Regulation of High-Affinity Nitrate Transporter Genes and High-Affinity Nitrate Influx by Nitrogen Pools in Roots of Barley[1]

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To investigate the regulation of *HvNRT2*, genes that encode high-affinity NO_3^- transporters in barley (*Hordeum vulgare*) roots, seedlings were treated with 10 mM NO_3^- in the presence or absence of amino acids (aspartate, asparagine, glutamate [Glu], and glutamine [Gln]), NH_4^+, and/or inhibitors of N assimilation. Although all amino acids decreased high-affinity $^{13}NO_3^-$ influx and *HvNRT2* transcript abundance, there was substantial interconversion of administered amino acids, making it impossible to determine which amino acid(s) were responsible for the observed effects. To clarify the role of individual amino acids, plants were separately treated with tungstate, methionine sulfoximine, or azaserine (inhibitors of nitrate reductase, Gln synthetase, and Glu synthase, respectively). Tungstate increased the *HvNRT2* transcript by 20% to 30% and decreased NO_3^- influx by 50%, indicating that NO_3^- itself does not regulate transcript abundance, but may exert post-transcriptional effects. Experiments with methionine sulfoximine suggested that NH_4^+ may down-regulate *HvNRT2* gene expression and high-affinity NO_3^- influx by effects operating at the transcriptional and post-transcriptional levels. Azaserine decreased *HvNRT2* transcript levels and NO_3^- influx by 97% and 95%, respectively, while decreasing Glu and increasing Gln levels. This suggests that Gln (and not Glu) is responsible for down-regulating *HvNRT2* expression, although it does not preclude a contributory effect of other amino acids.

The uptake of NO_3^- in terrestrial plants is mediated by at least three transport systems that coexist in the plasma membranes of root cells (for review, see Glass and Siddiqi, 1995; Crawford and Glass, 1998). These fall into two classes, referred to as low- and high-affinity transport systems (LATS and HATS, respectively). The HATS are further subdivided into constitutive (CHATS) and inducible (IHATS) systems (Siddiqi et al., 1990; Aslam et al., 1993). The LATS are involved in NO_3^- uptake at high external concentrations of NO_3^- (>0.2 mM), while the CHATS and IHATS are saturated at low external NO_3^-concentrations (approximately 100 μM). In barley (*Hordeum vulgare*) roots, LATS activity is expressed without prior exposure to NO_3^- (Siddiqi et al., 1990), and this transport system appears to be subject to down-regulation by accumulated N (Clement et al., 1978; Siddiqi et al., 1990). A gene considered to encode the LATS (*AtNRT1*, originally *CHL1*) was the first higher plant NO_3^- transporter gene to be cloned from Arabidopsis (Tsay et al., 1993). However, in contrast to the apparent constitutive nature of this transport in barley roots, the *AtNRT1* gene transcript is undetectable prior to exposure to NO_3^- in Arabidopsis (Tsay et al., 1993; Huang et al., 1996). This apparent anomaly is unexplained and warrants exploration. Recently, it has been proposed that the AtNRT1 protein may participate in both high- and low-affinity NO_3^- uptake (Wang et al., 1998; Liu et al., 1999).

In higher plants, genes considered to encode IHATS have been cloned from barley (Trueman et al., 1996; Vidmar et al., 2000), Arabidopsis (Filleur and Daniel-Vedele, 1999; Zhuo et al., 1999), *Nicotiana plumbaginifolia* (Quesada et al., 1997), and soybean (Amarashinghe et al., 1998). In barley, putative IHATS are encoded by a multigene family of seven to 10 members (Trueman et al., 1996). To date, four members of this family, originally named the *BCH* family and renamed *HvNRT2*, have been isolated from barley (Trueman et al., 1996; Vidmar et al., 2000). The *HvNRT2* genes encode proteins composed of 507 to 509 amino acids, with molecular masses of 54 to 55 kD, including 12 hydrophobic (transmembrane) regions that belong to the major facilitator superfamily (as do the other plant IHATS). It has been shown that the mRNA levels of these IHATS

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genes increase rapidly following the provision of NO_3^- (a process referred to as induction) to NO_3^--deprived plants (Trueman et al., 1996; Quesada et al., 1997; Amarashinghe et al., 1998; Filleur and Daniel-Vedele, 1999; Zhuo et al., 1999). This increase in transcript levels is correlated at the physiological level with increased NO_3^- influx when NO_3^- is first supplied (Siddiqi et al., 1989; Glass et al., 1990; Hole et al., 1990; Zhuo et al., 1999).

In barley, all four of the *HvNRT2* transcripts investigated are coordinately up-regulated following the provision of NO_3^- (Vidmar et al., 2000). Under quasi-steady-state conditions of NO_3^- supply, the highest levels of IHATS mRNA and $^{13}NO_3^-$ influx were obtained when the external NO_3^- concentration was maintained at 50 μM (Vidmar et al., 2000). In *N. plumbaginifolia*, genes involved in N acquisition and assimilation, *NpNRT2.1*, *NIA* (nitrate reductase [NR]), and *NII* (NR), were coordinately expressed under conditions of NO_3^- induction and N repression (Krapp et al., 1998). Furthermore, NR mutants of *N. plumbaginifolia* and Arabidopsis showed elevated levels of *NRT2.1* transcript (Krapp et al., 1998; Filleur and Daniel-Vedele, 1999; Lejay et al., 1999), which is consistent with the proposal that *NRT2.1* transcript abundance is regulated by feedback from reduced forms of N rather than from NO_3^- itself. Nevertheless, at the physiological level, the down-regulation of IHATS to a lower steady-state level following peak induction has been argued to result from effects of accumulated NO_3^- and/or a product(s) of its assimilation (Siddiqi et al., 1989; King et al., 1993). This conclusion was based on correlations between root $[NO_3^-]$ and $^{13}NO_3^-$ influx (Siddiqi et al., 1989), as well as data gained from NR double mutants of barley (King et al., 1993). Additional physiological evidence for a role of tissue NO_3^- in down-regulating NO_3^- influx came from studies by Ingemarsson et al. (1987) based upon the use of tungstate (an inhibitor of NR) and from Doddema and Otten (1979), based on kinetic studies of NO_3^- uptake in Arabidopsis. Evidence consistent with the down-regulation of NO_3^- influx by NH_4^+ (Aslam et al., 1996) and/or by amino acids (Doddema and Otten, 1979; Breteler and Siegerist, 1984; Muller and Touraine, 1992; Muller et al., 1995) has also been advanced.

The effects of NH_4^+ on NO_3^- uptake are more complex, due to the possibility of affecting NO_3^- uptake at a number of levels (transcript abundance, protein level, or direct effects of NH_4^+ on the NO_3^- transporter). This has resulted in a lack of consensus concerning the mechanism(s) of the NH_4^+ effects on NO_3^- fluxes. Using NO_2^- as a tracer of NO_3^-, Aslam et al. (1994) suggested that NH_4^+ increased NO_3^- efflux rather than diminished influx, while the use of $^{13}NO_3^-$ demonstrated that influx was strongly reduced (Glass et al., 1985; Lee and Drew, 1989; Kronzucker et al., 1999). A recent paper by Kronzucker et al. (1999) established that in barley roots, the provision of NH_4^+ in the external medium simultaneously decreased NO_3^- influx and increased efflux, the absolute effect upon influx being more significant. Moreover, this effect occurred within minutes of supplying NH_4^+, suggesting that NH_4^+ itself was acting directly upon the IHATS.

This direct effect of NH_4^+ on NO_3^- influx does not preclude long-term effects. For example, in longer term experiments, Breteler and Siegerist (1984) showed that Met sulfoximine (MSO), an inhibitor of Gln synthetase, relieved the inhibitory effect of NH_4^+ on NO_3^- uptake in dwarf bean. These authors concluded that the NH_4^+ effect arose from products of the assimilation of NH_4^+ rather than from NH_4^+ itself. However, King et al. (1993) observed no relief of NH_4^+ inhibition of NO_3^- influx by MSO in barley roots. Likewise, de la Haba et al. (1990) suggested that NH_4^+, and not its assimilation products, was responsible for inhibiting NO_3^- uptake into sunflower roots. Clearly, part of the confusion in the literature has resulted from the aforementioned multiple levels at which NH_4^+ is capable of inhibiting NO_3^- uptake. There is every reason to expect that NH_4^+ might have direct effects on the transport system as well as effects at the level of transcription via products of NH_4^+ assimilation.

Feeding plants with amino acids to mimic putative shoot signals and to investigate the effects of downstream metabolites of NO_3^- that might control NO_3^- uptake by roots has frequently been undertaken (Lee et al., 1992; Imsande and Touraine, 1994). Amino acids, provided to roots exogenously in the nutrient solution decreased NO_3^- uptake in Arabidopsis (Doddema and Otten, 1979), common bean (Breteler and Arnozis, 1985), soybean (Muller and Touraine, 1992), and wheat (Rodgers and Barneix, 1993). This down-regulation of NO_3^- uptake was usually preceded by a lag period of 3 h or more, indicating that the effects of amino acids on NO_3^- uptake were not direct or allosteric (Muller and Touraine, 1992). However, it is necessary to interpret the results of such experiments with caution, since the effects of particular amino acids may be influenced by the extent of their uptake, the extent of their biochemical interconversion and assimilation, and their effects upon the expression of the NO_3^- transport system. In some cases (e.g. Muller and Touraine, 1992), amino acids were applied to cotyledons or to skin flaps to simulate the "normal" pathway of amino acid delivery from shoot to root. The results of such experiments confirm those derived from exogenous application of amino acids, but it is still necessary to consider the extent of amino acid transport to the roots and the potential for interconversion of applied amino acids when attempting to identify critical regulatory compounds.

In this report we investigate the regulation of NO_3^- influx, *HvNRT2* transcript abundance, and changes

in NO_3^-, NH_4^+, and amino acid concentrations in roots during the down-regulation of NO_3^- influx. For this purpose, barley seedlings were exposed to NO_3^- with or without NH_4^+, amino acids (Asn, Asp, Gln, and Glu), and inhibitors of key enzymes of the N-assimilation pathway.

RESULTS

Effects of Treatment with 10 mM NO_3^- on $^{13}NO_3^-$ Influx, Tissue N Concentrations, and *HvNRT2* Transcript Accumulation in NO_3^--Deprived Plants

When seedlings that had previously been grown on N-free medium were fed 10 mM NO_3^-, $^{13}NO_3^-$ influx (measured at 50 μM) displayed a typical time course response, increasing from 0.42 to 2.9 μmol g^{-1} fresh weight h^{-1}, equivalent to a 7-fold increase of $^{13}NO_3^-$ influx, within the first 12 h. This confirms earlier reports to this effect (Siddiqi et al., 1989; Vidmar et al., 2000). Thereafter, influx decreased to 2.4 μmol g^{-1} fresh weight h^{-1} by the end of the experiment (at 48 h). Parallel northern-blot analysis showed that *HvNRT2* transcript was initially undetectable in root tissue, but increased dramatically within the first 6 h after exposure to NO_3^-, and then steadily decreased to undetectable levels by 24 h. Analysis of inorganic N (Fig. 1A) showed that the root NO_3^- concentration increased from 5.1 $\pm$ 2.7 μmol g^{-1} fresh weight at time 0 to 46.2 $\pm$ 11.2 μmol g^{-1} fresh weight by 6 h of NO_3^- provision. By 48 h, root NO_3^- had increased to 89.2 $\pm$ 8.5 μmol g^{-1} fresh weight. In contrast, NH_4^+ levels remained basically unchanged, varying between 3.5 and 4.5 μmol g^{-1} fresh weight (Fig. 1A) during the 48 h of exposure to 10 mM NO_3^-. Figure 1B shows that during the course of the experiment the concentrations of Gln, Glu, Asp, and Asn increased to maximum levels that were 10-, 9-, 13-, and 4-fold higher, respectively, than initial values. Gln and Glu concentrations increased rapidly during the first 12 h after the onset of NO_3^- supply, then decreased to slightly lower concentrations by 48 h.

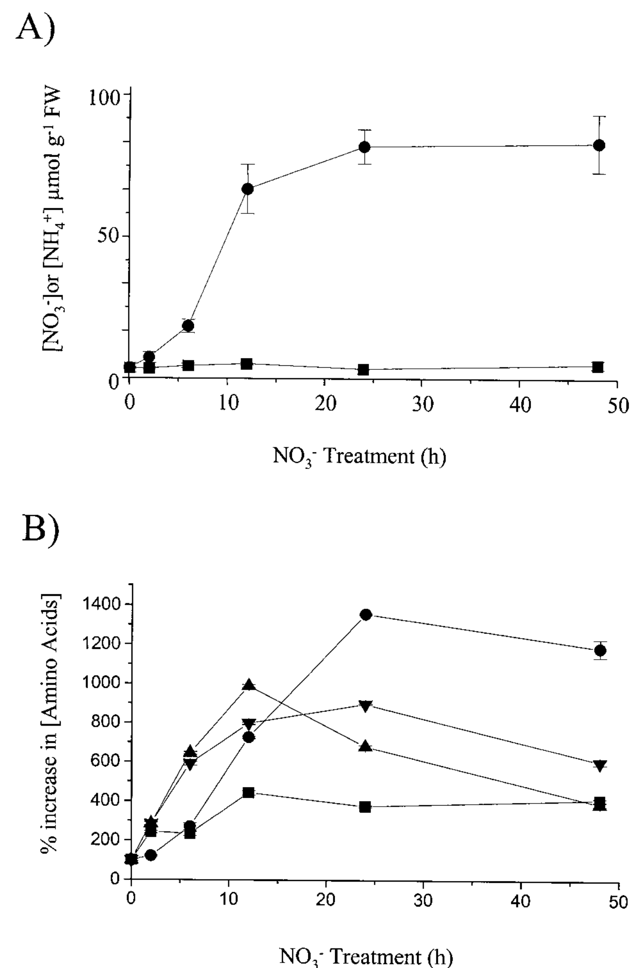

Figure 1. Plant inorganic and organic N concentrations as a function of feeding 10 mM NO_3^- to N-starved barley plants. Seven-day-old-seedlings were grown in one-tenth-strength N-free modified Johnson's solution, and then supplied with 10 mM NO_3^- for 0, 2, 6, 12, 24, and 48 h. A, Root concentrations of inorganic N: NO_3^- (●) and NH_4^+ (■). B, Root concentrations of amino acids as percentage of N-starved control Asn (■), Asp (●), Gln (▲), and Glu (▼).

Effects of Exogenously Applied Amino Acids on $^{13}NO_3^-$ Influx and *HvNRT2* Transcript Accumulation

$^{13}NO_3^-$ influx was reduced by all amino acids tested (Fig. 2A) in plants pretreated with 1 mM Gln, Glu, Asn, or Asp for 6 h during induction of NO_3^- influx with 10 mM NO_3^-. The strongest effect was due to Asp (89% inhibition), followed by Glu (79% inhibition), Asn (45% inhibition), and Gln (29% inhibition). Northern-blot analysis of RNA isolated from the same amino acid-treated roots showed a similar pattern for the abundance of *HvNRT2* transcript (Fig. 2B), except that Glu was more inhibitory than Asp. The exogenous application of each of the amino acids (Glu, Gln, Asp, and Asn) generally increased concentrations of all of the four amino acids in root tissue (Table I). For example, the application of Asp decreased root Asp 1.6-fold, while at the same time increasing Asn 2-fold, Glu 1.8-fold, and Gln 1.6- fold. By reference to the NO_3^- treatment, root NO_3^- concentrations were either increased by 40% by the Gln treatment, unaffected by the Glu treatment, or reduced by 43% and 32% respectively, after treatment with Asp and Asn (Table I). The changes of *HvNRT2* transcript levels were most strongly correlated with increases of Glu (r^2 = 0.92) and Gln (r^2 = 0.68) concentrations, while Asp and Asn concentrations were poorly correlated (r^2 values of 0.4 and 0.22, respectively).

Effects of Inhibitors of N Assimilation on $^{13}NO_3^-$ Influx and *HvNRT2* Transcript Accumulation

The effects of 0.5 mM tungstate, 1 mM MSO, and 0.25 mM aza-Ser (AZA), inhibitors of the enzymes

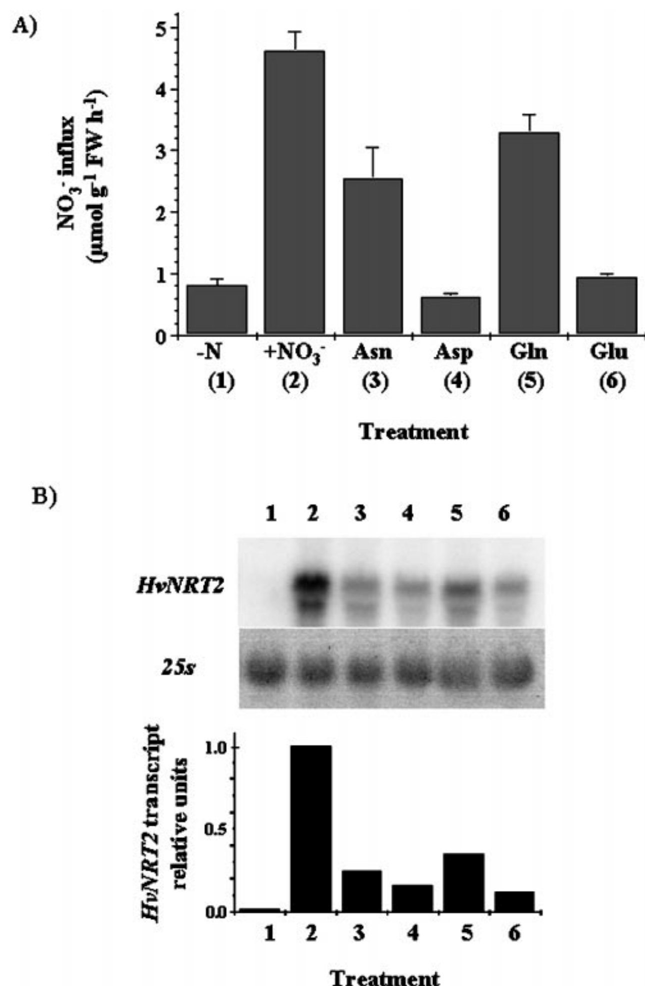

Figure 2. Effects of exogenous treatment with amino acids on $^{13}NO_3^-$ influx and *HvNRT2* transcript accumulation in roots. Seven-day-old-seedlings were grown in one-tenth-strength N-free modified Johnson's solution. A, $^{13}NO_3^-$ influx was measured at 50 μM. Each of the treatments consisted of four replicates. B, Northern-blot analysis of *HvNRT2* transcript from RNA isolated from barley roots. Treatments: lane 1, N starved; lanes 2 through 6, treated with 10 mM NO_3^- alone (lane 2) or with 10 mM NO_3^- plus amino acids (lanes 3–6) for 6 h. Lane 3, 1 mM Asn; lane 4, 1 mM Asp; lane 5, 1 mM Gln; and lane 6, 1 mM Glu. Quantification of transcript levels was by phosphor imager average of two experiments (standardized by 25S transcript). Transcript abundance was calculated in proportion to the 6 h-NO_3^- treatment (as 1 relative unit).

NR, Gln synthetase, and Glu synthase, respectively, were evaluated in separate experiments by supplying these inhibitors together with 10 mM NO_3^- for 6 h to N-starved plants. By examining the effects of these inhibitors on transcript abundance and NO_3^- influx, the role of these assimilates in regulating IHATS influx may be assessed. All three inhibitors decreased $^{13}NO_3^-$ influx compared with the control treatment, which was the 6 h of exposure to 10 mM NO_3^- (Fig. 3A). The AZA effect was the most dramatic, decreasing influx by 97%. Tungstate and MSO reduced NO_3^- influx by 44%. In contrast to its effect upon influx, tungstate increased *HvNRT2* transcript by 20% to 30% (Figs. 3B [lane 4] and 4 [lane 3]). This treatment failed to significantly affect NO_3^-, NH_4^+, Asp, or Glu concentrations, but decreased both Asn and Gln concentrations (Table II).

When exogenous Glu was added to the tungstate treatment solution, the *HvNRT2* transcript level was reduced by 59% compared with the tungstate-plus-NO_3^- treatment (Fig. 4). MSO had only a small effect upon *HvNRT2* transcript abundance when applied in the presence of NO_3^- as the sole source of N. Thus, transcript abundance was reduced by 15%, 7%, or increased by 22%, respectively, in separate experiments (Table III; Fig. 3B, lane 3, and Fig. 4, lane 4). When 1 mM Glu was added to the MSO treatment, a 62% decline in *HvNRT2* transcript abundance was observed (Fig. 4). AZA (Fig. 3B, lane 5) decreased transcript abundance by 95%. This was the largest observed effect on transcript abundance of any of the inhibitors used. As would be expected of inhibitors of Gln synthetase and Glu synthase activities, the MSO and AZA treatments increased root NH_4^+ levels by 4.5- and 2-fold, respectively (Table II), compared with the control treatment (plants treated with 10 mM NO_3^- without inhibitor). MSO treatment also decreased Gln, Asn, and Glu concentrations, but had little effect on the Asp concentration (Table II). Likewise, blocking Glu synthase with AZA increased Gln and Asn concentrations 1.4- and 1.3-fold, respectively, and reduced Asp and Glu to 0.45 and 0.1, respectively, of their control values (Table II). AZA and MSO treatments decreased concentrations of NO_3^- in root tissue by 80% and 25%, respectively (Table II).

Effects of Exogenous NH_4^+ on $^{13}NO_3^-$ Influx and HvNRT2 Transcript Accumulation

It was reported previously (Vidmar et al., 2000) that *HvNRT2* transcript abundance was not reduced during the first 2 h of exposure to 10 mM NH_4^+ supplied together with 10 mM NO_3^- to roots of barley plants. However, treatments of longer duration, e.g. 4 or 6 h, resulted in a dramatic decrease in the abundance of *HvNRT2*. This decrease was accompanied by a marked decrease of $^{13}NO_3^-$ influx, and it was concluded that either NH_4^+ itself or a product(s) of its assimilation was responsible for down-regulating *HvNRT2* mRNA levels. To explore this question further, we determined $^{13}NO_3^-$ influx, transcript abundance of *HvNRT2*, and NH_4^+ and amino acid concentrations in roots grown on NO_3^- alone or on NO_3^- together with NH_4^+ with or without MSO. These treatments were designed to investigate the effects of elevated root NH_4^+ concentrations and diminished Gln levels (plus MSO treatments) on $^{13}NO_3^-$ influx and *HvNRT2* transcript abundance (Table III). $^{13}NO_3^-$ influx under control conditions (corresponding to 6 h of NO_3^-) was reduced from 3.2

Table I. *Effects of exogenously supplied amino acids on inorganic and organic N levels in plant roots*

Values shown are the means of four independent replicates ± SD of the mean.

Treatment	Amino Acid				Inorganic N
	Asn	Asp	Gln	Glu	NO_3^-
	nmol g^{-1} fresh wt				*µmol g^{-1} fresh wt*
−N	0.82 ± 0.41	0.24 ± 0.02	0.71 ± 0.06	2.11 ± 0.81	5.1 ± 2.7
6 h-NO_3^-	1.32 ± 0.25	0.59 ± 0.14	3.41 ± 0.21	5.29 ± 1.07	46.2 ± 11.2
6 h-NO_3^- + Asn	4.02 ± 2.01	1.32 ± 0.22	4.62 ± 1.01	8.50 ± 2.13	26.2 ± 12.7
6 h-NO_3^- + Asp	2.58 ± 1.28	0.98 ± 0.16	5.52 ± 0.42	9.42 ± 2.61	31.5 ± 2.1
6 h-NO_3^- + Gln	2.51 ± 1.02	0.62 ± 0.21	5.02 ± 0.58	8.51 ± 2.61	64.8 ± 9.4
6 h-NO_3^- + Glu	1.46 ± 0.68	0.92 ± 0.11	6.82 ± 0.51	10.21 ± 1.94	47.5 ± 4.1

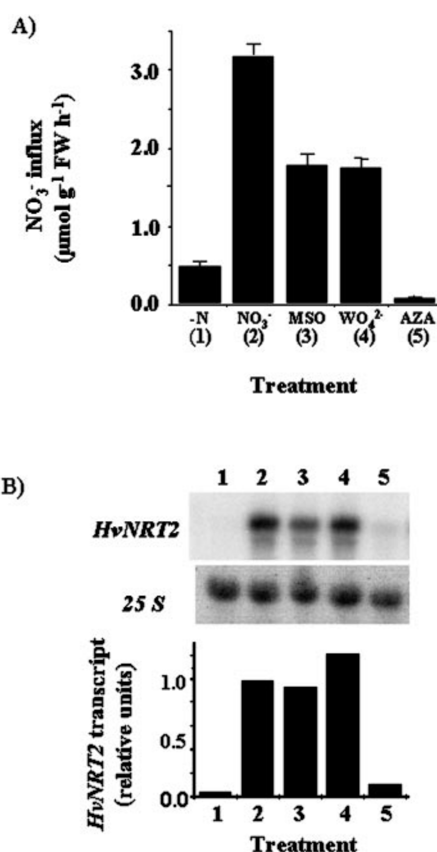

Figure 3. The effect of N assimilation inhibitors on $^{13}NO_3^-$ influx and *HvNRT2* transcript accumulation. Seven-day-old barley seedlings were grown in one-tenth-strength N-free modified Johnson's solution. A, $^{13}NO_3^-$ influx measured at 50 µM. Each treatment consists of four replicates. B, Northern-blot analysis of *HvNRT2* transcript accumulation in RNA isolated from barley roots. Treatments: lane 1, N starved; lanes 2 through 6, treated with 10 mM NO_3^- for 6 h; inhibitors were added to treatments in lanes 3 through 6. Lane 3, 1 mM MSO; lane 4, 0.5 mM tungstate; lane 5, 0.25 mM AZA. Quantification of transcript levels was by phosphor imager average of two experiments (standardized by 25S transcript). Transcript abundance was calculated in proportion to the 6 h-NO_3^- treatment (as 1 relative unit).

to 1.74 µmol g^{-1} fresh weight h^{-1}, with a concomitant 4.2-fold increase of root NH_4^+ concentration (4.5–18.9 µmol g^{-1} fresh weight) as a result of exposure to NO_3^- plus MSO (Table III). Exposure to NO_3^- plus NH_4^+ reduced $^{13}NO_3^-$ influx to 0.97 µmol g^{-1} fresh weight h^{-1}, and increased root NH_4^+ concentration 10.5-fold (4.5–47.2 µmol g^{-1} fresh weight), while NO_3^- plus both NH_4^+ and MSO reduced $^{13}NO_3^-$ influx to 0.84 µmol g^{-1} fresh weight h^{-1} and increased root NH_4^+ concentration 12.3-fold (4.5–55.2 µmol g^{-1} fresh weight). Additions of NO_3^- or NH_4^+ increased root Gln concentration compared with the minus-N treatment (Table III), while MSO had the opposite effect. Thus, root Gln increased from 0.71 to 3.41 nmol g^{-1} fresh weight after 6 h of NO_3^- treatment, while 6 h of NO_3^- plus MSO treat-

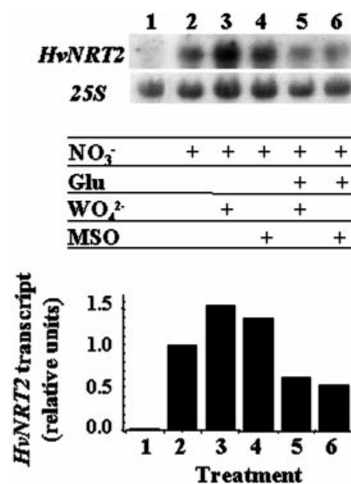

Figure 4. Effects of combinations of N-assimilation inhibitors, tungstate, and MSO in the presence of Glu and NO_3^- on HvNRT2 transcript accumulation in barley roots. Lane 1, Northern-blot analysis of RNA isolated from roots of N starved plants; lanes 2 through 6 contain RNA isolated from roots treated with 10 mM NO_3^-. Transcript abundance was calculated in proportion to the 6 h-NO_3^- treatment (as 1 relative unit). Lane 2, 10 mM NO_3^-; lane 3, 0.5 mM tungstate plus 10 mM NO_3^-; lane 4, 1 mM MSO plus 10 mM NO_3^-; lane 5, 0.5 mM tungstate plus 10 mM NO_3^- and 1 mM Glu; lane 6, 1 mM MSO plus 10 mM NO_3^- and 1 mM Glu. Quantification of transcript levels was by phosphor imager average of two experiments (standardized by 25S transcript).

Table II. *Effects of inhibitors of N assimilation on inorganic and organic N levels in plant roots*

Values shown are the means of four independent replicates ± SD of the mean.

Treatment	Amino Acid				Inorganic N	
	Asn	Asp	Gln	Glu	NO_3^-	NH_4^+
	nmol g^{-1} fresh wt				*µmol g^{-1} fresh wt*	
−N	0.82 ± 0.41	0.24 ± 0.02	0.71 ± 0.06	2.11 ± 0.81	5.1 ± 2.7	4.2 ± 0.2
6 h-NO_3^-	1.32 ± 0.25	0.59 ± 0.14	3.42 ± 0.21	5.29 ± 1.07	46.2 ± 11.2	4.5 ± 0.34
6 h-NO_3^- + Tungstate	0.61 ± 0.11	0.57 ± 0.21	0.65 ± 0.28	4.80 ± 0.72	42.5 ± 15.6	3.6 ± 0.47
6 h-NO_3^- + MSO	0.71 ± 0.41	0.54 ± 0.12	2.12 ± 0.81	2.90 ± 0.62	34.2 ± 7.4	18.5 ± 1.4
6 h-NO_3^- + AZA	1.78 ± 0.87	0.27 ± 0.12	4.88 ± 1.47	0.54 ± 0.24	8.4 ± 2.1	10.4 ± 2.1

ment reduced this value to 2.12 nmol g^{-1} fresh weight. Exposure to NO_3^- plus NH_4^+ for 6 h produced the highest value for root Gln concentration, 6.7 nmol g^{-1} fresh weight, while the addition of MSO reduced this value to 4.54 nmol g^{-1} fresh weight. Using the 6-h NO_3^- treatment as a control, these treatments reduced *HvNRT2* transcript levels to 0.85 (NO_3^- plus MSO treatment), 0.14 (NO_3^- plus NH_4^+ treatment), and 0.09 (NO_3^- plus NH_4^+ plus MSO treatment) relative units.

DISCUSSION

NO_3^- uptake is subject to regulation by both positive (induction) and negative (down-regulation) effects. The latter appear to depend upon the N status of the whole plant (for reviews, see Glass and Siddiqi, 1995; Crawford and Glass, 1998). It has been suggested that the cycling of amino acids between shoots and roots serves to provide the necessary information regarding whole-plant N status, enabling roots to regulate N uptake accordingly (Cooper and Clarkson, 1989; Muller and Touraine, 1992). Furthermore, when plants simultaneously absorb different N forms (e.g. NO_3^-, NH_4^+, and amino acids), there is a need to integrate information from several putative feedback signals. While the N-cycling model is based on physiological data, there is presently little information available regarding the molecular mechanism(s) responsible for translating these proposed signals into processes that modulate NO_3^- transport at root plasma membranes.

At the physiological level, it has long been known that NO_3^- is the signal for the induction of the IHATS (Jackson et al., 1973; Goyal and Huffaker, 1986; Aslam et al., 1996), although NO_2^- can also serve as an inducer for this process (Siddiqi et al., 1992; Aslam et al., 1996). The cloning of genes that encode this transport system has made it possible to show that this is equally the case at the transcriptional level (Trueman et al., 1996; Quesada et al., 1997; Amarashinghe et al., 1998; Zhuo et al., 1999; Filleur and Daniel-Vedele, 1999). Thus, transcript levels for these genes increase dramatically in response to the provision of NO_3^- in the media, and the expression patterns have been correlated with NO_3^- influx, giving indirect evidence that *NRT2* encode high-affinity transporters (Quesada et al., 1997; Lejay et al., 1999; Zhuo et al., 1999). Heterologous expression of the *NRT2* genes in an *Hansenula polymorpha* NO_3^- transport mutant defective in the *YNT1* gene (a member of the *CRNA* family) demonstrated that these genes encode functional NO_3^- transporters (Zhang et al., 1998).

By contrast, the identity of the N intermediate(s) responsible for the characteristic down-regulation of NO_3^- influx following peak induction has remained in controversy (see introduction). To investigate the factors responsible for this negative feedback control of IHATS and abundance of *HvNRT2* transcript, we designed a series of experiments according to three criteria. First, plants were maintained on minus-N media. These plants contained low concentrations of all N derivatives, allowing changes of N pools to be

Table III. *Effects of NH_4^+ and MSO on HvNRT2 transcript abundance, NH_4^+ concentrations, and Gln concentrations in plant roots*

Values shown are the means of four independent replicates ± SD of the mean. Plants were grown on one-tenth modified (−N) Johnson's solution, then treated for 6 h with the treatments shown below, using 10 mM NO_3^- ± 1 mM MSO, or 10 mM NO_3^- + 10 mM NH_4^+ ± 1 mM MSO. Transcript abundance was calculated using the ratio of *HvNRT2/25s* hybridizing signal, quantified on a phosphor imager.

Treatment	Relative Transcript Level	NO_3^- Influx	[NH_4^+]	[Gln]
		µmol g^{-1} fresh wt h^{-1}	*µmol g^{-1} fresh wt*	*nmol g^{-1} fresh wt*
−N	0.12	0.48 ± 0.12	4.2 ± 0.21	0.71 ± 0.06
6h NO_3^-	1.00	3.20 ± 0.25	4.5 ± 0.34	3.41 ± 0.21
6h NO_3^- + MSO	0.85	1.74 ± 0.21	18.9 ± 1.4	2.12 ± 0.81
6h NO_3^- + NH_4^+	0.14	0.97 ± 0.13	47.2 ± 3.1	6.70 ± 0.54
6h NO_3^- + NH_4^+ + MSO	0.09	0.84 ± 0.08	55.2 ± 2.8	4.54 ± 0.52

readily detected following provision of NO_3^-. Second, minus-N plants were exposed to high levels of NO_3^- (10 mM), so that NO_3^- might enter root cells via the LATS for NO_3^-, even if inhibitor treatments reduced high-affinity transport. Third, the treatments with amino acids and inhibitors of N-assimilatory enzymes were short-term (6 h) to minimize the possibility of secondary effects of these treatments.

Several possible signals for the down-regulation of IHATS have been proposed. These include root NO_3^-, root NH_4^+, and/or amino acids (Siddiqi et al., 1989; Lee et al., 1992; Muller and Touraine, 1992; King et al., 1993). At the molecular level, Quesada et al. (1997) demonstrated that *NpNRT2.1* transcript levels in NO_3^--grown plants decreased due to the supply of NH_4^+ or Gln. Unfortunately, these preliminary results failed to identify whether NH_4^+ itself or products of its assimilation were responsible for the observed effects. A study by Zhuo et al. (1999) using inhibitors of NO_3^- assimilation concluded that NH_4^+ and Gln were both active in the down-regulation of the Arabidopsis *AtNRT2.1* gene.

Time Course of the Down-Regulation of NO_3^- Influx

To investigate the down-regulation of NO_3^- influx in barley roots, we monitored five parameters: NO_3^- influx, *HvNRT2* transcript levels, and NO_3^-, NH_4^+, and amino acid concentrations of root tissue. The typical time profile of $^{13}NO_3^-$ influx was observed upon providing NO_3^- to plants previously starved of N (Siddiqi et al., 1989; Vidmar et al., 2000). This pattern correlated well with *HvNRT2* transcript accumulation during the first 6 h of NO_3^- provision (Vidmar et al., 2000), but thereafter transcript levels decreased to levels that were undetectable, while influx remained relatively high. Two possibilities might account for this anomaly. First, turnover rates of the IHATS protein may be relatively slow compared with those of the corresponding mRNA, and therefore the abundance of IHATS mRNA need not correlate with influx capacity. To evaluate this it will be necessary to make use of antibodies to the NRT2 protein. Second, other transporter types (CHATS or LATS) may contribute to the observed fluxes; e.g. CHATS activity has been demonstrated to increase 2- to 3-fold following exposure to NO_3^- (Aslam et al., 1993; Kronzucker et al., 1995). During this prolonged exposure to NO_3^-, NH_4^+ levels increased only very slightly (10%), whereas root NO_3^- concentrations increased 17-fold (Fig. 1A), following the same pattern as reported by Siddiqi et al. (1989). Gln, Glu, Asn, and Asp levels increased from 4- to 13-fold (Fig. 1B). The low levels of accumulated NH_4^+ suggest that, under normal conditions, NH_4^+ itself does not participate in the down-regulation of *HvNRT2* transcript abundance. However, the changes in root concentrations of both NO_3^- and amino acids are consistent with their involvement in this process.

Effects of NO_3^- on *HvNRT2* Transcript Accumulation and NO_3^- Influx

To investigate the role of NO_3^- in the down-regulation of *HvNRT2* transcript abundance, we made use of tungstate, a well-known inhibitor of the enzyme NR (Deng et al., 1989). The response of $^{13}NO_3^-$ influx to tungstate treatment has been described in *Lemna* by Ingemarsson et al. (1987). Growth at high external [NO_3^-] in the presence of tungstate increased cell NO_3^- concentration and reduced $^{13}NO_3^-$ influx. By contrast, $^{13}NO_3^-$ influx remained high when plants grown at low external [NO_3^-] were treated with tungstate, and the authors proposed that this provided evidence for the regulation of NO_3^- influx by NO_3^- itself. Any potential for indirect inhibitory effects of tungstate was controlled for by the high values of $^{13}NO_3^-$ influx reported in the low-NO_3^- plants. In our experiments using high-N plants, $^{13}NO_3^-$ influx was also substantially reduced by exposure to tungstate during induction (Fig. 3A, treatment 4). This observation suggests that NO_3^- may regulate *HvNRT2* expression in barley roots. However, compared with control plants, *HvNRT2* transcript levels actually increased by 20% to 30% in response to the same treatment (Fig. 3B, lane 4, and Fig. 4, lane 3), a finding that makes it unlikely that NO_3^- inhibits influx at *HvNRT2* transcript levels. The increased *HvNRT2* transcript level associated with tungstate treatment is consistent with a similar effect of tungstate on levels of NR mRNA in tobacco (Deng et al., 1989), and with elevated levels of *NRT2.1* transcripts observed in Arabidopsis and *N. plumbaginifolia* mutants lacking NR activity (Krapp et al., 1998; Filleur and Daniel-Vedele, 1999; Lejay et al., 1999).

These observations suggest that the down-regulation of both *NR* and *NRT2* transcript abundance depends upon reduced forms of N rather than upon NO_3^-. By adding Glu to the tungstate treatment (Fig. 4), the block of N assimilation was bypassed and *HvNRT2* transcript levels were reduced. This confirms the importance of reduced N in regulating levels of *HvNRT2* transcript, although the experiment failed to distinguish between Glu and products of Glu metabolism; the data presented in Table I demonstrate that the addition of Glu doubled the concentrations of Gln, Asn, and Glu. Table I reveals that the root NO_3^- concentration was virtually unaffected by tungstate treatment, although cytoplasmic NO_3^- may have increased under these conditions. Asp and Glu concentrations also remained constant following tungstate treatment, whereas Asn and Gln concentrations were reduced by 53% and 81%, respectively (Table II). The evidence therefore suggests that the elevated transcript abundance observed under these conditions may result from relief of negative feedback associated with lowered concentrations of Asn or Gln. If this is the case, the observed decrease of $^{13}NO_3^-$ influx associated with tungstate

treatment can only be accounted for by effects at a post-transcriptional level. This hypothesis is in agreement with physiological experiments using the *NAR1/NAR7* NR double mutants of barley that have 1% to 5% of wild-type NR activity (Warner and Huffaker, 1989; King et al., 1993). These experiments revealed that mutant plants expressed the typical pattern of IHATS induction on supplying exogenous NO_3^-, as well as the typical down-regulation of NO_3^- influx that follows peak induction. The hypothesis of post-translational effects of NO_3^- may also account for the findings of Ingemarsson et al. (1987), who also reported a reduction of $^{13}NO_3^-$ influx in *Lemna* following exposure to tungstate.

Effects of NH_4^+ on NO_3^- Uptake and *HvNRT2* Transcript Abundance

NH_4^+ has been demonstrated to inhibit NO_3^- influx into barley roots within minutes of NH_4^+ application (Lee and Drew, 1989). Likewise, Kronzucker et al. (1999) demonstrated that $^{13}NO_3^-$ influx decreased and $^{13}NO_3^-$ efflux increased immediately following the addition of NH_4^+ in barley roots. These observations suggest that inhibitory effects of NH_4^+ result from direct effects of NH_4^+ at the plasma membrane at least. In addition, Quesada et al. (1997), Krapp et al. (1998), and Filleur and Daniel-Vedele (1999) reported that transcript abundance of the *NRT2* family of genes was reduced by NH_4^+ treatment. However, the experimental design of the latter studies failed to distinguish between effects of NH_4^+ itself and/or products of NH_4^+ assimilation. This distinction can be achieved by using MSO (an inhibitor of the enzyme Gln synthetase), which typically increases root [NH_4^+] and reduces [Gln]. For example, in the study by Lee et al. (1992), MSO treatment elevated cytoplasmic [NH_4^+] from 8 to 80 mM. In the present experiments, MSO increased root [NH_4^+] 4-fold without significantly reducing transcript abundance (Table III) in plants supplied with NO_3^- as the sole source of N. When plants were supplied with NO_3^- and NH_4^+ in the absence or presence of MSO, transcript abundance declined to 14% and 9%, respectively, of the NO_3^- treatment alone. The strong reduction of transcript abundance in the absence of MSO might be attributed to either an effect of NH_4^+ or a product(s) of NH_4^+ assimilation (e.g. Gln). However, in the presence of MSO, the reduction in transcript level indicates that NH_4^+ itself may participate in regulating transcript abundance. These results demonstrate that *HvNRT2* transcript abundance is strongly reduced when tissue [NH_4^+] is elevated by inhibitors or by provision of a concentrated exogenous source of NH_4^+.

Nevertheless, since $^{13}NO_3^-$ influx decreased by 47% in the presence of NO_3^- plus MSO, without significant effects on transcript levels (Table III), it is possible that NH_4^+ may also act at a post-transcriptional level. Indeed, accumulated NH_4^+ has been suggested to act directly upon the high-affinity NH_4^+ transporter (Rawat et al., 2000).

Regulation of *HvNRT2* Transcript Abundance and NO_3^- Influx by Amino Acids

Both NO_3^- influx and *HvNRT2* transcript abundance declined in root tissue in response to exogenous amino acid treatments. The inhibitory effects of the four amino acids tested on $^{13}NO_3^-$ influx were as follows: Asp > Glu > Asn > Gln. *HvNRT2* transcript abundance was reduced according to a similar pattern: Glu > Asp > Asn > Gln, with the decrease being greater than 50% in all cases. Earlier studies have demonstrated that exogenous application of amino acids can inhibit NO_3^- uptake/influx, but there has been a lack of agreement as to which amino acids are most active (Breteler and Arnozis, 1985; Glass, 1988; Muller et al., 1995). Breteler and Arnozis (1985) found that exogenously supplied Arg, Asp, Cys, and Glu inhibited NO_3^- uptake in NO_3^--induced plants. Rodgers and Barneix (1993) demonstrated the same pattern of inhibition, noting that Gln had only a minor effect. In the report by Muller and Touraine (1992), amino acids were fed to root tissue either via the cotyledons, to mimic shoot signals (via the phloem), or by exogenous feeding. Regardless of the method used, several amino acids were inhibitory and, of these, Gln was not the most effective. Tillard et al. (1998) reported that transferring castor bean plants grown on NO_3^- to N-free medium resulted in a transient increase in NO_3^- influx, and that this increase was correlated with a 40% decrease in the amino acid concentration of the phloem sap (predominantly Gln and Ser), which is consistent with a role for Gln in down-regulating NO_3^- influx.

However, without examining the fate of exogenously applied amino acids with respect to their differential uptake and/or metabolism, it is impossible to identify the putative regulators of NO_3^- influx. For example, feeding maize plants either Asn or Gln increased endogenous levels of both of these amides (Sivasankar et al., 1997). Likewise, exogenous application of Gln, Glu, or Asn significantly increased root NH_4^+ concentrations greater than 4-fold and altered concentrations of other amino acids in roots of rice plants (Wang, 1994; A. Kumar, personal communication). In our amino acid-treated barley plants, exogenous application of Gln, Glu, Asp, or Asn increased root concentrations of the applied amino acid and also those of the other amines and amides (Table I). Thus, as a strategy to identify putative regulators of *HvNRT2* transcript abundance, this method is inadequate.

Inhibiting Glu synthase (using AZA) in plants supplied with NO_3^- reduced $^{13}NO_3^-$ influx and *HvNRT2* transcript levels by 97% and 95%, respectively. (Fig. 3, A and B). This was the most potent inhibitor em-

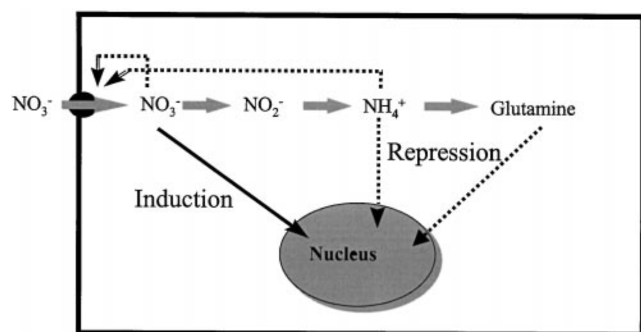

Figure 5. Proposed model for the regulation of IHATS NO_3^- transport and *HvNRT2* expression. Positive regulatory effects (induction) are shown by a solid line; negative effects are represented by dotted lines.

ployed in the present study. Compared with the NO_3^- control, AZA treatment also affected other measured parameters: root [NO_3^-] was reduced by 82%; root [NH_4^+] increased by 130%; root [Glu] decreased to approximately 10% of control; and [Gln] increased by 43% (Table II). The observed effects of AZA on *HvNRT2* transcript abundance might result from either decreasing the tissue concentration of NO_3^-, thus affecting induction, or from increasing concentrations of a feedback inhibitor (Gln or NH_4^+). The first hypothesis is unlikely, because the data in Table II reveal that significant quantities of NO_3^- were absorbed during AZA treatment. This is evident in the increase of root [NO_3^-] and in the larger increases of root [NH_4^+]. Moreover, concentrations of Asn and Gln were actually higher in the AZA treatments than in the NO_3^- controls. Both the elevated [NH_4^+] and elevated [amide] indicate that NR activity was at least equivalent to that of the controls, which is consistent with significant NO_3^- entry, induction of NR (and hence *HvNRT2*), and significant assimilation of NO_3^-.

The second hypothesis is supported, with respect to Gln, by the elevated Gln concentrations (and by the virtual elimination of Glu) associated with the AZA treatment (Table II). This treatment reduced the *HvNRT2* transcript level by 97% and $^{13}NO_3^-$ influx by 95%. If Glu were an important negative feedback regulator, its very low concentration (equivalent to 10% of controls) should have relieved the negative feedback effects on transcript and $^{13}NO_3^-$ influx. Support for a major role of Glu therefore relies upon the effects of exogenous application of Glu (Table I; Fig. 2). However, the data in Table I established that exogenous application of Glu increased root Gln 2-fold, compared with a 1.47-fold increase when Gln itself was supplied. The same was true for the exogenous application of Asp, which increased root Gln by 1.62-fold.

A critical role for Gln is also suggested by the results of the tungstate treatment. This was the only treatment that resulted in elevated levels of *HvNRT2* transcript, and also the only treatment that substantially reduced concentrations of root Gln (Glu levels were unchanged by this treatment). The argument for a role of root NH_4^+ is less convincing. During the time course experiment, root [NH_4^+] remained essentially constant despite a gradual diminution of *HvNRT2* mRNA (Vidmar et al., 2000). Likewise, the strong effects of NH_4^+ on *HvNRT2* abundance and $^{13}NO_3^-$ influx (Table III) can be explained as arising

Table IV. *Hypotheses concerning the regulation of IHATS expression and supporting observations*

Hypothesis	Observation
NO_3^- is not responsible for down-regulating *HvNRT2* transcript levels.	Tungstate treatment increased *HvNRT2* transcript abundance (Figs. 3B and 4).
NO_3^- may act post-transcriptionally.	Tungstate treatment decreased NO_3^- influx (Fig. 3A).
Elevated concentrations of NH_4^+ down-regulate *HvNRT2* transcript levels.	MSO decreased *HvNRT2* transcript levels, when applied together with NH_4^+ (Table III).
NH_4^+ exerts post-transcriptional effects.	MSO was capable of reducing NO_3^- influx substantially without significantly affecting *HvNRT2* transcript levels (Figs. 3, A and B, and 4).
Gln is the main down-regulator of *HvNRT2* transcript levels.	NH_4^+ treatment dramatically decreased *HvNRT2* transcript level when its conversion to Gln was not blocked by MSO (Table III).
	Addition of all amino acids increased root Gln levels, and decreased *HvNRT2* transcript levels (Table I; Fig. 2B).
	When root Glu and Gln concentrations were increased by exogenous application, *HvNRT2* transcript levels were inversely correlated with Glu and Gln concentrations.
	Tungstate treatment reduced root Gln by 81%, without affecting root Glu. This treatment produced a 20%–30% increase of *HvNRT2* mRNA.
	Treatments with AZA decreased root [Glu] by 90%, increased root [Gln] by 43%, and decreased *HvNRT2* transcript levels and $^{13}NO_3^-$ by 97% and 95%, respectively (Table II; Fig. 3).

from a conversion of NH_4^+ to Gln. However, when $[NH_4^+]$ becomes particularly high, as was evident when both NO_3^- and NH_4^+ were provided or when MSO was supplied together with NO_3^- and NH_4^+ (Table III), a case may be made for direct effects of NH_4^+ on transcript levels. A similar conclusion was reached in studies of Arabidopsis (Zhuo et al., 1999).

A model summarizing the main findings of the current experiments is provided in Figure 5, and the main hypotheses and supporting data are presented in Table IV.

MATERIALS AND METHODS

Plant Material

Seven-day-old barley (*Hordeum vulgare* cv Klondike) seedlings were used in all experiments. Seeds were surface-sterilized with 1% (v/v) hypochlorite solution and rinsed with de-ionized water. The seeds were placed on nylon mesh (pore size, 4 mm) which was fixed on to 20-mm (eight seeds) or 60-mm (25 seeds) plexiglass discs, depending on the experiment. The discs were placed on moist sand and seeds were covered with 10 mm of moist sand in the dark. After 3 d, seedlings were transferred to 40-L hydroponic tanks and grown in N-free 1/10-strength modified Johnson's solution for 4 d (Siddiqi et al., 1990). Prior to influx measurements, plants were treated with 10 mM KNO_3, with or without 5 mM $(NH_4)_2SO_4$ or 1mM amino acids, and with or without inhibitors of N assimilation. K concentrations of the growth media were monitored daily and a concentrated nutrient solution was supplied to the tanks in the same ratio as in the original modified Johnson's solution to prevent depletion. The pH of the solution was maintained at 6.2 ± 0.3 by the addition of excess $CaCO_3$ powder. Plants were grown in a controlled-environment chamber with a 16-h/8-h light/dark cycle at 20°C ± 2°C and 70% relative humidity. Light with a photon flux density at plant level equal to 300 μmol m^{-2} s^{-1} was provided by fluorescent tubes with a spectral composition similar to sunlight.

NO_3^- Influx

NO_3^- influx experiments were carried out essentially as described by Siddiqi et al. (1989). Seven-day-old barley plants, grown in hydroponic tanks and treated according to the particular experimental design, were transferred to 0.5-L of unlabeled influx solution containing 50 μM NO_3 for 5 min to equilibrate roots to the conditions to be employed for influx determination. They were then transferred to 0.5 L of influx solution containing 50 μM NO_3^- labeled with $^{13}NO_3^-$. After a 5-min influx period, plants were transferred back into a 0.5-L vessel of unlabeled solution for 3 min to remove unabsorbed tracer residing in the cell wall space. Roots and shoots were harvested separately and placed into 20-mL scintillation vials for counting in a Packard gamma counter (Minaxi δ, Auto-γ 5000 series, Packard, Downers Grove, IL). Production of $^{13}NO_3^-$ was as described by Kronzucker et al. (1995).

RNA Isolation and Northern-Blot Analysis

Total RNA was isolated using Trizol reagent (Life Technologies, Ontario, Canada), with two modifications. First, after the tissue was ground in a mortar and Trizol reagent was added at a ratio of 0.2 g of tissue to 1 mL of Trizol, the homogenate was centrifuged at 8,000*g* for 30 min to remove cellular debris. Second, after the total RNA was isolated, it was re-extracted with phenol:chloroform:iso-amyl alcohol (25:24:1), and precipitated with 0.3 M sodium acetate (final concentration) and two volumes of ethanol. Total RNA was separated on a 1.2% (w/v) agarose gel containing 1× 3-(*N*-morpholino)-propanesulfonic acid (MOPS) with 2.2 M formaldehyde, at 60 V for 3.5 h, then washed twice in water, and RNA transferred by capillary action to N^+ nylon membranes (Amersham, Quebec, Canada). The membranes were baked for 2 h at 80°C to fix the RNA, and then placed in prehybridization solution for 1 h. Following this procedure, membranes were transferred to hybridization solution with ^{32}P-labeled probe for 12 to 16 h. Prehybridization and hybridization solutions contained 6× SSC, 5× Denhardt's solution, 0.5% (w/v) SDS, and 20 μg/mL sonicated herring sperm DNA. Randomly labeled probes were made with the Prime-A-Gene kit (Promega, Madison, WI) using an internal fragment (*Afl*III–*Eco*RV) from *HvNRT2.3*, selected for its ability to recognize all known members of the *HvNRT2* family of cDNAs. Control levels of total RNA were probed using a fragment of the 25S gene on plasmid pV25S by digestion with *Xho*I. Membranes were washed as recommended by the manufacturer's instructions with 0.25× SSC buffer and 0.1% (w/v) SDS at 42°C for 15 min for the final wash.

NO_3^- Analysis

NO_3^- concentrations were determined from fresh tissues, extracted with boiling water at a ratio of 1 g of roots to 10 mL of water. The extracts were centrifuged at 8,000*g*, and the supernatant was filtered through a 0.45-μm filter. NO_3^- was analyzed using the cadmium-copper reduction method on a Technicon Autoanalyzer (Henricksen and Selmer-Olsen, 1970).

Amino Acid and NH_4^+ Measurements

Amino acids and NH_4^+ were extracted from root material in a buffer containing 58% (v/v) ethanol, 0.2 M formic acid, and 0.25 mM α-amino butyric acid as an internal standard by use of mortar and pestle at 4°C (Finnemann and Schjoerring, 1999). After centrifugation at 21,000*g* for 5 min and filtration through a 0.45-mm PVDF microcentrifuge tube filter (Whatman, Maidstone, UK), amino acids were measured with AccQ·Tag on HPLC (two 626 HPLC pumps; 4-mm Nova-pak C_{18} column, 3.9 × 150 mm, thermostatted at 39°C; 474 scanning fluorescence detector; 717_{plus} autosampler; 600S controller; all components Waters, Milford, MA). Mobile phase A consisted of 100 mM NaAc (Sigma-Aldrich, St. Louis), 5.4 mM triethylamine (Fluka, Buchs, Switzerland), and 3.5 mM EDTA (Sigma-Aldrich) adjusted to pH 5.7 with phosphoric acid. Mobile

phase B had a composition similar to that of A except for the pH, which was 6.7. Mobile phase C was acetonitrile (J.T. Baker, Amsterdam), and mobile phase D was ultrapure water (Milli-Q, Millipore, Bedford, MA; resistance 18.2 MΩ). All solutions were degassed before use. Gradient conditions were (v/v): 0.5 min with 90% A and 10% B; 16.5 min with 89% A, 10% B, and 1% C; 9 min with 80% A, 18% B, and 2% C; 6 min with 68% A, 27% B, and 5% C; 1.5 min with 63% A, 27% B, and 10% C; 3.5 min with 87.5% B and 12.5% C; 11 min with 87% B and 13% C; 0.1 min with 85% B and 15% C; 2.90 min with 60% C and 40% D; and 9 min with 90% A and 10% B. The initial flow rate was 1.0 mL min^{-1}, changing to 1.3 mL min^{-1} after 33.8 min. Standard curves were made using the appropriate concentrations of authentic amino acid standards (Sigma-Aldrich).

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