Plant Physiol. (1993) 102: 1279–1286

Feedback Regulation of Nitrate Influx in Barley Roots by Nitrate, Nitrite, and Ammonium[1]

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The short-lived radiotracer ^{13}N was used to study feedback regulation of nitrate influx through the inducible high-affinity transport system of barley (*Hordeum vulgare* L. cv Steptoe) roots. Both wild-type plants and the mutant line Az12:Az70 (genotype *nar1a;nar7w*), which is deficient in the NADH-specific and NAD(P)H-bispecific nitrate reductases (R.L. Warner, R.C. Huffaker [1989] Plant Physiol 91: 947–953) showed strong feedback inhibition of nitrate influx within approximately 5 d of exposure to 100 μM nitrate. The result with the mutant, in which the flux of nitrogen into reduced products is greatly reduced, indicated that nitrate itself was capable of exercising feedback regulation upon its own influx. This conclusion was supported by the observation that feedback in wild-type plants occurred in both the presence and absence of L-methionine sulfoximine, an inhibitor of ammonium assimilation. Nitrite and ammonium were also found to be capable of exerting feedback inhibition upon nitrate influx, although it was not determined whether these ions themselves or subsequent metabolites were responsible for the effect. It is suggested that feedback regulation of nitrate influx is potentially mediated through several nitrogen pools, including that of nitrate itself.

The influx of nitrate into roots of barley (*Hordeum vulgare* L.) may be mediated by at least three transport systems (Lee and Drew, 1986; Behl et al., 1988; Glass et al., 1990; Siddiqi et al., 1990; Aslam et al., 1992). At high external nitrate concentrations, a LATS operates and appears to be constitutive and essentially unregulated. At low external concentrations, two HATS appear to operate. One of these (the CHATS, with a K_m for nitrate of approximately 7 μM) appears to be constitutive, whereas the other (the IHATS, with a K_m of approximately 15–34 μM) is induced by exposure to nitrate (Lee and Drew, 1986; Aslam et al., 1992). The level of expression of the IHATS is subject both to induction, apparently at the genetic level, in the presence of nitrate and to a seemingly separate negative feedback regulation that responds, in some way, to the overall nitrogen status of the plant (Glass, 1988; Siddiqi et al., 1989; and refs. therein) and may involve repression at the genetic level.

There are several levels at which feedback regulation of the IHATS might be mediated. After nitrate is taken up into the root cells, it can be reduced to nitrite by NR, then to ammonium by NiR, and then it can be assimilated by the GS-GOGAT pathway, giving rise to Gln and ultimately all other amino acids and their metabolites (Lea et al., 1990). Alternatively, it can be transported unchanged into the vacuole for storage and later be released for reduction, or it can be translocated unchanged to the shoot to be reduced and assimilated or to be stored in the vacuole for later use (see reviews by Beevers and Hageman, 1980; Clarkson, 1986; Kleinhofs and Warner, 1990; and Solomonson and Barber, 1990, for an overview).

Previous studies have suggested that feedback regulation of the expression of the IHATS is mediated by some product of ammonium assimilation (Breteler and Siegerist, 1984; Lee and Rudge, 1986; Cooper and Clarkson, 1989; Lee et al., 1992) rather than by nitrate or ammonium per se. However, Siddiqi et al. (1989) emphasized the complexity of plant nitrogen metabolism and suggested that the existing evidence was insufficient to eliminate either nitrate or ammonium as sources of negative feedback. A model for feedback regulation involving only products resulting from ammonium assimilation may well be too simplistic, given that nitrogen metabolism is a complex process involving many compounds and several cellular compartments in both the root and shoot.

As an example, the transport of nitrate into the root vacuoles for storage is a major process that can accommodate a large proportion of the nitrate taken up into root cells (Clarkson, 1986). Vacuolar uptake and storage of nitrate would, in the short term, appear to bypass any feedback regulation mediated by either the root ammonium or amino acid pools, and yet in the longer term would make an important contribution to the plant nitrogen status. Similarly, transport of

[1] This research was supported by a Natural Sciences and Engineering Research Council of Canada research grant to A.D.M.G. B.J.K. acknowledges the support of Natural Sciences and Engineering Research Council of Canada and Izaak Walton Killam Memorial postdoctoral fellowships.

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Abbreviations: CHATS, constitutive high-affinity nitrate transport system; GOGAT, glutamine-2-oxoglutarate amidotransferase; GS, glutamine synthetase; HATS, high-affinity nitrate transport systems; IHATS, inducible high-affinity nitrate transport system; LATS, low-affinity nitrate transport system; MSO, L-methionine sulfoximine; NiR, nitrite reductase; NR, nitrate reductase; NRA, nitrate reductase activity.

nitrate for reduction in the shoot would bypass feedback regulation from reduced nitrogen in the roots. Are the fluxes into the vacuole and the xylem therefore merely an unregulated overflow from the flux into reduced products, or are they subject to feedback regulation as well? Siddiqi et al. (1989) speculated that tonoplast fluxes, under conditions where exogenous nitrate is unavailable, might influence the size of the cytoplasmic nitrate pool and, hence, actually regulate the flux of nitrate into reduced pools. Whether or not this is true, it seems logical that nitrate itself should be capable of exerting feedback regulation upon nitrate transport across both the plasma membrane and the tonoplast. However, evidence for such regulation is lacking. Siddiqi et al. (1989) found a negative correlation between nitrate influx and total root nitrate content when the latter was above a critical level, but they did not distinguish between the cytoplasmic and vacuolar nitrate pools or between effects arising from nitrate itself and effects arising from reduced nitrogen pools.

In the present study, we examined the possibility that nitrate itself exercises feedback regulation upon the IHATS. Among other techniques, we conducted influx experiments with the barley mutant line Az12:Az70 (genotype *nar*1a;*nar*7w; Warner and Huffaker, 1989), which lacks isozymes for the NADH- and NAD(P)H-dependent NRs. We used the short-lived isotope ^{13}N as a radiotracer, which permits the measurement of unidirectional uptake, or "influx," over short time periods (Siddiqi et al., 1989). The results indicate that feedback regulation occurs in the absence or near absence of products arising from nitrate reduction, strongly suggesting that nitrate itself contributes to such regulation. We speculate that feedback regulation is mediated through pools of several nitrogen compounds, including nitrate, possibly acting through a common regulatory mechanism.

MATERIALS AND METHODS

Seed Germination and Plant Growth

Seeds of barley (*Hordeum vulgare* L. cv Steptoe, either the wild type or the mutant line Az12:Az70 [genotype *nar*1a;*nar*7w; Warner and Huffaker, 1989]) were germinated in moist sand. After 4 to 5 d, when the shoots were 2 to 3 cm in height, individual seedlings were transferred to hydroponic culture (Siddiqi et al., 1989). Each seedling was placed in a holder consisting of 6 cm of clear Plexiglas tubing (i.d. 12 mm) and held loosely in place at the level of the seed by a foam plug near the bottom so that the shoot was supported by the Plexiglas tube. The holders were inserted into a piece of Plexiglas sheet drilled with holes of the appropriate size and cut to fit either 8- or 25-L hydroponic tanks. Each holder was held in place with an O-ring around the base, so that the roots were fully immersed in the nutrient solution. When desired, either individual plants in their holders or the entire Plexiglas sheet with all the plants could be transferred into new solutions with minimal disturbance.

Seed germination, plant growth, and all experiments were carried out in a controlled environment room at 20 ± 2°C and 70% RH, with continuous light having a spectral composition similar to sunlight, provided by fluorescent tubes at an intensity of 300 $\mu E\ m^{-2}\ s^{-1}$ at plant level.

Induction Treatments

The basic nutrient solution for plant growth prior to induction was aerated one-tenth strength modified Johnson's solution (Siddiqi et al., 1989), with the pH maintained at approximately 6.0 by the presence of $CaCO_3$. This solution was nitrogen free. For induction, plants were transferred at the appropriate time to new solutions supplemented with the desired concentration of $Ca(NO_3)_2$, $NaNO_2$, or $(NH_4)_2SO_4$.

For experiments with 100 μM nitrate and nitrite, where the induction time was varied over a 7-d period, the plants were transferred to the appropriate solutions in 25-L tanks. The solution concentrations of nitrate or nitrite were checked every 1 to 2 d and replenished as necessary. Due to the small biomass of individual seedlings, with 48 or fewer seedlings per tank, the daily depletion was typically 5% or less. Potassium was also checked every 1 to 2 d and was replenished as necessary. The remaining nutrients were replenished proportionally according to the rate of potassium depletion.

For experiments where the induction period was fixed at 24 h and the concentration of nitrate, nitrite, or ammonium was varied, the plants were transferred to 8-L tanks containing the appropriate solutions, with up to seven plants per tank. Significant depletion of nutrients did not occur over the 24-h period, so the nutrient concentrations were not routinely monitored. The induction treatments were staggered in time so that the treatments were completed and the plants were all of the same age (either 11 or 12 d) on the day of each experiment.

For certain experiments, minor modifications were made to the above procedures. These are described in the appropriate figure legends.

Measurement of Nitrate Influx

$^{13}NO_3^-$ was produced by proton irradiation of water on the Tri-University Meson Facility-ACEL CP42 cyclotron using 20-mega electron volt protons (Siddiqi et al., 1989). The contaminants ^{18}F, $^{13}NH_4^-$, and $^{13}NO_2^-$ were removed according to the procedure of Siddiqi et al. (1989) as modified by Glass et al. (1990). The purified isotope was made up to the desired volume (typically 3 to 4 L) in temperature-equilibrated influx solution consisting of one-tenth strength modified Johnson's solution with 50 μM $Ca(NO_3)_2$. The prewash/desorption solution was identical but unlabeled.

At the beginning of each experiment, the labeled solution and the unlabeled prewash and desorption solutions were distributed separately into square 500-mL plastic food containers, with aeration provided by lines inserted into the container bases. The plants in their individual holders were transferred to square pieces of Plexiglas sheet cut to fit over the influx containers, and with holes drilled to accommodate nine plants. The roots of each set of nine plants, comprising a total biomass of approximately 0.2 g, were first immersed in 500 mL of unlabeled solution for a 5-min prewash; then in 500 mL of labeled solution for a 10-min influx period; then back in the unlabeled solution for a 2-min desorption to remove label from the free space.

The roots and shoots were then excised and the roots were spun in a basket centrifuge to remove excess solution. Radioactivity in the roots and shoots was measured separately with a Packard γ-counter (Minaxi ∂, Auto-γ 5000 series), and the counts were corrected for decay. Finally, the roots were weighed and nitrate influx was expressed as μmol g^{-1} fresh weight of roots h^{-1}, based on the sum of the root and shoot counts and the measured specific activity of the influx solutions.

The potential depletion of nitrate from the labeled solutions during the 10-min uptake period was at most 5%, based on the maximum observed influx values, and typically was around 1 to 2%. Therefore, any reduction in influx due to declining nitrate concentration during the uptake period was minor. It is possible that a transient, rapid efflux of nitrate from the roots upon transfer into the uptake solutions could have significantly reduced the specific activity, resulting in a variable underestimate of influx. However, based on the results of a previous study in which efflux was measured (Siddiqi et al., 1989), and accounting for the lower root mass used in the present study, efflux is unlikely to have increased the nitrate concentration in the uptake solutions by more than 1 or 2%.

For the experiment where MSO was used to block ammonium assimilation, the incorporated label was fractionated by cation exchange using the method of Fentem et al. (1983), modified for use with ^{13}N. Before counting, the roots were rapidly weighed and frozen in liquid nitrogen. Immediately after counting, the roots were ground in 5 mL of ice-cold water with a mortar and pestle, and the slurry was filtered through Whatman No. 1 paper under vacuum. The filtrate was then passed sequentially through two 1-cm^3 Dowex AG 50W-X8 cation-exchange columns equilibrated in the Na^+ and H^+ forms. The filter paper, the resin from the two columns, and the column effluent were each counted separately. Respectively, they contained counts originating from insoluble material, ammonium, amino acids, and negatively charged and neutral species (predominantly nitrite and nitrate).

As a control, a portion of the influx solution, containing only $^{13}NO_3^-$, was subjected to the same procedure to ensure that a significant amount of label from negatively charged species was not retained on the filter paper or the resins.

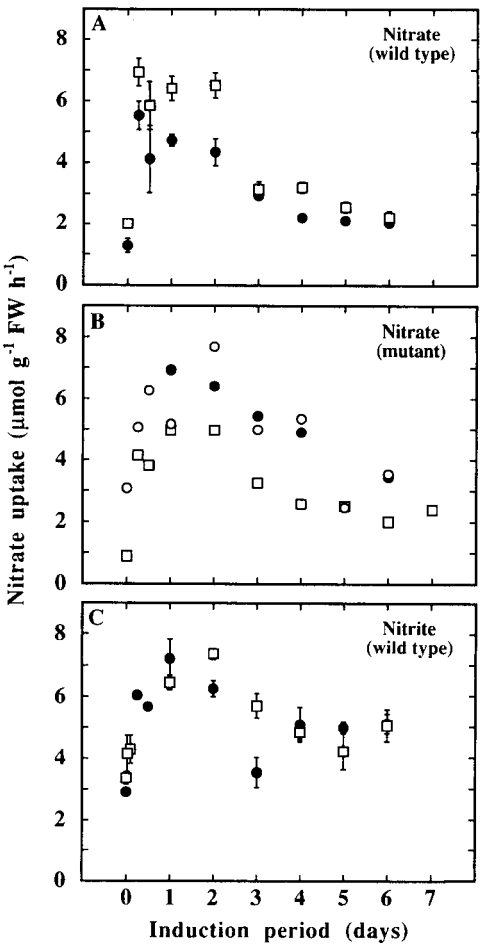

Figure 1. Changes in nitrate influx via the IHATS for plants induced for influx over various periods. The different symbols represent results from separate experiments. A, Wild-type plants exposed to 100 μM nitrate. B, Mutant genotype *nar*1a;*nar*7w exposed to 100 μM nitrate. C, Wild-type plants exposed to 100 μM nitrite. Each point on A and C represents the mean ± SE of three replicates, except the open squares on C, which are the means ± SE of four replicates. Error bars are not shown where they do not exceed the dimensions of the symbol. The points on B are the means of two replicates; therefore, the SE is not shown. The scatter in the data was comparable to that on A and C. FW, Fresh weight.

Chemicals and Assays

Nitrate in the induction solutions was measured as A_{210} following dilution with four parts of 5% perchloric acid (Cawse, 1967). Nitrite was measured colorimetrically after reaction with sulfanilimide and *N*-1-naphthylene-diamine-dihydrochloride as described by King et al. (1992). Potassium was determined with a flame photometer (model 443, Instrumentation Laboratory, Lexington, MA). Sulfanilimide, *N*-1-naphthylene-diamine-dihydrochloride, and MSO were purchased from Sigma, and Dowex AG 50W-X8 cation-exchange resin (100–200 mesh, H^+ form) was from Bio-Rad.

RESULTS

Figure 1A illustrates the pattern of nitrate influx in wild-type Steptoe barley exposed to 100 μM nitrate for periods ranging from 6 h to 6 d. Within 12 h there was a severalfold increase in nitrate influx, with the maximum rate (approximately 6 μmol g^{-1} fresh weight h^{-1}) maintained up to approximately 48 h. Subsequently, the rate declined sharply, reaching a final level of approximately 30% of the maximum rate.

Figure 1B shows a similar pattern for the *nar*1a;*nar*7w mutant. The maximum rate was similar to that of the wild type (Fig. 1A) and was obtained within 24 h. This rate was maintained over the period of 24 to 48 h, then declined to a level of approximately 30% of the maximum by 7 d.

Figure 1C shows the pattern of nitrate influx of wild-type plants exposed to 100 μM nitrite for various periods. The initial increase in influx and the maximum attained were

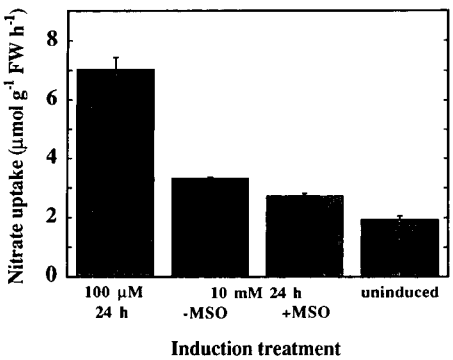

Figure 2. Nitrate influx for wild-type plants induced for influx by a 24-h exposure to 10 mM nitrate in the presence and absence of 0.25 mM MSO. Influx was measured in the presence of 100 μM nitrate. The uninduced rate and the rate for plants exposed to 100 μM nitrate for 24 h are shown for the purpose of comparison. Each bar represents the mean ± SE of three replicates. The cation-exchange fractionation of label for the −MSO and +MSO treatments is shown in Table I. FW, Fresh weight.

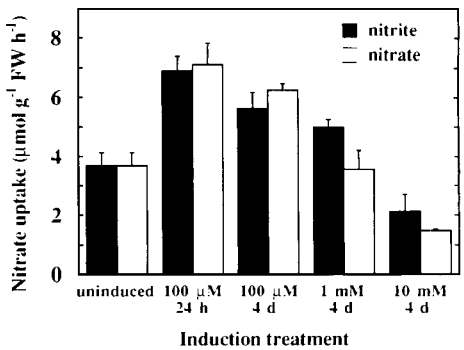

Figure 3. The concentration dependence of induction of nitrate influx in wild-type plants exposed to three different concentrations of nitrate or nitrite for 4 d. The uninduced rates and the rates from plants exposed to 100 μM nitrate or nitrite for 24 h are shown on the left for the purpose of comparison. Each bar represents the mean ± SE of four replicates. The experiments were repeated with essentially identical results (data not shown). FW, Fresh weight.

similar to that shown in Figure 1, A and B. After 48 h, the rate began to decrease, and it reached a level of 50 to 60% of the maximum by 6 d.

Figure 2 shows the effect on nitrate influx of exposure to 10 mM nitrate for 1 d in the presence and absence of 0.25 mM MSO. In both cases, influx was approximately 40% of the maximum, as obtained with 24 h of exposure to 100 μM nitrate, but was not as low as the uninduced rate. The presence of MSO failed to block the apparent negative feedback effect caused by provision of nitrate.

Table I shows the distribution of radiolabel in various fractions obtained from the +MSO and −MSO plants whose nitrate influxes are shown in Figure 2. MSO caused an 8-fold increase in the amount of label present in ammonium and reduced the amount of label in amino acids to 1.2% of the control level. With MSO, there was a slight increase in the label present in the nitrate and nitrite fraction and no change in the very small amount of label present in the insoluble fraction. The sample of influx solution; which contained label only in the form of nitrate, caused less than 1% cross-contamination of the ammonium, amino acid, and insoluble fractions when subjected to the same separation procedure as the plant material.

Figure 3 illustrates the response of nitrate influx in wild-type plants to three concentrations of nitrate or nitrite supplied over a 4-d period, with influx subsequently measured in 100 μM nitrate. Similar results were obtained for both ions. In comparison to the maximum rate, as obtained with 24 h of exposure to 100 μM nitrate or nitrite, the rates declined with increasing concentration of both nitrate and nitrite. The rates obtained at 10 mM nitrate or nitrite were less than 30% of the maximum rates and less than 50% of the uninduced rates.

Figure 4A shows nitrate influx of wild-type plants that were induced with 100 μM nitrate for 24 h then exposed to various concentrations of ammonium for 24 h in the continued presence of 100 μM nitrate. Ammonium was not present during the 10-min influx period. All concentrations of ammonium used, ranging from 10 μM to 1 mM, resulted in a strong reduction of nitrate influx relative to the control (which in this particular experiment was obtained by 48 h of exposure to 100 μM nitrate, rather than a 24-h exposure). The rates obtained with 0.2, 0.5, and 1 mM ammonium were less than 30% of the maximum rate and were comparable to the uninduced rate.

Figure 4B shows the effects of three concentrations of ammonium on nitrate influx of wild-type plants when ammonium was present only during the 5-min prewash and the 10-min influx measurement. The experiment was carried out after 24 h of induction with 100 μM nitrate. With 10 μM ammonium, slight inhibition was observed, and both 100 μM and 1 mM ammonium resulted in approximately 30% inhibition of nitrate influx.

Table I. *Percentage of label from $^{13}NO_3^-$ incorporated into various fractions of control and MSO-treated root systems*

Root material from three wild-type plants, which had been exposed to 10 mM nitrate in the presence and absence of 0.25 mM MSO before labeling with $^{13}NO_3^-$, was pooled and subjected to cation exchange as described in "Materials and Methods." For a control, a sample of the original influx solution, with approximately the same total radioactivity, was subjected to the same procedure. The influx data from the same experiment are shown in Figure 4.

Treatment	Percent of Label			
	Insoluble	NO_3^- or NO_2^-	NH_4^+	Amino Acids
−MSO	0.9	73.5	2.5	23.1
+MSO	0.9	78.7	20.2	0.3
Influx solution	0.0	98.7	0.6	0.7

DISCUSSION

The pattern of nitrate influx we observed for wild-type barley plants with varying lengths of induction is very similar

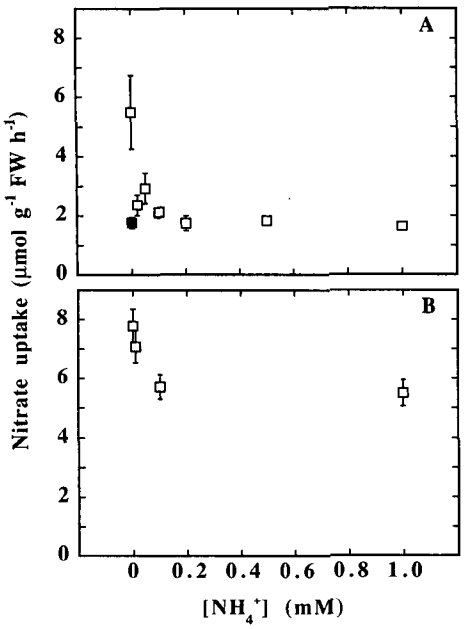

Figure 4. A, Nitrate influx for wild-type plants that were induced for influx with 100 μM nitrate for 24 h, then exposed to various ammonium concentrations ranging from 0 to 1 mM for 24 h, in the continued presence of 100 μM nitrate. The solid symbol represents the uninduced rate, shown for the purpose of comparison. Each point represents the mean ± SE of three replicates. Error bars are not shown where they do not exceed the dimensions of the symbol. B, Nitrate influx for wild-type plants that were induced for influx by exposure to 100 μM nitrate for 24 h and exposed to ammonium at three different concentrations during the 5-min prewash and the 10-min influx measurement only. Each point represents the mean ± SE of six replicates. FW, Fresh weight.

to the one observed by Siddiqi et al. (1989). We observed an initial increase in influx above the constitutive level ("induction," using the usual terminology), which was generally complete within 12 to 24 h, followed by a plateau and then a more gradual decline in activity (Fig. 1A). We refer to this decline as "negative feedback" or "feedback inhibition" after the terminology used by Siddiqi et al. (1989), without intending to imply a particular molecular mechanism. The relatively long time frame of the effect strongly suggests that it arises from repression of synthesis of nitrate transport protein(s) at the genetic level with subsequent degradation of the existing proteins (Clarkson, 1986), rather than from a short-term mechanism such as allosteric regulation of transport; however, to our knowledge, this issue has not been investigated.

Our results differ from those of Siddiqi et al. (1989) in one minor respect; namely, that the constitutive level of influx in our experiments with Steptoe barley, although somewhat variable, was consistently higher than that observed by Siddiqi et al. (1989) using Klondike barley. Because our influx experiments were conducted in the presence of a low nitrate concentration (100 μM), we consider it likely that the majority of transport activity observed in the absence of induction by nitrate is due to the CHATS, rather than the LATS (see Glass et al. [1990] and Siddiqi et al. [1990] for discussion of the differences between the two systems). It seems likely that the observed difference is simply due to a genetic difference in the level of the CHATS between the two varieties. However, we cannot rule out the possibility that cv Steptoe has a higher level of LATS activity than cv Klondike.

The pattern of induction and feedback inhibition for the mutant *nar*1a;*nar*7w (Fig. 1B) was remarkably similar to that observed for the wild type, in terms of both magnitude and time frame. The mutant is deficient in the NADH-specific and NAD(P)H-bispecific NRs (Warner and Huffaker, 1989); its residual NRA as determined by an in vitro assay was only 1 to 2% of that of wild type and its in vivo nitrate assimilation rate was 13% of that of wild type. We carried out an in vivo NR assay on some of the mutant plants used in these experiments (see King et al., 1992, for methodology) and found that NRA was approximately 10% of that of the wild type, which was close to the limit of detection for this method. We expect, therefore, that the flux of nitrogen into reduced products would be drastically reduced in the mutant under the conditions we used.

Breteler and Siegerist (1984), Lee and Rudge (1986), Cooper and Clarkson (1989), and most recently Lee et al. (1992) have suggested that feedback inhibition of nitrate uptake arises from a product of ammonium assimilation rather than from nitrate or ammonium. This suggestion arises in part from the observation that MSO, an inhibitor of GS, is able to overcome feedback inhibition of nitrate uptake, evidently by blocking the formation of reduced nitrogen products (Breteler and Siegerist, 1984; Lee et al., 1992). The latter study, which used a variety of approaches to the problem, was particularly convincing. We do not dispute the possibility that products of ammonium assimilation may exert negative feedback on nitrate influx. Nevertheless, we do not believe it is probable that such a mechanism can account for the feedback we have observed in the mutant *nar*1a;*nar*7w, where in vitro NRA and the in vivo rate of nitrate assimilation are extremely low (Warner and Huffaker, 1989). In this case, nitrate itself would appear to be the most likely agent capable of exerting feedback. In support of this suggestion, a previous study using *Lemna gibba* (Ingemarsson et al., 1987) showed that preloading of plants with nitrate, following inactivation of NR by tungstate, resulted in inhibition of both net nitrate uptake and nitrate influx, an effect that was later attributed to feedback inhibition by nitrate itself (Mattson et al., 1991).

It is interesting that the magnitude of the feedback effect was similar in both the mutant and wild-type plants. The mutant would be expected to accumulate more nitrate due to the fact that its nitrate influx is similar to that of wild type but its assimilation is impaired. However, Warner and Huffaker (1989) observed that the excess nitrate in the mutant accumulates in the leaves rather than in the roots. Therefore, it is possible that the root nitrate pools, and the feedback effects they exert, are similar in both the mutant and wild-type plants.

Further support for the suggestion that nitrate is capable of exerting feedback upon its own influx is provided by an experiment with MSO (Fig. 2). With Steptoe barley, 0.25 mM MSO was not capable of overcoming the feedback inhibition caused by a 24-h exposure to 10 mM nitrate, despite the fact that it virtually eliminated the flux of radiolabel into amino

acids (Table I). In this case, only nitrate, nitrite, or ammonium would seem to be possible agents for feedback inhibition, since in the absence of ammonium assimilation for 24 h the free amino acid pools would be severely depleted. We note that in another study (de la Haba et al., 1990) it was observed that MSO did not relieve the inhibition caused by ammonium addition, and in fact actually inhibited nitrate uptake when added alone. The authors suggested that ammonium itself rather than an assimilation product was the agent responsible for feedback.

At present, we cannot reconcile our results and those of de la Haba et al. (1990) with those of Breteler and Siegerist (1984) and Lee et al. (1992) regarding the effects of MSO. In our study, it was necessary to use a 24-h exposure to MSO to inhibit ammonia assimilation throughout the entire induction period with 10 mM nitrate, whereas the studies of Breteler and Siegerist (1984) and Lee et al. (1992) used exposures of 1.5 and 6 to 8 h, respectively. It has been reported that MSO, by reducing the leaf amino acid pools, can inhibit photosynthesis with exposures as short as 1 to 2 h (Achhireddy et al., 1983; Walker et al., 1984; Johansson and Larsson, 1986). Potentially, a reduction in photosynthetic rate could limit the energy supply for nitrate uptake, reducing nitrate influx and affecting our results; however, we do not believe this to be the case because the nitrate influx rate for the MSO-treated plants was only slightly lower than that of the control plants (Fig. 2). In this experiment, both sets of plants were exposed to 10 mM nitrate for 24 h, and the resulting feedback inhibition of nitrate influx could have masked possible inhibition resulting from toxic effects of MSO. Therefore, in a separate experiment, plants were exposed to 100 μM nitrate for 24 h to cause full induction of the IHATS, in the presence and absence of 0.25 μM MSO. Nitrate influx for the MSO-treated plants (5.54 $\pm$ 0.35 μmol g^{-1} fresh weight h^{-1}) was close to that of the control plants (6.21 $\pm$ 0.73 μmol g^{-1} fresh weight h^{-1}; means $\pm$ SE, $n = 3$), indicating that toxic effects of MSO, if any, were slight. In any case, the result with MSO, although not conclusive in itself, supports those obtained with the NR-deficient mutant, providing evidence that nitrate itself, at least under some conditions, is capable of exerting feedback regulation upon its own influx.

To extend this study in light of its discrepancy with the existing literature, we decided to examine possible feedback effects of nitrite and ammonium on nitrate influx in wild-type Steptoe barley. Nitrite is a particularly interesting candidate for regulation because it appears to be taken up by the same transporters as nitrate (Aslam et al., 1992) and is as capable of inducing the IHATS as is nitrate itself (Siddiqi et al., 1992). Figure 1C illustrates the pattern of nitrate influx with varying periods of induction with 100 μM nitrite. The pattern of induction and the level of influx reached were similar to those seen for nitrate (Fig. 1A). It is interesting that nitrite also caused feedback inhibition of influx, although to a somewhat lesser degree (Fig. 1C).

The concentration dependence of feedback inhibition by nitrate and nitrite over a 4-d period is shown in Figure 3. Both ions showed a very similar pattern, with inhibition showing a clear concentration dependence of similar magnitude. Therefore, it is interesting to consider whether the mechanism behind this concentration dependence could be similar for both ions. NR is usually considered the rate-limiting step for the nitrogen assimilation pathway; hence, the net rate of nitrate uptake can exceed the rate of nitrogen assimilation (Solomonson and Barber, 1990). In such circumstances, nitrate is capable of accumulating to high concentrations in the vacuole and of being translocated unchanged to the shoot (Clarkson, 1986), and it seems reasonable that both processes would be regulated with nitrate influx in a coordinated manner (Siddiqi et al., 1989).

In the present study, we did not measure the root nitrate pools. However, a previous study in which both the length of exposure to nitrate and the nitrate concentration were varied (Siddiqi et al., 1989) showed that for the barley cultivar Klondike, nitrate influx is positively correlated with the total root nitrate pool when this pool is small and is negatively correlated with it when it is large. As well, the cytoplasmic concentration of nitrate can vary over a considerable range (Siddiqi et al., 1991; King et al., 1992), providing a possible means of "sensing" the cellular nitrate status. All of the above factors would seem to favor a mechanism whereby nitrate itself can regulate its own influx. If this is the case, however, it is not clear whether the vacuolar or the cytoplasmic nitrate pool is ultimately responsible for the feedback effect.

Nitrite, in contrast to nitrate, is considered a toxic ion (Klepper, 1975) and does not accumulate in plant cells to nearly as great an extent due to the rate-limiting nature of NRA (Solomonson and Barber, 1990). Nevertheless, induction with nitrite results in measurable tissue nitrite accumulation, to cytoplasmic concentrations potentially as high as 2.8 mM (Siddiqi et al., 1992). Variations in cytoplasmic nitrite concentration, by influencing the flux through NiR and GS-GOGAT, could alter the pools of ammonium or its subsequent metabolites, any of which might then exert feedback inhibition. Alternatively, varying the cytoplasmic nitrite concentration could affect the expression of the IHATS in a more direct manner, similar to that which we propose for nitrate.

As a third possibility, oxidation of nitrite to nitrate could be responsible for the observed effects. Aslam et al. (1987) reported that nitrite in barley leaves is capable of being oxidized to nitrate, and hence of inducing NR, and that reagent-grade nitrite is contaminated to a slight degree with nitrate. In barley roots, we have consistently failed to observe conversion of nitrite to nitrate in either cv Klondike (Siddiqi et al., 1992) or cv Steptoe (present study, data not shown). We have also observed that exogenously supplied nitrate is able to induce both the IHATS and NR, whereas nitrite is not able to induce NR (Siddiqi et al., 1992). Therefore, we believe that it is unlikely that either nitrate production from nitrite in the roots or contamination of the nutrient solutions with nitrate is responsible for the induction and feedback inhibition of nitrate influx shown in Figure 1C.

Because we lacked access to NiR mutants and do not know of any specific inhibitors of NiR that could reasonably be used in vivo, we cannot determine with certainty whether nitrite itself is responsible for the observed feedback effect. However, we feel that this topic could be usefully pursued in future studies.

Finally, we examined the role of ammonium in exerting feedback upon the IHATS in wild-type plants (Fig. 4). In contrast to the results of Bloom and Sukrapanna (1990) and

Bloom et al. (1992), we found that ammonium itself was incapable of inducing the IHATS. A 24-h exposure of root systems to 100 μM or 1 mM ammonium resulted in nitrate influx values of 1.04 ± 0.07 and 1.01 ± 0.05 μmol g^{-1} fresh weight h^{-1}, respectively (mean ± SE, $n = 4$), compared with a rate of 1.19 ± 0.13 for uninduced plants and a rate of 7.70 ± 0.15 for plants induced with a 24-h exposure to 100 μM nitrate.

To study feedback inhibition by ammonium, therefore, it was necessary first to induce the IHATS with a 24-h exposure to 100 μM nitrate, then to expose the plants to various concentrations of ammonium in the continued presence of 100 μM nitrate. The results showed that ammonium is a very potent feedback inhibitor of nitrate influx (Fig. 4A), despite the fact that it was not present during the influx measurements. Concentrations ranging from 100 μM to 1 mM, supplied for 24 h, were as strongly inhibitory as 10 mM nitrate or nitrate supplied for 4 d (Fig. 3).

The study of feedback inhibition of expression of the IHATS by ammonium is complicated by the fact that ammonium is known to have rapid, reversible effects upon nitrate influx (Lee and Drew, 1989; Warner and Huffaker, 1989, and refs. therein). We found that inclusion of ammonium only during the 5-min prewash and the 10-min influx measurement caused a reduction of nitrate influx (Fig. 4B). Both 100 μM and 1 mM ammonium resulted in approximately 30% inhibition of influx. Discussion of the nature of this short-term inhibition is outside the scope of this study, and the topic has been thoroughly treated elsewhere (Lee and Drew, 1989). We note, however, that the magnitude of this short-term inhibition is insufficient to account for the longer-term effect shown in Figure 4A.

The feedback effect of ammonium upon nitrate influx could be due to the effect of a product of its assimilation, as proposed by Breteler and Siegerist (1984). However, if this is true, we find it puzzling that ammonium is so much more effective than nitrite in producing feedback, given the very high potential rates of nitrite reduction in root tissue (Beevers and Hageman, 1980). Unlike nitrate, nitrite and ammonium do not accumulate in roots to high levels and are not translocated to the shoot in large quantities, due to the high capacity of roots for their assimilation; however, both ions are taken up readily by barley roots at rather similar rates (see Bloom and Sukrapanna, 1990, and Aslam et al., 1992, for results for ammonium and nitrite, respectively). Therefore, the uptake and assimilation rates of each ion should be similar, and the feeding of one ion rather than the other at the same concentration should not greatly change the flux of nitrogen through GS-GOGAT and, hence, the pool sizes of later metabolites. This argument is by no means conclusive; however, in light of our results and those of de la Haba et al. (1990) described above, we reiterate the suggestion of Siddiqi et al. (1989) that it might be premature to eliminate ammonium itself as a potential candidate for feedback regulation.

In summary, this study has provided evidence that nitrate itself is capable of exerting feedback regulation upon the IHATS. In addition, nitrite and ammonium appear capable of feedback inhibition, although further studies will be required to show whether the effects are due to these ions themselves or subsequent assimilation products. It is possible that various nitrogen metabolites exert their feedback effects on nitrate influx through a common mechanism whose nature is at present unknown.

ACKNOWLEDGMENTS

We wish to thank Michael Adam and Tamara Hurtado at the Tri-University Meson Facility for the production of ^{13}N and Xiaoge Chen, Jarnail Mehroke, Herbert Kronzucker, and Miaoyuan Wang for essential assistance with the radioisotope experiments.

Received March 31, 1993; accepted May 16, 1993.
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