Expression Studies on *AUX1*-like Genes in *Medicago truncatula* Suggest That Auxin Is Required at Two Steps in Early Nodule Development

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Medicago truncatula contains a family of at least five genes related to *AUX1* of *Arabidopsis thaliana* (termed *MtLAX* genes for *Medicago truncatula*-like *AUX1* genes). The high sequence similarity between the encoded proteins and *AUX1* implies that the *MtLAX* genes encode auxin import carriers. The *MtLAX* genes are expressed in roots and other organs, suggesting that they play pleiotropic roles related to auxin uptake. In primary roots, the *MtLAX* genes are expressed preferentially in the root tips, particularly in the provascular bundles and root caps. During lateral root and nodule development, the genes are expressed in the primordia, particularly in cells that were probably derived from the pericycle. At slightly later stages, the genes are expressed in the regions of the developing organs where the vasculature arises (central position for lateral roots and peripheral region for nodules). These results are consistent with *MtLAX* being involved in local auxin transport and suggest that auxin is required at two common stages of lateral root and nodule development: development of the primordia and differentiation of the vasculature.

Additional keywords: legume–rhizobium symbiosis, organogenesis, signaling.

The role and mechanism of auxin action in plant organogenesis has been the object of intensive and sustained research over many decades. The recent identification of components of auxin transport, signal transduction, and action, mainly through genetic studies of *Arabidopsis thaliana*, has opened up new approaches to study the problem in this model plant and in other species (Leyser and Berleth 1999).

We are interested in the development of nodules in leguminous plants. These symbiotic organs develop in nitrogen-poor media in response to lipochitooligosaccharidic Nod factors (LCOs) produced by the appropriate symbiotic rhizobia bacte-

ria (*Azorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Sinorhizobium*, and *Rhizobium* spp.) (Brewin 1991; Hirsch 1992; Kijne 1992; Mylona et al. 1995; Schultze and Kondorosi 1998). Normally, nodule organogenesis and infection proceed hand in hand to produce nodules in which the bacteria are enclosed in the central tissue in an environment suited to the fixation of dinitrogen. In this way, the bacteria obtain a favorable niche and the plant benefits from a new source of nitrogen. This symbiotic association is important for the agronomic and environmental value of leguminous plants.

Nodule organogenesis may be separated from infection as shown by certain rhizobia strains (e.g., *exo*[−] mutants or transzeatin secreting strains) that elicit nodules without infecting the tissue (Cooper and Long 1994; Kapp et al. 1990) and certain genotypes of *Medicago sativa* that can spontaneously produce nodules (Truchet et al. 1989). Thus, nodule development is a plant-developmental program. Moreover, it may be similar to lateral root development because both occur postembryonically from preexisting roots. For the development of the indeterminate nodules of *Medicago*, *Pisum*, *Vicia*, and *Trifolium* spp., the similarity to lateral root development is very striking in that both structures possess an apical meristem emerge from the internal tissues of the root preferentially near the xylem poles. It is generally believed that these two organs have a different ontogeny, with lateral roots arising almost exclusively from the division of pericycle cells, whereas indeterminate nodules develop mainly from the inner cortical cells. This difference is not absolute because activation and division of the pericycle cells also is at the origin of nodule development (Timmers et al. 1999; Yang et al. 1993). There is an important difference in the development of the vasculature, however, with lateral roots containing a single and central vascular bundle, whereas nodules contain several peripheral vascular strands.

The question remains whether the similarities and differences in the ontogeny of these two organs require the same or different molecular signals. Auxin rather than cytokinin is clearly a major signal promoting lateral root development (Coenen and Lomax 1997). It is generally believed that this hormone is synthesized primarily in the shoot and transported in the xylem parenchyma to the apical regions of the root in an active process known as “polar auxin transport.” From here it is transported basipetally in the root epidermis and cortex

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(Leyser 1999). Plants treated with auxin or containing abnormally high levels of this hormone produce a greater number of lateral roots (Boerjan et al. 1995; Lomax et al. 1995), whereas auxin response mutants such as *AUX1*, *AXR1*, and *AXR4* (Hobbie and Estelle 1994) produce less laterals. Detailed developmental and genetic studies suggest that this hormone acts in at least two stages of lateral root development, and the acropetal polar transport and basipetal transport are believed to be involved (Celenza et al. 1995; Laskowski et al. 1995).

Although LCOs provoke nodule initiation, it has long been held that endogenous plant hormones such as cytokinins and auxins are involved in nodule development (Hirsch and Fang 1994; Hirsch et al. 1997). The fact that certain nodulin genes (genes expressed specifically in nodules compared with roots) can be induced in roots by cytokinin or in pseudonodules produced by auxin transport inhibitors provides molecular evidence for such a hypothesis (Hirsch et al. 1997). Recently, through the use of an auxin-sensitive promoter-reporter gene construct to measure auxin accumulation in *Trifolium repens*, Mathesius et al. (1998) proposed that inhibition of polar auxin transport by rhizobially produced LCOs is a very early event, preceding nodule formation. This hypothesis is supported by Boot et al. (1999), who showed that LCOs reduce the polar auxin transport capacity of *Vicia sativa* roots. Moreover, as the pattern of expression of the auxin-sensitive promoter is very similar in the early stages of nodule and lateral root development, Mathesius et al. (1998) suggest that these two organs share similar auxin requirements for organogenesis to proceed. This group extended their studies to show that the initiation of nodulation and lateral root development leads to similar activation of cortical cells such that, in the more mature parts of the root, rhizobia are able to "hijack" the cells activated during lateral root development in order to form a nodule (Mathesius et al. 2000).

The gene *AUX1* is a key gene for lateral root development and gravitropism in *A. thaliana* and encodes a membrane protein involved in auxin uptake (Bennett et al. 1996; Marchant et al. 1999). Its activity is required for vectorially located auxin efflux carriers such as PIN2 (also called EIR1, WAV6, or AGR1) to mediate gravitropic responses (Marchant et al. 1999). The *AUX1* gene is expressed in root tips and during lateral root initiation (Marchant et al. 1999). We took advantage of the isolation and knowledge of the role of this gene in *A. thaliana* to clone relatives from *Medicago truncatula* to further investigate the role of auxin in nodulation and the parallels with lateral root development.

RESULTS

Characterization of a *M. truncatula* gene family related to *AUX1*.

In order to isolate clones related to *AUX1*-like genes that potentially play a role in the legume-rhizobia symbiosis, cDNA libraries prepared from inoculated roots or 4-day-old nodules were screened with an *A. thaliana* *AUX1*-probe. Twenty-two and six clones were isolated from the two libraries, respectively (corresponding to an abundance of 1:13,600 and 1:30,000). These clones corresponded to at least five genes named *MtLAX1-5* (*M. truncatula*-like *AUX1* genes). The number of *MtLAX1-5* clones was 10, 10, 4, 1, and 1, respectively. Recent screening of the *M. truncatula* EST banks

revealed six related clones, one related to *MtLAX1* and five related to *MtLAX3*.

Clones containing the complete amino acid sequence of *MtLAX1-3* were sequenced entirely and revealed that the proteins contain between 465 to 484 residues that show 81 to 89% amino acid identity, with most of the differences occurring in the N-terminal 40 or C-terminal 20 amino acids (Fig. 1). Structural predictions suggest that they are plasma-membrane proteins, with an N-terminal signaling sequence and at least six transmembrane-spanning regions. Searches of the nonredundant and EST databases revealed that *MtLAX1-3* are highly similar to putative proteins that are closely related to *AUX1* and encoded by a set of genes from a range of dicot and monocot species. Blast and ProDom searches showed that they also share similarity to some plant amino acid permeases, suggesting that they are new members of the amino acid-auxin permease (AAP) family of channel-type transporters (Young et al. 1999).

In comparison to *AUX1*, *MtLAX1-3* show 87, 90, and 84% amino acid identity, respectively. All amino acids in *AUX1* in which point mutations affect *AUX1*-mediated gravitropism are conserved in the three *MtLAX* proteins, except Met305 (Fig. 1). This residue is conservatively changed to leucine in the three *M. truncatula* proteins, whereas a nonconservative change to arginine (mutant *AUX1-102*) leads to altered gravitropism (Marchant and Bennett 1998; M. J. Bennett, unpublished). The conclusions from these analyses are that *MtLAX1-3* most likely have similar subcellular locations (plasma-membrane) and biochemical functions (auxin uptake) as *AUX1*.

Sequencing of a number of independent cDNA clones of *MtLAX1* and *MtLAX2* led to several observations that suggest that the translation of the mRNAs may be regulated. First, clones that contain exceptionally long regions before the initiation codons were obtained, suggesting that the *MtLAX1* and *MtLAX2* mRNAs may have 5' untranslated regions (UTRs) of at least 607 and 432 bases, respectively. Second, four bases upstream of the initiating codon of *MtLAX1*, two clones showed either a small insertion (16 bp) or deletion (31 bp), the latter removing the initiation codon, so that translation would start 10 amino acids further downstream. It is not clear how these clones have arisen, but this observation suggests that there is some regulation on translation initiation. Variation in the length of the 3' regions of the clones suggest that the mRNA of both genes have a number of possible polyadenylation sites leading to 3' UTRs of 137, 216, or 315 bases for *MtLAX1* and 311 or 472 bases for *MtLAX2*. One clone of *MtLAX2* showed a completely different 3' region, leading to a replacement of the C-terminal 77 amino acids by a different sequence of 34 amino acids. The mRNA of this clone is likely to have arisen from lack of splicing because the divergence point contains the splicing consensus sequence GT and an intron occurs at this position in *AUX1*.

Reverse transcription polymerase chain reaction (RT-PCR) analysis of *MtLAX* mRNA abundance.

With the aim to determine which of the *MtLAX* genes may be associated with nodulation, RT-PCR was employed to measure specifically the abundance of the mRNAs in different organs (roots, nodules, leaves, stems, petioles, flowers, and shoot apices) and during nitrogen starvation and root nodula-

tion (Fig. 2). The technique relied on internal standards to quantitatively measure the abundance of each mRNA (Wang et al. 1989). In order to assess the experiments and technique, the abundance of mRNAs of the *MtPR10-1* and *MtENOD12* genes also were measured and compared with previous work. *MtPR10-1* is strongly and constitutively expressed in roots but weakly transcribed in nodules and aerial organs (Gamas et al. 1998; Pingret et al. 1998). *MtENOD12* is an early nodulin gene, expressed within 1 h after rhizobial inoculation (Pingret et al. 1998) and very highly expressed in young nodules (Pichon et al. 1992). Our technique allowed us to obtain results compatible with these previous observations. We found that the level of *MtPR10-1* mRNA is much higher in roots than in other organs of the plant, showing less than a 40% variation in roots during nitrogen starvation and rhizobial inoculation (Fig. 2). *MtENOD12* is very highly expressed in nodules, particularly young ones, and its expression could be detected in roots as early as 2 h following rhizobial inoculation. We also detected mRNA of this gene in stems, which has

not been reported previously as far as we know, although the pea *ENOD12* gene is expressed in this organ (Scheres et al. 1990). The samples used in our experiments allowed us to measure up to 100- and 2,000-fold differences in the abundance of mRNA for the *MtPR10-1* and *MtENOD12* genes, respectively, demonstrating the high sensitivity of this method.

By contrast, the abundance of *MtLAX1* and *MtLAX2* mRNA varied only by nine- and fourfold, respectively, in the different samples. The abundance of both of these mRNAs is highest in roots, flowers, and stems, with lower levels detectable in all other organ samples. In roots, the abundance of *MtLAX1* mRNA is approximately twice that of *MtLAX2*, but in nodules they are approximately equivalent. In the nodulation experiment, no changes in the abundance of the mRNAs could be detected after 5 days of nitrogen starvation of the roots. Following rhizobial inoculation, an increase in *MtLAX1* mRNA was observed at 4 h, followed by a decrease at 6 h, and the level then reattained its original value at 52 h (Fig. 2). Similar but more variable fluctuations were observed in *MtLAX2*

Fig. 1. Alignment of the full-length amino acid sequences deduced from cDNAs of three *Medicago truncatula* LAX genes (MtLAX1-3) and a comparison with the AUX1 sequence from *Arabidopsis thaliana* (AtAUX1). \$ = residues in AtAUX1, which give an agravitropic phenotype in AUX1 mutants. Shadings indicate the degree of homology at each position: black = 100%; dark gray = 75%; light gray = 50%.

Fig. 2. Abundance of mRNAs of four *Medicago truncatula* genes (*MtPR10-1*, *MtENOD12*, *MtLAX1*, and *MtLAX2*) in different organs and during nodulation. Plants were grown in aeroponic cultures with ammonium nitrate as a nitrogen source for approximately 3 weeks and different organs were harvested. Some plants were starved of combined nitrogen for 1 (–N 1d) or 5 days (–N 5d). Remaining plants were inoculated with *Sinorhizobium meliloti* (S.m), and whole root systems (up to 72 h) or nodules (5 to 20 days) were harvested at different time points. The abundance of mRNAs was determined by reverse transcript polymerase chain reaction analysis with internal standards for each gene. Bars show standard deviation determined from three to six replicate analyses. Similar letters for the expression of each gene indicate that the values obtained for that gene do not differ significantly at the $P = 0.05$ level.

mRNA abundance. Note that only up to 2.5-fold changes in the root abundance of the mRNAs for *MtLAX1* and *MtLAX2* were measured during these early time points.

Determinations of *MtLAX3* mRNA revealed that the abundance of mRNA was much lower than it was for *MtLAX2* in all of the RNA samples shown in Figure 2, and thus could not be reliably determined. We did not attempt to measure the expression of *MtLAX4* and *MtLAX5*, whose mRNAs are probably of even lower abundance in roots and nodules than appeared in *MtLAX3*, judging by the abundance of clones for the different genes in the cDNA libraries (see above).

From these RT-PCR experiments, it seems that the two *LAX* genes most highly expressed in roots and nodules, *MtLAX1* and *MtLAX2*, do show some variation in expression during early stages of nodulation, but neither gene appears to be specialized for a unique role in this process because they are both expressed in other parts of the plant. Moreover, the similar expression patterns of these two genes may suggest that they play similar and mutually redundant roles in the plant.

In situ hybridization of *MtLAX* gene expression in roots and nodules.

Alterations may occur in the pattern of gene expression in plant tissues that are not detectable by measuring the abundance of mRNAs in organ extracts. In order to determine whether *MtLAX* genes show spatial changes in expression during nodulation, we used in situ hybridization. Because the abundance of *MtLAX* mRNAs is very low, we used a probe corresponding to the three full-length cDNAs (*MtLAX1-3*). Because of the similarity in sequence, the probe did not discriminate among the various *MtLAX* mRNAs. Thus, our goal was to determine whether changes occur in the pattern of expression of auxin-uptake carriers during nodulation and root development, rather than detailing the expression patterns of individual *MtLAX* genes.

In primary roots, it is clear from serial section studies that *MtLAX* genes are expressed preferentially near the tips (Fig. 3B), with expression in the older regions being much weaker and detectable only in the vasculature. In the root-tip region, the major expression is seen in the central tissues of the elongation zone, in a region corresponding to the developing vasculature (Fig. 3B and D). Expression also is seen in the starch-filled cells of the root cap (Fig. 3E and F). Low but detectable expression is observed in the cortical cells of this tip region (Fig. 3B and data not shown).

During the early stages of lateral root development, a striking expression is seen in the proximal region of the primordium in the cells closest to the primary root's central cylinder (Fig. 3H). Following the establishment of the meristem and initiation of tissue differentiation, expression in the central region became localized to the elongated cells of the developing vasculature (Fig. 3L). Expression also is seen in the peripheral layer of the root tip (Fig. 3L), which may correspond to the developing root cap (Charlton 1996). Sections hybridized with a sense probe showed no specific signal (Fig. 3J), illustrating that the observed hybridization is specific for the *MtLAX* mRNAs.

Nodule development was initiated by spot inoculation with *Sinorhizobium meliloti* in the susceptible region of the root. Expression of the *MtLAX* genes is observed in the young, elongated nodule primordium (Fig. 4B) in cells close to the

root vasculature. As the primordium develops, the expression appears stronger in these cells, which are small, include small nuclei (Fig. 4D and F), and are rich in amyloplasts (Fig 4E). These cells are separated from larger cells with larger nuclei by a thin layer of cells that may represent the original root endodermis. We therefore suggest that the strongly expressing cells may be derived from divisions of the pericycle and, perhaps, other tissues of the vasculature (see below). Expression also is seen near the periphery of the developing nodule (Fig. 4D). Serial transverse sections across two developing nodules revealed that this peripheral expression is strongest at the base and tapers off toward the apex of the developing nodule (a section taken from near the base appears in Fig. 4H). In this peripheral zone, the nodule vascular bundles will develop. Sections of mature nodules did not give any signal above the background, illustrating that the role of *MtLAX* genes in nodule development occurs primarily in the early stages. Note that on nodule sections in early and late developmental stages, a sense probe did not give any clear signal (data not shown), suggesting that the above results are specific to the *MtLAX* mRNAs.

Transverse sections of the spot-inoculated roots clearly show that the nodules develop primarily near the xylem pole and the primordium appears to be constituted of cells arising from the inner cortex, endodermis, pericycle, and vascular parenchyma (Fig. 4I and J). Many of the cells of the nodule primordium are rich in amyloplasts (Fig. 4I). The cells closest to the root's central cylinder, probably derived from the xylem parenchyma and the pericycle, show the strongest expression of the *MtLAX* genes (Fig. 4J). We noticed that the root endodermis fluoresces at the opposite sides to the developing nodule, suggesting that it has been suberized and, perhaps, lignified (Schreiber et al. 1999; Wallace and Fry 1994), whereas no such fluorescence was observed at the side of the developing nodule (Fig. 4K). As suberization restricts the exchange of metabolites and signals between the vasculature and the cortex, we examined noninoculated roots to determine where suberization commenced. Transverse sections of roots at different distances from the root tip show that the endodermis does not fluoresce in the young region of the root used for spot inoculation (Fig. 4M) but rather in slightly older regions (Fig. 4L).

DISCUSSION

Comparison of LAX genes in *M. truncatula* and *A. thaliana*.

We have shown here that *M. truncatula* contains a family of at least five genes (*MtLAX1-5*) that are closely related to the *AUX1* gene of *A. thaliana* (Bennett et al. 1996). That all of these genes encode auxin import carriers, which was recently shown for *AUX1* (Marchant et al. 1999), is suggested by the high overall sequence identity of the encoded proteins (at least 80%), conservation of key amino acids required for *AUX1* activity, and similar predictions in plasma-membrane localization and topology (Fig. 1). In *A. thaliana*, *AUX1* is part of a family of four genes, with *AUX1* playing an essential role in gravitropic responses and conferring root-growth sensitivity to exogenous auxin (Bennett et al. 1996). Three related genes, *LAX1*, *LAX2*, and *LAX3*, also presumably encode auxin import carriers, yet play roles in other physiological processes as

well. Studies on *AUX1* mutant roots (Hobbie and Estelle 1994) and expression studies on *LAX3* (M.J. Bennett, *unpublished*) suggest that both of these genes are involved in lateral root development. In *M. truncatula*, we have shown here that the three *LAX* genes most highly expressed in roots also are expressed in other organs (Fig. 2) and hence may play pleiotropic roles related to auxin uptake. In this study, we have investigated the expression of these *MtLAX* genes particularly in roots and during nodulation, a process specific to legumes, in order to examine further the role of auxin in nodule organogenesis.

Expression and role of *MtLAX* genes in root development.

In primary roots of *M. truncatula*, *LAX* genes are expressed predominantly in the developing vasculature near the root tip as well as in the root cap, with low expression detectable in the cortical cells (Fig. 3). This root-tip pattern of expression is consistent with the hypothesis from the *A. thaliana* study that *LAX* proteins facilitate local auxin transport and, particularly in roots, the redistribution of auxin exiting from the polar auxin stream to the differentiating surrounding tissues (Marchant et al. 1999). Our detection of *MtLAX* gene expression in the root cap suggests that *LAX* proteins may be involved in transporting auxin in the cells perceiving, in addition to responding, to the gravitropic stimulus, an observation consistent with radiolabeling experiments on maize root caps (Young et al. 1990).

During lateral root development of *M. truncatula*, the *LAX* genes are strongly expressed, initially in the proximal part of the early primordium. At later stages, *LAX* expression is localized to the central developing vasculature and the peripheral region, which may represent the differentiating root cap (Fig. 3). In this way, the pattern of expression, seen in primary root tips, would be attained. Expression of *LAX* genes in the primordium of lateral roots is consistent with these genes, playing an important role in lateral root development, as shown for *AUX1* in *A. thaliana* (Hobbie and Estelle 1994). Moreover, expression in the developing vasculature suggests that local auxin transport mediated by *LAX* proteins is important for auxin-regulated vasculature differentiation (Aloni 1995).

Expression and role of *MtLAX* genes in nodulation.

Inhibition of polar auxin transport seems to be one of the first steps in nodule initiation and is effected by the exogenous addition or rhizobial production of Nod factors. Their effect may be mediated by flavonoids (Lomax et al. 1995; Mathesius et al. 1998). The subsequent accumulation of auxin at the inoculation site is hypothesized to be required for the initial divisions to form the nodule primordium (Mathesius et al. 1998). Expression of *LAX* genes in this primordium (Fig. 4) may be essential for facilitating auxin transport from the polar auxin transport stream to support the development of the nodule, particularly when polar auxin transport toward the root tip resumes approximately 2 days after inoculation (Mathesius et al. 1998). At a slightly later stage, perhaps after the meristem has been initiated, *LAX* genes are expressed in the peripheral regions of the developing nodule. This expression is different from the peripheral expression seen in developing lateral roots because it is more intern and, in contrast with the root expression, is stronger at the base, tapering toward the tip of the

developing nodule. Analogous with the expression in the central region of developing lateral roots where expression is clearly associated with the developing vasculature, we suggest that the peripheral LAX expression in the developing nodules

also may be associated with vasculature differentiation at a stage before the strands become defined. It is equally possible, however, that this expression is associated with other processes occurring in the periphery of the nodule such as the

Fig. 3. Pattern of expression of *MtLAX* genes in primary roots and developing lateral roots. In situ hybridization with an antisense (A to H, K and L) or sense (I and J) *MtLAX* probe with the structure of the sections shown in A, C, E, G, K, and I, bright field after staining with toluidine blue or in B, D, F, H, J, and L, dark field where the hybridization is revealed as white silver grains. A to F, Longitudinal section of a primary root showing two major and one minor zone of expression. C and D, Developing vasculature (triangle). E and F, Root cap (arrow). A and B, Cortex (arrow). Note in E, white starch-filled amyloplasts corresponding to the statoliths involved in graviperception. G to L, Sections through developing lateral roots showing in G and H, expression in the primordium (star) particularly close to the central cylinder and in K and L, the proximal regions (star), the central procambium (arrowhead), and the peripheral regions (arrow) of the developing lateral root. I and J, No signal is seen with a sense probe. Bars = 200 μ m (A and B), 100 μ m (C and D, G to J); 65 μ m (E and F), 54 μ m (K and L).

Fig. 4. A to J, Pattern of expression of *MtLAX* genes during early stages of nodule development. **K to M,** Development of the root endodermis. **A to J,** In situ hybridization with an antisense *MtLAX* probe. Structure of the sections shown in **A, C, E, and G,** bright field after staining with toluidine blue or in **B, D, F, H, and J,** dark field where hybridization is revealed as white silver grains. **A to H,** Longitudinal sections through spot-inoculated roots 2 to 3 days after inoculation showing expression in cells close to the vasculature in **A and B,** young primordium and **C and D,** slightly older primordium. **D,** Expression is seen at the periphery of the developing nodule (arrow). **E and F,** Boxed region in **C and D** was enlarged and reveals that the proximal higher expressing cells in the nodule primordium are smaller and have smaller nuclei (star) than the more distal lower-expressing cells. These cells (star) also contain many amyloplasts. **G and H,** Tangential section through two developing nodules reveals expression in the basal peripheral regions (arrow). **I and J,** Transverse section through a spot-inoculated root shows *MtLAX* expression mainly in the proximal regions of the nodule primordium (arrow), including cells derived from the vascular parenchyma. **K to M,** UV epifluorescence microscopy showing **K,** root endodermis (arrowheads) fluorescing at the sides opposite to the developing nodule (cross). In roots before spot inoculation the endodermis shows. **L,** Uniform fluorescence in the older regions of the root. **M,** No fluorescence near the root tips. Bars = 160 μ m (**A to D, G and H**), 70 μ m (**E and F**), 50 μ m (**I and J**), and 80 μ m (**K to M**).

establishment of the nodule endodermis (Brewin 1991). It is noteworthy that Mathesius et al. (1998) reported expression of an auxin-responsive promoter in the peripheral regions of developing clover nodules, which is consistent with our studies on *M. truncatula*.

Comparison of early lateral root and indeterminate nodule development.

Many similarities exist between the development of lateral roots and indeterminate nodules. Both organs develop postembryonically from near the xylem poles of preexisting roots. Moreover, there is evidence that activation and division of the pericycle is at the origin of both organs. For example, in alfalfa, Timmers et al. (1999) have shown that pericycle divisions precede inner cortical cell divisions in nodule initiation and, as shown initially in soybean (Yang et al. 1993), the pericycle expresses the early nodulin gene *ENOD40*. We have shown here that *LAX* gene expression in the nodule primordium appears to be associated with cells derived from divisions of the pericycle (Fig. 4), supporting an important role for the pericycle in nodule development in *M. truncatula*. Thus the differences in the ontogeny of lateral roots and indeterminate nodules may be more quantitative than qualitative, with divisions of the inner cortex providing the bulk of the nodule primordium, whereas the lateral root primordium is composed predominantly of pericycle-derived cells. We also have seen that the vasculature parenchyma divides during these initial stages of nodule primordium formation, which is similar to observations of lateral root formation in white clover (Mathesius et al. 1998).

There is increasing evidence that auxin is involved in the development of the nodule primordium, in addition to lateral root primordia. For lateral roots, there is clear genetic and physiological evidence that auxin is required in at least two stages: the initiation of pericycle divisions to form the primordium and the establishment and maintenance of the meristem (Celenza et al. 1995; Laskowski et al. 1995). Until recently, however, the role of auxin in nodulation has been less clear. The observations that polar auxin transport inhibitors provoke nodule-like structures that express some nodulin genes (Hirsch et al. 1997) and rhizobia via Nod factors inhibit polar auxin transport before primordium development (Boot et al. 1999; Mathesius et al. 1998) clearly suggest a role for auxin in the very early steps of nodulation. Our observation that *MtLAX* genes are expressed in the primordium of both organs suggests that the development of these organs have similar auxin requirements that may be met, at least in part, by transport via the *LAX* proteins.

Another important similarity between nodule and lateral root development is that both organs initiate preferentially in a region of the root proximal to the meristem and opposite the protoxylem poles (Charlton 1996; Laskowski et al. 1995; Mylona et al. 1995). In mature-root tissues, careful observation by Mathesius et al. (2000) has shown that nodules generally initiate only from developing lateral roots, which they termed "lateral root-associated nodules" or LRANs. The hijacking of lateral roots by rhizobia to form nodules, as aptly described by Mathesius et al. (2000), provides additional evidence of the similarities in the initial development of lateral roots and nodules, posing some interesting questions concerning the initiation of nodule development.

It has been hypothesized that nodule initiation requires opposing gradients of factors emanating from the vasculature and the rhizobia (Libbenga et al. 1973). One of the vasculature factors may be uridine, which was identified as the stele factor from pea able to initiate cortical cell division in the presence of plant hormones (Libbenga et al. 1973; Smit et al. 1995). Another factor may be auxin, as suggested above. The signal emanating from rhizobia may be either Nod factors or plant factors elicited by Nod-factor perception. Mathesius et al. (2000) suggest that the lack of nodule development in mature parts of the root is a result of the lack of production or perception of the rhizobia-derived and/or induced signal. We suggest that an alternative explanation is the lack of the vasculature signal. We have shown here that the endodermis does not fluoresce in the young regions of the root where nodules initiate but rather in slightly older regions where it fluoresces at the sides lacking developing nodules or lateral roots (Fig. 4J to L). This fluorescence may suggest a suberization and, perhaps, lignification of the cell walls which, together with the casparian strips between the endodermal cells, may effectively restrict the free flow of signals from the stele into the cortex in addition to inhibiting metabolite transport into the vasculature (Schreiber et al. 1999; Wallace and Fry 1994). LRANs may form because the lateral roots either allow an outflow of the vasculature factors required for nodulation or have advanced organ initiation past the stage where these factors are required, thus allowing rhizobia to initiate nodulation.

Model for auxin involvement in early nodule development.

In summary, we have shown that *M. truncatula* contains a small family of genes related to the *AUX1* auxin import family (*LAX* genes). These genes are expressed in the nodule primordium, providing further support for the role of auxin in nodulation. Expression of *LAX* genes in corresponding locations in the early stages of lateral root and nodule formation suggests that local auxin transport, mediated by *LAX* proteins, may be required at two common stages in the development of these two organs: the development of the primordia and the initiation and differentiation of the vasculature.

Our results and those of others (Boot et al. 1999; Mathesius et al. 1998; Mathesius et al. 2000) are consistent with the following model of auxin involvement in early nodule development (Fig. 5). Inhibition of polar auxin transport by Nod factors or Nod-factor-elicited messengers causes a local increase in auxin concentration at the site of nodule initiation. This increase in auxin promotes divisions in the pericycle and cortical cells and the nodule primordium is initiated. Expression of *LAX* proteins and, perhaps, other auxin transporters in the primordium forms a conduit for auxin, allowing polar auxin transport toward the root tip to resume without starving the nodule primordium of auxin. Nodulation initiation can occur efficiently only in regions of the roots where the endodermis is not yet suberized (the root-tip region) or in association with lateral roots where the endodermis is breached. Clearly, further cytological and physiological studies, coupled with studies of early nodulation mutants, is required to verify this model.

MATERIALS AND METHODS

Plant growth.

M. truncatula (line J5) was generally grown in aeroponic conditions, as described by Gamas et al. (1996). The plants

were grown for approximately 3 weeks with 5 mM ammonium nitrate containing medium, then starved of this nitrogen source for 5 days before inoculation with *Sinorhizobium meliloti* GMI 6526 (Ardourel et al. 1994). Whole root systems from two or three plants were collected at intervals, quickly frozen in liquid nitrogen, and stored at -80°C .

For the in situ hybridization experiments, seedlings were grown on agar plates and nodulation was induced by spot inoculation with *S. meliloti* GMI 6526, as described by Mathis et al. (1999). Root tips (about 1 cm) were harvested 2 to 3 days later, fixed, and embedded as described.

Isolation and characterization of *AUX1*-like *M. truncatula* cDNAs.

Approximately 180,000 PFU of a cDNA library prepared from 4-day-old nodules (Gamas et al. 1996) and approximately 300,000 PFU of a cDNA library from roots harvested 6, 24, and 48 h following rhizobial inoculation (Szybiak-Strozycka et al. 1995) were screened essentially as described by Gamas et al. (1996) with a radiolabeled *Pst*I-*Xba*I fragment isolated from an *A. thaliana* *AUX1* cDNA clone (Bennett et al. 1996). Hybridizing clones were plaque-purified, converted to plasmid clones in the vector pBluescriptSK⁻ (Stratagene, La Jolla, CA, U.S.A.), and classified by restriction mapping and DNA sequencing. Full-length cDNAs were sequenced on both strands by specific primers either manually with Sequenase (Amersham Pharmacia Biotech, Amersham, U.K.) or automatically with an ABI 373A automatic sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Sequence data were analyzed by the GCG software (Genetics Computer Group, Madison, WI, U.S.A.) and the NCBI Blast server. Protein localization predictions were made with the P-

SORT program (Nakai and Kanehisa 1992). Sequence alignments were performed with CLUSTALX (Thompson et al. 1997) and annotated with GeneDoc (Nicholas and Nicholas 1997).

RT-PCR.

Total RNA was extracted from plant tissue, essentially as performed by Jackson and Larkins (1976). Contaminating DNA was digested with 10 U of RNase-free DNaseI (Roche Diagnostics, Mannheim, Germany) at 37°C for 30 min. RNAs were then cleaned by phenol-chloroform extraction, precipitated with ethanol, and resuspended in 10 μl of water. To measure the abundance of specific mRNAs in the total RNA samples, we used internal standard RNAs (stRNAs) for quantification of the RT-PCR, as described by Wang et al. (1989). They were obtained by in vitro transcription with standard DNAs (stDNAs) as templates. These stDNAs contained small deletions in the cDNAs and were prepared by PCR with chimeric primers as follows. The forward chimeric primers were designed to introduce the T7 promoter in front of the stDNA, by adding 17 bases of its consensus sequence (TAATACGACTCACTATA) (Martin and Coleman 1987) in front of the forward primer chosen for the RT-PCR. The reverse chimeric primer was designed to introduce a 9- to 30-bp deletion at the 3' end of the fragment amplified by RT-PCR, as described by Celi et al. (1993), and consisted of the chosen reverse RT-PCR primer followed by a 20-to-22-bases sequence of the gene of interest located 9 to 30 bases upstream. The PCR fragments amplified with these chimeric primers were purified by gel electrophoresis and 50 ng were used for in vitro transcription with T7 RNA polymerase (Promega, Madison, WI, U.S.A.), following the supplier's protocol. The stRNAs were treated with 10 U of RNase-free DNase (Roche Diagnostics) for 10 min at 37°C .

Coupled RT-PCR was performed with Titan enzyme mix (avian myeloblastosis virus for the reverse transcription and Expand High-Fidelity enzyme mix for the PCR; Roche Diagnostics). Each tube contained 10 to 100 ng of total RNA, 0.01 to 100 ng of stRNA, 0.2 μl of the Titan enzyme mix, 1 U of RNase Inhibitor (Life Technologies, Paisley, U.K.), 5 mM dithiothreitol, 0.5 μM of a specific forward and reverse primer, 0.2 mM deoxynucleoside triphosphate (each), and 0.05 μCi of $\alpha^{32}\text{P}$ -dGTP in a total volume of 10 μl . For each sample, the ratio between total RNA and stRNA was optimized in preliminary experiments to obtain bands of the same order of intensity for the mRNA and stRNA. RT-PCR was performed in a thermal cycler with 30 min at 55°C (to reverse transcribe *MtPR10-1* and *MtENOD12*) or 60°C (to reverse transcribe *MtLAX1-3*), followed by a 2-min denaturation step at 94°C , and 35 PCR cycles (5 s at 94°C , 15 s at 60°C , 45 s at 68°C), and a final elongation step of 10 min at 68°C . Bouaboula et al. (1992) showed that cDNA and stDNA templates are amplified in a parallel manner throughout the PCR, up to the late, nonexponential phase. Using this procedure, the sizes of the RT-PCR products obtained from the mRNA and stRNAs were, respectively: *MtLAX1*, 248 and 239 bp; *MtLAX2*, 329 and 315 bp; *MtLAX3*, 130 and 101 bp; *MtENOD12*, 220 and 195 bp; *MtPR10-1*, 285 and 260 bp. RT-PCR products were separated on 5% polyacrylamide gels and radioactive bands were quantified by a phosphorimager and ImageQuant software (Amersham Pharmacia Biotech). The

Fig. 5. Model of changes in auxin transport during early nodule development. **A**, Uninoculated roots exhibit an acropetal polar auxin transport in the vasculature and a basipetal transport in the cortex and epidermis of the root tips. **B**, Inoculation with rhizobia that produce Nod factors results in an immediate but transient inhibition of polar auxin transport and the accumulation of auxin in all the tissues surrounding the blockage transport (gray dots) (Mathesius et al. 1998), stimulating cell divisions in the cortex and pericycle (rectangles). **C**, Expression of auxin transporters directs auxin transport to the developing nodule and the normal root polar auxin transport and basipetal auxin transport resumes.

picogram estimation of target mRNA per nanogram of total RNA was calculated by the formula $Q_{\text{mRNA}} = (Q_{\text{stRNA}} \times R) / Q_{\text{totalRNA}}$, where Q_{stRNA} is the quantity (in picograms) of stRNA in the tube, R the ratio of band intensities mRNA/stRNA, and Q_{totalRNA} the quantity (in nanograms) of total RNA in the tube. We observed that these absolute values varied slightly among replicate experiments, but the relative differences throughout the samples were conserved. Therefore, the levels of mRNA were expressed as percentage of the most abundant value (100%) in a series of samples. RNA samples from at least two independent experiments were analyzed for each condition and the abundance of each mRNA was measured in three to six replicate analyses of each RNA sample. Relative values were used to obtain the average and standard deviation for each mRNA in each sample and statistical analysis was performed (Student test with $\alpha = 0.05$).

In situ hybridization.

RNA probes for in situ hybridization were produced with an in vitro transcription kit (Promega) and ^{35}S -UTP αS (NEN Life Science Products Inc., Boston, MA, U.S.A.) diluted to a specific activity of 80 Ci of ^{35}S -UTP αS per mmol to provide a concentration of 12.5 μM UTP in the reaction mixture. DNA templates were full-length cDNA clones of *MtLAX1-3*, which were linearized at either side of the pBluescript vector and transcribed with either T7 or T3 RNA polymerase to produce antisense and sense RNA probes, respectively. The probes were degraded to about 200 bases in length by alkaline hydrolysis. Equal quantities of the three antisense or sense probes were mixed together and 100 μl (approximately 600,000 cpm) of these mixed probes were used per slide. Each slide contained either a whole nodule, root tip, or root segment cut into serial sections. The localization of expression of *MtLAX* genes was carried out, essentially as described by Mathis et al. (1999), except that the hybridization was carried out in 50% formamide at 42°C, the washing in 2 $\times$ SSC at room temperature, and the slides were exposed for 1 month. At least ten and five slides were used for antisense and sense probes, respectively, for each developmental stage.

Epi-fluorescence of root transverse sections.

Roots were fixed as described (Mathis et al. 1999) and stained with 0.2% eosine B in alcoholic solution. Sections from either paraplast-embedded material (7 μM) or nonembedded material (50 μM) were observed under UV illumination with an Axiphot II microscope (Carl Zeiss Inc., Thornwood, NY, U.S.A.).

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