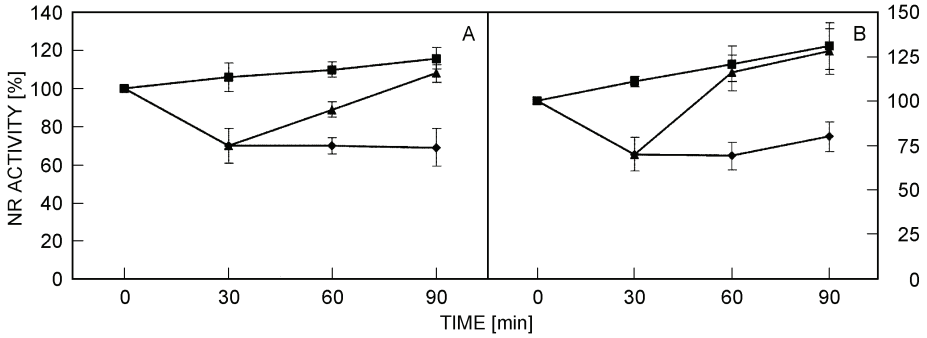

Fig. 2. Cucumber roots were incubated in 10 mM Mes-KOH + 5 mM KNO_3 for 90 min in an aerated buffer (*rhombs*) or in a buffer flushed with nitrogen (*squares*). Portions of the roots were aerated for 30 min and then flushed with nitrogen for the next 60 min (*triangles*). At the time indicated, desalted extracts were obtained from particular sets of roots and the maximum (*A*) and actual (*B*) NR activities were determined. The NR activity at time 0 was used as 100 % (*A* - 0.53 ± 0.02 and *B* - 0.29 ± 0.011 μmol(NO_2^-) g^{-1}(f.m.) h^{-1}). Means of 12 replications of 4 independent experiments. *Error bars* represent SD.

Table 1. Effect of different oxygen availability in roots environment (aeration or N_2 flushing for 30, 60 or 90 min) on NR activation state [%]. Activation state shows NR actual activity as a percentage of maximum enzyme activity.

Control	Air 30	Air 60	Air 90	N_2 30	N_2 60	N_2 90
49	40	32	38	56	58	60

The inhibitory effect of oxygen on the maximum and actual activities of NR in roots was reversible (Fig. 2*A*,*B*). When the roots which were aerated for 30 min were transferred to the buffer flushed with nitrogen, a progressive increase of the maximum NR activity and more rapid increase of the actual NR activity were observed (Fig. 2*A*,*B*). Lastly, after 60 min both activities reached the level found in the roots which were incubated in a buffer flushed 90 min only with nitrogen.

Effect of protein kinases and phosphatases inhibitors on changes in root NR activity under different oxygen availability: The rapid and reversible changes of NR activities which were observed in roots under different, external conditions suggested their posttranslational character. To explain if these changes involved reversible phosphorylation of the enzyme protein, studies with staurosporine, an inhibitor of serine-treonine protein kinases, and microcystin LR, an inhibitor of type 1 and type 2 protein phosphatases (PP1/PP2A) were performed. Staurosporine (2 μM) was applied to the buffer with roots flushed 30 min with air (Fig. 3). Feeding staurosporine to the roots did not prevent the air-induced decrease of maximum NR activity (Fig. 3*A*), but completely abolished the air-induced decrease of the actual NR activity (Fig. 3*B*). Subsequent treatment of aerated roots with N_2 increased both NR activities (Fig. 4), and after 60 min they reached the levels determined under N_2-flushing during the whole experiment (Fig. 4). Changes in the maximum enzyme activity under limited availability of oxygen were insensitive to 1 μM microcystin LR, (Fig. 4*A*) while N_2-induced reactivation of the actual NR activity was completely cancelled in the presence of this inhibitor (Fig 4*B*). The above data suggest that one or more phosphatases were stimulated by anoxia conditions and thus mediated the NR activation under the limited availability of oxygen.

However, it can not be excluded that in root tissues, besides reversible phosphorylation, some other mechanism(s) altering the NR activity under variable external O_2 status can also operate. To characterize those possibilities, the levels of ATP and pyridine nucleotides as well as the specific NR transcript in roots incubated in O_2- or N_2-flushed buffer were measured.

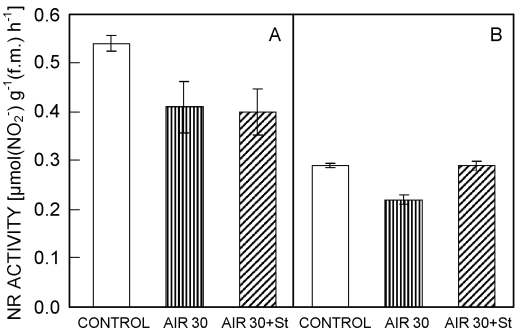

Fig. 3. Cucumber roots were aerated for 30 min in 10 mM Mes-KOH + 5 mM KNO with or without 2 μM staurosporine (St). The part of roots incubated without aeration was used as a control. After incubation, desalted extracts were obtained from particular sets of roots and the maximum (*A*) and the actual (*B*) NR activities were determined. Means of 6 replications of 2 independent experiments. *Error bars* represent SD.

Changes in ATP and pyridine nucleotide levels in roots treated in different oxygen availability: In roots incubated with nitrogen for 60 min the amount of ATP gradually decreased. ATP content decreased also in roots incubated 30 min in the buffer flushed with air, but this reduction was considerably lower (Fig. 5). Conversely, no considerable difference in the levels of oxidized nucleotides (NAD^+ and $NADP^+$) between roots aerated and those treated with nitrogen was found (Table 2). On

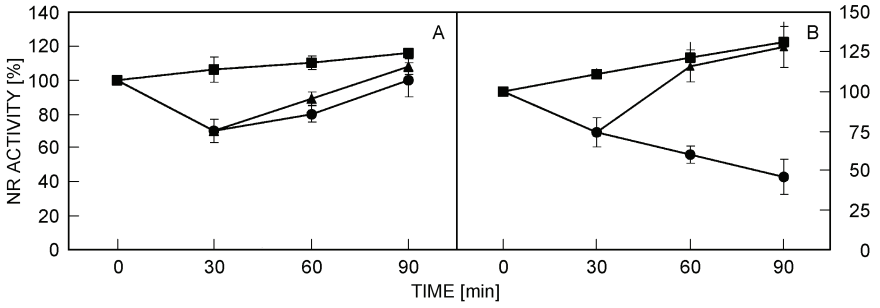

Fig. 4. Cucumber roots were incubated for 90 min in 10 mM Mes-KOH + 5 mM KNO_3 flushed with nitrogen (*squares*). The part of roots was aerated for 30 min and then flushed with nitrogen for the next 60 min in a buffer with (*circles*) or without (*triangles*) 1 μM microcystin LR (MC). At the time indicated desalted extracts were obtained from particular sets of roots and the maximum (*A*) and actual (*B*) NR activities were determined. The NR activity determined in cucumber roots at time 0 was used as 100 % (*A* - 0.98 and *B* - 0.46 μmol(NO_2^-) g^{-1}(f.m.) h^{-1}). Means of 12 replications of 4 independent experiments. *Error bars* represent SD.

Table 2. Contents of pyridine nucleotides [nmol g^{-1}(f.m.)] and their ratios in roots under different oxygen availability. Cucumber roots were incubated 30 min in 10 mM Mes-KOH pH 5.5 + 5 mM KNO_3 aerated or flushed with nitrogen. Afterwards, levels of pyridine nucleotides were determined as described in Material and Methods. Obtained data were used to analyze the ratio of oxidized to reduced forms of nucleotides. The presented results are means of 3 replicates.

	Air	N_2
NAD^+	7.220 ± 0.10	7.673 ± 0.17
NADH	0.496 ± 0.01	0.832 ± 0.06
$NADP^+$	6.087 ± 0.32	6.484 ± 0.40
NADPH	0.860 ± 0.04	1.229 ± 0.07
NAD^+/NADH	14.56	9.22
$NADP^+$/NADPH	7.08	5.28
Ox./red	9.81	6.87

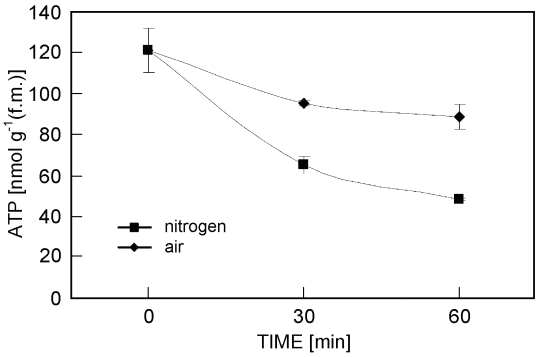

Fig. 5. Cucumber roots were incubated for 60 min in 10 mM Mes-KOH + 5 mM KNO_3 aerated or flushed with N_2. At the time indicated some of the roots were frozen in liquid nitrogen and 1 g of frozen tissue was used to determine the ATP content. Means of 3 replications. *Error bars* represent SD.

the other hand the amount of reduced nucleotides, NADH and also NADPH, significantly increased in roots incubated under limited oxygen conditions (Table 2). Moreover, the ratio of oxidized to reduced forms of nucleotides was distinctly lower under low oxygen availability (Table 2).

Effect of different oxygen availability on the expression of *CsNR* gene in roots: It was shown that the expression of *CsNR* did not significantly change after 15 min of treating the roots both with air and nitrogen. Following aeration or flushing of the incubation buffer with N_2 led to a decrease of *CsNR* transcript level in the roots (Fig. 6*A*,*B*). In addition, the expression of *CsNR* in roots treated with nitrogen was lowered more than in aerated roots.

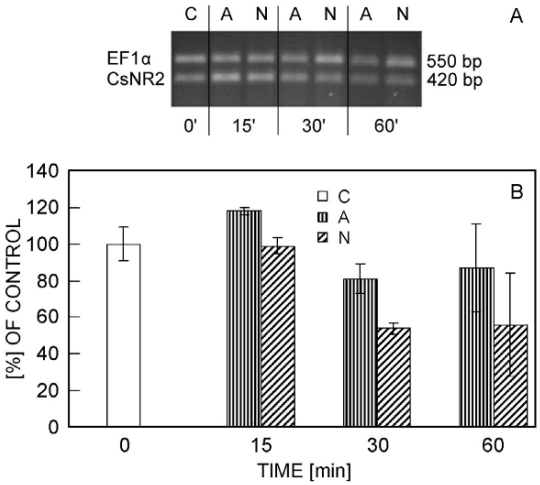

Fig. 6. Cucumber roots were incubated for 60 min in 10 mM Mes-KOH + 5 mM KNO_3 aerated or flushed with N_2. At the time indicated samples of roots were frozen in liquid nitrogen and 50 mg of frozen tissue was used for total RNA isolation. To determine the expression of *CsNR* gene, RT-PCR analysis was performed. The expression of *CsNR* in roots at the beginning of the experiment (C) was used as 100 %. *A* - gel image from electrophoresis on 1.5 % agarose gel, *B* - ratio of *CsNR* gene signal to EF1α gene signal (internal standard). A - aerated roots, N - roots flushed with nitrogen.

Discussion

The content of oxygen in the atmosphere and in plant leaves is rather high and constant. For this reason, O_2 is not a limiting factor for metabolism in green tissues. On the contrary, diffusion of gases in water and soil solutions is rather poor and for this reason plant roots growing in soil are often subjected to deficit aeration (Kaiser and Huber 1994). In the laboratory, rinsing of the roots with the nitrogen-flushed incubation buffer might easily mimic natural hypoxia conditions.

It has been shown that changes in many environmental conditions, *e.g.*, light/dark transition, treatments with respiratory inhibitors, sugar availability, or cell acidification often result in modulation of NR activity by the phosphorylation/dephosphorylation mechanism (Glaab and Kaiser 1993, Bortel *et al.* 1997, Kaiser *et al.* 1999, 2000). Our results demonstrated that oxygen availability to roots is also a factor affecting NR activity through protein phosphorylation. Incubation of cucumber roots in a buffer flushed with nitrogen increased the maximum and actual NR activities while aeration of the roots decreased both NR activities. The inhibitory effect of root aeration on the maximum and actual NR activities was similar and appeared already after 30 min of incubation. Moreover, this inhibition of both activities was rapidly reversed if the buffer was flushed with nitrogen. Modifications of actual NR activity during air or nitrogen treatment were sensitive to the inhibitors of protein kinases and phosphatases indicating the involvement of both enzymes in the modulation of NR activity under different oxygen availability. Similar data were

obtained earlier by Glaab and Kaiser (1993) for pea roots, by Bortel and Kaiser (1997) for barley roots and also by Kaiser and Huber (1997) for spinach leaves.

The treatment of roots with nitrogen significantly decreased the ATP content in tissues (Fig 5). According to Glaab and Kaiser (1993) the low concentration of endogenous ATP could limit the activity of specific protein kinases catalyzing NR phosphorylation. However, experimental data of Bortel and Kaiser (1997) as well as Kaiser *et al.* (1999) have shown that changes in the cellular adenine nucleotides under limited availability of oxygen had no direct effect on activities of protein kinases and phosphatases. Also previous data of Bachmann *et al.* (1995) indicated that specific NR kinases have a high affinity to ATP and a decrease of the ATP content under partial anoxia was not sufficient to inhibit the activity of NR kinases (Kaiser *et al.* 1999). Nevertheless, it cannot be excluded, that the reduction of ATP content during anoxia could directly affect plasma membrane and tonoplast H^+-ATPases (Ricard *et al.* 1994), because both enzymes have considerably lower affinity to ATP than NR kinases. Diminishing the activity of membrane proton pumps can lead to the temporary decrease of the cytoplasmic pH under anoxia (Saint-Ges *et al.* 1991, Ricard *et al.* 1994). In turn, it was shown by Bachmann *et al.* (1995) that a drop in the pH of cytosol significantly diminishes the activity of specific NR kinases in spinach leaves. Therefore we assume that limited oxygen availability favors dephosphorylations of the nitrate reductase protein following the increase of actual activity of the enzyme (De la Haba *et al.* 2001).

Contrary to the actual activity of NR, the changes in its maximum activity, determined in the presence of EDTA, did not depend on the reversible phosphorylation of enzyme protein. This was proven in experiments with staurosporine and microcystin LR. None of inhibitor had an effect on the maximum NR activity under different aeration. We could not exclude that a decrease of maximum NR activity observed after 30 min of root aeration was the result of partial degradation of the enzyme protein. Although transferring aerated roots to O_2-deficient conditions resulted in rapid (within 1 h) increase in NR activity, such fast enzyme reactivation ruled out the possibility that NR activation by anoxia was due to the *de novo* synthesis of enzyme protein, because time as long as 4 to 8 h is required to synthesize and process a new, active NR protein (Li and Oaks 1993). Consistent with that are the results of *CsNR* gene expression. Although flushing roots with nitrogen led to a decrease of NR mRNA level in tissue, it was not positively correlated with NR activity measured in those conditions (Fig. 6). The aeration of the roots did not distinctly change the *CsNR* expression. Hence, some post-translational modifications of the enzyme, other than protein phosphorylation, must be involved in NR modification under anoxia. Recently, evidence has been presented that some similar, post-translational modulations of leaf NR caused by anaerobiosis were the result of its hysteretic behaviour (Huber and Huber 1995, Kaiser and Huber 1997) induced by changes in tissue level of pyridine nucleotides (Lillo and Ruoff 1992, Lillo 1994). Although understanding of such regulation is still limited, Huber and Huber (1995) have suggested that binding of nucleotides to the allosteric side of NR caused the conformational changes which modulate enzyme activity. Also Lillo and Ruoff (1992) have shown that NAD(P)H was responsible for structural conversion of NR to the form with higher activity. To explain if pyridine nucleotides are the target of the anoxia-induced alteration of maximum NR activity, we measured their level in cucumber roots. It was found, that the induction of nitrate reductase activity under anoxia was correlated with an increase of the reduced forms of pyridine nucleotides (NADH and NADPH). Simultaneously the content of oxidized nucleotides was similar in both combinations. The lowered content of reduced pyridine nucleotides in aerated roots might be the result of competition between the respiration and nitrate reduction for these forms of nucleotides. In these conditions NADH produced in tricarboxylic acid cycle is fully consumed in the mitochondrial respiration chain and its transport to the cytoplasm is strongly limited. Thus, the cytosolic NADH content decreased considerably and could affect the maximum NR activity (Kaiser *et al.* 2000). On the other hand in anoxia conditions, higher reduction of nitrate by NR and accompanied oxidation of NADH to NAD^+ may serve as an important reaction recycling oxidized nucleotides when respiration is limited (Lea *et al.* 2004, Lillo *et al.* 2004).

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