

***ENOD12* Gene Expression as a Molecular Marker for Comparing *Rhizobium*-Dependent and -Independent Nodulation in Alfalfa**

M. Pichon, E.-P. Journet, F. de Billy, A. Dedieu, T. Huguet, G. Truchet, and D. G. Barker

Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes, INRA-CNRS, BP 27, 31326 Castanet-Tolosan Cédex, France

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Certain genotypes of alfalfa are able to nodulate in the absence of *Rhizobium* (Nar), resulting in the formation of non-nitrogen fixing root structures which possess histological features of *Rhizobium*-elicited nodules. To study the spatio-temporal expression pattern of the early nodulin *ENOD12* gene during Nar ontogeny, we have crossed an alfalfa genotype exhibiting a pronounced Nar phenotype with transgenic alfalfa expressing a *MtENOD12*-GUS chimeric gene fusion. Following nitrogen deprivation, and in the absence of *Rhizobium*, GUS activity can first be detected in foci of dividing cells in the inner root cortex, corresponding to early stages of Nar development. Such Nar primordia are initiated exclusively in the older part of the root system, in contrast to *Rhizobium*-elicited nodulation, and the majority of mitotic foci arrest at an early stage of development. At later stages, *ENOD12* is transcriptionally active in a sub-meristematic region of the spontaneous nodule, analogous to the localisation of *ENOD12* transcripts in N₂-fixing nodules. Based on the spatio-temporal pattern of Nar development throughout the root system, we find no evidence for systemic autoregulation of spontaneous nodulation. These results show that *Medicago ENOD12* gene expression can serve as a molecular marker for spontaneous nodule ontogeny in alfalfa and we conclude that, despite certain similarities, significant regulatory and developmental differences exist between *Rhizobium*-dependent and -independent nodulation.

Additional keywords: autoregulation, combined nitrogen deprivation, early nodulin gene, Nar ontogeny, spontaneous nodulation, transgenic *Medicago*.

The formation of nitrogen-fixing nodules on the roots of leguminous plants results from the symbiotic association with soil bacteria known as rhizobia. In the case of the temperate legume alfalfa, the microsymbiont *Rhizobium meliloti* penetrates into the root cortex via root hairs after the induction of a specialized tubular structure of plant origin called the infection thread. Concomitant with bacterial infection, nodule organogenesis is initiated as inner root cortical cells are mito-

tically reactivated, leading to the formation of the nodule primordium. The development of an apical meristem and the release of *Rhizobium* from the infection thread result in the subsequent formation of the mature N₂-fixing nodule. Alfalfa nodules are cylindrical, and longitudinal sections reveal a series of developmental stages behind the persistent meristem which include a zone of infection, a starch-rich interzone, a nitrogen fixing zone, and a zone of senescence (Vasse *et al.* 1990).

In 1989, Truchet *et al.* showed that certain alfalfa plants could develop non-nitrogen fixing structures on their root systems under strictly axenic conditions. This phenomenon was termed "Nodulation in the Absence of *Rhizobium*" (the Nar phenotype) or "spontaneous nodulation" (Truchet *et al.* 1989; Caetano-Anollés *et al.* 1990a). Microscopic studies of Nar⁺ alfalfa roots showed that the ontogeny of spontaneous nodulation initiates with cell divisions in the inner cortex (Joshi *et al.* 1991). Fully developed Nar nodules possess histological features characteristic of indeterminate nodules, including a persistent apical meristem, peripheral vascular bundles, an outer nodule endodermis and a surrounding cortex (Truchet *et al.* 1989; Joshi *et al.* 1991). Furthermore, the presence of nitrate in the growth medium efficiently inhibits the formation of these root-derived structures, as also occurs in the case of *Rhizobium*-induced nodulation (Truchet *et al.* 1989; Caetano-Anollés *et al.* 1990a).

The Nar phenomenon demonstrates that *Rhizobium* is not absolutely required for nodule morphogenesis, and consequently that Nar⁺ alfalfa plants possess all the genetic information needed to develop genuine root nodule structures. Considerable variability exists between such plants in terms of the distribution, morphology, and timing of appearance of Nar nodules, although *Rhizobium*-elicited nodulation appears to be unmodified in Nar⁺ plants (Truchet *et al.* 1989). The Nar⁺ phenotype is conserved during clonal propagation (Truchet *et al.* 1989) and transmitted through meiosis (Caetano-Anollés and Gresshoff 1992; T. Huguet, unpublished), thus arguing that plant genetic determinants are involved. A genetic model has been proposed for the control of Nar trait inheritance in alfalfa cv. "Vernal" (Caetano-Anollés and Gresshoff 1992).

Studies of *Rhizobium*-elicited nodulation have revealed that a number of plant genes are expressed in specific tissues related to different stages of the symbiotic interaction. So-called "early" nodulin genes are transcribed during infection and early nodule developmental stages, whereas "late" nodu-

Corresponding author: David G. Barker.

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lin gene expression is more directly associated with the N_2 -fixation process itself. So far, little is known about nodulin gene expression during spontaneous nodule ontogeny. Truchet *et al.* (1989) demonstrated that transcripts corresponding to one of the early nodulin genes, *ENOD2*, were present in such nodules, and Hirsch *et al.* (1992) subsequently localized these transcripts to nodule parenchyma tissue. This is identical to the tissue-specific localization previously described for *ENOD2* gene expression in *Rhizobium*-elicited nodules (van de Wiel *et al.* 1990). The finding that transcripts corresponding to the late nodulin leghemoglobin could not be detected in spontaneous nodules is consistent with the absence of N_2 -fixing tissues in these structures (Truchet *et al.* 1989).

In contrast to *ENOD2*, the early nodulin gene *ENOD12* is expressed in the central tissue of the nodule, and *in situ* hybridization studies on *Rhizobium*-elicited pea nodules has shown that *PsENOD12* transcripts localize to the zone of infection which lies between the meristematic zone and the N_2 -fixation zone (Scheres *et al.* 1990). We have reported similar results for *Medicago* nodules using a hybridization probe derived from the *Medicago truncatula* *MtENOD12* gene (Pichon *et al.* 1992). To facilitate studies of the spatio-temporal expression pattern of the *MtENOD12* gene during early stages of the symbiotic association, we constructed transgenic alfalfa (*M. varia*) plants expressing a fusion between the *MtENOD12* promoter and the *gusA* (*uidA*) reporter gene. Histochemical staining for GUS activity confirmed that *ENOD12* expression occurs in root and nodule tissues which are closely associated with infection events (Pichon *et al.* 1992). Indeed, transcriptional activation of the *MtENOD12* gene can first be detected in non-infected epidermal cells which lie just behind the growing root tip in the zone of root hair emergence, and we have recently shown that this very early response is directly dependent upon the activity of the extracellular lipo-oligosaccharides of *Rhizobium* known as Nod factors (Journet *et al.* 1994).

In this paper, transgenic Nar^+ alfalfa harboring the *MtENOD12*-GUS chimeric gene fusion are used to study whether the infection-related *ENOD12* gene is also expressed during Nar ontogeny. We find that GUS activity is present in Nar primordia which develop in the inner root cortex following deprivation of exogenous combined nitrogen. At later stages, and despite the lack of bacterial infection, *ENOD12* is transcribed in a well-defined submeristematic zone of the Nar nodule. By means of this convenient molecular marker, we have been able to map both early and late stages of Nar development within the entire root system and to show that most Nar primordia are arrested at an early stage of development. These findings have allowed us to compare and contrast the nodulation processes which occur either in the absence or presence of the microsymbiont *Rhizobium*. Preliminary results relating to this work appeared in the Proceedings of the 9th International Congress on Nitrogen Fixation held in Cancun, Mexico, December 1992.

RESULTS

Transgenic alfalfa plants with an enhanced Nar^+ phenotype.

When the *M. varia* genotype A2 or its transgenic derivatives carrying the *MtENOD12*-GUS fusion (Pichon *et al.*

1992) were grown in aeroponic conditions, only a very limited number of Nar nodules developed on the root system even after lengthy (4 wk) periods of combined nitrogen deprivation. To facilitate the analysis of *MtENOD12* expression during spontaneous nodulation in alfalfa, a representative primary transformant line (V5) was crossed with a *M. sativa* genotype (NSL-D) which has a pronounced Nar^+ phenotype (see Materials and Methods). Approximately 75% of the plants resulting from this cross expressed the *MtENOD12*-GUS fusion at a clearly detectable level and rapidly formed Nar nodules upon nitrogen deprivation. However, significant variation was observed between individual plants, both in terms of the level of GUS expression and the frequency of spontaneous nodulation. This is consistent with both the genetic complexity of tetraploid allogamous alfalfa and the presence of multiple copies of the chimeric gene fusion in the V5 parental line (Pichon *et al.* 1992). As mentioned earlier, plants with Nar^+ phenotypes are not modified with regards to *Rhizobium*-elicited nodulation (Truchet *et al.* 1989). We have confirmed that NSL-D \times V5 progeny nodulate with identical kinetics, similar frequencies and within the same region of the root as previously described for alfalfa (Bhuvaneswari *et al.* 1981; Caetano-Anollés and Gresshoff 1991a).

ENOD12 is expressed in foci of dividing cells in the inner root cortex.

In three independent experiments, a total of 21 GUS-positive NSL-D \times V5 progeny plants were grown aeroponically for a 2- to 3-wk period before combined nitrogen deprivation. Whole root systems of randomly sacrificed plants were subsequently harvested at regular intervals and treated for histochemical localization of GUS activity. Twenty-four hours after nitrogen deprivation, we were able to observe the development of GUS-staining foci of dividing cells in the inner root cortex of the most reactive plants (Fig. 1A). Two days later, all of the plants examined possessed such foci (Fig. 1B), which were only very rarely observed in the root systems of plants continuously supplied with combined nitrogen. GUS activity was clearly associated with clusters of cells (ranging from two to over 20 cells per cluster) derived from anticlinal followed by periclinal divisions within the inner cortex (Fig. 1C). Transverse sections of the root confirmed that these dividing cells were located either in the innermost cortical cell layer in direct contact with the endodermis or in the adjacent cortical layer (Fig. 1D). Furthermore, these foci were generally located opposite protoxylem poles (Fig. 1D), the usual position for the development of N_2 -fixing root nodules in response to *Rhizobium* (Phillips 1971; Libbenga and Harkes 1973). These and subsequent findings (see below) suggested that such mitotic foci correspond to the initial stages of Nar development. Finally, it should be emphasized that GUS expression was not observed in cortical tissue outside the mitotic foci (Fig. 1A, B, and D).

Rhizobium-elicited nodulation is generally initiated close to the growing root tip (Bhuvaneswari *et al.* 1981; Caetano-Anollés and Gresshoff 1991a). However, during the first days following combined nitrogen removal, we observed that GUS-staining foci were formed exclusively in the oldest region of the primary root. Subsequently, and in a progressive manner along with root growth, new foci were initiated in adjacent regions lower down on the primary root as well as in the old-

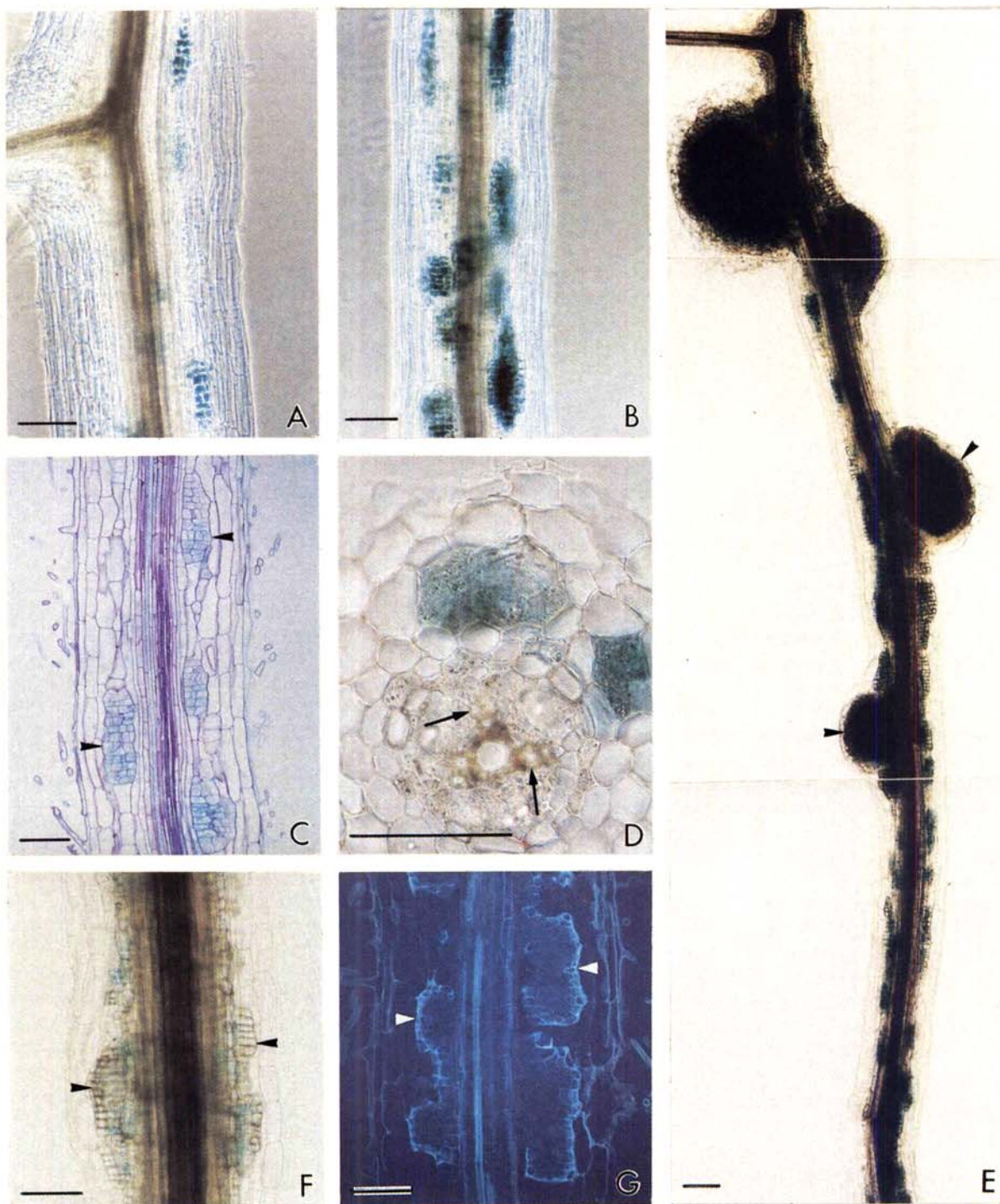


Fig. 1. Histochemical analysis of GUS activity in roots of transgenic alfalfa plants carrying the *MtENOD12*-GUS chimeric gene, following combined nitrogen deprivation. After staining with X-Gluc, the samples were observed microscopically under phase contrast (**A and B**), bright field (**C-F**), and UV-fluorescence (**G**). Bars: 100 μ m, except **E**: 200 μ m. **A and B**, GUS-positive foci observed in whole root after 24 (**A**) and 72 hr (**B**) of combined nitrogen deprivation. **C**, 4- μ m-thick longitudinal section of root fragment embedded in Technovit and stained with toluidine blue. Clusters of GUS-positive cells (arrowheads) are seen in the inner cortex. **D**, 80- μ m-thick root transverse section. GUS-positive foci in inner cortex are located opposite protoxylem poles (arrows). **E**, High density of GUS-staining foci in whole root fragment 4 days after nitrogen removal. Arrowheads point to developing Nar nodules. **F**, Region of the oldest part of the root 3 days after nitrogen removal. Note the decrease of GUS-staining in the foci and the thickening of the cell walls (arrowheads). **G**, 4- μ m-thick section of Technovit-embedded material observed by fluorescence microscopy. Autofluorescence (arrowheads) is seen in the outermost cell layer of the foci.

est parts of lateral roots (not shown). Although this pattern of mitotic foci development was identical for all the plants examined, the density of foci was very variable between individuals. For the plants with the highest densities, foci were virtually contiguous with each other along and around the root stele (Fig. 1E). Although spontaneous nodulation was

relatively infrequent for *M. varia* A2 plants and their transgenic derivatives, the location of these few nodules and the more abundant Nar mitotic foci appeared to be identical to that observed for the plants resulting from the NSL-D \times V5 cross (not shown).

By comparing the frequency of foci development within

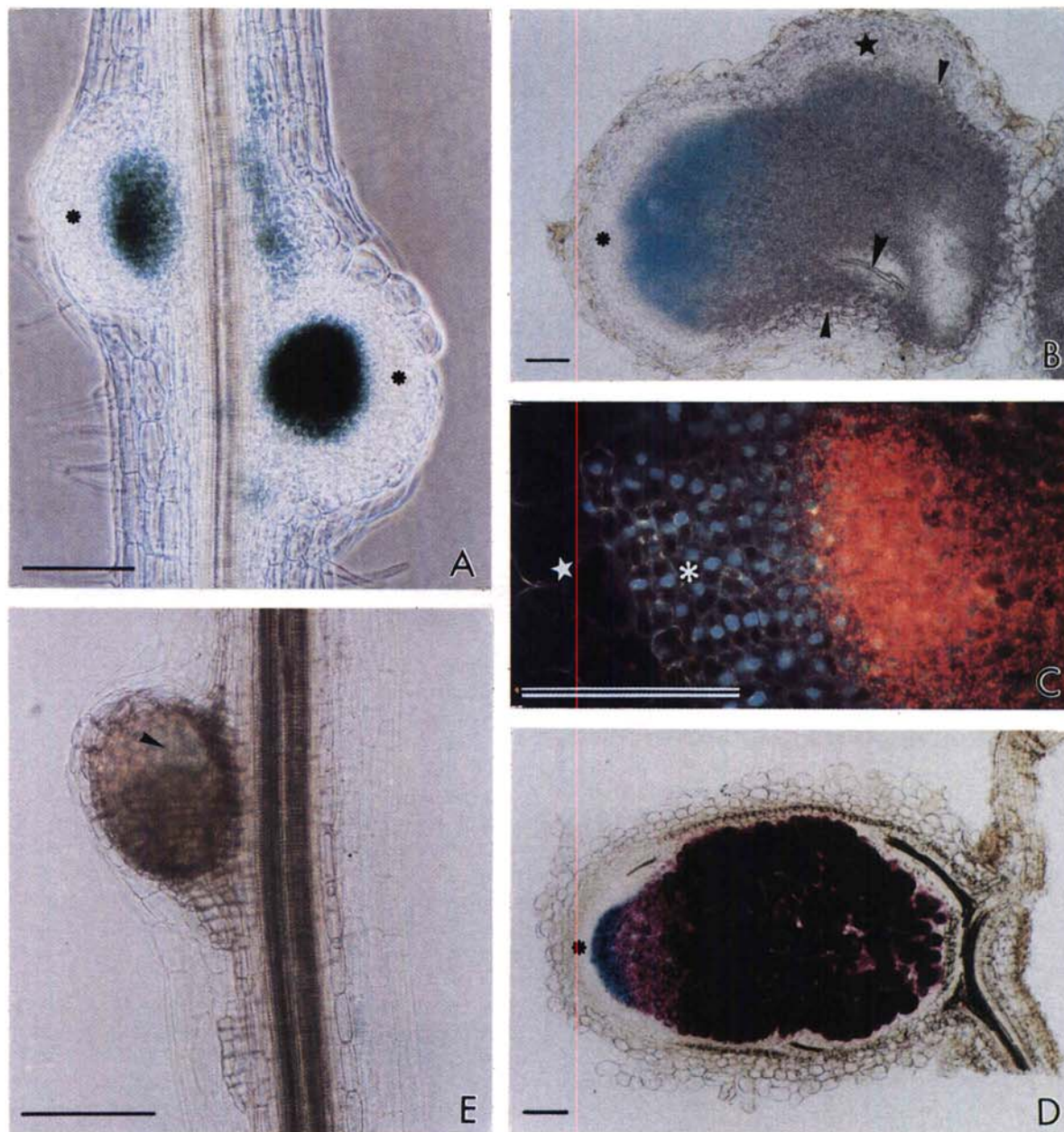


Fig. 2. Histochemical analysis of Nar nodules in transgenic alfalfa plants carrying the *MtENOD12*-GUS chimeric gene. Bars: 200 μ m, except C: 100 μ m. **A**, Developing Nar nodules observed on whole root. Intense GUS staining is observed in the central region of the nodule behind the meristematic region (asterisks). **B**, 80- μ m-thick section (stained for 2 hr) of a mature elongated Nar nodule harvested 15 days after combined nitrogen removal. GUS-staining is observed in the tissue located immediately behind the meristematic region (asterisk). The nodule cortex (star), the nodule endodermis (small arrowheads) and a vascular bundle (large arrowhead) are visible. **C**, 1- μ m-thick section of an Epon-embedded mature Nar nodule. The distal part of the nodule has been viewed using a combination of UV-fluorescence and dark-field microscopy modes. Nuclei can be identified by the strong blue autofluorescence. The GUS-staining (X-Gluc as substrate) region is colored in red as a consequence of observing the resin-embedded semi-thin section by dark-field microscopy, and localizes reporter gene activity to a zone proximal to the meristem (asterisk). The star indicates the GUS-negative nodule cortex. **D**, 80- μ m-thick section of a N_2 -fixing nodule harvested 17 days after inoculation of transgenic *Medicago varia* with *Rhizobium meliloti* RCR 2011 bearing the *hemA-lacZ* chimaeric gene (see Materials and Methods). The section was double-stained for GUS (20-min reaction) and β -galactosidase activities. The blue stain (X-Gluc as substrate) corresponds to *MtENOD12*-GUS expression in a narrow zone proximal to the meristem (asterisk) and the red stain (Magenta-gal as substrate) localizes symbiotic bacteria. **E**, Arrested Nar nodule. Note the very faint GUS staining (arrowhead) inside the nodule.

the root system with the frequency of subsequent Nar nodule development (see below), it became clear that, for the majority of plants, most of the foci arrested at early stages of development. The transition of older actively dividing foci into arrested foci was already visible on most plants approximately 3 days after the removal of combined nitrogen. This transition was accompanied by a reduction in the intensity of GUS staining and a thickening of cell walls (Fig. 1F). Observation under UV light showed that the thickening of cell walls was associated with a strong autofluorescence and occurred especially in the outer cell layer of the foci (Fig. 1G). At later times, GUS activity could no longer be detected in such arrested foci which could nevertheless be readily identified within whole root segments because of the prominent cell wall thickenings of the outer cell layer (not shown). Aborted development was also observed among those foci which subsequently initiated in younger regions of the root system.

***ENOD12* is expressed throughout spontaneous nodule development.**

The relatively small proportion of the cortical mitotic foci which continued to divide actively gave rise to developing spontaneous nodules that eventually emerged from the root cortex. GUS activity was detected at all stages of this developmental process. The first rounded Nar nodules were clearly visible approximately 3–4 days after nitrogen removal (Figs. 1E and 2A) with uniform GUS staining throughout the central tissue. The peripheral tissues of such nodules, as well as the apical meristematic region, were devoid of GUS activity (Fig. 2A). This is analogous to the pattern of *MtENOD12* expression found at early stages of N_2 -fixing nodule development (Pichon *et al.* 1992).

Elongation of Nar nodules could first be seen from about 7–10 days after nitrogen removal and multilobed structures with a coralloid morphology were occasionally observed after 1 mo. Longitudinal sections of Nar nodules harvested 15 days after nitrogen removal (Fig. 2B) revealed a characteristic histological structure, including an apical meristem and a starch-rich central tissue as previously described by Truchet *et al.* (1989) and Joshi *et al.* (1991). Histochemical staining showed that GUS activity was located in the distal part of the central tissue, below the meristem (Fig. 2B and C). GUS staining was not observed in the nodule meristem itself, the nodule cortex, the nodule endodermis, and nodule tissue surrounding the vascular bundles (Fig. 2B). For comparative purposes, Figure 2D shows a section from a mature *Rhizobium*-elicited nodule which has been stained for both GUS activity (*MtENOD12*-GUS fusion) and β -galactosidase activity (localization of *Rhizobium*). *ENOD12* gene expression occurs in submeristematic zones of the two nodule types, even though there is no bacterial infection in Nar nodules. However, judging from the difference in the times required for the histochemical staining of nodule sections (compare Fig. 2B and D), it is probable that the level of *MtENOD12* expression in Nar nodules is significantly lower than in normal N_2 -fixing nodules. Finally, it should be underlined that GUS activity was not detected in the root epidermis at any stage of Nar ontogeny.

For each transgenic plant examined, Nar nodules always developed in root regions where foci were already present and the spatio-temporal pattern of Nar nodule formation was clearly correlated with that of the mitotic foci. However, the

conversion rate of foci into nodules varied considerably between individual plants. As was previously reported for the parental Nar clone NSL-D (Truchet *et al.* 1989), nodules were unclustered and evenly distributed along the root system. It is important to emphasize that the further development of Nar nodules in younger parts of the root did not appear to be influenced by the presence of either aborted foci or developing nodules in older regions of the root. We have also observed Nar nodules that appeared to be arrested at an early developmental stage, based on the partial or total loss of GUS activity in their central tissue (Fig. 2E). Thin sectioning revealed that these nodules were surrounded by a continuous, peripheral endodermis (not shown). Finally, and in contrast to N_2 -fixing nodule development, there did not appear to be a strict synchrony in the growth of non-arrested neighboring Nar nodules, suggesting either a variable timing for Nar initiation or variable developmental rates.

DISCUSSION

In this paper, spontaneous nodulation in alfalfa has been analyzed using transgenic plants which express the reporter gene *gusA* (*uidA*) under the regulatory control of the early nodulin *ENOD12* gene promoter. We find that the *MtENOD12*-GUS gene fusion is a convenient molecular marker for all stages of Nar development, thereby greatly facilitating the analysis of the spatio-temporal ontogenesis of such root-derived structures and also allowing us to more clearly distinguish similarities and differences between spontaneous nodulation and the nodulation process elicited by *Rhizobium*.

Ontogeny and spatio-temporal distribution of Nar primordia.

GUS-staining mitotic foci, elicited in the root cortex of transgenic NSL-D \times V5 progeny, develop as a direct response to the removal of exogenously supplied combined nitrogen. Foci development can be observed within 24 hr for the most responsive plants and is normally located within the inner root cortex, already identified as the site of Nar ontogenesis for *Medicago sativa* 'Vernal' (Joshi *et al.* 1991). Moreover, we find that foci are generally located opposite protoxylem poles (Fig. 1D), analogous to the location of the majority of the indeterminate *Rhizobium*-elicited nodule primordia (Phillips 1971; Libbenga and Harkes 1973). Furthermore, there is an excellent correlation between the spatio-temporal distribution of such foci and Nar nodulation throughout the root system. We therefore conclude that the mitotic foci expressing *ENOD12* are indeed "Nar primordia." However, it is important to note that, whereas *Rhizobium*-elicited primordia are generally initiated in the younger part of the root at sites of root hair infection (Bhuvaneshwari *et al.* 1981; Caetano-Anollés and Gresshoff 1991a), Nar primordia are formed exclusively in more mature regions of the root. Since a similar location of Nar primordia has also been observed for the parental *M. varia* transgenic line V5, it is likely that this finding can be generalized to other spontaneously nodulating alfalfa lines.

It has been shown that purified Nod factors of *Rhizobium* can provoke cortical cell divisions and nodulation in alfalfa, thus leading to the hypothesis that these lipo-oligosaccharide

signal molecules excreted by *Rhizobium* are responsible for inner root cortical activation during early stages of bacterial infection (Truchet *et al.* 1991). Since we can rule out a direct role for *Rhizobium*-derived Nod factors in eliciting Nar primordia and since the two types of nodulation are initiated in quite different regions of the root, it may indeed be that the molecular mechanisms responsible for nodule organogenesis differ between *Rhizobium*-elicited and spontaneous nodulation.

Comparison of *MtENOD12* expression during spontaneous and *Rhizobium*-induced nodulation.

What are the conclusions that can be drawn by comparing and contrasting the tissue-specific expression patterns of the early nodulin gene *ENOD12* during various stages of spontaneous and *Rhizobium*-induced nodulation? First, it is important to emphasize that GUS activity could not be detected in epidermal cells of the root system of Nar⁺ transgenic alfalfa, either prior to or during combined nitrogen deprivation. This contrasts with the early expression of the *MtENOD12*-GUS gene fusion that can be observed in the developing root epidermis following inoculation with the microsymbiont *R. meliloti* (Pichon *et al.* 1992) or after addition of NodRm factors (Journet *et al.* 1994) and is consistent with the observation that spontaneous nodulation does not involve morphological changes at the root surface such as root hair curling (Truchet *et al.* 1989; Joshi *et al.* 1991). Therefore, the response of differentiating root epidermal cells to *Rhizobium* or Nod factors is truly characteristic of the symbiotic interaction, both at the molecular and morphogenetic levels.

This study of spontaneous nodulation in transgenic alfalfa has revealed that the *MtENOD12*-GUS gene fusion is transcribed at a clearly detectable level during the earliest cell divisions in the inner cortex leading to the formation of nodule primordia. Following inoculation of transgenic alfalfa with *Rhizobium*, pronounced GUS staining in the root cortex is first detected in outer cortical cell layers at sites of infection (Pichon *et al.* 1992), and is subsequently associated with infection threads progressing towards the inner cortex (Pichon 1993). However, we have so far been unable to detect reporter gene expression during the initial inner cortical cell divisions which generate the nodule primordium. Further evidence that the *MtENOD12* gene is not transcriptionally activated at sites of *Rhizobium*-elicited inner cortical cell division has recently come from studies in which transgenic alfalfa were inoculated with a *R. meliloti* *exoA* strain (J. A. Leigh, D. G. Barker, E. P. Journet, and G. Truchet, unpublished). In no case were we able to detect GUS activity in nodule primordia elicited by this infection-deficient mutant. Equally, nodule primordia which develop opposite aborted infection sites during normal nodulation of alfalfa (Vasse *et al.* 1993) do not express the *MtENOD12*-GUS chimaeric gene at detectable levels (results not shown). The significance of this intriguing difference in gene expression between spontaneous and *Rhizobium*-elicited nodulation is unclear, but may be related to the different locations of the two types of primordia within the root as discussed above. Finally, it should also be pointed out that the tissue-specificity of *ENOD12* gene expression during early stages of *Rhizobium*-induced nodulation may differ between legumes, since it has been reported for pea that *PsENOD12* mRNA accumulates in

the nodule primordium before infection threads penetrate deep into the cortex (Scheres *et al.* 1990).

Expression of the *MtENOD12*-GUS fusion was detectable at all stages during the development of Nar primordia into nodules. GUS activity was initially present throughout the homogeneous central tissue located proximal to the newly formed meristem (Fig. 2A) and then subsequently restricted to a submeristematic region in more mature Nar nodules (Fig. 2B and C). This *MtENOD12* expression pattern is reminiscent of that found in N₂-fixing nodules (Pichon *et al.* 1992; Fig. 2D), taking into account the close similarity between the histological structures of the two types of nodules. Could the same mechanism of *MtENOD12* activation operate both in N₂-fixing and Nar nodules? Based on our previous observations showing that expression of the *MtENOD12* gene in N₂-fixing nodules of alfalfa and *M. truncatula* initiates in a narrow submeristematic region that we termed the "preinfection zone" (Pichon *et al.* 1992), it is tempting to speculate that the two types of nodules possess truly analogous zones immediately below the meristem. If so, then the triggering of *MtENOD12* transcription ahead of the infection zone in the nodule might not be a consequence of bacterial signaling but rather be due to a state of cell differentiation corresponding to a preparation for infection. Finally, the higher level of *ENOD12* expression in *Rhizobium*-elicited nodules as compared with spontaneous nodules can probably be attributed to the infection process itself, since we have previously observed enhanced gene expression at sites of *Rhizobium* infection during early stages of the symbiotic interaction (Pichon *et al.* 1992).

Studies using transgenic *Medicago* plants have revealed that, in addition to the nodulation-related expression described above, the *MtENOD12* gene is also transcribed at sites of secondary root emergence (Pichon 1993; Journet *et al.* 1994). However, in this case, expression of the *MtENOD12*-GUS fusion is limited to very early stages of root formation, and appears to locate to stele-derived cells which are differentiating into vascular tissue that will connect the central cylinder to the developing lateral root. Thus, although the expression of *MtENOD12* is not symbiosis-specific within the alfalfa root, this gene can serve as a useful molecular marker for both root and nodule ontogeny, providing that gene expression is monitored at the histological level.

Is spontaneous nodulation subject to autoregulation?

The formation of nitrogen-fixing nodules on leguminous plants is controlled by feedback mechanisms that regulate the overall level of nodulation (for a review, see Caetano-Anollés and Gresshoff 1991b). In alfalfa, the existence of a primary systemic feedback control of nodulation has been most clearly revealed by experiments using split-root systems (Kosslak and Bohlool 1984), showing that the inoculation of one side of the root inhibits nodulation on the other side (Kosslak and Bohlool 1984; Caetano-Anollés and Bauer 1988). Such systemic regulation appears to operate in the youngest part of the root, at a stage preceding *Rhizobium* infection (Caetano-Anollés and Gresshoff 1991a). Experiments with transgenic alfalfa (NSL-D × V5) inoculated with *R. meliloti* suggest that these plants are subject to similar regulatory mechanisms, since root hair infection in the youngest part of the root is suppressed as early as 48 hr following inoculation (Pichon 1993). The fact that nodules are infected does not appear to

be an essential requirement for the expression of such systemic feedback mechanisms, since the development of empty non N_2 -fixing nodules induced by an exopolysaccharide mutant of *R. meliloti* can act to partially limit the subsequent development of N_2 -fixing nodules which are elicited after a delayed inoculation with the wild-type strain (Caetano-Anollés *et al.* 1990b).

With regard to spontaneous nodulation in alfalfa, a partial, albeit significant, inhibition of *Rhizobium*-elicited nodulation was also observed when plants which had previously developed spontaneous nodules were inoculated with a wild-type strain of *R. meliloti* (Caetano-Anollés *et al.* 1990a). On the other hand, spontaneous nodulation is very efficiently suppressed following an early inoculation with the wild-type *Rhizobium* strain (Caetano-Anollés *et al.* 1990a; T. Huguet, unpublished). However, until now, the question as to whether Nar nodulation is itself autoregulated has not yet been addressed. We find that, despite the arrest of the majority of Nar foci and the low frequency of conversion of Nar primordia into nodules, both foci induction and Nar nodule development occur continuously throughout plant growth in the absence of exogenous combined nitrogen. Taken together, these results lead to the two following conclusions. 1) The fact that Nar⁺ plants can continuously develop spontaneous nodules (this study) strongly suggests that neither Nar primordia nor mature Nar nodules are involved in eliciting feedback control of spontaneous nodulation. 2) Differences in the extent of feedback control of nodulation can be correlated with the nature of the developing nodule: Nodules elicited by wild-type *Rhizobium* appear to be more "efficient" in eliciting feedback control of nodulation than empty nodules either elicited by mutant *Rhizobium* strains or having developed spontaneously.

The results presented in this article imply a reassessment of the relationship between Nar and *Rhizobium*-induced nodulation. Similarities between both nodulation processes, as deduced from this study and others previously published (Truchet *et al.* 1989; Caetano-Anollés *et al.* 1990a; Joshi *et al.* 1991; Hirsch *et al.* 1992), concern the nature of the root tissue giving rise to nodule primordia (inner root cortex opposite protoxylem poles), the overall histology of the nodules, the transcription of the early nodulin gene *ENOD2* in nodule parenchyma tissue, the transcription of the *ENOD12* gene in submeristematic nodule tissues, and the inhibition of nodulation by combined nitrogen. However, two unexpected differences have now emerged in relation to nodule ontogeny and autoregulation. Ontogenetically, the earliest stages of Nar vs. normal N_2 -fixing nodulation differ, both in terms of their location within the root system (mature vs. differentiating, respectively) and in relation to the expression of the *ENOD12* marker gene in nodule primordia. Secondly, a striking difference has emerged from the fact that, in contrast to wild-type nodulation, feedback control does not appear to operate during spontaneous nodulation. Further research is now required to understand the significance of these differences in relation to the nodule development process.

MATERIAL AND METHODS

Plant material and growth conditions.

A number of independent primary transformant lines of *Medicago varia* A2 (Deak *et al.* 1986) had previously been

obtained following the introduction of the *MtENOD12*-GUS chimeric gene by means of an *Agrobacterium tumefaciens* transformation protocol and subsequent regeneration via somatic embryogenesis (Pichon *et al.* 1992). Transformant lines were propagated vegetatively and grown under the appropriate conditions to induce flowering (3,000 lx; 16 hr/8 hr photoperiod; 20° C). Transgenic alfalfa plants with an enhanced Nar⁺ phenotype were obtained by manually crossing the representative *M. varia* transgenic line V5 (paternal parent) with the self-sterile *M. sativa* 'Gemini NSL-D' genotype (maternal parent) which has a pronounced spontaneous nodulation phenotype (Truchet *et al.* 1989).

The NSL-D × V5 progeny seeds were surface sterilized, germinated, and grown in aeroponic conditions in the presence of 5 mM ammonium nitrate (Barker *et al.* 1988). After 2–3 wk, combined nitrogen was removed from the growth medium and plants were either grown in the absence of *Rhizobium* in order to trigger spontaneous nodulation, or inoculated with the *R. meliloti* strain GMI6526 (Ardourel *et al.* 1994). This strain, which harbors the pXLGD4 plasmid, and hence constitutively expresses the chimaeric *hemA::lacZ* gene fusion (Leong *et al.* 1985), is a derivative of the wild-type RCR2011. Whole root systems, root segments, or nodules were collected at various times after combined nitrogen removal and treated as described below.

Histochemical localization of β -glucuronidase (GUS) activity.

Histochemical staining for GUS activity (Jefferson *et al.* 1987) was performed on whole root segments or nodules using the histochemical substrate X-Gluc (X-glcA cyclohexylammonium salt; Biosynth AG, Staad, Switzerland) and the protocol described in Pichon *et al.* (1992). Stained intact samples or slices (80- μ m-thick; Microcut H1200; Bio-Rad) were briefly cleared with sodium hypochlorite to improve the contrast between stained and non-reactive tissues (Boivin *et al.* 1990) and observed with an Olympus Vanox light microscope using bright-field, dark-field, or phase contrast optics.

Alternatively, nodules were sliced into 80- μ m-thick sections prior to staining for GUS activity. In this case, prefixation of intact samples was carried out with 1% *p*-formaldehyde and the GUS reaction medium contained potassium ferrocyanide and ferricyanide at the higher concentration of 2 mM. Incubation times were short (10 min to 2 hr). No qualitative differences in the tissue-specific location of GUS activity were found between these sections and those obtained using material that had been stained prior to sectioning. It was therefore concluded that the slower penetration of the histochemical substrate into intact root nodules does not lead to artefactual staining patterns for the *MtENOD12*-GUS fusion.

For the localization of GUS activity at the cellular level, stained roots or nodules were 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 either Epon resin (Merck) or Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany). Sections (1–4 μ m thick) were observed using bright-field microscopy after counterstaining with 0.02 % toluidine blue in 1 % sodium borate, dark-field microscopy, or under UV illumination for detection of autofluorescent cell material.

Double histochemical staining for GUS and β -galactosidase activities.

Following GUS histochemical staining with X-Gluc as substrate, certain samples were further processed to stain for the presence of *Rhizobium* expressing β -galactosidase. These samples were rinsed and equilibrated for 15 min at room temperature in Z' buffer (100 mM NaPO₄, pH 7.4, 10 mM KCl, 1 mM MgSO₄; Teeri *et al.* 1989), fixed with 1,25% glutaraldehyde in Z' buffer for 1 hr (Boivin *et al.* 1990) in order to inactivate the endogenous plant β -galactosidases, rinsed twice and stained at 28° C for 1–16 hr with 0.8 mg/ml Magenta-gal or Salmon-gal (Biosynth AG) in Z' buffer containing 5 mM each of potassium ferricyanide and ferrocyanide. Whole samples or sections were then observed by microscopy as indicated above.

This double-staining protocol provides a powerful investigative tool for studying the relationship between the expression of a plant promoter-GUS gene fusion and the presence of either symbiotic or pathogenic bacteria in adjacent plant tissues. Such an approach (with Magenta-glucuronide/X-gal as substrates) has proved to be very useful in studies of *ENOD12* gene expression as a function of *Rhizobium* infection at the alfalfa root surface (results not shown) and the activation of the tobacco *hsr203J*-GUS gene fusion in response to invading *Pseudomonas solanacearum* (Pontier *et al.* 1994).

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