

Effect of nitrate pulses on the nitrate-uptake rate, synthesis of mRNA coding for nitrate reductase, and nitrate-reductase activity in the roots of barley seedlings

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Abstract. Using pulses of nitrate, instead of the permanent presence of external nitrate, to induce the nitrate-assimilating system in *Hordeum vulgare* L., we demonstrated that nitrate can be considered as a trigger or signal for the induction of nitrate uptake, the appearance of nitrate-reductase activity and the synthesis of mRNA coding for nitrate reductase. Nitrate pulses stimulated the initial rate of nitrate uptake, even after subsequent cultivation in N-free medium, and resulted in a higher acceleration of the uptake rate in the presence of nitrate than in its absence.

Key words: *Hordeum* – Nitrate pulse – Nitrate uptake (induction) – Nitrate reductase mRNA

Introduction

It has been reported several times that nitrate is required for the induction of a NO_3^- -uptake system (Minotti et al. 1969; Goyal and Huffaker 1986; Behl et al. 1988; review by Glass 1988). Usually this was concluded from experiments in which plants were initially exposed to nitrate and the induction of the NO_3^- -uptake system was monitored over several hours as an increasing rate of NO_3^- uptake. During a time period of 6–8 h the net rate of NO_3^- uptake was reported to increase five- to tenfold (Warner and Huffaker 1989). In most cases induction occurred after a lag period which might represent the time necessary for the incorporation of functional carriers into the plasma membrane. However, in some studies, this reported delay might have been the consequence of insufficient time resolution of the uptake experiments. In the experiments presented in this paper, we have tried to eliminate this factor by analyzing samples every 7 min.

Reports from different laboratories show that external nitrate was present throughout the experiments on induc-

tion of NO_3^- uptake. Therefore, there is no evidence as to whether nitrate acts strictly as a signal or as a nitrogen source, or both. Furthermore, the amount of nitrate necessary to obtain induction could not be estimated from such experiments. Pulses of nitrate were used by McKown and McClure (1988) and resulted in an accelerated uptake of nitrate in excised root systems. However, the pulses used were longer and the concentrations higher than in the experiments presented here. No data were presented concerning the expression of nitrate reductase (NR) after such pulses, although the relation between NO_3^- uptake and NR activity is close in algae (Tischner and Lorenzen 1981) and most higher plants (Jackson et al. 1973).

The aim of this paper is to demonstrate the effects of very low amounts of NO_3^- which were applied as a pulse and initiated persistent events leading to an accelerated NO_3^- uptake in intact barley seedlings. Such remarkably low amounts of external nitrate have not previously been used in nitrate-uptake studies. Evidence is presented that such low amounts of NO_3^- affect the pools of mRNA coding for NR. The data are interpreted as indicating that NO_3^- operates as a signal in these experiments.

Material and methods

Plant culture. Barley (*Hordeum vulgare* L. cv. CM72 Numar, obtained from the Plant Growth Laboratory, University of California, Davis, USA) seeds were cultivated hydroponically as reported by Aslam et al. (1973) in N-free quarter-strength Hoagland solution (Hoagland and Arnon 1950). The seedlings were grown for 8 d in a growth chamber with a daily regime of 16 h light and 8 h darkness. The relative humidity was 65% to 70% and the photon fluence rate was $400 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$.

Application of NO_3^- pulse. Ten seedlings (approx. 1 g FW) were drained carefully for 30–40 s to remove the nitrogen-free medium and transferred into NO_3^- -containing nutrient solution (concentrations as indicated in the *Results* section) for different periods of time. After the NO_3^- treatment, plants were rinsed three times each for 10 min in 1 l of fresh N-free medium to remove NO_3^- from the free space in cell walls. After rinsing, the plants were kept in fresh N-free medium for the time indicated in the figure legends.

Abbreviation: NR = nitrate reductase

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Induction experiments. Plants were removed from the N-free medium and placed in a nutrient solution containing 0.7 mM NO_3^- . The time course of nitrate uptake was recorded according to Goyal and Huffaker (1986).

Estimation of NO_3^- content. The seedlings were ground and extracted in water. The NO_3^- concentrations were measured using a high-performance liquid chromatography (HPLC) system (Goyal and Huffaker 1986).

Isolation of polyadenylated mRNA and hybridization. The polyadenylated mRNA was isolated from 50 g of root tissue according to Sambrook et al. 1989, and an aliquot of 10 μg per dot was loaded onto a nylon membrane (Hybond; Amersham Inc., Braunschweig, FRG) using a dot-blot apparatus (SRC 96D-Minifold I; Schleicher & Schüll, Einbeck, FRG). After prehybridization the random-primed probe (1.3-kb insert in *PUC12* coding for NADH-NR of barley, obtained from Dr. A. Kleinhoffs, Botany Department, Washington State University, Pullman, USA) was added. The hybridization was performed at 37°C in the presence of 50% formamide (Sambrook et al. 1989). The membrane was washed twice (0.3 M NaCl, 0.33 M Na-citrate = 1 \times SSC, 0.1% sodium dodecyl sulfate; 15 min; room temperature and subjected to autoradiography on Hyperfilm- β -max (Amersham). The dots visible after developing the autoradiographs were identified and cut out of the membrane, and the radioactivity was measured (PW 4540 liquid scintillation analyser; Philips, Eindhoven, The Netherlands). The counts per minute were taken as a measure of the amount of mRNA per dot.

Preparation of NR and determination of activity. The preparation of both, plasmalemma-bound and soluble NR and the estimation of enzyme activity was as reported earlier (Ward et al. 1988).

Calculations. Nitrate-uptake rates were calculated on a plant fresh-weight basis using a personal computer-based calculation program (Goyal and Huffaker 1986). All data presented are the average of at least three to five separate experiments with deviations of less than 6%.

Results

The effect of nitrate pulses on nitrate content and uptake. The nitrate content of whole barley seedlings was positively correlated with the NO_3^- concentration applied during the pulses (Table 1). Depending on the NO_3^- concentration during the pulse, a higher initial NO_3^- uptake rate and an accelerated increase of the uptake rate with time compared with untreated plants was observed. This response was dependent on the concentration of NO_3^- during the pulse (Fig. 1). As for NO_3^- -pulsed plants, control plants displayed only a short (less than 15 min) lag-phase for induction of NO_3^- uptake. Treatment of plants with NO_3^- pulses, where both time and concentration were varied, but where their product remained constant, resulted in identical responses (Fig. 2).

The time in N-free medium following the NO_3^- pulse also affected the response, the optimum time being 4 h (Fig. 3). An effect on the initiation of NO_3^- uptake induction was also obtained with chlorate pulses (Fig. 4). Pulses of NO_2^- resulted in a slightly lower response compared with that noted for chlorate, and there was no effect on the initiation of NO_3^- -uptake induction with pulses of either ammonium or glutamine over a variety of concentrations and time (data not presented).

Table 1. The effect of nitrate pulses of various concentrations on the nitrate content of barley seedlings. The CaSO_4 -grown seedlings were harvested immediately after the nitrate pulse

Nitrate pulse	Nitrate content ($\text{nmol} \cdot (\text{g FW})^{-1}$)
10 min, 17 μM NO_3^-	27
10 min, 35 μM NO_3^-	59
10 min, 70 μM NO_3^-	116

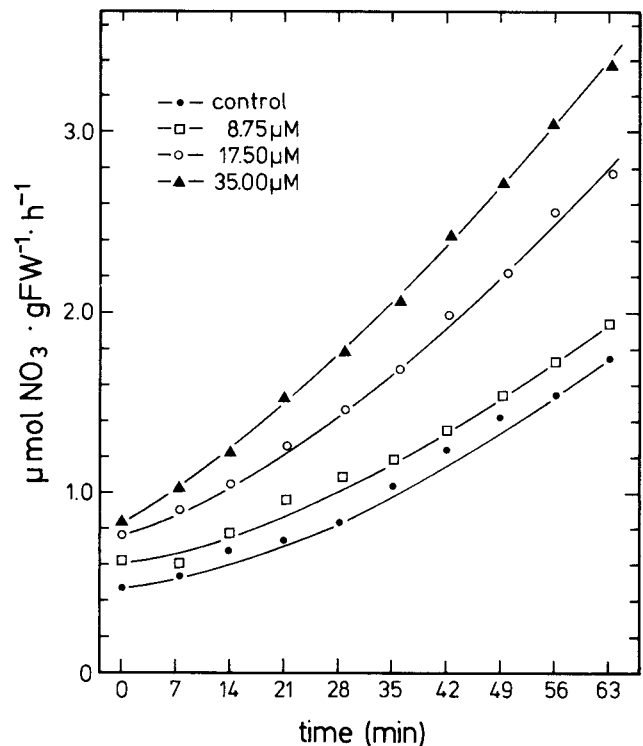


Fig. 1. The effect of NO_3^- pulses on NO_3^- uptake of barley seedlings. Seedlings grown without nitrogen for 10 d were treated with an NO_3^- pulse of 10 min (NO_3^- concentrations as indicated). After three washes in N-free medium (10 min each) the plants were transferred into N-free nutrient for 4 h. Then NO_3^- uptake was monitored in a 0.7 mM NO_3^- solution

The effect of NO_3^- pulses on NR expression and activity. The application of NO_3^- pulses to CaSO_4 -grown barley seedlings resulted in an increase of the NR activity during the time in N-free medium (soluble activity) with a lag of 15 min (Fig. 5). A similar profile was obtained for the plasmalemma-bound NR activity which increased without any delay (Fig. 5). After reaching a maximum value this NR activity declined. Also, the content of mRNA coding for NR was increased by NO_3^- pulses from a basic level, probably as a result of transcriptional initiation and/or a reduction in the rate of degradation. The synthesis of mRNA was initiated and continued to a maximum amount after 60 min in N-free medium and then declined (data not shown). Pulses of 50 μM , 200 μM and 500 μM NO_3^- resulted in 35%, 55%, and 82% of the final ($t_{60 \text{ min}}$) content after 30 min (Fig. 6).

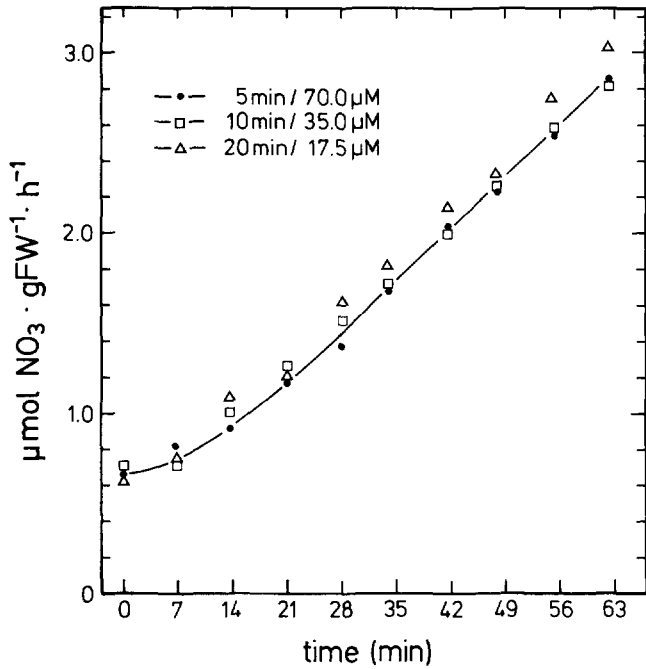


Fig. 2. The effect of NO_3^- pulses of 5 min ($70.0 \mu\text{M}$), 10 min ($35.0 \mu\text{M}$) and 20 min ($17.5 \mu\text{M}$) on NO_3^- uptake of barley seedlings. Plant growth and treatment post NO_3^- pulse were as indicated in the legend of Fig. 1

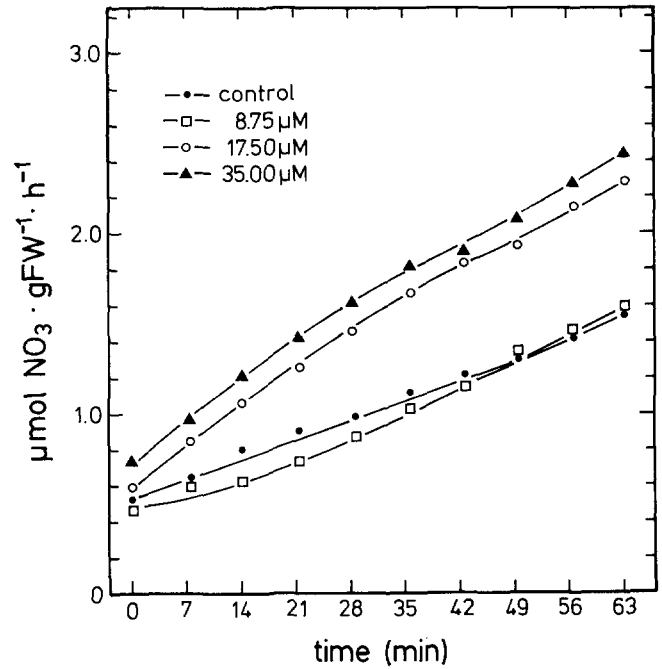


Fig. 4. The effect of 10-min ClO_3^- pulses on NO_3^- uptake of barley seedlings kept in N-free medium for 2 h after the pulse. Plant growth and treatment post ClO_3^- pulse were as indicated in the legend of Fig. 1

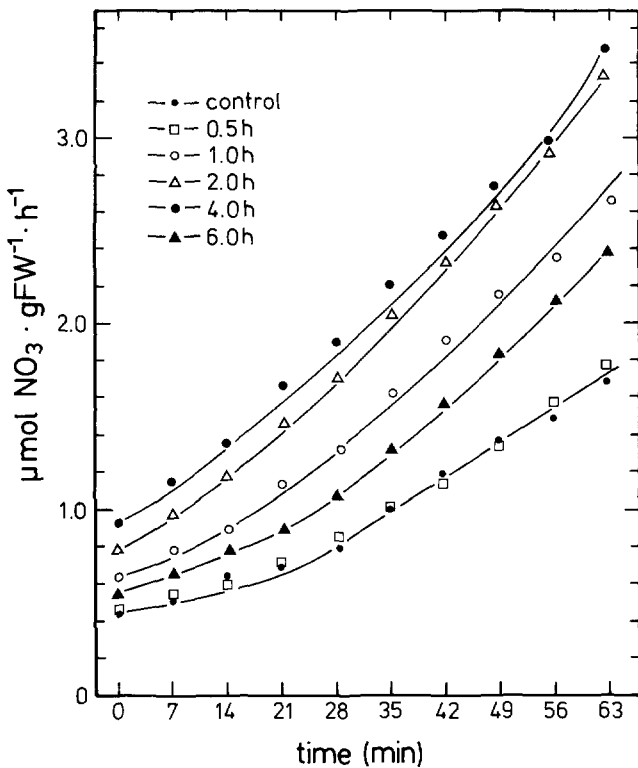


Fig. 3. The effect on NO_3^- uptake of barley seedlings of various periods of time in N-free nutrient medium after an NO_3^- pulse (10 min, $35 \mu\text{M}$ NO_3^-). Plant growth and treatment post NO_3^- pulse were as indicated in the legend of Fig. 1

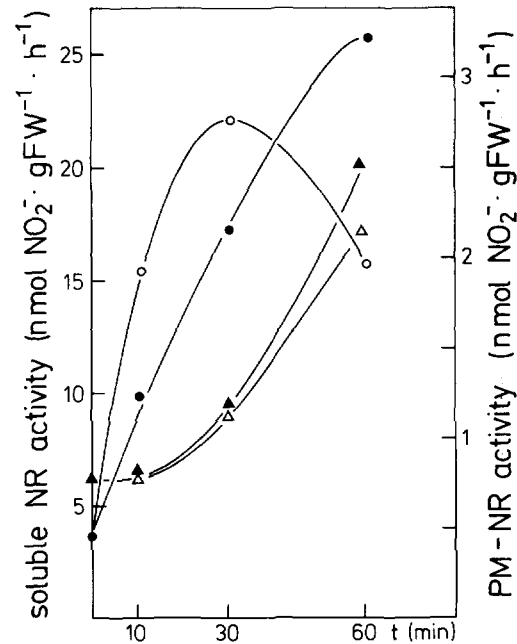


Fig. 5. The effect of NO_3^- pulses on plasma-membrane-bound NR (\circ, \bullet) and soluble NR (Δ, \blacktriangle). Barley seedlings grown without nitrogen for 10 d were treated with 10-min pulses of NO_3^- (\bullet, \blacktriangle : $200 \mu\text{M}$; \circ, Δ : $500 \mu\text{M}$). After three washes in N-free medium (10 min each) the plants were transferred into N-free nutrient. Samples were taken at the time indicated

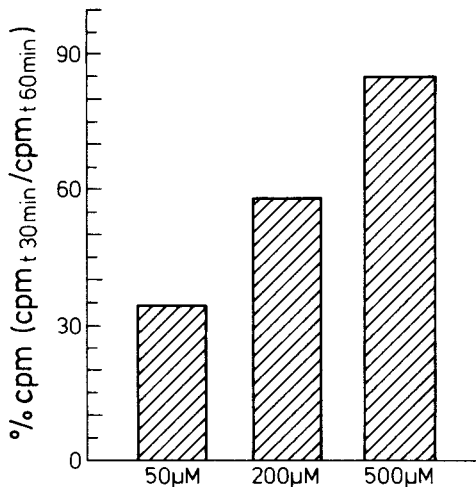


Fig. 6. The effect of 10-min NO₃⁻ pulses (concentrations as indicated) on the synthesis of mRNA coding for NR. After the pulse the plants were transferred into N-free medium. Samples of roots were taken after 30 min and 60 min, respectively. Dot-blot of the mRNA were hybridized with a ³²P-labelled NR probe. The counts per minute were taken as measure of the amount of NR-coding mRNA. The data are expressed as the percentage of cpm after 30 min relative to cpm after 60 min (100%)

Discussion

The experiments reported here were performed with intact barley seedlings and not with excised roots (McKown and McClure 1988) in order to keep the root-shoot interaction intact. The low dose of NO₃⁻ used in the pulses and the frequency of estimation of nitrate-uptake rate supports the interpretation that here nitrate can be regarded as a signal rather than a nitrogen source.

The application of NO₃⁻ pulses increased the nitrate content of N-free cultivated barley seedlings. The amount of nitrate taken up was very low (27–116 nmol · g FW⁻¹, depending on the concentration during the pulse) and insufficient for appreciable protein synthesis. However, treatment of CaSO₄ grown barley seedlings with such NO₃⁻ pulses resulted in an increase of soluble NR activity during the time in N-free medium. This shows that the external presence of nitrate is not necessary for this increase. A similar observation was obtained for the plasmalemma-bound NR (Ward et al. 1988; Tischner et al. 1989) which increased without any delay. This indicates a preference for synthesis of mainly plasma-membrane NR directly after the NO₃⁻ pulses. Later, the balance between plasmalemma-bound and soluble NR activities shifted strongly in favour of the soluble form. The NR activities measured were not as high as reported by Aslam and Huffaker (1982) because of the lack of sufficient nitrogen for protein synthesis.

The content of mRNA coding for NR was also increased by NO₃⁻ pulses from a basic level. The synthesis of mRNA was initiated and continued to a maximum amount after 60 min in N-free medium. Pulses of 50 μM, 200 μM and 500 μM NO₃⁻ resulted in 35%, 55% and 82% of the final (t_{60 min}) content after 30 min. These data indicate that the expression of the NR-coding gene is

accelerated by higher NO₃⁻ concentrations during the pulse. Probably, the rate of mRNA degradation is also reduced, causing an increase in the mRNA level. An effect of the permanent presence of nitrate on the expression of the genes encoding the nitrate-assimilating system has been reported by Caboche and Rouze (1990); Privalle et al. (1989) and Galangau et al. (1988). However, as we have demonstrated here, the permanent presence of external nitrate is not necessary for the initiation of NR gene expression.

The data presented demonstrate that nitrate pulses cause an acceleration of the NO₃⁻-uptake rate. They also indicate that the lag-phase of several hours that has previously been reported (Goyal and Huffaker 1986; Mäck and Tischner 1986) might be due to a lower time resolution in the uptake experiments. In one recent study of barley plants by Bloom and Sukrapanna (1990), no lag-phase of NO₃⁻-uptake induction was found. Jackson et al. (1973) also reported a steady increase of the NO₃⁻-uptake rate after exposure of corn plants to nitrate. In our experiments, NO₃⁻ pulses, in which both time and concentration were varied such that their product remained constant, resulted in identical responses.

The optimal response found after 4 h in N-free medium following the NO₃⁻ pulse indicates that the nitrate-induced events for initiation of NO₃⁻ uptake persist and continue even when nitrate is not available in the medium. The decrease of the effect after more than 4 h in the N-free medium possibly indicate the turnover of a protein or precursor involved in NO₃⁻ transport. The lack of any exogenous nitrogen source may also cause the observed decline (Mäck and Tischner 1986). A stimulating effect on the initiation of NO₃⁻ uptake was also obtained with chlorate pulses. This anion has been frequently used as a nitrate analog in experiments in which identical rates of chlorate and NO₃⁻ uptake were observed (e.g. Deane-Drummond 1984). Based on our experiments, we suggest that chlorate acts similarly to nitrate in the initiation process and therefore may not only be an analog to nitrate at the level of the uptake system but also at the transcriptional level. Pulses of NO₂⁻ resulted in a slightly lower response compared with that noted for chlorate. From our data, we cannot determine if NO₂ itself or NO₃⁻ produced from NO₂⁻ oxidation is responsible for the observed effect. Such an oxidation was suggested to occur in the leaves of barley (Aslam and Huffaker 1989).

No effect on the initiation of NO₃⁻-uptake induction was found when pulses of either ammonium or glutamine were used at a variety of concentrations and for various times. These results are contrary to those reported by Bloom and Finazzo (1986) and Bloom and Sukrapanna (1990), but are in agreement with several other reports (Tischner and Lorenzen 1981; Ullrich et al. 1981; Warner and Huffaker 1989).

The low amount of nitrate taken up during the pulse may support the method of signal transduction suggested by Redinbaugh and Campbell (1991). These authors implied that a hypothetical nitrate sensor at the plasma membrane activates a hypothetical regulator, which then also induces the expression of the nitrate-uptake system.

Probably even the lowest amount of nitrate we found in the plants after a pulse (27 nmol · g FW⁻¹ after a 10-min pulse with 17 μM NO₃⁻) was enough to be recognized by the sensor and to initiate indirectly the expression of the gene coding for nitrate uptake. This effective NO₃⁻ content is several magnitudes lower than that calculated by McKown and McClure (1988) for excised root systems.

Past studies (Goyal and Huffaker 1986; Behl et al. 1988) indicate that the induction of an NO₃⁻-uptake system depends on de-novo protein synthesis. In our experiments, only very limited amounts of NO₃⁻ were available in the cells for reduction (by the basic NR activity always present; Behl et al. 1988; Mäck and Tischner 1986) and, later, for protein synthesis. We speculate that the acceleration of nitrate-uptake induction which occurs when higher amounts of NO₃⁻ are applied in the pulse might, because of the low availability of nitrogen in the nutrient medium, lead to the accumulation of a precursor, possibly mRNA, thereby limiting the efficiency of protein synthesis during and after the pulse. These interpretations are in agreement with data reported by Siddiqi et al. (1989) and Hole et al. (1990) who presented evidence for the requirement of external nitrate for the induction of nitrate influx. We have previously demonstrated (Behl et al. 1988) that even in the presence of sufficient storage material (carbohydrate and protein) in the seed, the capacity of a constitutive uptake system remains at a low level, (basic uptake system), as long as no NO₃⁻ is supplied. In addition, the appearance of nitrate reductase can be detected appreciably later than nitrate uptake from the medium.

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