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Development of Accelerated Net Nitrate Uptake[1]

EFFECTS OF NITRATE CONCENTRATION AND EXPOSURE TIME

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ABSTRACT

Upon initial nitrate exposure, net nitrate uptake rates in roots of a wide variety of plants accelerate within 6 to 8 hours to substantially greater rates. Effects of solution nitrate concentrations and short pulses of nitrate (≤1 hour) upon 'nitrate-induced' acceleration of nitrate uptake in maize (*Zea mays* L.) were determined. Root cultures of dark-grown seedlings, grown without nitrate, were exposed to 250 micromolar nitrate for 0.25 to 1 hour or to various solution nitrate concentrations (10–250 micromolar) for 1 hour before returning them to a nitrate-free solution. Net nitrate uptake rates were assayed at various periods following nitrate exposure and compared to rates of roots grown either in the absence of nitrate ($CaSO_4$-grown) or with continuous nitrate for at least 20 hours. Three hours after initial nitrate exposure, nitrate pulse treatments increased nitrate uptake rates three- to four-fold compared to the rates of $CaSO_4$-grown roots. When cycloheximide (5 micrograms per milliliter) was included during a 1-hour pulse with 250 micromolar nitrate, development of the accelerated nitrate uptake state was delayed. Otherwise, nitrate uptake rates reached maximum values within 6 hours before declining. Maximum rates, however, were significantly less than those of roots exposed continuously for 20, 32, or 44 hours. Pulsing for only 0.25 hour with 250 micromolar nitrate and for 1 hour with 10 micromolar caused acceleration of nitrate uptake, but the rates attained were either less than or not sustained for a duration comparable to those of roots pulsed for 1 hour with 250 micromolar nitrate. These results indicate that substantial development of the nitrate-induced accelerated nitrate uptake state can be achieved by small endogenous accumulations of nitrate, which appear to moderate the activity or level of root nitrate uptake.

A wide variety of plant systems develop an accelerated rate of nitrate uptake following initial exposure to nitrate (3, 9, 11 for reviews). The development of accelerated nitrate uptake appears to be substrate 'induced' and to require protein synthesis; it is sensitive to inhibitors of the synthesis of RNA and protein (10) as well as to *p*-fluorophenylalanine (21). Whether the development and maintenance of accelerated nitrate uptake requires direct or indirect involvement of protein synthesis remains uncertain. After initial exposure, nitrate may induce synthesis of specific membrane transport proteins and other proteins, which alter the activity of membrane proteins. Alternatively, nitrate uptake may be accelerated indirectly by nitrate as a consequence of derepression of genes for nitrate assimilation enzymes.

Several observations indicate that only small quantities of nitrate were necessary to initiate and sustain induced or accelerated nitrate uptake. Jackson *et al.* (10) reported that decapitated maize (*Zea mays* L.) seedlings with less than 5 μmol nitrate g^{-1} FW[2] developed accelerated nitrate uptake rates. Barley (*Hordeum vulgare* L.) seedlings also developed accelerated nitrate uptake after extended exposure to 10 μM nitrate, at which negligible net nitrate uptake occurred (4). The time needed to reach a constant rate of nitrate uptake by dwarf bean (*Phaseolus vulgaris* L.) was independent of solution nitrate (10–1000 μM) concentration (2). Nitrate deprivation of fully induced maize roots caused a slow loss (50% in 32 h) of the estimated induced state of nitrate uptake (17). During this period, root nitrate decreased from about 120 to 15 μmol g^{-1} FW resulting in a decrease of the estimated net flux of presumably vacuole nitrate into the cytoplasm of 2.3 to 0.55 μmol g^{-1} FW h^{-1}.

In the present work, experiments were conducted to further characterize the physiological responses of maize roots to initial nitrate supply. Unlike previously published work (*e.g.*, 2, 4, 10, 20, 21), which has examined the development of accelerated nitrate uptake under a continuous nitrate supply following initial nitrate exposure, we investigated the effects of a single nitrate pulse on the induction of nitrate uptake. A preliminary report of this work was presented previously (16).

MATERIALS AND METHODS

Plant Material. Root systems from 5-d-old dark-grown maize (*Zea mays* L., B73 × Mo17) seedlings decapitated below the first node were used. The endosperm remained attached to a portion of the mesocotyl, thereby providing an endogenous source of assimilates for the root. Surface-sterilized (12.5% bleach, v:v, for 5 min) caryopses were germinated in darkness at 30°C between germination paper saturated with 0.1 mM $CaSO_4$. After 3 d, seedlings with 12- to 16-cm long primary roots were selected for the experiments. Shoot and seminal roots were excised and cultures of five seedling roots each were transferred to 15-L containers of aerated 0.25 or 1.0 mM $CaSO_4$ at 30°C. Root cultures remained in this solution for 2 d before initiation of experiments unless otherwise indicated. The number of cultures did not exceed 35 per container during this period.

Nitrate Uptake 'Induction' Experiments. A series of experiments were conducted at 30°C using various initial nitrate treatments to 'induce' accelerated net nitrate uptake. The induction experiments included treatment with a continuous 10 μM nitrate exposure; a 1.0-h pulse to 10, 50, and 250 μM nitrate; exposure

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[2] Abbreviations: FW, fresh weight; DW, dry weight.

to cycloheximide (5 μg ml^{-1}) during a 1.0-h pulse to 250 μM nitrate; and pulse durations of 0.25, 0.5, and 1.0 h with 250 μM nitrate. Root cultures for control treatments were exposed to one or more of the following aerated solutions at 30°C: 0.25 mM $CaSO_4$, 1.0 mM $CaSO_4$, and 250 μM nitrate as $Ca(NO_3)_2$ plus 1.0 mM $CaSO_4$ for a 20-h period to achieve an induced nitrate uptake state.

For the continuous 10 μM nitrate induction experiment, 4.5-d-old root cultures were transferred to a 15-L container receiving a continuous flow (1.0 L min^{-1}) of fresh 10 μM KNO_3 nutrient solution. The basal nutrient composition of this solution and that used for nitrate uptake assays in this experiment was: 0.05 mM K_2SO_4, 0.4 mM KH_2PO_4, 0.25 mM $MgSO_4$, 0.25 mM $CaSO_4$, 18 μM Fe as Fe-EDTA, and 40% full-strength micronutrient concentration recommended by Hoagland and Arnon (8). Control root cultures were maintained on 0.25 mM $CaSO_4$. Four replicate root cultures were used at each nitrate uptake assay and then harvested. The roots were rinsed in nitrate-free solutions before and after each nitrate uptake assay.

All nitrate-pulse treatments were with KNO_3 plus 1.0 mM $CaSO_4$ solutions. Following nitrate pulses, the roots of each culture were rinsed with deionized water and then with fresh 1.0 mM $CaSO_4$. After rinsing the roots, cultures were returned to vessels containing the original 1.0 mM $CaSO_4$ solutions used to culture the root systems. Unless otherwise indicated, four replicate root cultures of each nitrate-pulse treatment and control treatment were subsequently assayed for nitrate uptake and harvested.

At each harvest, roots exposed to nitrate were rinsed in three containers each with 1.0 L of 1.0 mM $CaSO_4$ at room temperature over a 1-min period.

Nitrate Uptake Assay. Measurements of nitrate uptake activity were initiated by transfer of each root culture to a 250-ml container of aerated 250 μM KNO_3 plus 1.0 mM $CaSO_4$ at 30°C. Depending on the age of the root cultures, the ratio of root mass to nitrate uptake solution was about 4.6 to 8.4 mg FW ml^{-1}. The nitrate uptake assay was for 1 h. Nitrate uptake was determined by solution depletion of nitrate. Solution nitrate concentrations were spectrophotometrically measured directly by UV absorption at 210 nm or colorimetrically after dissimilatory reduction of nitrate to nitrite with *Escherichia coli* (15).

Tissue Nitrate. Primary root tissues were frozen, lyophilized, and weighed. Unless indicated, treatment replicates of the root cultures at each harvest were combined before grinding the dried tissue for nitrate analysis. Tissue nitrate was extracted with water (97°C for 1 h), and an aliquot of the extract was measured for nitrate after conversion to nitrite (15).

RESULTS

Within 12 h of exposure to a continuous flow of 10 μM nitrate nutrient solution, net nitrate uptake rates of maize roots increased four- to sixfold (Fig. 1). The measured magnitude of accelerated nitrate uptake depended on the external nitrate concentration in which nitrate uptake was assayed. The nitrate uptake rate assayed 12 h after initial exposure to 10 μM nitrate nutrient solution was markedly greater than in roots continuously cultured on 1.0 mM $CaSO_4$ (Fig. 1B). Net nitrate uptake rates of individual cultures assayed with 10 μM nitrate nutrient solution (Fig. 1A), however, were consistently about threefold less than the rates of roots assayed with 250 μM nitrate solution (Fig. 1B). Nevertheless, the nitrate uptake development patterns were similar for measurements made at both nitrate concentrations (*cf.* Fig. 1, A and B). During this period, the 22% relative increase in root DW (from 18.9 ± 0.1 to 23.0 ± 0.3 mg root^{-1}) was much less than the relative increase in nitrate uptake rate (>500% for 250 μM nitrate assay).

Net nitrate uptake rates of roots determined during a 24 h nitrate-free period following a 1 h pulse with 250 μM nitrate were compared with rates of roots of the same age either grown continuously in $CaSO_4$ or previously cultured with 125 μM $Ca(NO_3)_2$ for 20 h. Within 6 h after the nitrate pulse, net nitrate uptake rates increased to maximum values, which were about fourfold greater than those of $CaSO_4$-grown roots but about 25% less than the nitrate uptake rates of roots exposed to nitrate for 26 h (Fig. 2). Thereafter, rates of nitrate-pulsed roots progressively declined to a level approximately 1.5-fold greater than those of $CaSO_4$-grown roots at 24 h after the nitrate pulse (Fig. 2).

Root nitrate concentrations of 5.0, 52, 38, 19, and 14 μmol g^{-1} DW were measured at the end of the respective assay periods at 0, 3, 6, 12, and 24 h for nitrate-pulsed roots. These changes

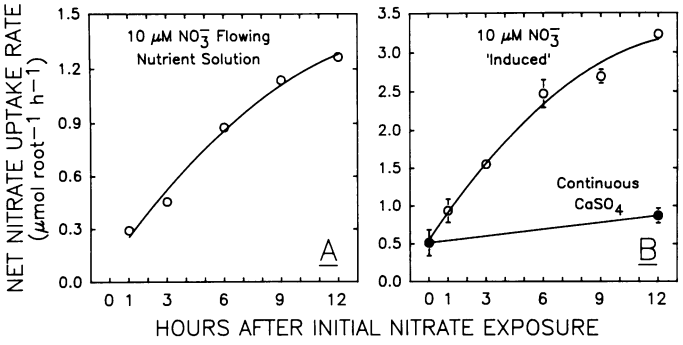

FIG. 1. Net nitrate uptake rates of maize roots continuously exposed to aerated 10 μM nitrate nutrient solution. Rates were determined (panel A) by measuring the flow rate and nitrate concentration of the inlet and outlet stream of a container holding the root cultures or (panel B) by transferring individual cultures to aerated 250 μM nitrate nutrient solution at the times indicated and measuring solution nitrate depletion after 1 h. Root DW (FW) increased from about 19 (230) to 23 (280) mg root^{-1} between the first and last assay periods, respectively. Closed symbols in panel B represent rates of roots continuously cultured in 0.25 mM $CaSO_4$. Bars in panel B indicate ±SE exceeding symbol size.

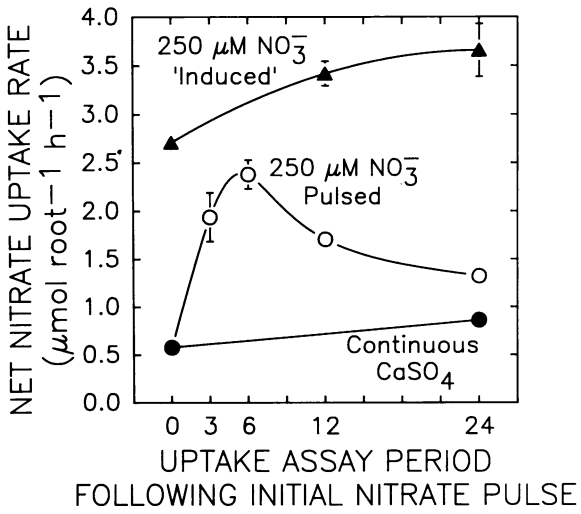

FIG. 2. Net nitrate uptake rates of roots returned to 10 mM $CaSO_4$ after a 1-h pulse with 250 μM nitrate compared with rates of roots grown with and without nitrate. Roots grown with nitrate were exposed to 125 μM $Ca(NO_3)_2$ for 20 h prior to the time of nitrate pulsing. Roots grown without nitrate were continuously exposed to 1.0 mM $CaSO_4$. Nitrate uptake rates were determined by solution depletion of nitrate after 1-h incubation in solutions initially 250 μM KNO_3. Root DW (FW) increased from about 25 (300) to 34 (420) mg root^{-1} between the first and last assay periods, respectively. Bars indicate ±SE exceeding symbol size.

in tissue nitrate concentrations were loosely correlated with changes in nitrate uptake rates of the nitrate-pulsed roots (*cf.* Fig. 2). The $CaSO_4$-grown roots had 7.9 μmol nitrate g^{-1} DW at the end of the 24-h assay period. Tissue nitrate concentrations of roots continuously treated with 250 μM nitrate increased from 300 to 580 μmol g^{-1} DW at the end of the 0- and 24-h assay periods, respectively.

The presence of cycloheximide (5 μg ml^{-1}) during a 1-h pulse with 250 μM nitrate delayed the development of accelerated nitrate uptake rates (Fig. 3). During the nitrate pulse, the presence of cycloheximide had a relatively small inhibitory effect upon the initial nitrate uptake rate (Fig. 3). This observation was enforced by the similarity of tissue nitrate concentrations immediately following the nitrate pulse (7.0 and 7.8 μmol g^{-1} DW for roots pulsed in the presence and absence of cycloheximide, respectively). At 3 h after the nitrate pulse, nitrate uptake rates of roots pulsed with nitrate in the absence of cycloheximide were about fourfold greater than the rates of those pulsed in the presence of cycloheximide or of those roots grown continuously on $CaSO_4$. Nitrate uptake rates of cycloheximide-treated roots assayed 6 h after the nitrate pulse, however, accelerated to a level about threefold greater than the rates of $CaSO_4$-grown roots, but they were still less than those of roots pulsed with nitrate in the absence of cycloheximide (Fig. 3).

Accelerated nitrate uptake development patterns following 250 μM nitrate-pulse durations of 0.25 and 0.5 h were similar to the acceleration pattern of roots pulsed for 1 h (Fig. 4). The magnitude of nitrate uptake acceleration for roots receiving the shortest pulse (0.25 h) was somewhat less than that of roots receiving the longest pulse (1 h). Nitrate uptake rates of roots assayed 6 h after a nitrate pulse for 0.25 h were about 80% of the rates of roots pulsed for 1 h, but the rates for the shortest pulse were about twofold greater than those of $CaSO_4$-grown roots. Following the 3-h uptake assay period root nitrate concentrations were 42, 41, and 54 μmol g^{-1} DW for the 0.25-, 0.5-, and 1-h pulse treatments, respectively, and 19 μmol g^{-1} DW for the continuous $CaSO_4$ treatment. After the 6-h assay period, root nitrate concentrations were 41, 48, and 58 μmol g^{-1} DW for the 0.25-, 0.5-, and 1-h pulse treatments, respectively, and 16 μmol g^{-1} DW for $CaSO_4$ treated roots.

Roots pulsed with 10, 50, and 250 μM nitrate for 1 h and assayed 3 h after the pulse had accelerated nitrate uptake rates

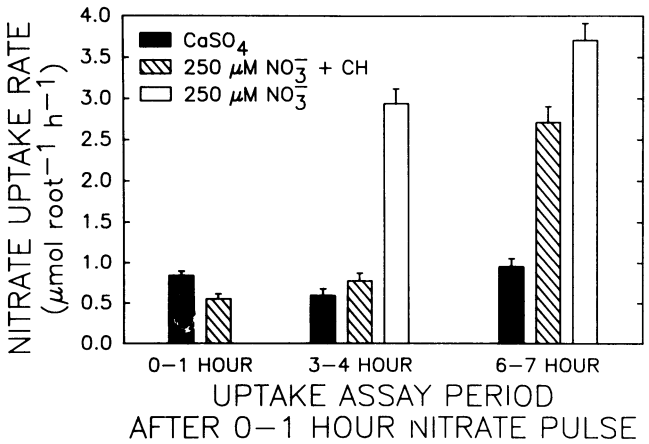

FIG. 3. Effect of cycloheximide (5 μg ml^{-1}, CH) during a 1-h pulse with 250 μM nitrate on the development of accelerated nitrate uptake rates. Details of the nitrate pulse, $CaSO_4$ chase, and nitrate uptake assay were as described for Figure 2. Cycloheximide was included only in the pulse solutions for the CH treatment. Root DW (FW) increased from about 29 (360) to 33 (400) mg $root^{-1}$ between the first and last assay periods, respectively. Bars indicate SE.

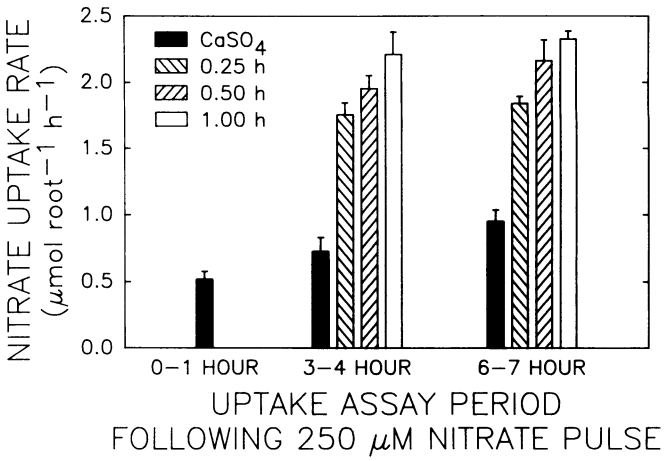

FIG. 4. Net nitrate uptake rates of $CaSO_4$-grown roots compared with the rates of roots pulsed with 250 μM nitrate for varying periods. Nitrate pulsed roots were returned to 1.0 mM $CaSO_4$ for 3 or 6 h before assaying uptake. Rates were assayed as described for Figure 2. Root DW (FW) increased from about 25 (300) to 29 (350) mg $root^{-1}$ between the first and last assay periods, respectively. Bars indicate SE.

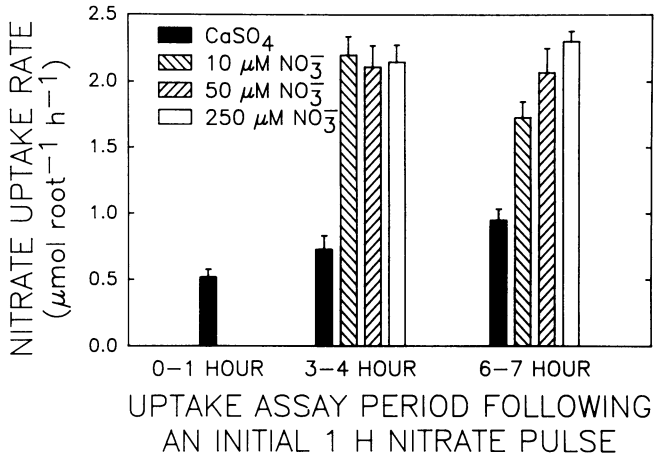

FIG. 5. Effect of solution nitrate concentration during a 1-h pulse on net nitrate uptake of roots returned to 1.0 mM $CaSO_4$ for varying periods. Rates were compared with those of 1.0 mM $CaSO_4$-grown roots. Rates were assayed as described for Figure 2. Root DW (FW) increased from about 25 (300) to 29 (350) mg $root^{-1}$ between the first and last assay periods, respectively. Bars indicate SE.

which were threefold greater than the rates of $CaSO_4$-grown roots (Fig. 5). For the assay period 6 h after the pulses, nitrate uptake rates of roots pulsed with 10 μM nitrate decreased to a level that was only 80% greater than that of $CaSO_4$-grown roots. After the 3-h uptake assay period, nitrate concentrations of roots pulsed with 10 μM nitrate were similar to those following the 6-h assay period (45 *versus* 48 μmol g^{-1} DW, respectively).

DISCUSSION

An extensive body of literature has been published on the nitrate specificity and the physiology of the development of the accelerated nitrate uptake phase following initial nitrate exposure. Nutritional, environmental, and genetic effects on development of accelerated uptake have been reviewed (3, 9, 11, 12). Traditionally, reported studies of nitrate uptake 'induction' after initial nitrate exposure have been conducted with a continuous

nitrate supply. Jackson *et al.* (10) and Deane-Drummond (4) have hypothesized that small quantities of root nitrate are sufficient to initiate a substantial increase in nitrate uptake. Data from our experiments with nitrate pulses clearly support this hypothesis.

The development of accelerated nitrate uptake did not require a continuous supply of nitrate. The degree of acceleration and the maintenance of accelerated nitrate uptake rates, however, depended upon the solution nitrate concentration and the duration of external nitrate exposure (Figs. 4 and 5). Treatments that exposed root cultures to either a continuous supply of 10 μM nitrate (Fig. 1) or a 1-h pulse to 250 μM nitrate (Fig. 2) caused an acceleration of net nitrate uptake. After a 1-h pulse with 250 μM nitrate followed by exposure to nitrate-free solution, net nitrate uptake rates accelerated within 6 h before declining. Compared to the rate assayed at 6 h, the nitrate uptake rates assayed at 12 and 24 h after the pulse were 30 and 40% less, respectively (Fig. 2). Also, the rates attained at 6 h after the pulse (Fig. 2), even though significantly greater than those of $CaSO_4$-grown roots, were not as great as the uptake rates attained by roots continuously exposed to either 10 μM nitrate for 12 h (Fig. 1B) or to 250 μM nitrate for 20 h or more (Fig. 2).

Assuming that root nitrate concentrations are correlated with duration of a nitrate pulse, external nitrate concentration during a pulse, and the elapsed time of nitrate deprivation after a pulse, then our data suggest that the level or activity of accelerated nitrate uptake was moderated by low internal concentrations of nitrate. Based on data reported in this paper and several unreported experiments, the average nitrate concentration and DW of roots from seven similar experiments immediately following a pulse of 250 μM nitrate for 1 h were 14.3 $\pm$ 1.7 μmol g^{-1} DW and 24.9 $\pm$ 1.0 mg $root^{-1}$. Using the conversion factor of 82 mg DW g^{-1} FW (our unpublished data) to change tissue nitrate concentrations to a FW basis, an estimate of the bulk nitrate concentration of the maize root cells at the end of a 1-h exposure to 250 μM nitrate would be about 1.3 mM. Whether the cytoplasmic and vacuole nitrate concentrations would be equivalent at low levels of nitrate is unknown. Recent evidence, however, indicates higher nitrate concentrations in the vacuole than the cytoplasm of tissues grown with nitrate (14, 18). Disregarding intercellular localization effects, the estimated cytoplasmic nitrate concentration after the nitrate pulse would probably not exceed 1.3 mM. At low external ion concentrations, however, the epidermal cell layer of roots are major sites of ion accumulation (1, 13, 22). Because low solution nitrate concentrations were used in these studies and epidermal root cells may have accumulated more nitrate than cortical cells, this estimate of cytoplasmic nitrate concentration may represent a minimum for epidermal and outer cortical root cells. Further research is needed to determine the limits within which changes in nitrate concentrations of specific root cells and cell compartments are correlated with changes in accelerated or induced nitrate uptake.

Inclusion of cycloheximide, an inhibitor of protein synthesis at the translation level, in nitrate-pulse solutions caused a delayed development of accelerated nitrate uptake (Fig. 3). This observation is in agreement with earlier suggestions (11) that the development of nitrate-induced acceleration of nitrate uptake in roots requires protein synthesis. The inhibitory effect of cycloheximide, however, was only temporary. The temporary inhibition may be due to a loss of cycloheximide from root cells involved in nitrate uptake. A similar but more rapid alleviation of inhibition after the removal of cycloheximide from uptake solution has been reported for 'augmented' potassium influx into aerated maize root segments (7). Nevertheless, the full nature of the apparent requirement for protein synthesis remains to be determined. A direct involvement through nitrate-induced synthesis of nitrate specific membrane transport proteins or proteins that modulate nitrate transport activity may be associated with the accelerated nitrate uptake state. Also, an indirect involvement of nitrate-induced synthesis of nitrate assimilatory enzymes may contribute to the total driving force for accelerated nitrate uptake.

At least two recent reports support the possible involvement of the synthesis or activation of transport proteins in the induction of the accelerated nitrate uptake state. Morgan *et al.* (20) observed a more rapid development of accelerated nitrate uptake than *in situ* nitrate reduction upon initial exposures of maize seedlings to nitrate. McClure *et al.* (19) reported enhanced ^{35}S-methionine *in vivo* labeling of a 31 kD membrane polypeptide in a tonoplast- and endoplasmic reticulum-enriched fraction of 8-h 10 mM nitrate-induced roots. The labeled polypeptide appeared to turn over very slowly after removal of external nitrate (19), which would be consistent with the observed decay pattern of the accelerated nitrate uptake following nitrate deprivation (see Fig. 6 in Ref. 17). Alternatively, other reports suggest a contribution and prerequisite of nitrate reductase for the development of the accelerated induced state of nitrate uptake. Deane-Drummond (5) reported that tungstate treatment, which renders nitrate reductase in an inactive state, inhibited the induced development of nitrate uptake associated with an 18-h nitrate pretreatment of previously nitrogen deprived cells of *Chara corallina*. Furthermore, Deane-Drummond and Jacobsen (6) observed that nitrate pretreatment enhanced influx of the nitrate analogue $^{36}ClO_3^-$ into nitrogen-starved roots of wild type *Pisum sativum* L. seedlings; the pretreatment, however, did not enhance $^{36}ClO_3^-$ influx in mutant seedlings defective in nitrate reductase. Interestingly, the influx rates of the mutant seedlings that were not pretreated were already accelerated and exceeded those of the comparably treated wild type (see Table I in Ref. 6).

Nitrate pulse treatments such as those used in our experiments should be useful for evaluating biochemical aspects of induction of nitrate uptake, since definite transitions in the expression of accelerated nitrate occur. Demonstration of correlative changes in synthesis of specific root proteins with changes in nitrate uptake activities during development and decay of the accelerated nitrate uptake state awaits further research.

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