The *LATD* Gene of *Medicago truncatula* Is Required for Both Nodule and Root Development

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The evolutionary origins of legume root nodules are largely unknown. We have identified a gene, *LATD*, of the model legume *Medicago truncatula*, that is required for both nodule and root development, suggesting that these two developmental processes may share a common evolutionary origin. The *latd* mutant plants initiate nodule formation but do not complete it, resulting in immature, non-nitrogen-fixing nodules. Similarly, lateral roots initiate, but remain short stumps. The primary root, which initially appears to be wild type, gradually ceases growth and forms an abnormal tip that resembles that of the mutant lateral roots. Infection by the rhizobial partner, *Sinorhizobium meliloti*, can occur, although infection is rarely completed. Once inside *latd* mutant nodules, *S. meliloti* fails to express rhizobial genes associated with the developmental transition from free-living bacterium to endosymbiont, such as *bacA* and *nex38*. The infecting rhizobia also fail to express *nifH* and fix nitrogen. Thus, both plant and bacterial development are blocked in *latd* mutant roots. Based on the *latd* mutant phenotype, we propose that the wild-type function of the *LATD* gene is to maintain root meristems. The strong requirement of both nodules and lateral roots for wild-type *LATD* gene function supports lateral roots as a possible evolutionary origin for legume nodules.

Additional keywords: bacteroid, nodule development, nodule evolution.

Formation of nitrogen-fixing legume nodules is a highly orchestrated procedure requiring the interaction of plant and bacteria to form a specialized structure, the root nodule, an organ morphologically and functionally unlike any existing plant structure. The evolutionary origin of legume root nodules has stimulated much speculation. *Rhizobium* nodulation is limited to the legume family: with one exception, only legumes nodulate. What is the origin of legume nodules? The fossil record of nodule evolution is poor, but phylogenetic analysis predicts three independent origins of nodulation within the legume lineage (Doyle et al. 1997).

The rapid appearance of root nodules in the legume lineage would suggest the recruitment of genes and pathways from an existing role in development or physiology to an additional function in nodulation (Harris 2002; Szczygłowski and Amyot 2003; Wilkens 2002). The recent identification of genes with a dual function in nodule formation and plant development, such as *Crinkle*, *Har1/Nts-1*, *Sickle*, and *Astray*, provide evidence for such recruitment. Analysis of these pleiotropic nodulation

genes has revealed connections between nodule formation and other aspects of plant development, namely ethylene signaling, root architecture, the shoot apical meristem, and light signaling (Nishimura et al. 2002; Penmetsa and Cook 1997; Schauser et al. 1998; Szczygłowski et al. 1998; Tansengco et al. 2003; Wopereis et al. 2000).

Unlike most plant organs, legume root nodules are not derived from a meristem. Rather, nodules form on the sides of roots when cortical cells respond to the bacterial nodulation signal by reentering the cell cycle and dividing. This growing primordium is invaded by rhizobial infection structures (infection threads) and bacteria ultimately are released into primordium cells. In indeterminate nodules (those that form a persistent nodule meristem), rhizobia are released into cells that have ceased dividing and a meristem forms at the end of the distal tip of the nodule. The meristem takes over growth of the nodule, producing new cells that are infected by growing infection threads. Vasculature develops in the periphery of the nodule, surrounding the central zone containing infected cells and connecting with the vascular system in the root. As the nodule grows, it eventually ruptures the overlying epidermis and emerges from the root.

Over the years, people have speculated that nodules may have evolved by modification of an existing developmental program. Actinorhizal nitrogen-fixing nodules appear to be modified lateral roots, but their relationship to legume nodules is unresolved. The actual organ or structure that legume nodules are based on is less clear; enough so that they have been suggested to have evolved *sui generis* (Hirsch and LaRue 1997). A number of other possible origins have been proposed for legume nodules: lateral roots, modifications of stems, and carbon storage organs (Caetano-Anolles et al. 1993; Hirsch and LaRue 1997; Nutman 1948; Sprent 1989).

Nodules have been compared most often to lateral roots as a result of their similar development. Like nodules, lateral roots form on the sides of roots adjacent to xylem poles, developing a meristem at the distal tip and rupturing overlying tissue layers as they emerge from the primary root. However, unlike lateral roots, legume nodules lack a root cap and have a peripheral vasculature like stems, rather than the central vasculature of roots and actinorhizal nodules. In addition, nodule and lateral root primordia are formed primarily from different tissues: the nodule from cortex and lateral roots from pericycle (Brewin 1991; Hirsch 1992). Although nodules and lateral roots derive primarily from different tissue layers, the situation is more complex: in fact, pericycle cells are activated and divide during nodule formation in *Medicago truncatula* (Timmers et al. 1999) and both pericycle and endodermal cells have been observed to divide in pea (Bond 1948). A further similarity between nodule and lateral root development in legumes is the involvement of cortical cells in the formation of legume lateral

roots (Charlton 1996). Cortical cells adjacent to lateral root primordia are activated in *M. truncatula* and often divide (Mathesius et al. 2000). Thus, the same root tissue layers are involved in nodule and lateral root development in legumes, but to different degrees. Finally, the accumulation of starch granules and the presence of transfer cells in nodules raised the possibility that legume nodules may have evolved from carbon storage organs (Caetano-Anolles et al. 1993).

The evidence for a root origin of nodules has been supported further by the identification of genes, such as the AUX1-like genes *MtLAX1*, *MtLAX2* (de Billy et al. 2001), *MtANN1* (De Carvalho-Niebel et al. 2002), *M. sativa* cyclinA2;2 (Roudier et al. 2003), and the early nodulin (*enod*) genes *ENOD11*, *ENOD12*, and *ENOD40*, that are highly expressed in both developing nodules and developing root tissue: lateral root primordia and the primary root tip (Hirsch et al. 2001; Journet et al. 1994, 2001; Papadopoulou et al. 1996). In addition, experiments in white clover indicate that cortical cells activated in lateral root formation can be induced to form nodule primordia even in mature regions of the root (Mathesius et al. 2000).

Together, these observations suggest that nodule evolution has involved extensive recruitment of preexisting developmental pathways. Although evidence exists to imply a lateral root origin for nodules, the developmental pathways that have been recruited are yet to be defined. Furthermore, much of the evidence suggesting a lateral root origin for nodule evolution is insubstantial and, as yet, no genetic links between these two structures have been defined. Here we describe the *LATD* gene of *M. truncatula*, which is required for both lateral root and

nodule development, as well as for maintenance of the primary root meristem. *LATD* provides the strongest evidence to date for shared genetic components during both nodule and lateral root development. Based on the mutant phenotypes, we propose that *LATD* functions in the maintenance of root meristems.

RESULTS

Isolation and initial genetic characterization of the *latd* mutant.

The lateral root organ-defective (*latd*) mutant was isolated in a screen for nodulation defective mutants of *M. truncatula* (see Materials and Methods). In addition to its nodule phenotype, these mutants had short, stunted lateral roots and a short primary root with few root hairs (Fig. 1). This mutant phenotype was stably inherited in the M_3 and M_4 generations and remained constant after two backcrosses. The *latd* nodule phenotype segregated 3:1 wild-type/mutant, consistent with a monogenic recessive mutation (183:555 nod-/nod⁺; $\chi^2 = 0.0072$, $P > 0.9$).

To ask whether the root and nodule defects cosegregate, we examined the segregation of mutant phenotypes in the F_2 plants from the second backcross. For this analysis, we focused on nodule maturity, root hairs, and the lateral root phenotype. We did not include primary root length as a criterion, because the length of wild-type roots and *latd* mutant roots overlap. Although 95% of wild-type primary roots are longer than *latd* roots, 5% fall within the *latd* length distribution (data not shown). We analyzed cosegregation in a population of 738 F_2

Fig. 1. Phenotypes of wild-type and *latd* mutant plants. **A**, Plants grown in soil in greenhouse for 4 weeks (not vernalized). Plant on the left is wild type (A17), plant on the right is the *latd* mutant. **B** and **D**, Wild type; **C** and **E**, *latd*. **B** and **C**, Plants grown on BNM medium (Ehrhardt et al. 1992) containing 0.1 μ M L- α -(2-aminoethoxyvinylglycine) (AVG) to increase nodulation frequency, 17 days postinoculation with Rm1021. **D** and **E**, Root hairs from 3-day-old plants grown on BNM medium (no AVG). Scale bars = 100 μ m. **F**, Primary root length of wild-type and *latd* mutant plants grown on BNM plates (no AVG). Error bars represent standard error of the mean.

individuals and found that 73% were wild-type for all three phenotypes (rh⁺, lat⁺, and nod⁺). Of the remaining 27%, we found that most were mutant for all three phenotypes. Progeny testing of individuals with questionable phenotypes confirmed that most were *latd* homozygotes exhibiting all three mutant phenotypes. The five remaining F₂ individuals segregated approximately 25% *latd* and 75% wild-type progeny, indicating that they were heterozygous for the *latd* mutation. This observation suggests that *latd* heterozygotes occasionally may exhibit a subset of mutant phenotypes. In no case did we observe a re-appearance of the mixed phenotype in the progeny. Thus, we conclude that the root hair, nodule, and lateral root phenotypes in the *latd* mutant cosegregate, suggesting that they are due to either a single mutation or two or more tightly linked mutations.

The shoot of *latd* mutant plants appears to be generally wild type. There is no fasciation of the stem, and morphology of leaves and flowers appear to be wild type. Pods, although small, are fertile. The size of the shoot, however, remains smaller than that of wild type, with few branches (data not shown). It is not clear whether the small shoot is a direct result of the *latd* mutation, or whether it is a secondary effect due to poor nutrient uptake by the small root system and few root hairs.

The *latd* mutants are defective in nodule formation.

The *latd* mutant nodules are small and white, consistent with a lack of full differentiation (Fig. 1B and C). Mature nodules are characterized by their ability to fix nitrogen. To test whether the *latd* nodules were functional, we checked for nitrogenase activity by measuring the reduction of acetylene to ethylene. We found that, at 20 days postinoculation (dpi), *latd* mutant nodules failed to reduce acetylene, whereas all wild-type plants contained nitrogen-fixing nodules (Table 1). As a negative control, we also examined wild-type plants inoculated with rhizobia containing a disruption of the *fixJ* gene, which is absolutely required for nitrogen fixation (David et al. 1988). Plants inoculated with *fixJ* mutant bacteria exhibited the same low values for acetylene reduction as *latd* plants inoculated with wild-type *Rhizobium* bacteria.

Rhizobial infection is incomplete in *latd* mutants.

Because the *latd* mutant nodules did not fix nitrogen, we asked whether they were infected with *Rhizobium* bacteria. In order to visualize the bacteria within the developing nodules, we infected *latd* mutants with *Sinorhizobium meliloti* 1021/pXLGD4, a strain that constitutively expresses the *lacZ* gene from a *hemaA::lacZ* fusion, and then stained for β -galactosidase activity (Boivin et al. 1990). We found that the *latd* mutant was infected by this strain, but that the infection was not as advanced as for wild-type roots of the same age (Fig. 2A and C). By 11 dpi, all stages of infection were present in inoculated *latd* roots, except the final stage in which rhizobia appear to fill the nodule. Instead, the most advanced infections in *latd* roots had infection threads that branched within the

nodule primordium, but individual infection threads still could be distinguished and overall staining was faint (Fig. 2F).

To determine whether *Rhizobium* infection of *latd* mutants was blocked at the final stage or merely delayed, we allowed infection to progress further before counting infection events. We found that, by 17 dpi, infection of *latd* mutants still lagged behind the wild type and *latd* mutants continued to exhibit an accumulation of stage 5 infection events (data not shown). In contrast, wild-type plants inoculated in parallel had approximately five times more “filled” nodules (stage 6) than stage 5 events. At both 11 and 17 dpi, *latd* mutant roots had approximately half the number of infection events as the wild type: *latd* mutant roots had an average of 25.2 infection events at 11 dpi and 27.4 at 17 dpi, compared with 41.3 at 11dpi and 43.6 at 14 dpi for wild-type plants. Thus, not only is the progress of infection altered in *latd* mutants, but the total number of infection events is decreased. The similar numbers of infection events for 11 and 17 dpi indicate that few new infection events occurred after 11 dpi in either wild-type or *latd* mutant plants and that the *latd* mutant did not catch up to the wild type during this time. In contrast, mycorrhizal formation is normal in *latd* mutants (M. Harrison, *personal communication*).

The *latd* mutation blocks gene expression changes associated with development of the rhizobial endosymbiont.

During the process of nodule formation, the rhizobial partner makes the transition from a free-living bacterium to an endosymbiont. This process happens in stages as the bacterium infects the root through an infection thread, is released into a vesicle, the symbiosome, in the host cell, and there differentiates into a bacteroid capable of fixing nitrogen. The developmental changes the infecting rhizobia undergo are accompanied by changes in gene expression (Oke and Long 1999a and b). In order to probe the developmental stage of *latd* nodules, we inoculated mutant seedlings with *S. meliloti* strains containing different nodule-expressed promoters fused to the *uidA* gene encoding β -glucuronidase (GUS). We chose promoter-GUS fusions that are expressed in different regions of the nodule and are associated with different stages of bacterial development. This approach has proven successful in characterizing different symbiotic mutants of pea (Voroshilova et al. 2001). We examined the expression of GUS fusions to five promoters: *nodF* (an early marker); *nex1*, *nex38*, and *bacA* (associated with the transition from free-living to bacteroids); and *nifH* (a late gene that encodes a subunit of the nitrogenase enzyme complex). Expression of the *nodF::uidA* fusion is activated in free-living rhizobia by flavonoids secreted by the host root and continues throughout infection and in the developing nodule, where it is highest in the meristematic region (Kalinowski and Long 1996; Sharma and Signer 1990). The *bacA* gene is required for survival of rhizobia within the nodule. *bacA* is expressed at low levels in free-living bacteria and

Table 1. Acetylene reduction of wild-type and *latd* mutant plants

Plant genotype ^a	Bacterial inoculum	Acetylene reduction (nmol plant ⁻¹ h ⁻¹) ^b	No. of plants assayed ^c	Standard deviation
A17	1021	2	5	1.3
A17	<i>fixJ2.3::Tn5-233</i>	0.0015	4	0.00122
<i>latd</i>	1021	0.0010	6	0.00077
Latd ⁺ siblings	1021	3	6	1.4

^a The *latd* mutants were selected from a segregating population derived from a heterozygous individual. Phenotypically wild-type (Latd⁺) siblings from the same population were assayed as controls.

^b Plants were assayed for acetylene reduction 3 weeks after inoculation.

^c All plants assayed formed nodules or nodule primordia.

throughout the nodule, although expression is highest in the infection zone (Glazebrook et al. 1993). The *nex1* and *nex38* promoters were identified in a screen for promoters that can functionally substitute for the *bacA* promoter, and are expressed in approximately the same region of the nodule as *bacA* and at very low levels in free-living rhizobia (Oke and Long 1999a; V. Oke, *personal communication*). *nifH* is expressed late in bacteroid development in a band across the nodule that corresponds to the nitrogen fixation zone (Sharma and Signer 1990).

In order to examine progression of rhizobial development within *latd* mutant nodules, we inoculated *latd* roots with the rhizobial reporter gene fusions and stained for GUS activity. Although *latd* mutant nodules have only very limited infection by *S. meliloti*, it was possible to follow expression of bacterial genes using GUS activity. We found that the *nodF::uidA* fusion, which also is expressed in free-living rhizobia, is expressed in *latd* mutant nodules with approximately the same timing as the

wild type. Expression of the *nex1::uidA* fusion, however, was induced only in a subset of *latd* mutant nodules, even at time-points when all wild-type nodules expressed the fusions. The *latd* mutant nodules never expressed the *nex38::uidA*, *bacA::uidA*, and *nifH::uidA* fusions (Table 2). Thus, *latd* mutant nodules are able to support rhizobial differentiation through the transition to *nex1* expression, but do not support later development characterized by full expression of *nex1* and induction of *nex38*, *bacA*, and *nifH* expression.

The *latd* mutation causes disorganization of the primary root meristem and blocks root growth.

In addition to its effect on nodulation, the *latd* mutation affects root architecture. Although morphology of the seedling root appears normal in the first few days after germination, the primary root grows slowly and rapidly becomes significantly shorter than wild-type roots of the same age. Lateral roots that form are short and misshapen, rarely exceeding 1 mm in length

Fig. 2. Progress of rhizobial infection of *latd* and wild-type roots. Plants were grown on BNM plates (Ehrhardt et al. 1992) containing 0.1 μ M L- α -(2-aminoethoxyvinylglycine) to increase nodulation frequency. Roots were inoculated at 3 days with Rm1021/pXLGD4 (containing a *hemA::lacZ* fusion) and infection was monitored by staining whole mount roots with X-gal, staining *Sinorhizobium meliloti* blue. **A**, The number of infection events of different stages were counted in wild-type and *latd* mutant roots at 11 days postinoculation (dpi). Each point represents the average of 3 to 5 roots. **B**, Different stages of rhizobial infection: 1, spot in the curl of a root hair; 2, infection thread in a root hair; 3, infection thread reaches the root cortex; 4, infection thread branches in the root cortex; 5, infection thread reaches the nodule primordium; 6, bacterial release into the nodule primordium (individual infection threads no longer visible.) **C** and **D**, Whole mount wild-type and **E** and **F**, *latd* mutant roots 10 dpi with Rm1021/pXLGD4, magnified $\times 25$. Wild-type roots show **C**, clear branching of infection threads within nodule primordia, while **D**, larger nodules (those emerging from the epidermis) display a darkly stained central region no longer containing obvious infection threads. **E**, The *latd* nodule primordia are generally smaller and rarely contain branched infection threads. **F**, Larger *latd* nodules rarely break the epidermis and exhibit faint blue staining, representing a small number of infection threads. Note **E**, brown pigmentation of several root cortical cells and **F**, nodule primordium cells in *latd* roots.

(Fig. 1). Furthermore, the tips of both lateral and primary roots are altered. Although *latd* primary root tips appear normal at 3 days, by 13 days more than half of *latd* mutant primary roots appear different from the wild type: mutant root tips are misshapen, mature root hair cells are found close to the root tip, and there is no obvious root cap. By 22 days, 100% of *latd* homozygotes exhibit abnormal primary root tips (Fig. 3A). This change in root tip morphology coincides with a cessation of *latd* primary root growth (Fig. 1F).

We observed that distance between the first root hair (marking the beginning of the differentiation zone) and the tip of *latd* primary roots gradually shrinks until, by 22 days, this distance is 0.68 ± 0.02 mm in *latd* mutant roots compared with 0.96 ± 0.03 mm in wild-type plants (Fig. 3A, yellow bar). The region measured comprises the root cap, the root apical meristem, and the elongation zone. In order to determine which of these zones is reduced in *latd* mutant roots, we examined longitudinal sections of 24-day-old root tips (Fig. 3B through I). We found that all three regions of *latd* root tips (the root cap, the root apical meristem, and the elongation zone) were severely abnormal with respect to morphology, size, and cell shape. Some *latd* root tips were so severely disorganized that it was not possible to identify regions corresponding to these zones (Fig. 3H and I). In less severely affected root tips, the zones were still identifiable, but each lacked characteristic features (Fig. 3D through G).

The meristem of wild-type roots is easily identifiable: organized, parallel files of cells terminate at apparent initials, small, cytoplasmically dense cells (Fig. 3C, arrow). In contrast, *latd* primary root tips appear strikingly different. Even less severely affected root tips have disorganized cell files ending in a region that lacks obvious initials and small, cytoplasm-rich cells. Rather, all cells in this region are much larger and contain small to prominent vacuoles (Fig. 3D and E). More severely affected root tips terminate in spherical bulbs of root tissue composed of rounded or asymmetric cells with no obvious axis and prominent vacuoles (Fig. 3H and I).

The elongation zone of wild-type roots is characterized by short, cytoplasmically dense cells close to the meristem, gradually changing distally to longer cells with an increasingly prominent vacuole (Fig. 3B). Extensively vacuolated cells in the distal region appear to expand radially, effectively widening the root. Only at this point do root cells begin to differentiate. In *latd* mutant roots, however, fully vacuolated, radially expanded cells are seen close to the root tip (Fig. 3D and E). Strikingly, these cells are significantly shorter than vacuolated wild-type cells at the beginning of the differentiation zone (compare Fig. 3B and D). Consistent with the observation that these cells exhibit features of cells in the differentiation zone, cells with

characteristic xylem cell wall thickenings often are visible close to the root tip in *latd* mutants (Fig. 3D and H, short arrows) but not in the wild type. In addition, the cell files in this region are disorganized: cells are not evenly aligned, cell shape is rounded and asymmetric, and cell files start and end (Fig. 3D, arrowheads). In more severely affected root tips, cell files are difficult to discern at all (Fig. 3F and H, stele and cortex, respectively). There is also an apparent loss of polarity in *latd* cortical cells. In wild-type roots, cortical cell nuclei are positioned along the outer longitudinal wall. In *latd* mutant roots, cortical cell nuclei are positioned randomly with respect to both the root surface and the root axis (i.e., longitudinal versus end walls; data not shown).

The most apical region of the root tip, the root cap, is severely affected in *latd* mutants. The root cap is a dynamic structure that protects the root apical meristem (RAM), perceives gravity, and produces border cells. In wild-type plants, mature columella cells (central root cap) are elongated along the axis of the root and contain prominent amyloplasts at the apical end of the cells (Fig. 3C, arrowheads). These cells are cytoplasm rich and contain no obvious vacuole. The *latd* columella cells are rounded cells that appear to be dissociating from each other. There are no apparent amyloplasts and the vacuole occupies almost the entire cell (Fig. 3E and G). Except for their rounded shape, *latd* root cap cells more closely resemble border cells, the root cap cells that are periodically sloughed off into the rhizosphere (Fig. 3C, cells adjacent to root). In addition, the number of “columella” cell layers is highly variable in *latd* mutant root tips. Some plants have a reduced number of these cells (Fig. 3E), some have an increased number of cell layers (although the layers are not as wide, Fig. 3G) and, in some individuals, these cells appear to be absent (Fig. 3I). Similarly, lateral root cap cells do not form an even, single layer in *latd* mutants as they do in the wild type (Fig. 3B). Rather, *latd* mutants have ragged or multiple layers of lateral root cap cells (often sloughing off to form border cells) (Fig. 3D and F) or appear to completely lack this cell type (Fig. 3H).

The *latd* mutation blocks elongation of lateral roots and causes disorganization of the lateral root tip.

Lateral roots first appear at approximately 13 days in both wild-type and *latd* mutant plants under these growth conditions. The tips of *latd* lateral roots briefly appear to be wild type; however, by 10 days after emergence from the primary root, they develop the misshapen *latd* phenotype and cease growth (Fig. 4A through D). The *latd* lateral roots only rarely develop root hairs. Staining with the nuclear dye 4',6-diamidino-2-phenylindole (DAPI) reveals a striking lack of organization in the *latd* mutant

Table 2. Percentage of nodules expressing different bacterial β -glucuronidase (*gus*) fusions at different days postinoculation^a

GUS fusion	Plant genotype	Days postinoculation (%) ^b				
		7	10	13	17	24
<i>nodF</i>	A17	100 (30)	100 (42)	100 (57)	100 (54)	100 (51)
	<i>latd</i>	100 (22)	100 (34)	100 (25)	100 (50)	100 (33)
<i>nex1</i>	A17	51 (61)	97 (67)	99 (70)	100 (57)	100 (33)
	<i>latd</i>	0 (19)	27 (30)	24 (41)	32 (28)	22 (41)
<i>nex38</i>	A17	15 (48)	52 (63)	67 (45)	85 (27)	100 (37)
	<i>latd</i>	0 (14)	0 (46)	0 (30)	0 (5)	0 (24)
<i>bacA</i>	A17	61 (51)	79 (53)	87 (39)	95 (61)	100 (38)
	<i>latd</i>	0 (27)	0 (23)	0 (36)	0 (15)	0 (62)
<i>nifH</i>	A17	0 (18)	5 (22)	24 (17)	72 (46)	100 (22)
	<i>latd</i>	0 (10)	0 (10)	0 (10)	0 (9)	0 (9)

^a Nodules with any staining at all were counted as positive. Plants inoculated with Rm1021 (containing no *gus* fusions) in parallel never stained with the GUS substrate. Plants were grown on 0.1 μ M L- α -(2-aminoethoxyvinylglycine) to increase the frequency of nodulation.

^b Number in parentheses following the percentage indicates number of nodules examined for each data point. Timepoints with greater than 95% of the nodules showing staining are marked in bold. Timepoints with no nodules staining are indicated by italics.

lateral roots compared with the wild type. Wild-type lateral roots are highly organized, with an almost white meristematic region (an area of small, dividing cells packed closely together), a root cap, and organized files of nuclei leading back up the root (Fig. 4E). In contrast, *latd* lateral roots appear to lack an area of densely packed nuclei where the meristem should be and an obvious root cap (Fig. 4F). Thus, tips of *latd* lateral roots appear to share many characteristics of *latd* primary root tips that have ceased growth (Fig. 3).

The *latd* mutation reduces the length and density of root hairs.

The earliest observable difference in the growth of *latd* roots is the reduction in number and length of root hairs. In fact, when grown on 1 μ M L- α -(2-aminoethoxyvinylglycine) (AVG), *latd* roots are almost completely devoid of root hairs (data not shown). This observation suggested that *latd* root hair formation is responsive to ethylene levels. To determine whether *latd* mutant roots would produce root hairs of wild-

Fig. 3. A comparison of *latd* and wild-type primary root tips. **A**, Progression of wild-type and *latd* primary root tips over time. Newly germinated wild-type and *latd* mutant plants were grown on BNM plates (Ehrhardt et al. 1992), and the root tip of a given individual was photographed at different times after plating (number indicates number of days after plating). Several individual wild-type and *latd* mutants were followed over 22 days. Shown are root tips from a representative wild-type and *latd* individual. Note that the *latd* root tip becomes increasingly abnormal over time: long root hairs appear close to the root tip and the root tip becomes more misshapen and less regular (days 20 and 22). Yellow bar indicates distance between the first observed root hair and the root tip for these individuals at 22 days. White scale bar = 100 μ m. Longitudinal sections through 24-day-old **B** and **C**, wild-type and **D–I**, *latd* primary root tips. **C**, **E**, **G**, and **I** are higher magnifications of the root cap and meristematic regions of the root tips in sections **B**, **D**, **F**, and **H**, respectively. Short arrows indicate xylem cells. Long arrow indicates meristem. Arrowheads in **C** indicate amyloplasts. Arrowheads in **D** indicate termination of cell file. Scale bar: **B** = 100 μ m and **C** = 10 μ m.

type length and density if provided with more ethylene, we grew *latd* mutants on increasing concentrations of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate biosynthetic precursor to ethylene. Formation of ACC is usually the rate-limiting step in ethylene formation, and ACC is rapidly metabolized to ethylene (McKeon et al. 1995). We found that *latd* root hairs, like the wild type, increase in length as the concentration of ACC increases. However, *latd* root hairs never reach the length of wild-type root hairs grown under the same conditions (Fig. 5). This suggests that *latd* mu-

tants are able to respond to ethylene and, most likely, the defect in root hair growth is independent of ethylene effects.

The *latd* mutation maps to linkage group 1.

We mapped the *LATD* gene using a genetic map created with polymorphisms between the A17 and A20 ecotypes (Choi et al. 2004). We first established a mapping population by crossing the *latd* mutant to A20 and scoring the F₂ individuals for the *latd* mutant phenotype. In order to identify linkage to a chromosome, we analyzed 15 to 30 *latd* mutant F₂ individuals

Fig. 4. Lateral root phenotypes of **A** and **B**, *latd* mutants and **C** and **D**, wild-type plants grown on 0.1 μ M L- α -(2-aminoethoxyvinylglycine). **A** and **C**, Lateral roots 3 days after emergence from the primary root and **B** and **D**, the same roots 1 week later at 10 days after emergence. Note that the *latd* mutant root grows very little between day 3 and 10, but appears increasingly abnormal. The sharp reduction of root hairs on the lower part of the wild-type root in **D** is the result of the root initially growing off the plate into the air and then returning to the media. *Medicago truncatula* roots in the air have very low numbers of root hairs. DAPI (4',6-diamidino-2-phenylindole) staining of **E**, whole-mount wild-type and **F**, *latd* mutant lateral roots focused on the center of the root. Bar = 100 μ m.

from the mapping population with three representative markers from each linkage group. Initial linkage was found with the marker NCAS, which is located in the middle of linkage group 1. Subsequent mapping with 95 *latd* homozygotes from the mapping population showed that NCAS maps 26.9 cM from the *LATD* locus. Analysis of the mapping population using other markers linked to NCAS identified several close markers. R15J11L, in particular, showed tight linkage and we were unable to separate it from the *latd* mutation by recombination in a population of 440 *latd* homozygotes (Fig. 6).

DISCUSSION

The evolutionary origin of nitrogen-fixing legume root nodules has long been a source of controversy. Comparative developmental analyses suggested lateral roots as the underlying model for legume nodules (Hirsch and LaRue 1997). This model has been supported by molecular data indicating shared gene expression between nodules and developing lateral roots (de Billy et al. 2001; De Carvalho-Niebel et al. 2002; Hirsch et al. 2001; Journet et al. 1994, 2001; Papadopoulou et al. 1996; Roudier et al. 2003) and developmental observations of lateral root-associated nodules (Mathesius et al. 2000) and aborted nodules respecified as lateral roots (Ferraioli et al. 2004). Here, we provide genetic evidence supporting a lateral root origin for legume nodules. We show that the *LATD* gene of *M. truncatula* is required for the normal function of three meristems: that of the primary root, lateral roots, and nodules. It does not appear to affect the function of the shoot meristem. These observations suggest that the function of root and nodule meristems is under common genetic control, and that the *LATD* gene plays a key role in this pathway. In addition, we show that development of the infecting rhizobial symbiont is blocked in *latd* mutants, indicating that rhizobial development is linked to a functioning nodule meristem.

The *LATD* gene is required for maintenance of the primary root meristem.

Newly germinated *latd* roots are able to grow, indicating a functional RAM. However, growth soon slows, and the root tip appears increasingly abnormal (Figs. 1 and 3). Simultaneous with the decrease in root growth, the distance between the first

Fig. 5. Length of wild-type and *latd* mutant root hairs on different concentrations of 1-aminocyclopropane-1-carboxylic acid (ACC). Plants were grown on plates containing BNM medium (Ehrhardt et al. 1992) with different concentrations of ACC (A17, $n > 19$; *latd*, $n > 5$) and root hairs 2 to 3 cm above the root tip were analyzed. Two root hairs (visible from base to tip) were measured on each root and the average root hair length of each condition plotted. Error bars represent standard error of the mean.

root hair (beginning of the differentiation zone) and the root tip diminishes, reflecting a decrease in the size of the elongation zone and meristem (Fig. 3A). In particular, the gradient of root hair development at the tip is lost, with mature root hairs close to the tip rather than developing root hairs, suggesting an absence of newly differentiating cells. By 24 days, the root tip is highly disorganized, lacking clear cell files, elongation zone, and columella. The meristem is not identifiable microscopically and growth ceases. The function of a meristem is to provide a source of dividing cells and to inhibit differentiation of cells in the meristem. The RAM of *latd* primary roots loses the ability to do both: the root no longer grows and mature xylem and root hair cells can be found in what should be the elongation zone (Fig. 3A, C, and E). Even cells in the region of the meristem have features of differentiating cells: a larger cell size and clear vacuoles (Fig. 3F, H, and I).

A number of mutations have been identified that affect root meristem growth, primarily in *Arabidopsis*: *distorted architecture 1*, *fasciata 1* and 2, and *tonsoku*, and *root meristemless 1* and 2 (Cheng et al. 1995; Kaya et al. 2001; Para and Sundås-Larsson 2003; Suzuki et al. 2004), but also in tomato, *defective embryo and meristems* (Keddie et al. 1998). In general, these mutants are characterized by disorganization of

Fig. 6. The *LATD* gene maps to the top of linkage group 1 (LG1). Genetic markers used in mapping are shown with the genetic distance between them indicated to the left of the chromosome in centimorgans (cM). To the right of the chromosome, the number of *latd/latd* homozygotes recombinant for these markers is shown over the total number of *latd/latd* homozygotes tested. The *LATD* gene maps above DK140L; however, in the absence of recombinants between R15J11L and *LATD*, we cannot place the *LATD* gene relative to R15J11L.

the meristematic region, the presence of differentiated cells close to the quiescent center, and, often, the absence of clearly discernable quiescent cells. The tips of *latd* primary roots are more disorganized than any previously described root meristem mutant, but this may be because most other mutants were characterized at an earlier stage of seedling growth than *latd*. Most of the mutations affect activity of the shoot apical meristem (SAM) as well as the RAM, indicating a central role in cell proliferation or meristem function. Only the root meristemless (*rml*) mutations *rml1* and *rml2* specifically affect root meristem function, as the *latd* mutation does (Cheng et al. 1995). *RML1* has been shown to encode the first enzyme in glutathione biosynthesis, γ -glutamylcysteine synthetase, and can be rescued by the addition of reduced glutathione (Vernoux et al. 2000). Interestingly, reducing the reduced glutathione or profilin levels in *Arabidopsis* results in plants with short primary roots and short root hairs (Ramachandran et al. 2000; Sanchez-Fernandez et al. 1997). Thus, the short root hair length and decreased root hair density in *latd* mutants (Figs. 1 and 5) may simply be a reflection of decreased meristematic activity in the *latd* root tip. It is unlikely that the *LATD* gene is the *M. truncatula* homolog of *RML1*, because addition of reduced glutathione does not appear to rescue the *latd* mutant phenotype (Y. Liang and J. M. Harris, *unpublished data*) and *RML1* activity has been shown to be essential for both initiation and maintenance of cell division in the postembryonic root, whereas our results indicate a role for the *LATD* gene only in maintenance of RAM activity. In this respect, *LATD* more closely resembles *FASCIATA1* and 2, which also are required for meristem maintenance but not initiation.

Like many meristem-defective mutants, the *latd* mutant also exhibits defects in the root cap (Kaya et al. 2001; Para and Sundås-Larsson 2003; Suzuki et al. 2004). The relationship of the root cap to the RAM is not just protective, as was once thought, but may also be instructive. Genetic ablation of root cap cells in *Arabidopsis* leads to a cessation of root growth, with differentiated cells appearing close to the root apex, thus mimicking meristem defective mutants (Tsugeki and Fedoroff 1999). Analysis of the RAM in pea suggests that the portion of the meristem that produces root cap cells is under separate control from the remainder of the root meristem, which gives rise to root tissue. As columella cells differentiate into border cells and dissociate from the root, the root cap meristem is stimulated to produce more cells (Brigham et al. 1998).

The root cap is severely affected in *latd* mutants. By 24 days, none of the roots examined had differentiated columella cells (Fig 3H and I). Rather, the cells in this position all resembled border cells in the process of dissociating. The most severely affected roots had no root cap cells at all (Fig. 3F). Border cells are considered the end fate of cells produced by the root cap meristem (Brigham et al. 1998); therefore, it may be that, in *latd* mutants, as border cells dissociate, they are no longer replaced by the meristem. Because the meristem requires the presence of root cap cells in order to function, it may be that the loss of root cap cells exacerbates the existing meristem defects, leading to a complete cessation of root growth.

A common meristematic program for roots and nodules.

Our observations that the *LATD* gene is required for normal function of both root meristems and of the nodule meristem but not for the shoot apical meristem, suggest that nodules have more in common with roots than shoots and argues against a stem origin for legume nodules. The strong requirement of both nodules and lateral roots for *LATD* gene function supports the model that nodules may have evolved from a lateral root blueprint.

Analysis of the *latd* mutant root phenotype suggests that the wild-type *LATD* gene may be required for maintenance of root and nodule meristems, rather than their establishment. This is clearly the case for the primary root meristem, which functions for several days, with the growth rate slowing, and finally stopping, at day 22 (Fig. 1). Nodule and lateral root meristems form postembryonically on the side of a root. These meristems appear more strongly affected than that of the primary root, with little or no growth occurring after the organ emerges from the root. In addition, DAPI staining reveals that the lateral root tip is highly disorganized with no obvious meristem or root cap. Malamy and Benfey (1997) have shown that, in *Arabidopsis*, lateral root emergence is a function of the primordium and does not require meristem activity, being driven largely by cell expansion. Only after emergence from the parent root does the newly forming lateral root meristem direct lateral root growth. Because *latd* mutant lateral roots are arrested shortly after emergence from the parent root, it is not clear whether the lateral root meristem is ever functional. However, *latd* lateral roots become increasingly disorganized and misshapen in the week after emergence (Fig. 4), suggesting that some cell division is occurring, although in a disorganized fashion. It is not known when the nodule meristem begins to function in wild-type nodules. A meristem-like region is visible in developing nodules before emergence from the root, but, like lateral root meristems, it may not function at that stage. Thus, in *latd* nodules and lateral roots, it is not clear whether the defect is maintenance of meristematic competence, as it is in the primary root, or whether it is a failure to establish a functional meristem in the developing primordia.

Coordinate development of plant and bacterial symbiotic partners.

The *latd* mutant nodules are defective not only in development of the plant organ but also in the development of the infecting *Rhizobium* bacteria. Infection of *latd* nodules is limited, with an accumulation of early infection stages and no nodules exhibiting later infection stages (Fig. 2). These nodules do not fix nitrogen (Table 1) and do not express the *nifH* gene, which encodes a subunit of the nitrogenase enzyme complex and is an indicator of mature nodules. Studies in pea have shown that rhizobia in Fix^- nodules do not express bacterial genes associated with the process of nitrogen fixation (Voroshilova et al. 2001). In this report, we looked at an earlier stage and examined expression of three genes associated with the developmental transition from free-living *Rhizobium* bacteria to endosymbiotic bacteroid: *bacA*, *nexI*, and *nex38* (Glazebrook et al. 1993; Oke and Long 1999a; V. Oke, *personal communication*). Of these three genes, only one, *nexI*, is expressed in *latd* mutant nodules, but staining is faint and is never seen in more than 32% of nodules examined (Table 2). The failure to express rhizobial genes associated with bacteroid development indicates that, once inside the plant, rhizobial development is dependent on progression of the plant developmental program. The inability of the *Rhizobium* bacteria to infect further suggests the lack of an appropriate substrate in *latd* nodules or the absence of a plant signal. It may be simply that cells competent for infection are not formed and, thus, the infecting *Rhizobium* bacteria are trapped within the infection thread and unable to differentiate. Alternatively, some aspect of plant nodule development may produce a signal that the infecting *Rhizobium* bacterium monitors before activating a differentiation program. The *nexI* gene encodes a protein with homology to the peroxiredoxin family of antioxidants (Oke and Long 1999a). Perhaps expression of *nexI* normally is triggered when the bacterium encounters a strongly oxidative environment. Recent studies point to a modified oxidative burst, similar to that which

often accompanies pathogen recognition, during the early stages of *Rhizobium* infection (Matamoros et al. 2003). Whether the block to infection in *latd* mutants is due to the lack of an appropriate substrate or the absence of a plant signal, our results suggest the existence of a checkpoint which blocks rhizobial infection and development if the plant host has not developed sufficiently. Interestingly, infection by mycorrhizal fungi and subsequent arbuscule formation is unaffected in *latd* mutants (M. Harrison, *personal communication*). This is consistent with a model in which the *LATD* gene is not required for symbiosis per se but, rather, for the establishment or maintenance of the nodule meristem, whose function may be a necessary prerequisite for further rhizobial infection and differentiation.

The map location excludes *latd* from being allelic with most of the described nodulation mutants. The previously unmapped *nip* mutation results in a similar phenotype to *latd*. The *nip* mutation fails to complement the *latd* mutation and appears also to be linked to the R15J11L marker (Veereshlingam et al. 2004; H. Veereshlingam and R. Dickstein, *personal communication*). Although *nip* mutants have a weaker phenotype, it seems likely that the *latd* and *nip* mutations are alleles of the same gene. Identifying the sequence of the *LATD* gene and determining a physiological role for its gene product may help to clarify the function of *LATD* in nodule formation and root development. Analysis of *LATD* gene function in legumes and nonlegumes may shed some light on the process by which ancestral legumes altered their root developmental program to accommodate a rhizobial symbiont.

MATERIALS AND METHODS

Bacterial strains and general growth conditions.

Bacterial strains used in this study are listed in Table 3. All strains are isogenic with *S. meliloti* 1021 and were grown at 30°C in Luria Bertani medium supplemented with the appropriate antibiotic when necessary: streptomycin (Sm) at 500 µg/ml, neomycin (Nm) at 50 µg/ml, spectinomycin (Sp) at 50 µg/ml, and tetracycline (Tc) at 2 µg/ml.

Plant growth conditions.

M. truncatula seed were scarified for 5 min in concentrated sulfuric acid, washed twice in sterile water, surface sterilized for 3 min in undiluted Clorox bleach, then washed five to eight times in sterile water. Seed then were imbibed for 3 h of shaking at room temperature and germinated overnight in the dark at room temperature in a damp, inverted petri dish sealed with parafilm. Young seed (less than 2 months old) were left in water at 4°C for 10 to 14 days after imbibition to increase the frequency of germination.

For assessing root and nodule phenotypes, germinated seedlings were transferred aseptically to 245-by-245-mm petri dishes (24085, Nunc, Rochester, NY, U.S.A.) containing BNM agar at

pH 6.5 (Ehrhardt et al. 1992), sealed with surgical tape (1535-1; 3M Health Care, St. Paul, MN, U.S.A.), and placed at a near-vertical position in a Conviron growth chamber (model MTR30; Winnipeg, Manitoba, Canada). Where indicated, the ethylene inhibitor AVG was added to the media after autoclaving (A 1284; Sigma-Aldrich, St. Louis). Plants to be grown for seed were either planted directly in soil (Metromix 360; Scotts-Sierra Horticultural Products Company, Marysville, OH, U.S.A.) or transplanted from plates to soil and covered with a dome for a week. Plants in soil were grown either in growth chambers or a greenhouse. Growth chambers were kept at 20 or 22°C for 16-h days at a light intensity of 195 mE m⁻² s⁻¹ and either 50 or 90% humidity. Natural light in the greenhouse was supplemented with high-pressure sodium and metal halide bulbs to achieve a 16-h day. Temperatures were maintained between 24 and 28°C during the day and 19 and 21°C at night.

Plants on plates were flood inoculated with *S. meliloti* 1021 3 days after plating, unless otherwise noted. An overnight culture of *Rhizobium* bacteria was washed and resuspended in 10 mM magnesium sulfate to a final volume of 10 times the original culture volume. The resuspended *Rhizobium* culture (10 ml) was used to flood the roots of plants growing on a plate, the excess was removed, and the plate was resealed and returned to the growth chamber. Plants in soil that had not been inoculated and all *latd* plants were fertilized every 2 to 4 weeks with Miracle-Gro according to the manufacturer's instructions.

latd Isolation and genetic characterization.

Four thousand M₂ seed from two pools (bulks B and F) each consisting of approximately 320 ethylmethane sulphonate-mutagenized M₁ individuals (gift from V. Penmetsa and D. Cook, University of California at Davis) (Penmetsa and Cook 2000), were scarified, germinated, transferred to large petri plates, and inoculated as described above. The *latd* mutation was observed segregating (3:1, wild-type/mutant) in the progeny of a putative mutant (isolated from bulk B) which did not have a strong nodule or root phenotype. It is likely that the original mutant was heterozygous for the *latd* mutation, and the mutant was maintained as a heterozygote until after the first backcross due to the low fertility of the unbackcrossed line.

The starting population for the mutagenesis was the A17 wild-type line, and A17 was used as the wild type for all experiments. The *latd* mutant was backcrossed two times to A17. The χ^2 test and cosegregation analysis was done on the progeny of the second backcross. Phenotypic characterization was done with the twice-backcrossed mutant. For mapping, the once-backcrossed *latd* mutant was crossed to the A20 line and mapped relative to CAPs, dCAPs, and microsatellite polymorphisms (Choi et al. 2004).

DAPI staining of whole mount roots.

Roots were fixed and partially cleared in 1:3 ethanol/acetic acid for 16 h, then washed in phosphate-buffered saline (PBS)

Table 3. Bacterial strains used in this study

Strains ^a	Relevant characteristics ^b	Reference or source
Rm1021	Wild type, Sm ^R	Meade et al. 1982
Rm1021/pXLGD4	<i>hemA::lacZ</i> , Sm ^R , Tc ^R	Boivin et al. 1990
DW386	<i>nodF::uidA^c</i> , Sm ^R , Nm ^R	Wais et al. 2002
VO2196	<i>bacA::uidA^c</i> , Sm ^R , Nm ^R , Sp ^R	V. Oke (<i>personal communication</i>)
SN66	<i>nex1::uidA^c</i> , Sm ^R , Nm ^R	V. Oke (<i>personal communication</i>)
AM005	<i>nex38::uidA^c</i> , Sm ^R , Nm ^R	V. Oke (<i>personal communication</i>)
CSB357	<i>nifH::uidA^c</i> , Sm ^R , Nm ^R	C. Starker and S. Long (<i>personal communication</i>)
VO2638	<i>fixJ2.3::Tn5-233</i>	Oke and Long 1999a

^a All strains are in the Rm1021 background.

^b Sm = streptomycin, Tc = tetracycline, Nm = neomycin, and Sp = spectinomycin.

^c Transcriptional fusions to the *uidA* gene.

for 30 min, stained with DAPI in PBS at 2 mg/ml for 60 min and washed twice in PBS for 30 min (Ehrhardt 1993). Roots were mounted in PBS and observed on a Nikon Epithet light microscope using a UG-1 excitation filter and a UV10 dichronic mirror and a L-420 barrier filter (all from Technical Instruments, Burlingame, CA, U.S.A.). Roots were photographed using 160T Kodak film.

Analysis of nodule morphology and function.

Nitrogen fixation of individual plants was assayed using acetylene reduction as described previously (Oke and Long 1999a). Plants were grown individually on slants of BNM agar in 18-by-150-mm tubes and flood inoculated. To increase the nodulation frequency, 0.1 μ M AVG was added to the media.

To examine *Rhizobium* infection, plants were harvested at specified days after inoculation with *S. meliloti* 1021/pXLGD4 and fixed and stained for β -galactosidase activity as described previously (Boivin et al. 1990), then treated with 20% Clorox bleach for 5 min and washed five times with water before mounting. Stained roots were examined on a Nikon Epithet light microscope.

To examine bacterial gene expression in plantar, nodules infected with *S. meliloti* strains carrying promoter-GUS fusions were sectioned and stained for GUS activity as described previously (Swanson et al. 1993). In order to carry out a time course of nodule development, only large nodules from the top of the modulated portion of the root were chosen for analysis. Nodule sections were examined on a Zeus Stem 2000C dissecting microscope and photographed with an Olympus DP11 digital camera. The same microscope-camera setup also was used to photograph fresh nodules, root tips, and lateral roots.

Root tip sections.

Root tips of 24-day-old plants grown on BNM agar and inoculated with Rm1021 at 3 days were fixed by immersion in fixative (3.5% glutaraldehyde, 1% paraformaldehyde in 0.1 M Millonig's phosphate buffer, pH 7.2) overnight at 4°C. After three washes in Millonig's buffer (5 min each), the pieces were postfixed in 1% osmium tetroxide in Millonig's buffer for 45 min at 4°C. The tissue was rinsed again in Millonig's buffer, followed by dehydration in a graded ethanol series, then cleared in propylene oxide and embedded in Spurr's epoxy resin. Semithin sections (1 μ m) were cut with glass knives on a Reichert ultracut microtome and stained with methylene blue-azure II. Digital images were captured using an Olympus BX50 upright light microscope (Olympus America, Inc., Lake Success, NY, U.S.A.) with an attached Optronics MagnaFire digital camera (Optical Analysis Corp., Nashua, NH, U.S.A.) and associated MagnaFire software (version 2.0). Exposure was set appropriately using a clip detect mode, an infrared cut filter was selected to filter out infrared light from the subject, and a white balance was done on a blank area of the slide.

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