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Nitrate reductase regulation in tomato roots by exogenous nitrate: a possible role in tolerance to long-term root anoxia

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Abstract

The mechanism of nitrate reductase (NR) regulation under long-term anoxia in roots of whole plants and the putative role of nitrate in anoxia tolerance have been addressed. NR activity in tomato roots increased significantly after 24 h of anaerobiosis and increased further by 48 h, with a concomitant release of nitrite into the culture medium. Anoxia promoted NR activation through dissociation of the 14-3-3 protein inhibitor and NR dephosphorylation. After 24 h of anoxia, the total amount of NR increased slightly up to 48 h. However, NR-mRNA levels remained constant between 0 h and 24 h of root anoxia and decreased after 48 h. This is probably due to the inhibition of NR degradation and the accumulation of its native form. NR was slightly dephosphorylated in the absence of oxygen and nitrate. Under anoxia, NR dephosphorylation was modulated by nitrate-controlled NR activity. In addition, the presence of nitrate prevents anoxic symptoms on leaves and delays wilting by 48 h during root anoxia. In the absence of nitrate, plants withered within 24 h, as they did with tungstate treatment, an inhibitor of NR activity. Thus, anoxia tolerance of tomato roots could be enhanced by nitrate reduction.

Key words: Anoxia, nitrate, nitrate reductase, nitrite, NR regulation, tomato roots.

Introduction

Nitrate reductase (NR) is the key enzyme in the overall process of nitrate assimilation by plants (Botrel *et al*., 1996; Datta and Sharma, 1999; Campbell, 2001; de la Haba *et al*., 2001). Nitrate absorbed by roots is reduced to nitrite by nitrate reductase and then nitrite is reduced by nitrite reductase to ammonium ion. Ammonium is thereafter incorporated into amino acids by the glutamine synthetase–glutamine-2-oxoglutarate transaminase (GS–GOGAT) enzyme system (Aslam *et al*., 2001), providing plant autotrophy for nitrogen (Heller *et al*., 1998). Under stress conditions, nitrite is also reduced to nitric oxide by nitrate reductase. Nitric oxide has various toxic effects and constitutes a signalling substance, so its effects should be broader. To limit the toxic effects of nitrite on root tissues, the NR activity is highly regulated through its degradation, activation, or inactivation.

Various studies indicate that nitrate reductase synthesis is promoted by nitrate, light, phytochrome, and photosynthesis (Appenroth *et al*., 2000; de la Haba *et al*., 2001). Nitrate reductase activity is modulated both by phosphorylation and by binding to a specific adapter protein known as 14-3-3 (Weiner and Kaiser, 1999, 2000). In spinach, phosphorylation of nitrate reductase on the 543-serine residue is the first step of enzyme inactivation (Kanamaru *et al*., 1999). Complete inactivation occurs when the phosphorylated form is associated with the 14-3-3 protein. This binding is initiated by divalent cations such as Mg^{2+} and Ca^{2+} (Weiner and Kaiser, 2001). Nitrate reductase activation is related to its dissociation from the 14-3-3 protein as well as to its dephosphorylation (Huber *et al*., 1996; Chung *et al*., 1999). However, information on nitrate reductase regulation in roots is scarce. In the case of barley, the enzyme is activated by uncouplers, mannose feeding, or anoxia (Botrel *et al*., 1996; de la Haba *et al*., 2001). Anoxic root tissues have a higher level of NR activity than aerated controls (Glaab and Kaiser, 1993), but the mechanisms regulating NR in anoxic roots are not well defined and the role of NR activation remains to be unravelled.

Nitrate requirement for induction of nitrate reductase is now well established: the enhancement of NR activity is regulated by the metabolic pool of nitrate localized in the

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cytoplasm under normoxic conditions (Ferrari *et al.*, 1973; Aslam *et al.*, 1976). The metabolic or active pool of nitrate is smaller than the large non-metabolic storage pool contained within the vacuole (Martinoia *et al.*, 1981). The concentration of nitrate in the cytoplasmic pool is lower and more constant than in the vacuolar pool (Zhen *et al.*, 1991). In barley root cells under normoxia, the exogenous concentration of nitrate determines the level of nitrate ions in the metabolic and non-metabolic pools (Aslam *et al.*, 2001). Exogenous nitrate supplied to the medium of anaerobic excised rice roots increased the adenylic energy charge and lowered the catabolic reducing charge (Reggiani *et al.*, 1985). Both factors could contribute to root survival and suggest a possible role of nitrate reduction in anoxia tolerance.

Anoxia studies were generally carried out during short periods (Botrel *et al.*, 1996; de la Haba *et al.*, 2001) on non-intact plants like maize (Xia *et al.*, 1995; Subbaiah and Sachs, 2003) or intact non-crop plants such as *Arabidopsis* (Ellis *et al.*, 1999; Paul *et al.*, 2004). The studies reported here were conducted on the effects of long-term anoxia (over 24 h) on tomato plants grown in soilless culture. In the same experimental conditions, the presence of nitrite was detected in the nutrient solution 16 h after the beginning of anoxia (Morard *et al.*, 2000). This result was corroborated on excised tomato roots (Morard *et al.*, 2004*b*) and also with sterile *in vitro*-cultured root tissues of tomato. Nitrate reduction is, therefore, a biochemical process linked to root cell metabolism and is not related to micro-organism activity (Morard *et al.*, 2004*c*).

The aim of this study was to determine the effect of long-term oxygen deprivation on NR expression and regulation. The role of nitrate on NR modulation and resistance to root anoxia was also investigated. In this paper, the following points were studied and discussed: (i) NR activity in oxygen-deprived roots, (ii) regulation of NR in anoxic conditions, and (iii) role of nitrate supplied in anoxia tolerance.

Materials and methods

Plant growth conditions

Experiments were conducted on 7-week-old tomato plants (*Lycopersicon esculentum* Mill.) cv. Rondello at the 10–12 leaf stage (1st flush). Plants were grown hydroponically in a culture room with a relative humidity of 50% and photoperiod of 14/10 h light/dark (170 $\mu E\ m^{-2}\ s^{-1}$) provided by Philips daylight fluorescent tubes. The day/night temperature was 20/18 °C. Tomato seeds were germinated on filter paper moistened with distilled water for 1 week at 23 °C in the dark. After 7 d, plants were grown individually in a separate PVC tank containing 15 l of nutrient solution over 6 weeks. The nutrient solution contained macronutrients: 2.25 mM KNO_3, 0.25 mM $Ca(NO_3)_2$, 0.35 mM KH_2PO_4, and 0.075 mM $MgSO_4$; micronutrients: 268.6 μM EDTA-Fe, 8.9 μM $MnSO_4$, 24.1 μM H_3BO_3, 1.7 μM $ZnSO_4$, 3.9 μM $CuSO_4$, and 0.1 μM Na_2MoO_4 (Morard *et al.*, 2004*a*). The solution was renewed every 2 weeks or as soon as the pH

reached 7 and/or when the conductivity was reduced by half with respect to the initial value. In each experiment, the mean root fresh weight expressed per litre of nutrient solution was 2.7 ± 0.5 g.

Root anoxia

After 5 weeks, plants were transferred to 8.0 l PVC tanks containing culture medium. Shortage of oxygen was imposed progressively by sealing the PVC tank with an airtight cover, by stopping aeration, and by including an airtight seal between the root and shoot. Oxygen dissolved in the nutrient solution being thus depleted, anoxia is reached when the root system has taken up all the oxygen dissolved in the nutrient solution (Morard and Silvestre, 1996). Total depletion occurs approximately 10 h after the beginning of oxygen shortage (Morard *et al.*, 2000). By contrast, control plants were continuously bubbled with air.

Three separate sets of experiments were conducted. In the first one, whole tomato plants grown in complete medium were deprived of oxygen for 24 h and 48 h, respectively, and harvested after each period. Control plants were continuously aerated. In a second set of experiments, root anoxia was conducted in the absence of nitrate for 48 h on medium containing solely 0.22 mM K_2SO_4, 2.16 mM KH_2PO_4, 0.25 mM $CaSO_4$, and 0.075 mM $MgSO_4$ with micronutrients. Plants were harvested after 48 h of anoxia. The aerated controls were grown on nitrate-depleted medium. In a third set of anoxia experiments, sodium molybdate was substituted by 40 μM sodium tungstate in the nutrient medium to inhibit NR activity (Deng *et al.*, 1989). In this case, ammonium was included in the medium so as to maintain nitrogen assimilation. The composition of this solution was 2 mM NH_4Cl, 0.25 mM $Ca(NO_3)_2$, 2.25 mM KNO_3, 0.075 mM $MgSO_4$, 0.35 mM KH_2PO_4, with micronutrients without molybdate and with tungstate. Control medium contained the same macronutrients and the composition of micronutrients was with molybdate and without tungstate.

Determination of anionic concentrations

Anion and cation concentrations in nutrient solution were periodically analysed using a Dionex high-pressure liquid ionic chromatograph DX-100 (Ionpac CS-12A and AS4A-SC, Dionex Co., Sunnyvale, CA, USA). To determine intracellular ammonium concentrations, roots were pressed at 4 °C using a hydraulic press at 27 MPa; the root juice (2 ml) was centrifuged at 10 000 *g* for 5 min and proteins were precipitated using 10 μl of 20 mM HCl. Supernatants were collected and ammonium concentrations determined by Dionex high pressure liquid ionic chromatography DX-100 (Ionpac CS-12A, Dionex Co., Sunnyvale, CA, USA). Nitrite concentrations were measured in the culture medium by spectrophotometry at 540 nm: a volume of 600 μl of solution was diluted with 1.4 ml of distilled water and 500 μl of 0.058 M sulphanilic acid, 0.0027 M naphthyl ethylene diamine, and 1.7 M phosphoric acid were added.

Nitrate reductase activity assay and nitrite analysis

Nitrate reductase activity was measured in fresh root tissues after 24 h and 48 h of oxygen deprivation as well as in aerated control plants. The roots were pressed at 4 °C; 600 μl of root juice were diluted with 1 ml of 0.1 M phosphate buffer pH 7.5, 0.1 M KH_2PO_4, 1 mM EDTA, 14 mM mercaptoethanol containing anti-protease cocktail (Sigma, L'Isle d'Abeau Chesnes, France), and 1 mM chymostatin, in the presence of 200 μl of 1.4 mM NADH and 200 μl of 0.1 M KNO_3 at 30 °C in the dark. After 30 min, the reaction was stopped with 100 μl of 1 M zinc acetate, 1 ml of 1% sulphanilic acid, and 1 ml of 0.2% naphthyl ethylene diamine (Sall, 1987). Nitrite concentration was measured at 540 nm. NR activity was determined by the difference between the quantity of nitrite in root extracts after 30 min of reaction and the initial quantity. The protein content of the enzyme extracts was measured according to the method of Bradford (1976).

NR activity is expressed in μmol nitrite min^{-1} g^{-1} of protein in root extracts.

Western blot and immunoprecipitation analysis

Protein was quantified both in normal aerated plant roots (controls) and in oxygen-deprived roots over a period of 24–48 h. Two grams of fresh root were ground to a fine powder in liquid nitrogen and suspended in 2 ml of extraction buffer containing a plant anti-protease cocktail (Sigma, L'Isle d'Abeau Chesnes, France) and 1 mM chymostatin to stabilize the NR. After continued grinding in a Potter homogenizer until thawed, the suspension was centrifuged for 15 min at 4 °C at 13 500 g. Supernatants containing cytosolic proteins were then collected. For western blot analysis, an equal amount of extracts (35 μg of total protein) was boiled in denaturing SDS (0.4%) buffer for 10 min. Proteins were separated by polyacrylamide–SDS gel electrophoresis and transferred to nitrocellulose membrane (Laemmli, 1970). Total NR was detected using monoclonal antibodies against squash (*Cucurbita maxima* L. cv. Burgess Buttercup) NR (Hyde *et al*., 1989). The immune complex was recognized by mouse IgG antibodies coupled to peroxidase. Bound proteins were detected by enhanced chemiluminescence. Quantification was performed with a Bio-Rad scanner and Molecular Analysis Software (Bio-Rad laboratories, Richmond, CA).

For immunoprecipitation assay, 2 g of fresh roots were ground to a fine powder in liquid nitrogen and suspended in 2 ml of RIPA buffer pH 7.5 (PBS, 1×, 1% Igepal, 0.5% sodium deoxycholate, and 0.1% SDS) and the plant anti-protease cocktail. After grinding in a Potter homogenizer until thawed, the suspension was centrifuged for 20 min at 4 °C at 13 500 g. Supernatants containing cytosolic proteins were diluted to 650 μg ml^{-1} and 1 ml was incubated for 1 h with 1 μg of monoclonal NR antibodies (*Cucurbita maxima* L. cv. Burgess Buttercup) and then mixed with 20 μl of mouse IgG-protein-A-agarose beads on a rotating plate for 12 h at 4 °C. Immunoprecipitates were collected by centrifugation for 20 min at 12 000 g. The pellets were washed three times with RIPA buffer and resuspended in 15 μl electrophoresis sample buffer, boiled and resolved by 12% SDS–PAGE. pNR protein was detected using anti-phosphoserine antibody and 14-3-3 NR protein using a 14-3-3 antibody. Bound proteins were detected by enhanced chemiluminescence.

RNA extraction

RNAs were extracted from roots of aerated plants (controls) and from roots following 24 h and 48 h of oxygen deprivation. RNAs were also extracted from tomato and tobacco leaves. Leaves were collected in the morning and in the evening to constitute positive controls with a high NR expression and negative controls with a low NR expression. Fresh roots (2 g) were ground to a fine powder in liquid nitrogen after which 750 μl of hot extraction buffer (65 °C) containing 4 M LiCl, 1 M TRIS–HCl pH 8, 0.5 M EDTA, and 0.7 M SDS, was added to each sample and mixed with 750 μl of phenol saturated with 10 mM TRIS–HCl, pH 8, and 1 mM EDTA. This mixture was homogenized for 30 s then 750 μl chloroform-isoamylalcohol (24:1, v:v) was added and the mixture vortexed. After centrifugation at 10 000 g for 5 min, the aqueous phase was mixed with 1 vol. of 4 M LiCl. RNAs were allowed to precipitate overnight at 4 °C and collected by centrifugation at 15 000 g for 10 min. The pellets were dissolved in 250 μl water, 25 μl 3 M sodium acetate, pH 5.6, and RNAs were precipitated with 2 vols of absolute ethanol at −20 °C for 1 h. After centrifugation, the RNA pellets were washed with 70% ethanol and dried (Verwoerd *et al*., 1989). RNAs were then analysed by northern blot analysis.

Preparation of cDNA probes

The NR cDNA (1.6 kbp PT13-29 NR) probe was kindly provided by Dr C Meyer (INRA, Versailles, France). cDNA inserts for hybridization were prepared by *Eco*RI digestion and gel-purified using standard protocols (Ausubel *et al*., 1995). DNA probes were radioactively ^{32}P-labelled by random primer method using the Megaprime DNA Labelling Systems kit (Amersham), as specified by the manufacturer.

Northern blot hybridization

Total RNA was isolated from leaf tissue as described by Verwoerd *et al*. (1989). Total RNA (15 μg) was separated on 1.2% (w/v) agarose gel containing 8% (v/v) formaldehyde. RNAs were transferred to Hybond N$^+$ nylon membrane (Amersham) and fixed via UV irradiation. Hybridization was performed at 65 °C in buffer containing 7% (w/v) SDS, 0.3 M sodium phosphate (pH 7.2), and 1 mM EDTA (pH 7.5) with the addition of 50 ng of denatured probes. After hybridization, the filters were washed at room temperature, twice in 2× SSC buffe+0.1% SDS for 10 min, and once in 0.1× SSC buffer+0.1% SDS at 65 °C for 10 min.

Statistics

Experiments were replicated two to eight times (see legend of figures). Results are expressed as means ±SD. Data were analysed by one-way analysis of variance, and multiple comparison of each treatment was calculated applying the Tukey method (Keppel, 1973).

Results

Measurement of nitrate reductase activity, nitrate uptake and nitrite release under root anoxia

Oxygen deprivation appeared about 10 h after the beginning of the experiment. Nitrate and nitrite concentrations were analysed in nutrient solution during the experiment (55 h). Data were calculated by the difference between initial concentration and each value of the time-course. Positive values (nitrate) corresponded to cumulated root uptake and negative values (nitrite) corresponded to cumulated release to the nutrient medium.

In aerated conditions, cumulated nitrate uptake increased time-dependently and reached higher values after 25 h (Fig. 1A). In anoxic conditions, roots continuously absorbed nitrate and cumulated uptake increased after 30 h (Fig. 1B). So, at the end of the experiment the total uptake of nitrate was 72% higher than the aerated control. Moreover, nitrite release appeared strongly in oxygen-deprived nutrient solution (Fig. 1B). In aerated nutrient solution, no nitrite was detected (Fig. 1A).

To analyse the long-term regulation of NR under asphyxia, plant material was sampled after 24 h and 48 h of root oxygen deprivation in soilless culture. To ensure reproducibility between experiments, NR activity was measured 3 h after the beginning of the light period. Actually, NR activity is maximum in the morning and then decreases at night (Jones *et al*., 1998; Lillo *et al*., 2001). NR activity of tissues increased regularly during the time-course of the experiment and reached its highest value 48 h after the beginning of anoxia (Fig. 2A). NR activity was 20-fold higher than the aerated control. NR enhancement was related to a strong release of nitrite in root tissues (Fig. 2B). Anoxia induced
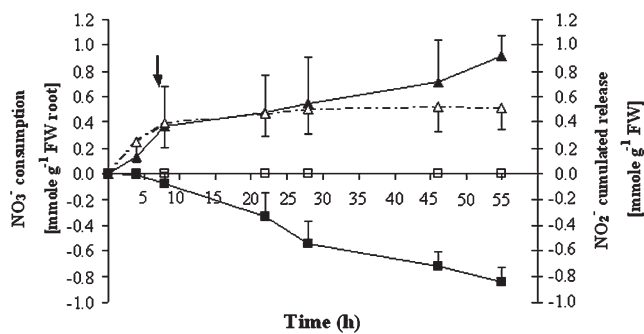
Fig. 1. Time-course of nitrate consumption with (open triangles) or without (closed triangles) aeration and cumulated nitrite released to nutrient solution with (open squares) or without (closed squares) aeration over a 55 h period. Nitrate and nitrite were expressed as cumulated quantities (mmoles of ion for 1 g fresh weight of root). Positive values correspond to root consumption and negative values to cumulated nitrite release into nutrient medium. Culture medium was not renewed during the experiment. The arrow indicates the lack of oxygen in root medium. Values represent the mean ±SD of five separate experiments.

a non-significant increase of ammonium content in roots (Fig. 2C).

NR regulation under long-term root anoxia

Immunoprecipitation and western blot analysis were performed to investigate whether NR protein was phosphorylated or bound to 14-3-3 protein under anoxic stress. Anoxic conditions promoted NR dephosphorylation whereas the phosphorylated form predominated in the aerated controls. Densitometric profiles of the western blot showed a decrease to 52% of phosphorylated-NR after 24 h of anoxia and to 35% after 48 h compared with the control (Fig. 3D). The analysis of 14-3-3 protein binding to NR revealed that anoxia also decreased the total amount of the 14-3-3 NR complex (Fig. 3D). The densitometric profiles of the 14-3-3 NR complex were reduced time dependently to 75% and 67% at 24 h and 48 h, respectively. Concomitantly with the study of post-translational regulation, the total quantity of NR [NR+phosphorylated-NR (pNR) plus phosphorylated-NR 14-3-3 bound (pNR 14-3-3)] was analysed with western blotting (Fig. 3C). The total amount of NR was increased by 66% at 24 h and 83% at 48 h (Fig. 3D).

Regulation of NR-mRNA level during root anoxia

The level of NR-mRNA expression under root anoxia was assayed by northern blot on roots and leaves using a tobacco NR probe (Fig. 4). Tobacco leaves constitute a reference to test the NR-cDNA probe in homologous hybridization conditions. NR-mRNA of tomato leaves were also assayed as a reference for NR-mRNA levels in roots. The level of NR-mRNA hybridization of the tobacco NR-labelled probe with positive (RNA of tomato and tobacco leaves collected in the morning) and negative controls (RNA of tomato and tobacco leaves collected in the evening) was observed (Fig. 4A). In these heterologous hybridization conditions, the
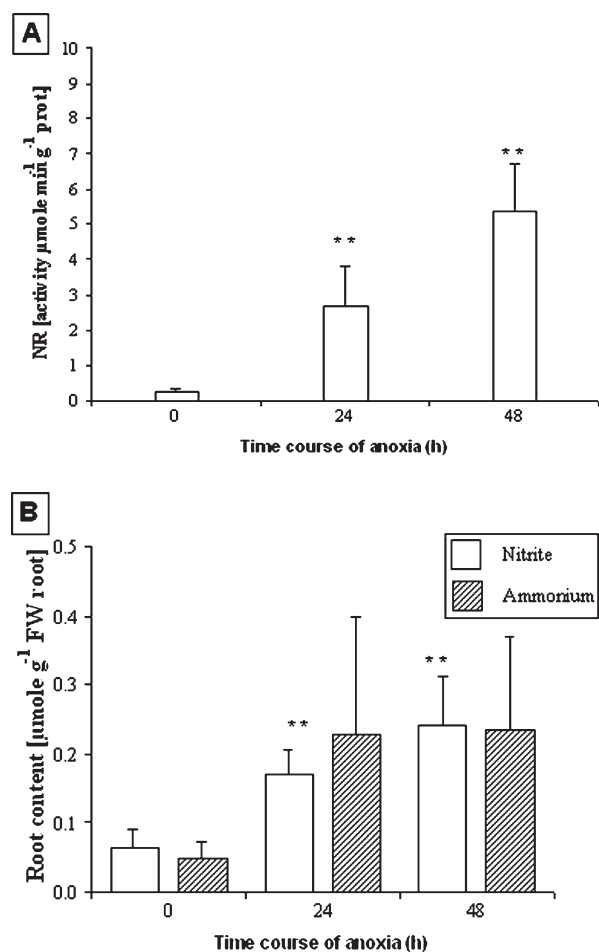

Fig. 2. Time-dependent change in NR activity (A) and nitrite and ammonium contents (B) in root tissues under anoxia. Results are presented as the means ±SD of five separate experiments. Two asterisks indicate significant variation at $P<0.01$.

signal intensity was lower in tomato (positive control) than in tobacco. However, a strong difference in NR-transcript expression in tomato leaves was observed between the evening and the morning. The results in Fig. 4B revealed that the NR-transcript level in tomato roots remained constant between 0 h and 24 h of anoxia. Then, at 48 h of anoxia, the level of NR-transcript dramatically decreased suggesting a lack of new NR synthesis.

NR regulation by nitrate during root anoxia

A nitrate-depleted culture medium was used to determine the role of nitrate in NR regulation during long-term root anoxia. NR activity and NR-phosphorylation were analysed in 48 h oxygen-deprived roots with or without nitrate. There was a strong increase of NR (>500%) with anoxia in the presence of nitrate (Fig. 5A). In the absence of nitrate and in the presence of oxygen, NR activity decreased to 48% compared with aerated roots in the presence of nitrate (Fig. 5A). After 48 h, the NR activity of anoxic roots
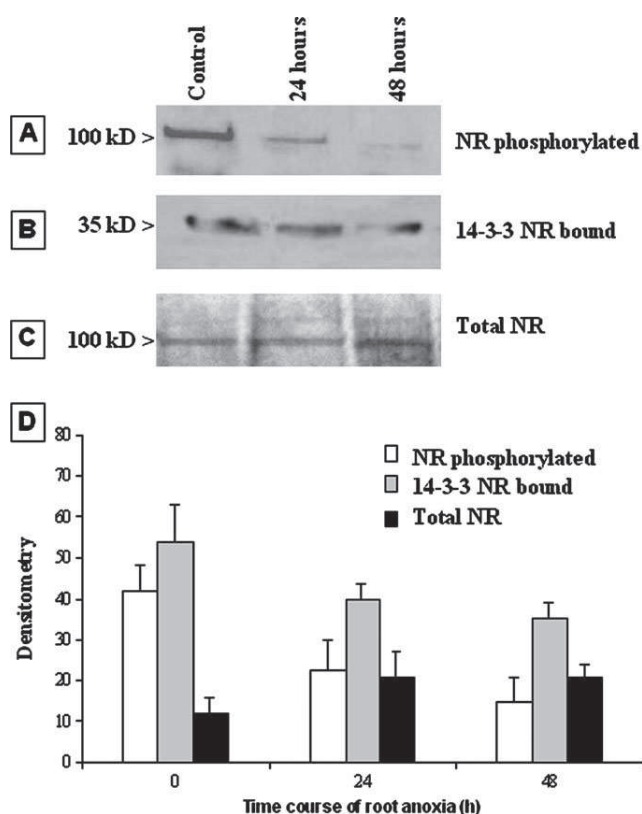
Fig. 3. Time-dependent effect of whole plant-root anoxia on NR regulation. Analysis of NR phosphorylation (A) and NR binding to 14-3-3 protein (B) were performed by immunoprecipitation and western blot. NR expression (NR free+NR-P+NR-14-3-3 bound) (C) was estimated by western blot. (D) Histogram of quantitative densitometric analysis (relative units) of phosphorylated NR, NR bound to 14-3-3 and total NR protein expression. Histograms represent means of three separate experiments.

deprived of nitrate was reduced to 37% versus anoxic roots in the presence of nitrate. These results support cytoplasmic NR activity that is dependent on nitrate. In the presence of nitrate, NR was strongly dephosphorylated in oxygen-deprived roots compared with the control (Fig. 5B). In the absence of nitrate, the level of phosphorylated NR was slightly decreased in oxygen-deprived roots versus control. After 48 h of anoxia, the level of NR phosphorylation was four times higher in the absence than in the presence of nitrate (Fig. 5C).

Morphological state of the plant in different conditions of root anoxia

To determine the role of nitrate and nitrate reductase in tolerance to root anoxia, the morphological state of plants under oxygen deprivation was analysed (i) in the presence of nitrate, (ii) in the absence of nitrate, and (iii) in the presence of tungstate in the culture media (Fig. 6).

Tungstate is assumed to replace molybdenum in the NR enzyme complex rendering it inactive. Tungstate supply prevents nitrate reduction (Notton and Hewitt, 1971). At the
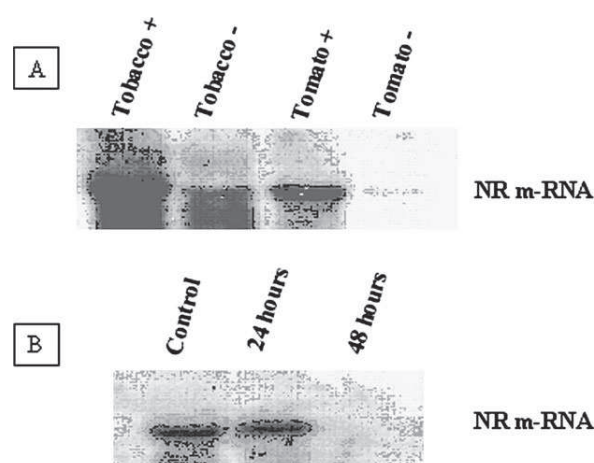
Fig. 4. Northern blots showing the expression of NR m-RNAs in tobacco and tomato leaves collected in the morning (positive control) and in the evening (negative control) (A), and in aerated or 24 h and 48 h oxygen-deprived roots (B). Total RNA (15 μg per lane) was hybridized to NR cDNA radiolabelled probes.

same time, plants were supplied with non-toxic concentrations of ammonium in the root medium to support nitrogen assimilation. In fact, concurrent use of tungstate and ammonium appears to be an appropriate means of restricting NR activity while maintaining nitrogen assimilation (Deng *et al.*, 1989).

The changes in leaf appearance of whole plants after 24, 48, and 72 h of root anoxia were noted (Fig. 6). Firstly, the non-toxic effects of tungstate were tested: in aerated conditions, 3 d absence of nitrate (Fig. 6A) or in the presence of tungstate with ammonium (Fig. 6B), did not generate any leaf symptoms.

After 24 h of oxygen deprivation, leaves from plants without nitrate (Fig. 6D) or with tungstate (Fig. 6E) showed the early symptoms of anoxia such as wilting and corky necrotic spots. Tomato plants with nitrate supplied did not present any leaf symptoms (Fig. 6F).

After 48 h of oxygen deprivation, necrosis was more pronounced on leaves of plants grown without nitrate (Fig. 6G) or with tungstate (Fig. 6H) than on plants with nitrate (Fig. 6I). The last one showed early wilting symptoms.

At 72 h of anoxia, leaves of plants without nitrate (Fig. 6J) or with tungstate (Fig. 6K) were curled up with necrotic symptoms. With the same oxygen deprivation, leaf necrosis of plants fed with nitrate (Fig. 6L) was slight.

Nitrate reduction by NR delayed plant wilting and leaf necrosis for about 48 h.

Discussion

Nitrate reductase activity in oxygen-deprived roots

In anoxic conditions only, nitrite appeared in the nutrient solution about 10 h after the beginning of the experiment
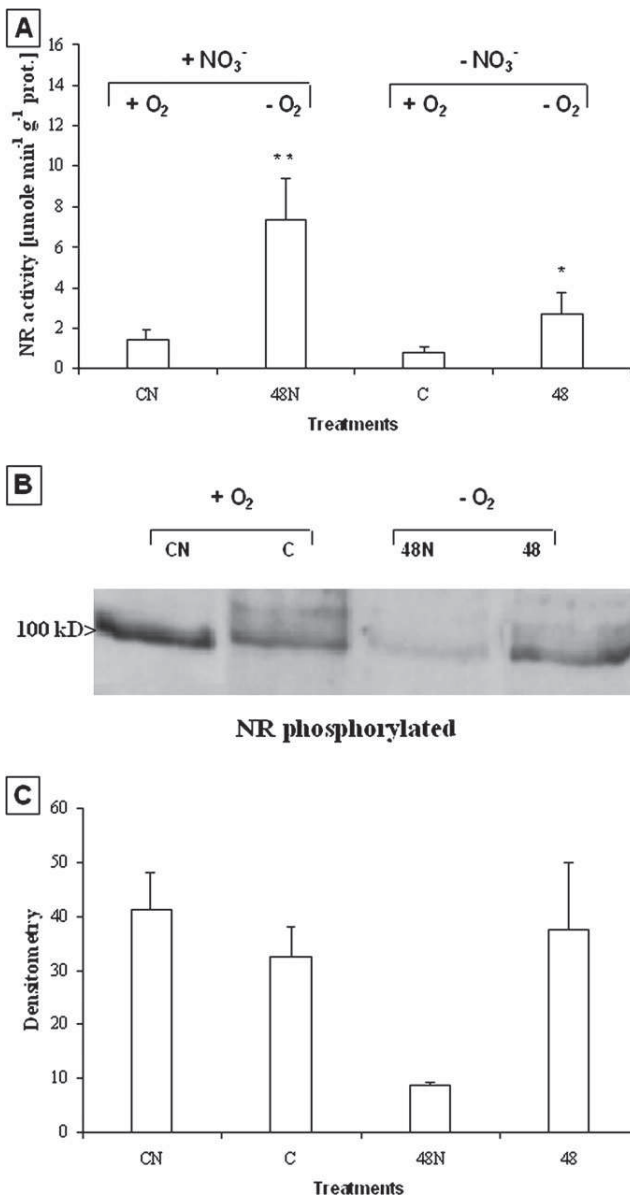
Fig. 5. (A) NR activity measured with nitrate in aerated conditions (CN) and after 48 h anoxic conditions (48N). NR activity measured without nitrate in aerated conditions (C) and after 48 h in anoxic conditions (48). Histograms represent mean ±SD of three separate experiments, two asterisks indicate a significant increase of NR activity at *P*<0.01. (B) Analysis of NR phosphorylation and NR expression in plants treated as described above. Each western blot is representative of three separate experiments. (C) Histogram of quantitative densitometric analysis (relative units).

(Fig. 1B). This phenomenon is related to the lack of oxygen as a result of root respiration (Morard *et al.*, 2000). Nitrate uptake was enhanced compared with the aerated control. These results suggest that NR activity was stimulated by anoxic conditions. NR activity was therefore measured in root juice pressed from whole tomato roots. The activity of nitrate reductase increased time-dependently under anoxic
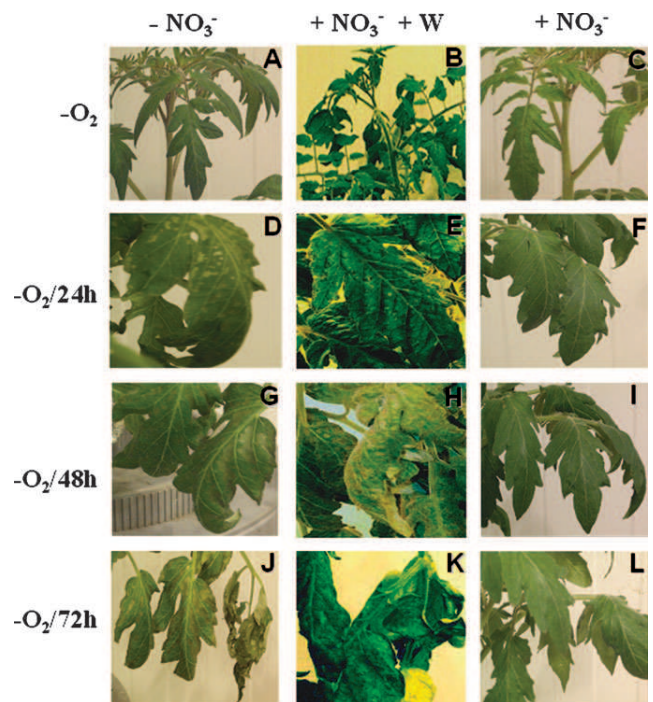
Fig. 6. Morphological changes of tomato leaves in aerated (A–C) and oxygen-deprived (D–L) roots in different conditions. (A, D, G, J) Plants cultivated without nitrate; (B, E, H, K) plants cultivated with nitrate and tungstate supplied with ammonium; (C, F, I, L) plants cultivated with nitrate. Each photograph is representative of three separate experiments.

conditions (Fig 2A). Concomitantly, nitrite content increased in root tissues (Fig. 2B), but not to a great extent because, in roots, nitrite is rapidly released into the nutrient medium (Fig 1A). A constant root ammonium concentration was observed during the time-course of anoxia (Fig. 2C). These results suggest that, under anoxia, nitrite was not highly accumulated into amino-acid synthesis by the roots. Nitrite release by anaerobic tissues is not a new finding. Ferrari *et al.* (1973) and Botrel *et al.* (1996) also noticed this phenomenon. Nevertheless, the role of nitrite release by oxygen-deprived plants remains unclear. To gain an insight into the role of nitrate reduction and nitrite leakage, NR regulation must be investigated.

Regulation of nitrate reductase in anoxic conditions

Normally, NR is inactivated by both phosphorylation and binding to a 14-3-3 protein inhibitor (Weiner and Kaiser, 1999, 2000). However, it has been suggested that in posttranslational modulation of NR, phosphorylated NR also constitutes an active form of the protein (Kaiser and Huber, 2001). These results indicate that anoxia induced NR activation by both dissociation with the 14-3-3 protein inhibitor and dephosphorylation of the NR protein (Fig. 3). After 48 h of anoxia, the respective decreases in NR phosphorylation and binding to the 14-3-3 protein inhibitor of the enzyme were very similar (26% and 36%, respectively).

These data suggest a relationship between NR phosphorylation and binding to the 14-3-3 protein inhibitor and, on the other hand, NR enzymatic activity under anoxia. The data imply that *in vivo*, in whole plant roots, the active form of the NR induced by anoxia is the native form.

Concomitantly with NR regulation, the expression of NR-mRNA was analysed by northern blots at 24 h and 48 h of root anoxia (Fig. 4). Data showed that anoxia did not increase NR-mRNA synthesis. Surprisingly, NR-mRNA was very low at 48 h of root anoxia, indicating that oxygen-deprivation strongly disturbed root metabolism. During anoxia, a general decrease in mRNA translation and an activation of the expression of anoxic genes producing anaerobically induced proteins (ANPs) has been observed (Sachs *et al.*, 1980, 1996). Normal protein synthesis was inhibited under anoxia and 10–20 ANPs appear constituting 70% of the total. The majority of the genes induced encode for enzymes involved in starch, glucose mobilization, glycolysis, and fermentation. The anaerobic response element (ARE) regulates some of these genes (Walker *et al.*, 1987). In maize, hypoxic stress rapidly induces the expression of these genes within four h. More recently Huq and Hodges (1999) identified a novel gene family called anaerobically inducible early (AIE) in a very flood-tolerant variety of rice. Tomato is not particularly adapted to long-term root anoxia. Tomato plants die under a prolonged period of root oxygen deprivation unlike tolerant species (Morard and Silvestre, 1996). However, under this study's experimental conditions, tomato was able to tolerate a prolonged period of root anoxia for up to four days. It was assumed that rapid induction of AIE genes did not occur in tomato and that NR did not constitute an ANP gene product. Nevertheless, although NR-mRNA synthesis decreased at 48 h of root anoxia, the total amount of NR remained constant between 24 h and 48 h and NR activity strongly increased. It is known that the NR association with the 14-3-3 protein inhibitor is necessary for its degradation (Kaiser and Huber, 2001). These results demonstrate that the NR–14-3-3 complex is gradually dissociated during anoxia (Fig. 3). Several factors could explain NR dissociation with the 14-3-3 protein inhibitor under anoxia. Kaiser and Brendle-Behnisch (1995) demonstrated that cellular acidification prevented its binding with NR. Under anoxia, other studies described cellular acidification (Roberts *et al.*, 1984). Both events argue for an arrest of NR degradation under anoxia. Overall, these data suggest a strong inhibition of NR degradation within 48 h and this could constitute a possible adaptative response.

Following this conclusion, the effect of nitrate on NR regulation was assayed in the presence or absence of oxygen. The results of Fig. 1 pointed out that nitrate uptake was strongly increased in oxygen-deprived roots compared with aerated roots. In this study's conditions, NR activity was maximum when nitrate was provided in the culture medium, independently of the presence of oxygen (Fig. 5).

Nevertheless, in the absence of nitrate, NR activity was much higher in oxygen-deprived than in aerated roots. Tomato plants can, therefore, tolerate nitrate shortage, but NR activity is then limited. In the absence of nitrate in the culture medium, the nitrate in cell vacuoles remains the only, although not easily available, pool for nitrate reductase activity. The nitrite content in aerated tomato roots (0.08 ± 0.001 µmol g^{-1} root FW) was analogous with nitrite content in nitrate-depleted aerated and anoxic roots (0.1 ± 0.01 and 0.12 ± 0.05 µmol g^{-1} root FW, respectively). So, nitrate is not a limiting factor for nitrogen assimilation during the time-course of the experiment (48 h). In these conditions, the high levels of phophorylated-NR observed suggest that nitrate plays a key role in NR post-translational regulation.

Role of nitrate supplied in anoxia tolerance

According to previous agronomic observations (Arnon, 1937; Malavolta, 1954; Guyot and Prioul, 1985), nitrate supplied to roots enhanced the plant's tolerance to anoxia. Confirmation of these finding was studied with observation of the aerial parts during tomato root anoxia, particularly the visual changes on leaves over the period from 24 h to 72 h (Fig. 6). Nitrogen nutrition can be changed in two different ways, either deprivation of nitrate supplied or blocking of NR activity. These results clearly demonstrated that the absence of nitrate accelerated the appearance of anoxic symptoms over 24–48 h (Fig. 6D, G). Likewise, when NR activity was blocked by tungstate, anoxic symptoms appeared 24–48 h earlier in anoxic plants in the presence of nitrate (Fig. 6E, H). In oxygen-deprived plants with nitrate, no symptoms appeared 24–48 h after the beginning of anoxia (Fig. 6F, I). Symptoms appeared only after 72 h (Fig. 6L). Overall, these results indicated that plant tolerance to anoxia was dramatically decreased by the absence of nitrate reduction to nitrite. Moreover, visual symptoms indicate that root anoxia is highly stressful for the aerial part of tomato plants. The principal effects of anoxia on leaves are first, stomatal closure, then epinastic curvature and wilting, and finally corky necrotic spots. Morphological changes on leaves are linked with a signal transduction pathway from roots for aerial plant parts. This phenomenon occurs under the influence of hormone signals and is related to the change of ethylene concentration interacting with gibberellins (Musgrave *et al.*, 1972), auxins (Cookson and Osborne, 1978) and cytokinins (Takei *et al.*, 2002). Stomate closing was promoted by an increase of abscisic acid in leaves (Zhang and Davies, 1987) and related to decreased water uptake (Bradford and Hsiao, 1982). In anoxia, hydraulic conductivity of root was also inhibited by gating of aquaporins, related to cytosolic acidosis (Tournaire-Roux *et al.*, 2004).

On root tissues, the first consequence of anoxia is a lowering and then a stopping of aerobic respiration. The

mitochondrial electron-transport chain is also stopped and hence the synthesis of ATP is inhibited (Blokhina, 2000). In these conditions, fermentative pathways such as lactic and ethanolic fermentation are promoted and affect cell energy charge and NADH/NAD$^+$ ratio (Chrikova and Belonogova, 1991). Too low an energy charge leads to the death of root cells by the degradation of the mitochondrial ultrastructure and this was observed in pumpkin roots after 10–15 h of anoxia (Vartapetian *et al.*, 2003). This phenomenon cannot be reversed by reaeration. Mitochondrial ultrastructure can be preserved by the hydrolysis of starch reserves and the activation of fermentation enzymes that limit the effects of anoxia. Moreover, ADH activity could be enhanced by the appearance of oxidative stress due to NADP(H) activation when oxygen disappears (Baxter-Burrell *et al.*, 2002). In these experimental conditions, root peroxidase activity, expressed in mM oxidized guaiacol μg^{-1} protein min^{-1}, was enhanced time-dependently from 8.3 ± 3.3 to 11 ± 3.3 after 24 h anoxia and to 45 ± 10.6 after 72 h anoxia.

One of the major consequences of the reduction of aerobic respiration is the ATP shortage for membrane ATPases, resulting in the depolarization of the root cell membrane (Buwalda *et al.*, 1988). This cell phenomenon induced a fast K$^+$ efflux out of the roots of cucumber (Bertoni *et al.*, 1993) and of tomato (Morard *et al.*, 2000) in the same experimental conditions. Likewise, the fermentative pathway and passive leakage of H$^+$ and anions from the vacuole into the cytoplasm lead to an acidification of cell cytoplasm reducing tolerance to anoxia (Roberts *et al.*, 1984; Kaiser and Huber, 2001; Drew and Armstrong, 2002). However, in anoxic conditions, H$^+$ extrusion by maize roots increases when the cytoplasmic pH falls (Xia and Roberts, 1996), regulation of cytoplasmic pH, limiting cell acidosis, enhanced root cell survival (Drew, 1997). Moreover, cytosolic acidification promotes nitrate reductase activity, resulting in nitrite release out of root cells (Stoimenova *et al.*, 2003). One hypothesis is that nitrate reduction could prevent a pH fall by the extrusion of H$^+$ as H$^+$NO$_2^-$ like nitrite transport as described in the chloroplast (Shingles *et al.*, 1996). In accordance with work on pH, these results showed a strong nitrite release over 10 h and a pH decrease from 6 ± 0.08 to 5.4 ± 0.1 was recorded in the nutrient solution after 48 h of anoxia.

In conclusion, these studies demonstrate that (i) root anoxia induces activation rather than new synthesis of NR and (ii) nitrate reduction improves plants' resistance to anoxia. Likewise, under oxygen deprivation, NR displayed functions other than nitrogen assimilation: it enabled NAD$^+$ regeneration and prevented the pH from dropping to life-threatening levels. To confirm these hypotheses, further studies under anoxia are necessary, in particular, investigations into the effects of the presence or absence of nitrate on the NADH/NAD$^+$ ratio and on intracellular pH variations.

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