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A putative transporter is essential for integrating nutrient and hormone signaling with lateral root growth and nodule development in *Medicago truncatula*

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Genbank accession GQ401665 contains the *LATD/NIP* sequence.

SUMMARY

Legume root architecture involves not only elaboration of the root system by the formation of lateral roots but also the formation of symbiotic root nodules in association with nitrogen-fixing soil rhizobia. The *Medicago truncatula LATD/NIP* gene plays an essential role in the development of both primary and lateral roots as well as nodule development. We have cloned the *LATD/NIP* gene and show that it encodes a member of the *NRT1*(*PTR*) transporter family. *LATD/NIP* is expressed throughout the plant. p*LATD/NIP-GFP* promoter–reporter fusions in transgenic roots establish the spatial expression of *LATD/NIP* in primary root, lateral root and nodule meristems and the surrounding cells. Expression of *LATD/NIP* is regulated by hormones, in particular by abscisic acid which has been previously shown to rescue the primary and lateral root meristem arrest of *latd* mutants. *latd* mutants respond normally to ammonium but have defects in responses of the root architecture to nitrate. Taken together, these results suggest that *LATD/NIP* may encode a nitrate transporter or transporter of another compound.

Keywords: nodule development, lateral root development, root architecture, symbiotic nitrogen fixation, *Medicago truncatula, NRT1*(*PTR*) gene family.

INTRODUCTION

The plasticity of root development provides a mechanism by which plants can modulate their root architecture in response to changing environmental conditions. Modulation of root architecture is more complicated in legumes than in other plants, because legumes form two kinds of root organs: lateral roots, made by all higher plants, and symbiotic root nodules, formed in conjunction with nitrogen-fixing rhizobia. Lateral root formation and nodulation are tightly regulated by environmental inputs, hormone signaling, and signals exchanged between the root and rhizobia. Both lateral roots and nodules develop from an organ primordium that forms a new meristem at its tip. In lateral roots and indeterminate nodules the new meristem is persistent, while in determinate nodules it is transient (Mathesius, 2008).

Nodulation begins with signal exchange between the legume host roots and rhizobia, followed by invasion of the root by rhizobia. In *Medicago truncatula*, plant-derived infection threads that initiate in curled root hairs lead

rhizobia through epidermal cells and across several cell layers, and deposit them into symbiosomes in newly divided inner cortical cells through an endocytotic process (Brewin, 2004; Crespi and Frugier, 2008a; Oldroyd and Downie, 2008). Within the symbiosomes, the rhizobia mature into bacteroids, capable of nitrogen fixation. Only mature bacteroids express the *nif* genes required for symbiotic nitrogen fixation (Fischer, 1994). Although legume nodules are an important source of fixed nitrogen, they are also powerful sinks, consuming significant amounts of photosynthate.

Hormonal control of legume root architecture has several unique features. An important aspect is the differential regulation of lateral root formation and nodulation. Regulatory controls exist to restrict the number of symbiotic nodules on the legume root system. Many of these controls have opposite effects on lateral root formation, making it possible to induce lateral root formation without simultaneously stimulating nodule formation. In this way the root system can be expanded without adding more photosynthate-consuming nodules. The hormones ethylene, jasmonic acid, auxin and abscisic acid (ABA) all inhibit nodule initiation but stimulate the formation of legume lateral roots. Conversely, cytokinin signaling stimulates nodulation but inhibits lateral root formation (reviewed in Oldroyd and Downie, 2008). Another unique feature of legume root architecture is its altered response to ABA compared to non-legumes. Whereas high concentrations of ABA (1 or 10 μM) inhibit lateral root formation in Arabidopsis (De Smet *et al.*, 2006), they stimulate lateral root formation in both nodulating and non-nodulating legumes. This altered ABA response must have been acquired at the base of the legume lineage, coincident with the acquisition of a predisposition for nodulation (Liang *et al.*, 2007).

Nitrogen, an essential nutrient, plays a profound regulatory role in plants, including regulation of lateral root formation and nodulation. Inorganic nitrogen can be taken up from the environment by primary and lateral roots as either ammonia or nitrate, and rhizobial bacteroids within nodules fix atmospheric dinitrogen into ammonium. Nitrogen fixation is a highly energy-consuming process and the presence of adequate bioavailable nitrogen in the soil has a potent inhibitory effect on both nitrogen fixation and nodule initiation. Root architecture is modulated by nitrate, which acts by regulating lateral root initiation. The effects of nitrate on root branching, however, depend on whether nitrate is sensed globally by the root system or as a localized patch. High global levels of nitrate repress lateral root initiation in Arabidopsis, whereas locally high nitrate levels stimulate it (Zhang *et al.*, 2000; Forde, 2002).

Nitrate is transported into plant cells by members of both the NRT1(PTR) and NRT2 families of transporters (reviewed in Tsay *et al.*, 2007). In Arabidopsis, the AtNRT1.1 and AtNRT2.1 nitrate transporters play key roles in regulating lateral root formation in response to environmental conditions. AtNRT2.1 appears to act as a sensor of external nitrate levels as well as acting as a high-affinity transporter (Remans *et al.*, 2006a), and it is possible that AtNRT1.1, which functions as a dual-affinity transporter, may play a similar sensing role (Remans *et al.*, 2006b; Ho *et al.*, 2009; Wang *et al.*, 2009). Legume genes required for the regulation of root architecture and nodulation by nitrogen have yet to be identified, and nitrogen sensing and signaling in legumes may present unique features because of the presence of nitrogen-fixing nodules.

The *LATD*/*NIP* gene is required for the establishment and maintenance of three root meristems, that of the primary root, lateral roots and symbiotic root nodules. Three allelic *latd* and *nip* mutants exhibit defects in root architecture and nodulation (Veereshlingam *et al.*, 2004; Bright *et al.*, 2005; Teillet *et al.*, 2008). During nodulation in *latd* and *nip-1* mutant plants, rhizobia are able to infect mutant roots through infection threads, but are unable to be endocytosed into symbiosomes. In the less severe *nip-3* mutant, rhizobia become endocytosed, but the number of infected cells remains limited. Nodules in all three mutants are characterized by an accumulation of brown pigments, identified as polyphenolics by histochemical staining in *nip-1*. Addition of the hormone ABA can rescue the *latd* root meristem defects, restoring normal growth. *latd* mutants synthesize normal levels of ABA, but have additional ABA-insensitive phenotypes, suggesting that *LATD*/*NIP* functions in the ABA response (Liang *et al.*, 2007).

Here we show that *LATD*/*NIP* encodes a member of the NRT1(PTR) family of transporters. *LATD*/*NIP* gene function is required in the root, and is expressed in the meristem and elongation zone of the root apex and in the meristem and infection zone of nodules. We find that expression of *LATD*/*NIP* is regulated by cytokinin, auxin and ABA, but not by nitrate. We also show that *latd*/*nip* mutants have a decreased response to nitrate for the regulation of root architecture, suggesting a link between *LATD*/*NIP* and nitrate signaling.

RESULTS

Root control of the *nip-1* root and nodule phenotypes

Most root and nodulation phenotypes in legumes are controlled by the root, but some, notably the *sunn* supernodulation phenotype, are regulated by the shoot (Penmetsa *et al.*, 2003). To determine whether the *nip-1* root and nodule phenotypes are controlled by the root or the shoot, grafting experiments were carried out. Reciprocal grafts were made between *nip-1*, in the A17 ecotype background, and the wild-type ecotype A20, and the grafted plants were inoculated with *Sinorhizobium meliloti*. This enabled phenotypic analysis coupled with genotyping of the relevant tissue to unequivocally identify the root and shoot as mutant or wild type. Results from at least three replicates of each combination demonstrated that *nip-1* root and nodule phenotypes

are controlled by the root genotype (Figure S1 in Supporting Information).

Identification of the *LATD/NIP* locus by positional cloning

A positional cloning approach was carried out to determine the identity of the *LATD*/*NIP* gene. Previously, the *LATD*/*NIP* gene was mapped to the top of linkage group 1 (LG1) and shown to be tightly linked to the genetic marker R15J11L (Bright *et al.*, 2005). At the time positional cloning was initiated, the *M. truncatula* physical map in this region included several bacterial artificial chromosome (BAC) restriction fingerprint contigs that contained co-segregating co-dominant genetic markers (Cannon *et al.*, 2005), but was incomplete, necessitating a chromosome walk (see Experimental Procedures). Using a large F_2 mapping populations, *LATD*/*NIP* was mapped between markers164N9 and 78L20; marker M17 was never separated from *LATD*/*NIP* by recombination (Figure S2). These three markers localize to overlapping BACs mth2-164P8 and mth2-78L20, placing *LATD*/*NIP* on one of these BACs.

To determine which gene in the two BACs corresponded to *LATD*/*NIP*, the predicted genes on these BACs were screened for altered expression levels in *latd* and *nip* homozygotes using quantitative (q)RT-PCR. Expression of gene AC157646_18 on BAC mth2-164P8 was decreased in homozygous *latd* mutant plants (Figure 1a). Sequencing AC157646_18 revealed a premature STOP codon in the *latd* mutant (Figure 1b) suggesting that the low transcript levels in *latd* are due to nonsense-mediated decay. The *nip-1* and *nip-3* alleles were found to have wild-type transcript levels of AC157646_18; their mutations were determined to be amino acid substitutions (Figure 1b). The severity of the lateral root elongation phenotype observed in the *latd*/*nip* allelic series correlates with the type of mutation, STOP versus substitution, detected (Figure S3).

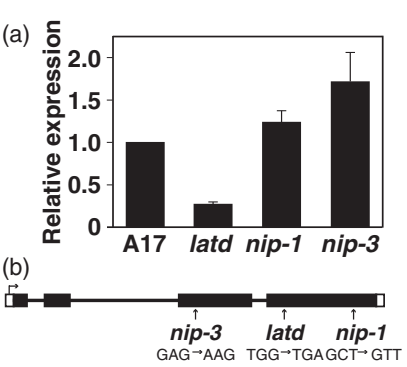

Figure 1. Identification of the *LATD*/*NIP* gene.
(a) Expression analysis of *LATD*/*NIP* in *latd* and *nip* mutant roots by quantitative RT-PCR. Expression of the *LATD*/*NIP* gene in *latd* mutants is significantly lower than in the wild type, but expression in the *nip-1* and *nip-3* mutants is normal.
(b) Exon/intron gene model showing positions of mutations in the *LATD*/*NIP* gene.

Complementation of *latd/nip* mutants by the wild-type *LATD/NIP* gene

To confirm that the mutations in AC157646_18 (*LATD*/*NIP*) in *latd* and *nip-1* plants were the cause of the root and nodule defects, we asked whether a wild-type genomic copy could complement the mutant phenotype. We transformed roots using *Agrobacterium rhizogenes* containing one of two complementation vectors (Boisson-Dernier *et al.*, 2001). The complementation vector used to transform *latd* mutant roots, pCY33, contained a wild-type copy of *LATD*/*NIP* with a 2.6 kb upstream region, full-length coding fragment, including introns, and 0.5 kb downstream of the stop codon. *latd* mutants fail to produce elongated lateral roots (Bright *et al.*, 2005); all of the *latd* roots transformed with the *LATD*/*NIP*-containing vector grew long lateral roots, whereas none of the plants transformed with the empty vector exhibited lateral root elongation of more than 2 mm (Figure 2, Table 1). *latd* primary roots have a major defect in their ability to maintain the root apical meristem (Liang *et al.*, 2007). The *LATD*/*NIP*-transformed *latd* roots had wild-type root tip morphology (Figure 2i). In contrast, *latd* roots transformed with empty vector had a disorganized root tip, similar to that of untransformed *latd* primary roots (Figure 2h). Leaves from each of the *LATD*/*NIP*-transformed *latd* plants were genotyped to demonstrate that they were homozygous for the mutant allele (Figure S4), confirming that *LATD*/*NIP* rescued the *latd* mutant phenotypes in the roots. For complementation of *nip-1*, a second complementation vector was made, pRD100, containing the 2.8 kb upstream, coding region including introns and 0.8 kb downstream genomic regions of *LATD*/*NIP*. Phenotypically wild-type roots and nodules were observed on *nip-1* plants transformed with this vector, but not the empty vector (Table 1).

In addition to the primary and lateral root defects, plants carrying mutations in *LATD*/*NIP* are unable to form functional nitrogen-fixing nodules. The nodules that do form are small, exhibit brown pigmentation and are ineffective for nitrogen fixation (Veereshlingam *et al.*, 2004; Bright *et al.*, 2005; Teillet *et al.*, 2008). Nodules that form on *latd* or *nip-1* roots transformed with empty vector resemble those on untransformed *latd* or *nip-1* plants (Figure 2e,k). In contrast, nodules that develop on complemented *latd* roots are large, elongated and exhibit the pink coloration that is a hallmark of Fix+ nodules (Figure 2f). To visualize rhizobial differentiation inside the nodule, transformed roots were inoculated with *S. meliloti* carrying a *nifH::*GUS reporter gene that is a reporter for mature bacteroids. *nifH::*GUS is not expressed in *latd* mutant nodules (Bright *et al.*, 2005); however, nodules that develop on mutant roots transformed with *LATD*/*NIP* have a wild-type pattern of *nifH::*GUS staining (Figure 2c inset, compare with the wild-type, Figure 2a inset), indicating that the infected rhizobia are differentiating into

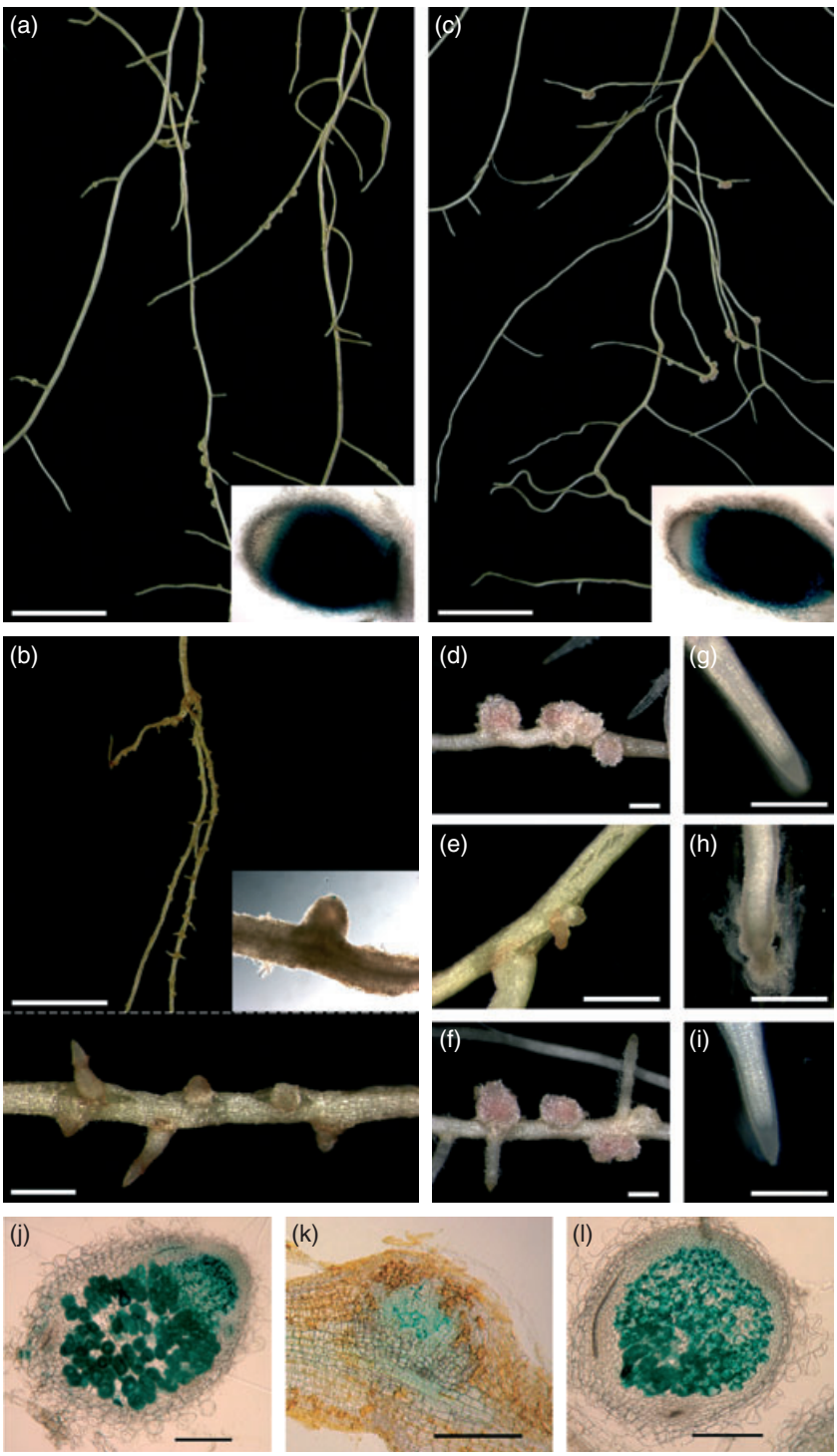

Figure 2. Complementation of *latd* and *nip-1* mutant roots with wild-type *LATD*/*NIP*.
(a, d, g) Wild-type roots transformed with the complementing vector (pCY33). (b, e, h) *latd* mutant transformed with the empty vector pReDRootII. (c, f, i), *latd* mutant transformed with pCY33.
(a–c) Whole root architecture of composite plants at 14 days post-inoculation (dpi) with a *Sinorhizobium meliloti* strain carrying *nifH*:GUS, including a close-up of the uncomplemented *latd* root (below dashed line) and nodule (as the inset) in panel (b). *nifH*:GUS staining is shown as an inset in panels (a) and (c).
(d–f) Nodules at 14 dpi.
(g–i) Primary root tip 3 weeks after transformation and prior to *S. meliloti* inoculation.
(j–l) *nip-1* and wild-type control nodules inoculated with *S. meliloti* carrying a constitutive *hemA:lacZ* gene, sectioned and stained with X-Gal. (j) Wild-type nodule transformed with empty vector pCAMBIA2300. (k) *nip-1* nodule transformed with empty vector pCAMBIA2300. (l) *nip-1* nodule transformed with pRD100.
Scale bars: (a–c) 1.0 cm; (d–i) and inset (b) 0.1 cm; (j)–(l) 0.2 mm.

bacteroids. To examine infection phenotypes in the *nip-1* mutant, in which infection has been more closely examined (Veereshlingam *et al.*, 2004), *nip-1* mutant roots were transformed with the complementation vector pRD100, with an empty vector as control, and inoculated with rhizobia expressing the constitutive *hemA*::*lacZ* gene (Boivin *et al.*, 1990). Ten days after inoculation, nodules were sectioned and examined for the extent of infection. Figure 2(l) shows that the *LATD*/*NIP* gene restores normal rhizobial infection to *nip-1* nodules (compare with the wild-type in Figure 2j), while the empty vector does not (Figure 2k). Together, these results demonstrate that AC157646_18 is able to rescue the root and nodule defects in *latd*/*nip* plants, and thus corresponds to the *LATD*/*NIP* gene.

Table 1 Complementation of *latd* and *nip-1* mutant roots with wild-type *LATD*/*NIP*

	Plants with wild-type lateral roots	Plants with *latd*/*nip* lateral roots	Plants with wild-type nodules	Plants with *latd*/*nip* nodules	Plants with no nodules
latd + pRedRootII	0/9	9/9	0/9	5/9	4/9
latd + pCY33	13/13	0/13	12/13	0/13	1/13
nip-1 + pCAMBIA2300	0/8	8/8	0/8	8/8	0/8
nip-1 + pRD100	7/9	2/9	6/9	2/9	1/9

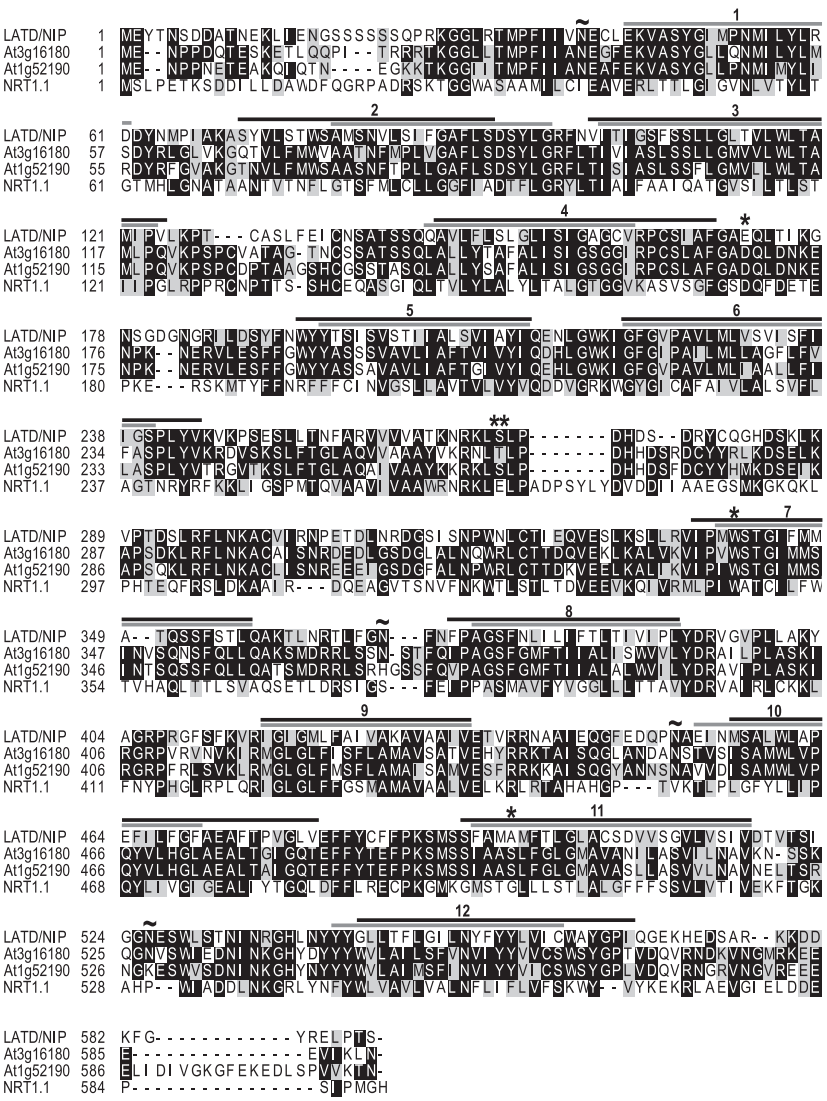

Figure 3. Comparison of deduced amino acid sequence of *LATD*/*NIP* with the closest Arabidopsis homologs (*At3g16180*, *At1g52190*) and *NRT1.1* (*At1g12110*).
Single asterisks show the positions of the mutations in *latd*, *nip-1* and *nip-3*. The bars show the positions of the predicted transmembrane domains in NRT1.1 (gray) and LATD/NIP (black), which are numbered according to those predicted for NRT1.1. Double asterisks mark the position of the predicted cAMP/cGMP dependent phosphorylation site in LATD/NIP. The tildes show the position of the four predicted *N*-glycosylation sites, all of which are predicted on loops facing away from the cytosol.

The *LATD/NIP* gene is a member of the *NRT1(PTR)* transporter family

BLAST analysis (Altschul *et al.*, 1990) of the 591 amino acids encoded by the *LATD*/*NIP* gene revealed that its closest homologs are encoded by Arabidopsis *At1g52190* and *At3g16180* (Figure 3), in the *NRT1*(*PTR*) family of transporters. The *NRT1*(*PTR*) family has 53 members in Arabidopsis and 80 in rice; several Arabidopsis NRT1(PTR) proteins have been extensively studied and shown to transport nitrate or di/tripeptides (Tsay *et al.*, 2007). Neither *At1g52190* nor *At3g16180* has been well-studied, but *At1g52190* has been reported in a review to encode a nitrate transporter (Tsay *et al.*, 2007), although the data are unpublished. To visualize the relationship of *LATD*/*NIP* to the Arabidopsis *NRT1*(*PTR*) genes, we carried out a phylogenetic analysis (Figure S5). *LATD*/*NIP* belongs to clade IV of the *NRT1*(*PTR*) family that, thus far, has been shown to encode low-affinity influx and

efflux nitrate transporters, although most members are uncharacterized biochemically (Segonzac *et al.*, 2007; Tsay *et al.*, 2007).

Most proteins in the NRT1(PTR) family, including At1g52190 and At3g16180, are predicted to be plasma membrane proteins with 12 conserved transmembrane domains (TMDs) with the N-terminus predicted inside. Interestingly, LATD/NIP is predicted by the HMMTOP program to have 11 TMDs with the first conserved TMD missing (Figure 3) (Tusnády and Simon, 1998, 2001), with the N-terminal domain predicted outside, thus conserving the overall topology. For LATD/NIP, the conserved TMD numbering is used here. NRT1(PTR) members commonly have a long cytosolic loop consisting of approximately 100 amino acid residues between TM segments 6 and 7, with at least one phosphorylation site. Using Prosite (Sigrist *et al.*, 2002; Hulo *et al.*, 2008), a RKLS cAMP-dependent protein kinase site was found for LATD/NIP in this loop which is conserved in At1g52190 (Figure 3). Potential *N*-glycosylation sites were found (Figure 3) as well as other potential phosphorylation and *N*-myristoylation sites (not shown).

Modulation of root architecture by inorganic nitrogen

Modulation of root architecture by available nitrogen provides a flexible way to alter root development in response to changing concentrations of a key nutrient (Zhang *et al.*, 2000; Forde, 2002; Walch-Liu *et al.*, 2006; Walch-Liu and Forde, 2008). Because the LATD/NIP demonstrates strong sequence similarity to known nitrate transporters (Figures 3 and S5) and is required for primary and lateral root development, we investigated the effect of inorganic nitrogen on root architecture in the *latd* mutant. To characterize the effects of inorganic nitrogen on primary root length and lateral root density, *latd* plants were grown on a nitrogen-free medium for 1 week, transferred to increasing concentrations of nitrate or ammonium and grown for 3 weeks. In parallel, *latd* plants were also transferred to a medium containing potassium chloride to control for the altered salt concentration of the nitrogen-salt-containing medium. Wild-type plants responded to both forms of nitrogen with a decrease in primary root length compared to the salt control, while *latd* primary root growth was insensitive to nitrate at all concentrations tested. *latd* primary roots responded to the ammonium chloride treatments in a manner similar to the wild type, with a decrease in length as ammonium chloride concentrations increased (Figure 4a). These effects may be caused by ammonium, but the possibility that they may be caused by chloride cannot be ruled out (compare 50 mM KCl with 50 mM NH_4Cl). Nitrate does not have a significant effect on the lateral root density of wild-type plants in this assay. Although there was an apparent increase in lateral root density of *latd* mutants on nitrate, this was found not to be statistically significant, indicating that *latd* plants, like the wild type, do not show altered lateral root density in response to global increases in nitrate (Figure 4b). Ammonium, however, had a powerful stimulatory effect on lateral root development in both wild-type and *latd* plants at all concentrations tested (Figure 4b).

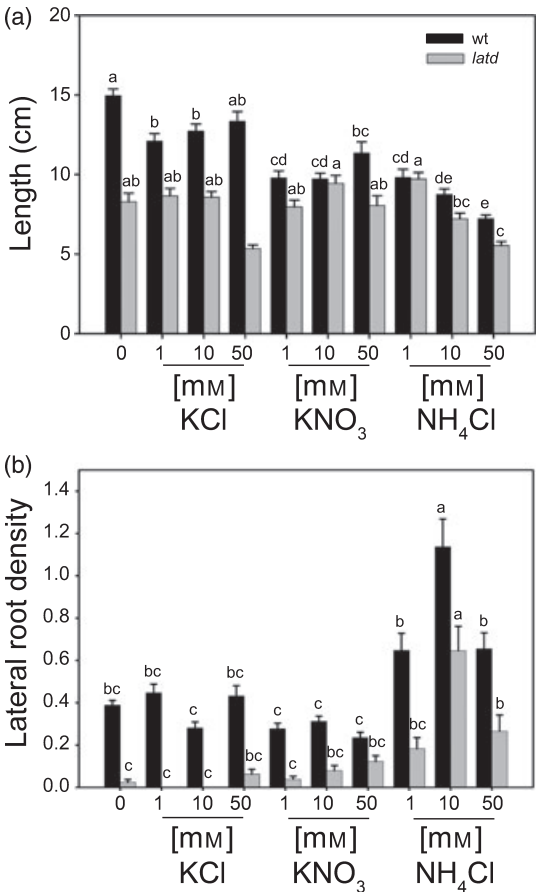

Figure 4. Effect of inorganic nitrogen on root architecture in wild-type and *latd* mutant plants.
(a) Primary root length.
(b) Lateral root density; the number of lateral roots cm^{-1} of primary root length. The mean ± SEM of three experimental repeats is presented (n = 30). Statistics presented reflect analysis of each genotype separately and across all nitrogen treatments.

***LATD/NIP* is expressed in root and nodule meristems**

LATD/*NIP* transcript accumulation was examined by qRT-PCR and found to be present in all tissues examined (Figure 5). These qRT-PCR results agree well with *LATD*/*NIP* expression measured on the *M. truncatula* Affymetrix chip (Benedito *et al.*, 2008), where *LATD*/*NIP* is represented by two probe sets; the data are available at http://bioinfo.noble.org/gene-atlas/v2/ and is compiled in Table S1.

To examine the spatial localization of *LATD*/*NIP* expression in root systems, we constructed a *LATD*/*NIP* promoter–*GFP* transcriptional fusion (p*LATD*/*NIP-GFP*) and expressed it in roots. p*LATD*/*NIP-GFP* expression was found to be enriched at the tips of both primary and lateral roots

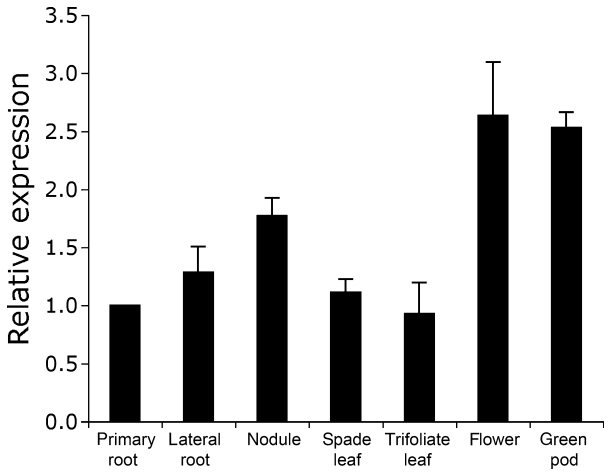

Figure 5. Tissue specific *LATD*/*NIP* expression.
The presence of *LATD*/*NIP* transcript was assessed via quantitative RT-PCR using RNA collected from the indicated organs of 16-day-old seedlings (primary root, lateral root, nodule and spade leaf) and 6-week-old plants (trifoliate leaves, flowers and green pods). Seedlings were inoculated with *Sinorhizobium meliloti* on day 3. Expression is normalized to Ub10 and calibrated to primary root.

including the root cap, meristem and proximal portion of the elongation zone (Figure 6a–h). To confirm the staining pattern, we used qRT-PCR to measure *LATD*/*NIP* transcript levels: we observed a five-fold increase in expression in the primary root tip versus the remainder of the root (Figure 6i). Similar, but not identical, results were obtained by transcription profiling (Holmes *et al.*, 2008), where a 1.7-fold difference in *LATD*/*NIP* expression was seen between the 3 mm root tip and 1 cm root section adjacent to the root tip (Table S1). We observed a similar spatial pattern in nodules: 12 days after inoculation with *S. meliloti*, p*LATD*/*NIP-GFP* expression was found in both the meristematic and distal infection zones of the developing nodule (Figure 6j–m).

LATD/NIP expression is regulated by multiple hormones

To gain insights into the role of *LATD*/*NIP* root and nodule development, we examined regulation of *LATD*/*NIP* expression by nitrate and hormones. Several nitrate transporters in the *NRT1*(*PTR*) family are regulated by nitrate (Wang *et al.*, 2003, 2004); thus we assessed the effect of nitrate on *LATD*/*NIP* transcript abundance in primary roots by qRT-PCR. We found that a 2-h treatment with nitrate had little effect on *LATD*/*NIP* expression (Figure 7a). In profiling experiments, Ruffel *et al.* (2008) examined transcript accumulation after split roots were grown for 4 days in differing concentrations of nitrate or ammonium or when nitrogen was fixed through nitrogen fixation (Ruffel *et al.*, 2008). *LATD*/*NIP* transcript levels were found to be relatively constant in these different nitrogen status conditions (Table S1).

The *LATD*/*NIP* gene functions downstream of ABA to regulate root growth, stomatal closure and germination (Liang *et al.*, 2007). In order to further characterize the interaction of ABA and *LATD*/*NIP*, we examined its regulation by ABA. We found that ABA treatment led to a decrease in *LATD*/*NIP* expression in both the primary root tip and proximal root sections, suggesting that ABA negatively regulates *LATD*/*NIP* (Figure 7b). Since lateral root formation is strongly affected in the *latd* and *nip* mutants, we also examined the effect of auxin (IAA), which plays a major role in stimulating lateral root development, on *LATD*/*NIP* expression. We found that auxin treatment led to a sharp decrease in *LATD*/*NIP* transcript abundance, whereas treatment with the cytokinin benzyladenine resulted in a two-fold increase (Figure 7b). These results indicate strong hormonal control of *LATD*/*NIP* at the level of gene expression.

DISCUSSION

Here, we report the cloning of the *LATD*/*NIP* gene that is required for root and nodule meristem function, rhizobium infection and ABA responses (Veereshlingam *et al.*, 2004; Bright *et al.*, 2005; Liang *et al.*, 2007; Teillet *et al.*, 2008). The *latd* and *nip* allelic mutants have defects in primary and lateral root development, nodulation and, in the case of the most severe *latd* allele, root hair formation and stomatal responses to ABA. We used a map-based cloning strategy to identify the *LATD*/*NIP* gene and found that it encodes a putative transporter of the *NRT1*(*PTR*) family. We showed that *LATD*/*NIP* function is required in the root for regulation of root and nodule phenotypes and that the gene is expressed in root and nodule meristems and modulated by hormones that regulate meristem activity.

LATD/*NIP* is a member of the large *NRT1*(*PTR*) transporter family. Our phylogenetic analysis placed *LATD*/*NIP* in clade IV of this family: although many are still uncharacterized biochemically, thus far several genes in clade IV encode nitrate transporters, including both influx and efflux transporters (Segonzac *et al.*, 2007; Tsay *et al.*, 2007; Almagro *et al.*, 2008). In addition to transporting nitrate, members of the NRT1(PTR) family in other clades have been shown to transport di- or tripeptides, amino acids and dicarboxylic acids (Tsay *et al.*, 2007). The non-legume nodule-specific transporter in the NRT1(PTR) family, *Alnus glutinosa* (alder) AgDCAT1, has been found to transport dicarboxylates (Jeong *et al.*, 2004); it is embedded in a section of the phylogenetic tree that contains characterized nitrate transporters (Figure S5 and Tsay *et al.*, 2007). Thus, the substrate cannot be predicted by phylogenetic relationships and it is possible that LATD/NIP transports compounds other than or in addition to nitrate.

Although the substrate of LATD/NIP is still unknown, the mutations in the gene may provide some insight into structure and function. The mutation in *latd* is a STOP mutation, suggesting that the more severe phenotype of *latd* compared with the *nip-1* and *nip-3* alleles may be caused by nonsense-mediated decay (Figures 1, 3 and S3;

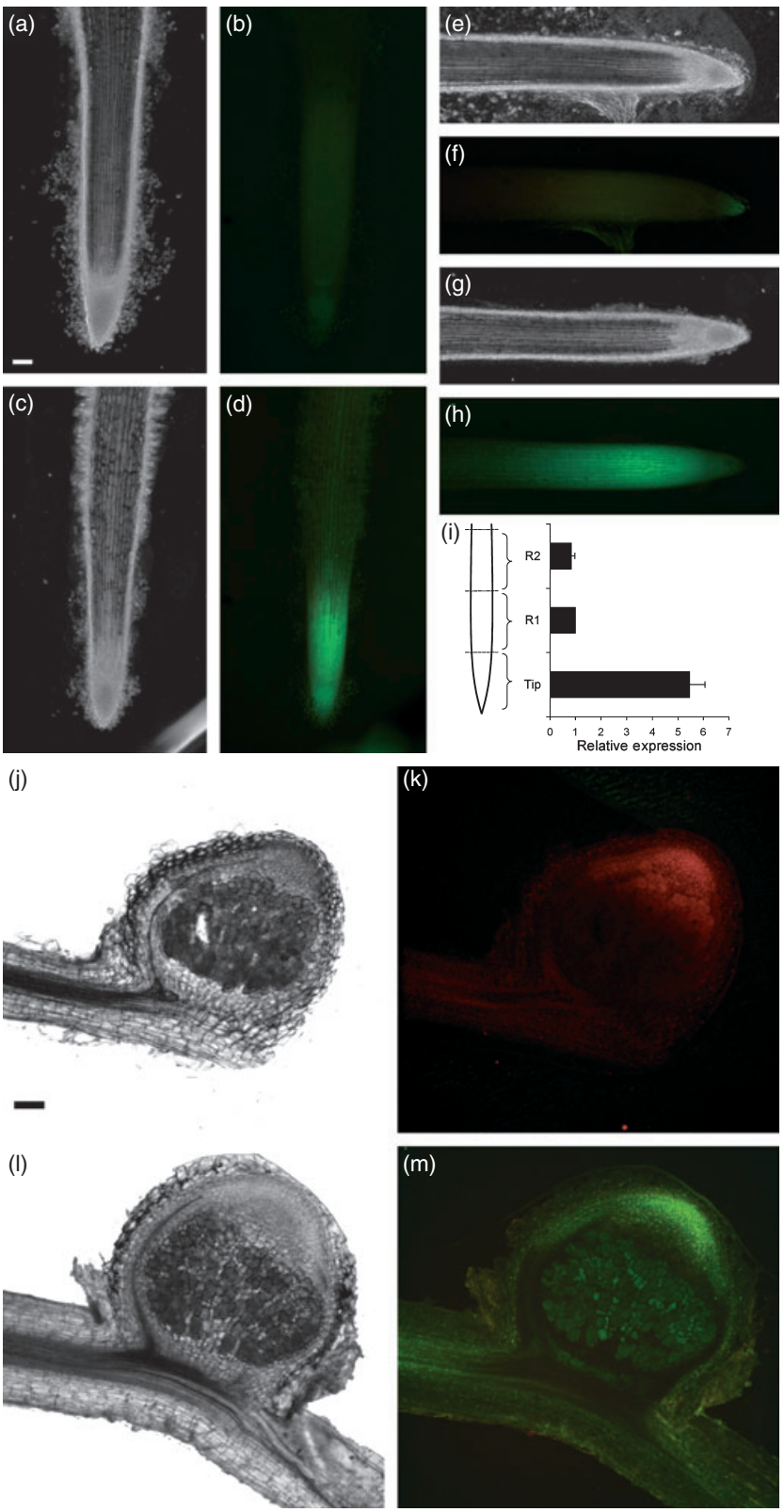

Figure 6. p*LATD*/*NIP-GFP* expression in primary and lateral roots and nodules of wild-type *Medicago truncatula*.
(a–d) Primary roots. (e–h) Lateral roots. (j–m) Nodules.
Panels (a), (b), (e), (f), (j) and (k) are empty vector controls while (c), (d), (g), (h), (l) and (m) are transformed with the *pLATD*/*NIP-GFP* fusion. Panels (a), (c), (e), (g), (j) and (l) are dark- and brightfield images and (b), (d), (f), (h), (k) and (m) are fluorescence images. Panels (b), (d), (f) and (h) are epifluorescence and show the green channel only. Panels (k) and (m) are confocal images and are merged red (from the *dsRed* gene on the vector) and green channels. The individual channels are shown in Figure S6. The scale bars in panels (a) and (j) are 0.1 cm and are the same for all root and nodule panels, respectively. (i) *LATD*/*NIP* transcript abundance was measured using quantitative RT-PCR by isolating RNA from three sequential 0.5 cm long sections of the primary root starting with the tip followed by root sections R1 and R2. The R1 section was used as the calibrator.

Veereshlingam *et al.*, 2004; Bright *et al.*, 2005; Teillet *et al.*, 2008). The positions of the substitution mutations in *nip-1* and *nip-3* were found in conserved sequences of proteins in the NRT1(PTR) family. For *nip-3*, the E171K substitution is within a conserved [DEN]-Q-[LFYI] motif in a predicted cytosolic domain just after TMD 4 (Figure 3). The acidic E

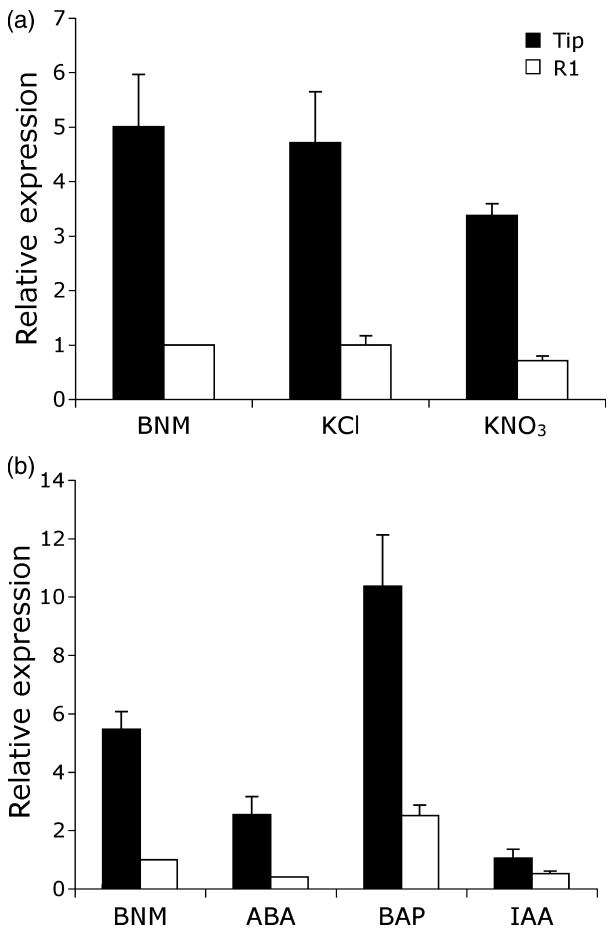

Figure 7. Regulation of *LATD*/*NIP* gene expression by nitrate and hormones. Quantitative RT-PCR analysis comparing tip and R1 sections (see Figure 6) that were collected 2 h after the specified treatments or with no treatment (buffered nodulation medium, BNM).
(a) *LATD*/*NIP* expression after treatment with 10 mM nitrate or KCl salt control.
(b) *LATD*/*NIP* expression after treatment with 10 μM of the indicated hormone. Plants were grown in growth pouches on N-free BNM medium for 6 days prior to treatment. The R1 section of BNM-only control roots is used as the calibrator for each experiment.

residue may help this short cytosolic domain project away from the acidic head groups on the membrane, which would be abolished by the basic K residue in the mutant. For *nip-1*, the A497V substitution is found in TMD11; this position is occupied by a small A, S or G residue in 50 of 53 of the NRT1(PTR) family members in Arabidopsis as well as in most other NRT1(PTR) members sequenced to date (data not shown). The larger V residue probably disrupts a substructure maintained by the smaller residue in the wild-type LATD/NIP protein; the severity of the *nip-1* allele indicates that this A residue is important for function.

Transporters have been found to have important roles in nodulation in legumes. Legume expressed sequence tag (EST) and gene expression profiling projects have focused on developing and fixing nodules and have reported hundreds of genes encoding putative transport proteins expressed in legume nodules (Fedorova *et al.*, 2002; El Yahyaoui *et al.*, 2004; Kuster *et al.*, 2004; Benedito *et al.*, 2008). During the early stages of legume nodulation, flavonoid inhibition of auxin transport has been shown to be a requirement for nodule structure formation in indeterminate nodulators, e.g. in *M. truncatula* (Wasson *et al.*, 2006), but not during determinate nodule formation (Subramanian *et al.*, 2007). Later in nodulation, the rhizobia invade host cells and become surrounded by the symbiosome membrane through which all plant–rhizobia molecular commerce must cross (Udvardi and Day, 1997; White *et al.*, 2007). A sulfate transporter that is essential for nitrogen fixation in lotus (Krusell *et al.*, 2005), soybean (*Glycine max*) and *Lotus japonicus* N70 anion transporters with a preference for nitrate (Vincill *et al.*, 2005) and the above-mentioned *A. glutinosa* AgDCAT1 dicarboxylate transporter all reside on the symbiosome membrane. Additionally, a plasma-membrane localized ammonium transporter was found to be important for nitrogen-fixing activity of lotus nodules and is thought to be involved in recovery of ammonium derived from symbiotic nitrogen fixation and lost from cells via diffusion prior to its being assimilated (Rogato *et al.*, 2008).

Lateral root development in Arabidopsis is modulated by nitrate transporters from two different families: NRT1.1, a dual-affinity transporter from the NRT1(PTR) family, and NRT2.1 a high-affinity transporter from the NRT2 family (Tsay *et al.*, 1993; Wang *et al.*, 1998; Cerezo *et al.*, 2001; Filleur *et al.*, 2001). Plants with mutations in NRT1.1 or NRT2.1 have defects in the response of their root architecture to changes in nitrate availability. This response is independent of nitrate uptake, leading to the model that both NRT1.1 and NRT2.1 function as nitrate sensors, separable from their abilities to transport nitrate across the plasma membrane. (Little *et al.*, 2005; Remans *et al.*, 2006a,b; Wang *et al.*, 2009). Consistent with this model, *NRT1.1* is expressed in lateral root primordia and emerging lateral roots (Huang *et al.*, 1996). Curiously, *NRT2.1* is not expressed in lateral root primordia or newly emergent lateral roots generated in response to low nitrate conditions, suggesting that for NRT2.1 the sites of nitrate perception and response are distinct (Remans *et al.*, 2006a). Expression of both *NRT1.1* and *NRT2.1* is upregulated by nitrate (Wang *et al.*, 2003, 2004). Like *NRT1.1*, *LATD*/*NIP* is expressed in root meristems and emerging lateral roots, but apparently unlike either *NRT1.1* or *NRT2.1*, its expression appears not to be induced by nitrate (Figures 6 and 7, Table S1) in the conditions in which this has been studied (this study and Ruffel *et al.*, 2008). *latd* mutant plants respond normally to ammonium for lateral root growth, but are not responsive to nitrate (Figure 4), suggesting that LATD/NIP might perceive and/or transport nitrate.

A key step in lateral root formation is new meristem activation at the apex of the nascent organ; regulation of this step provides an important control of root architecture. In Arabidopsis, lateral root meristem activation occurs after

emergence from the primary root and is regulated by nitrate, signaling through ABA. Abscisic acid plays a negative role in meristem activation in Arabidopsis (Zhang *et al.*, 2007). However, ABA promotes activation of lateral root meristems in the *latd* mutant (Liang *et al.*, 2007), consistent with our previous finding that, in legumes, ABA stimulated lateral root number (Liang and Harris, 2005).

Nodulation mirrors many aspects of lateral root development. Like lateral roots, nodules are initiated by cell divisions in internal cell layers in differentiated roots (Hirsch, 1992). Legumes that form indeterminate nodules, thought to be the ancestral condition (Doyle, 1998), form a persistent meristem at the apex of the developing organ. In more basal legumes, such as peanut, lateral root and nodule primordia are always associated (Hirsch, 1992), as is nodulation of more mature parts of the root system in white clover (Mathesius *et al.*, 2000). Further support for shared aspects of development comes from the finding that miR166 is expressed in both lateral root and nodule meristems and its alteration disrupts the development of both organs (Boualem *et al.*, 2008). Our data show that *LATD*/*NIP* is required for the development of nodules and lateral roots (Veereshlingam *et al.*, 2004; Bright *et al.*, 2005; Teillet *et al.*, 2008) and that it is expressed in the nodule and root meristems (Figure 6), suggesting a common role for *LATD*/*NIP* in establishing and maintaining these meristems.

latd, *nip-1* and *nip-3* mutants exhibit defects in rhizobial invasion and differentiation as well as in nodule development, with rhizobia trapped in abnormal infection threads (Veereshlingam *et al.*, 2004; Bright *et al.*, 2005; Teillet *et al.*, 2008). The least severe *nip-3* allele has nodules containing rhizobium-infected cells, although the number of infected cells is reduced, as are cells that are apparently meristematic (Teillet *et al.*, 2008). Although we do not at present understand the mechanisms contributing to these defects, we do note that nodule meristematic activity has been shown to be spatially restricted by miRNA transcription in the infection zone (Combier *et al.*, 2006). The phenotypes of the *latd* and *nip* mutants suggest that reciprocal control of the infection zone by the nodule meristem is possible.

Hormonal control of root meristem function provides a mechanism for plants to coordinate development of the root system. In this way, plants can modulate lateral root development in response to stress or nutrient availability. Nodules are tremendous consumers of photosynthate produced by the shoot, and hormonal control of nodulation provides an efficient way to turn nodules on or off. Thus, it is not surprising that *LATD*/*NIP*, with a role in meristem function, would be regulated by hormones that regulate root and nodule formation.

Auxin and cytokinin are key regulators of root and shoot meristems as well as important modulators of nodule initiation (Mathesius *et al.*, 1998, 2000; Lohar *et al.*, 2004; Murray *et al.*, 2007; Crespi and Frugier, 2008b; Ding and Oldroyd, 2009; Moubayidin *et al.*, 2009). We found that both auxin and cytokinin regulate *LATD*/*NIP* expression in opposite directions in the root: auxin inhibits and cytokinin stimulates expression (Figure 7b). Interestingly, the effect of auxin and cytokinin on *LATD*/*NIP* expression is the reverse of their roles in lateral root development; in that process, auxin stimulates and cytokinin inhibits (reviewed in Fukaki and Tasaka, 2009). Together with the analysis of the mutant phenotypes, these observations are consistent with a model in which too little or too much LATD/NIP activity is detrimental to lateral root formation. Similarly, ABA, which is required for the function of root meristems in *M. truncatula* and which acts upstream of *LATD*/*NIP* (Liang *et al.*, 2007), can also be inhibitory to meristem function at high concentrations. Those same high concentrations of ABA also repress *LATD*/*NIP* expression (Figure 7b).

What might LATD/NIP transport? How does its substrate regulate meristem function? Does LATD/NIP function intersect with known miRNA systems that modulate nodule and lateral root meristem activity in *M. truncatula*? A biochemical and cell biological analysis of LATD/NIP function is necessary to address these questions. Ultimately, a molecular understanding of LATD/NIP will give us insight into how meristem activation and function in both roots and nodules is initiated and regulated.

EXPERIMENTAL PROCEDURES

Plant growth conditions

Plants were grown on Metro Mix 360 (Sun Gro, http://www.sungro.com/) in MTR30 Conviron (http://www.conviron.com/) growth chambers at 20°C, 50% humidity and a 16-h light period of 125 μmol m^{-2} sec^{-1} using cool white fluorescent bulbs. For growth on different nitrogen concentrations, seeds were plated on 10 × 10 cm Petri plates containing N-free Buffered Nodulation Medium (BNM; Ehrhardt *et al.*, 1992) plus 1.2% agar, and grown for 6 days. Seedlings were transferred to 24 × 24 cm plates (Nunc, http://www.nuncbrand.com/) containing BNM supplemented with the indicated salt concentrations and grown for an additional 3 weeks. Plates were sealed with surgical tape (cat. no.1530-1, 3M Micropore, http://www.3m.com/) and the lower portion of the plate was covered with aluminum foil. For nodulation studies, plants were grown in aeroponic chambers, starved for N for 5 days and inoculated with *S. meliloti* ABS7 harboring pXLGD4 (Boivin *et al.*, 1990), as previously described (Veereshlingam *et al.*, 2004).

Variance analysis

All pair-wise comparisons per genotype were analyzed by one-way analysis of variance using Tukey's test at $P = 0.05$ using SPSS (release 16.0.1 for Mac; SPSS Inc., http://www.spss.com/).

Grafting

Seeds were scarified with concentrated H_2SO_4, sterilized with concentrated bleach, imbibed for 6 h at room temperature (RT) (25°C), then overnight at 4°C, and germinated overnight at RT in the dark on modified Fahraeus medium (Limpens *et al.*, 2004) solidified with 0.9% Phytoblend agar (Caisson Laboratories, Inc., http://www.caissonlabs.com/) on Petri dishes. The seedlings were placed on

fresh media and grown for 5 days at 21°C with 16 h/8 h light/darkness. Seedlings were transferred to new Petri dishes with Fahraeus medium in the bottom half, with the roots between the medium and the dish. The roots were cut at the hypocotyl and tape was used to affix the shoot of the relevant plant to the Petri dish, immediately on top of a root section. The bottom halves of the dishes were covered with aluminum foil and returned to 21°C with 16 h/8 h light/darkness. After 2–3 weeks, when the shoot/root junction was healed, the plants were placed in aeroponics chambers and inoculated with *S. meliloti*. Nodulation was evaluated at 10–15 days post-inoculation. Genotypes of roots and shoots were scored with the 4L4 genetic marker to discriminate mutant (A17 background) from wild type (WT; A20 background).

Positional cloning of *LATD/NIP*

LATD/*NIP* mapped to the top of linkage group 1 (Bright *et al.*, 2005), an area with spotty coverage in the *M. truncatula* genome. For fine mapping, F_2 progeny from *latd* × A20 and *nip-1* × A20 mapping crosses were examined for segregation of the *latd*/*nip* phenotype and scored with PCR-based genetic markers. Existing markers from the Medicago Genome Project (Choi *et al.*, 2004) as well as new ones made during the chromosome walk were used (Table S2). These were simple sequence repeat (SSR) markers and co-dominant; therefore, both chromosomes in each individual were scored. Once markers flanking *LATD*/*NIP* were identified, all F_2 progeny were scored for recombination and phenotypically wild-type recombinants were checked in the F_3 generation for segregation of *latd*/*nip* progeny. Gaps in the physical map were filled by chromosome walking using BLAST searches to identify BAC end sequence (BES) and BAC sequence (http://www.medicago.org/) overlaps with other existing sequenced BACs. In cases where no BAC sequence was available, hybridization of probes from specific BAC sequences to filters containing BAC DNA (CUGI, http://www.genome.clemson.edu/) was used to identify overlapping BACs. Overlaps were verified by multiple, co-migrating PCR products and confirmed by new genetic markers. Those BACs representing a minimal tiling path were sent to the Medicago Genome Project for sequencing.

Phylogenetic analysis

Arabidopsis and alder members of the *NRT1*(*PTR*) family were as reported (Tsay *et al.*, 2007). Arabidopsis sequences from TAIR (http://www.arabidopsis.org/) were aligned with ClustalX 2.0.11 (Thompson *et al.*, 1997) and visually edited with MacClade 4.08 (Maddison and Maddison, 2005). Maximum parsimony (MP) analysis was performed with MEGA4 (Tamura *et al.*, 2007) using the Close-Neighbor-Interchange algorithm with search level 3. The bootstrap consensus tree was inferred from 10 000 replicates. Partitions reproduced in fewer than 50% of bootstrap replicates were collapsed. All alignment gaps were treated as missing data. Bayesian inference of phylogeny was performed using MrBayes version 3.1.2 (Huelsenbeck *et al.*, 2001; Ronquist and Huelsenbeck, 2003). Four chains were initiated in two independent runs for 10 000 generations sampling one tree every 100 generations.

Construction of complementation and localization binary vectors

Table S3 contains the sequences of primers used. Plasmid pCY33- was made by amplifying a 6.1-kb genomic fragment containing *LATD*/*NIP* from A17 genomic DNA using primers 18F and 18R, digesting with *Pac*I and *Kpn*I and ligating into the RedRootII binary vector (Limpens *et al.*, 2004; R. Guerts, pers. comm.). Plasmid pRD100 was made by amplifying a 6.4-kb genomic A17 fragment containing *LATD*/*NIP* using primers 10F and 2R. The resulting fragment was digested with *Eco*RI and *Sac*I and ligated into pCAMBIA2300 (http://www.cambia.org/). For the p*LATD*/*NIP-GFP* construct, *GFP* cDNA from pEGAD (Cutler *et al.*, 2000) was amplified using GFP L and GFP R primers and inserted into the multiple cloning site of pGEM EasyA (Promega, http://www.promega.com/). Subsequently, a 3-kb *LATD*/*NIP* promoter fragment was amplified from A17 genomic DNA with Mt promoter L and Mt promoter R primers and cloned upstream of GFP using *Kpn*I and *Age*I sites. Then, the p*LATD*/*NIP-GFP* cassette was excised using *Kpn*I and *Pac*I and inserted into pRedRootII creating pCY44.

Agrobacterium rhizogenes transformation

Hairy root transformations were performed as described (Limpens *et al.*, 2004; Pislariu and Dickstein, 2007). Root architecture, nodulation and p*LATD*/*NIP-GFP* expression were scored 3 weeks post-inoculation.

Histochemical staining of nodules

Nodules infected with *S. meliloti* carrying the *nifH*-GUS reporter were bisected and stained overnight at 37°C in 1 mM X-Gluc, 50 mM sodium phosphate buffer, pH 7.0, and 0.02% SDS (Swanson *et al.*, 1993). Nodules were visualized on a Leica MZ8 dissecting microscope (Leica Microsystems, http://www.leica.com/). Images were captured using a 11.2 Color Mosaic camera and Spot Basic software (Diagnostics Instruments Inc., http://www.diaginc.com/). Nodules infected with *S. meliloti* carrying the *hemA-lacZ* reporter were sectioned and stained as previously described (Veereshlingam *et al.*, 2004; Pislariu and Dickstein, 2007).

Fluorescence and confocal microscopy

For primary and lateral roots, fluorescence imaging of dsRED and p*LATD*/*NIP-GFP* expression was visualized using an Olympus IX70 inverted brightfield microscope (Olympus America Inc., http://www.olympusamerica.com/). Images were captured with QImaging Retiga 2000R camera (QImaging, http://www.qimaging.com/) and QCapture Pro software version 5.1.1.14 (Media Cybernetics Inc., http://www.mediacy.com/). For nodules, 50 μm sections (Vibratome, http://www.vibratome.com/) were imaged by confocal microscopy using a Zeiss 200M microscope (Zeiss, http://www.zeiss.com/) attached to a CSU-10 Yokogawa confocal scanner with Hamamatsu camera (McBain Instruments, http://www.mcbinst.com/).

Quantitative RT-PCR

Total RNA was isolated using RNeasy (Qiagen, http://www.qiagen.com/) and contaminating DNA was removed using DNA-free (Applied Biosystems/Ambion, http://www.ambion.com/). For first-strand cDNA synthesis, 500 ng of RNA was reverse transcribed with Superscript II (Invitrogen, http://www.invitrogen.com/). Quantitative PCR was performed using the ddCt method after validation of primers (Sun *et al.*, 2006; Ding *et al.*, 2008). Reaction conditions for a 25 μl reaction included 0.25 μl cDNA, 60 nM of each primer and 12.5 μl SYBR Green Master Mix (Sigma, http://www.sigmaaldrich.com/). *LATD*/*NIP* primers used were AW684429 L and AW684429 R. Control primers used were Clathrin (MtCLA F and MtCLA R) and Ubiquitin10 (Ub10 L and Ub10 R). The primer sequences are in Table S3.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Reciprocal grafting establishes that the *latd/nip* phenotype is controlled by the root.

Figure S2. Integrated physical and genetic map in the region containing *LATD*/*NIP*.

Figure S3. Root architecture of the *latd*/*nip* mutant alleles.

Figure S4. Genotype of shoots of composite *latd* plants with roots complemented by *LATD*/*NIP* and control constructs.

Figure S5. Phylogenetic analysis of the amino acid sequences of *LATD*/*NIP*, Arabidopsis members of the *NRT1*(*PTR*) family, and *AgDCAT1*.

Figure S6. p*LATD*/*NIP-GFP* expression in nodules.

Table S1. *LATD/NIP* Affymetrix gene expression results.

Table S2. The PCR primers for genetic markers used in fine map to clone *LATD*/*NIP*.

Table S3. The PCR primers used for complementation, localization vectors and quantitative RT-PCR.

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