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Isolation and Characterization of *HvNRT2.3* and *HvNRT2.4*, cDNAs Encoding High-Affinity Nitrate Transporters from Roots of Barley[1]

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Two full-length cDNAs, *HvNRT2.3* and *HvNRT2.4*, were isolated from roots of barley (*Hordeum vulgare*), using reverse transcriptase-PCR and RACE-PCR. The corresponding polypeptides, consisting of 507 amino acids (molecular masses of 54.6 kD), belong to the major facilitator superfamily (MFS), and are closely related (>87% identity) to those encoded by *HvNRT2.1* and *HvNRT2.2* (formerly *BCH1* and *BCH2*, respectively) from roots of barley. The latter are considered to encode inducible high-affinity NO_3^- transporters (Trueman et al., 1996). *HvNRT2* transcripts were undetectable in NO_3^--deprived plants. Following exposure to either NO_3^- or NO_2^-, transcript abundance and $^{13}NO_3^-$ influx increased to a maximum by 6 to 12 h, then declined in *HvNRT2.1*, *HvNRT2.2*, and *HvNRT2.3*. The pattern of *HvNRT2.4* transcript abundance was different, remaining high after achieving peak abundance. When external NO_3^- concentrations were varied from 0 to 500 μM under steady-state conditions of NO_3^- supply, *HvNRT2* transcript accumulation and $^{13}NO_3^-$ influx were highest in 50 μM NO_3^- -grown plants. When NH_4^+ was provided together with NO_3^-, transcript accumulation during the first 2 h was similar to that due to NO_3^- alone, but by 4 h the transcript level was significantly reduced. *HvNRT2* transcript was undetectable in leaf tissues.

The absorption of NO_3^- by root cells is mediated by at least three kinetically distinct and thermodynamically active transport systems that are localized in the plasma membranes of root cells. The three transport systems were first characterized on the basis of their different responses to external NO_3^- concentrations and by their different NO_3^- inducibility (for review, see Glass and Siddiqi, 1995; Crawford and Glass, 1998). The constitutive high-affinity transport system (CHATS) is a low-capacity, high-affinity transporter that is expressed without the necessity of prior exposure to NO_3^- (Behl et al., 1988; Siddiqi et al., 1990; Aslam et al., 1992). This transporter represents the main pathway for NO_3^- entry into roots from low external NO_3^- on first exposure to NO_3^-, and is therefore critical for the induction of the high-capacity, high-affinity inducible transport system (IHATS). This transport system can be induced by either NO_3^- or NO_2^- (Siddiqi et al., 1992; Aslam et al., 1996). A notable feature of the time course of this induction is that it is typically followed by down-regulation of NO_3^- influx to a much lower steady-state level. While it is very evident that the induction of the IHATS is mediated by NO_3^-, the subsequent down-regulation has been attributed to NO_3^-, NH_4^+, and/or various amino acids (Ingemarsson et al., 1987; Siddiqi et al., 1990; Lee et al., 1992; Muller and Touraine, 1992).

At high external NO_3^- concentrations (>200 μM), a low-affinity transport system (LATS) becomes apparent. This system, like CHATS, is expressed in barley (*Hordeum vulgare*) plants grown in the complete absence of NO_3^- and shows no evidence of saturation, even at NO_3^- concentrations as high as 50 mM (Siddiqi et al., 1990). All three transporter systems bring about a rapid depolarization of the membrane electrical potential difference when exposed to exogenous NO_3^-, which is consistent with the hypothesis that the free energy for active transport of NO_3^- is provided by the proton motive force via a $2H^+$:$1NO_3^-$ symport (Ullrich and Novacky, 1981; McClure et al., 1990; Glass et al., 1992; Meharg and Blatt, 1995; Wang and Crawford, 1996). At present, the only genetic information on the CHATS comes from the isolation of a chlorate-resistant mutant (*chl8*) from Arabidopsis (Wang and Crawford, 1996). Physiological studies of this mutant showed that plants grown in submerged cultures without NO_3^- failed to show the typical pattern of NO_3^- uptake or depolarization of membrane electrical potential differences when exposed to NO_3^-, suggesting an absence of normal CHATS expression. By contrast, the IHATS and LATS activities of this mutant were normal.

Genes that are thought to encode the LATS and IHATS transporters have been isolated from various higher plants. In the case of LATS, this was accomplished by screening Arabidopsis mutants with chlorate (a toxic analog of NO_3^-). This resulted in the isolation and characterization of chlorate-resistant mutants (Doddema and Telkamp, 1979). Of these, only one (B1) was affected in NO_3^- transport (the other mutants were defective in the synthesis of molybdate cofactor and NO_3^- reductase). Using the same screening method, Tsay et al. (1993) isolated a T-DNA-tagged mutant that mapped to the same locus as B1. A T-DNA tagged *CHL1* gene and the wild-type homolog were subsequently isolated. The expression of *CHL1 cDNA* in *Xenopus* oocytes resulted in the accumulation of NO_3^-

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and the subsequent depolarization of the oocyte membrane upon exposure to NO_3^-. These results provide more direct evidence that the protein encoded by the *CHL1* gene is capable of NO_3^- transport (Tsay et al., 1993). Physiological analysis of the *chl1* deletion mutant by Touraine and Glass (1997) and Arabidopsis transformed with *chl1* under the control of the 35S promoter (Huang et al., 1996) was interpreted to indicate the existence of two low-affinity NO_3^- transport systems in Arabidopsis. These transport systems and/or their respective genes appear to be differentially regulated by ammonium or products of ammonium assimilation.

The first gene encoding an inducible high-affinity NO_3^- transporter from eukaryotic organisms was cloned from *Aspergillus nidulans* (Johnstone et al., 1990; Unkles et al., 1991). The *crnA* mutant (Tomsett and Cove, 1979) was defective in NO_3^- uptake in conidiospores and young mycelia (Brownlee and Arst, 1983). The *crnA* gene, which was able to restore NO_3^- uptake in this mutant (Johnstone et al., 1990; Unkles et al., 1991), encodes a protein that is 507 amino acids long and contains 12 membrane-spanning regions. This protein is a member of the major facilitator superfamily (MFS) (Trueman et al., 1996), a superfamily of membrane proteins that contains the conserved amino acid motif of (D/N) RXGR(R/K) and $IX_2RX_3GX_3G$ between membrane spanning domains 2 and 3 (Henderson, 1991).

A number of genes that are homologous with *crnA* has been cloned from other eukaryotes. These include *YNT1* from *Hansenula polymorpha* (Perez et al., 1997), *CrNRT2.1* and *CrNRT2.2* from *Chlamydomonas reinhardtii* (Quesada et al., 1994), *HvNRT2.1* and *HvNRT2.2* (formerly *BCH1* and *BCH2*) from barley (Trueman et al., 1996), *NpNRT2.1* from *Nicotiana plumbaginifolia* (Quesada et al., 1997), *GmNRT2* from soybean (*Glycine max*) (Amarashinghe et al., 1998), and *AtNRT2.1* and *AtNRT2.2* from Arabidopsis (Zhuo et al., 1999). All of the above are thought to encode high-affinity NO_3^- transporters and belong to the MFS.

In barley the isolation of a *BCRNA* fragment by PCR using oligonucleotides directed at a conserved MFS motif led to the isolation of the first *HvNRT2.1* and *HvNRT2.2* genes encoding putative NO_3^--inducible high-affinity transporters in higher plants (Trueman et al., 1996). Northern-blot analysis of nitrogen-starved barley plants showed that the *HvNRT2* transcript accumulated rapidly in roots following provision of NO_3^- (Trueman et al., 1996). This is in agreement with physiological data showing that NO_3^- influx can increase up to 30-fold in the high-affinity range upon NO_3^- treatment (Siddiqi et al., 1989). Subsequently, in both *N. plumbaginifolia* and Arabidopsis, levels of *NpNRT2.1* and *AtNRT2.1* transcripts decreased when the NO_3^- supply was maintained beyond the period of peak induction (Krapp et al., 1998; Zhuo et al., 1999). This pattern of expression correlates with the overshoot of high-affinity NO_3^- transport and the subsequent decline to a lower steady-state level referred to above (Siddiqi et al., 1989). Reduced nitrogen forms such as NH_4^+ or Gln, which are known to diminish NO_3^- uptake when applied in the presence of NO_3^-, decreased *NpNRT2.1* and *AtNRT2.1* transcript levels in roots of *N. plumbaginifolia* and Arabidopsis, respectively (Quesada et al., 1997; Krapp et al., 1998; Zhuo et al., 1999). By use of metabolic inhibitors, particularly Met sulfoximine and azaserine, which block the enzymes Gln synthetase and GOGAT, respectively, it was suggested that both NH_4^+ and Gln were active in the down-regulation of *AtNRT2.1* expression (Zhuo et al., 1999).

In barley, the genome organization may allow for the presence of as many as seven to 10 members of the *HvNRT2* gene family (Trueman et al., 1996). Due to the considerable physiological data available for this species, barley is an important model system in which to investigate the mechanism of transcriptional regulation of NO_3^- transport. In this report, we describe the isolation of two new cDNAs, *HvNRT2.3* and *HvNRT2.4*, which are closely related to *HvNRT2.1* and *HvNRT2.2*, and the isolation of the 5′ upstream region of *HvNRT2.1*, *HvNRT2.2*, and *HvNRT2.3*. We have also characterized the expression pattern of the *HvNRT2* family of genes and $^{13}NO_3^-$ influx in response to the provision of various nitrogen sources in parallel experiments.

MATERIALS AND METHODS

Plant Material

Seven-day-old seedlings of barley (*Hordeum vulgare* cv Klondike) were used in all experiments. Seeds were surface-sterilized with 20% (v/v) commercial bleach solution and rinsed with de-ionized water. The seeds were placed on a nylon mesh (pore size, 4 mm) fixed onto 20-mm (8 seeds) or 60-mm (25 seeds) plexiglass discs, depending on the experiment. The discs were placed in moist sand in the dark, and the seeds were covered to a depth of 10 mm. After 3 d the seedlings were transferred to 40-L hydroponic tanks and grown in nitrogen-free one-tenth-strength modified Johnson's solution (Siddiqi et al., 1989) for 4 d more. Depending on the experiment, nitrogen was supplied in the form of NO_3^-, NO_2^-, or NH_4^+. The K^+ concentration was monitored daily and the concentrations of K^+ and other nutrients were restored by the addition of a concentrated stock solution to maintain them at constant levels. The pH of the solutions was maintained at 6.2 ± 0.3 by the addition of excess $CaCO_3$ powder. Plants were maintained in a controlled environment chamber with a 16-h/8-h light/dark cycle at $20°C \pm 2°C$ and 70% relative humidity. Light (photon flux density at plant level of approximately 300 μmol m^{-2} s^{-1}) was provided by fluorescent tubes with a spectral composition similar to sunlight.

RNA and DNA Isolation

Total RNA was isolated using Trizol Reagent (Life Technologies/Gibco-BRL, Cleveland) 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 per milliliter 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 again extracted with phenol:chloroform:iso-amyl alcohol (25:24:1) and precipitated with sodium acetate (final concentration 0.3 M) and 2

volumes of ethanol. An mRNA isolation kit (FastTrack, Invitrogen, Carlsbad, CA) was used according to the manufacturer's instructions. Genomic DNA was isolated as described by Asubel et al. (1995).

cDNA and Genomic Library

Messenger RNAs isolated from roots of 7-d-old barley seedlings treated for 2 and 6 h with 10 mM KNO_3 were used as the template for cDNA synthesis. A cDNA synthesis kit (Marathon, CLONTECH Laboratories, Palo Alto, CA) was used for the construction of a cDNA library. A DNA walking kit (PromoterFinder, CLONTECH Laboratories) was used for the construction of a barley genomic DNA library.

Northern-Blot Analysis

Total RNA was separated on a 1.2% (w/v) agarose gel containing 1× MOPS buffer (20 mM 3-[*N*-morpholino] propanesulfonic acid, 8 mM sodium acetate and 1 mM EDTA) with 2.2 M formaldehyde, at 60 V for 3.5 h, then washed twice in water, and the RNA was transferred by capillary action to nylon membrane (Hybond N+, Amersham-Pharmacia Biotech, Uppsala). The membrane was baked for 2 h at 80°C to fix the RNA, and was then placed in prehybridization solution for 1 or 4 h (random labeled probe or oligonucleotide probe, respectively). Membranes were next exposed to hybridization solution with ^{32}P-labeled probe for 12 to 16 h. For random-labeled probes, prehybridization and hybridization solutions were 6× SSC, 5× Denhardt's solution, 0.5% (w/v) SDS, and 20 μg mL^{-1} sonicated herring sperm DNA, respectively. Random-labeled probes were made with a kit (Prime-A-Gene, Promega, Madison, WI) using an internal fragment from the *HvNRT2.3* gene from plasmid pBCH3 by digestion with *Eco*RV and *Afl*III.

Control levels of total RNA were probed with a fragment of the 25S gene on plasmid pV25S by digestion with *Xho*I. Membranes were washed according to the manufacturer's instructions with 0.25 SSC and 0.1% (w/v) SDS at 42°C for 15 min for the final wash. Oligonucleotide probing, prehybridization solution, and hybridization solution consisted of 50% (v/v) formamide, 6× SSC, 0.01% (w/v) SDS, and 0.05 mg mL^{-1} salmon sperm DNA. Prehybridization was at 37°C for 4 h, while hybridization was for 12 to 16 h. Washing of the membrane consisted of 2× 15-min washes at room temperature with 2× SSC and 0.05% (w/v) SDS. The oligonucleotides used as probes were DX 46: 5′CTGTAGTTCAGTACTTGTACATAGG for the *HvNRT2.1* gene; DX48: 5′CACTGTACGTGTACACAGGTAAAG for the *HvNRT2.2* gene; BCH3: 5,GGTCCAAATGGAGGTGGAGG for the *HvNRT2.3* gene; and BCH4: 5′ CAAAATTTGAAACTTATACGTGTAGG for the *HvNRT2.4* gene. T4 DNA kinase (Life Technologies, Gaithersburg, MD) and [α-^{32}P]ATP (Amersham-Pharmacia Biotech) were used for end-labeling of oligonucleotides. G-25 spin columns (Pharamacia, Montreal, Canada) were used to separate unincorporated [^{32}Pα]ATP from reaction mixtures.

Isolation and Screening of *HvNRT2.3* and *HvNRT2.4* cDNAs by Reverse Transcriptase-PCR and RACE-PCR

The isolation of *HvNRT2.3* was by 5′- and 3′-RACE-PCR. Oligonucleotide BCRNA-7 (5′GTATGGGTGTGCCTTCCT) was used for the 3′ prime race, while for 5′-RACE-PCR and isolation of a full-length cDNA, BCH3 (5′TGCCTTATACCTGCTGCTGGGGTG) was used. The cDNA template was fabricated using the cDNA synthesis kit. 5′- and 3′-RACE-PCR conditions were 7 min at 94°C then 35 cycles of 94°C for 45 s, 62°C for 45 s, with a 5-min extension period at 72°C.

The isolation of *HvNRT2.4* was by 5′- and 3′-RACE-PCR. The oligonucleotide DZ44: 5′ GGACTAGCAGCGGGT was used in the initial 3′-RACE-PCR. RACE-PCR conditions were 94°C for 7 min, then 35 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 4 min. The PCR reaction products were purified and then separated on a 1.2% (w/v) agarose gel. The digested DNA was then transferred to nylon membrane (Hybond N+, Amersham-Pharmacia Biotech) for Southern analysis. Positive PCR products were cloned into pCR2.1 (Invitrogen) and sequenced. Oligonucleotide BCH4 (5′ CAAAATTTGAAACTTATACGTGTAGG) was used for the isolation of 5′-RACE-PCR product.

Full-length clones for *HvNRT2.3* and *HvNRT2.4* were generated by PCR using a high-fidelity PCR system (Expand Long, Boehringer Mannheim/Roche, Basel). For *HvNRT2.4,* oligonucleotides used were BCH3-5, 5′GGTCCAAATGGAGGTGGAGG and BCH3, while for *HvNRT2.4,* oligonucleotides BCH4-5, 5′CTCAGTAGATATGGAGGTGAGGC and BCH4 were used. The full-length PCR products were subcloned into pCR2.1 (Invitrogen). After restriction endonuclease analysis of the resulting cDNAs, resulting overlapping fragments were subcloned into pBlueScript I KS+ (Stratagene, La Jolla, CA). Sequences were determined on both strands using M13 forward and reverse primers. For regions where subclones could not be generated, specific oligonucleotides were designed for sequencing.

Isolation of Upstream Region of *HvNRT2.1*, *HvNRT2.2*, and *HvNRT2.3*

A DNA walking kit (PromoterFinder, CLONTECH Laboratories) was used for the isolation of the upstream region of *HvNRT2.1*, *HvNRT2.2*, and *HvNRT2.3*. Specific oligonucleotides were designed to hybridize with the 5′-untranslated region (UTR). For *HvNRT2.1*, the oligonucleotides were GB1 (5′CAACAACTAGAAGCAGCTAATGGTGGC) and GB1-2 (5′GTTGCAGCTCTTGAGCTTGGCTTGCAA); for *HvNRT2.2*, GB2 (5′TCGAGCTAGCTAGCTTAGTCGCACTGG) and GB2-2 (5′GTGTGTCTTTAATGGTGGTTGCTGCTG); and for *HvNRT2.3*, GB3 (5′ GGACCTTGCTTGATCGAGCTAGTCTCC) and GB3-2 (5′ GGAGCTAGCTTGCTTGATCAGCTGCAG). All PCR reactions used a high-fidelity PCR system (Boehringer Mannheim/Roche). For the first round of PCR the oligonucleotide AP1 was used (PromoterFinder DNA walking kit) with GB1, GB2, or GB3, at 92°C for 3 min, then 30 cycles of 92°C for 25 s, 65°C for 30 s, and 68°C for 10 min. The amplicon was diluted one-tenth,

and then a second round of PCR (nested) was conducted using AP2 (PromoterFinder DNA walking kit) and GB1-2, GB2-2, or GB3-3. The resulting PCR products were purified using the Gene Clean Kit (BIO101, Vista, CA), and cloned into pCR2.1.

NO_3^- Influx

NO_3^- influx experiments were carried out essentially as described by Siddiqi et al. (1989). Barley plants were grown on sand for 3 d, transferred to hydroponic tanks for 4 d, and then exposed to various media according to the experimental design. Plants were then transferred to 0.5-L vessels containing unlabeled uptake solution for 5 min so as to bring the root epidermal and cortical apoplasm to the same NO_3^- concentration as was used for the influx determination. After this pretreatment they were transferred to 0.5-L vessels containing a 50 μM NO_3^- influx solution labeled with $^{13}NO_3^-$ for a period of 5 min. Thereafter, plants were transferred back to the 0.5-L vessel of unlabeled nutrient solution for 3 min to remove tracer from the cell wall. Roots and shoots were harvested separately and placed into 20-mL scintillation vials for counting in a γ-counter (Minaxi δ Auto-γ 5000 series, Packard Instruments, Meriden, CT). $^{13}NO_3^-$ was produced as described by Kronzucker et al. (1995).

RESULTS

Isolation of *HvNRT2.3* and *HvNRT2.4* cDNAs

The isolation of *HvNRT2.3* (accession no. AF091115) was accomplished by RACE-PCR. Oligonucleotides directed to the *BCRNA* fragment (Trueman et al., 1996) were designed and subsequently used for 5′- and 3′-RACE PCR. The sequencing of the 3′-RACE-PCR product indicated a new member of the *HvNRT2* family of genes in barley, which we designated as the 3BCH3 fragment. The 5′-RACE-PCR product was a contaminant of *HvNRT2.1*. Therefore, a new oligonucleotide was designed based on sequence data from the 3′-UTR of the 3BCH3 fragment. 5′-RACE-PCR resulted in the isolation of a full-length cDNA. The cDNA sequence revealed that it was a new member of the *HvNRT2* family of genes in barley. This cDNA, which is 1,822 bp in length, was designated *HvNRT2.3*.

A different strategy was used in the isolation of the *HvNRT2.4* (accession no. AF091116) cDNA. We designed an oligonucleotide encoding protein consensus sequences found in the *CrNRT2.1*, *CrNRT2.2*, *HvNRT2.1*, and *HvNRT2.2* cDNAs. This consensus sequence represents amino acid positions 166 to 174 of the HvNRT2.1 protein. This amino acid motif has the sequence GLAAGWGNM, which is conserved among the NO_3^-/NO_2^- subgroup of the MFS (Trueman et al., 1996). The cDNA library used for 3′-RACE-PCR was digested with a number of restriction endonucleases that digest within *HvNRT2.1* and *HvNRT2.2* cDNAs. This was done to rule out the possibility that *HvNRT2.1* and *HvNRT2.2* cDNAs would be amplified. The 3′-RACE-PCR products were transferred to nylon membrane and probed with *HvNRT2.1* cDNA at medium stringency. The resulting RACE-PCR products that hybridized with the *HvNRT2.1* probe were cloned and sequenced. The sequencing data indicated that one of the RACE-PCR products was another member of the *HvNRT2* gene family in barley. This fragment is designated 3′HvNRT2.4. An oligonucleotide specific to the 3′-UTR of this gene was designed and synthesized. 5′-RACE-PCR resulted in the isolation and cloning of *HvNRT2.4* cDNA. This cDNA is 1,705 bp in length.

Protein Structure, Genetic Analysis, Comparison of the Nucleotide, and Protein Sequences

The predicted HvNRT2.3 and HvNRT2.4 proteins are 507 amino acids in length, with molecular masses of 54.6 kD. The pIs of the HvNRT2.3 and HvNRT2.4 proteins are 8.21 and 8.54, respectively. Both predicted proteins contain 12 membrane spanning regions and have the MFS conserved sequence of (D/N) RXGR(R/K) and $IX_2RX_3GX_3G$ (Henderson, 1991; Marger and Saier, 1993). We analyzed the predicted protein sequences of HvNRT2 proteins with PROSITE program (Bairoch et al., 1997) and found possible sites for protein phosphorylation (see asterisks, Fig. 1). HvNRT2.3 and HvNRT2.4 proteins have three possible protein kinase C phosphorylation sites (Woodgett et al., 1986; Kikkawa et al., 1988) at positions 28 to 30, 381 to 383, and 484 to 486, with residue compositions of SFR, SRR, and SER, respectively. HvNRT2.3 and HvNRT2.4 proteins also have three casein kinase II sites (Pinna, 1990) at positions 453 to 456, 463 to 466, and 482 to 485, with residue compositions of TEEE, SEEE, and SRSE, respectively. The phosphorylation sites are all on the predicted cytoplasmic face of the NRT2 proteins. These phosphorylation sites were also present in HvNRT2.1. The predicted localization of HvNRT2.3 and HvNRT2.4 proteins using the PSORT program (Nakai and Kanehisa, 1992) was the plasma membrane.

We analyzed and compared the protein and nucleotide sequences of the four known members of the *HvNRT2* multigene family, and compared them with each other and with other NO_3^- transporters. Figure 1 shows the alignment of the predicted protein sequences, while Figure 2 shows the phylogenetic relationships of the four predicted HvNRT2 proteins compared with the NO_3^- transporters NpNRT2.1, AtNRT2.1, CrNRT2.1, GmNRT2, YNT1, and CRNA. The amino acid sequences of the predicted proteins of the HvNRT2 family shows >87% identity, with HvNRT2.2 being the most divergent. The highest level of sequence divergence was observed at 5′- and 3′-UTRs of all four cDNAs. In comparing the predicted protein sequence of *HvNRT2* genes from barley with the other plant *NRT2* genes, we found a stretch of 21 amino acids near the amino terminus of the NRT2 proteins (*GmNRT2*, *AtNRT2.1*, and *NpNRT2.1*) that was not present in the HvNRT2 proteins. Analysis of this sequence predicts a possible protein kinase C phosphorylation site (TGR), and/or a casein kinase II phosphorylation site (TGRE) (Woodgett et al., 1986; Kikkawa et al., 1988; Pinna, 1990).

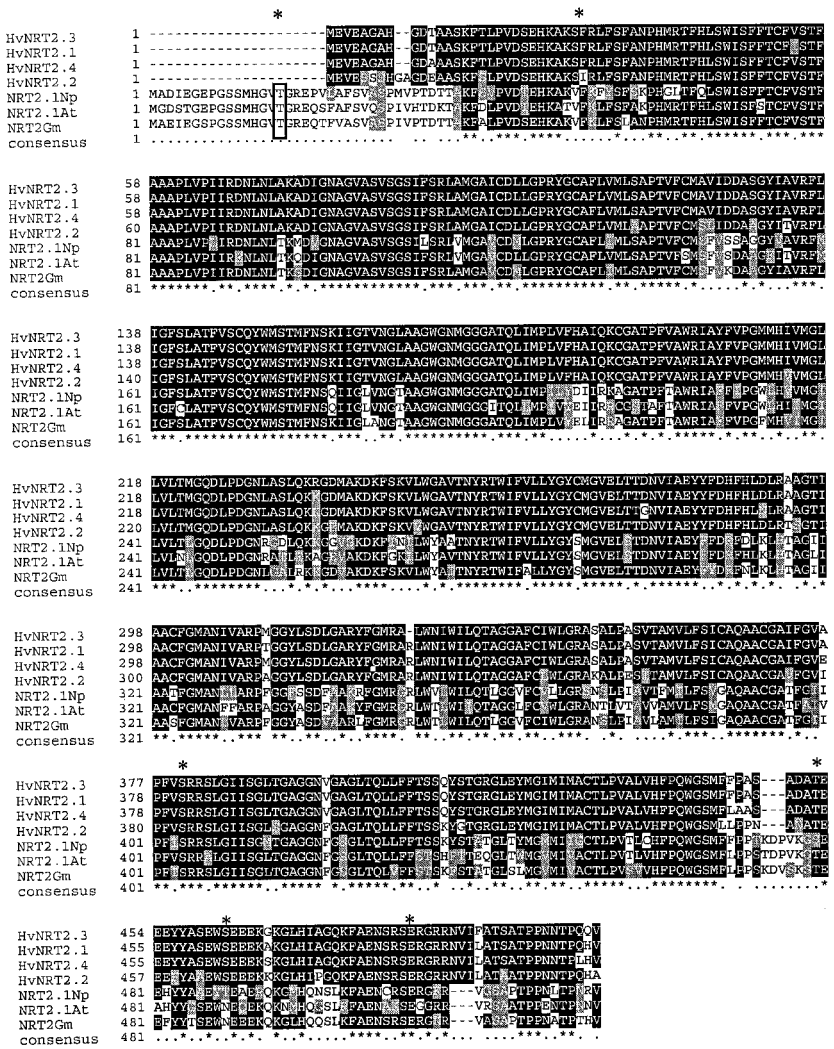

Figure 1. Predicted amino acid sequence of *HvNRT2.3* (accession no. AF091115) and *HvNRT2.4* (accession no. AF091116) with alignment of eight full-length sequences representing the following other inducible high-affinity NO_3^- transporters: *HvNRT2.1* (accession no. U34198), *HvNRT2.2* (accession no. U34290), *NpNRT2.1* (accession no. Y08210), *AtNRT2.1* (accession no. Z97058), *GmNRT2.1* (accession no. AF047718), *CRNA* (accession no. U34382), *YNT1* (accession no. Z69783), and *CrNRT2.1* (accession no. Z25438). The alignment was made using Multialign (Smith et al., 1996; BCM launcher, Baylor College of Medicine, Houston). Possible sites for protein phosphorylation are indicated by asterisks.

Isolation, Analysis, and Comparison of 5′ Upstream Regions for *HvNRT2.1*, *HvNRT2.2*, and *HvNRT2.3*

We isolated the 5′ upstream region of *HvNRT2.1* (accession no. AF189727), *HvNRT2.2* (accession no. AF189728), and *HvNRT2.3* (accession no. AF189729) (DNA fragments 535, 635, and 1,436 bp in length, respectively) by the use of a DNA walking kit. The TATA boxes were located at −48, −37, and −45 for *HvNRT2.1*, *HvNRT2.2*, and *HvNRT2.3*, respectively. In comparing the promoter sequences of *HvNRT2.1*, *HvNRT2.2*, and *HvNRT2.3*, we found that *HvNRT2.1* had 65% homology with *HvNRT2.3* and 58% homology with *HvNRT2.2*. By comparison, *HvNRT2.2* had 53.2% homology with *HvNRT2.3*. We found one stretch of DNA present in the promoter sequence of *HvNRT2.1*, *HvNRT2.2*, and *HvNRT2.3*, which was highly homologous, (domain I) 16/19 (84.2% identity) with the consensus sequence TGATTCCGTNNGNTGCAAT. If we specifically compared the areas adjacent to domain I of *HvNRT2.1* and *HvNRT2.3*, this domain increased both in DNA size and homology 29/33 (87.8% homology). We also found another stretch of DNA, domain II, with 62/69 identical nucleotides (89.8% homology). In Arabidopsis a putative cis-acting NO_3^--inducible element (NIE) containing a core sequence (A[G/C] TCA) preceded by an AT-rich region is considered to be involved in the induction of the NO_3^- reductase genes by NO_3^- (Hwang et al., 1997). In barley the *HvNRT2* promoters were found to contain this core sequence. *HvNRT2.1* has one copy of the core sequence at −430, *HvNRT2.2* has two copies at −120 and −299, and *HvNRT2.3* has four copies at −684, −764, −994, and −1,267 from the transcription start site.

Time Profile of NO_3^--Induced mRNA of the *HvNRT2* Multigene Family and $^{13}NO_3^-$ Influx

The effects of two NO_3^- concentrations (1 and 10 mM) on the expression of *HvNRT2* multigene family and NO_3^- influx was investigated for various pretreatment times (0–48 h). Northern-blot analysis using an internal fragment of *HvNRT2.3* (which was able to recognize all members of the *HvNRT2* family), showed that 1 mM NO_3^- induced the accumulation of *HvNRT2* transcript in roots to their highest

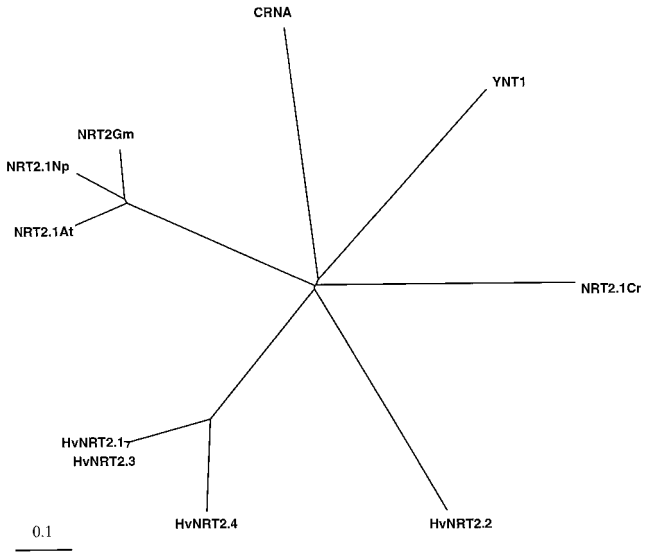

Figure 2. Phylogeny of predicted amino acid sequence of inducible high-affinity NO_3^- transporters. The phylogeny was obtained with the PileUp program (Genetics Computer Group) with *HvNRT2.1*, *HvNRT2.2*, *HvNRT2.3*, *HvNRT2.4*, *AtNRT2.1*, *NpNRT2.1*, *GmNRT2.1*, *CRNA*, *YNT1*, and *CrNRT2.1*.

level within 3 h of treatment. Thereafter, transcript levels steadily decreased to undetectable levels by 24 h (Fig. 3a). The same overall pattern was observed for the 10 mM NO_3^- treatment. *HvNRT2* mRNA accumulation peaked at 6 h, and then decreased to undetectable levels by 24 h (Fig. 3b). In short-term experiments in which plants were supplied with 10 mM NO_3^-, *HvNRT2* transcript accumulation was observed within 30 min of the onset of NO_3^- treatment (data not shown). In parallel experiments, using 50 μM external NO_3^- to measure high-affinity NO_3^- influx (Fig. 3c), influx increased 20-fold from the onset of 1 mM NO_3^- pretreatment to a maximum value at 9 h, then decreased. Nevertheless, even at 48 h, influx remained relatively high, despite the fact that transcript abundance had decreased to undetectable levels. This may indicate the participation of other transport systems in the measured influx. The pattern of response to pretreatment with 10 mM NO_3^- was essentially similar to the 1 mM pretreatment (Fig. 3c).

To investigate the expression of each of the known *HvNRT2* cDNAs, oligonucleotide probes directed to the 3′-UTR were designed and used in northern-blot analysis. Figure 4 shows the accumulation of specific *HvNRT2* mRNAs in nitrogen-starved plants following exposure to 1 mM NO_3^- for 0, 3, 6, 9, 12, 24, and 48 h. Accumulation of *HvNRT2.1*, *HvNRT2.2*, and *HvNRT2.3* transcripts in roots peaked at 3 to 6 h, then declined to undetectable levels by 12 to 24 h. By contrast, *HvNRT2.4* transcript levels, which increased within 3 h of NO_3^- feeding, remained at elevated levels for the duration of the experiment (48 h). To visualize transcript abundance of each of the different *HvNRT2* homologs, x-ray film was exposed to *HvNRT2* northern blots for the following times: *HvNRT2.1*, 12 h; *HvNRT2.2*, 48 h; *HvNRT2.3*, 16 h; and *HvNRT2.4*, 96 h.

Effect of Various External NO_3^- Concentrations on *HvNRT2* Transcript Levels and NO_3^- Influx

To investigate the effect of external NO_3^- concentrations on *HvNRT2* transcript levels and NO_3^- influx under steady-state culture conditions, barley plants were grown in hydroponic solutions containing 0, 10, 50, 100, and 500 μM NO_3^- for 4 d. Both *HvNRT2* transcript levels (northern-blot analysis, Fig. 5a) and $^{13}NO_3^-$ influx from 50 μM NO_3^- (Fig. 5b) were subsequently monitored in roots of these plants. NO_3^- influx varied from 0.54 μmol g^{-1} fresh weight h^{-1} in plants maintained at 0 external NO_3^- to its maximum rate (5.32 μmol g^{-1} fresh weight h^{-1}) for plants maintained at 50 μM NO_3^-. At higher NO_3^- concentrations, influx decreased to 3.15 μmol g^{-1} fresh weight h^{-1} in plants maintained at the 500 μM level. The abundance of *HvNRT2* transcript followed the same pattern.

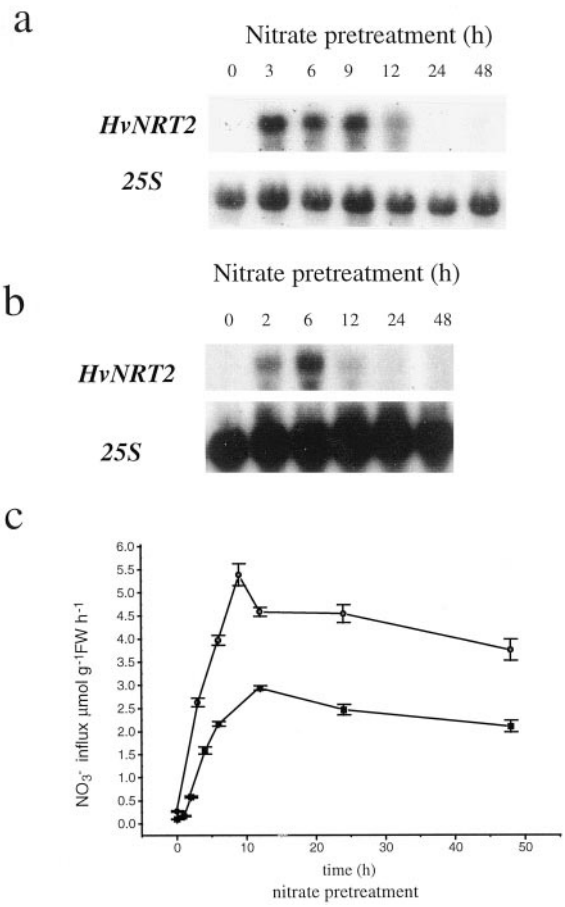

Figure 3. Time course effects of NO_3^- pretreatment on root *HvNRT2* transcript abundance and $^{13}NO_3^-$ influx. a, Northern blot from roots of plants exposed to 1 mM KNO_3 for the times shown. b, Northern blot from roots of plants exposed to 10 mM KNO_3 for the times shown. Twenty micrograms of total RNA was introduced into each lane and washed at medium stringency. Northern blots were probed with the *25S* ribosomal subunit to ensure equal loading of RNA. c, $^{13}NO_3^-$ influx values measured at 50 μM NO_3^- for plants pretreated with 1 (●) or 10 (■) mM NO_3^- for the times shown.

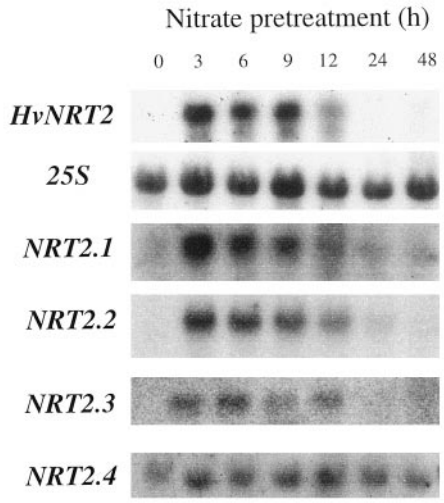

Figure 4. Northern-blot analysis of members of the *HvNRT2* family of genes. Time course effects of pretreating nitrogen-starved plants with 1 mM NO_3^- for 0, 3, 6, 9, 12, 24, and 48 h. Twenty micrograms of total RNA was introduced into each lane, probed with *HvNRT2.3* internal fragment (which recognizes all known *HvNRT2* homologs), and then washed at medium stringency (panel 1). Northern blots were probed with the *25S* ribosomal subunit to ensure equal loading of RNA. Northern blots were also probed with specific oligonucleotides directed to *HvNRT2.1*, *HvNRT2.2*, *HvNRT2.3*, and *HvNRT2.4* transcripts, and washed at high stringency.

Effect of NO_2^- and NH_4^+ on Transcript Levels of the *HvNRT2* Multigene Family

Figure 6 shows the effect of simultaneously providing 10 mM NH_4^+ on the accumulation of *HvNRT2* transcript during induction of NO_3^- transport by 10 mM NO_3^-. Two hours after providing NH_4^+ and NO_3^- together, no effect on *HvNRT2* transcript accumulation was apparent, compared with plants supplied with NO_3^- only. However, by 4 and 6 h, respectively, transcript abundance decreased dramatically. In parallel experiments, we measured $^{13}NO_3^-$ influx at 50 μM NO_3^- in plants supplied with both NO_3^- and NH_4^+ for 6 h. $^{13}NO_3^-$ influx in these plants and in nitrogen-starved plants remained at low levels.

The capacity of exogenously supplied NO_2^- to induce *HvNRT2* transcript accumulation in nitrogen-starved barley seedlings was also investigated (Fig. 6). Supplying 10 mM NO_2^- increased the accumulation of *HvNRT2* transcripts in roots of barley seedlings, but at a slower rate than in the NO_3^- treatment.

DISCUSSION

Characterization of *HvNRT2* cDNAs and 5′ Upstream Regions

In this study, reverse transcriptase-PCR and RACE-PCR were employed for the isolation of two new putative high-affinity NO_3^- transporter genes (*HvNRT2.3* and *HvNRT2.4*) from barley. The coding regions of these genes are highly conserved with respect to each other and to *HvNRT2.1* and *HvNRT2.2* (Trueman et al., 1996), with greater than 87% homology at the protein level. Both *HvNRT2.3* and *HvNRT2.4* have the conserved motif of the major facilitator superfamily, and, possibly, protein kinase C and casein kinase II phosphorylation sites. The location and number of sites are constant for all of the known NRT2 proteins, and the sites appear to be located on the cytoplasmic face of each protein. The major difference between the NRT2 proteins from barley and those from *N. plumbaginifolia*, Arabidopsis, and soybean is a deletion of 21 amino acids in the NRT2 proteins at the amino terminus. In this deletion are located protein kinase C and casein kinase II phosphorylation sites. This may indicate that there are differences in post-translation modification of the barley NRT2 proteins. The role of these phosphorylation sites was not assessed in this study, but it is interesting that protein kinase C and casein kinase II sites were present in barley NRT2 proteins, as well as in the N terminus (21 amino acid sequence) of the other NRT2 proteins. In barley, Southern-blot analysis demonstrated that there are seven to 10 *NRT2* homologs (Trueman et al., 1996), while in *N. plumbaginifolia*, soybean, and Arabidopsis, there appear to be only two

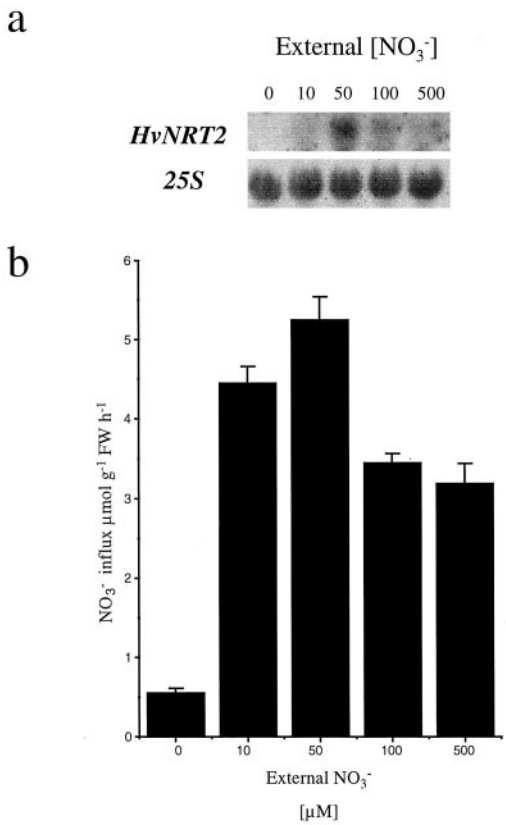

Figure 5. Effect of external NO_3^- supply on transcript abundance of *HvNRT2* genes and $^{13}NO_3^-$ influx in roots of barley plants grown on 0, 10, 50, 100, and 500 μM NO_3^-. a, Northern-blot analysis of *HvNRT2* transcript abundance; 20 μg of total RNA was introduced into each lane and washed at medium stringency. To ensure equal loading into the lanes, RNA was probed with the *25S* ribosomal subunit and washed at high stringency. b, NO_3^- influx was measured at 50 μM NO_3^- after 4 d of growth at the NO_3^- concentrations shown.

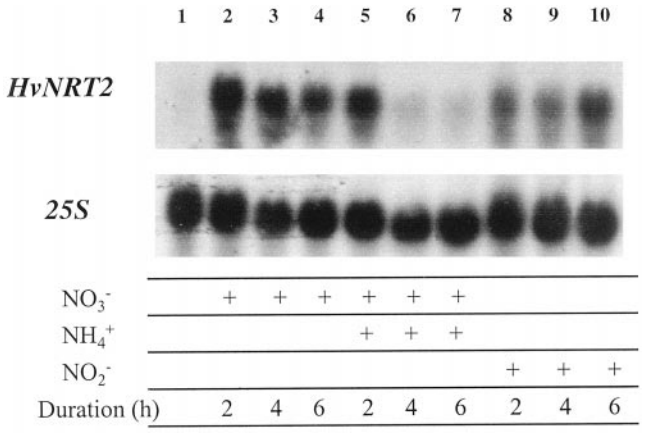

Figure 6. Effects of the duration of NO_3^-, NO_3^- plus NH_4^+, and NO_2^- pretreatment on *HvNRT2* transcript abundance. Lane 1, Plants grown in nitrogen-free medium; lanes 2 through 4, 10 mM NO_3^--supplied plants; lanes 5 through 7, 10 mM NH_4^+ and 10 mM NO_3^--co-supplied plants; and lanes 8 through 10, 10 mM NO_2^--supplied plants for times shown. Twenty micrograms of total RNA was introduced into each lane and probed with *HvNRT2.3* internal fragment and washed at medium stringency. Northern blot was probed with the *25S* ribosomal subunit to ensure equal loading of RNA.

copies of the gene (Quesada et al., 1997; Amarashinghe et al., 1998; Zhuo et al., 1999). Why barley should possess seven to 10 copies of this gene family is unknown.

Analysis of the 5′ upstream region of *HvNRT2.1*, *HvNRT2.2*, and *HvNRT2.3* revealed that these sequences are less conserved (>53% homology). One region of DNA (domain I) was conserved in the promoter sequences of *HvNRT2.1*, *HvNRT2.2*, and *HvNRT2.3*. Also present was domain II, a region of DNA comprising 69 bp with a homology of 89%. The roles of these sequences are unknown, but they may function in the regulation of the *NRT2* genes. The NIE core elements, demonstrated to be present in variable copy number in the *HvNRT2* promoter (see "Results" section), may participate in NO_3^- induction of these genes (Hwang et al., 1997). Unlike the core sequences of Arabidopsis, the barley *HvNRT2* and NR (*NAR1* and *NAR7*) promoters are not preceded by an AT-rich region.

Time Profile of *HvNRT2* Transcript Levels and NO_3^- Transport

The substantial increase of high-affinity NO_3^- uptake following first exposure to NO_3^- has been referred to as NO_3^- induction (Jackson et al., 1973; Goyal and Huffaker, 1986). This process typically increases rates of net NO_3^- uptake several-fold (Warner and Huffaker, 1989). Using the *Klondike* barley and $^{13}NO_3^-$ to measure plasma membrane influx, Siddiqi et al. (1989) demonstrated a 28-fold increase. It is evident that the increase of influx associated with induction is governed by the constitutive value of influx associated with CHATS activity and the extent of the IHATS flux. In *Steptoe* barley, the CHATS activity was already substantially higher than in *Klondike*, and therefore the increase of influx associated with induction was substantially less in *Steptoe* than in *Klondike* (King et al., 1993). In the present experiments using 1 mM or 10 mM NO_3^- treatments, both influx and *HvNRT2* transcript levels increased with the same time dependence. Peak activities of both transcript and influx occurred during the first 6 to 12 h, after which they declined. Plants pretreated at 10 mM NO_3^- had lower values of influx than those treated with 1 mM NO_3^-, and this probably reflects a greater down-regulation by larger internal nitrogen pools in the 10 mM NO_3^--treated plants. This decline of *HvNRT2* transcript has been shown for other cDNAs encoding putative NO_3^- transporters when plants were pretreated with NO_3^- for >12 h as in *N. plumbaginifolia* (Quesada et al., 1997) and Arabidopsis (Zhuo et al., 1999).

The investigation of the effects of NO_3^- treatment on individual members of the *HvNRT2* family of genes revealed that these genes are coordinately induced in root tissue with approximately the same pattern in time as observed using a single *HvNRT2* (*HvNRT2.3*) probe, which recognized all members of this family. Nevertheless, the transcript abundances varied among the *HvNRT2* homologs in the following order: *HvNRT2.1* = *HvNRT2.3* > *HvNRT2.2* > *HvNRT2.4*. This result does not preclude differential expression by different cell types of the root (e.g. endodermis, stele, root hairs, etc.). In tomato, expression of the *LeNRT1.1* gene, which encodes a low-affinity NO_3^- transporter, is predominately localized in the root cylinder, while *LeNRT1.2* is predominately localized in root hairs (Lauter et al., 1996). *NpNRT2.1* has also been shown to be highly expressed in epidermal and endodermal cells at the root tip, and in lateral root primordia and epidermis of mature roots (Krapp et al., 1998). Likewise, there was a differential expression of the *AtNRT1* gene in Arabidopsis, the gene being expressed in outer layers of the root tip and in progressively deeper layers with increasing distance from the root tip (Huang et al., 1996). Clearly, a significant question remains as to the functional roles of the multiple representatives of the *HvNRT2* family of genes.

The long-term effect of exposure to external NO_3^- (0, 10, 50, 100, or 500 μM) prior to influx measurements revealed that both *HvNRT2* transcript levels and influx were highest in plants grown with 50 μM NO_3^- (Fig. 5). The reported K_m of IHATS, 25 to 100 μM, varies with genotype and as a function of NO_3^- pretreatment (Siddiqi et al., 1990). The present correlation between *NRT2* transcript levels and influx values confirms earlier observations to this effect in Arabidopsis (Zhuo et al., 1999) and provides further evidence that the *NRT2* genes encode components of the inducible high-affinity transport systems for NO_3^- influx.

Effects of External NO_2^- or NH_4^+ on NO_3^- Influx and *HvNRT2* Transcript Accumulation

To investigate whether nitrogen signals other than NO_3^- may be involved in the induction of the *HvNRT2* genes, NO_2^- was supplied in place of NO_3^- during the standard induction treatment. Furthermore, to determine whether NH_4^+ might impact upon the induction process, 10 mM NH_4^+ was provided together with 10 mM NO_3^- in separate experiments (Fig. 6). Although NO_2^- is rarely present in

soil solution, under laboratory conditions it is able to induce both NO_2^- and NO_3^- uptake (Siddiqi et al., 1992; King et al., 1993; Aslam et al., 1996), albeit at a slower rate. NO_2^- at 10 mM increased *HvNRT2* transcript levels within 2 h of treatment (Fig. 6). It may be that the slower response to NO_2^- is due to a slower transduction of the NO_2^- signal or to lower rates of NO_2^- uptake. Alternatively, perhaps NO_2^- was responsible for toxic effects at the concentration provided.

When NH_4^+ was provided together with NO_3^-, *HvNRT2* transcript accumulation was unaffected for the first 2 h of the treatment, but by 4 to 6 h, there was virtually no transcript present. It is thought that NH_4^+ may: (a) inhibit NO_3^- influx directly at the transport step through effects at the plasma membrane (Glass et al., 1985; Lee and Drew 1989; King et al., 1993; Aslam et al., 1996), and (b) function as a signal resulting in repression of IHATS transcription either through effects of NH_4^+ itself or after its conversion to amino acids. Earlier physiological studies suggested that NH_4^+ effects on NO_3^- uptake resulted from downstream assimilation products of NH_4^+ (Breteler and Siegerist, 1984; Lee and Drew, 1989; Muller and Touraine, 1992). Strong support for this hypothesis comes from treatments with Met sulfoximine, which have resulted in a release from the inhibitory effects of exogenous NH_4^+ (Breteler and Siegerist, 1984; Lee and Drew, 1989). However, other studies have demonstrated a failure of Met sulfoximine to relieve the inhibitory effects of NH_4^+ during application of Met sulfoximine (King et al., 1993; Aslam et al., 1996). In the present experiments high levels of NO_3^- pretreatment (10 mM) were employed to ensure that NO_3^- would enter the root by both the high- and low-affinity transport systems, notwithstanding an inhibitory effect of NH_4^+ on high-affinity NO_3^- uptake. This would distinguish between low levels of induction of NO_3^- influx arising from inadequate internal [NO_3^-] and low levels of induction resulting from down-regulation of *HvNRT2* expression by NH_4^+ or its assimilation products.

The present results (Fig. 6) appear to show that induction by 10 mM NO_3^- proceeded normally in the first 2 h of NH_4^+ treatment despite the presence of exogenous NH_4^+. It may be that there is insufficient buildup of cytosolic NH_4^+ or products of its assimilation to cause any negative effects on induction in the first hours of exposure to NH_4^+. In a study of *AMT1* expression in Arabidopsis, 9 h of exposure to 5 mM NH_4NO_3 were required before *AMT1* expression was reduced to approximately 20% of its original value (Rawat et al., 1999). By this time root Gln concentrations had increased approximately 5-fold, and this nitrogen fraction was responsible for the largest change of root nitrogen composition under these conditions. Likewise, in experiments examining the expression of *AtNRT2.1* in roots of Arabidopsis, Zhuo et al. (1999) demonstrated that transcript abundance was reduced to levels corresponding to those of uninduced plants after 3 h of azaserine treatment. Azaserine blocks the activity of Glu synthase, reducing Glu and increasing Gln concentrations. This observation is consistent with a potent effect of Gln on *AtNRT2.1* transcript abundance. However, the same study revealed that treatments with Met sulfoximine reduced transcript abundance to approximately 10% of control (induced) plants, indicating that NH_4^+ itself may also act with negative effects at the level of transcription.

In summary, transcripts of members of the *HvNRT2* gene family are induced by both NO_3^- and NO_2^-. The promoter sequence contains a core NIE domain, which has been implicated in the induction pathway of NR genes in Arabidopsis. Also present in the promoter sequences of *HvNRT2.1*, *HvNRT2.2*, and *HvNRT2.3* genes was a common domain I whose function is presently unknown. The presence of NH_4^+ during the first hours of induction appears not to affect *HvNRT2* transcript accumulation, suggesting that cytosolic [NH_4^+] may need to build to a sufficiently high level (by 4–6 h) before transcript abundance is affected. Alternatively, the result may indicate that downstream metabolites such as Gln are more important regulators of *NRT2* expression. Transcript levels of all known members of the *HvNRT2* gene family (*HvNRT2.1*, *HvNRT2.2*, *HvNRT2.3*, and *HvNRT2.4*) at first increased following provision of NO_3^-, then, as is the case for NO_3^- influx, down-regulation occurred (with the exception of *HvNRT2.4*, which remained constant for the duration of the experiment). The strong correlation between patterns of *HvNRT2* expression and high-affinity NO_3^- influx provides further support for the identification of the *HvNRT2* genes as participants in high-affinity NO_3^- influx in barley roots.

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