Rhizobium meliloti Elicits Transient Expression of the Early Nodulin Gene ENOD12 in the Differentiating Root Epidermis of Transgenic Alfalfa

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To study the molecular responses of the host legume during early stages of the symbiotic interaction with Rhizobium, we have cloned and characterized the infection-related early nodulin gene MtENOD12 from Medicago truncatula. In situ hybridization experiments have shown that, within the indeterminate Medicago nodule, transcription of the MtENOD12 gene begins in cell layers of meristematic origin that lie ahead of the infection zone, suggesting that these cells are undergoing preparation for bacterial infection. Histochemical analysis of transgenic alfalfa plants that express an MtENOD12 promoter-β-glucuronidase gene fusion has confirmed this result and further revealed that MtENOD12 gene transcription occurs as early as 3 to 6 hr following inoculation with R. meliloti in a zone of differentiating root epidermal cells which lies close to the growing root tip. It is likely that this transient, nodulation (nod) gene-dependent activation of the ENOD12 gene also corresponds to the preparation of the plant for bacterial infection. We anticipate that this extremely precocious response to Rhizobium will provide a valuable molecular marker for studying early signal exchange between the two symbiotic organisms.

INTRODUCTION

The symbiotic interaction between prokaryotic rhizobia and leguminous plants leads to the formation of novel plant organs known as root nodules. Within these organs, the microsymbiont uses photosynthate-derived energy to convert atmospheric nitrogen into ammonia, a form of fixed nitrogen that can be assimilated by the plant host. A complex interplay between the legume host and its bacterial partner is required to assure the induction and subsequent development of the nitrogen-fixing root nodule.

In the case of temperate legumes such as pea and alfalfa, the first morphological event marking the initial symbiotic interaction with Rhizobium is the characteristic curling of root hairs into so-called "shepherd's crooks." Specialized tubular structures known as infection threads then convey the microsymbiont from the root hair tip toward the root inner cortex, where the initiation of cell division has already led to the formation of the nodule primordium. As infection threads penetrate and ramify within the primordium, releasing bacteria into the central tissue, a zone of apical meristematic activity directs outward growth of the differentiating nodule. Mature pea and alfalfa nodules are cylindrical in shape because these nodules, known as indeterminate, possess persistent apical meristems. A longitudinal section through such a nodule reveals the entire series of developmental steps that correspond to the concerted codifferentiation of the two symbiotic partners. The apical (or distal) meristematic zone I is followed consecutively by the prefixing zone II (zone of infection), the amyloplast-rich interzone II/III, the nitrogen-fixing zone III, and finally zone IV of senescence (Vasse et al., 1990).

Recent research has shown that multiple signal exchange is essential for the correct recognition between the plant and the microsymbiont (for reviews, see Long, 1989; Fisher and Long, 1992; Verma, 1992). In particular, rhizobial nodulation (nod) genes, whose transcription requires plant flavonoids, are responsible for the synthesis of extracellular lipooligosaccharides that mediate the specific symbiotic interaction with the legume host (Dénarié and Roche, 1992). In the case of R. meliloti, sulfated and acetylated lipooligosaccharides have been purified from the supernatants of luteolin-induced cell cultures (Lerouge et al., 1990), and it has been demonstrated that these so-called Nod factors are biologically active in specific root hair deformation assays on the host plant alfalfa. Furthermore, these same signaling molecules are also capable of eliciting cortical cell divisions and the formation of genuine nodules on alfalfa roots (Truchet et al., 1991). Interestingly, the sulfate group appears to be necessary for the expression of host specificity because nonsulfated factors are not active on alfalfa,
but are now able to elicit symbiotic responses on the roots of common vetch, a nonhost for \textit{R. meliloti} (Roche et al., 1991).

A detailed analysis of the plant response to these bacterial signal molecules requires the identification of plant genes that can serve as molecular markers for the recognition, infection, and nodule organogenesis triggering processes. The pioneering work of Bisseling and coworkers (reviewed in Nap and Bisseling, 1990) has shown that certain plant genes are specifically expressed in a variety of plant tissues that are involved in early stages of rhizobial infection and nodule development. It was shown that transcription of the pea early nodulin gene \textit{ENOD12}, which encodes a (hydroxy)proline-rich protein, takes place within root cortical cells that either contain or lie ahead of the advancing infection thread (Scheres et al., 1990). Transcripts of \textit{ENOD12} were also found within cells of the nodule primordium prior to infection thread penetration and within the infection zone of the mature root nodule. These same authors further showed that \textit{ENOD12} mRNA could be detected in total RNA extracts of pea root hairs 24 to 48 hr after infection with \textit{R. leguminosarum} and that this response was dependent on functional \textit{nod} genes. The fact that \textit{ENOD12} transcription could also be elicited in root hairs following treatment with cell-free supernatants of flavonoid-induced bacterial cultures provided further evidence for the role of Nod factors in the expression of this early plant symbiotic gene (Scheres et al., 1990).

In recent years, research in our laboratory has focused on the nitrogen-fixing symbiosis between \textit{R. meliloti} and a variety of plants from the genus \textit{Medicago}, including alfalfa and the diploid autogamous \textit{M. truncatula} (Barker et al., 1990). The availability of a wide range of \textit{R. meliloti} mutants that have altered symbiotic properties and the means of purifying the corresponding Nod factors from these mutant strains make this a most attractive system with which to study the regulation of plant genes in response to bacterial lipooligosaccharide signals. Furthermore, certain alfalfa genotypes are amenable to transformation and regeneration procedures (Deak et al., 1986; Chabaud et al., 1988), thus enabling us to construct transgenic plants expressing reporter genes under the regulatory control of the plant gene of interest. In this way, the transcriptional activity of the gene can be evaluated both at the cellular level and throughout the intact root system using a simple histochemical staining procedure (Jefferson et al., 1987).

In this study, we describe the isolation and characterization of a single-copy gene, homologous to pea \textit{ENOD12}, that was obtained by screening a genomic library of \textit{M. truncatula}, that was obtained by screening a genomic library of \textit{M. truncatula}. The tissue-specific transcription of this early nodulin gene has been studied by both in situ hybridization and the analysis of transgenic alfalfa plants expressing reporter genes under the regulatory control of the plant gene of interest. In this way, the transcriptional activity of the gene can be evaluated both at the cellular level and throughout the intact root system using a simple histochemical staining procedure (Jefferson et al., 1987).

**RESULTS**

**Identification of an \textit{M. truncatula} Gene Homologous to Pea \textit{ENOD12}**

Figure 1 shows that several hybridizing bands of variable intensity can be observed when a pea \textit{ENOD12} cDNA fragment is used to probe restriction digests of \textit{M. truncatula} genomic DNA under low-stringency conditions. Genomic clones corresponding to each of the major hybridizing bands were isolated by screening an \textit{M. truncatula} genomic DNA library constructed in the phasmid vector pGY97 (Vincze and Kiss, 1990). By means of DNA-DNA hybridization and partial sequence analysis (see below and results not shown), we were able to conclude that only one of these clones (pMt12) contained a gene that is homologous to the pea \textit{ENOD12} probe. Details of the four other \textit{M. truncatula} genes that were isolated by this screening procedure will be presented elsewhere.
Figure 2. Restriction Map and DNA Sequence of the *M. truncatula* ENOD12 Gene.

(A) Partial restriction map of the 8.8-kb insert of the genomic clone pMt12 containing the *MtENOD12* gene (see Methods). The coding region of *MtENOD12* is represented by the thickened horizontal line, and the accompanying arrow shows the direction of transcription. A horizontal bar indicates the 0.5-kb SphI-BamHI restriction fragment that was used for hybridization studies. The 2.3-kb promoter fragment that was amplified by PCR to generate the gusA transcriptional fusion (see Methods) is shown by the horizontal dashed line bordered by asterisks. Abbreviations for restriction sites are as follows: B, BamHI; C, Clal; H, HindIII; M, MscI; N, Ncol; Sp, SphI; B/S, BamHI/Sau3A junction.

(B) DNA sequence lying between the SphI and MscI restriction sites and covering the *MtENOD12* coding region. The deduced amino acid sequence has been annotated to indicate the putative signal peptide (italics), the peptide cleavage site (vertical arrow), and the proline-rich pentapeptide repeat motifs (double-underlining). A potential TATA element within the promoter region has been underlined. Nucleotide numbering is relative to the A residue (+1) of the initiator ATG codon; there is no "O position. Amino acid numbering is shown in parentheses.

The partial restriction map of the pMt12 insert is presented in Figure 2A, showing both the location of the coding region and the direction of transcription of *MtENOD12*, as determined by sequence analysis (see below). Genomic gel blot hybridization of *M. truncatula* DNA with the 0.5-kb SphI-BamHI fragment, which covers part of the coding region and the 5' noncoding region of *MtENOD12* (Figure 2), showed that this single-copy gene lies within the 12-kb EcoRI and 6-kb HindIII genomic fragments (Figure 1) and, thus, corresponds to the most intense hybridization signal seen with the pea *ENOD12* probe. Govers et al. (1991) have shown that, in the case of the pea (*Pisum sativum*) genome, there are two closely related *ENOD12* genes, *PsENOD12A* and *PsENOD12B*, and that both genes have the same organ-specific pattern of expression. The fact that *MtENOD12* is a single-copy gene has greatly simplified the analysis of *MtENOD12* transcription by means of specific DNA and RNA hybridization probes (see below). In contrast, several hybridizing bands can be seen when alfalfa genomic DNA is hybridized with the *MtENOD12* probe (Figure 1). This probably reflects the allelic heterozygosity commonly found with the tetraploid allogamous alfalfa and serves to illustrate the advantage of using the diploid autogamous *M. truncatula* for such molecular studies.

Sequence Analysis of the *MtENOD12* Gene

The nucleotide sequence of the coding strand of *MtENOD12* and the deduced amino acid sequence are presented in Figure 2B. No alternative open reading frame of significant length could be identified on either strand. As is the case for the pea *ENOD12* proteins (Govers et al., 1991), the ATG initiation codon
of MtENOD12 is followed by 23 amino acids that, according to the rules of Von Heijne (1983), are likely to serve as a membrane-translocation signal peptide. The remaining coding sequence (79 amino acids) is mainly composed of the repeating pentapeptide unit PPXXX, a structural feature that has also been found in a family of hydroxyproline-rich cell wall proteins of soybean known as SbPRPs (Hong et al., 1990).

The homologies between the Medicago and the two pea ENOD12 proteins are shown in Figure 3A. It is striking that only a single gap of 7 amino acids has to be introduced into the C-terminal region of MtENOD12 to optimize the alignment with PsENOD12A. The two regions of maximum homology correspond to the signal peptide sequence (83% nucleotide and 71% amino acid identities) and the proline-rich repeat region (76% nucleotide and 69% amino acid identities). The greater nucleotide sequence homology coupled with the near perfect alignment of the two sequences strongly suggest a common evolutionary origin for these two genes. Closer inspection of the proline repeat region of MiENOD12 and PsENOD12A reveals five well-conserved repeats of the decamer sequence PPVPN/KPPV/KE. Despite the extensive deletion within the PsENOD12B coding region, it is clear that the two putative pea proteins are more closely related to each other than to the Medicago protein.

A comparison between the 5' flanking sequence of MtENOD12 and that of PsENOD12B (the only upstream sequence currently available) reveals three stretches of quite striking homology within 140 bp upstream from the translation initiation site (Figure 3B). The first stretch runs from positions -1 to -29 (76% conservation), the second from -43 to -105, including the putative TATA element (81%), and the third from -111 to -141 (68%). Numbering is based on the Medicago sequence. Interestingly, it has already been shown that upstream sequences of the PsENOD12B gene, which correspond to the central conserved stretch, can also be aligned with equivalent promoter regions of the small family of soybean genes that encode the SbPRPs (Govers et al., 1991). However, as these authors have pointed out, the considerable differences in both developmental and tissue-specific expression patterns shown by ENOD12 and these three soybean PR genes (Wyatt et al., 1992) make it unlikely that this conserved region could be a determinant of organ-specific regulation.

Spatial-Temporal Expression of MtENOD12 during Nodule Development

Studies on the variation in ENOD12 mRNA levels during nodule development in pea had revealed two particular characteristics. First, gene transcription could be detected earlier during nodule development as compared with previously described nodule-specific genes, and second, the abundance of ENOD12 transcripts decreases as the young immature nodule develops into the mature nitrogen-fixing nodule (Scheres et al., 1990).

When M. truncatula plants are grown in aeroponic conditions (see Methods), nodules first become visible on the root system between 3 and 4 days following inoculation with R. meliloti, and nitrogenase activity is first detectable 2 to 3 days later. Figure 4 shows the profile of MtENOD12 mRNA abundance in total RNA extracts prepared from nodules of M. truncatula harvested between 4 and 8 days after inoculation. The level of MtENOD12 transcripts is relatively high at 4 to 5 days postinoculation and then drops rapidly by a factor of approximately 10-fold as the nodule continues to mature and begins to fix atmospheric nitrogen. As is the case for the pea nodule, this is in striking contrast to the profiles observed for transcripts encoding either the abundant oxygen-buffering protein leghemoglobin or the nodule parenchyma-specific protein ENOD2 (van de Wiel et al., 1990), both of which remain at constantly high levels as the nodule reaches maturity (Figure 4). A control hybridization with a human ubiquitin probe has been included to show that RNA loadings were approximately equal.
The tissue-specific location of these transcripts within the nodule using in situ hybridization. Sections of *M. truncatula* nodules, harvested either 4 days or 3 weeks after inoculation, were hybridized with a [35S]-labeled antisense RNA probe. Control experiments with sense probes routinely gave low, even backgrounds (results not shown). Figure 5A shows that a relatively uniform hybridization signal is present within the central tissue of the 4-day-old nodule. At this early stage of development, the nodule is approximately spherical in shape with a central tissue in which ramifying intercellular infection threads penetrate the plant tissue. Our results showed that all cells within the central tissue of the 4-day-old nodule express the *MtENOD12* gene, which is in line with the results obtained for *ENOD12* mRNA localization in immature pea nodules (Scheres et al., 1990).

In situ hybridization experiments carried out on sections of 3-week-old mature nitrogen-fixing nodules of *M. truncatula* showed that *MtENOD12* transcripts are present at the distal end of the prefixation zone II, corresponding to a region of the nodule where bacteria are being released from the infection threads (Figure 5B). When examining the apical region of the 3-week-old Medicago nodule at a higher magnification, it is possible to distinguish meristematic cells undergoing cell division (Figure 5C). While *MtENOD12* transcripts are absent in these actively dividing cells, a hybridization signal is clearly visible in the two to three cell layers in which infection threads are not yet present. These results suggest that *MtENOD12* transcription is initiated in the proximal cell layers of zone I that have ceased to divide, but that are not yet part of the prefixation zone II where infection takes place. The hybridization signal drops to background levels at the proximal end of prefixing zone II where bacteroids and plant cells are rapidly differentiating. This occurs prior to the amyloplast-rich interzone region (results not shown), which we have previously shown to be the site of leghemoglobin gene transcriptional activation (de Billy et al., 1991).

Expression of a Transcriptional *MtENOD12* Promoter-\textit{gusA} Fusion in Nodules of Transgenic Alfalfa

To complement the in situ hybridization studies and to facilitate the analysis of *MtENOD12* gene expression during earlier stages of the symbiotic interaction, we decided to introduce an *MtENOD12* promoter-\textit{gusA} fusion into transgenic Medicago plants. The 2.3 kb of DNA lying immediately upstream of the *MtENOD12* ATG translation initiation codon was cloned in front of the *Escherichia coli* gus\textit{A} coding region in such a way as to generate a precise transcriptional fusion (see Methods). This chimeric construction was then introduced into *M. varia* A2 plant tissue by means of an *Agrobacterium tumefaciens*-leaf disc transformation protocol (Chabaud et al., 1988). Regeneration of whole plants via somatic embryogenesis led to the isolation of 20 kanamycin-resistant plants that were phenotypically indistinguishable from the nontransformed line. Genomic DNA gel blot analysis of 12 of these regenerated plants provided...
Figure 5. In Situ Hybridization of *M. truncatula* Nodule Sections (7 µm) with an *MtENOD12* 35S-Labeled Antisense Probe.

(A) Four-day-old immature nodule showing a uniform pattern of hybridization within the central tissue. Silver grains appear as bright spots when viewed by dark-field microscopy. Bar = 50 µm.

(B) Three-week-old mature nodule. The hybridization signal is maximal at the distal end of the prefixation zone II (large asterisk), which corresponds to the infection zone of the nodule. Note the low level of hybridization at the proximal end of this zone (arrows). The meristematic zone is indicated by the small asterisk. Bar = 200 µm.

(C) Bright-field microscopic image of a 3-week-old nodule. *MtENOD12* transcripts (silver grains now appear as dark spots) are present in the two to three cell layers of the *preinfection* zone (star, see Discussion), which lie proximal to actively dividing meristematic cells. The arrowhead indicates a cell in anaphase. Note that infection threads (arrows) are not yet present in the cells of the *preinfection* zone. Bar = 20 µm.

direct evidence for the successful integration of between two and five copies of the reporter gene fusion (results not shown).

To examine the expression pattern of the *MtENOD12-gusA* fusion following inoculation with *Rhizobium*, primary transformants were taken through several cycles of vegetative propagation and then 4- to 5-cm-long cuttings were grown for about 3 weeks in aeroponic conditions until the root systems were well developed. *R. meliloti* was added to the liquid growth medium lacking combined nitrogen, and samples of the roots were examined for GUS activity at regular intervals after inoculation. Two of the 20 plants were scored negative for GUS activity throughout the experiment, and the remaining 18 responded in a qualitatively identical fashion.

Nodules first became visible on the transgenic alfalfa root system approximately 3 to 4 days following *Rhizobium* inoculation, showing that *M. varia* and *M. truncatula* have very similar kinetics of nodule development. These immature nodules developed an intense indigo blue coloration when treated with the histochemical GUS substrate X-gluc, and subsequent sectioning showed that the GUS activity was distributed uniformly throughout the central tissue (results not shown). Figure 6A shows that at a slightly later stage of nodule development (4 to 5 days postinoculation) the blue staining zone had clearly moved to the distal end of the central tissue. Expression of the *gusA* fusion is clearly visible in tissue that lies distal to the zone where infection threads are visible. This correlates well with the localization of *MtENOD12* mRNA by in situ hybridization (Figure 5C). The very pale coloration that is present in certain peripheral cells of the nodule may be due to limited diffusion from the intensely staining regions.

The distal localization of GUS activity in nitrogen-fixing and elongating indeterminate alfalfa nodules can be clearly seen in stained whole root segments (Figure 6B). Thick sections (80 µm) of GUS-stained mature nodules have also been stained with potassium iodide to reveal starch-containing cells (Figure 6C), showing that the *MtENOD12* promoter fusion is no longer being expressed in the central cell layers that immediately precede the amyloplast-rich interzone region.

Expression of the *MtENOD12* Promoter-*gusA* Fusion during the Earliest Stages of the Symbiotic Interaction

Having established that the expression of the *ENOD12* promoter-*gusA* fusion in transgenic alfalfa nodules was very similar to the pattern of gene transcription in *M. truncatula*


of nitrogen starvation in the absence of *Rhizobium*. Furthermore, inoculation with a non-nodulating strain of *R. meliloti* carrying a mutation in the nodA gene failed to elicit any reporter gene expression within the apical region of the root (results not shown), demonstrating that this early response is indeed dependent on the activity of the bacterial nod genes.

Histochemical staining of transgenic alfalfa root segments 48 to 72 hr after inoculation with *R. meliloti* revealed a number of discrete, dark blue-colored loci within the mature root hair region (Figure 7E). Preliminary observations have shown that certain early symbiotic events, such as root hair deformation, root hair infection, and cortical cell division, do indeed take place within these reactive loci, where GUS activity can now also be detected within the root cortex (results not shown). However, a detailed cytological study will be required to identify those cell types that express the *gusA* fusion and to correlate events occurring on the root surface with those that take place within the cortex. With the exception of these intensely staining loci, all the surrounding epidermal cells (including root hairs) were without detectable GUS activity. Harvesting and staining root segments at intermediate time points (results not shown) have confirmed that this region corresponds to the

Figure 6. GUS Activity in Root Nodules of Transgenic *M. varia* Expressing the *M. ENOD12 Promoter-gusA* Gene Fusion.

(A) Section (1 to 2 μm thick) of a 5-day-old immature nodule. Reporter gene activity is present in distal cell layers (arrows) that lie ahead of the prefixation zone II (asterisk). The blue coloration is most intense within the distal part of the prefixation zone, where it is possible to visualize sections through infection threads (arrowheads). Bar = 100 μm.

(B) Histochemical staining of a whole nodulated root segment harvested 7 days postinoculation. GUS activity is clearly visible at the distal end of the nodules. The arrowhead indicates a localized region of the root where earlier stages of the symbiotic interaction are visible (see also Figure 7E). Bar = 250 μm.

(C) A section (80 μm thick) of a mature nitrogen-fixing nodule harvested 3 weeks after inoculation. A gradient of GUS activity can be observed within prefixation zone II (star), decreasing from the distal end toward the proximal end. The section was cleared with sodium hypochlorite and then stained with potassium iodide (Vasse et al., 1990) to reveal the amyloplast-rich cells of interzone II/III (arrowheads). Bar = 200 μm.
Figure 7. Expression of the MtENOD12 Promoter-gusA Fusion in Transgenic M. varia Roots during Early Stages of the Symbiotic Interaction.

(A) Pattern of GUS activity throughout the zone of epidermal cells close to the growing root tip 20 hr after inoculation with R. meliloti. Bar = 250 μm.
(B) Reactive epidermal cells of the root elongation zone (marked by a single arrowhead in [A]). Bar = 125 μm.
(C) Reactive zone of root hair emergence (double arrowheads in [A]). Bar = 125 μm.
(D) Zone of mature root hairs (triple arrowheads in [A]) in which GUS activity cannot be detected. Bar = 500 μm.
(E) GUS activity within discrete loci of the mature root hair zone of the transgenic alfalfa root harvested 72 hr after inoculation. Note that, with the exception of the intensely stained regions, GUS activity is absent throughout the remaining root epidermis. Bar = 700 μm.

maturation of the zone which had previously stained uniformly for GUS activity close to the root tip (Figures 7A to 7C). We therefore concluded that, for the majority of differentiating root epidermal cells, this early transcription of the MtENOD12 gene is a transient phenomenon (compare Figures 7A and 7E).

DISCUSSION

Detailed molecular and cellular analyses of the events that occur during the earliest stages of the symbiotic interaction between the host legume and the corresponding rhizobial partner require the identification of genes that can act as markers for the plant response. With this goal in mind, and wishing to focus our studies on the Medicago–R. meliloti symbiosis for the reasons discussed earlier (see Introduction), we have cloned and characterized an M. truncatula gene, MtENOD12, that appears to be both structurally and functionally homologous to the pea early nodulin gene ENOD12 (Scheres et al., 1990). These authors have proposed that the protein encoded by the pea ENOD12 gene is most probably a (hydroxy)proline-rich cell wall protein. Their argument is based on the presence of a putative N-terminal transmembrane signal sequence and by drawing an analogy with the small family of SbPRPs, which are composed almost entirely of a proline-rich pentapeptide repeat motif (Hong et al., 1990). The fact that the homology between the deduced pea and Medicago ENOD12 amino acid sequences is greatest within both the signal peptide (71%) and the domain of proline-rich repeats (69%) (Figure 3A) further argues for the cell wall localization of this nodulation-related protein.
Medicago ENOD12 Gene Transcription

By means of in situ hybridization experiments, we have shown that, in both immature and mature nitrogen-fixing nodules of *M. truncatula*, MTENOD12 transcription is maximal in the zone in which infection threads are spreading and releasing bacteria into host cells. Those cells that are "infected" will subsequently differentiate into the enlarged Rhizobium-filled cells of the nitrogen-fixing zone, whereas the uninfected cells will remain small in size and develop large vacuoles. By examining the apical meristematic region of the Medicago nodule in greater detail, we have been able to observe that MTENOD12 transcription is in fact initiated within a narrow zone, composed of two or three cell layers, which is immediately adjacent and proximal to the actively dividing meristematic cells, but clearly lacking infection threads (Figure 5C). We propose that the term "preinfection zone" be used to describe this narrow band of cells lying between the meristematic cells and the infection thread region. That MTENOD12 transcription should be triggered within this zone is interesting in light of the fact that, during early stages of infection, pea ENOD12 transcripts have been found in cortical cells that lie ahead of the progressing infection thread (Scheres et al., 1990), thus leading to the hypothesis that "diffusible" signal molecules originating from the infection thread are responsible for ENOD12 gene activation at a distance. Our observations would suggest that a similar gene activation mechanism exists in the developing nodule, where the cells that lie immediately ahead of the infection thread region are also preparing for subsequent infection. This would also explain why all cells within this zone contain ENOD12 transcripts irrespective of whether they become infected or remain uninfected.

One of the principle advantages of studying symbiosis-related plant gene expression in species of the genus Medicago lies in the possibility of obtaining transgenic alfalfa via A. tumefaciens transformation and somatic embryogenesis. With the notable exception of Lotus (Petit et al., 1987), alfalfa is the only legume that is currently amenable to such routine transformation and regeneration procedures. Based on the assumption that gene regulatory mechanisms would be highly conserved between closely related species of the genus Medicago, we have introduced a chimeric gene composed of a 2.3-kb MTENOD12 promoter fragment fused to the coding region of the gusA reporter gene into the high-frequency regenerating line of alfalfa, *M. varia* A2. A qualitatively homogeneous response, in terms of reporter gene expression, was obtained from 18 of the 20 transgenic plants tested following inoculation with *R. meliloti*. More importantly, the distribution of GUS activity within the nodules that formed on the roots of these transgenic plants correlated remarkably well with the localization of MTENOD12 mRNA, as determined by in situ hybridization analyses on sections of *M. truncatula* nodules (Figure 5). The level of GUS activity was found to be maximal within the invasion zone of both immature and mature nitrogen-fixing nodules. Furthermore, expression of the chimeric reporter gene also appeared to be initiated in preinfection cell layers which precede the zone of infection thread proliferation (Figure 6A). These results provide convincing evidence that the 2.3-kb MTENOD12 promoter fragment contains all the information necessary for regulated expression of the Medicago ENOD12 gene and that our reporter gene assay in transgenic alfalfa provides a valid means of evaluating the expression patterns of this early symbiotic gene.

When transgenic alfalfa plants were used to examine the expression of the MTENOD12 gene during the earliest stages of the symbiotic interaction, we discovered that reporter gene activity could first be detected in roots as little as 3 to 6 hr following inoculation with *R. meliloti*. Furthermore, GUS activity was present not only in young developing root hairs but throughout all epidermal cells of a region extending from just behind the growing root tip as far as the beginning of the mature root hair region (Figure 7A). The relatively uniform pattern of staining suggests that all cells on the outer surface of the root and lying within this zone respond to the presence of the bacterial symbiont. Because a nodulation-deficient mutant of *R. meliloti* carrying a Tn5 insertion in the nodA gene does not elicit this reaction, we can reasonably conclude that this corresponds to a nod gene-dependent symbiotic response.

The fact that ENOD12 gene expression should be triggered in this actively differentiating region of the root is of considerable interest because it is now well established for alfalfa (and indeed for most other legumes so far examined) that the events that lead to subsequent nodule formation are generally initiated within the part of the root that lies between the elongating root tip and the zone of root hair emergence (Bhuvaneswari et al., 1981; Caetano-Anolles and Gresshoff, 1991). Even when successful infections are initiated within the more mature region of the root, these are usually restricted to a zone no greater than 1 cm distant from the point of first root hair emergence. The striking correlation with the pattern of early reporter gene expression suggests that the transcription of the ENOD12 gene parallels the differentiation of a zone that is undergoing preparation for subsequent *Rhizobium* infection.

Epidermal cells that form root hairs are known as trichoblasts, and during this differentiation process, polar tip growth is established only after cells have initiated a round of cell division and have arrested in cytokinesis (for a review, see Kijne, 1991). We can speculate that, in response to rhizobial signals, specific symbiosis-related proteins, such as ENOD12, may be incorporated into the developing root hair cell wall, thus rendering the root hairs susceptible to *Rhizobium* infection. Modifications in the cell wall of the root hair could have an important role in several stages of the infection process including bacterial attachment, localized cell wall degradation, and the development of the infection thread. In the case of pea, it has been shown that the mRNA population of root hairs is significantly modified following infection by *R. leguminosarum*, with the appearance of at least one new mRNA species and a significant enhancement in the levels of a second mRNA (Gloudemans et al., 1989). However, the identity and subcellular localization of the corresponding proteins have not yet been determined.

Could the rhizobial signals that trigger this very early reaction in epidermal cells close to the root tip be the same
symbiotic Nod factors recently identified as extracellular lipooligosaccharides? Interestingly, Nod factors are able to specifically induce root hair branching of the host legume (Lerouge et al., 1990). Morphologically, this branching process corresponds to the formation of a new growth tip on the side of the root hair and is, therefore, analogous in certain respects to root hair growth during trichoblast differentiation. Because it has been shown that Rhizobium Nod factors are able to stimulate root hair development (Roche et al., 1991) and also to induce ENOD12 gene expression in pea root hairs (Scheres et al., 1990), these molecules could well be responsible for initiating changes in the number and composition of the root hairs that will permit subsequent Rhizobium infection. The purification of sulfated lipooligosaccharides from the parental strain of R. meliloti (NodRm-IV [Ac,S]), which specifically elicit alfalfa root hair branching, and the corresponding nonsulfated derivatives purified from nodH mutants (NodRm-IV [Ac]), which have lost this capacity (Roche et al., 1991), will now enable us to examine directly how these molecules influence ENOD12 gene transcription in relation to the infection process.

The zone of epidermal and root hair cells that stained uniformly for GUS activity continued to be clearly visible near the root tips of transgenic alfalfa plants until approximately 24 hr after inoculation with R. meliloti. During the period 24 to 72 hr postinoculation, as the distance between the reactive zone and the root tip gradually increased due to root growth, a small percentage of root hairs within this zone began to stain very intensely for GUS activity, while the remaining root hairs and epidermal cells rapidly lost their blue coloration (Figure 7E). Preliminary analysis suggested that GUS activity was also present within inner regions of the root cortex at this stage (Figure 7E and results not shown), most probably corresponding to the development of the nodule primordium, as described for the pea ENOD12 gene (Scheres et al., 1990).

Taken together, our findings suggest the following scenario. Within hours of the addition of Rhizobium, the Medicago ENOD12 gene is activated transiently within epidermal cells of the reactive zone close to the root tip. This rapid response leads to the differentiation of root hairs that are susceptible to infection by Rhizobium. Root hairs that are infected continue to express the ENOD12 gene, and in this case, the level of transcription is enhanced.

Such a series of events is interesting for the following reasons. First, we can draw a correlation between the expression of MtENOD12 in epidermal cells preparing for infection thread initiation (this study), the expression of the pea ENOD12 gene in cortical cells and cells of the nodule primordium that lie ahead of the infection thread (Scheres et al., 1990), and the expression of the Medicago gene in cells of the preinfestation zone of the differentiating nodule prior to infection thread penetration (this study). Could Nod factors be involved in all of these processes? The fact that the appropriate factors can elicit both cortical cell divisions and nodule organogenesis in alfalfa roots (Truchet et al., 1991) and cortical cell divisions in Vicia roots (Spaink et al., 1991) provides indirect evidence that this may indeed be the case. Second, our findings suggest that recognition of the lipooligosaccharide factor precedes differentiation of the root hair. If so, then it is easier to appreciate how several different determinants of host specificity may be involved in the preliminary stages of the symbiotic interaction. The production and specific recognition of Nod factors would provide the initial trigger to the process, leading subsequently to the expression of other specificity determinants, such as lectins (Diaz et al., 1989), involved at later stages of the interaction (e.g., at the surface of the root hair). The identification and localization of Nod factor receptors will clearly be of considerable importance in understanding the role(s) played by these molecules during symbiosis.

In addition to providing new insights into the nature of the plant response during the earliest stages of the Rhizobium-legume interaction, transgenic alfalfa expressing the ENOD12-gusA fusion should also prove useful for monitoring the plant reaction to a variety of R. meliloti mutants with altered symbiotic characteristics. In particular, the very precocious expression of reporter gene activity in root epidermal cells should provide an excellent marker for studying the response to mutants that produce modified Nod factors, and may even lead to a convenient and sensitive assay for the lipooligosaccharide factors themselves.

METHODS

Plant Material and Growth Conditions

Plants of Medicago truncatula cv Jemalong were grown aeroponically and inoculated with the wild-type Rhizobium meliloti RCR2011, as previously described (Gallusci et al., 1991). Cuttings of the M. varia genotype A2 (Deak et al., 1986) were kindly provided by G. B. Kiss (Szeged, Hungary) and were propagated vegetatively in axenic culture on SH agar medium (Schenk and Hildebrandt, 1972) containing 1% sucrose. For nodulation experiments, cuttings of transgenic M. varia plants were grown aeroponically using the same conditions as for M. truncatula. After 2 to 3 weeks, plants were inoculated with either R. meliloti RCR2011 or the control non-nodulating nodA mutant strain (GM15386; Debeli et al., 1986). Whole root fragments or nodules were collected at various times after inoculation and treated as described below.

Purification of Nucleic Acids and Filter Hybridization

The isolation and purification of high molecular weight genomic DNA from leaves of M. truncatula cv Jemalong, M. sativa cv Gemini, and Pisum sativum cv Rondo were carried out as described in Barker et al. (1988). Total RNA was extracted from M. truncatula nodules harvested at various times after inoculation with R. meliloti according to Lullien et al. (1987). Electrophoresis of restricted genomic DNA on non-denaturing agarose gels and of denatured total RNA on 6% formaldehyde-agarose gels was performed according to Sambrook et al. (1989). Transfer to GeneScreen membranes (Du Pont—New England Nuclear) and subsequent hybridization at 37°C in the presence of 50% formamide and 10% dextran sulfate were carried out following the manufacturer's instructions. After hybridization, blots were...
washed in 2 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS at temperatures between 55 and 65°C. Radioactive probes were prepared by the oligolabeling procedure (Feinberg and Vogelstein, 1983) using a 32P-dCTP, and unincorporated nucleotides were subsequently removed by spin dialysis through Sepharose CL 6B (Pharmacia, Sweden).

Isolation of a Genomic Clone Containing the *M. truncatula* ENOD12 Gene

The construction and screening protocol of the genomic library of *M. truncatula* leaf DNA prepared in the phasmid vector pGY97 (Vincze and Kiss, 1990) has already been described (Gallusci et al., 1991). A pea ENOD12 cDNA probe (Scheres et al., 1990), kindly provided by T. Bisseling (Wageningen, The Netherlands), was used to identify positive clones within the library. Hybridization conditions were the same as those used for genomic DNA gel blot analysis (see above). One of these clones, pMt12, was partially sequenced and found to contain a gene (MtENOD12) whose coding sequence is highly homologous to pea ENOD12 (see text). This clone has a total insert size of approximately 8.8 kb, and Figure 2A shows the partial restriction map of the insert and the location of the MtENOD12 gene.

DNA Sequencing

The sequence of the Sphl-Msc1 DNA fragment that covers the MtENOD12 coding region and immediate flanking sequences (Figure 2) was determined by subcloning short restriction fragments (200 to 300 bp) into the multipurpose vector pUC19. To obtain the sequence of both DNA strands, the dideoxy chain termination reaction (Sanger et al., 1977) was carried out using double-stranded templates (Murphy and Kavanagh, 1988) and polymerase priming from both ends of the pUC19 polylinker. The junction sequences between adjacent fragments were confirmed either by using overlapping clones or by sequencing from synthetic oligonucleotide primers. The nucleotide sequence data reported in this paper has been submitted to EMBL, GenBank, and DDBJ as accession number X68032.

In Situ Hybridization

For the preparation of the single-stranded RNA probes, the 0.5-kb Sphl-BamHI restriction fragment from the MtENOD12 genomic clone was subcloned into pBlueScript SK+ (Strategene). Synthesis and partial hydrolysis of radiolabeled sense and antisense RNA were carried out as described in de Billy et al. (1991). In situ hybridizations on 7-μm-thick sections using 32P-labeled RNA probes were performed as described previously (de Billy et al., 1991), except for the addition of a 24-hr prehybridization step using the standard hybridization buffer minus dextran sulfate.

Construction of a Transcriptional MtENOD12 Promoter–gusA Fusion

As a first step, the 2.3-kb DNA fragment lying upstream of the MtENOD12 ATG translation initiation codon (Figure 2) was amplified using the polymerase chain reaction (PCR) to introduce appropriate restriction sites at either end of the fragment. The genomic clone pMt12 was used as a template for a PCR, using the phosphorylated forward primer P1 (5′-TTAGGAATTCT[EcoRI]ATATATGAGGCGGAG-3’) and reverse primer P2 (5′-GGAGGCCATTG[Ncol]TAATAGTATAATT-3’); the bases underlined in the primer sequences correspond to substitutions in the genomic sequence. Twenty cycles of amplification were performed (94°C for 1 min, 57°C for 1 min, 72°C for 3 min) under otherwise standard PCR conditions (Gelfand and White, 1990). The PCR product was then blunt-ended using T4 DNA polymerase (Sambrook et al., 1989) and cloned into Smal-linearized pUC19 to obtain pUC19-prMt12.

Sequence analysis of five such clones failed to reveal a single error within the 300-nucleotide stretch that lies immediately upstream of the ATG codon, suggesting a very low error frequency for the Taq DNA polymerase under our experimental conditions. Because of the presence of a second Ncol site within the MtENOD12 promoter (Figure 2), it was necessary to carry out a partial digestion of pUC19-prMt12 after linearization with EcoRI to recover the full-length 2.3-kb EcoRI-Ncol fragment. This promoter fragment was then cloned between the EcoRI and Ncol polylinker sites of pCC OGUS (Axiau et al., 1989) so that subsequent digestion with EcoRI and NsiI would yield a DNA fragment containing the MtENOD12 promoter fused to the gusA coding sequence with a 3′ flanking polyadenylation signal (cauliflower mosaic virus 35S). After adding an NsiI site to the 3′ terminus, the resultant fragment was cloned between the unique EcoRI and SfiI sites of the binary vector plP100 to give plP100-prMt12. Plasmid plP100 (a kind gift of P. Ratet, Gif-sur-Yvette, France) is a derivative of pBin19 (Bevan, 1984). As a result of the preceding manipulations, the MtENOD12 promoter sequence upstream of the β-glucuronidase (gusA) coding region is identical to that in the MtENOD12 gene itself, except for the substitution of two C residues for the two A residues at positions −1 and −2. The resultant junction sequence –ACCATG, which has been verified by sequencing plP100-prMt12, satisfies the preferences shown by plant genes for the three nucleotide positions that precede the ATG codon (Cavener and Ray, 1991).

Transformation of *M. varia* and Recovery of Transgenic Plants

The binary vector plP100-prMt12 was mobilized into the *Agrobacterium tumefaciens* LBA4404 (Hoekema et al., 1983) according to the procedure described by Hoekema et al. (1978), with three freeze-thaw cycles, and transformed *A. tumefaciens* colonies were selected by growth on 50 μg/mL kanamycin. Leaf segments of *M. varia* A2 were transformed via *A. tumefaciens*, and somatic embryogenesis was induced on kanamycin-resistant callus tissue as described by Chabaud et al. (1988). Embryos were matured on the modified UM medium of Strickland et al. (1987), with the addition of 5 g/L charcoal (T. Huguet, personal communication). The regenerated primary transformants were grown in axenic culture on SH medium (Schenk and Hildebrandt, 1972) with 1% sucrose and propagated by taking cuttings. Patterns of reporter gene expression from the MtENOD12 promoter–gusA fusion were unaltered even after numerous cycles of vegetative propagation.

Histochemical Localization of GUS Activity

Histochemical staining for GUS activity was performed as previously described (Jefferson et al., 1987) with the following modifications. Whole root fragments or nodules were excised from the plant and prefixed by vacuum infiltration with an ice-cold solution of 0.3% p-formaldehyde in 0.1 M potassium phosphate buffer, pH 7.0, followed by incubation on ice for 45 min. After two washes in the phosphate buffer, whole
organisms were immersed in the GUS substrate solution containing 1 mM X-gluc (5-bromo-4-chloro-3-indolyl glucuronide, cyclohexylammonium salt; Biosynth AG, Staad, Switzerland), 5 mM EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 0.1 M potassium phosphate buffer, pH 7.0. Incubation was performed in the dark at 37°C for periods of time between 4 and 24 hr, depending upon the intensity of the coloration. After rinsing in phosphate buffer, stained tissues were observed either as whole specimens or as sections (80 μm thick; Micropat H 1200; Bio-Rad) with an Olympus Vanox light microscope using bright-field optics. To improve the contrast between stained and nonreactive tissues, the samples were briefly cleared with sodium hypochlorite (Bovin et al., 1990). Using these methods, endogenous GUS activity was never observed in either root or root nodule tissues from untransformed alfalfa plants.

For the localization of GUS activity at the cellular level, nodules were dissected from stained nodulated root segments and then, successively, postfixed for 1 hr in 2.5% glutaraldehyde buffered with 0.2 M sodium cacodylate, pH 7.2, rinsed in the same buffer, dehydrated in an alcohol series, and embedded in Epon resin (Merck, Darmstadt). Sections (1 to 2 μm thick) were counterstained with basic fuchsin (2% in distilled water) and observed by bright-field microscopy.

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