Activation and Deactivation of Nitrate Reductase Isozymes in the Leaves of Barley Plants under Light and Dark Conditions

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Abstract: Addition of NO_3^- to the incubation medium of dark-grown NO_3^- -starved barley seedlings (*Hordeum vulgare* L. cv. Steptoe) resulted in a greater accumulation of NO_3^- in leaves and roots more than those of illuminated-grown seedlings. These observations might be related to the role of NO_3^- transportation and assimilation. Also, NO_3^-induced both NADH-specific nitrate reductase (NR) isozyme in leaves and NAD(P) H- bispecific NR in roots. The NR activation state was markedly increased in presence of 200 µM staurosporin, a kinase inhibitor, and 50 µM arsenate, an inhibitor of ATP synthesis, in the incubation medium, while NR greatly decreased in presence of 250µM microcyctin, a phosphatase inhibitor. Addition of 20µM cyclohexamide, inhibitor of protein synthesis, to the incubation medium, in the dark resulted in an increase of both actual nitrate reductase (NR_{act}) and maximum nitrate reductase (NR_{max}) in the leaves. These observations suggested that activation/deactivation of leaf NR might be attributed to phosphorylation/ dephosphorylation of enzyme protein rather than inhibition of enzyme protein synthesis.

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Keywords: Activation state; nitrate reductase; activation; protein phosphorylation; NR_{max}= maximum NR capacity of the dephospho form; NR_{act} = actual NR activity

1. Introduction

Nitrate reduction takes place in both roots and shoots but is spatially separated between the cytoplasm where the reduction takes place and plastids/chloroplasts where nitrite reduction occurs. **(Meyer and Stitt, 2001).** Nitrate reductase (NR) is known to be under complex regulation that occurs at both transcription and posttranscriptional levels (**Crawford, 1995; Scheible et al., 1997a; Pérez et al., 2005).**The NR in most higher plant species is NADH specific (EC 1.6.6.1), but some species also have an NAD (P)H-bispecific NR (EC 1.6.6.2) (**Kleinhofs and Warner, 1990; Li and Oaks, 1993).**At the enzyme activity level the NADH and NAD(P)H NR isozymes are differently regulated by various environmental factors such as nitrate resupply, light, temperature, pH, CO_2, O_2, water potential, nitrogen source and phytohormons which induces a global changes of NR gene expression **(Lea et al., 2004; Gojon et al., 2009; Krapp et al., 2011).** In addition, **Provan and Lillo (1999)** have reported that in higher plants, NR is rapidly inactivated/ activated by phosphorylation/ dephosphorylation in response to environmental stimuli and various treatments as sugars, cytosolic acidification, and anaerobiosis.

Kaiser and Huber (2001) suggested that NR exists basically in three states: free NR, phosphorylated NR (pNR) and (phosphor-NR-inhibitor protein complex). It has been shown that catalysed by protein kinases, NR can be phosphorylated on a serine residue within the hinge 1 region, creating a binding site for 14-3-3 proteins. In the presence of mM concentrations of free Mg^{2+} or other divalent cations, binding of 14-3-3 proteins to pNR inactivates pNR (**Bachmann et al., 1996; Campbell, 1999; Weiner and Kaiser, 2000; Mackintosh and Meek, 2001).** Dephosphorylation is catalysed by type 2A protein phosphatase (PP2A) (**Mackintosh, 1992; Huber et al., 2002; Saber, 1996).** The balance between the activities of kinases and phosphatases acting on NR and pNR determines the phosphorylation status of the enzyme. (**Engelsberger and Schulze, 2012).**

Generally, the phosphorylated form of NR is thought to predominate over the dephosphorylated NR in darkness and vice versa. **(Mackintosh, 1992; Huber et al., 2002).** In addition to 14-3-3 proteins, Mg^{2+}, Ca^{2+}, or polyamines have repeatedly been shown to be necessary for the inactivation of NR, and the effects of these ions on NR inactivation are complex (**Provan et al., 2000; Athwal and Huber 2002; Kaiser and Huber, 2001).** The aim of this study was to evaluate the role of phosphorylation and dephosphorylation processes in activation of NADH-specific NR isozyme in the leaves of wild type Steptoe plants grew under light and dark conditions.

2. Materials and methods

Seed Germination and Plant Growth

Barley (*Hordeum vulgare* L., cv Steptoe) seeds

was soaked and aerated in 1% sodium hypochlorite for 5 min then rinsed in distilled water and placed on moistened paper in the dark for 24 h in an environmentally controlled room. After 24 h seeds were spread over a plastic net placed 3 cm above the surface of a 2 L of 0.5 mM $CaCl_2$. The seeds were covered with wet filter paper and kept in the dark at 25°C. After 48 h the seedlings, from which the endosperm were removed, were transferred to a N-free nutrient solution (pH 5.6) containing 0.2 mM $CaCl_2$, 0.2 mM $MgSO_4$, 2 mM KH_2PO_4, 0.025 mM Fe-EDTANa_2, and micro-elements as described by **Mae and Ohira, (1981)**. Barely seedlings were germinated and grown with the same nutrient solution in a growth cabinet under12h light and 12h dark conditions. At day 9 after germination, 2.3mM KNO_3^- was applied with nutrient solution to the N-deficient seedlings. The seedlings were divided into two sets; the first was incubated for 10h at 25°C under light illumination ($250\mu Em^{-2}s^{-1}$), as previously described by **(Sueyoshi et al., 1999)**, while the second set was kept for 10h in darkness.

Estimation of accumulated NO_3^-

Nitrate was extracted from the dried tissues with deionized water (5ml g^{-1} fresh weight basis) by heating at 80°C for 20 min and the concentration was determined by the capillary electrophoresis (Quanta 4000E, Millipore Corp., MA, USA) with a fused silica capillary tube (70µm ×60 cm) as previously described by **Sato et al., 1998 ; Kawachi et al., 2002.** Nitrate concentration was detected by optical density at 185nm.

***In vitro* nitrate reductase assay**

Extraction

NR extraction was carried out according to the method of **(Aslam et al., 2001)**.Samples were homogenized with an extraction buffer (2 ml /g fresh weight) containing 50 mM Hepes-KOH (pH 7.7), 25 mM NaF, 1 mM $Na_4P_2O_7$, 10 mM 2-mercaptoethanol, 10 µM leupeptin and 1 mM EDTA. The homogenate was centrifuged at 15,000 rpm for 20 min and the resulting supernatant was used for the NR assay.

Activity measurement

The NADH-dependent NR activity was assayed in 450 µl of reaction mixture (50 mM Hepes-KOH (pH 7.7), 5 µM leupeptin, 2 mM KNO_3, 10 µM FAD, 1 mM EDTA and 0.2 mM ß-NADH) as described by **Dailey et al., 1982b**. In the case of the NADPH-dependent NR assay, a reaction mixture containing 0.1 mM ß-NADPH, 0.1 mM ß-$NADP^+$ and 0.2 mM glucose-6-phosphate instead of ß-NADH was used, in order to allow rapid regeneration of NADPH **(Savidov et al., 1998).** The reaction was carried out at 30°C for 15 min and stopped by the addition of 50µl of 1 M zinc acetate. After removal of the precipitate by centrifugation, equal volumes of 1% sulfanilamide and 0.02% *N*-(1-naphthyl) ethylene diamine dichlorohydrate were added to the supernatant, after which the absorbance was measured with a spectrophotometer (UV-1600, SHIMADZU Co., Ltd., Kyoto, Japan) at 540 nm **(Botrel et al.,1996).** NR_{act} and NR_{max} were estimated according to the method of **(Kaiser and spill, 1991).**The calibration curve was prepared using sodium nitrite solution.NR activity state was defined by the ratio of the actual activity (NR_{act}) to the total NR activity (NR_{max}) **(MacKintosh et al., 1995).**

Electrophoresis and immune-blotting analysis

Sodium dodecyl sulfate (SDS) -polyacrylamide gel electrophoresis was performed in 10% gel by the method of (**Laemmli, 1970).** After electrophoresis, the proteins in the gels were transferred onto poly-vinylidene, difluoride (PVDF) membranes (Nihon Millipore, Tokyo, Japan). The NR protein on the membranes was detected with anti NR antibody **(Kojima et al., 1995).**

Statistical analysis

Each treatment was analyzed with at least three replicate tissue samples. Results are the means of three replicates. SEs exceeding the symbol size is indicated by bars.

3. Results and Discussion

Net NO_3^- accumulation in Steptoe seedlings grown under light condition was 55% and 50% lower in leaves and roots than those of plants grown under darkness, respectively (Table 1). These observations may be related to the rate of NO_3^- transportation and the capacity of NO_3^- assimilation. It has been proposed that NO_3^- reduction in the leaves might regulate NO_3^- transport to the roots and that is mainly correlated with NO_3^- assimilation in roots i.e depend on NR activity. **(Ben Zioni et al., 1971; Oji et al., 1989; Forde, 2000; Abdel-Latif et al., 2004).** In connection with these events, the lower activity of NADH-NR isozyme (Figure 1a) in the leaves of dark-grown barley plants may result in a suppression of NO_3^- transport and increase of NO_3^- accumulation.

It is shown that the NADH-NR specific in leaves and the NAD(P)H-NR bispecific isozymes in roots are differentially regulated by nitrate and light conditions (Figure 1 a, b). The isozyme NAD(P)H-NR was not expressed in the Steptoe leaves but it was induced by NO_3^- in the roots. **Dailey et al (1982a)** reported that in barley plants, both NR isozymes are active in the roots, while only one isozyme (NADH-NR) is active in the leaves. Similar observations were reported by **Li and Oaks (1993)** working on maize and **Abdel-Latif et al (2004)** working on barley. In addition, **Kaiser and Huber (2001)** suggested that the expression of NR was induced by NO_3^- and the NR activity altered by the change in light and dark conditions, pH, CO_2 concentration and anoxia.

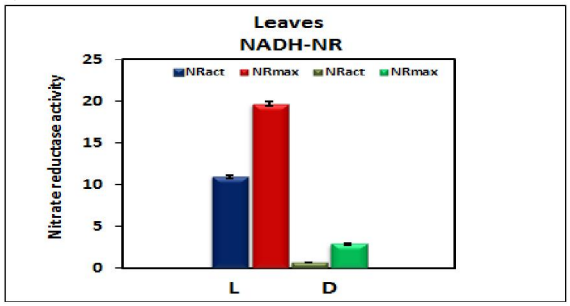
In the present study, the NR_{act} was 56% of NR_{max} in the leaves in 10h- illuminated barley leaves, this was declined to 23% in the dark-grown plants (Figure 1a). Similarly in roots, the NR activation state of NADH-NR and NADPH-NR isozymes decreased from 39% and 45% to 8% and 14%, respectively. (Figure 1 b, c). Many authors (**Kaiser and Spill, 1991; Kojima et al.,1995; Su et al., 1996; Man et al., 1999)** have reported that the NR activation state in leaves is mainly lower in the dark than in the light.

It is concluded that during darkness, NR in leaves is phosphorylated in the hinge 1 region on serin 543 **(Bachmann et al., 1996a)** by a divalent cation dependent protein kinase **(Bachmann et al., 1995; Kaiser et al, 1999)** and is inactivated after binding of an inhibitor protein **(Spill and Kaiser, 1994; Glaab and Kaiser, 1995)**, which belongs to the family of 14-3-3 proteins **(Bachmann et al., 1996b; Moorhead et al., 1996).** Therefore, the lower NR activation state in Steptoe leaves under the dark conditions, in this study, may be attributed to suppression of NO_3^- transport and/or increasing of NR phosphorylation.

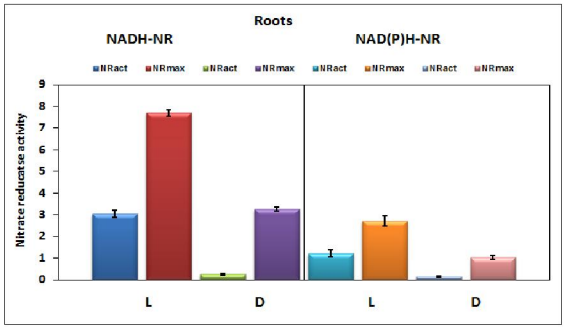
Supplementation the incubation medium of barley plants with 200 µM staurosporin, a kinase inhibitor, in presence or absence of light resulted in an increase to 91% and 12% in NR_{act} and NR_{max} isozymes, respectively. (Table 2). At the same time, the NR activation state was markedly increased, revealing the decrease of phosphorylation process of NR protein due to inhibition of kinase activity. In contrast, presence of 250µM microcystin, a phosphatase inhibitor, under light or dark conditions led to 54% and 37% decreased, respectively, in NR_{max} of Steptoe leaves, suggesting the inhibition of dephosphorylation process of NR. **Mackintosh (1992) and Huber et al (2002)** have reported that phospho-NR is dephosphorylated and activated by a type 2A protein phosphatse, while **Saber (1996)** showed an increase of NR and acid phosphatase activities in broad bean leaves under illumination conditions and the both activities were markedly declined when light was turned off. He concluded that the stimulation of NR activity in light and dark may be related to phosphorylation/dephosphorylation processes as well as binding with specific protein inhibitor. Several NR-protein inhibitors were isolated from different plants grown under light and dark conditions. **Sherrard et al (1979)** identified three forms of endogenous NR-protein inhibitors from wheat leaves, while **Spill and Kaiser (1994)** isolated an NR-inactivator protein with a M.M of 67KDa from spinach leaves. In addition, **Saber (1996)** identified a protein inhibitor for NR in broad bean leaves with a M.M of 58KDa, whereas **Athwal et al (1998)** and **Kaiser and Huber (2001)** suggested that Mg^{2+} was a prerequisite for binding the specific protein inhibitor 14-3-3s to NR protein.

Engelsberger and Schulze (2012) concluded that the balance between the activity of kinases (phosphorylation) and phosphatase activity (dephosphorylation) acting on NR and NR-protein inhibitor controls the phosphorylation status of NR enzyme. Previously **Lillo et al (2003)** proposed the possibility that the NR level increase was due to stability of NR protein. Thus, the activation and inactivation of NR in barley leaves, in the present study, may be attributed to phosphorylation/ dephosphorylation processes depending upon the ATP level and rate of binding with protein inhibitor.

(a)



(b)



(c)

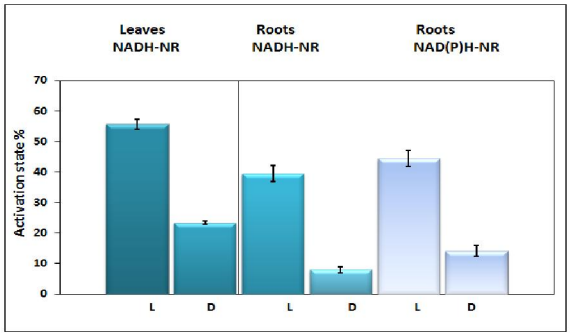


Figure 1. Nine-d-old starved barley Steptoe seedlings were supplied with nutrient solution supplemented with 2.3mM KNO_3^-, and the plants grew for 10h in light and dark. Samples were taken for estimation of NADH-specific NR and NAD (P) H-bispecific NR isozymes activities (a, b) and NR activation state (c). The enzyme activity was expressed as µ mole NO_2^- $g^{-1}f.w^{-1}.h^{-1}$. Results are the means of three replicates ± SE.

Table 1. Nitrate accumulation in the leaves and roots of barley plants. Nine-d-old starved barley seedlings were grew in nutrient solution supplemented with 2.3mM KNO_3^- for 10h in the light or dark. Results are the means of three replicates ± SE.

Treatment	Leaf Root NO_3^- accumulation (µ mol.plant^{-1})	
10h Light	0.795 ± (0.07)	0.493± (0.07)
10h Dark	1.446 ± (0.09)	0.985 ± (0.09)

Table 2. Effect of staurosporin and microcyctin on the NR activity in the leaves of barley plants. Nine-d-old barley seedlings grown hydroponically on 2.3mM KNO_3^- nutrient solution supplemented with 250µMmicrocyctin for 10h under light and dark conditions. Leaf samples were taken for estimation of NR activity. Results are the means of three replicates ± SE.

Conditions	NR_{act} (µ mol.g.f.wt^{-1}.h^{-1})	NR_{max} (µ mol.g.f.wt^{-1}.h^{-1})	NR Activation state
10h L, Control	9.15 ± (0.10)	21.66 ± (0.41)	0.42
10hL, + Staurosporin	17.52 ±(0.29)	24.15 ± (0.45)	0.73
10h L, +Microcyctin	9.46 ± (0.39)	11.49 ± (0.26)	0.82
10h D, Control	0.75 ± (0.06)	2.23 ± (0.10)	0.34
10hD, + Staurosporin	6.57 ± (0.32)	11.13 ± (0.11)	0.59
10h D, +Microcyctin	0.78 ± (0.03)	0.82 ± (0.02)	0.95

Table 3. Effect of arsenate on NR activity in the leaves of barley seedlings grown in dark. Nine-d-old barley seedlings grown hydroponically on 2.3mM KNO_3^- nutrient solution supplemented with 50µM arsenate for 10h in dark. Results are the means of three replicates ± SE.

Conditions	NR_{act} (µ mol.g.f.wt^{-1}.h^{-1})	NR_{max} (µ mol.g.f.wt^{-1}.h^{-1})	NR Activation state
10h D, Contro	0.79 ± (0.05)	2.59 ± (0.19)	0.31
10h D+ Arsenate	4.12 ± (0.32)	9.46 ± (0.22)	0.44

Table 4. Effect of cycloheximide on the NR activity in the leaves of barley plants. Nine-d-old barley seedlings grown hydroponically on 2.3mM KNO_3^- nutrient solution supplemented with 20µM cyclohexamide for 10h under light and dark conditions. Leaf samples were taken for estimation of NR activity. Results are the means of three replicates ± SE.

Conditions	NR_{act} (µ mol.g.f.wt^{-1}.h^{-1})	NR_{max} (µ mol.g.f.wt^{-1}.h^{-1})	NR Activation state
10h L+Cycloheximide	12.84 ± (0.29)	28.97 ± (0.57)	0.44
10h D+ Cycloheximide	4.94 ± (0.04)	9.61 ± (0.31)	0.51

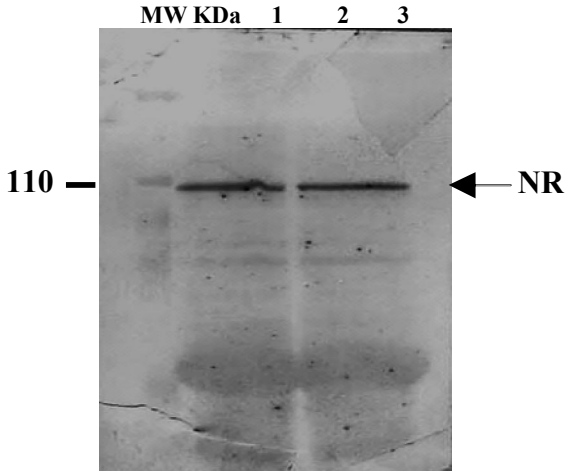


Figure 2. Immunochemical identification of the NR protein on western blots from SDS-PAGE gels of crude extracts of nine-d-old barley seedlings grown hydroponically on 2.3mM KNO_3^- nutrient solution supplemented with 20µM cyclohexamide for 10h in light or dark. Lane 1: marker; Lane 2: 10 h light; Lane 3 10h dark.

Data in Table 3 demonstrate that addition of 50µM arsenate (an inhibitor of ATP synthesis) in the incubation medium of barley plants grown in the darkness resulted in a 6-fold increase in NR_{act} and 3.5-fold increase in $NR_{max,}$ and hence NR activation state in the leaves. These observations revealed the decrease of ATP liberated, during respiration, may result in a decrease of phosphorylation of NR protein, and therefore preventing the binding of phosphorylated enzyme protein with the specific NR protein inhibitor**. Kaiser and Huber (1994a) and Kaiser et al., (1999)** showed that NR was inactivated *in vitro* by incubation with ATP, suggesting that the enzyme was phosphorylated in presence of Mg^{2+} and hence bind with specific protein inhibitor. Furthermore, **Douglas et al., (1995)** reported that proteolytic activity may result in a truncated NR that does not bind specific NR protein inhibitor. In the present study, addition of 20µM cyclohexamide, an inhibitor of protein synthesis, to the incubation medium of dark grown Steptoe seedlings resulted in a marked increase in NR_{act} and NR_{max} isozymes in the leaves (Table 4). These observations are in agreement with those reported by **(Kaiser and Huber 1997)** indicating the degradation of specific protein which bind with NR protein causing

an increase of NR activity rather than NR protein degradation. In connection with this view Figure 2 show a distinct band of NR enzyme in presence or absence of cyclohexamide in the leaves of dark grown plant. These findings led to the expectation that inhibition the synthesis of specific protein inhibitor(s) preventing the binding with enzyme protein rather than inhibitor of NR protein synthesis and/or degradation resulting in an enhancement of NR activity in Steptoe barley leaves.

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