**MtLAX2, a Functional Homologue of the Arabidopsis Auxin Influx Transporter AUX1, Is Required for Nodule Organogenesis**

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Most legume plants can form nodules, specialized lateral organs that form on roots, and house nitrogen-fixing bacteria collectively called rhizobia. The uptake of the phytohormone auxin into cells is known to be crucial for development of lateral roots. To test the role of auxin influx in nodulation we used the auxin influx inhibitors 1-naphthoxyacetic acid (1-NOA) and 2-NOA, which we found reduced nodulation of *Medicago truncatula*. This suggested the possible involvement of the AUX/LAX family of auxin influx transporters in nodulation. Gene expression studies identified MtLAX2, a paralogue of Arabidopsis (*Arabidopsis thaliana*) AUX1, as being induced at early stages of nodule development. *MtLAX2* is expressed in nodule primordia, the vasculature of developing nodules, and at the apex of mature nodules. The *MtLAX2* promoter contains several auxin response elements, and treatment with indole-acetic acid strongly induces *MtLAX2* expression in roots. *mtlax2* mutants displayed root phenotypes similar to Arabidopsis aux1 mutants, including altered root gravitropism, fewer lateral roots, shorter root hairs, and auxin resistance. In addition, the activity of the synthetic DR5-GUS auxin reporter was strongly reduced in *mtlax2* roots. Following inoculation with rhizobia, *mtlax2* roots developed fewer nodules, had decreased DR5-GUS activity associated with infection sites, and had decreased expression of the early auxin responsive gene *ARF16a*. Our data indicate that MtLAX2 is a functional analog of Arabidopsis AUX1 and is required for the accumulation of auxin during nodule formation in tissues underlying sites of rhizobial infection.

**INTRODUCTION**

Legume plants form a symbiotic relationship with a group of soil bacteria called rhizobia, leading to the formation of specialized root organs called nodules. Within the nodules, the rhizobia are taken up into the cells where they reduce atmospheric nitrogen to ammonia, which promotes plant growth and productivity. Several previous studies have indicated an important role for the plant hormone auxin in nodule formation. We show that chemical inhibitors of auxin uptake reduced nodulation in the model legume *Medicago truncatula*. We studied the gene expression of the auxin influx carrier AUX/LAX family in *M. truncatula* and found that one member, *MtLAX2*, showed increased expression at the very early stages of nodule formation. Comparison of *MtLAX2* with other LAX genes indicated that it is the counterpart of *AUX1*, a gene that has been shown to be involved in root branching in the nonlegume Arabidopsis (*Arabidopsis thaliana*). *M. truncatula* mutants with a defective *MtLAX2* gene showed reduced responses to auxin and had fewer lateral roots and nodules compared to wild-type plants. Our findings indicate that MtLAX2-mediated auxin accumulation is important for nodule formation in legumes.
Plants integrate internal developmental cues and environmental signals to regulate root growth including the production of lateral roots for anchoring in the soil and nutrient foraging. One example of this is the formation of lateral roots in response to low nitrogen availability. The formation of lateral roots is governed by the growth hormone auxin at every stage (Lavenus et al., 2013). In Arabidopsis, oscillations in auxin signaling in the basal root meristem are correlated with future sites of lateral root emergence suggesting that initiation sites are “primed” (De Smet et al., 2007). Moreover, localized auxin signaling precedes, and is required for, the initial divisions of lateral root founder cells in the pericycle (De Smet et al. 2007; Dubrovsky et al., 2008; Laskowski et al., 2008). Both initiation and subsequent emergence of lateral roots is associated with localized increases in auxin concentration, which depend on members of the AUX-LAX family of auxin transporters (Marchant et al., 2002; Swarup et al., 2008; Swarup and Péret, 2012). A second example of root developmental responses conditioned by plant nutrient status is nodulation. Nodules are specialized lateral organs that form on roots of legumes and actinorhizal plants during symbiosis with nitrogen-fixing soil bacteria. Despite gross functional and anatomical differences, lateral roots and nodules possess some common features: cell divisions in the pericycle occur during their formation (Malamy and Benfey, 1997; Timmers et al., 1999; Lucas et al., 2013; Xiao et al., 2014); both lateral roots and indeterminate nodules feature a persistent meristem, and both organ types initiate opposite protoxylem poles, a phenomenon that, at least for nodulation, depends on ethylene signaling (Heidstra et al., 1997; Casimiro et al., 2001; Pennetsa et al., 2003; Lohar et al., 2009). Nevertheless, important differences exist: in nodule development, the pericycle divisions are accompanied by divisions in the cortex. These cortical divisions give rise to the majority of the cells in mature nodules, whereas lateral roots are comprised mainly of pericycle-derived cells (Xiao et al., 2014; Herrbach et al., 2014; de Billy et al., 2001). Developing lateral roots possess a centrally located vasculature while legume nodules develop multiple vascular strands on the periphery of the nodule (Guan et al., 2013). Interestingly, actinorhizal nodules, which are evolutionarily more ancient than legume nodules, feature a central vasculature (Péret et al., 2007). Furthermore, knockdown of several M. truncatula PLETHORA family members, encoding transcription factors that have been linked to auxin biosynthesis in Arabidopsis (Aida et al., 2004; Pinon et al., 2013; Yamaguchi et al., 2016), reduced nodulation and impaired nodule-meristem function in M. truncatula (Franssen et al., 2015). Based on these observations we can predict further overlap in the genes involved in the formation of nodules and lateral roots, but that different timing, levels, and location of expression of these genes will be important in determining which lateral organ is formed.

To date, studies on the hormonal regulation of nodule development have mainly focused on cytokinin auxin and ethylene, which can act as either positive or negative regulators of nodulation (for review, see Miri et al., 2016; Guinel, 2015). In particular, cytokinin signaling has been shown to be both necessary and sufficient for nodule formation, being required for the timely division of cortical cells leading to primordia formation (Murray et al., 2007; Tirichine et al., 2007; Gonzalez-Rizzo et al., 2006). Studies using various markers indicate that increased auxin signaling occurs at the site of nodule primordium formation in determinate and indeterminate nodules and in meristems of indeterminate nodules (Mathesius et al., 1998; Pacios-Bras et al., 2003; Suzuki et al., 2012, 2013; Breakspear et al., 2014; Roux et al., 2014). Additionally, transcripts of members of the AUX/LAX gene family, which encode auxin influx transporters, are expressed in nodule primordia (de Billy et al., 2001). Furthermore, physiological studies of the hypernodulated mutants sunn and sickle revealed that increased nodulation in these mutants is correlated with increases in auxin transport rates and auxin content of roots (Prayitno et al., 2006; van Noorden et al., 2006). Despite this large and growing body of circumstantial evidence implicating auxin in nodulation, relatively few functional studies have been carried out. Application of auxin transport inhibitors to roots can induce formation of “pseudonodules” on some legumes and RNAi silencing in M. truncatula of a set of PIN genes, which encode auxin efflux transporters, supports a role for changes in auxin distribution in nodule formation (Allen et al., 1953; Hirsch et al., 1989; Rightmyer and Long, 2011; Huo et al., 2006). While progress has been made, our understanding of the role of auxin in nodulation is limited and has been hindered by the lack of available auxin signaling and transport mutants. Here we characterize the role of MtLAX2, a functional analog of the auxin influx transporter AtAUX1. We report that mtlax2 mutants are compromised in nodulation, indicating a role for auxin influx in nodule formation in legumes.

RESULTS

Auxin Transport Inhibitors Block Nodule Development but Not Nod-Factor Signaling

To investigate the importance of influx transporter-driven auxin movement in nodulation, we used the auxin transport inhibitors 1-naphthoxyacetic acid (1-NOA) and 2-NOA, which, at low concentrations, can block auxin entry into plant cells, but do not themselves act as auxins (Delbarre et al., 1996). M. truncatula A17 seedlings pretreated for 24 h with 1-NOA or 2-NOA were inoculated with Sinorhizobium meliloti (Rm2011) and then allowed to grow for 7 d. Both 1-NOA and 2-NOA decreased the total number of nodules by 50% (Fig. 1A, left) and also decreased primary root length (Supplemental Fig. S1A). Reduction in nodule numbers was not simply a consequence of having shorter roots, as nodule density was also reduced compared to control seedlings (Fig. 1A, right). The growth of S. meliloti Rm2011 was

MtLAX2 Is Required for Nodulation in M. truncatula
unaffected by the presence of these auxin transport inhibitors (Fig. 1B).

Since nodule organogenesis requires the Nod factor signaling pathway, we tested whether auxin transport inhibitors interfere with signal transduction thereby affecting nodule initiation. First, we tested whether the symbiotic marker gene ENOD11 was activated by Nod factors in the presence of these auxin transport inhibitors. Three-day-old ENOD11pro-GUS transgenic seedlings pretreated for 24 h with 1-NOA or 2-NOA were transferred to a fresh solution containing the auxin transport inhibitor and 1 nM Nod factors for an additional 24 h. GUS staining of these seedlings revealed that 1-NOA and 2-NOA did not block induction of ENOD11 (Fig. 1C). Next, we checked whether Nod factor-induced calcium spiking was affected; calcium oscillations induced by 1 mM Nod factor were not affected by either 1-NOA or 2-NOA (Fig. 1D). We also tested effects of indole-3-acetic acid (IAA) and the auxin efflux transport inhibitor, 1-naphthylphthalamic acid (NPA) and found that both reduced nodule numbers (Supplemental Fig. S1B). Neither IAA nor NPA inhibited ENOD11 gene activation or perturbed Nod factor-induced calcium oscillations (Supplemental Fig. S1, C and D). Neither IAA nor NPA affected the growth of S. meliloti in liquid culture (Supplemental Fig. S1E). These results suggest that Nod factor signaling is not affected by auxins and auxin transport inhibitors and therefore inhibition of auxin influx may directly affect nodule development.

MtLAX2, a Parologue of the Auxin Importer AtAUX1, Is Induced by S. meliloti

We investigated the expression of the AUX-LAX family of auxin influx transporters in M. truncatula root segments spot inoculated with rhizobia (Rm2011). The MtLAX family comprises five genes (described by Schnabel and Frugoli, 2004) and the expression of one member, MtLAX2 (Medtr7g067450), was the only LAX gene significantly upregulated 16 h after inoculation compared to mock inoculated roots (Fig. 2A). Phylogenetic analysis indicates that MtLAX2 is paralogous to Arabidopsis AUX1 (Fig. 2B) and that it is most closely related to AtAUX1, as was previously reported by Schnabel and Frugoli (2004). This was confirmed by comparison of the chromosomal regions containing MtLAX2 to Arabidopsis, which revealed extensive microsynteny to the AtAUX1 and AtLAX2 regions (Supplemental Fig. S2).

Next, we investigated the spatial expression patterns of MtLAX2 in roots using the MtLAX2 promoter driving the GUS reporter. This revealed that like AtAUX1, MtLAX2 is expressed in the root meristem, lateral root primordia, and vascular bundles of uninfected roots (Supplemental Fig. S3, A–D). Upon infection with S. meliloti, we found that MtLAX2 expression was associated with proliferative cell divisions subtending sites of apparently successful infections (Fig. 2, C and D). However, not every root hair undergoing infection or curling in response to S. meliloti was associated with underlying cortical MtLAX2pro-GUS expression. This

**Figure 1.** Inhibiting auxin influx affects nodule numbers but not Nod factor signaling. A, Average nodule number (left) and nodule density (right) on M. truncatula seedlings grown in the constant presence of auxin influx inhibitors, either 50 µM 1-NOA or 5 µM 2-NOA, 7 dpi with S. meliloti (Rm2011). n = 19, 17, 20 for control, 1-NOA, and 2-NOA, respectively. Student’s t test ***P < 0.001 and *P < 0.01. Error bars depict SEM. B, Growth of Rm2011 in presence of 1-NOA and 2-NOA at the above concentrations. Bars depict SEM. C, Representative images showing staining of M. truncatula seedlings carrying the ENOD11pro-GUS reporter after 24 h pretreatment with 50 µM 1-NOA and 5 µM 2-NOA with or without 1 µM Nod factor (NF) treatment for an additional 24 h. Scale bar 800 µm. D, Calcium oscillations initiated in root hairs of M. truncatula seedlings treated with 1 µM Nod factor with addition of 50 µM 1-NOA or 5 µM 2-NOA. Numbers of root hairs testing positive for calcium spiking after treatment are indicated to the right.
MtLAX2 Is Required for Nodulation in M. truncatula

Isolation of mtlax2 Mutants

Using the Tnt1 retrotransposon insertion population available for M. truncatula we screened for lines containing insertions in MtLAX2. The gene structure of MtLAX2 is highly similar to that of AtAUX1, having eight exons (Fig. 3A). We identified two lines with independent insertions in exon four (Fig. 3A), which we designated mtlax2-1 (NF14494) and mtlax2-2 (NF16662). Sequencing of the Tnt1 insertion sites confirmed that mtlax2-1 and mtlax2-2 have insertions at 1,144 and 1,242 bps downstream of the ATG start codon, respectively. MtLAX2 expression was tested in homozygous mutants using semi-quantitative reverse transcription (RT-PCR). Full-length transcripts could not be detected at 22 PCR cycles (Fig. 3B) in either mutant line. At higher cycle numbers weak bands were produced in the mutants using gen specific primers. Sequencing of these products indicates that in both cases the different Tnt1 insertions result in aberrant splicing creating premature stop codons (Supplemental Fig. S4, B and C) generating the same truncated protein. Using a Tnt1-specific primer and a gene-specific primer we further confirmed that the MtLAX2 mRNAs are interrupted by Tnt1 sequences in each case (Supplemental Fig. S4A). Sequencing of these products indicates that in both cases the different Tnt1 insertions result in aberrant splicing creating premature stop codons (Supplemental Fig. S4, B and C) generating the same truncated protein. The transcripts produced would encode proteins missing several transmembrane regions that are present in the amino acid transport domain (Supplemental Fig. 4D). These findings indicate that mtlax2-1 and mtlax2-2 are very likely null mutants.

MtLAX2 Is a Functional Analog of AUX1

Mutations in aux1 in Arabidopsis cause development of fewer lateral roots, agravitropic growth in roots, and shorter root hairs (Marchant et al., 2002; Bennett et al., 1996; Pitts et al., 1998; Rahman et al., 2002). To test
if mtlax2 mutations cause similar phenotypes, wild-type and mtlax2 mutant seedlings were grown for 7 d in soil to compare root phenotypes. The mtlax2 mutants had shorter primary roots, fewer lateral roots, and a 50% reduction in lateral root density (Fig. 3C). The mtlax2 mutants were also insensitive to root growth inhibition by auxin when watered with 1 μM IAA for 2 weeks (Fig. 3D). Gravitropic responses measured 48 h post a 90° gravistimulus were compromised in the mtlax2 mutants, which had increased growth angles (mean = 60–72°) compared to wild type (mean = 35°; Fig. 3E; t tests, P < 1 × 10⁻⁵). Root hair length, scored on 10-d-old seedlings, was reduced by 25% to 30% in the lax mutants (Fig. 3F).

Given that the mtlax2 mutant phenocopies many of the aux1 root defects, we next attempted to complement the Arabidopsis aux1 mutant using MtLAX2 expressed from the AtAUX1 promoter. Forty independent transgenic lines carrying AtAUX1pro-MtLAX2 were isolated, but none recovered wild-type gravitropic responses or sensitivity to the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D; Supplemental Fig. S5), despite having stable expression of the transgene (Supplemental Fig. S6).
MtLAX2 Is Required for Nodulation but Not Arbuscular Mycorrhization

As MtLAX2 was highly expressed during root nodulation (Fig. 2), we also investigated whether mtlax2 mutants exhibited nodule developmental defects. One week postinoculation (wpi) with rhizobia the number of nodules was reduced by one-half in both mtlax2-1 and mtlax2-2 mutants grown in soil (Fig. 4A). The average number of nodules was also reduced in mtlax2 mutants grown on plates (Supplemental Fig. S7A, left) and this was correlated with a reduction in nodule density (Supplemental Fig. S7A, right), suggesting that the reduced nodulation in the mutant was not simply a consequence of reduced root length. A time course experiment using soil-grown plants revealed that the reduction in nodulation was significant 1 and 3 wpi but not at 4 wpi (Fig. 4B). Tests of nodulation in a segregating population of mtlax2-2 revealed that at 2 wpi, plants homozygous for mtlax2-2 had reduced nodulation, whereas heterozygotes and plants wild type for LAX2 were indistinguishable from the wild-type control (Fig. 4C); this demonstrates that the mtlax2-2 mutation is recessive and that MtLAX2 facilitates optimum nodule formation.

At 4 wpi, nodules formed on the mtlax2 mutant appeared morphologically wild type-like; that is, they were elongated and pink (Supplemental Fig. S7B). Similarly, longitudinal sections through the cortex of mtlax2 nodules (2 wpi) revealed wild type-looking symbiosomes and typical infection threads (Supplemental Fig. S7C), suggesting establishment of functional nitrogen fixation in the mutants. The expression of MtLAX2-GUS at the nodule apex (Fig. 2, G and H) suggested a potential role in nodule growth. To test this, we measured nodule length at 2 wpi and found that it was reduced in mtlax2 mutant lines (Fig. 4D). Hence, MtLAX2 appears to be required to facilitate nodule elongation, in addition to formation.

Much of the molecular machinery required for establishing nodulation and arbuscular mycorrhization is shared, and auxin has recently been implicated in interactions with arbuscular mycorrhiza (Hanlon and Coenen, 2011). To address this possibility, we tested the ability of the mtlax2-1 mutant to be colonized by arbuscular mycorrhizal fungi (Rhizophagus irregularis) and found that it was wild type-like (Supplemental Fig. S7D). Hence, MtLAX2 appears to not be required to facilitate arbuscular mycorrhization.

MtLAX2 Is Auxin Inducible and Is Required for Auxin Signaling in the Root

Many genes involved in nodulation are induced by purified Nod factors, but published data indicate that no members of the AUX-LAX family are induced by Nod factors, suggesting that the induction of MtLAX2 by S. meliloti might be indirect (Breakspear et al., 2014). In silico analysis of the 2-kb upstream promoter region of the MtLAX2 gene revealed the presence of three TGTCTC auxin responsive elements and two truncated TGTCT motifs (Fig. 5A) that the AUXIN RESPONSIVE FACTOR (ARF) family of transcription factors have been shown to bind (Ulmasov et al., 1999; Walcher and Nemhauser, 2012). To test whether MtLAX2 is auxin-inducible, we treated M. truncatula seedlings with either 1 μM IAA or its structural analog benzoic acid and monitored MtLAX2 gene expression using quantitative RT (qRT)-PCR. IAA, but not benzoic acid or the dimethyl sulfoxide carrier, strongly increased the expression of MtLAX2 (Fig. 5B). To determine whether the

Figure 4. Nodulation phenotypes of mtlax2 mutants. A, Numbers of pink and white nodules on the mtlax2 mutants 1 wpi with S. meliloti (Rm1021). Student's t test *P < 0.05, **P < 0.01, ***P < 0.001; n = 29, 28, and 29 for control, mtlax2-1, and mtlax2-2, respectively. Bars depict SEM. B, Average nodule number on wild type (R108) compared to mtlax2-1 and mtlax2-2 mutants 2 to 4 wpi with S. meliloti (Rm1021). Student's t test, *P ≈ 0.05; n = 17–22. Bars represent SEM. C, Average nodule number for different genotypes in a population segregating for mtlax2 compared to wild type (WT) (R108). Student's t test *P < 0.05; n = 38, 38, 83, and 19, respectively. D, Average nodule length on wild type and mtlax2 alleles inoculated with S. meliloti. Student's t test *P < 0.05 and **P < 0.01. For 1 wpi, n = 46, 49, and 31 and 2 wpi, n = 51, 24, and 53 for wild type, mtlax2-1, and mtlax2-2. Bars represent SEM.
observed expression of MtLAX2 in nodules was dependent on legume-specific cis elements, we analyzed a transgenic Pisum sativum GUS reporter line (AtAUX1pro-GUS) in which the GUS gene was driven by the Arabidopsis AUX1 promoter. This analysis revealed expression throughout the nodule primordia and at the nodule apex, a similar pattern to that of MtLAX2 (Supplemental Fig. S8, A–C).

MtLAX2 is predicted to increase intracellular auxin levels and thereby increase auxin signaling in those cells. To test this hypothesis, we crossed mtlax2-1 to a transgenic line carrying the DR5-GUS auxin reporter. In wild-type seedlings, auxin-regulated GUS reporter staining was strong throughout root apical meristems and in epidermal cells above the root apex (Fig. 5C, first). In mtlax2, the staining was much less than wild type and was predominantly limited to the root tip columella tissues with weak staining in neighboring cells (Fig. 5C, second). In seedlings treated with 1 μM IAA for 3 h, wild type (as expected) showed increased staining due to DR5-GUS; an increase was also seen in the mtlax2-1 mutant, but the increase was much less than that seen in wild type (Fig. 5C, third and fourth). We therefore conclude that auxin signaling in mtlax2 roots is strongly reduced.

MtLAX2 Is Required for Auxin Signaling Responses during Early Stages of Nodulation

During nodulation, the DR5-GUS marker accumulates in root hairs undergoing infection (Breakspear et al., 2014). Using the DR5 reporter as a readout of the auxin signaling pathway, we used the mtlax2-1 DR5-GUS line to query whether early hormone responses at sites of nodule formation were altered relative to the wild-type control. Seven-day-old seedlings were infected with lacZ-marked S. meliloti and differentially stained for β-galactosidase (LacZ) and GUS activity 2 d postinoculation (dpi). The number of infection events associated with DR5-GUS staining was reduced in the mtlax2 mutant (wild type = 9.7 ± 1.50 [SEM] infections/plant, mtlax2-1 = 2.1 ± 0.5, P = 0.0006, Student’s t test, n = 10 for wild type and n = 17 for mtlax2-1). In addition, in comparison to wild type, the extent of GUS staining in the vicinity of infections was reduced in mtlax2-1 (Fig. 5D).

Figure 5. mtlax2-1 shows aberrant auxin responses. A, Three full auxin responsive elements (TGTCTC) and two TGTCT motifs are present in the 2-kb promoter region of MtLAX2. B, Expression of MtLAX2 in M. truncatula roots upon a 3-h treatment with IAA (1 μM) or its structural analog benzoic acid (BA; 1 μM), as measured by qRT-PCR. Data are average of three biological replicates consisting of eight seedlings each. Student’s t test ***P < 0.001. C, Comparison of wild type (WT) (R108) and mtlax2-1 carrying the DR5-GUS reporter with and without IAA treatment. The mtlax2 mutant exhibits less GUS staining than wild type (R108), and this difference is enhanced after treatment with 1 μM IAA. Scale bars represent 100 μM. D, Representative images of mtlax2-1 and wild-type soil-grown seedlings with the DR5-GUS reporter 2 dpi with Rm1021. The mtlax2-1 mutant shows reduced DR5-GUS staining in the vicinity of infected root hairs (arrowheads) compared to wild type (R108). Scale bars represent 100 μM. E, Expression of ARF16a in the wild type and mtlax2 mutant backgrounds 2 dpi with Rm1021 relative to controls inoculated with SL44 (S. meliloti nodΔD1ABC) using qRT-PCR. Student’s t test *P < 0.05, **P < 0.01. Three to four biological replicates were used per genotype each consisting of five seedlings each. Expression values were normalized using UBIQUITIN and TIP41.
To further investigate the role of MtLAX2 in auxin responses during nodulation, we tested the expression of the early auxin responsive gene Auxin Response Factor 16a (ARF16a), which is highly expressed in emerging nodules and in infected root hairs (Breakspear et al., 2014). Seven-day-old seedlings were inoculated with Rm1021, and the root tissue was harvested at 2 dpi. To improve sensitivity, the lateral and primary root tips were removed since ARF16a is highly expressed in these tissues. ARF16a transcript levels were then monitored using qRT-PCR. A significant increase in ARF16a levels in response to rhizobial inoculation was observed in wild type but not in the mtlax2 mutants, suggesting that accumulation of auxin signaling genes at sites of infection require the auxin influx carrier MtLAX2 (Fig. 5E). Promoter-GUS analysis of ARF16a shows its expression strongly overlaps with that of MtLAX2 and both are expressed in the nodule primordia, the nodule vascular bundle, and the apex of mature nodules (Supplemental Fig. S9; Breakspear et al., 2014). However, ARF16a is strongly expressed in infected epidermal cells (Breakspear et al., 2014), a pattern that we did not observe for MtLAX2, indicating that the two genes are at least in part differentially regulated. Expression of two other infection-induced auxin responsive markers identified in Breakspear et al., 2014, MtGH3.1 and MtIAA9 (Supplemental Fig. S6E), show a similar expression pattern to that of MtARF16a and appear to be expressed more highly in wild type compared to mtlax2-1 and mtlax2-2 upon infection. However, these differences were not statistically significant.

**DISCUSSION**

The plant hormone auxin represents a critical signal during root growth and development. The AUX/LAX family of auxin influx transporters, including AUX1 and LAX3, have been shown to play key roles during primary and lateral root development (Bennett et al., 1996; Marchant et al., 2002; Swarup et al., 2008). We report that MtLAX2 plays an important role in the formation of root nodules and lateral roots in legumes, indicating common requirements for auxin influx activity for both forms of lateral organs.

During nodulation, MtLAX2 was expressed in the cortex below sites of rhizobial infection and in the developing nodule primordium. The latter finding is consistent with the report by de Billy et al. (2001), where in situ hybridization was used to study LAX gene expression. In mature nodules, expression was limited to the nodule apex, including the meristem and the distal infection zone. Expression was also seen in nodule vascular tissues in developing nodules. Nonsymbiotic expression of MtLAX2 was seen in root tips, lateral root primordia, and vascular tissues, matching the patterns observed for Arabidopsis AUX1 (Swarup et al., 2001; Péret et al., 2012). Phylogenetic and synteny analyses indicate that MtLAX2 is paralogous to AtAUX1 and in support of this, MtLAX2 has the same gene structure consisting of eight exons, a feature that distinguishes AtAUX1 and AtLAX1 from the AtLAX2/AtLAX3 clade (Swarup, and Péret, 2012). The ataux1 and mtlax2 mutants have highly similar phenotypes, including decreased primary root growth, fewer lateral roots, decreased gravitropic growth, shorter root hairs, and auxin resistance, indicating they are functionally analogous. It appears therefore that MtLAX2 was recruited into nodulation from the existing root development pathway. This is similar to genes such as the PLTs that are not symbiosis specific but are further involved in root developmental programs (Franssen et al., 2015). However, the gravitropic growth and the root hair elongation phenotypes of the mtlax2 mutants are less severe than observed for Arabidopsis aux1 mutants (Pitts et al., 1998; Fig. 3). This may be due to functional redundancy in *M. truncatula*, possibly with the close homolog MtLAX1, which is expressed at similar or higher levels than MtLAX2 in the root (Medicago Gene Atlas; Benedito et al., 2008; de Billy et al., 2001). Despite good expression, the AtAUX1pro-MtLAX2 transgene failed to complement ataux1-22. It was reported previously that aux1 could not be complemented by other Arabidopsis LAX family members due to improper intracellular trafficking of these proteins in cell types normally expressing AUX1 (Péret et al., 2012). We propose that while MtLAX2 and AtLAX1 are paralogues and are functional analogs in the context of root development, they have acquired differences at the amino acid level that have caused them to be biochemically inequivalent.

qRT-PCR data show that absence of MtLAX2 interferes with early induction of the gene encoding the ARF16a transcription factor (Fig. 5D). The reduced expression of ARF16a may be a direct consequence of decreased auxin accumulation in cells of the nodule primordia in lax2 mutants. Members of the ARF family have been shown to bind to AREs (AUXIN RESPONSE ELEMENTS) to promote (ARF activators) or repress (ARF repressors) gene expression (Ulmasov et al., 1997, 1999; reviewed by Li et al., 2016). Several ARF family members are expressed in mature nodules (Roux et al., 2014). These ARFs, which include eight predicted ARF activators, are expressed in a gradient across the nodule zones, with the highest expression seen in the nodule apex (meristem, distal infection zone), coincident with expression of cyclin genes (data from Roux et al., 2014; summarized in Murray, 2016). The strong overlap of MtLAX2 expression with auxin signaling genes such as ARF16a and cell division markers in the nodule apex is consistent with auxin’s role in cell division; mutations in MtLAX2 resulted in slower growing nodules (Fig. 4, A–D). Furthermore, MtLAX2 expression was found to be strongly induced by exogenous IAA, consistent with the presence of several AREs in the MtLAX2 promoter. This is supported by the recent findings of Herrbach et al. (2017) who reported that MtLAX2 is induced by the auxin analog 1-naphthaleneacetic acid, but not by the application of Nod factors. In Arabidopsis, two studies found AtAUX1 to be auxin inducible (Paponov
et al., 2008; Vanneste et al., 2005), but this induction was not seen in a later study (Péret et al., 2012). The strong inducibility of MtLAX2 by auxin, and the fact that the AtAUX1 promoter was functional in pea nodules, opens the possibility that regulation of MtLAX2 in nodulation could be, at least in part, regulated by auxin.

The expression of AUX1 orthologs has been studied using promoter-GUS assays in two actinorhizal plants: Casuarina glauca, which forms infection threads in curled root hairs, and Discaria trinervis, which does not form infection threads. The reported pattern of expression for the promoter of DmAUX1 in D. trinervis (Imanishi et al., 2014) is similar to what we find for MtLAX2 of in M. truncatula, with expression in the nodule meristem but not in nodule infected cells. Intriguingly, the CgAUX1 promoter gave the same pattern of expression in D. trinervis, despite its very different pattern of expression in C. glauca, where it was expressed in infected root hair cells and infected cells of the nodule but not in the nodule primordium (Péret et al., 2007). This indicates that regulation of AUX1 homologs is host specific. However, despite clear evidence for activation of auxin signaling in M. truncatula root hairs (Breakspear et al., 2014), we did not observe expression of MILAX2 in epidermal cells containing infection threads. Together, these results suggest that while presumably important, the presence of auxin itself is not sufficient to activate all auxin responsive genes in a given tissue/cell type. Instead, specific activation of gene expression could vary depending on which auxin signaling module is activated, as has been reported for lateral root development (Goh et al., 2012).

Based on these results we suggest that LAX2’s role in the enhancement of auxin signaling in infected root hairs is indirect.

Expression of MILAX2 at the site of primordia formation and in the nodule meristem implicate MtLAX2 in localized accumulation of auxin at these sites. However, it cannot be excluded that MtLAX2 effects on nodulation are also partly through its expression in other tissues. A role has been demonstrated for AtAUX1 in leaf vascular tissues for shoot-root long distance auxin transport contributing to lateral root formation and MtLAX2 is also expressed in leaves (Medicago Gene Atlas; Marchant et al., 2002). Similarly, MtLAX2 activity in aerial tissues may contribute to the shoot-to-root loading of auxin, resulting in lower auxin responses in mtlax2 roots as evidenced by the decreased expression of DR5-GUS in the mutant (Fig. 5B).

Over the last decade and a half, numerous studies using auxin markers point to a central role for this hormone in nodulation. Advancement of our understanding of nodule development will require an understanding of how these changes in auxin distribution and signaling are achieved. Here we have demonstrated the requirement for MtLAX2 in the auxin responses associated with rhizobial infection and subsequent nodule formation. However, in addition to auxin influx, auxin efflux has also been shown to be important for nodulation and lateral root formation (Huo et al., 2006; Marhavý et al., 2013; Benková et al., 2003). In lateral root formation, a model has been proposed in which the auxin efflux carrier PIN3 channels auxin to the pericycle founder cell prior to the initial division (Marhavý et al., 2013). It is possible that LAXs and PINs similarly cooperate to increase auxin levels to initiate the divisions leading to nodule formation. Candidates for this role are MtPIN2, MtPIN3, and MtPIN4, which, when knocked down, result in reduced nodule number (Huo et al., 2006). But is auxin required to trigger the first divisions of nodule initiation? Normally, nodules form only at sites associated with successful rhizobial infections, but in mutants with gain-of-function alleles in either components of Nod factor signaling, or in cytokinin perception, nodules spontaneously form in the absence of rhizobia (Tirichine et al., 2006, 2007; Singh et al., 2014; Gleason et al., 2006). This indicates that the site of nodule formation is determined by the plant, and that Nod factor signaling is directly upstream of the events leading to auxin accumulation and cell divisions. Indeed, increased expression of auxin markers is associated with spontaneous nodule formation (Suzaki et al., 2012), and we have shown a strong inhibition of nodule formation by NPA, confirming earlier work (Prayitno et al., 2006; Takenashi et al., 2011a). In contrast, transient treatment with high concentrations of NPA can induce the formation of nodule-like structures, so-called pseudonodules (Allen et al., 1953; Hirsch et al., 1989; Rightmyer and Long, 2011). Furthermore, inoculation of legume roots with rhizobia causes inhibition of polar auxin transport (Boot et al., 1999; Prayitno et al., 2006; Wasson et al., 2006), suggesting that an interruption of auxin flow may be required for nodule formation in indeterminate nodules. Evidence suggests that flavonoids, which appear to act as inhibitors of auxin efflux, may mediate localized inhibition of auxin transport during nodulation (Jacobs and Rubery, 1988; Mathiesius et al., 1998; Brown et al., 2001; Wasson et al., 2006), and it has been shown that this phenomena is dependent on the cytokinin receptor CRE1 and flavonoid biosynthesis (Plet et al., 2011). Moreover, flavonoids and other auxin transport inhibitors can rescue nodulation in cre1 mutants, indicating that flavonoids act downstream of cytokinin signaling to modulate auxin transport (Ng et al., 2015). Fitting with this model, flavonoids are produced at higher levels in the root hair differentiation zone where nodules form (Djordjevic et al., 1987; Peters and Long, 1988), and key flavonoid biosynthetic genes are specifically upregulated in nascent nodule primordia (Breakspear et al., 2014; Liu et al., 2015). One possible scenario is that flavonoid production below infection sites causes localized auxin accumulation, which further leads to increased expression of MILAX2, increasing auxin levels and leading to nodule formation. This work, through identification of LAX2, advances our understanding of the events leading to changes in auxin accumulation in nodulation and provides an essential tool to conduct future studies to understand hormone interactions during nodulation.
**Materials and Methods**

**Plant Growth Conditions and Nodulation Assays**

*Medicago truncatula* ecotypes Jemalong A17, Jester A17, and R108 seedlings were used in this study. All mutants and transgenic plants described were derivatives of either ecotype. Plants were grown in a 3:1 mixture of terragreen and sharp sand or in John Innes Cereal Mix (loam based). Plants were watered regularly as needed and kept in controlled environment chambers with a 16-h photoperiod at 20°C and 80% humidity. For nodulation assays, germinated seedlings were transferred to 4-cm-diameter pots filled with sterile 1:1 mixture of terragreen and sharp sand, and seedlings were allowed to grow for 1 week before inoculation. *Sinorhizobium meliloti* Rm1021 was inoculated at the base of each seedling using a 1-mL suspension (OD_{600} between 0.02 and 0.05 in water) added after 1 week after germination, and nodules were counted at different time points. The spot inoculation technique is described separately below.

**Inhibitor Treatment and Rhizobial Infection Assays**

Seedlings were either grown on distilled water agar or basic nodulation medium (BNM) using a filter paper sandwich method as described (Breakspear et al., 2014). Seedlings were grown vertically on 1.5% agarose slants between two filter paper squares. The required volume of chemical in the solvent carrier was mixed in 50 mL of melted agarose medium per plate. For the infection assays, germinated seedlings were pretreated on the inhibitor-containing medium for 24 h before inoculation with 1 mL of *S. meliloti* Rm1021 suspension as above. Seedlings were then grown for 7 d at 25°C in controlled environment chambers with a 16-h photoperiod, histochemically stained using X-gal and X-gluc, and infection threads were scored by light microscopy (Nikon Eclipse 800).

**Calcium Spiking Measurements**

Analysis of calcium spiking was done in the presence of the chemicals and 1 × 10^{-6} M Nod factor as described previously (Sun et al., 2015).

**Spot Inoculation and Auxin Treatment of *M. truncatula* Roots**

Jester A17 seeds were grown for 2 d on BNM medium supplemented with 1 μM aminophosphoyvinyl-Gly (Sigma) at 22°C 16 hours light/8 hours dark. *S. meliloti* Rm1021 was grown in minimal medium supplemented with 5 μM luteolin (Sigma) and diluted to a final concentration of 0.02 OD_{600} using Fahraeus plant medium. The mock treatment consisted of Fahraeus plant medium with luteolin added and then diluted the same as the inoculant. Approximately 1 μL of the *S. meliloti* suspension or mock treatment was inoculated onto the infection zone, and 16 h later, these were dissected as 2- to 3-mm segments. About 50 to 60 segments were collected per biological replicate, frozen in liquid nitrogen, and stored at −80°C. RNA was extracted using the RNeasy Micro Kit (Qiagen), and cDNA was prepared from 0.5 μg total RNA using iScript cDNA SYNTHESIS KIT according to the manufacturer’s instructions (Bio-Rad).

For treatment with auxin, overnight-germinated seedlings were grown on water-agarose medium for 3 d. Eight plants per replicate were transferred for 3 h to small petri dishes containing liquid BNM containing 1 μM IAA or the solvent control. Root tips of the treated seedlings were removed, and only the root zone with root hairs was collected for RNA extraction.

**RNA Purification from Roots and Quantitative PCR**

RNA was isolated using RNeasy plant mini kit (Qiagen) following the manufacturer’s protocol. The eluted RNA was treated with DNase (Ambion) and the quality evaluated by agarose gel electrophoresis. Then 1 μg of total RNA was used for cDNA synthesis using Superscript III (Invitrogen). These cDNA samples were diluted 20-fold in sterile double distilled water. Forward and reverse primers were added at a final concentration of 0.2 μM, to which 5 μL of the diluted cDNA and 10 μL of SYBR Green Taq Ready Mix (Sigma) were added, making a reaction volume of 20 μL. A minimum of three biological replicates was used per treatment and qPCR reactions performed using the Biорad CFX96 real-time system.

**Gene Cloning**

A 2.983-bp fragment upstream of *MtLAX2* start site was amplified from *M. truncatula* A17 genomic DNA using Phusion High-Fidelity DNA Polymerase (NEB). The fragments were cloned into pDONR201 using Gateway BP Clonase II enzyme mix (Invitrogen) and recombined into the pkGWFS7 Gateway destination vector by LR reaction to create *MtLAX2-*GUS. For cloning the *MtLAX2* cDNA, the 1,455-bp coding region was PCR amplified from a root cDNA library as described above. The primers used in this study are listed in Supplemental Table S1 (Primers).

**Generation of Stably Transformed Hairy Root Lines of *M. truncatula***

Plant roots transformed with promoter-GUS constructs were generated in *M. truncatula* A17 background by hairy root transformation as described (Breakspear et al., 2014). The composite plants were transferred to water agar with 100 μM aminophosphoyvinyl-Gly or to a mixture of equal amounts of sand and terragreen 4 weeks after transformation. The roots were then inoculated with Rm201 pXLCDA (lacZ). The roots were harvested at different time points for GUS staining, and some samples were then stained with Magental-gal (Melford) for visualization of Rm201 pXLCDA as previously reported (Pichon et al., 1994).

**Histochemical GUS Assays**

GUS staining was performed in 50 mM sodium phosphate buffer (pH 7.0) containing 1 mg/mL X-Gluc (Melford), 1 mM EDTA, and 1% Triton-X100. Samples were stained overnight and then washed with fresh buffer before imaging. For sectioning, nodule samples were fixed in 2.5% glutaraldehyde and 1% osmium tetroxide in 30% Suc. These were made into paraffin blocks (for GUS staining), and some samples were then stained with Magenta-gal (Melford) and left overnight in 30% Suc. Sections were made using the cryostar NX70 (Thermo Scientific) and images taken with a Nikon Eclipse E800 light microscope.

**Generation of *Pisum sativum* AtAUUX1-GUS Transgenic Lines**

The AtAUUX1-GUS construct (Marchant et al., 1999) was transformed into *P. sativum* using Agrobacterium tumefaciens. Transgenic shoots were selected using 5 μg/mL phosphinothricin, grafted onto wild-type shoots, and allowed to set seed. These lines were also confirmed by a PCR reaction for presence of AtAUUX1-GUS. The copy number of the Bar gene was analyzed by Southern blot as described by Sambrook et al. (1989). Lines homozygous for the construct were propagated and seedlings of the progeny analyzed.

**insertional Mutant Screening and Genotyping**

*Tnt1* retrotransposon insertions in *MtLAX2* were screened using a nested PCR approach (Cheng et al., 2011) selecting for lines with insertions in exons. R2 progeny were genotyped using primers *MtLAX2_Tnt1E* and TnIR for the *mtlax2-1* allele by hand pollination as described in the *Medicago truncatula* handbook.

**Phylogenetic Tree Construction**

Amino acid sequences for *MtLAX1-MtLAX5* were originally described by Schnabel and Frugoli (2004). Gene model and accession numbers are provided in Supplemental Table S2. Phylogenetic analysis was done using Phylogeny.fr suite of programs as follows (Dereeper et al., 2008). Amino acid sequences were aligned using MUSCLE 3.7 and refined using Gblocks 0.9b1. The phylogenetic tree was created using PhyML 3.0 and the tree was rendered using TreeDyn 198.3.

**Root Hair and Nodule Measurement**

Root hair length was measured using the Leica DFC 420 stereo microscope. Five germinated seedlings of *M. truncatula* per plate were treated and placed on Plant Physiol. Vol. 174, 2017.
between filter paper sandwiches on square petri dishes. The seedlings were grown for 7 d under long days (16 h). To measure the root hair length, the top filter paper was removed with forceps without moving the roots and placed under the microscope. Five consecutive root hair on each side of the root were measured from the base to the tip of the hair using the Leica application suite Version 4.2.0 software. Nodule length was measured similarly from tip to base after straightening them out to the same plane.

Bacterial Growth

*S. mellioti* Rm2011 hemA lacZ (Plasmid pXLCD4) was grown overnight in TY medium with appropriate antibiotics, the OD<sub>600</sub> was measured, and the CFU (colony forming units) calculated. This culture was diluted in fresh tryptone yeast extract (TY) medium to 10<sup>-5</sup> CFU. In a 48-well plate, 360 μl of sterile TY medium was added to 40 μl of the diluted culture containing the desired dilution of the chemical to be tested. A minimum of 5 replicates was used per treatment and the wells were randomly assigned per treatment. The plate was shaken at 28°C in an Infinite 2000 plate reader measuring OD<sub>600</sub> at hourly intervals for up to 70 h. The data were analyzed using Microsoft Excel 2010.

Mycorrhizal Colonization Assay

Seedlings were germinated as described above and then transferred to pots containing equal parts soil green and sand mixed with nurse inoculum containing roots of *Urtica dioica* (Allium schoenoprasum) plants infected with spores of the mycorrhizal fungus *Rhizophagus irregularis*. Plants were allowed to grow for 4 to 5 weeks before harvesting the root tissue. Roots were then washed and a root sample was collected for analysis. The fungus was visualized using an ink staining protocol (Q. Zhang et al., 2010) and quantified using the grid intersection method (McGregor et al., 1990).

Complementation Assay

For genetic complementation of aux1, *MtLAX2* cDNA was PCR amplified and fused with the Arabidopsis *Aux1* promoter (1.7 kb) and terminator (0.3 kb) in pORFaux1 (Péret et al., 2012) between Xhol and BamHI sites to create the plasmid pAML2. pAML2 was sequenced to check for PCR errors. Transformation of *A. tumefaciens* C58 (C58) and the Arabidopsis *AUX1* genes (genome version MtV4.0) were made using BLAST searches. The NCBI gene accessions are as follows: Medtr4g073770.1 (AY115845.1). MtLAX2 (Medtr4g073770.1) was grown overnight in TY medium was added to 40 μl of the diluted culture containing the desired dilution of the chemical to be tested. A minimum of 5 replicates was used per treatment and the wells were randomly assigned per treatment. The plate was shaken at 28°C in an Infinite 2000 plate reader measuring OD<sub>600</sub> at hourly intervals for up to 70 h. The data were analyzed using Microsoft Excel 2010.

Synteny Analysis

Homology between the Arabidopsis *Aux1* region (obtained from the TAIR database) and regions containing *M. truncatula LAX* genes (genome version MtV4.0) were made using BLAST searches. The NCBI gene accessions are as follows: Medtr5g0882220.2 (AY115841.1), Medtr7g067450.1 (AY115843.1), Medtr3g072870.1 (AY115842.1), Medtr4g153590.2 (AY115844.1), and Medtr4g073770.1 (AY115845.1).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers *MtLAX1 (Medtr5g0882220.2) AY115841.1, MtLAX2 (Medtr7g067450.1) AY115843.1, MtLAX3 (Medtr3g072870.1) AY115842.1, MtLAX4 (Medtr4g153590.2) AY115844.1, MtLAX5 (Medtr4g073770.1) AY115845.1.*

Supplemental Data

The following supplemental data are available.

Supplemental Figure S1. Effect of auxin and auxin influx inhibitors on root growth and nodulation in *M. truncatula*.

Supplemental Figure S2. *MtLAX2* and *AtAux1* are paralogues.

Supplemental Figure S3. Expression pattern of *MtLAX2*.

Supplemental Figure S4. *mtlax2-1* and *mtlax2-2* mutations result in mis-spliced transcripts containing premature stop codons.

Supplemental Figure S5. *MtLAX2* does not restore aux1 mutant phenotype.

Supplemental Figure S6. RT-PCR confirmation of *ataux1-22* lines complemented with *MtLAX2*.

Supplemental Figure S7. Symbiotic phenotypes of *mtlax2* mutants.

Supplemental Figure S8. GUS staining in nodules of *P. sativum* stably transformed with *AtAux1pro:GUS*.

Supplemental Figure S9. Dynamic expression pattern of *ARF16a* during nodule development.

Supplemental Table S1. List or primers used in this study. All sequences are provided in the 5′ to 3′ orientation.

Supplemental Table S2. Gene identifiers and accession numbers for AUX-LAX genes mentioned in this study.

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