***MtENOD20*, a Nod Factor-Inducible Molecular Marker for Root Cortical Cell Activation**

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The spatio-temporal expression pattern of the *Medicago truncatula* *ENOD20* gene during early stages of nodulation has been analyzed with transgenic alfalfa (*M. varia*) expressing a p*MtENOD20*-*GUS* chimeric fusion. Our results show that transcriptional activation of this gene occurs initially in dividing inner cortical cells corresponding to sites of nodule primordium formation and subsequently in root hairs containing infection threads. Use of *Sinorhizobium meliloti* *nod* gene mutants that uncouple nodule organogenesis from infection has confirmed that early *MtENOD20* transcription is tightly linked to cortical cell activation (CCA). Furthermore, these experiments have revealed that an *S. meliloti* *nodH* mutant, defective in Nod factor sulfation and epidermal cell activation, is nevertheless able to elicit low-level CCA. Purified *S. meliloti* Nod factors trigger *MtENOD20* transcription very rapidly (within 12 to 24 h) in the root cortex, and studies with transgenic alfalfa show that Nod factors are able to elicit gene expression coupled to CCA at concentrations as low as 10^{-11} M. Finally, we have made use of a range of synthetic lipo-chitooligosaccharides to show that fatty acid chain length is an important structural parameter in the capacity of Nod factors to elicit CCA. Taken together, our results suggest that p*MtENOD20*-*GUS* transgenic lines should prove valuable tools in future studies of *Rhizobium* and Nod factor-elicited CCA.

Additional keywords: early nodulin, *GUS* reporter gene.

The symbiotic association between leguminous plants and soil bacteria known as rhizobia leads to the formation of complex root structures (nodules) in which the bacteria reduce atmospheric nitrogen to ammonia, thus providing the host plant with a privileged source of combined nitrogen. Early stages of root nodulation involve developmental host responses that are related either to root penetration by the bacterium (bacterial infection) or to the initiation of nodule organogenesis within the cortex. These responses result from a molecular dialogue between the two organisms, and in particular the synthesis of specific lipo-chitooligosaccharide (LCO) signaling molecules (known as Nod factors) by the microsymbiont (for recent reviews see Dénarié et al. 1996; Heidstra and Bisseling 1996; Long 1996). Nod factors possess multiple biological activities

that are related either to rhizobial infection (e.g., hair deformation) or nodule organogenesis (e.g., cortical cell activation [CCA]), and these molecules are of fundamental importance to successful host/bacterial recognition and root nodulation.

One of the best-studied N_2 -fixing symbiotic associations is that formed between *Sinorhizobium meliloti* and *Medicago* species such as alfalfa or the model legume *M. truncatula*. *S. meliloti* Nod factors (NodRm) consist of a chito-tetraose backbone, C6-sulfated at the reducing end and both O-acetylated and N-acylated (majority C16:2 fatty acid) at the non-reducing end (Lerouge et al. 1990). It has been shown that purified NodRm elicits both root hair deformation in alfalfa at concentrations as low as 10^{-12} M (Roche et al. 1991) and CCA leading to visible root outgrowths down to 10^{-9} M (Truchet et al. 1991). Use of both *S. meliloti* *nod* gene mutants (*nod* genes encode the enzymes responsible for Nod factor synthesis) and purified, structurally altered Nod factors has revealed that substitutions at both ends of the NodRm sugar backbone are important for biological activity (reviewed in Dénarié et al. 1996).

The identification of plant genes (encoding so-called early nodulins) that are activated during the early stages of root infection/nodulation can facilitate the study of the molecular and cellular responses of the plant to rhizobial Nod factors (see review in Heidstra and Bisseling 1996). With this goal in mind we recently described the initial characterization of two new early nodulin genes (*MtENOD16* and *MtENOD20*), isolated by low-stringency screening of an *M. truncatula* genomic library with a pea *ENOD5* cDNA probe (Greene et al. 1998). Sequence analysis showed that both of these genes encode extracellular proteins belonging to a small family of early nodulins (including Ps/VsENOD5 and GmN315/ENOD55) with structural homology to copper-binding phytochans.

In this article, we present a detailed study of the nodulation-related expression pattern of the *MtENOD20* gene, making use of transgenic alfalfa lines into which we have introduced a chimeric gene consisting of a 2.1-kb-long *MtENOD20* promoter fragment fused to the β -glucuronidase (*GUS*) reporter gene. We show that, following *S. meliloti* inoculation, transcription of the *MtENOD20* gene is initially induced in the root inner cortex at sites of nodule primordium formation, and subsequently during host cell infection. We have exploited these transgenic lines, in combination with *S. meliloti* *nod* gene mutants and purified Nod factors (both biological and synthetic), to establish the relationship between *MtENOD20*

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gene expression and CCA and to demonstrate the utility of this new molecular marker in Nod factor structure/function studies.

RESULTS

Construction of the p*MtENOD20*-*GUS* fusion and introduction into *Medicago varia* A2.

In order to study the spatio-temporal expression pattern of the *MtENOD20* gene during both early and late stages of nodulation, we constructed a transcriptional fusion between the 2.1-kb DNA fragment lying immediately upstream of the *MtENOD20* ATG initiation codon and the coding region of the *GUS* reporter gene (see Materials and Methods). This chimeric gene was then cloned into the binary vector pLP100 (Szabados et al. 1995) and introduced via *Agrobacterium tumefaciens*-mediated leaf-disk transformation into the highly embryogenic alfalfa *Medicago varia* A2 (Deak et al. 1986). A total of 22 independent kanamycin-resistant transgenic lines were regenerated following somatic embryogenesis of transformed callus tissue. Primary transformants were then grown in aeroponic chambers and inoculated with *Sinorhizobium meliloti* RCR2011 (Rosenberg et al. 1981). Preliminary analyses of root and nodule tissues at various times after inoculation revealed qualitatively identical patterns of GUS activity for 17 out of the 22 lines. Tissues from the remaining 5 lines were negative for GUS staining. The detailed studies described below were performed with representative lines that expressed high levels of the p*MtENOD20*-*GUS* fusion. S₁ seed obtained by self-fertilization was used for experiments in growth pouches.

Expression of *MtENOD20* during *S. meliloti* root infection and nodulation.

Prior to inoculation, GUS activity was undetectable in cortical, epidermal, and meristematic root tissues of transgenic p*MtENOD20*-*GUS* lines. On the other hand, weak staining was occasionally observed in root vascular tissues of 10-day-old, pouch-grown plants. Apart from the root, the only transgenic plant tissue in which we have been able to detect GUS activity is the seed endosperm (data not shown).

Following inoculation with *S. meliloti* RCR2011, expression of the p*MtENOD20*-*GUS* fusion could first be detected in the root cortex after approximately 24 to 36 h (Fig. 1A). At this stage, root hair infection was not yet detectable and GUS staining was strictly limited to discrete cortical loci within the nodulation-susceptible region of the root (see Materials and Methods). Thin sectioning revealed that reporter gene expression was associated with localized regions of cell division within the root inner cortex (Fig. 1B), corresponding to nodule primordium development. The size and intensity of these GUS staining regions increased significantly by 48 to 72 h post inoculation (hpi), with the additional appearance of reporter gene activity in associated root hairs displaying shepherd's crook curling, characteristic of root hair infection (Fig. 1C and D). Sections through stained cortical regions 72 hpi again showed that GUS activity was present in regions of nodule development (Fig. 1E). Note that the *MtENOD20* gene is not expressed in tissues of emerging lateral root primordia (Fig. 1E). Finally, observation of many root systems harvested and stained for GUS

activity between 24 and 48 hpi have confirmed that *MtENOD20* expression in the cortex always precedes infection-related expression in root hairs.

At the stage when nodules became clearly visible as root outgrowths (approximately 4 days post inoculation) GUS staining was very intense in the central tissues of these young developing nodules, which mainly consist of cells undergoing infection by the highly ramified infection thread network (Fig. 1F). Adjacent to these emerging nodules, GUS staining was also clearly visible at localized sites in peripheral root tissues (Fig. 1F and G). Double-staining for β -galactosidase activity to detect the presence of the bacterium (see Materials and Methods) showed that infection threads were present in the epidermal GUS-staining cells (Fig. 1H). Note that the red coloring of the infection thread is masked by the overlaying blue staining of the GUS activity in the cytoplasm. We presume that these events correspond to unsuccessful infections in which progression of the infection thread has been arrested either in epidermal or outer cortical cell layers, as previously reported by Penmetsa and Cook (1997) and Vasse et al. (1993), respectively.

Sectioning and subsequent staining of mature nitrogen-fixing nodules (Fig. 1I) revealed that reporter gene activity is present exclusively in central tissues. GUS staining is totally absent in nodule meristematic tissue, and can first be detected in distal zone II where infection initiates, increasing in intensity as infected cells differentiate and enlarge toward the proximal end of this zone (for the definition of nodule zones see Vasse et al. 1990). Following the amyloplast-rich interzone II-III, staining becomes generally weak and patchy throughout the nitrogen-fixing zone III, and at present we do not know the reason for this irregular staining pattern.

Various attempts were made to confirm that the endogenous *MtENOD20* gene is activated rapidly in the *M. truncatula* root following *S. meliloti* inoculation. Unfortunately, both Northern (RNA) analysis (with total RNA extracts) and in situ hybridization proved insufficiently sensitive to detect *MtENOD20* transcript induction during the first 24 to 48 hpi. We therefore made use of the reverse transcriptase-polymerase chain reaction (RT-PCR) approach, and to maximize sensitivity PCR was performed with specific primers located on either side of the single *MtENOD20* intron, thus allowing us to distinguish between amplified fragments of genomic or cDNA origin (see Materials and Methods). The RT-PCR experiment illustrated in Figure 2A shows that *MtENOD20* mRNA is absent in non-inoculated *M. truncatula* roots, and can first be detected (albeit at a low level) as early as 24 hpi with *S. meliloti* RCR2011. This and the subsequent increases in transcript levels observed 48 to 72 hpi correlate well with the timing and extent of reporter gene activity observed in the transgenic *M. varia* expressing p*MtENOD20*-*GUS*. The quality of the cDNAs used in the various PCRs was controlled by performing parallel amplifications with primers specific for the constitutively expressed *MtPR10-1* gene (Gamas et al. 1998; and see Materials and Methods).

In conclusion, our results argue that expression of the *MtENOD20* gene can be linked to at least two distinct early stages of nodulation. Expression is first detected during nodule primordium formation in the root inner cortex, and then subsequently in both root hairs and nodule tissues that contain infection threads.

Fig. 1. Histochemical localization of β -glucuronidase (GUS) activity throughout various stages of root nodulation in transgenic *Medicago varia* expressing the pMtENOD20-GUS fusion. **A**, Whole root segment close to the root tip 36 h post inoculation (hpi) with *Sinorhizobium meliloti* RCR2011. Arrows indicate cortical sites staining positively for GUS activity. There is an increase in staining intensity as the root develops (zone of root hair emergence is at left-hand end of the segment). Bar = 100 μ m. **B**, Section (4 μ m thick) of a Technovit-embedded root 36 hpi. GUS staining (colored red under dark-field observation) is localized mainly to inner cortical cell layers. Arrows indicate anticlinal divisions. Bar = 25 μ m. **C**, Whole root segment 48 hpi. GUS activity is visible in the cortex (asterisk) and in associated root hairs with characteristic shepherd's crook curling (arrows). Bar = 50 μ m. **D**, Stereomicroscopic image of whole root segment 72 hpi illustrating relationship between GUS staining in the cortex and in associated infected root hairs. Bar = 200 μ m. **E**, Longitudinal root section (4 μ m thick) 72 hpi viewed under dark-field optics. GUS activity (red coloration) localizes to developing nodule primordia (asterisks), but is totally absent in a lateral root primordium (star). Bar = 50 μ m. **F**, Whole root segment 4 days post inoculation showing intense GUS staining in inner tissues of emerging nodules. Arrows indicate staining in peripheral tissues corresponding to arrested infection events. Bar = 200 μ m. **G**, GUS staining in infected root hair where infection thread progression has arrested within the epidermal cell. Bar = 50 μ m. **H**, As for G, except that tissue has been double-stained for GUS (blue) and β -galactosidase (red) to localize the bacteria (expressing the constitutive *hemA-lacZ* fusion) in the infection thread (arrow). The red coloring is masked by the overlying blue GUS staining. Bar = 50 μ m. **I**, Longitudinal section (80 μ m thick) through a mature 10-day-old nitrogen-fixing nodule. GUS staining is confined to central tissues of the nodule and extends from infection zone II down through nitrogen-fixing zone III. The nodule meristem is marked by an asterisk; arrows indicate amyloplast-rich interzone II-III layer. Bar = 100 μ m.

CCA and associated *MtENOD20* gene expression elicited by *S. meliloti* *nodH* and *nodF/nodL* mutants.

Mutations that inactivate key genes within the *S. meliloti* nodulation (*nod*) locus result in modifications to the structure of the symbiotic Nod factor molecule and thereby generate mutants with a variety of infection/nodulation phenotypes. Certain of these mutants are reported to entirely lack the capacity to induce symbiotic responses in the host plant, whereas others elicit modified epidermal/cortical responses. In order to further evaluate the relationship between *MtENOD20* expression and early stages of nodulation we inoculated transgenic alfalfa plants expressing the *pMtENOD20-GUS* fusion with various *S. meliloti* *nod* mutants.

As a control we initially tested a strain with a polar mutation in the *nodA* gene. This mutation abolishes synthesis of all three enzymes encoded by the *nodABC* operon, together responsible for elaborating the Nod factor LCO core structure (reviewed in Dénarié et al. 1996). As expected, inoculation with the *nodA* mutant failed to elicit expression of the *MtENOD20* gene in either epidermal or cortical tissues (Table 1). The second type of *S. meliloti* strain tested was mutated in the host-specificity gene *nodH*. Nod factors synthesized by a *nodH* mutant lack a critical sulfate substitution on the C-6 position at the reducing end of the sugar backbone (Roche et al. 1991). A variety of bioassays have previously shown that *nodH* mutants are unable to elicit ei-

ther symbiotic epidermal responses (root hair curling/*MtENOD12* expression) or root nodulation on the normal host plant alfalfa (Debellé et al. 1986; Journet et al. 1994). Nevertheless, reporter gene activity was detected in roots of transgenic alfalfa as early as 48 hpi with a strain carrying a Tn5 insertion in the *nodH* gene. At 72 hpi, GUS staining was clearly observed as discrete patches in regions of the root normally susceptible to nodulation at the moment of inoculation (see Materials and Methods) and was localized essentially to inner cortical tissues (Fig. 3A). Approximately one third of inoculated plants stained positively (albeit weakly) for inner cortical GUS expression 48 hpi, compared with a 100% response for the wild-type strain (Table 1). From 72 hpi onward, the percentage of plants with detectable GUS activity increased to 80% with an accompanying increase in the intensity of staining. Detailed observation of the responsive zones at 72 hpi revealed a tight correlation between GUS staining and the presence of both cortical cell divisions and features such as centrally positioned nuclei and amyloplast accumulation (Fig. 3A and data not shown). This type of symbiotic response has been termed cortical cell activation (CCA) and is typically observed under conditions of defective infection, such as that found following inoculation with the *nodF/nodL* double mutant (Ardourel et al. 1994; see below). Identical results were obtained for a second *nodH* mutant, this time carrying a deletion within the coding region of the gene (Table 1), confirming that Nod factor sulfation-defective *S. meliloti* mutants are indeed capable of eliciting CCA. Significantly, these regions of CCA do not develop further, and in no case did we observe external swellings of the root. Furthermore, morphological responses such as root hair deformations were not observed in epidermal tissues located above regions of CCA, and reporter gene activity was never detected in root hairs of plants inoculated with *nodH* mutants. Finally, we have recently observed similar patterns of reporter gene expression in cortical cell layers following *nodH* mutant inoculation of *M. truncatula* plants transformed with the identical *pMtENOD20-GUS* construct (data not shown).

The third *S. meliloti* mutant examined, defective in both the *nodF* and *nodL* genes, produces Nod factors that are doubly modified at the nonreducing end of the sugar backbone. These LCOs lack the *O*-acetyl group and are *N*-acylated with *cis*-vaccenic acid ($\Delta 11$ -C18:1) instead of the normal $\Delta 2,9$ -C16:2 fatty acid chain (Ardourel et al. 1994). *nodF/nodL* mutants are defective in infection and nodulation, but nevertheless are able to elicit striking multiple root hair deformations and an extensive CCA response (Ardourel et al. 1994). Figure 3B shows a GUS-stained segment of a transgenic *M. varia* root 4 days after inoculation with a *nodF/nodL* mutant. Consistent with our

Fig. 2. Transcriptional activation of *MtENOD20* in *Medicago truncatula* roots following *Sinorhizobium meliloti* inoculation and Nod factor addition. *M. truncatula* plants in growth pouches were (A) inoculated with *S. meliloti* RCR2011 or (B) treated with 5×10^{-8} M NodRm. Secondary roots were harvested at the indicated time points, total RNA extracted, and *MtENOD20* transcript levels evaluated by reverse transcriptase–polymerase chain reaction analysis (see Materials and Methods). The additional higher molecular weight band in the 24-h Nod factor-treated sample corresponds to amplification of contaminating genomic DNA (see Materials and Methods). Parallel amplification of *MtPR10-1* mRNA was used as a control for quality and quantity of RNA samples.

Table 1. Percentage of β -glucuronidase (GUS)-positive transgenic plants following inoculation with *Sinorhizobium meliloti* *nod* mutants

<i>S. meliloti</i> strain	Time following inoculation (days)			
	2	3	5	10
RCR2011 (parent)	100	100	100	100
RCR2011 <i>nodA</i> ::Tn5	0	0	0	0
RCR2011 <i>nodH</i> ::Tn5	30	80	80	80
RCR2011 Δ <i>nodH</i>	20	80	80	80
RCR2011 Δ <i>nodF/nodL</i> ::Tn5	40	70	90	100

findings for the *nodH* mutants, regions showing clear CCA responses also stained intensely for GUS activity. At this stage, no staining was observed in the highly deformed root hairs adjacent to CCA-responsive regions of the root (Fig. 3B), in line with the infection-defective phenotype of this mutant (Ardourel et al. 1994). Despite similar kinetics for the percentage of plants responding to *nodH* and *nodF/nodL* mutants following inoculation (Table 1), it should be emphasized that the cortical response to the *nodF/nodL* mutant was generally more extensive and more intense with regard to GUS staining, and that regions of CCA elicited by this mutant often led to visible root swellings 7 to 10 days after inoculation (data not shown).

***MtENOD20* expression in root tissues in response to *S. meliloti* Nod factors.**

Our results with wild-type and mutant strains suggested that *MtENOD20* expression could serve as a useful marker for early symbiotic responses, and in particular those involving CCA. Since purified Nod factors are capable of eliciting root nodule organogenesis in alfalfa (Truchet et al. 1991), we decided to examine Nod factor responses in roots of transgenic plants expressing *pMtENOD20-GUS*. Initial experiments were carried out with 10^{-8} M NodRm, since relatively high concentrations of the *S. meliloti* Nod factor (10^{-9} to 10^{-7} M) are required to observe nodulation-related root deformations (Truchet et al. 1991). We found that reporter gene activity could be readily detected in inner cortical root tissues 24 to 48 h following addition of 10^{-8} M NodRm to transgenic plants grown in pouches (Fig. 3C). GUS staining localized to zones within the root corresponding to the nodulation-susceptible region of the root at the moment of Nod factor addition. Figure 3C also clearly illustrates the typical Nod-factor-induced root hair deformations (most frequently hair branchings), which occur in regions of the epidermis located above the zones of cortical staining. Note that GUS activity is not associated with these root hair deformations, again consistent with our conclusion that *MtENOD20* expression in root hairs is dependent upon infection thread formation. Detailed observations of the GUS-staining cortex revealed the presence of both cortical cell divisions and starch-containing cells (data not shown), underlining once more the relationship between symbiotic CCA and *MtENOD20* expression. Approximately two thirds of pouch-grown plants scored positive for cortical GUS staining as early as 24 h following addition of 10^{-8} M NodRm, with the percentage of positively staining plants increasing to 100% 48 h later (Table 2).

The kinetics of Nod-factor-dependent *MtENOD20* mRNA accumulation were then examined with the sensitive RT-PCR approach described above. Figure 2B shows that *MtENOD20* transcripts can be detected within 12 h following addition of 5×10^{-8} M NodRm to *M. truncatula* roots. Differences in the timing of mRNA accumulation elicited by *S. meliloti* and purified Nod factors (compare Figure 2A and B) are probably due to the relatively high concentration of NodRm used in this experiment and to the fact that the Nod factors must first be synthesized in the bacteria.

The rapidity of *pMtENOD20-GUS* gene activation in the cortex in response to 10^{-8} M NodRm encouraged us to investigate the reactivity of the root cortex to much lower concentrations of Nod factor. Dose-response experiments with

MtENOD20 expression as a qualitative measure of CCA showed that Nod factors are in fact active down to 10^{-11} M, although the percentage of responding plants is significantly lower at subnanomolar concentrations of the LCO elicitor (Table 2). Note that only differences of greater than 10% in the percentage of GUS-staining plants can be considered statistically significant (see Materials and Methods). We also observed that the extent and intensity of individual responding regions were generally reduced at these low Nod factor concentrations.

CCA elicited by modified Nod factors.

It has previously been shown that modifications to the structure of the *S. meliloti* Nod factor can result in significant alterations in the capacity of this signaling molecule to elicit visible, nodulation-related root deformations in the alfalfa root (Truchet et al. 1991). We therefore attempted to use transgenic *pMtENOD20-GUS* alfalfa plants as a sensitive semiquantitative assay system for evaluating the effects of such structural modifications on early cortical cell responses. For comparative purposes all Nod factor variants were tested at a concentration of 10^{-8} M and reporter gene expression was monitored during a period of 2 to 5 days after addition of the elicitor. Following the earlier experiments with *nodF/nodL* and *nodH* mutants, we initially tested the corresponding modified factors purified from these two strains. As expected, and in line with results previously presented by Ardourel et al. (1994) the non-O-acetylated *cis*-vaccenic acid factor produced by the *nodF/nodL* mutant was indeed able to elicit cortical *MtENOD20* gene expression (Table 3). On the other hand, and rather to our surprise, we were unable to detect reporter gene activity in roots of transgenic plants treated with the purified nonsulfated factor synthesized by the *nodH* mutant. This negative result was confirmed by testing a total of more than 30 plants in several independent experiments and with a higher (up to 10^{-7} M) nonsulfated factor concentration (data not shown). Different hypotheses explaining this discrepancy between the CCA-inducing activities of the *nodH* mutant and the purified nonsulfated factor will be discussed later.

Since removing the *O*-acetyl group and replacing the fatty acid C16:2 by C18:1 did not appear to be critical for Nod-factor-dependent CCA we decided to vary the length of the fatty acid chain. In these experiments we made use of a range of chemically synthesized, O-sulfated LCOs lacking the *O*-acetyl group and with linear mono-unsaturated fatty acid chains ranging from 0 to 16 carbon atoms in length (Demont-Caulet et al., *in press*). The results presented in Table 3 show that a major reduction in the percentage of reactive plants was observed when the fatty acid chain was shortened to half the normal length. As with very low concentrations of NodRm (see above), the extent and intensity of the responding regions were also reduced in response to C8:1 N-acylated molecules. When the fatty acid moiety was totally removed, leaving a sulfated chitin tetramer [CO-IV (S, NH₂)], we observed a total loss of CCA activity (Table 3), and it was subsequently confirmed that this is also the case at the higher concentration of 10^{-7} M (data not shown). These results suggest that the presence and length of the acyl chain play a key role in the capacity of the *S. meliloti* Nod factor to elicit the CCA response in alfalfa.

DISCUSSION

The *MtENOD20* gene is transcribed during both nodule organogenesis and host cell infection.

The expression pattern of the *M. truncatula* *ENOD20* early nodulin gene during root nodulation has been studied at the cellular level with *M. varia* plants transformed with a promoter-*GUS* gene fusion. The validity of such a transgenic plant approach has already been demonstrated for several other early nodulin genes including *ENOD12* (Pichon et al. 1992; Bauer et al. 1996), *ENOD40* (Fang and Hirsch 1998), and *Mtlec1&3* (Bauchrowitz et al. 1996), and is particularly appropriate when gene expression is limited to discrete sub-populations of cells within a given organ. Our results show that the p*MtENOD20*-*GUS* fusion is first activated during the earliest stages of nodule organogenesis in the alfalfa root inner cortex, approximately 24 h following *S. meliloti* inoculation, and we have used RT-PCR to confirm that the endogenous *MtENOD20* gene follows similar activation kinetics. Analysis of histochemically stained tissues has revealed a strict correlation between reporter gene activity and the presence of cell divisions in the inner cortex, corresponding to sites of nodule

primordium formation (Fig. 1B), although at this stage we have no information concerning the precise temporal relationship between cell division and gene expression.

In addition to nodule primordium-related gene expression, reporter gene activity was also detected in the root epidermis at sites of primordium development (Fig. 1C and D). *GUS* staining appeared to be strictly limited to root hairs containing infection threads and our results with *S. meliloti* nodulation mutants and purified Nod factors together suggest that *MtENOD20* expression in root hairs is indeed infection dependent (Fig. 3A–C). The relative timing of *MtENOD20* activation in the cortex and epidermis is in line with previous observations that CCA precedes root hair infection (Calvert et al. 1984; Dudley et al. 1987; Bauchrowitz et al. 1996).

It should be recalled that the *MtENOD20* gene encodes a protein that belongs to a small family of early nodulins (including *MtENOD16*, *Ps/VsENOD5*, and *GmN315/ENOD55*) with structural (but probably not functional) homology to phytocyanins (Greene et al. 1998). In situ hybridization studies have shown that the *Ps/VsENOD5* genes are expressed in root cortical cells carrying infection threads and in infected cells of the nodule, but not in the nodule pri-

Fig. 3. Localization of β -glucuronidase (*GUS*) activity in transgenic *Medicago varia* roots in response to either *Sinorhizobium meliloti* *nod* mutants or purified Nod factors. **A**, Root segment of transgenic *M. varia* 72 h post inoculation with RCR2011 *nodH::Tn5*. Anticlinal cell divisions in the inner cortex (white arrows), and centrally located nuclei with surrounding amyloplasts (arrowheads) are typical characteristics of cortical cell activation (CCA; Ardourel et al. 1994). Dark arrows indicate undamaged root hairs above the zone of CCA that are not deformed. Bar = 100 μ m. **B**, Transgenic *M. varia* root 4 days post inoculation with RCR2011 Δ *nodF/nodL::Tn5*. Cortical *GUS* staining associated with CCA is very intense, and root hairs exhibit the striking deformations typical for this mutant (arrows). Bar = 100 μ m. **C**, Root segment showing a complete zone of *GUS* staining 48 h after the addition of 10^{-8} M NodRm to transgenic *M. varia*. Reporter gene activity is localized essentially to the inner cortex. Unstained root hairs throughout the entire zone (but not outside) display characteristic Nod-factor elicited deformations such as branching (arrows). Bar = 100 μ m.

mordium prior to infection (Scheres et al. 1990; Vijn et al. 1995). We have reached similar conclusions for the *MtENOD16* gene based on a transgenic plant/reporter gene approach (V. Vernoud, *unpublished results*). The soybean *N315/ENOD55* genes are also expressed in infected cells of the nodule (de Blank et al. 1993; Kouchi and Hata 1993). These findings suggest that the expression of all members of this gene family can be correlated with developmental events related to host cell infection by the microsymbiont. On the other hand, *MtENOD20* is the only gene member identified so far that is also activated during nodule organogenesis in the root cortex.

We have previously proposed that the extracellular proteins encoded by this gene family may be components of the cell wall matrix, with possible roles in cell wall assembly/reorganization (Greene et al. 1998). Such a function would certainly be compatible with both the requirements for infection thread progression/penetration and the likely restructuring of the cell wall in the developing primordium. All members of this early nodulin family possess a Pro-rich domain, highly variable in length, sequence, and composition, located on the C-terminal side of the conserved globular domain. However, it is striking that *MtENOD20* possesses by far the largest (hydroxy)proline-rich domain of the entire family, consisting essentially of hydroxyproline-rich plant glycoprotein (HRGP) structural motifs (Greene et al. 1998). It remains to be determined whether there is a relationship between this lengthy HRGP-like domain and the unique expression pattern of the *MtENOD20* gene.

***MtENOD20* gene expression is associated with CCA in the absence of infection.**

Symbiotic *MtENOD20* expression has been further investigated in experiments exploiting *S. meliloti* *nod* mutants with nodulation-defective phenotypes resulting from structural modifications to the Nod factor. The *S. meliloti* *nodF/nodL* mutant produces Nod factors (NodRm-IV[S,C18:1]) modified at the nonreducing end of the sugar backbone and is infection-defective (Ardourel et al. 1994). However, this mutant is still able to elicit both elaborate root hair deformations and a striking cortical cell response characterized by starch accumulation, the distribution of amyloplasts around cortical cell nuclei, and limited cell divisions, that has been termed cortical cell activation (CCA). The *pMtENOD20-GUS* fusion is strongly expressed at sites of CCA resulting from inoculation of transgenic alfalfa with a *nodF/nodL* mutant (Fig. 3B). This is consistent with activation of the transgene during early stages of nodule primordium development (see above) and also at sites of CCA elicited by both the *S. meliloti* *nodH* mu-

tant and purified Nod factors (Fig. 3A and C, respectively; see below). In this paper, RT-PCR experiments have been used to demonstrate rapid induction of the endogenous *MtENOD20* gene in response to both *S. meliloti* inoculation and purified Nod factor treatment (Fig. 2). Although RT-PCR was not used to confirm endogenous *MtENOD20* expression elicited by the *nodF/nodL* and *nodH* mutants, it is clear from the use of histochemical GUS localization that *pMtENOD20-GUS* expression in the inner cortex is always associated with differentiation processes related to symbiotic CCA.

S. meliloti *nodH* mutants synthesize Nod factors that lack the terminal reducing end *O*-sulfate substitution (Roche et al. 1991) and until now have been considered unable to elicit symbiotic responses on the normal host plant alfalfa. However, we have discovered that *nodH* mutants are capable of activating *MtENOD20* expression coupled to CCA (Fig. 3A), although less efficiently than the *nodF/nodL* mutant. It is probable that this symbiotic response was not previously reported for *nodH* mutants (Truchet et al. 1991; Ardourel et al. 1994) because of both its relatively low frequency and the limited extent of CCA, which never develops as far as visible root swellings. On the other hand, purified non-sulfated *S. meliloti* factors were not able to elicit *MtENOD20* expression and the CCA response in transgenic alfalfa lines, even when applied at relatively high concentrations (up to 10^{-7} M). One possible explanation for this apparent paradox is that the *nodH* mutant synthesizes a second molecule that is either sufficient itself for low-level CCA stimulation, or acts in synergy with the nonsulfated Nod factor. A somewhat analogous situation has been described for *Bradyrhizobium japonicum* *nodZ* mutants that produce nonfucosylated, pentameric Nod factors that lack biological activity, and yet nodulate soybean normally (Stacey et al. 1994; Stokkermans et al. 1995). However, it is also possible that plant chitinases are responsible for the failure of exogenously supplied, nonsulfated factors to elicit CCA, since Staehelin et al. (1994) have demonstrated that the absence of the sulfate group makes the *S. meliloti* Nod factors particularly sensitive to chitinase degradation. Furthermore, Goormachtig et al. (1998) have recently identified a *Sesbania rostrata* chitinase-encoding gene that is transcribed very early during nodulation in cortical tissues adjacent to the developing primordium. If the chitinase hypothesis is correct, then the ability of the *nodH* mutant to initiate CCA suggests that the continued synthesis of Nod factors by the bacterium at the root surface is necessary for this activity.

Significantly, root hair deformations are not observed in epidermal tissues above sites of *nodH* mutant-elicited CCA

Table 2. Percentage of β -glucuronidase (GUS)-positive plants as a function of the concentration of exogenously *Sinorhizobium meliloti* Nod factor

NodRm concentration ^a (M)	Length of Nod factor treatment (days)			
	1	2	3	5
10^{-7}	80	90	100	100
10^{-8}	60	80	100	100
10^{-9}	60	80	90	90
10^{-10}	40	70	80	80
10^{-11}	30	50	60	60
10^{-12}	0	0	0	0

Table 3. Percentage of β -glucuronidase (GUS)-positive plants as a function of lipo-chitoooligosaccharide (LCO) structure

Elicitor (10^{-8} M) ^a	Length of elicitor treatment (days)		
	2	3	5
NodRm-IV(Ac, S, C16:2) (parental strain)	80	100	100
NodRm-IV(Ac, C16:2) (<i>nodH</i> mutant)	0	0	0
NodRm-IV(S, C18:1) (<i>nodF/nodL</i> mutant)	80	90	90
LCO-IV(S, C16:1)	70	90	90
LCO-IV(S, C12:1)	70	80	80
LCO-IV(S, C8:1)	30	30	50
CO-IV(S, NH ₂)	0	0	0

^a Abbreviations: Ac, *O*-acetyl; S, *O*-sulfate.

(Fig. 3A), in line with previous reports that the *nodH* mutant is unable to induce symbiotic epidermal responses (Debellé et al. 1986), including expression of the *MtENOD12* gene (Journet et al. 1994). Assuming that the CCA activity of the *nodH* mutant is due to the nonsulfated factor, this suggests that the absence of the sulfate moiety is more critical for the epidermal response. These results also imply that root cortical responses can be elicited without apparent activation of epidermal tissues and therefore favor the hypothesis that different Nod factor perception mechanisms are involved in initiating early symbiotic responses in the cortex and epidermis.

***MtENOD20* as a molecular marker for Nod factor-elicited CCA.**

When purified NodRm factor is added to transgenic alfalfa roots *pMtENOD20-GUS* expression associated with localized sites of CCA can be detected within 24 h (Fig. 3C). As with *S. meliloti*-elicited expression, we have confirmed the rapid induction of endogenous *MtENOD20* transcripts with RT-PCR amplification of total RNA extracted from Nod factor-treated *M. truncatula* roots (Fig. 2B). Use of transgenic plants has further demonstrated that *MtENOD20* expression linked to CCA can be detected at NodRm concentrations as low as 10^{-11} M (Table 2). We have previously proposed that the physiological concentration of Nod factors produced by the micro-symbiont at the root surface is most probably in the subnanomolar range (Journet et al. 1994). If this is indeed the case then our results show for the first time that exogenously applied Nod factors are capable of initiating CCA at physiological levels.

We have made use of a series of synthetic, non-O-acetylated LCOs with variable acyl chain lengths to show that the length of the lipid chain is of critical importance for eliciting gene activation coupled to CCA in the cortex. Even at the relatively high concentration of 10^{-8} M, shortening the fatty acid to half the normal length (C8:1) results in a major reduction in CCA activity, while nonacylated, sulfated chitin tetramers are totally devoid of activity (Table 3). These findings are consistent with a recent Nod factor structure/activity study employing the same synthetic LCOs and alfalfa nodulation as a semiquantitative assay (Demont-Caulet et al., *in press*). Our experiments with biological (NodRm-IV[S,C18:1]; *nodF*/*nodL* mutant) and synthetic (LCO-IV[S,C16:1]) molecules show that, when tested at 10^{-8} M, removal of the O-acetyl group and modifications to the number and position of the double bonds within the N-acyl chain have no detectable effect on the efficiency of CCA induction (Table 3). Nevertheless, it will be important to repeat these experiments when synthetic, O-acetylated LCOs become available and to use a lower range of LCO concentrations in order to increase the sensitivity of the assay.

These findings for alfalfa are in line with previous reports showing that the exogenous addition of either unsubstituted or O-acetylated chitin oligomers to vetch and soybean fails to elicit cortical cell divisions and nodule primordium formation (Spaink et al. 1991; Minami et al. 1996; Schlaman et al. 1997). However, when microtargeted into the cortex, O-acetylated chitin oligomers appear to be capable of eliciting changes in the root auxin balance characteristic of nodule initiation in clover (Mathesius et al. 1998) and inducing cortical cell divisions in vetch in the presence of uridine (Schlaman et

al. 1997). Taken together, these findings imply that the fatty acyl moiety may be necessary for LCO transport across the epidermal cell layer, and are consistent with different sites of perception for Nod factor-elicited epidermal and cortical responses, as discussed above.

MtENOD20 now joins a small group of early nodulin genes including *ENOD40* (Vijn et al. 1995; Minami et al. 1996; Fang and Hirsch 1998), *Mtlec1&3* (Bauchrowitz et al. 1996), and certain *ENOD12* genes (Vijn et al. 1995; Bauer et al. 1996) whose nodulation-related expression in cortical tissues can be elicited by both *Rhizobium* and purified Nod factors. However, compared with that of the majority of the other early nodulins, *MtENOD20* gene expression within the root can be considered essentially specific to the nodulation process. This is illustrated both by the absence of detectable *MtENOD20* transcription in noninoculated *M. truncatula* roots (even after 38 cycles of RT-PCR) and the specificity of GUS staining with respect to symbiotically activated tissues in transgenic *M. varia* roots (with the exception of a low background vascular staining for a minority of pouch-grown plants). Analogous transgenic plant/reporter gene studies have shown that *MsENOD40* and *MsENOD12A* genes are expressed in lateral root primordia (Bauer et al. 1996; Fang and Hirsch 1998) and *Mtlec1&3* in inner root tissues independently of nodulation (Bauchrowitz et al. 1996).

Until now the organogenetic activity of Nod factors in the majority of legumes has generally been studied by means of cytological analyses. This approach often requires lengthy (1 to 3 weeks) exposures to high concentrations of Nod factor, and is particularly difficult to exploit when the resulting CCA is limited to only a few rounds of division. The results presented in this article indicate that transgenic plants expressing the *pMtENOD20-GUS* fusion should provide both a sensitive and rapid assay for monitoring Nod factor-elicited CCA, and at the same time a convenient marker for distinguishing between root and nodule primordia. The identical transgene has recently been introduced into the homologous host *M. truncatula* in order to facilitate the detailed characterization of plant mutants that are defective in early stages of nodulation.

MATERIALS AND METHODS

Construction of a transcriptional *MtENOD20* promoter-*GUS* fusion.

In order to create a precise transcriptional fusion between the promoter region of the *MtENOD20* gene and the coding region of the *Escherichia coli* GUS reporter gene, a PCR strategy similar to that described in Pichon et al. (1992) was used. Briefly, a genomic *MtENOD20* clone (Greene et al. 1998) was used as template for PCR with a forward primer (5'-CTACTAAGCTT(*EcoRI*)CTTCGGTGAAGACC-3') and reverse primer (5'-AGAAGCCATGG(*NcoI*)TCATGATTCT-3') in order to amplify a 2.15-kb promoter DNA fragment (single underlined bases correspond to substitutions required to create the labeled restriction sites and double underlining indicates the *MtENOD20* translation initiation codon). After digestion with *EcoRI* and *NcoI* the *MtENOD20* promoter fragment was cloned between the *EcoRI* and *NcoI* sites within the polylinker region of the vector pBSGUS, in order to generate a precise transcriptional fusion. The vector pBSGUS is a pBluescript hybrid that was constructed by cloning the

EcoRI/PstI GUS coding fragment from pCCOGUS (Axelos et al., 1989) into the corresponding polylinker sites of pBluescript KS⁺ (Stratagene, La Jolla, CA). The p*MtENOD20-GUS* fusion was then excised from the pBS vector by *HindIII/SacI* digestion, and cloned between the corresponding unique sites in the binary vector pLP100 (Szabados et al. 1995). The junction between the promoter fragment and the GUS coding region was confirmed by DNA sequencing.

Transformation of *M. varia* and regeneration of transgenic plants.

The introduction of the p*MtENOD20-GUS* fusion into the *M. varia* genotype A2 (Deak et al. 1986) was performed essentially as described in Pichon et al. (1992). Scarified leaflets were co-cultivated for 4 days with *Agrobacterium tumefaciens* LBA4404 containing the pLP100-p*MtENOD20-GUS* binary vector. Somatic embryogenesis was induced on kanamycin-resistant callus, with the first embryos appearing 1 month after co-culture. For maturation and plantlet formation, embryos were transferred to hormone-free medium supplemented with maltose, proline, and charcoal. Regenerated primary transformants, each derived from an individual leaf callus, were grown on axenic medium containing 1% sucrose at 25°C with a 16-h photoperiod (65 $\mu\text{E s}^{-1} \text{m}^{-2}$) and were propagated by cuttings. To obtain transgenic *S*₁ seeds, primary transformants were transferred to pots and grown under the appropriate conditions to induce flowering and pod formation (25°C, 16-h photoperiod, 230 $\mu\text{E s}^{-1} \text{m}^{-2}$).

S. meliloti inoculation and Nod factor addition to transgenic plants.

For reporter gene expression studies during symbiosis, cuttings of primary *M. varia* transformants were grown under aeroponic conditions as described in Bauchrowitz et al. (1996). After 2 to 3 weeks of growth, combined nitrogen was removed from the medium and plants were inoculated with the *S. meliloti* strain RCR2011 (approximately 5×10^5 bacteria per ml). Whole root segments or nodules were collected at various times after inoculation for GUS histochemical staining (see below).

Transgenic *S*₁ seeds from two representative primary transformant lines were used both for experiments with *S. meliloti* symbiotic mutants and for tests with purified Nod factors or synthetic LCOs. Seeds were surface sterilized (8 min H_2SO_4 , followed by 2 min 12% sodium hypochlorite) and germinated for 36 h in the dark at 25°C. Transgenic seedlings (five plants per pouch) were then placed in plastic growth pouches (Mega International, Minneapolis) as described in Journet et al. (1994). Following the development of secondary roots (7 to 10 days later), roots were either inoculated with *S. meliloti* (approximately 5×10^6 bacteria per root) or treated with the appropriate Nod factor/LCO by direct addition into the growth pouch.

Bacterial strains, purified Nod factors, and synthetic LCOs.

Transgenic plants in pouches were inoculated with the parental *S. meliloti* strain RCR2011 and with the following four mutant strains: RCR2011 *nodA::Tn5*#2208 (GMI 5383, Debelle et al. 1986), RCR2011 *nodH::Tn5*#2313 (GMI5431, De-

bellé et al. 1986), RCR2011 $\Delta(\text{nodH})8$ (GMI5619, Debelle et al. 1988), RCR2011 $\Delta(\text{nodF})13\text{nodL::Tn5}$ (GMI6628, Ardourel et al. 1994). *S. meliloti* strains were grown at 28°C in standard TY medium supplemented with 6 mM CaCl_2 and appropriate antibiotic selection.

S. meliloti Nod factors purified from the corresponding overproducing parental and mutant strains (Roche et al. 1991) were kindly provided by F. Maillat (J. Dénarié's group, LBMRPM, Castanet-Tolosan). Sulfated LCO-IV with variable length fatty acid chains (C8:1, C12:1, and C16:1) and sulfated chitin tetramer lacking the acyl chain and with a free NH_2 [CO-IV(S, NH_2)] were a kind gift of J.-M. Beau and were synthesized as described in Demont-Caulet et al. (*in press*). Serial dilutions of Nod factors and LCOs were carried out in sterile water.

Histochemical localization of GUS activity and the semiquantitative assay.

Histochemical staining for GUS activity was performed on whole root segments for up to 24 h with the substrate X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide, cyclohexylammonium salt; Biosynth AG, Staad, Switzerland) as described in Journet et al. (1994). Nodules were sliced into 80- μm -thick sections (Microcut H1200; Bio-Rad, Hercules, CA) prior to staining for GUS activity. In this case, pre-fixation of intact samples was carried out with 0.5% para-formaldehyde and incubation times in GUS reaction medium were generally limited to between 15 min and 2 h. Stained intact samples or slices were briefly cleared with sodium hypochlorite as described in Pichon et al. (1992) and observed with a Zeiss Axio-phot light microscope (Carl Zeiss, Oberkochen, Germany). For localization of GUS activity at the cellular level, stained roots were post-fixed for 1 h in 2.5% glutaraldehyde buffered in 0.1 M potassium phosphate (pH 7.0), dehydrated in an alcohol series and embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany). Sections (4 μm thick) were observed by bright- and dark-field microscopy after counter-staining with basic fuchsin (0.5% in distilled water). Double staining for GUS and β -galactosidase activity after inoculation with a RCR2011 strain carrying a constitutive *hema-lacZ* fusion (Ardourel et al. 1994) was performed according to Pichon et al. (1994).

In our semiquantitative assay, transgenic plants were scored positive for GUS staining in the cortex on the basis of a clear and unambiguous signal located at or below the region of the root that is normally susceptible to nodulation at the moment of bacterial inoculation. This susceptible region lies between the root tip and the zone of root hair emergence (Bhuvaneswari et al. 1981; Caetano-Anollés and Gresshoff 1991), and the use of growth pouches enabled us to monitor root growth following either *S. meliloti* inoculation or Nod factor addition and thus to position GUS expression in relation to the susceptible zone. This approach eliminated the potential risk of confusing *Rhizobium*/Nod factor responses with the development of spontaneous nodule primordia in the older region of the root (see Pichon et al. 1994). Since approximately 20% of the transgenic alfalfa *S*₁ plants generated after selfing were GUS negative, the percentage values presented in Tables 1–3 have been corrected to take this into account. At least 15 to 20 plants were tested for each experimental condition, and each series of experiments was repeated at least

twice. In the experiments with *nodH* mutants, a total of over 150 plants were tested. Percent values have been rounded up to the nearest 10% and statistical analysis has shown that, in the majority of cases, differences of 10% cannot be considered significant.

RT-PCR assay to detect *MtENOD20* transcripts.

For each time point (0, 12, 24, 48, and 72 h), secondary roots of 30 pouch-grown *M. truncatula* cv. Jemalong plants, either inoculated with *S. meliloti* 2011 or treated with purified Nod factors, were harvested and ground in liquid nitrogen. Total RNA was isolated by a standard phenol/sodium dodecyl sulfate protocol and quantified by spectrophotometry and agarose gel electrophoresis. Prior to RT-PCR, genomic DNA was eliminated by treating 1- to 5- μ g aliquots of total RNA with 10 U of DNase I in RT buffer (50 mM TrisHCl, 75 mM KCl, 3 mM MgCl₂, pH 8.3) at 37°C during 30 min, followed by phenol/chloroform extraction and ethanol precipitation. First-strand cDNA synthesis with 1 to 3 μ g of total RNA was performed as described in Pingret et al. (1998). Primers were chosen on either side of the single *MtENOD20* intron (Greene et al. 1998) in order to distinguish cDNA from genomic amplified products. A 283-bp *MtENOD20* cDNA fragment was amplified during 38 cycles (94°C/30 s, 62°C/30 s, 72°C/30 s) between positions 123 and 570 (relative to the ATG codon) of the genomic sequence (GenBank accession no. X99467) with forward primer 5'-AACTCGACATGCACTCACCCG-3', and reverse primer 5'-GGAAGAAAGTACGGGAGCTACC-3'. Under our experimental conditions an exponential increase of PCR product was still observed after 38 cycles. The parallel amplification of a constitutively expressing gene in roots, *MtPR10-1* (Gamas et al. 1998; GenBank accession no. Y08726), allowed us to control for equivalent cDNA levels in samples. *MtPR10-1* cDNA was amplified during 25 cycles according to Pingret et al. (1998). PCR products were analyzed by Southern blotting with the 1-kb *HindIII/SstI* fragment of *MtENOD20* and the complete cDNA for *MtPR10-1*.

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