Effects of a Nod-factor-overproducing strain of Sinorhizobium meliloti on the expression of the ENOD40 gene in Melilotus alba

Walter F. Giordano, Michelle R. Lum, and Ann M. Hirsch

Abstract: We have initiated studies on the molecular biology and genetics of white sweetclover (Melilotus alba Desr.) and its responses to inoculation with the nitrogen-fixing symbiont Sinorhizobium meliloti. Early nodulin genes such as ENOD40 serve as markers for the transition from root to nodule development even before visible stages of nodule formation are evident. Using Northern blot analysis, we found that the ENOD40 gene was expressed within 6 h after inoculation with two different strains of S. meliloti, one of which overproduces symbiotic Nod factors. Inoculation with this strain resulted in an additional increase in ENOD40 gene expression over a typical wild-type S. meliloti strain. Moreover, the increase in mRNA brought about by the Nod-factor-overproducing strain 24 h after inoculation was correlated with lateral root formation by using whole-mount in situ hybridization to localize ENOD40 transcripts in lateral root meristems and by counting lateral root initiation sites. Cortical cell divisions were not detected. We also found that nodulation occurred more rapidly on white sweetclover in response to the Nod-factor-overproducing strain, but ultimately there was no difference in nodulation efficiency in terms of nodule number or the number of roots nodulated by the two strains. Also, the two strains could effectively co-colonize the host when inoculated together, although a few host cells were occupied by both strains.

Key words: ENOD40, Nod factor, Melilotus, Sinorhizobium, symbiosis.

Introduction

Rhizobia are symbiotic bacteria that elicit on the roots of specific legume hosts the formation of new organs (nodules), within which the bacteria proliferate, differentiate into bacteroids, and subsequently fix atmospheric nitrogen into ammonia. The host plant and rhizobia continually exchange molecular signals prior to nodule initiation and as the nodule develops (Fisher and Long 1992). Flavonoids released by plant roots or seeds act as the first signals, serving as chemoattractants to the bacteria and also inducing rhizobial (nod) genes (see review by Perret et al. 2000). The products of the nod genes are involved in the synthesis and secretion...
of lipo-chitooligosaccharide molecules known as Nod factors, which trigger the earliest stages of nodule development, including root hair deformation, cortical cell division, nodule morphogenesis, and the expression of several early nodulin (ENOD) genes (see reviews by Bladergroen and Spanik 1998; Downie and Walker 1999). Nod factor application also leads to an increase in nod-gene-inducing flavonoids in the host, demonstrating that there is a positive feedback between the host and the symbiont (see references in Hirsch 1992). Each rhizobial species produces Nod factors with distinctive substitutions that are recognized by a particular legume host (Dénaire et al. 1996; Perret et al. 2000).

In spite of the fact that Nod factor is essential for the earliest stages in the development of nitrogen-fixing nodules, only ineffective, bacteria-free nodules are induced by adding purified Nod factor. However, to date, few legumes (Medicago sativa, Glycine soja, Phaseolus sp., Acacia sp., and Lotus corniculatus) have been rigorously tested for their responses to Nod factor alone, leaving it unresolved as to the universality of this response (see references in Hirsch 1999). Bacterial entry and proliferation as well as the induction of nitrogenase activity are required for the development of effective nitrogen-fixing nodules. It is likely that other signals and responses are required for rhizobial invasion, division, and differentiation (Gage and Margolin 2000). However, Nod factor is clearly involved in these stages because Rhizobium leguminosarum bv. viciae mutants lacking host-specific nodulation genes could not stimulate nodule or infection thread formation on either the homologous host vetch (Vicia sativa) (Walker and Downie 2000) or the heterologous host alfalfa (Medicago sativa), even though the latter contained an introduced pea lectin gene (van Rhijn et al. 2001).

This study focuses on white sweetclover (Melilotus alba Desr.), which like alfalfa is nodulated by Sinorhizobium meliloti, one of the best genetically characterized rhizobial species (Galibert et al. 2001). White sweetclover exhibits many of the same characteristics and patterns of gene expression as does alfalfa, but unlike alfalfa, white sweetclover is an autogamous diploid and is genetically tractable (Hirsch et al. 2000). Nodules formed on white sweetclover are indeterminate, similar to those developed on alfalfa, pea, and vetch.

As a prelude for studying gene expression in white sweetclover symbiotic mutants, the differences in ENOD40 gene expression in uninoculated and inoculated wild-type M. alba roots were investigated. ENOD40 was chosen as a marker because it is one of the earliest nodulins to be expressed upon Rhizobium inoculation. ENOD40 homologs have been cloned from a number of legumes as well as from nonlegumes including rice, a monocotyledon (Kouchi et al. 1999), and thus, ENOD40 is not a nodulin in the strict sense. Not only is it expressed in nonlegumes, but it is also expressed in regions of the plant other than nodules, such as emerging lateral roots, leaf marginal meristems, the stem pro cambium (Asad et al. 1994; Papadopoulos et al. 1996), and other nonsymbiotic tissues (Flemetakis et al. 2000), even in the absence of Rhizobium. These data indicate that the ENOD40 gene product may be involved in several plant developmental processes. ENOD40 is also expressed in mycorrhizal roots (van Rhijn et al. 1997) and is induced by cytokinins (Fang and Hirsch 1998). In addition, transgenic Medicago truncatula plant roots that overexpress ENOD40 exhibit accelerated nodulation when inoculated with S. meliloti, suggesting that ENOD40 may be directly correlated with the induction of nodules (Charon et al. 1999).

In this study, two effective rhizobial strains, one a typical wild-type strain and a second strain that overproduces symbiotic Nod factors, were chosen. The latter strain was used to test whether there was a higher level of ENOD40 expression in the inoculated roots and, if so, to see whether this enhanced expression could be correlated with increased nodulation on white sweetclover as suggested by the results of Charon et al. (1999). Based on the literature and our previous experiments with alfalfa, we predicted that ENOD40 gene expression would increase with higher levels of Nod factor. Here, we report on the tests of these predictions.

Materials and methods

Plant material

For RNA isolation, white sweetclover (U389) seeds were surface sterilized after scarification and germinated on screens in Magenta jars (Magenta Corp., Chicago, Ill.) containing quarter-strength Hoagland’s medium without nitrogen (Löbler and Hirsch 1993). Two days after germination, the seedlings were inoculated with either Rm1021 or GMI6032 (see below). For studies of nodule kinetics and occupancy, white sweetclover seeds were surface sterilized and aseptically germinated in a plastic dish pan containing vermiculite and perlite (2:1) and watered with quarter-strength nitrogen-free Hoagland’s medium as previously described (Hirsch et al. 1989). Three days after germination, the seedlings were inoculated. For the nodule kinetics experiments, roots from 10 plants were harvested every 5 days postinoculation (dpi), and for the nodule occupancy experiments, nodules from approximately 15 plants were analyzed every 3 days starting at 10 dpi. For the in situ hybridization studies and to determine the number of lateral roots, white sweetclover seeds were surface sterilized after scarification and germinated in Petri dishes containing water–agar and then transferred 3 days later to quarter-strength nitrogen-free Hoagland’s medium with 0.8% Phytagar (Invitrogen). Five days after germination, seedlings were mock inoculated with water or inoculated with Rm1021 or GMI6032. For the in situ hybridization analysis, the roots were collected 24 h postinoculation (hpi) and prepared as described below. For the counting experiments, seedling roots were collected 24 hpi, fixed in FAA (formalin – acetic acid – alcohol) overnight, and then cleared in 10% KOH for 2 h at 37°C.

Bacterial strains

White sweetclover plants were inoculated with wild-type S. meliloti (Rm1021) (Meade et al. 1982) or with S. meliloti strain GMI6032, which carries the plasmid pGMI149, resulting in the overproduction of symbiotic Nod factors (Lerouge et al. 1990). pGMI149 carries the complete set of common and specific nod genes as well as three regulatory genes, nodD1, nodD3, and syrM.

The bacterial cultures were grown in Rhizobium defined medium (Vincent 1970) to an OD600 of 0.8–1.0. For
Rm1021, the medium was supplemented with streptomycin (100 µg/mL) and for GM16032 with tetracycline (10 µg/mL). One millilitre of bacteria resuspended in quarter-strength Hoagland’s medium minus nitrogen was added to a Magenta jar, and 10 mL of bacteria was used to inoculate each row of plants growing in the plastic dish pans.

For studies on nodule occupancy, rhizobia harboring plasmids that drive the expression of the autofluorescent proteins GFP (green fluorescent protein) and DsRed (red fluorescent protein) were used. We generated a GFP-labeled GM16032 strain by introducing the plasmid pHc60 (Cheng and Walker 1998) into GM16032 by a triparental mating strategy using the helper plasmid pRK2013 (Ditta et al. 1980). Selection for the successful introduction of the plasmid was by fluorescence because GM16032 is tetracycline resistant. Fluorescent colonies were restreaked for several generations on selective medium and reexamined for fluorescence to ensure that the introduced plasmid was stable. A DsRed-labeled Rm1021 strain (Rm1021/pDG77) was generously provided by Dan Gage (Bringhurst et al. 2001). Sweetclover plants were inoculated with a 1:1 mixture of the two strains, GFP-labeled GM16032 and DsRed-labeled Rm1021. This made the interaction between the plant and the rhizobia easy to follow because of the green or red fluorescent proteins constitutively expressed by the bacterial strains.

RNA isolation and Northern hybridization

To examine the temporal patterns of MaENOD40 gene expression, entire inoculated root systems were harvested at different time points after inoculation, frozen immediately in liquid nitrogen, and stored at –70°C until use (Löbler and Hirsch 1993). Total RNA was isolated using RNA STAT-60 (Tel-Test “B” Inc., Friendswood, Tex.). For Northern analysis, RNA was size fractionated on formaldehyde agarose gels; 10 µg RNA were loaded per lane. After transfer to Nytran membranes (Schleicher and Schuell, Keene, N.H.), the blots were hybridized with radiolabeled [α-32P]dATP probes derived from a 580-bp Psi1 fragment of the MsENOD40-2 cDNA clone. The blots were also hybridized with the Msc27 probe (Kapros et al. 1992), which was used as a control to check for equal loading. All probes were prepared using random hexamer priming DNA labeling. Blots were prehybridized for 2–4 h at 55°C in 50% (v/v) formamide – 5X Denhardt’s solution – 5X SSPE – 0.5% sodium dodecyl sulfate (SDS) (1X SSPE = 150 mM sodium chloride – 10 mM sodium phosphate, pH 7.4, – 1 mM EDTA). The hybridization was done in the same solution for another 16 h. At the end of the hybridization period, the membrane was washed twice in 1X SSC – 0.5% SDS (1X SSC = 150 mM sodium chloride – 15 mM sodium citrate, pH 7.0) for 10 min at 37°C and then twice in 0.1X SSC – 0.5% SDS for 15 min at 55°C. The filters were exposed to X-ray film at ~70°C for 1–7 days. The hybridization signals were quantified using an Ultrascan XL laser densitometer (Pharmacia, Piscataway, N.J.).

Reverse transcription – polymerase chain reaction (RT–PCR)

For RT–PCR, total RNA (1 µg) from white sweetclover roots was used as a template for cDNA synthesis using 0.5 mM dNTPs, 25 µg oligo-dT(12-18) µL, 2 units ribonuclease inhibitor µL, and 10 units Superscript II reverse transcriptase µL (GIBCO/BRL, Grand Island, N.Y.) at 42°C for 60 min. The reaction was inactivated by heating at 75°C for 15 min. The products were diluted and used as template for the PCR amplification, which was carried out using 1 µM of each specific primer, 0.2 mM dNTPs, 2.5 mM MgCl2, and 2.5 units of Taq DNA polymerase (GIBCO/BRL). The Msc27 primers were 5′-GGAGGTGGAGGAAGTGG-3′ and 5′-CACCAACAAAAGAATTGAAGG-3′ and generated a 310-nucleotide (nt) product. The ENOD40 primers were 5′-CCTCTAAACCATCCATCAAG-3′ and 5′-AGTCAGCTGCAAATTGAGTGA-3′, which generated a 534-nt product. Amplification was performed using an initial 5-min cycle at 94°C followed by 30 cycles of 30 s of denaturation at 94°C, 30 s of primer annealing at 55°C, 90 s of elongation at 72°C, and a final 10 min at 72°C for extension. The PCR products were size fractionated on a 1.5% agarose gel and visualized with ethidium bromide.

In situ hybridization

For preparing antisense and sense RNA fragments of the ENOD40 gene, the MsENOD40-2 cDNA clone was digested with BamHI and XhoI and the probes were produced from T3 and T7 RNA polymerase promoters, respectively, labeled with digoxigenin by using the DIG RNA-labeling kit from Boehringer-Mannheim (Ridgefield, Conn.), and then degraded to approximately 150 nt long before hybridization (McKhan and Hirsch 1993). Entire white sweetclover roots, which were harvested 24 hpi with GM16032 or Rm1021, were fixed, dehydrated, and hybridized to digoxigenin-labeled antisense or sense RNA probes according to a procedure derived from De Almeida Engler et al. (1998). Photographs were taken with Kodak Ektachrome Tungsten 160 film on a Zeiss Axiophot microscope, scanned into a computer, and composites made using Adobe Photoshop.

Fluorescence microscopy

Observation of root colonization and nodule occupancy was made with a Zeiss Axiophot light microscope equipped with fluorescence and using a band-pass blue filter combination (450–496 nm). Confocal images were taken using a Bio-Rad MRC1024ES (krypton–argon) confocal laser scanning microscope attached to a Nikon Eclipse E800 light microscope using settings for observation of FITC (488 nm) and TRITC (568 nm). Images were processed by Adobe Photoshop.

Results

ENOD40 gene expression

To obtain a baseline for ENOD40 gene expression in M. alba, RNA was isolated at 6 and 24 hpi from uninoculated roots or from roots inoculated with Rm1021 and then analyzed. We also utilized GM16032, an S. meliloti strain that overproduces Nod factors (Lerouge et al. 1990), to study the effect of augmenting the production of Nod factor on ENOD40 gene expression. Almost undetectable basal expression levels were observed in uninoculated control roots at both time points (Fig. 1). In contrast, ENOD40 gene expression was elevated in both sets of inoculated roots as
Spatial expression pattern of \textit{ENOD40} gene expression at 24 hpi and determination of lateral root number

The temporal analysis of \textit{ENOD40} gene expression demonstrated that there was a significant difference between GMI6032- and Rm1021-inoculated white sweetclover roots at 24 hpi. We then asked whether a difference could be detected between the treatments with regard to the spatial expression patterns. Earlier studies using in situ hybridization analysis of paraffin-embedded and sectioned alfalfa roots (Asad et al. 1994) showed that at 24 hpi, \textit{ENOD40} was expressed in the pericycle and procambial cells of the root. In some roots, transcripts were also detected in nuclei of epidermal and cortical cells adjacent to the site of inoculation (Asad et al. 1994).

A whole-mount in situ hybridization (WISH) method was used to make the comparisons between comparably treated Rm1021- and GMI6032-inoculated roots (Fig. 2). The sense probe demonstrated that there was no signal above background for either uninoculated (data not shown) or inoculated roots (Fig. 2A). When the antisense probe was utilized on uninoculated roots, only basal expression of \textit{ENOD40} was observed (Fig. 2B).

At 24 hpi, root hairs showed some deformation, and some radial cell expansion was observed behind the root tip, but there was no evidence of cortical cell divisions, nor were infection threads detected. Antisense \textit{ENOD40} probes hybridized to meristematic cells of the main root tips and also strongly labeled lateral root initiation sites in the inoculated roots (Figs. 2C–2F). The pattern of \textit{ENOD40} expression in the root meristems was similar to that found in alfalfa roots marked with \textit{MsENOD40} promoter – GUS fusions and treated with either 10^{-6} M cytokinin or 10^{-8} M Nod factor for 4 days (Fang and Hirsch 1998). Some signal was also detected in the tissues of the central stele, most likely the pericycle and internal parenchyma cells (Figs. 2C and 2D), and this result, too, was similar to that reported earlier for alfalfa (Fang and Hirsch 1998).

In contrast with the Northern blot analysis illustrated in Fig. 1 where \textit{ENOD40} was shown to be upregulated three- and sixfold, respectively, over the uninoculated controls, we could not detect a significant visual difference in the intensity of the color reaction in the acropetal 1-cm regions of the Rm1021- or GMI6032-inoculated roots collected for the WISH analysis. We reasoned that the increased signal observed using Northern blot analysis for Rm1021- and GMI6032-inoculated roots might be due to an increased activation of the pericycle or to the initiation of more lateral root primordia 24 hpi in the GMI6032-inoculated roots. The number of pre-emergent and emergent lateral roots in the Rm1021- and GMI6032-inoculated as well as in the mock-inoculated roots was counted. Roots inoculated with GMI6032 had significantly more pre-emergent and emergent lateral roots (2.56 ± 0.22 lateral roots/cm) than did roots inoculated with Rm1021 (1.94 ± 0.17 lateral roots/cm). In addition, inoculation with either strain of rhizobia resulted in significantly more lateral root initiation than in the mock-inoculated roots (1.25 ± 0.16 lateral roots/cm).

Nodule kinetics

Next, we tested whether GMI6032, because it produces more Nod factor than Rm1021, was more effective in nodulating white sweetclover roots than Rm1021. Overall, the roots inoculated with GMI6032 showed an increased rate of nodulation in comparison with the roots infected with Rm1021 (Fig. 3A). For example, at 10 dpi, 100% of the plant roots were nodulated by GMI6032 whereas only 60% were nodulated by Rm1021 (Fig. 3B). The number of nodules per root system was also significantly higher 15 dpi. However, at the end of the experiment, there was no significant difference in the final nodule numbers for roots inoculated with Rm1021 compared with those inoculated with GMI6032 (Figs. 3A and 3B). There were also no morphological differences between the nodules induced by the two strains (data not shown).

To determine whether the initial increase in nodule number upon GMI6032 inoculation was correlated with a concomitant increase in \textit{ENOD40} transcript levels compared
with Rm1021-inoculated roots, RT-PCR experiments were performed on RNA isolated from white sweetclover roots 10 and 15 dpi. As expected, ENOD40 gene expression was enhanced in roots inoculated with either strain in comparison with the control roots. Moreover, GMI6032 inoculation augmented ENOD40 gene expression in roots significantly above that following Rm1021 inoculation at both 10 and 15 dpi (Fig. 3C).

Detection of rhizobial strains in plant roots and nodules

Next, the faster-nodulating strain, GMI6032, was tested to determine whether it could colonize white sweetclover nodules better than Rm1021. Originally, nodule occupancy was assessed by squashing the nodules co-inoculated with GMI6032 and Rm1021 and selecting for antibiotic resistance, but this gave us equivocal results (data not shown). We reasoned that a visual assessment of the nodules was a truer test of nodule occupancy than nodule squashes, so we inoculated white sweetclover roots with GFP-labeled GMI6032 and DsRed-labeled Rm1021.

Inoculation of plants with both strains resulted in the same temporal development of nodules, as illustrated in Fig. 3A. The nodules were either examined whole or sectioned with a razorblade and then analyzed using confocal microscopy. Figure 4A illustrates the outer surface of the

Fig 2. Localization of ENOD40 gene transcripts in entire white sweetclover (Melilotus alba) roots. In situ hybridization was performed in (B) uninoculated roots, (C and E) roots inoculated with Rm1021 or (A, D, and F) roots inoculated with GMI6032. Digoxigenin-labeled ENOD40 sense (Fig. 2A) and antisense (Figs. 2B–2F) probes were used. Scale bar = 100 µm.
root and shows that both rhizobial strains attach to the root surface. Infection threads were observed containing either GFP- or DsRed-expressing rhizobia or both (Figs. 4A–4C). In the infection threads that contained GMI6032 expressing GFP as well as Rm1021 expressing DsRed, the rhizobia were not randomly intermixed (Fig. 4C).

Discussion

Previously in alfalfa, it was shown that ENOD40 is expressed at least twofold over background levels following inoculation with S. meliloti Rm1021 or by adding Nod factor to uninoculated roots (Fang and Hirsch 1998). In this report, these observations are extended to white sweetclover, where a threefold accumulation of ENOD40 transcripts over the uninoculated roots was measured. In addition, S. meliloti strain GMI6032, which carries a plasmid resulting in the overproduction of symbiotic Nod factors, elicited a sixfold increase in ENOD40 transcript accumulation over the uninoculated controls. These data indicate that the ENOD40 response is correlated with Nod factor production by the rhizobia. Similar data have been reported by a number of laboratories for different legumes (Minami et al. 1996; Niwa et al. 2001).

WISH analysis showed that in white sweetclover roots, ENOD40 was expressed in the root meristems, lateral root initials, and also the central stele, locations determined previously to be sites of ENOD40 expression in alfalfa by in situ hybridization analysis of sectioned tissue (Asad et al. 1994) and by GUS staining (Fang and Hirsch 1998). However, in contrast with the Northern blot results presented in Fig. 1, WISH analysis suggested that there were similar levels, based on the intensity of the color reaction, of ENOD40 gene expression in roots inoculated with Rm1021 versus GMI6032 strains. This apparent contradiction between the Northern and WISH results is explained in part by the difference in lateral root numbers between Rm1021- and GMI6032-inoculated roots. Roots inoculated with GMI6032 produced significantly more lateral roots than uninoculated roots or roots inoculated with Rm1021. In the tissue collected for Northern analysis, lateral root primordia were probably included in the 24-hpi-source tissue for the RNA analyzed on the blots because entire root systems were collected. For the difference in inoculated versus uninoculated roots, increased expression of ENOD40 in the activated central tissues was detected by the WISH analysis. Thus, our data are in agreement with Compaan et al. (2001), who reported that an upregulation of ENOD40 gene expression in inoculated roots precedes cortical cell division in response to rhizobia. However, based on the WISH results and the counts of lateral root primordia, it appears that ENOD40 gene expression in white sweetclover in response to Nod factor is also strongly correlated with lateral root initiation. In contrast, Compaan et al. (2001) did not report any mitotic activity in the pericycle of alfalfa roots even 48 hpi.
Our results also differ from those of Charon et al. (1997) who found that transgenic *Medicago truncatula* plants, which expressed high levels of ENOD40, did not exhibit any difference in the number of lateral root primordia from the nontransgenic controls. However, these plants produced high levels of ENOD40 constitutively and were uninoculated whereas the white sweetclover plants in our experiments were untransformed and inoculated. Furthermore, the *Medicago truncatula* transgenic lines with high constitutive levels of ENOD40 expression underwent cortical cell divisions spontaneously (Charon et al. 1997).

Later studies on these same transgenic *Medicago truncatula* plants that overexpress ENOD40 demonstrated that they exhibited accelerated nodulation when inoculated with *S. meliloti* (Charon et al. 1999). We found that inoculation with GMI6032, which overproduces Nod factors, thereby enhancing ENOD40 gene expression, accelerated nodulation on white sweetclover. The percentage of plant roots nodulated and the number of nodules per root system differed between the roots inoculated with the two rhizobial strains up to 20 dpi. After that time, there was no statistical difference between the two strains in terms of nodule numbers or percentage of roots nodulated. The overexpressing ENOD40 *Medicago truncatula* lines also exhibited faster nodulation, but the difference between the controls and the transgenic lines in terms of nodule numbers persisted until 31 dpi (Charon et al. 1999).

A consequence of the initial accelerated rate of nodulation in response to GMI6032 might result in this strain occupying more nodules than Rm1021. Most nodule occupancy assays require re-isolation of the bacteria from nodules followed by identification of the species present, usually by testing for antibiotic resistance (Johnston and Beringer 1975). When this approach was used, approximately 50% of the nodules

---

**Fig. 4.** White sweetclover (*Melilotus alba*) roots co-inoculated with DsRed-labeled Rm1021 and GFP-labeled GMI6032. (A) Infection threads containing only Rm1021, but GFP-labeled GMI6032 cells are found on the root surface. (B) Infection threads containing either DsRed-labeled Rm1021 or GFP-labeled GMI6032. (C) Infection threads containing both Rm1021 and GMI6032. (D) An entire nodule. Where the two strains overlap, the fluorescence is yellow. (E) Section of a co-inoculated nodule showing the red fluorescence of Rm1021, the green fluorescence of GMI6032, and the yellow fluorescence of simultaneous colonization (arrows) of a host cell by Rm1021 and GMI6032. Very few host cells fluoresce yellow, indicating that the two different symbionts usually occupy distinct host cells. Bar = 100 µm.
examined contained GM16032 and 50% of the nodules contained Rm1021, a result that was difficult to interpret. As this type of method is based on the recovery of culturable bacteria from crushed nodules, we may have overlooked the majority of symbiotic colonizers of the nodule, for example, bacteria that had differentiated into bacteroids and consequently were not culturable. Therefore, a more direct method was sought to monitor the two strains that were co-inoculated. Hence, we used rhizobia harboring plasmids that drive expression of autofluorescent proteins. These proteins allow the simultaneous expression of multiple fluorescent markers, which can be distinguished from each other with the appropriate filter sets (Yang et al. 1998). This approach enabled us to see that both strains of rhizobia inhabited the same nodule and occasionally even the same infection thread. These results correlate with those obtained with different rhizobia strains (Stuurman et al. 2000). However, most white sweetclover nodule cells contained one strain or the other; few host cells harbored both strains. The present study does not clarify at this time whether GM16032 has a competitive advantage over Rm1021 in terms of nodule occupancy; a better way to quantify green versus red fluorescence in nodules is needed. It does show, however, that the overproduction of Nod factor by GM16032 does not impair its ability to interact with roots. This contrasts with bacteria mutated in Nod factor or exopolysaccharide biosynthesis, which are much less competitive than their wild-type counterparts (Klein et al. 1988; Lamrabet et al. 1999).

In summary, the results of this study demonstrate a direct correspondence between Nod factor production, ENOD40 gene expression, and lateral root development in the M. alba – S. meliloti symbiosis. The Nod-factor-overproducing strain, GM16032, induced ENOD40 gene expression almost twice the level as Rm1021, indicating that Nod factor production is directly related to this process. It also significantly stimulated lateral root formation in white sweetclover, accounting for the enhanced ENOD40 transcript accumulation in GM16032- versus Rm1021-inoculated roots. The data also show that increased Nod factor production accelerates nodule formation in white sweetclover, but ultimately the timing of nodule formation and degree of nodule occupancy appear to be similar for Rm1021 and GM16032. The fact that increased Nod factor augments ENOD40 gene expression strongly suggests that other plant genes may be similarly upregulated. We are currently testing this hypothesis.

Acknowledgements

We thank J. Dénarié (Toulouse, France) for S. meliloti strain GM16032, D. Gage (Storrs, Conn.) for Rm1021/pDG77, and H.P. Cheng (Stony Brook, N.Y.) for pHC60. We thank Nancy A. Fujishige for help with the WISH protocol. This research was supported by UC-BIOSTAR grant S98-86 to A.M.H. and by a CONICET (Argentina) fellowship to W.F.G.

References


Fang, Y., and Hirsch, A.M. 1998. Studying early nodulin gene expression almost twice the level for the enhanced ENOD40 transcript accumulation in GM16032- versus Rm1021-inoculated roots. The data also show that increased Nod factor production accelerates nodule formation in white sweetclover, but ultimately the timing of nodule formation and degree of nodule occupancy appear to be similar for Rm1021 and GM16032. The fact that increased Nod factor augments ENOD40 gene expression strongly suggests that other plant genes may be similarly upregulated. We are currently testing this hypothesis.

Acknowledgements

We thank J. Dénarié (Toulouse, France) for S. meliloti strain GM16032, D. Gage (Storrs, Conn.) for Rm1021/pDG77, and H.P. Cheng (Stony Brook, N.Y.) for pHC60. We thank Nancy A. Fujishige for help with the WISH protocol. This research was supported by UC-BIOSTAR grant S98-86 to A.M.H. and by a CONICET (Argentina) fellowship to W.F.G.

References


