Functional Assessment of the *Medicago truncatula* NIP/LATD Protein Demonstrates That It Is a High-Affinity Nitrate Transporter

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The *Medicago truncatula* NIP/LATD (for Numerous Infections and Polyphenolics/Lateral root-organ Defective) gene encodes a protein found in a clade of nitrate transporters within the large NRT1(PTR) family that also encodes transporters of dipeptides and tripeptides, dicarboxylates, auxin, and abscisic acid. Of the NRT1(PTR) members known to transport nitrate, most are low-affinity transporters. Here, we show that *M. truncatula nip/latd* mutants are more defective in their lateral root responses to nitrate provided at low (250 μM) concentrations than at higher (5 mM) concentrations; however, nitrate uptake experiments showed no discernible differences in uptake in the mutants. Heterologous expression experiments showed that MtNIP/LATD encodes a nitrate transporter: expression in *Xenopus laevis* oocytes conferred upon the oocytes the ability to take up nitrate from the medium with high affinity, and expression of MtNIP/LATD in an Arabidopsis *chl1(nrt1.1)* mutant rescued the chlorate susceptibility phenotype. *X. laevis* oocytes expressing mutant Mtnip-1 and Mtlatd were unable to take up nitrate from the medium, but oocytes expressing the less severe Mtnip-3 allele were proficient in nitrate transport. *M. truncatula nip/latd* mutants have pleiotropic defects in nodulation and root architecture. Expression of the Arabidopsis NRT1.1 gene in mutant Mtnip-1 roots partially rescued Mtnip-1 for root architecture defects but not for nodulation defects. This suggests that the spectrum of activities inherent in AtNRT1.1 is different from that possessed by MtNIP/LATD, but it could also reflect stability differences of each protein in *M. truncatula*. Collectively, the data show that MtNIP/LATD is a high-affinity nitrate transporter and suggest that it could have another function.

All plants require nitrogen (N) as an essential nutrient and are able to acquire N from nitrate (NO$_3^-$) and ammonium (NH$_4^+$) in the soil. Nitrate acquisition begins with its transport into root cells, accomplished by NO$_3^-$ transporters. Soil NO$_3^-$ concentrations can vary by 5 orders of magnitude (Crawford, 1995), and to cope with the variability, plants have evolved both high-affinity (HATS) and low-affinity (LATS) transport systems. These are encoded by two gene families: the phylogenetically distinct NRT1(PTR) and NRT2 families. Members of these families also participate in the movement of NO$_3^-$ throughout the plant and within plant cells (Miller et al., 2007; Segonzac et al., 2007; Tsay et al., 2007; Almagro et al., 2008; Lin et al., 2008; Fan et al., 2009; Li et al., 2010; Barbier-Brygoo et al., 2011; Wang and Tsay, 2011; Xu et al., 2012). Proteins in the Chloride Channel (CLC) transporter family also transport NO$_3^-$; these transporters are associated with cytosol-to-organelle NO$_3^-$ movement (Zifarelli and Pusch, 2010).

NRT1(PTR) is a large family of transporters, comprising 53 members in Arabidopsis (*Arabidopsis thaliana*), 84 members in rice (*Oryza sativa*), with NRT1 (PTR) members known in several other species (Tsay et al., 2007; Zhao et al., 2010). In addition to transporting NO$_3^-$ coupled to H$^+$ movement, members of the NRT1(PTR) family have been found to transport dipeptides or tripeptides, amino acids (Waterworth and Bray, 2006), dicarboxylic acids (Jeong et al., 2004), auxin (Krouk et al., 2010b), and/or abscisic acid (Kanno et al., 2012). Only a small number of NRT1 (PTR) proteins have been functionally studied compared with the large number that exist in higher plants; thus, the number of biochemical functions ascribed to this family may expand.

Of the NRT1(PTR) members known to transport NO$_3^-$, most are LATS transporters. An important exception is Arabidopsis NRT1.1(CHL1), a dual-affinity transporter that is the most extensively studied NRT1 (PTR) protein. AtNRT1.1(CHL1) was identified initially on the basis of its ability to confer chlorate toxicity.

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1 This work was supported by the National Science Foundation (grant nos. IOS-0923756 to R.D. and IOS-0923668 to D.J.S.).
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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Rebecca Dickstein (beccad@unt.edu).

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www.plantphysiol.org/cgi/doi/10.1104/pp.112.196444

906 *Plant Physiology*, October 2012, Vol. 160, pp. 906–916, www.plantphysiol.org © 2012 American Society of Plant Biologists. All Rights Reserved.
resistance and was the first of this family to be cloned (Doddema et al., 1978; Tsay et al., 1993). AtNRT1.1 (CHL1) is an essential component of NO$_3^-$ transport and NO$_3^-$ signaling pathways, with important roles regulating the expression of other NO$_3^-$ transporters and root architecture (Remans et al., 2006; Walch-Liu et al., 2006; Walch-Liu and Forde, 2008). Its expression is inducible by NO$_3^-$ (Huang et al., 1996). Reversible phosphorylation is essential to its ability to switch between LATS and HATS activity (Liu and Tsay, 2003). In addition, AtNRT1.1(CHL1) acts as a NO$_3^-$ sensor (Muños et al., 2004; Ho et al., 2009; Wang et al., 2009) and has been shown to transport auxin in a NO$_3^-$ concentration-dependent manner (Krouk et al., 2010b). It has been suggested that AtNRT1.1’s ability to transport auxin may be part of its NO$_3^-$-sensing mechanism (Krouk et al., 2010a, 2010b; Gojon et al., 2011).

Most legumes and actinorhizal plants have the additional ability to form symbiotic N-fixing root nodules with soil bacteria, enabling them to thrive in NO$_3^-$- and NH$_4^+$-depleted environments. Legume nodule formation commences with signal exchange between the plant and rhizobia, followed by root cortical cell divisions, invasion of the root by rhizobia inside plant-derived infection threads, and subsequent endocytosis of rhizobia into newly divided plant host cells, forming symbiosomes. Within symbiosomes, the rhizobia differentiate into bacteroids that are capable of N fixation. The recent availability of three sequenced legume genomes will add to our knowledge of this important gene family (Sato et al., 2008; Schmutz et al., 2010). Benedito et al. (2010) recognized 111 non-redundant Medicago truncatula sequences corresponding to genes in the 2.A.17 transporter class, containing NRT1(PTR) genes (Benedito et al., 2010). In Lotus japonicus, 37 members of the NRT1(PTR) family were identified by an in silico search (Crisculo et al., 2012). The recent availability of three sequenced legume genomes will add to our knowledge of this important gene family (Sato et al., 2008; Schmutz et al., 2010; Young et al., 2011). In faba bean (Vicia faba), two NRT1(PTR) transporters have been studied; one was demonstrated to transport dipeptides in yeast, while the second was found to be phylogenetically close to a soybean NRT1(PTR) (Miranda et al., 2003). In alder (Alnus glutinosa) nodules, the NRT1(PTR) transporter AgDCAT localizes to the symbiotic interface and transports dicarboxylic acid from the cytosol toward its symbiotic partner, Frankia spp. (Jeong et al., 2004). The M. truncatula MtNRT1.3 transporter was shown to be a dual-affinity NO$_3^-$ transporter; MtNRT1.3 is up-regulated by the absence of NO$_3^-$ (Morère-Le Paven et al., 2011).

The M. truncatula NIP/LATD (for Numerous Infections and Polyphenolics/Lateral root-organ Defective) gene encodes a predicted NRT1(PTR) transporter (Harris and Dickstein, 2010; Yendrek et al., 2010), and the three known nip and latd mutants have pleiotropic defects in nodulation and root architecture. Mtnip-1, containing a missense (A497V) mutation in one of the NIP/LATD protein’s transmembrane domains, is well characterized with respect to nodulation phenotypes (Veereshlingam et al., 2004). Mtnip-1 develops nodules that initiate rhizobial invasion but fail to release rhizobia from infection threads. Its nodules lack meristems and accumulate polyphenolics, a sign of host defense. Mtnip-1 plants also have defective root architecture (Veereshlingam et al., 2004). The Mltad mutant has the most severe phenotype, caused by a stop codon (W341STOP) in the middle of the NIP/LATD putative protein (Yendrek et al., 2010). Mltad has serious defects in root architecture, with a nonpersistent primary root meristem, lateral roots (LRs) that fail to make the transition from LR primordia to LRs containing a meristem, and defects in root hairs. Mltad also has defective nodules (Bright et al., 2005). The missense Mtnip-3 mutant (E171K) is the least affected, forming invaded nodules with meristems and polyphenol accumulation. Mtnip-3 has nearly normal root architecture (Teillet et al., 2008; Yendrek et al., 2010).

Here, the function of the MtNIP/LATD protein is investigated. We find that MtNIP/LATD is a high-affinity NO$_3^-$ transporter and provide evidence suggesting that it may have an additional biochemical function.

RESULTS

Mtnip Mutants’ Nitrate Phenotypes

Because of MtNIP/LATD’s similarity to low-affinity NO$_3^-$ transporters in the NRT1(PTR) family (Yendrek et al., 2010), one hypothesis is that it may be a low-affinity NO$_3^-$ transporter. Bioavailable N is known to suppress nodulation and to inhibit N fixation in mature N-fixing nodules (Streeter, 1988; Fei and Vessey, 2009). It is also possible that Mtnip/latd mutants would develop functional root nodules in conditions of NO$_3^-$ insufficiency. To test whether Mtnip-1 was altered in the suppression of nodulation by bioavailable N, Mtnip-1 mutant plants were cultivated in 1 and 10 mM KNO$_3$ and also in 5 mM NH$_4$NO$_3$ in the presence of Sino-rhizobium melliloti. As shown in Table I, Mtnip-1 formed low numbers of nodules under these conditions compared with no-N conditions, similar to the wild type (A17), suggesting normal suppression by bioavailable N sources. The few nodules that formed in Mtnip-1
Table 1. Nodulation in the absence and presence of N sources

In each experiment, wild-type A17 and Mtnip-1 plants were grown in the same aeroponic chamber in the given N regime, as described in “Materials and Methods.” For each experiment, seven to 11 plants of each genotype were evaluated. Nodules were counted at 15 dpi. Data are presented as the mean number of nodules per plant ± se.

| Experiment | N Conditions | A17 Nodule No. | Mtnip-1 Nodule No. |
|------------|--------------|----------------|-------------------|
| 1          | No NO3       | 31.9 ± 3.2     | 20.8 ± 1.6        |
| 2          | No NO3       | 19.9 ± 4.1     | 13.25 ± 0.8       |
| 3          | 1 mm KNO3    | 7.8 ± 0.7      | 2.2 ± 0.4         |
| 4          | 1 mm KNO3    | 5.3 ± 0.6      | 1.1 ± 0.3         |
| 5          | 5 mm NH4NO3  | 1.7 ± 0.6      | 0.7 ± 0.2         |
| 6          | 5 mm NH4NO3  | 5.7 ± 0.6      | 2.8 ± 0.5         |
| 7          | 10 mm KNO3   | 3.4 ± 0.8      | 0.6 ± 0.3         |
| 8          | 10 mm KNO3   | 4.0 ± 1.0      | 0.7 ± 0.4         |

1 mm KNO3, 10 mm KNO3, or 5 mM NO3·NH4 had an Mtnip-1 nodule phenotype.

To examine whether the mutants had defects in NO3 uptake, we grew Mtnip-1 and Mtnip-3 mutants, with wild-type A17 as a control, in two different concentrations of KNO3: 250 µM and 5 mM. Uptake was measured by monitoring the depletion of NO3 from the medium. As can be seen in Figure 1, no differences in NO3 uptake were observed in the mutants, suggesting that NO3 LATS and HATS are functioning in these plants. However, because measurement of the depletion of NO3 from the medium is less sensitive than measuring NO3 influx, subtle changes in NO3 uptake may not have been detected in this experimental system.

In Arabidopsis, growth of plants in different NO3 concentrations is known to affect root architecture (Zhang and Forde, 2000; Linkohr et al., 2002). To determine if M. truncatula nip mutants’ LR phenotype was affected by NO3, we grew Mtnip-1, Mtnip-3, and A17 in the presence of 0 µM, 250 µM, and 5 mM KNO3 and examined root growth parameters after 2 weeks. Wild-type A17 produced longer LRs in both NO3 concentrations tested than it did at 0 NO3 and slightly shorter LRs in 5 mM as compared with 250 µM KNO3 (Fig. 2). LR lengths of both Mtnip-1 and Mtnip-3 were significantly shorter than those of the wild type in all conditions tested, with one exception; in 5 mm KNO3, the average LR lengths of Mtnip-3 were similar to those of A17. Both Mtnip-1 and Mtnip-3 mutants had longer LRs when grown in 5 mm KNO3 (Fig. 2C) than they did in 250 µM KNO3 (Fig. 2B) and when grown in the absence of NO3 (Fig. 2A). These data are consistent with an MtNIP/LATD function in high-affinity, low-concentration NO3 uptake or response.

MtNIP/LATD Protein Transports Nitrate, But Not His, in Xenopus laevis Oocytes

To test whether the MtNIP/LATD protein transports NO3, we expressed MtNIP/LATD in X. laevis oocytes and assayed them for the acquisition of NO3 transport activity. Transport activity was initially assessed at two different NO3 concentrations to categorize transporter affinity and at pH 5.5 and 7.4 to test pH dependence. As shown in Figure 3, A and B, oocytes expressing MtNIP/LATD were capable of significant NO3 uptake above the water-injected control oocytes at both low (250 µM) and high (5 mM) NO3 concentrations at pH 5.5. However, at pH 7.4, there was no significant NO3 uptake (Fig. 3, C and D), indicating that transport is H+ coupled. We compared MtNIP/LATD NO3 transport with that of the dual-affinity AtNRT1.1 transporter and found that MtNIP/LATD had slightly lower nitrate transport than AtNRT1.1 at 250 µM NO3. At 5 mM NO3, MtNIP/LATD transported approximately 20% as much nitrate as did AtNRT1.1 (Supplemental Fig. S1), suggesting that MtNIP/LATD is not dual affinity. To determine MtNIP/LATD’s Km, we measured NO3 uptake in MtNIP/LATD-injected oocytes compared with water-injected control oocytes over NO3 concentrations ranging from 50 µM to 10 mM. MtNIP/LATD displays saturable kinetics for NO3 uptake, with a Km of 160 µM (Fig. 3, E and F). The data show only one saturation point (Fig. 3E) and thus indicate that MtNIP/LATD has single, high-affinity NO3 transport.

Because the NRT1(PTR) family BnNRT1.2 NO3 transporter also transports His (Zhou et al., 1998), a rat

Figure 1. Nitrate uptake in M. truncatula. Wild-type A17, Mtnip-1, and Mtnip-3 plants were placed in solutions containing 250 µM (A) or 5 mM (B) nitrate. Nitrate uptake was monitored by its depletion from the medium at 2-h intervals. Data are plotted for one biological replicate ± se.
NRT1(PTR) family member transports both peptides and His (Yamashita et al., 1997), and Arabidopsis AtPTR1 and AtPTR2 peptide transporters also transport His (Tsay et al., 2007), we assessed His uptake in MtNIP/LATD-injected oocytes compared with water-injected control oocytes. At pH 5.5 and 1 mM His, we observed no His transport (Supplemental Table S1).

Proteins Encoded by Two Mtnip/LATD Mutant Alleles But Not a Third Allele Are Defective in Nitrate Transport in the Oocyte System

The finding that MtNIP/LATD transports NO₃⁻ opens the possibility that the defects observed in the Mtnip/latd mutants result from defective NO₃⁻ transport. To determine if the proteins encoded by the available defective MtNIP/LATD genes are capable of NO₃⁻ transport, we tested them in the X. laevis oocyte system. The results demonstrate that the missense Mtnip-1 and truncated Mtlatd proteins are defective in NO₃⁻ transport at 5 mM NO₃⁻, while the Mtnip-3 protein is capable of transport at this higher concentration (Fig. 4). Oocytes expressing Mtnip-3 protein as well as Mtnip-1 protein were assessed for high-affinity transport at 250 μM NO₃⁻; since the Mtlatd protein, encoded by a gene with a nonsense codon in the middle of MtNIP/LATD, failed to transport NO₃⁻ at 5 mM NO₃⁻, it was not tested for transport at 250 μM NO₃⁻. Mtnip-3 was capable of transport at 250 μM, while Mtnip-1 was not. Because the Mtnip-3 mutant has defective root architecture, aberrant nodulation, and fixes far less N than the wild type (Teillet et al., 2008), it may be defective in another function besides NO₃⁻ transport.

MtNIP/LATD Expression in the Arabidopsis chl1-5 Mutant Restores Chlorate Sensitivity

Although MtNIP/LATD transports NO₃⁻ in oocytes, it could be argued that it may not function as a

Figure 2. LR lengths of M. truncatula plants grown in different conditions. Wild-type A17 (black bars), Mtnip-1 (gray bars), and Mtnip-3 (horizontally striped bars) were grown in liquid buffered nodulation medium with no added NO₃⁻ (A), with 250 μM KNO₃ (B), or with 5 mM KNO₃ (C), with the medium changed every other day. LR lengths were measured after 2 weeks. Data are shown for one biological replicate ± se (n = 5). Replicates gave similar results. Asterisks mark LR lengths from plants grown at 250 μM KNO₃ and 5 mM KNO₃ that are significantly different from the same genotype grown at 0 mM KNO₃, using Student’s t test at P < 0.05.

Figure 3. Nitrate uptake in X. laevis oocytes expressing Mtnip/LATD. Oocytes were microinjected with Mtnip/LATD mRNA (black bars, +) or water as a negative control (gray bars, −) incubated for 3 d, and then placed for the indicated times in medium containing 250 μM or 5 mM NO₃⁻ at pH 5.5 or 7.4. The oocytes were rinsed, lysed, and assayed for NO₃⁻ content. A and C, Treatment with 250 μM NO₃⁻; B and D, Treatment with 5 mM NO₃⁻. A and B, pH = 5.5. C and D, pH = 7.4. Data are shown for one biological replicate ± se (n = 3–5 batches of 4–6 oocytes per batch). Asterisks mark NO₃⁻ uptake significantly different from the negative control, using Student’s t test at P < 0.05. Similar results were obtained in more than five repetitions of the experiment. E, Michaelis-Menten plot of oocyte NO₃⁻ uptake. Mtnip/LATD-injected oocytes (squares) or water-injected oocytes (circles) as control were incubated for 3 h in 50 μM to 10 mM NO₃⁻ in batches of five and assayed for NO₃⁻ uptake. Results for two biological replicates are indicated by the black and gray symbols, with error bars showing se. All NO₃⁻ uptake was significantly different from the negative control, using Student’s t test at P < 0.05, except for that at 50 μM. F, Hanes-Woolf plot of averaged NO₃⁻ uptake data, in Mtnip/LATD-injected oocytes minus water-injected oocytes, presented in E. These data were used to calculate the Kₘ of 160 μM.
NO$_3^-$ transporter in planta. The NO$_3^-$ transporter activity of MtNIP/LATD was further tested by studying its ability to complement the well-characterized Arabidopsis chl1-5 mutant, containing a large deletion in the AtNRT1.1 gene (Muños et al., 2004). This mutant was originally isolated on the basis of its resistance to the herbicide chlorate, which is taken up through the AtNRT1.1 transporter and reduced by NO$_3^-$ reductase to toxic chlorite (Doddema et al., 1978; Tsay et al., 1993). A construct containing MtNIP/LATD cDNA under the control of the constitutive Arabidopsis EF1a promoter (pAtEF1a-MtNIP/LATD) was introduced into Atchl1-5 plants, with plants transformed by AtNRT1.1 cDNA regulated by the same promoter (pAtEF1a-AtNRT1.1) serving as a positive control. Atchl1-5/pAtEF1a-MtNIP/LATD was found to be sensitive to chlorate, similar to the positive control Atchl1-5 plants transformed with pAtEF1a-AtNRT1.1 and wild-type ecotype Columbia (Col-0). Negative control mutant Atchl1-5 plants were resistant to chlorate, as expected (Fig. 5; Supplemental Fig. S2). Wild-type Col-0 and the Atchl1-5 plants constitutively expressing either AtNRT1.1 or MtNIP/LATD showed reductions in fresh weight and chlorophyll content after chlorate treatment compared with the resistant Atchl1-5 plants (Table II). A second independent transformed line of Atchl1-5 transformed with pAtEF1a-MtNIP/LATD was also constructed and found to be chlorate sensitive as well (Supplemental Fig. S2). Therefore, we conclude that since MtNIP/LATD transports the NO$_3^-$ analog chlorate in planta, it is extremely likely to transport NO$_3^-$ in planta as well.

**AtNRT1.1, But Not AgDCAT1, Partially Rescues the M. truncatula nip-1 Phenotype**

Since MtNIP/LATD restored chlorate sensitivity to the Arabidopsis chl1-5 mutant, we tested whether AtNRT1.1 would restore the M. truncatula nip-1 mutant to its wild-type phenotype. At the time that this experiment was performed, AtNRT1.1 was the only NRT1(PTR) member known to be a high-affinity (dual-affinity) NO$_3^-$ transporter (Tsay et al., 2007). We used composite M. truncatula plant hairy roots (Boisson-Dernier et al., 2001) transformed with pAtEF1a-AtNRT1.1 as our test system. Composite plants were grown in aeroponic chambers in the absence of NO$_3^-$, inoculated with *S. meliloti*, containing a large deletion in the AtNRT1.1 gene (Muños et al., 2004). This mutant was originally isolated on the basis of its resistance to the herbicide chlorate, which is taken up through the AtNRT1.1 transporter and reduced by NO$_3^-$ reductase to toxic chlorite (Doddema et al., 1978; Tsay et al., 1993). A construct containing MtNIP/LATD cDNA under the control of the constitutive Arabidopsis EF1a promoter (pAtEF1a-MtNIP/LATD) was introduced into Atchl1-5 plants, with plants transformed by AtNRT1.1 cDNA regulated by the same promoter (pAtEF1a-AtNRT1.1) serving as a positive control. Atchl1-5/pAtEF1a-MtNIP/LATD was found to be sensitive to chlorate, similar to the positive control Atchl1-5 plants transformed with pAtEF1a-AtNRT1.1 and wild-type ecotype Columbia (Col-0). Negative control mutant Atchl1-5 plants were resistant to chlorate, as expected (Fig. 5; Supplemental Fig. S2). Wild-type Col-0 and the Atchl1-5 plants constitutively expressing either AtNRT1.1 or MtNIP/LATD showed reductions in fresh weight and chlorophyll content after chlorate treatment compared with the resistant Atchl1-5 plants (Table II). A second independent transformed line of Atchl1-5 transformed with pAtEF1a-MtNIP/LATD was also constructed and found to be chlorate sensitive as well (Supplemental Fig. S2). Therefore, we conclude that since MtNIP/LATD transports the NO$_3^-$ analog chlorate in planta, it is extremely likely to transport NO$_3^-$ in planta as well.

**Figure 4.** Nitrate uptake in *X. laevis* oocytes expressing MtNIP/LATD or mutant Mtnip-latd mRNAs. Oocytes were microinjected with MtNIP/LATD mRNA, mutant mRNA, or water as a negative control, incubated for 3 d, and then placed for the indicated times in medium containing 5 mM or 250 μM nitrate, at pH 5.5, and assayed for nitrate uptake. A, Treatment with 5 mM nitrate. B, Treatment with 250 μM nitrate. C, Treatment with 5 mM nitrate. Oocytes expressing wild-type (WT) MtNIP/LATD (black bars), Mtnip-1 (dark gray bars), Mtnip-3 (horizontally striped bars), Mtlatd (hatched bars), or water as a negative control (gray bars) are shown. Data are shown for one biological replicate ± SD (n = 3–5 batches of 4–6 oocytes per batch). Asterisks mark nitrate uptake that is significantly different from the negative control, using Student’s t test at P < 0.05. Similar results were obtained in more than three repetitions of the experiment.

**Figure 5.** MtNIP/LATD complements the chlorate-insensitivity phenotype of the Arabidopsis chl1-5 mutant. Arabidopsis chl1-5 plants were transformed with a construct containing MtNIP/LATD cDNA under the control of the constitutive Arabidopsis EF1a promoter, pAtEF1a-MtNIP/LATD, or a positive control construct containing the Arabidopsis AtNRT1.1 gene under the control of the same promoter, pAtEF1a-AtNRT1.1. The plants were treated with chlorate, a NO$_3^-$ analog that can be converted to toxic chlorite after uptake, as described by Tsay et al. (1993). A, Arabidopsis Col-0 plant. B, Arabidopsis chl1-5 plant. C, Arabidopsis chl1-5/pAtEF1a-AtNRT1.1 plant. D, Arabidopsis chl1-5/pAtEF1a-MtNIP/LATD plant. Bars = one-quarter inch. The MtNIP/LATD gene was able to confer chlorate sensitivity on Arabidopsis chl1-5 plants, similar to the AtNRT1.1 gene.
obvious meristem, and did not differentiate into the zones that are the hallmark of N-fixing nodules (Fig. 7). Control plants transformed with pAtEF1α-MtNIP/LATD had wild-type phenotype nodules (Fig. 7F) and showed comparable rescue of nodulation and root architecture phenotypes as those transformed with pMtNIP/LATD-MtNIP/LATD (Supplemental Fig. S3), showing that use of the Arabidopsis EFIα promoter is not a limiting factor for complementation of the MtNIP-1 phenotype. Overall, the experiment shows that although AtNRT1.1 partially restores the root architecture phenotype, it is not able to restore normal nodulation to the Mtnip-1 plants.

We also tested whether the gene encoding the alder symbiotic AgDCAT1 dicarboxylate transporter could restore normal root development or nodulation to Mtnip-1 plants, using a similar approach as for AtNRT1.1, in composite transformed plants. Mtnip-1 plants expressing AgDCAT1 had a phenotype indistinguishable from Mtnip-1 plants transformed with an empty vector; in contrast, Mtnip-1 plants expressing MtNIP/LATD were restored to the wild type. Wild-type A17 plant phenotypes were unaffected by AgDCAT1 expression (Supplemental Fig. S3).

DISCUSSION

The major finding of the work reported here is that MtNIP/LATD protein is a NO$_3^-$ transporter, and its NO$_3^-$ transport activity partially correlates with root architecture development. Our results also suggest that MtNIP/LATD has another unknown activity that is important to MtNIP/LATD’s biological roles in nodule development and in the modulation of root architecture.

Data for MtNIP/LATD NO$_3^-$ transport come from two complementary experimental approaches. When MtNIP/LATD is expressed in the heterologous X. laevis oocyte system, it enables the oocytes to transport NO$_3^-$ in a pH-dependent manner, demonstrating that NO$_3^-$ transport is H$^+$ driven (Fig. 3), similar to other NO$_3^-$ transporters in the NRT1(PTR) family (Tsay et al., 1993). NO$_3^-$ uptake was characterized as having a $K_m$ of 160 $\mu$m, making it a HATS. A second line of evidence that MtNIP/LATD is a NO$_3^-$ transporter is that its gene’s constitutive expression can complement the Arabidopsis chl1-5 mutant, containing a deletion spanning the AtNRT1.1 gene (Muñoz et al., 2004). Atchl1-5 mutants expressing MtNIP/LATD are susceptible to chlorate, indicating that MtNIP/LATD confers on them the ability to take up the herbicide chlorate, a NO$_3^-$ analog, from the medium (Fig. 5). Both approaches showing that MtNIP/LATD transports NO$_3^-$ indicate that the direction of NO$_3^-$ transport is from outside to inside cells.

One might expect Mtnip/latd mutants to exhibit defects in NO$_3^-$ uptake. We measured NO$_3^-$ uptake from medium by Mtnip-1 and Mtnip-3 mutants and control wild-type M. truncatula A17 and observed no differences between the plants in NO$_3^-$ uptake at either 250 $\mu$m or 5 mm NO$_3^-$, representative of HATS and LATS, respectively (Fig. 1). This suggests that MtNIP/LATD is not a rate-limiting transporter for NO$_3^-$ uptake.

![Figure 6](image-url)
into plant tissue. MtNIP/LATD is expressed in primary and LR tips (Yendrek et al., 2010); if MtNIP/LATD’s primary biological role is to transport NO\textsubscript{3}⁻, it may constitute only a small portion of NO\textsubscript{3}⁻ transport in M. truncatula roots. It is also possible that in Mtnip/latd mutants, the plant compensates by up-regulating the activity of another transporter. Another possibility is that MtNIP/LATD’s transport function may be critical for redistribution of NO\textsubscript{3}⁻ within the plant. We found that Mtnip-1 is apparently normal for NO\textsubscript{3}⁻ suppression of nodulation (Table I), implying that MtNIP/LATD may not be involved in this pathway (Streeter, 1988; Fei and Vessey, 2009), and/or that there are other NO\textsubscript{3}⁻ transporters that can compensate for this function.

Previously, the effects of 10 and 50 mM KNO\textsubscript{3} on primary root length and LR density in Mlatd mutants were monitored; Mlatd plants, like the wild type, do not show altered LR density in response to global increases in NO\textsubscript{3}⁻ (Yendrek et al., 2010). Here, we examined LR lengths of Mtnip-1 and Mtnip-3 plants grown in 0 \(\mu\text{M}\), 250 \(\mu\text{M}\), or 5 mM KNO\textsubscript{3} and found that the LR length phenotype was rescued for Mtnip-3 and partially rescued for Mtnip-1 at the 5 mM KNO\textsubscript{3} level but not at 250 \(\mu\text{M}\) KNO\textsubscript{3} (Fig. 2), suggesting that MtNIP/LATD might have a more biologically important role at lower NO\textsubscript{3}⁻ concentrations than at higher ones. However, these experiments did not control for the effects of salt concentration on root architecture, which could have affected abscisic acid levels, leading to changes in LR lengths (Liang and Harris, 2005; Liang et al., 2007), and thus are only suggestive.

Even though NO\textsubscript{3}⁻ uptake was not limited in Mtnip/latd mutants compared with wild-type plants, NO\textsubscript{3}⁻ uptake differed when these alleles were expressed in oocytes and assayed for nitrate transport. Nitrate uptake experiments in the oocyte system showed that the protein encoded by the weakest allele, Mtnip-3, transported NO\textsubscript{3}⁻ indistinguishably from the wild type, while the proteins encoded by the two more severe alleles, Mtnip-1 and Mlatd, did not (Fig. 4). The Mtnip-3 mutant has a phenotype: it forms Fix+/− nodules that accumulate polyphenolics and has minor defects in root architecture, in primary root length (Teillet et al., 2008; Yendrek et al., 2010) and LR length (Fig. 2). Thus, there is a correlation between MtNIP/LATD’s ability to transport NO\textsubscript{3}⁻ and Mtnip/latd mutants’ abilities to form and maintain nodule and root meristems and to form nodules invaded intracellularly by rhizobia. Despite that NO\textsubscript{3}⁻ transport by Mtnip-3 protein is indistinguishable from the transport by wild-type MtNIP/LATD, Mtnip-3 has a root and nodule phenotype. This indicates that MtNIP/LATD may have another function besides NO\textsubscript{3}⁻ transport.

To further address the possible link between MtNIP/LATD and NO\textsubscript{3}⁻ transport, we expressed AtnRT1.1 in Mtnip-1 roots. We observed that the AtnRT1.1-transformed Mtnip-1 plants are partially restored for their root architecture phenotype but not for the nodulation phenotype. The use of pAtEF1a is not a limiting factor for phenotype rescue, since MtNIP/LATD, expressed under the control of the same promoter, was able to restore both the root architecture phenotype (data not shown) and the nodulation phenotype (Fig. 7). No effect was observed when AtnRT1.1 was expressed in wild-type A17 plants (Figs. 6 and 7). This suggests that AtnRT1.1 protein’s dual-affinity NO\textsubscript{3}⁻ transport activity affects Mtnip-1 root architecture and supports the idea that MtNIP/LATD’s NO\textsubscript{3}⁻ transport activity has a role in modulating root developmental responses. Additionally, the lack of full complementation of the root architecture defects by AtnRT1.1 and the noncomplementation of the nodulation phenotype by AtnRT1.1 suggest that there is a function of MtNIP/LATD that is different from that of AtnRT1.1. Alternatively, it is
possible that posttranslational regulation of AtNRT1.1 in *M. truncatula* differs from that of MtNIP/LATD, especially in nodules, and that this is the cause of AtNRT1.1’s inability to complement *Mtnip-1*’s nodulation phenotype. Because AtNRT1.1 has been demonstrated to transport auxin in the absence of NO3⁻ (Krouk et al., 2010b) and NO₃⁻ was only provided during plant transformation in these experiments, another possible explanation for the observed effects on *Mtnip*’s roots is that the partial restoration of normal root architecture was brought about by AtNRT1.1-induced changes in auxin concentration. When normally expressed in Arabidopsis, AtNRT1.1 is thought to prevent auxin accumulation at LR tips by mediating basipetal auxin transport in LRs, thus halting LR growth (Krouk et al., 2010b); this is the opposite of what we observed in the *Mtnip-1* mutant expressing AtNRT1.1 (Fig. 6). Because our experiments used a constitutive promoter to express AtNRT1.1, it is possible that the perturbation of auxin gradients within the roots caused the observed changes in root architecture. It is also curious that the nodule phenotype of *Mtnip-1* plants transformed with AtNRT1.1 is different from that of *Mtnip-3*. If the ability of *Mtnip-3* nodules to form a meristem and allow rhizobia to invade intracellularly is related to *Mtnip-3* protein’s ability to transport NO3⁻, one would expect to find the same phenotype in *Mtnip-1* plants transformed with AtNRT1.1 as in *Mtnip-3*, which is not the case. This finding further supports the idea that MtNIP/LATD protein’s activity is more than simply NO3⁻ transport and is also not explained by the spectrum of activities inherent in AtNRT1.1.

What role could MtNIP/LATD-mediated NO3⁻ transport play in nodulation, root architecture development, or their regulation? MtNIP/LATD NO3⁻ transport could supply N at low NO3⁻ concentrations to dividing plant and bacterial cells early in nodulation, and also to differentiated nodules, at the dividing and endoreduplicating cells present in zones I and II, where *MtNIP/LATD*’s promoter is active (Yendrek et al., 2010). The MtNIP/LATD promoter is also active in primary root meristems, LR meristems and surrounding tissue, and MtNIP/LATD NO3⁻ transport could have a similar role there. In this case, the supply of NO3⁻ to these tissues would provide the N required for basic cellular functions required by dividing cells, possibly facilitating the transition from primordium to self-sustaining meristem by these nascent LR organs.

Alternately, MtNIP/LATD could transport NO3⁻ as a precursor to the potent signaling molecule nitric oxide (NO) early in nodulation and LR development. NO has been detected in *M. truncatula* nodule primordia not containing intracellular rhizobia, suggesting an active NO pathway in these cells, as well as in infection threads, where NO could come from either symbiotic partner (del Giudice et al., 2011). NO has also been detected in LR primordia in tomato (*Solanum lycopersicum*; Correa-Aragunde et al., 2004). We note, however, that nodulation occurs in environmental conditions where N is limiting; indeed, our laboratory conditions for nodulation occur in N starvation. We supply 0 μM NO3⁻ during nodulation, and only the trace NO3⁻ expected to be in the micromolar range, present as contaminants in nutrient media and glassware are available. If NO₃⁻ is supplied to dividing nodule cells, it must come from seed NO₃⁻ stores, which should be close to depleted by the time nodules are forming or be remobilized from other N-rich components within the plant. Another possibility is that MtNIP/LATD NO3⁻ transport could participate in a proposed NO3⁻-NO respiration pathway in nodules (Horchani et al., 2011; Meilhoc et al., 2011). There are several observations that argue against this: *Mtnip-3*, with functional NO3⁻ transport (Fig. 4), has abnormal nodules (Teillet et al., 2008), and *MtNIP/LATD*’s promoter is active in nodule meristems and in the invasion zone but not in the N-fixing zone (Yendrek et al., 2010), suggesting that MtNIP/LATD may be absent in the N-fixing zone.

Because MtNIP/LATD may have another function besides NO3⁻ transport, it is plausible that the other function is responsible for some of the *Mtnip/latd* mutants’ phenotypes. Here, we have presented data suggesting that neither His nor dicarboxylates are substrates for MtNIP/LATD transport (Supplemental Table S1; Supplemental Fig. S3). We and others have speculated that MtNIP/LATD may be a NO3⁻ sensor (Harris and Dickstein, 2010; Yendrek et al., 2010; Gojon et al., 2011). If it is a NO3⁻ sensor, we predict that it may be responsible for high-affinity NO3⁻ sensing. This is because it is a high-affinity transporter and because the root architecture phenotypes are partially rescued by high, but not low, NO3⁻ concentrations (Fig. 2). It is also possible that MtNIP/LATD is able to transport hormones like AtNRT1.1 (Krouk et al., 2010b) and AtNRT1.2 (Kanno et al., 2012) and that this is responsible for the other function(s) of MtNIP/LATD.

**MATERIALS AND METHODS**

**Plant Growth Conditions**

*Meditago truncatula* A17 (wild type) and nodulation mutants were grown in aeroponic chambers with Lullien medium (Lullien et al., 1987), starved for N for 5 d, and inoculated with *Sinorhizobium medicae* strain AB137 (Bekki et al., 1987) or *Sinorhizobium meliloti* strain RM2011 (Rosenberg et al., 1981) harboring pXLGD4 (Boivin et al., 1990; Pemollet and Cook, 1997), as described previously (Veereshlingam et al., 2004; Pislariu and Dickstein, 2007). For experiments where *M. truncatula* plants were grown in the presence of N sources, the relevant N sources were added to Lullien medium from the beginning of the experiment and inoculations were done with *AB137/pXLGD4*. At 15 dpi, plants were stained with 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside acid (X-Gal) to identify nodules. *Arabidopsis thaliana* (Arabidopsis thaliana) plants were grown as described (Srivastava et al., 2008) at 22°C with a 16/8-h light/dark regime.

**Nitrate Uptake Studies in *M. truncatula***

A17, *Mtnip-1*, and *Mtnip-3* seedlings were surface sterilized, germinated, placed on buffered nodulation medium (Ehrhardt et al., 1992) solidified with agar, pH 5.8, supplemented with 5 mM NH4NO3, and grown for 7 d at 22°C.
with a 16/8-h light/dark regime, with roots shielded from the light. Plants were transferred to N-free buffered nodulation medium agar and grown for 3 d to starve them for N. Plants were placed in liquid buffered nodulation medium supplemented with either 250 μM or 5 mM KNO₃. Samples from the medium were collected at the indicated times and assayed for NO₃⁻ using the Cayman (no. 780001) NO₃⁻/NO₂⁻ assay kit following the manufacturer's instructions.

Construction of Oocyte Expression Vectors
RNA was extracted from M. truncatula A17 plants and mutant Mtnip-1, Mtld, and Mtlnp-3 using the RNeasy kit (Qiagen). First-strand cDNA was transcribed using oligo(dT) and SuperScript III reverse transcriptase (Invitrogen), and the wild-type MtNIP/LATD constructs were verified using primers Chl_1F and Chl_1R, cloned into PCR8/GW/TOPO, and then moved into pMS004 (see above) using Chl_1F and Chl_1R, cloned into PCRS/GW/TOPO, and then moved into pCO2/GW (a gift from Dr. John Ward) downstream of an SP6 promoter. All clones were verified by DNA sequencing.

Binary Vectors
MtNIP/LATD cDNA was amplified with NIPCF2 and NIPCODBst1R, digested with NcoI and BstEI, and ligated into a vector containing the AIEFla promoter engineered to contain a 5′ BamHI site and a 3′ NcoI site, creating pMS004. Subsequently, the AIEFla-MtNIP/LATD cDNA fragment was cloned into the BamHI and BstEI sites of binary vector pCAMBIA2301. AINRKT1.1 cDNA was amplified from total cDNA made from wild-type Col-0 Arabidopsis mRNA using primers CHL11F and CHL11R, digested with BglII and BstEI, and cloned into NcoI and BstEI-digested pMS004 to obtain pMS008. The pAIEFla-AINRKT1.1 was then subcloned into the BamHI and BstEI sites of pCAMBIA2301. For the pMtNIP/LATD-MtNIP/LATD construct, the pAIEFla in pMS004 was replaced by a 34-kb upstream region of MtNIP/LATD amplified using NIP 10F and NIP PR1 primers, followed by EcoRl and NcoI/BglII digestion to create the pMS006 clone. Then, the pMS006 EcoRI/BstEI fragment was moved into the EcoRI/BstEI site of pCAMBIA2301. AgDCAT1 cDNA was PCR amplified from pETI.2-AgDCAT1 (a gift of Dr. K. Pavlovskii) using DC-EII and DC-NleI primers and cloned into the BamHI and NleI sites of pMS014 vector, to create pMS015. Then, the pMS015 EcoRI/BstEI fragment was moved into the EcoRI/BstEI site of pCAMBIA2301 to create the pMtNIP/LATD-AgDCAT1 construct. Suplemental Table S2 contains the primer sequences.

Nitrate and His Uptake in Xenopus laevis Oocytes
Capped mRNA was transcribed in vitro from linearized plasmid using SP6 or T7 RNA polymerase (mMESSAGE mMACHINE; Ambion). Collagenase-promoter engineered to contain a 5′ digested with HindIII and a 3′ NcoI site, creating clones were verified using primers pCHL_01 and pCHL_01R (Supplemental Table S2) via PCR with Phusion high-fidelity DNA polymerase (New England Biolabs). The resulting 1,776-bp cDNAs were transcribed using oligo(dT) and SuperScript III reverse transcriptase (Invitrogen), and the wild-type MtNIP/LATD constructs were verified using primers Chl_1F and Chl_1R, cloned into PCR8/GW/TOPO, and then moved into pCO2/GW (a gift from Dr. John Ward) downstream of an SP6 promoter. All clones were verified by DNA sequencing.

UPLC-ESI-MS/MS Analysis
[^3]C₆His was quantified using a precolumn derivatization method with 6-aminquinolyl-N-hydroxysuccinimidyl carbamate (AQC) combined with UPLC-ESI-MS/MS. AQC derivatization was performed using the AccQTag derivatization kit (Waters) according to the manufacturer’s protocol. UPLC-ESI-MS/MS analysis was carried out on a Waters Acquity UPLC system interfaced to a Waters Xevo TQ mass spectrometer as described (Salazar et al., 2012). Briefly, the AQC-derivatized [^3]C₆His was separated on a Waters Acquity UPLC Ultra column (2.1 mm i.d. × 100 mm, 1.7-μm particles) using AccQTag Ultra eluents (Waters) and a gradient described earlier (Salazar et al., 2012). The sample injection volume was 1 μL, the UPLC column flow rate was 0.1 mL min⁻¹, and the column temperature was 55°C. Mass spectra were acquired using positive ESI and the multiple reaction monitoring mode, with the following ionization source settings: capillary voltage, 1.99 kV (ESI+); desolvation temperature, 650°C; desolvation gas flow rate, 1.00 L h⁻¹; source temperature, 150°C. Argon was used as the collision gas at a flow rate of 0.15 mL min⁻¹. The collision energy and cone voltage were optimized for the derivatized [^3]C₆His using the IntelliDart software (collision energy ≤ 26 eV; cone voltage = 20 V). The most sensitive parent-daughter ion transition of derivatized His (mass-to-charge ratio 332.1 > 171.0) was selected for quantitation. The mass spectrometer response was calibrated by injecting AQC-derivatized [^3]C₆His standard solutions of known concentration. The UPLC-ESI-MS/MS system control and data acquisition were performed with Waters MassLynx software. Data analysis was conducted with TargetLynx software (Waters).

Transformation of Atchl1-5 Plants with the MtNIP/LATD Expression Construct
The MtNIP/LATD expression construct (pAIEFla-MtNIP/LATD) was transformed into the Agrobacterium tumefaciens GV3101(pMP90) strain by electroporation. Positive colonies were selected by colony PCR, verified by restriction digestion, and transformed into the Atchl1-5 mutant (Tsay et al., 1993) by the floral dip method (Clough and Bent, 1998). Seeds were collected, and transformed plants were selected on kanamycin medium. Two independent homozygous transformed lines of Atchl1-5 transformed with pAIEFla-MtNIP/LATD were selected.

Chlorate Sensitivity Test
Plants were grown in vermiculite:perlite (1:1 mixture) under continuous illumination at 25°C to 27°C. Plants were irrigated every 2 to 3 d with medium containing 10 mM KH₂PO₄ (pH 5.3), 5 mM KNO₃, 2 mM MgSO₄, 1 mM CaCl₂, 0.1 mM FeEDTA, 50 μM H₂BO₃, 12 μM MnSO₄, 1 μM ZnCl₂, 1 μM CuSO₄, and 0.2 μM Na₂MoO₄. At 5 to 7 d post germination, plants were irrigated every 2 to 3 d with medium containing 2 mM NaClO₃ without NO₃⁻. Three days after ClO₃⁻ treatment, plants were switched to irrigation medium lacking ClO₃⁻ and NO₃⁻. Plants were examined 7 to 10 d after ClO₃⁻ treatment for necrosis and bleaching symptoms characteristic of chlorate toxicity (Wilkinson and Crawford, 1991), and their fresh weights and chlorophyll contents were obtained. Digital color photographs of the plants were obtained, corrected for color balance (Supplemental Fig. S2), converted to grayscale, and corrected for contrast (Fig. 5) using Photoshop (Adobe Software).

Chlorophyll content was determined from approximately 10 mg of fresh leaves that were weighed, frozen in liquid N₂, ground to a fine powder, added to tubes with 100 μL of water and 8 mL of 96% ethanol, and mixed. The tubes were kept at 25°C overnight, mixed again, and the particulate absorbance was recorded at 648.6 and 664.2 nm. Total chlorophyll was calculated as described (Lichtenthaler, 1987).

M. truncatula Root Transformation
Vectors containing the expression constructs were transformed into Agrobacterium rhizogenes ARqua1 strain (Quandt et al., 1993) by the freeze-thaw method (Högen and Willmitzer, 1988). Positive ARqua1 colonies were transformed into Minip-1 and A17 plants by the needle-poking method (Mortier et al., 2010).

Analysis of LRs and Nodules in Transformed Plants
Root nodules were analyzed at 12 dpi. They were stained with X-Gal for lacZ, present in pXLGD4 plasmid in s. medicae AB857 and mounted in 2.5% low melting point agarose: 50-μm sections were obtained using a 1000 Plus Vibratome (Vibratome) and observed by light microscopy. LRs were inspected visually and with a dissecting microscope.
The MIN/P/LATD sequence may be found under GenBank accession no. GQ401665.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Comparison of nitrate uptake in oocytes expressing MIN/P/LATD versus AtNRT1.1.

Supplemental Figure S2. MIN/P/LATD complements the chlorate-insensitivity phenotype of the Arabidopsis chl-1-5 mutant.

Supplemental Figure S3. AgDCAT1 does not complement the Minlp-1 mutant.

Supplemental Table S1. MIN/P/LATD-expressing oocytes do not take up His.

Supplemental Table S2. Sequences of oligonucleotide primers used in this study.

ACKNOWLEDGMENTS

We thank Pudur Jagadeeswaran, Tina Machu, and their laboratories for help with the X. laevis oocyte experiments, John Ward for vector pO02/GW and suggestions about oocytes, Jeanne Harris for helpful discussions, Brian Ayre and his laboratory for help with Arabidopsis transformation, Nigel Crawford for Arabidopsis chl-1-5 seeds, Katharina Pawlowski for the AgDCAT1 cDNA, and Jyoti Shah and Jeremy Murray for critical reading of the manuscript.

Received February 27, 2012; accepted August 1, 2012; published August 2, 2012.

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