Analysis of promoter activity of the early nodulin Enod40 in Lotus japonicus

Grønlund, M.; Roussis, A.; Flemetakis, E.; Quaedvlieg, N.E.M.; Schlaman, W.R.M.; Schlaman, H.R.M.

MPMI Vol. 18, No. 5, 2005, pp. 414–427. DOI: 10.1094/MPMI-18-0414. © 2005 The American Phytopathological Society

e-Xtra*

Analysis of Promoter Activity of the Early Nodulin *Enod40* in *Lotus japonicus*

Mette Grønlund,[1] Andreas Roussis,[1] Emmanouil Flemetakis,[2] Nicolette E. M. Quaedvlieg,[1] Helmi R. M. Schlaman,[1] Yosuke Umehara,[3] Panagiotis Katinakis,[2] Jens Stougaard,[3] and Herman P. Spaink[1]

[1]Institute of Biology, Leiden University, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands; [2]Department of Agricultural Biotechnology, Agricultural University Athens, Iera Odos 75, 11855 Athens, Greece; [3]Department of Molecular Biology, Aarhus University, Gustav Wieds Vej 10, 8000 Aarhus C, Denmark

Submitted 4 October 2004. Accepted 9 January 2005.

Our comparative studies on the promoter (pr) activity of *Enod40* in the model legume *Lotus japonicus* in stably transformed *Gus*A reporter lines and in hairy roots of *L. japonicus* demonstrate a stringent regulation of the *Enod40* promoter in the root cortex and root hairs in response to Nod factors. Interestingly, the *L. japonicus Enod40-2* promoter fragment also shows symbiotic activity in the reverse orientation. Deletion analyses of the *Glycine max* (*Gm*) *Enod40* promoter revealed the presence of a minimal region –185 bp upstream of the transcription start. Stable transgenic *L. japonicus* reporter lines were used in bioassays to test the effect of different compounds on early symbiotic signaling. The responses of pr*GmEnod40* reporter lines were compared with the responses of *L. japonicus* (*Lj*) reporter lines based on the *LjNin* promoter. Both reporter lines show very early activity postinoculation in root hairs of the responsive zone of the root and later in the dividing cells of nodule primordia. The *LjNin* promoter was found to be more responsive than the *GmEnod40* promoter to Nod factors and related compounds. The use of pr*GmEnod40* reporter lines to analyze the effect of nodulin genes on the *GmEnod40* promoter activity indicates that *LJNIN* has a positive effect on the regulation of the *Enod40* promoter, whereas the latter is not influenced by ectopic overexpression of its own gene product. In addition to pointing to a difference in the regulation of the two nodulin genes *Enod40* and *Nin* during early time points of symbiosis, the bioassays revealed a difference in the response to the synthetic cytokinin 6-benzylaminopurine (BAP) between alfalfa and clover and *L. japonicus*. In alfalfa and clover, *Enod40* expression was induced upon BAP treatment, whereas this seems not to be the case in *L. japonicus*; these results correlate with effects at the cellular level because BAP can induce pseudonodules in alfalfa and clover but not in *L. japonicus*. In conclusion, we demonstrate the applicability of the described *L. japonicus* reporter lines in analyses of the specificity of compounds related to nodulation as well as for the dissection of the interplay between different nodulin genes.

Corresponding author: H. P. Spaink; Telephone: +3171-5275065; Fax: +3171-5275088; E-mail: spaink@rulbim.leidenuniv.nl

Current address of M. Grønlund: Biotechnology Group, Department of Plant Biology, Danish Institute of Agricultural Sciences, Thorvaldsensvej 40, opg 8, 2., 1871 Frederiksberg C, Denmark.

Current address of A. Roussis: Center for Human and Clinical Genetics, Leiden University Medical Center, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands.

Current address of Y. Umehara: National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan.

*The *e*-Xtra logo stands for "electronic extra" and indicates the HTML abstract available on-line contains three supplemental figures and one supplemental table not included in the print edition.

The establishment of symbiosis between rhizobia and leguminous plants involves extensive signaling between the two partners. Major components of the signal exchange, which leads to the formation of a new plant organ, the nodule, are plant flavonoids, isoflavonoids, and rhizobial signal molecules. The rhizobial signal molecules' Nod factors consist of an acetylated chitin oligomeric backbone with various substituents (Bladergroen and Spaink 1998; Denarie et al. 1996; Perret et al. 2000; Spaink 1998, 2000). The signal exchange between the two symbiotic partners is highly specific and many plant responses required for establishment of a successful symbiosis depend on the chemical structure of the Nod factor (D'Haeze and Holsters 2002; Roche et al. 1991). For example, nodulation of *Vicia sativa* depends on the presence of a highly unsaturated fatty acyl moity (Spaink et al. 1991), which might function in the transport of the Nod factor into the plant tissue (Schlaman et al. 1997). In *Lotus japonicus*, an acetyl-fucose substituent on the *Mesorhizobium loti* Nod factor seems to be important for the specificity. *NodZ* and *nolL* genes, encoding a fucosyltransferase (Quinto et al. 1997) and acetyltransferase (Berck et al. 1999; Scott et al. 1996), respectively, were introduced into a *Rhizobium leguminosarum* bv. *viciae* strain, which normally nodulates vetch and pea. This caused the production of an acetyl-fucosylated *R. leguminosarum* Nod factor, enabling the modified *Rhizobium* strain to nodulate *L. japonicus* (Pacios-Bras et al. 2000).

During the different steps of symbiosis, several plant genes, the so-called "nodulin" genes, are specifically expressed or upregulated in different spatio-temporal expression patterns (Mylona et al. 1995; Oldroyd and Downie 2004; Schultze and Kondorosi 1998; Stougaard 2000). To supplement in situ hybridization studies and to facilitate the analysis of nodulin gene expression during early stages of nodule development, transgenic legume reporter lines have been produced whereby the activity of a particular nodulin promoter is visualized by the fusion to a reporter gene, mostly the β-glucuronidase (*Gus*) gene. Transgenic reporter lines also have proven useful for studying the specificity in legume–*Rhizobium* sp. signaling by visualizing the symbiotic plant response or responses for a given stage of the symbiosis.

One of the nodulin genes for which expression studies have been performed in detail is the nodule inception gene (*Nin*), initially identified by forward genetics (Schauser et al. 1999). Transposon-tagged *L. japonicus nin* mutants are nodulation-minus mutants and are characterized by excessive root hair curling in response to the compatible symbiont being incapable of initiating infection thread formation and cortical cell division. By use of the transposon tag, the *LjNin* gene was cloned and shown to encode a putative transcription factor, containing a leucine-zipper DNA-binding or dimerization domain, a putative nuclear localization domain, and acidic domains that may function in transcription activation (Schauser et al. 1999). In situ RNA localization studies showed that *LjNin* is expressed in the dividing cells of the nodule primordia and in the root stele adjacent to nodules, as well as in central tissue and vascular bundles of mature nodules (Schauser et al. 1999). Transgenic *L. japonicus* reporter lines carrying a construct with the *LjNin* promoter fused to the *Gus*Aint gene (*prLjNin* reporter lines) show promoter activity in root hairs of the responsive zone of the root as early as 3 h postinoculation (hpi) with *M. loti* strain R7A and Nod factors thereof, and later in the dividing cells of nodule primordia (Y. Umehara, *unpublished*). The transgenic pr*LjNin L. japonicus* reporter lines were used in crosses with *nfr1* mutants, visualizing the lack of early epidermis response in these mutants (Radutoiu et al. 2003). The nodulin genes (*Nfr1* and *Nfr5*) are predicted to encode transmembrane receptor kinases and are necessary for recognition of compatible rhizobial microsymbiont because *nfr1* and *nfr5 L. japonicus* mutants are completely unresponsive to Nod factors (Madsen et al. 2003; Radutoiu et al. 2003). *nfr1* mutants carrying the pr*LjNin* promoter reporter construct are unable to induce promoter activity in the invasion zone, where activation normally is seen in a wild-type background, upon inoculation with *M. loti* Nod factor and *M. loti* (Radutoiu et al. 2003).

Enod40 is another, intensively studied early nodulin gene, yet is without defined function. The *Enod40* gene is found in all legumes studied so far, as well as in nonlegumes such as tobacco (Matvienko et al. 1996), rice (Kouchi et al. 1999), tomato (Vleghels et al. 2003), rye grass, barley (Larsen 2003), and *Zea mays* (Compaan et al. 2003). The *Enod40* genes lack a long open reading frame (ORF) but share two short ORFs. It still is not known whether *Enod40* acts as peptides, possibly as a regulatory RNA, or both. The finding of synthetic ENOD40 peptides binding sucrose synthase in soybean (Röhrig et al. 2002) supports a peptide function. Although there are indications for ENOD40 peptide function, there also are results supporting a role of *Enod40* as a regulatory RNA, or maybe a combination of both. Experimental data support the existence of a secondary structure of *Gmenod40* (Girard et al. 2003). Further support for a role of the *Enod40* RNA came recently when a novel RNA-binding protein, MtRBP1 was found to interact with the *MtEnod40* RNA. In *Medicago truncatula*, MtRBP1 is localized in nuclear speckles, but it accumulates in the cytoplasm of *Enod40* expressing cells during nodulation, where *Enod40* RNA and MtRBP1 colocalize in cytoplasmic granules. The cytoplasm accumulation of MtRBP1 is dependent on *Enod40* association (Campalans et al. 2004).

Enod40 is expressed both early and late in symbiosis, making it a very suitable nodulin gene to use in transgenic reporter lines. In alfalfa, there are two *Enod40* genes, and their corresponding promoters were tested for response to phytohormones in transgenic reporter alfalfa lines (Fang and Hirsch 1998). Whereas the *MsEnod40-1* promoter is active mainly under symbiotic conditions, the *MsEnod40-2* promoter also is active in root and stem vascular tissue. Both promoters are activated by Nod factor and 6-benzylaminopurine (BAP) in similar patterns in epidermal cells and in cortical cell division foci induced by both Nod factor and BAP (Fang and Hirsch 1998). The auxin transport inhibitor induces pseudonodules in which the *MsEnod40-2* promoter is active, whereas the symbiotic *MsEnod40-1* promoter is not. Furthermore, the pr*MsEnod40-1* transgenic alfalfa reporter plants were used for promoter analyses, identifying the regions essential for obtaining symbiotic promoter activity in either Nod factor- or BAP-induced nodule primordia (Fang and Hirsch 1998).

In this article, the activity of soybean and *L. japonicus Enod40* promoters in the model legume *L. japonicus* (Handberg and Stougaard 1992) is described. The isolation and activity of the *GmEnod40-2* promoter first was described in transgenic hairy roots of *Vicia hirsuta*, using a *GmEnod40-2-Gus* reporter gene construct (Roussis et al. 1995). Promoter activity was analyzed 14 days postinoculation and was found in the root pericycle opposite nodules and strongly in the nodule vasculature, corresponding to the *GmEnod40* gene expression pattern described by in situ hybridization analyses (Kouchi and Hata 1993; Yang et al. 1993), except that *Enod40* transcripts also were found in the uninfected cells of the central tissue of soybean nodules, whereas no promoter activity was found in this tissue of transgenic *V. hirsuta* nodules (Roussis et al. 1995). Later, the same *GmEnod40(-2)* promoter was used as reference promoter in reporter gene constructs in *L. japonicus* (Martirani et al. 1999; Santi et al. 2003), as control in a T-DNA tagging experiment, to judge the suitability of hairy roots for nodulation studies, and to compare nodulin promoter activities between actinorhizal plants and legumes, respectively. In the first case, the authors described the *GmEnod40* promoter activity in transgenic roots of composite *L. japonicus* plants or in regenerated transgenic plants; in such roots, the promoter was found to be active in the root vascular tissue at the base of nodules, in the cells of nodule primordia, and in the pericycle cells of the nodule vasculature (Martirani et al. 1999), corresponding to the pattern observed in hairy roots of *V. hirsuta*. In the case of transgenic root cultures, excised from the shoots, the *GmEnod40* promoter was found to be active at the base of emerging lateral roots, whereas this nonsymbiotic activity was not observed in composite or regenerated plants, leading the authors to speculate on the existence of a shoot-derived inhibiting factor influencing the promoter activity (Martirani et al. 1999).

In the comparisons between *Enod40* promoter activity in actinorhizal plants and legumes, Santi and associates (2003) described *GmEnod40* promoter activity in the root pericycle at infection sites, in nodule primordia, and in the vasculature of transgenic nodules on hairy roots of composite *L. japonicus* plants, whereas they did not observe any nonsymbiotic promoter activity (Santi et al. 2003). In contrast to these results, when testing the *Enod40* promoter from the actinorhizal plant *Casuarina glauca*, activity was found in tips of primary and lateral root primordia in transgenic hairy roots of composite *L. japonicus* plants (Santi et al. 2003).

In this article, we describe the use of the reference *GmEnod40* promoter for comparison of promoter activities between the two *L. japonicus Enod40* promoters and the *GmEnod40* promoter in hairy roots as well as in stably transformed *L. japonicus* reporter lines. Furthermore, we describe a promoter deletion analysis of the *GmEnod40* promoter in hairy roots, indicating that a –185 bp promoter fragment is sufficient to drive symbiotic expression of the reporter gene.

The pr*GmEnod40* reporter lines showed that, in addition to being active at the stages of nodule development where cell division foci have been established as well as in young and mature nodules, as described previously (Martirani et al. 1999; Santi et al. 2003), the *GmEnod40* promoter also is active very early (a few hours) postinoculation, with either its compatible

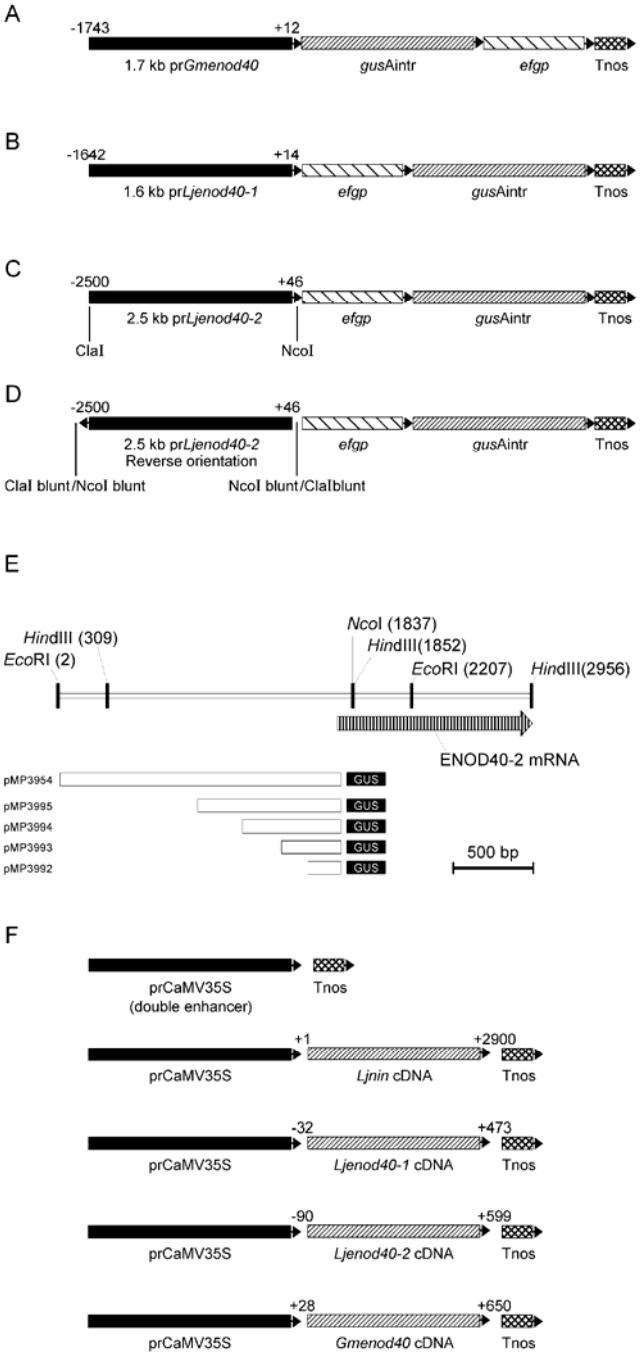

Fig. 1. Binary vector constructs for promoter analysis or overexpression of nodulin genes. **A,** pr*GmEnod40-Gus*Aint*/Gfp*; **B,** pr*LjEnod40-1-Gfp/Gus*Aint; **C,** pr*LjEnod40-2-Gfp/Gus*Aint; **D,** pr*LjEnod40-2*REV-*Gfp/Gus*Aint; **E,** deletion mutants of pr*GmEnod40*-2; **F,** prCaMV35S-*GmEnod40*, prCaMV35S-*LjEnod40*-1, prCaMV35S-*LjEnod40*-2, prCaMV35S-*LjNin,* and control (prCaMV35S alone).

symbiont or Nod factors. The early promoter activity allowed us to use the pr*GmEnod40* reporter lines in bioassays testing the effect of different compounds on early symbiotic responses in the determinate nodulator *L. japonicus*, and compare these to the responses obtained using the *L. japonicus* pr*LjNin* reporter line. In addition, the pr*GmEnod40* reporter lines were used to analyze the effect of other nodulin genes on the promoter activity.

RESULTS

Expression patterns of stable *Enod40* reporter lines of *L. japonicus*.

The progeny of 15 plant lines containing a *GmEnod40* reporter construct (Fig. 1A) were tested for *Gus* expression in batches of whole (T2) plants 3 weeks postinoculation (wpi) with *M. loti* strain R7A. In five lines (Table 1), GUS staining was observed only in *Rhizobium*-inoculated plants in the root epidermis of the responsive zone, in the root pericycle at the base of nodule primordia 3 to 5 days postinoculation (dpi), in the dividing cells of the nodule primordia, and in the developing and mature nodule vasculature. In the progeny of seven other lines (Table 1), the same symbiotic promoter activity was found; however, additionally, these lines exhibited symbiosis-independent GUS staining in the vascular tissue of the whole plant. The data for all plant lines, summarized in Table 1, give the impression of a correlation between the number of stably integrated T-DNA copies and the occurrence of general vascular staining because this was observed only in plants with a single insert of the T-DNA. Similar results were observed for the few transgenic lines containing the *LjEnod40-1* reporter construct.

From the *GmEnod40* reporter lines, two representative lines, each showing one of the two different promoter responses, were chosen for further analysis of the T2, T3, and T4 generations. Line *Lj*3637.19b, carrying a single T-DNA insertion, showed stable inheritance of the vascular and symbiotic responses in the homozygous T2, T3, and T4 offspring. These lines showed symbiotic responses in the epidermis as early as 24 hpi. Early responses were very difficult to analyze due to the relatively strong activity in the vascular system. Line *Lj*3637.33 had two T-DNA insertions, not directly linked. This line showed only symbiotic promoter activity and was used for time course analysis of the early promoter activity in the root epidermis of the responsive zone, after inoculation with either *M. loti* R7A or its purified Nod factors (van Spronsen et al. 2001). The first epidermis or root hair response appeared between 4 and 6 hpi in a few cells (Fig. 2A) of all staining plants in the T2 generation. The epidermis staining region broadened up to 3 dpi (Fig. 2A). Pericycle staining, cortical cell division, and cortical cell or primordium staining was observed in *Rhizobium* spp.-inoculated plants 5 dpi (Fig. 2B), while Nod factor-treated plants still showed only root epidermis staining (Fig. 2A) until 10 dpi, when cortical cell division had initiated

Table 1. Overview of occurrence of staining in stably transformed reporter lines of *Lotus japonicus*

No. of T-DNA insertions	No. of independent, transformed plant lines	Nonstaining plant lines	Symbiotic staining	Nonsymbiotic staining
pr*GmEnod40-Gus*Aint*/Gfp*				
1	8/15	1	7	7
2	6/15	2	4	0
4	1/15	0	1	0
pr*LjEnod40-1-Gfp/Gus*Aint				
1	2/3	0	2	2
3	1/3	0	1	1[a]

[a] Most roots do not show root vascular staining, but it does occur in some lateral roots.

and staining in pericycle and nodule primordium set in (Fig. 2A). The results (Table 2) showed that this staining pattern was not stably inherited in all T3 offspring plants. Although the pericycle staining at the base of the primordia always was inherited in a Mendelian fashion, the early epidermal staining was lost in some of the lines. A few of the T3 lines that did retain the original staining pattern showed stable inheritance of this phenotype in the T4 generation. Because of the unexpected loss of the original staining patterns in some lines, we analyzed representative offspring lines with Southern hybridization of T2, T3, and T4 plants (data not shown). The results showed that, in all analyzed lines (Table 2), the hybridization patterns were identical to the parental lines (data not shown).

We used in situ RNA hybridization to analyze the expression of the endogenous *LjEnod40-1* gene in wild-type plants and the selected pr*GmEnod40* reporter lines *Lj*3637.33 and *Lj*3637.19b. The results (Fig. 3) show that, for both the wild-type plants and the transgenic *Lj*3637.19b and *Lj*3637.33 lines, endogenous *LjEnod40-1* transcripts could be detected only in the nodules and in the part of the root vascular tissue facing the nodules. Comparisons with GUS staining patterns in the same lines showed that the signals obtained by in situ RNA hybridization corresponded to the symbiotic promoter activity observed after staining for GUS activity in the double integration line *Lj*3637.33 (Fig. 3).

Effects of inducing compounds on stable transgenic *Enod40* and *LjNin* reporter lines.

The offspring of the double integration line *Lj*3637.33 showing a stable GUS staining pattern, including early epidermis responses, was used in bioassays testing the symbiotic effect of different compounds on *L. japonicus* and the effective concentration window of Nod factors. Responses of the *GmEnod40* promoter in *L. japonicus* were compared with early responses in a transgenic *L. japonicus* pr *LjNin* reporter line (Radutoiu et al. 2003), (Y. Umehara, *unpublished*). The *LjNin* promoter was more responsive to the different compounds than the *GmEnod40* promoter (Table 3). Plants carrying the pr*GmEnod40* reporter construct responded only to Nod factors of the compatible symbiont *M. loti* R7A, at a relatively high concentration. In contrast, the *LjNin* promoter activity was observed at a 100-fold lower Nod factor R7A concentration; additionally, the *LjNin* promoter was active in response to Nod factors from the noncompatible symbiont *R. leguminosarum* bv. *viciae* strain RBL5560 (Spaink et al. 1989). A 1,000-fold higher concentration of Nod factor of *R. leguminosarum* than for Nod factor R7A was required to obtain the same visual response. Furthermore, the *LjNin* promoter also responded, in some conditions, to a mix of chitin tetramer and pentamer at high concentrations. We also tested 10- and 100-fold lower concentrations, but only observed weak responses at 10^{-5} M. The effect of chitin was tested in different conditions such as changing media, temperature, and light; using liquid assays; or sand inoculating plants on solid medium. In all conditions, the response was weak and variable without a regular, consistent cellular pattern, and was observed in only some plants. Nevertheless, this weak response never was observed in uninoculated plants. Because the Nod factors of *M. loti* are 4-O-acetyl-fucosylated, we tested a mixture of fucosylated and O-acetylated chitin tetramers and pentamers and found a similar weak

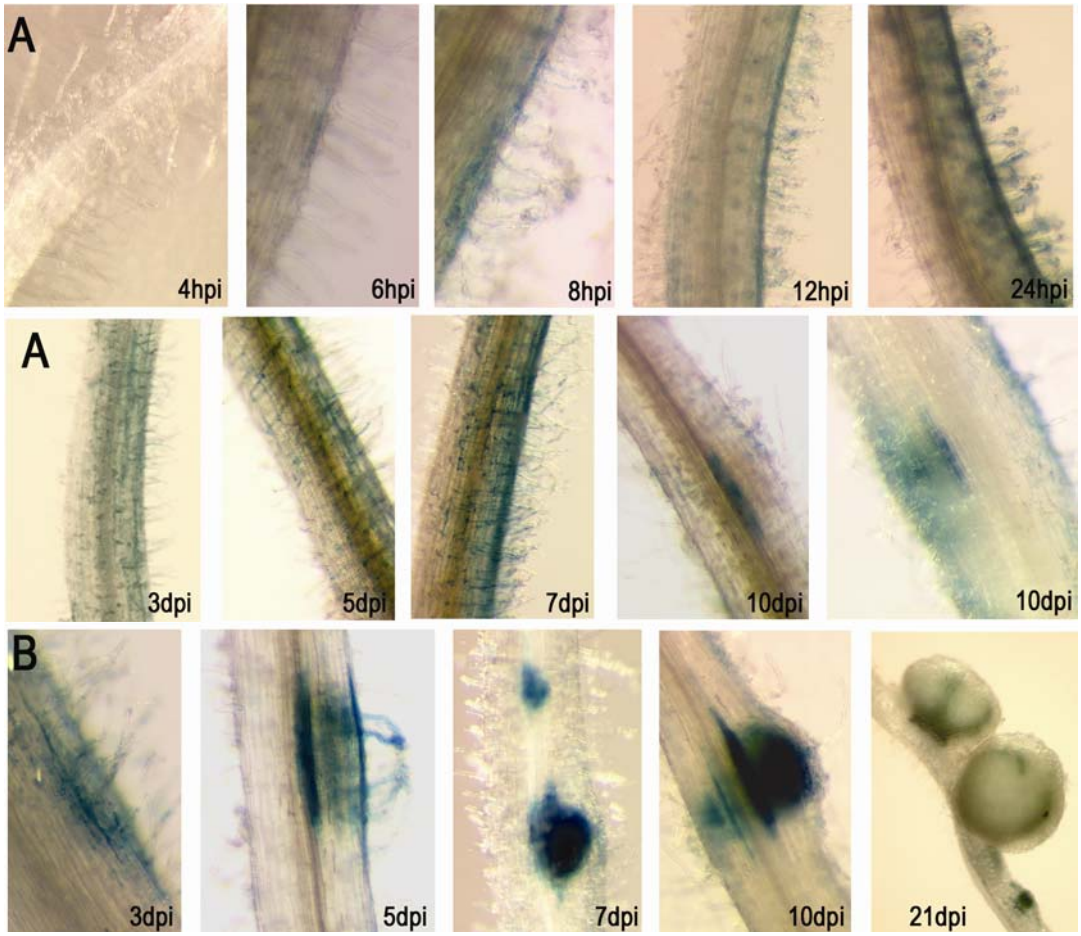

Fig. 2. Time course of early symbiotic staining. **A,** Staining in *Lj*3637.33 T2 plants 4 h to 10 days postinoculation (dpi) after *Mesorhizobium loti* R7A Nod factor application. **B,** Staining in *Lj*3637.33 T2 plants 3 to 21 dpi after *M. loti* R7A inoculation.

promoter activity of *Ljnin* with a 100-fold lower concentration compared with the response to nondecorated chitin. It was impossible to quantify the GUS response to compounds other than the compatible Nod factor due to the weak and variable root hair response. Quantitative single root hair measurements were not feasible; therefore, the responses were qualitatively described as + for full response (as for compatible Nod factor), and ± for weak, variable response in few root hairs (Table 3). We want to emphasize that although, in several cases, weak and variable responses were observed, the difference between the two promoters was consistent and nontreated plants never showed staining.

We also tested the plant hormones cytokinin BAP and auxin (indole-3-acetic acid) (IAA) for their ability to activate the *GmEnod40* or *LjNin* promoters in transgenic *L. japonicus* reporter lines. It previously has been shown that *MsEnod40* can be induced in alfalfa roots by the synthetic cytokinin BAP (Fang and Hirsch 1998; Hirsch and Fang 1994). We found no effect on treating the *L. japonicus* pr*GmEnod40* and pr*LjNin* reporter lines with IAA or BAP alone, under test conditions similar to those used for alfalfa. Furthermore, induction of *Enod40* expression by BAP (shown by in situ hybridization) was observed in white clover (Mathesius et al. 2000), although at a 100-fold higher concentration than the one used for alfalfa (Fang and Hirsch 1998). We tested BAP in conditions similar to those described for white clover on the *L. japonicus GmEnod40* reporter line, but observed a significant reduction in the root growth (and a lack of GUS staining). It also should be mentioned that, in previous studies with the pr*Ljnin* reporter lines in a different test system, where older plants were treated longer with the more stable auxin naphthalene-1-acetic acid, the *LjNin* promoter was activated in a root system with excessive lateral root formation (Y. Umehara, *unpublished*).

A pharmacological approach using the G protein activator mastoporan and *MtEnod12-Gus* transgenic alfalfa plant lines suggested a function of heterotrimeric G proteins in the Nod factor signaling pathways. An epidermal response in the transgenic alfalfa *MtEnod12* reporter lines similar to Nod factor-treated *MtEnod12* plants was observed when treated with Mastoporan (Pingret et al. 1998) and the possible involvement of G proteins in the Nod factor signaling pathways later was supported by a different pharmacological approach by Engstrom and associates (2002). When the transgenic *L. japonicus GmEnod40* and *LjNin* reporter lines were treated with Mastoporan, no promoter activity was observed.

Table 2. Staining patterns in offspring of the pr*GmEnod40* reporter line *Lj3637.33*

	Symbiotic staining		
Parental line	**Epidermis**	**Pericycle**	**Nodule**
T1:33	18/25	25/35	25/35
T2:33-1b	12/21	40/56	35/56
T3:33-1b-1	0/6	0/6	0/6
T3:33-1b-4	7/9	7/8	7/8
T2:33-2b	5/25	26/29	24/29
T3:33-2b-1	0/10	4/7	0/7
T3:33-2b-2	0/8	7/8	0/8
T2:33-4	0/10	15/16	13/16
T2:33-5b	25/36	49/59	49/59
T3:33-5b-1	0/9	0/9	0/9
T3:33-5b-2	7/8	6/8	6/8
T3:33-5b-3	7/8	6/6	5/6
T3:33-5b-4	2/8	11/15	9/15
T3:33-5b-5	7/7	5/5	5/5
T3:33-5b-6	0/9	0/10	0/10
T2:33-6b	0/21	27/39	27/39[a]
T3:33-6b-1	0/5	3/3	0/3
T3:33-6b-3	0/9	1/6	0/6
T2:33-8	0/7	9/10	0/10
T2:33-pl1	0/10	0/11	0/11
T2:33-pl2	0/12	20/29	7/29
T3:33-pl2-1	0/7	2/5	0/5
T3:33-pl2-2	0/9	8/9	0/9
T3:33-pl2-3	0/6	1/5	0/5
T3:33-pl2-4	0/10	6/8	0/8
T3:33-pl2-6	0/8	5/15	0/15
T2:33-pl3	0/12	7/10	7/10
T2:33-pl4	0/11	0/10	0/10
T2:33-pl5	0/12	10/12	1/12
T3:33-pl5-1	0/9	4/14	0/14
T3:33-pl5-4	1/9	5/16	1/16
T3:33-pl5-6	0/9	4/17	0/17
T3:33-pl5-8	0/10	8/9	0/9

[a] 19 of 27 staining only in nodule vasculature, not dividing cells.

Promoter analysis using a hairy root system.

Hairy roots were induced on two of the stable pr*GmEnod40* reporter lines, *Lj3637 33-10b* and *Lj3637 33-5b*, by transformation with a wild-type *Agrobacterium rhizogenes* LBA1334 strain (Offringa et al. 1986) in order to compare the promoter activity in stably transformed lines with the activity in hairy roots. Hairy roots of composite plants from both lines stained only upon inoculation with *M. loti* strain R7A and with the same symbiotic staining pattern as observed in inoculated roots of the stable transformants, seemingly not disturbed by the altered hormone balance in the hairy roots. These results encouraged us to use the hairy root system for additional promoter analyses.

Three different constructs containing either the *GmEnod40-2* promoter (Fig. 1A), the *LjEnod40-1* promoter (Fig. 1B), or the *LjEnod40-2* promoter (Fig. 1C) fused to the *Gus*Aint and *Gfp* reporter genes were introduced into wild-type *L. japonicus* plants by *A. rhizogenes* LBA1334. In this system, general root vascular staining was observed in most of the hairy roots in addition to the symbiotic staining, with no correlation to T-DNA insertion numbers (data not shown). The symbiotic staining appeared the same for the three different promoter fusions, indicating that the two *L. japonicus Enod40* promoters as well as the heterologous *Glycine max Enod40* promoter are regulated in a similar manner during nodulation of *L. japonicus*.

Table 3. Responses of stably transformed *Lotus japonicus* pr*GmEnod40*- and pr*LjNin* reporter lines upon application of different compounds tested in bioassays

		Response of reporter lines[a]	
Compound[b]	**Concentration (M)**	**prGmEnod40**	**prLjNin**
NF R7A	10^{-11}	–	–
NF R7A	10^{-10}	–	±
NF R7A	10^{-9}	–	±
NF R7A	10^{-8}	±	+
NF R7A	10^{-7}	+	+
NF R.leg	10^{-7}	–	±
Chitin IV/V	10^{-5}	–	±
CO-fuc/OAc-CO	10^{-7}	–	±
GlcNAc	10^{-6}	–	–
BAP	10^{-6}	–	–
BAP/NF R7A	$10^{-6}/10^{-7}$	±	±
IAA	10^{-6}	–	–
Mastoporan/Ca^{2+}	$10^{-6}/10^{-3}$	–	–

[a] Response: + = optimal staining compared with rhizobia-inoculated plants, – = absence of response, and ± = very weak response; only a few plants stain in a few cells.

[b] NF R7A = Nod factors isolated from *Mesorhizobium loti* strain R7A; NF R. leg = Nod factors isolated from *Rhizobium leguminosarum* strain RBL5560; Chitin IV/V = mixture of chitin tetramers and pentamers; CO-fuc/OAc-CO = mixture of fucosylated and O-acetylated chitin oligosaccharide; GlcNAc = N-acetylglucosamine; BAP = 6-benzylaminopurine; and IAA = indole-3-acetic acid.

We also tested a construct containing the *LjEnod40-2* promoter in reverse orientation (Fig. 1D), fused to the *Gfp* and *Gus*Aint reporter genes. This construct, likewise, was introduced into *L. japonicus* by *A. rhizogenes* LBA1334 transformation and the resulting hairy roots were tested for *Gus* expression 4 and 8 wpi as well as in a few uninoculated roots. Composite plants resulting from transformations with pr*LjEnod40-2*REV reporter construct had, in general, fewer

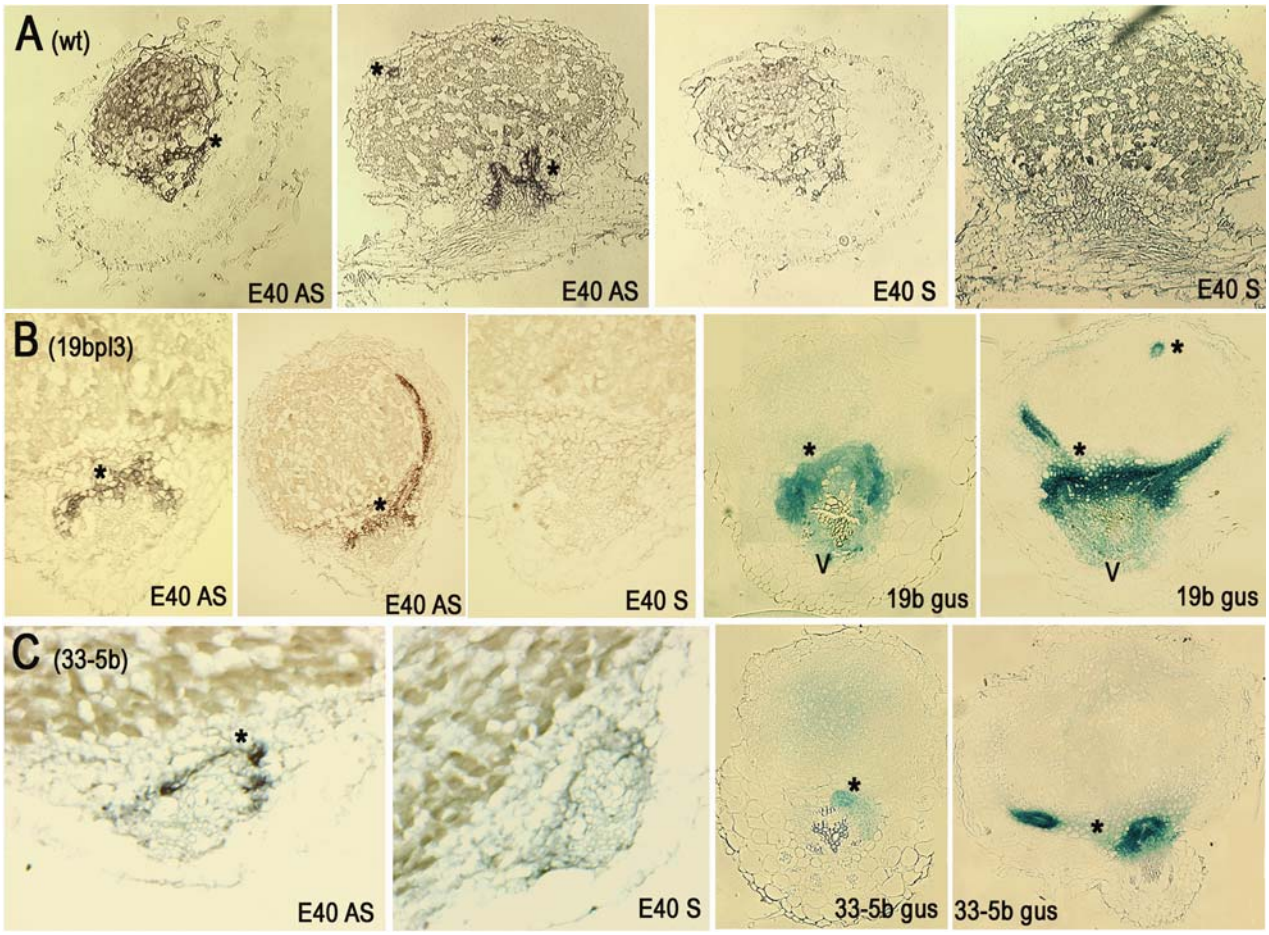

Fig. 3. Comparison of endogenous *LjEnod40*-1 expression and promoter activity in transgenic pr*GmEnod40-Gus*Aint/*Gfp* reporter lines. In situ hybridization signals of *LjEnod40-1* in nodules of **A,** wild-type, B, *Lj*3637.19bpl3, and **C,** *Lj*3637.33-5b plants compared with β-glucuronidase (GUS) localization in nodule sections of **B,** *Lj*3637.19bpl3 and **C,** *Lj*3637.33-5b. Pictures marked with "E40AS" are sections probed with antisense *LjEnod40-1* in in situ hybridization, showing endogenous *LjEnod40* expression in the root pericycle connected to nodules as well as in nodule vasculature (asterisks). Comparable signals were obtained by in situ hybridization studies for-wild type *Lotus japonicus* plants and for the transgenic pr*GmEnod40* reporter lines; no signal was observed in root vascular tissue that was not connected to the nodule. No signals were seen in control sections, probed with sense *LjEnod40-1* ("E40 S"). In comparison, sections of GUS-stained nodules are included for the transgenic lines "19b gus" and "33-5b gus", showing the nonsymbiotic root vascular (V) staining in the transgenic *Lj*3637.19bpl3 line and the symbiotic GUS staining pattern (asterisks) comparable to the in situ signals obtained for wild-type plants and for the transgenic *Lj*3637.33-5b line.

Table 4. Summarizing data of staining patterns in hairy roots on wild-type plants transformed with the pr*LjEnod40*-2REV reporter construct and wild-type plants transformed with the *GmEnod40* promoter deletion constructs[a]

Construct	R7A	Root vascular	Nodule	Nodule minus root vascular	Negative
LjEnod40-2-*Gfp*/*Gus*Aint	+	26/32	29/32	7/32	13/45
	–	6/11	…	…	5/11
LjEnod40-2REV-*Gfp*/*Gus*Aint	+	18/36	29/36	19/36	29/65
	–	8/18	…	…	10/18
GmEnod40 Δ-1700	+	22/48	31/48	9/48	9/57
	–	10/21	…	…	11/21
GmEnod40 Δ-894	+	40/40	6/40	0/40	26/66
	–	3/8	…	…	5/8
GmEnod40 Δ-624	+	34/34	3/34	0/34	38/72
	–	8/9	…	…	1/9
GmEnod40 Δ-374	+	53/53	19/53	0/53	22/75
	–	7/9	…	…	2/9
GmEnod40 Δ-185	+	43/43	7/43	0/43	24/67
	–	2/9	…	…	7/9

[a] Summary of data from composite plants with hairy roots induced by LBA1334 carrying the respective constructs, screened 4 and 8 weeks post inoculation with *Mesorhizobium loti* R7A. Uninoculated composite plants were screened after 4 weeks of growth after transfer to pots. Numbers give the ratio of composite plants having one or more hairy roots staining in the specified tissue out of the total number of staining composite plants. The last column gives the ratio of composite plants, which did not show any staining in the hairy roots, out of the total number of composite plants screened.

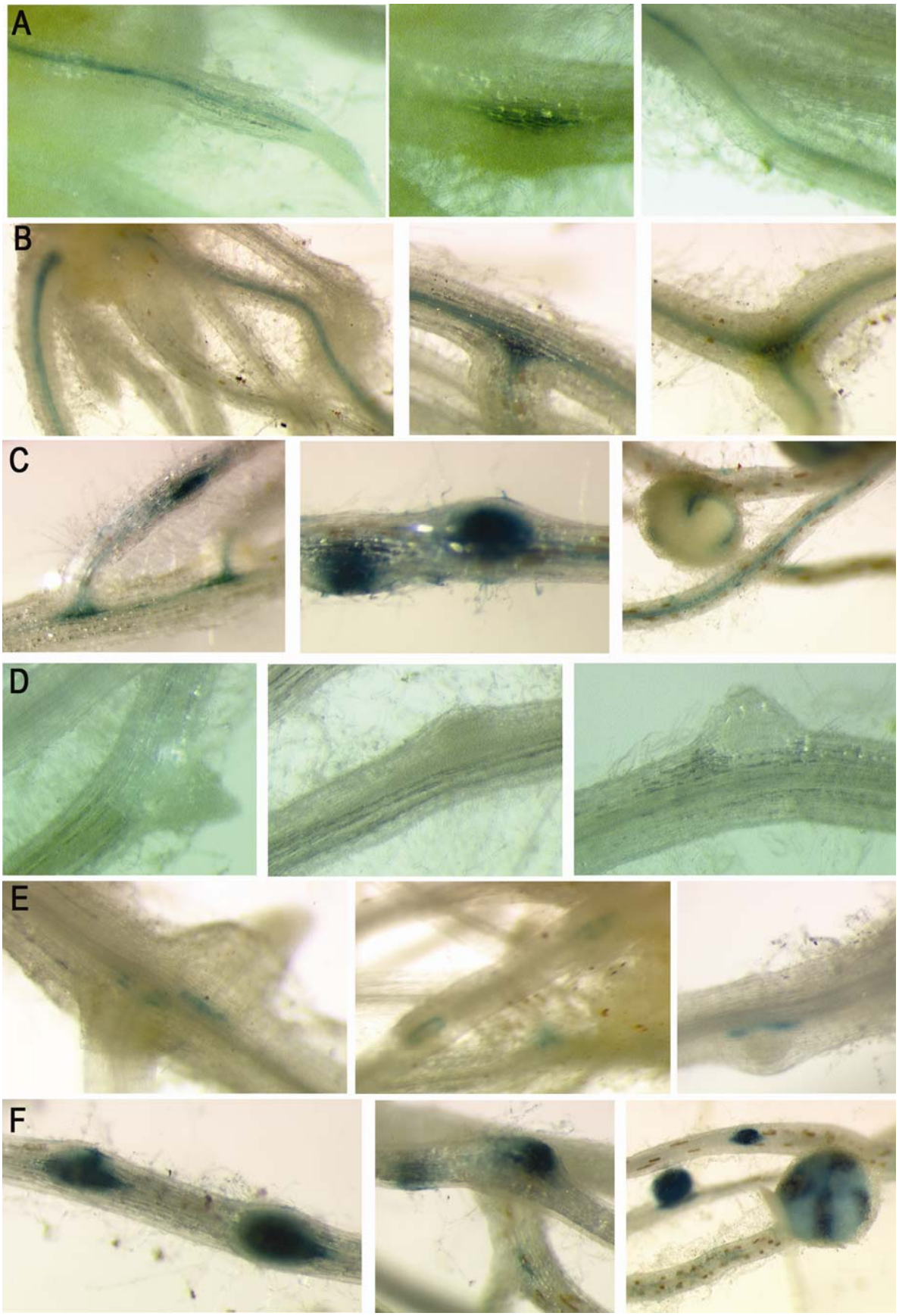

Fig. 4. *GmEnod40* promoter activity in hairy roots on stable lines of *Lj*3637.33-5b. **A–C,** Hairy roots transformed with the prCaMV35S-*LjNin* construct. **D–F,** Hairy roots transformed with the control construct prCaMV35S in pPZP211 vector or transformed with wild-type *Agrobacterium rhizogenes* LBA1334. **A** and **D,** β-Glucuronidase (GUS) stained 2 weeks after transformation, directly from hairy root emergence medium. **B** and **E,** GUS stained after 10 days or 4 weeks of growth, respectively, in clay support and minimal medium, uninoculated. **C** and **F,** GUS stained after 10 days or 4 weeks of growth, respectively, in clay support and minimal medium, inoculated with *Mesorhizobium loti* R7A.

hairy roots showing any *Gus* expression compared with the control transformation of a construct containing pr*LjEnod40-2* reporter construct (Table 4). Staining of pr*LjEnod40-2*REV reporter construct containing hairy roots showed a pattern of promoter activity similar to that of the roots transformed with the "forward promoter" construct, whereas the intensity of the staining was reduced using the reverse promoter (Table 4). Another difference observed was the frequency of nonsymbiotic staining in the root vascular tissue. Whereas almost all (26/32) of the hairy roots containing the forward promoter construct exhibited intense root vascular staining, only half (18/36) of those transformed with the "reverse promoter" construct showed weak root vascular staining. Interestingly, these data indicate that the *LjEnod40-2* promoter also is capable of driving the expression of a reporter gene from a reverse orientation.

Deletion analyses of the *G. max Enod40* promoter were performed in hairy roots of *L. japonicus*. Four different deletion variants of the *GmEnod40* promoter were constructed (Fig. 1E) by amplifying regions of the 1.7-kb promoter fragment used for stable transformation. The primer sets all included the same 3′ end, including the putative start ATG of peptide I, translationally fused to the *Gus* reporter gene, varying only in the length of the upstream sequence. None of the deletion constructs affected the nonsymbiotic root vascular staining (Table 4). Instead, a significant effect was observed in the frequency of hairy roots showing symbiotic staining when introducing the deletion constructs. The different deletion constructs with promoter fragments of –894 to –185 bp all showed a few hairy roots with GUS stain in some nodules, with a frequency comparable between them but lower than for the –1.7-kb promoter construct (Table 4), indicating that the –1.7-kb to –894-bp promoter fragment contained a positive regulatory promoter element. On the other hand, even a –185-bp promoter fragment still was able to drive symbiotic expression of the reporter gene, suggesting that this small promoter element contained all the regulatory information needed for the tight symbiotic regulation of the *Enod40* promoter, as well as for the more nonspecific root vascular activity observed. The same promoter deletions were used in yeast one-hybrid experiments with the aim of identifying upstream factors activating the *Enod40* promoter screening a nodule cDNA library of *L. japonicus* (provided by C. Poulsen, Aarhus, Denmark). Unfortunately, several screenings did not result in any positive clones.

Effect on the *GmEnod40* promoter activity of overexpression of the *LjNin* gene in hairy roots of the symbiotic staining reporter line.

T3 plants from the stable transgenic line *Lj3637 33-5b* retained the original GUS staining pattern of symbiotic staining and, when tested for promoter activity in hairy roots, showed the same activity in hairy roots as in normal roots. These plants were used to introduce different constructs for overexpression of the nodulin genes *LjNin*, *LjEnod40-1*, *LjEnod40-2,* and *GmEnod40* (Fig. 1F) in hairy roots, in order to study the possible effect of these genes on the *GmEnod40* promoter activity in *L. japonicus*. The cDNAs for the respective genes were inserted into the binary vector pPZP111 behind the CaMV35S promoter double enhancer (Quaedvlieg et al. 1998) and introduced into the stable transgenic plants by *A. rhizogenes* transformation. The induced hairy roots were tested for *Gus* expression in both uninoculated roots, which should not stain in the stable transgenic line, and in plants inoculated with *M. Loti* R7A, in order to investigate possible effects on the *GmEnod40* promoter activity. Over-expression of the three *Enod40* genes did not alter the promoter activity compared with the control plants (Table 5), indicating a lack of auto-regulation of the *GmEnod40* promoter. In contrast, overexpression of *LjNin* caused an induction (or enhancement) of *GmEnod40* promoter activity in the root vascular tissue in both uninoculated and nodulated hairy roots (Fig. 4A through C; Table 5). We found that, when testing the composite plants for *Gus* expression directly from the hairy root induction medium, approximately 2 weeks after transformation, the control plants showed no GUS staining (Fig. 4D), whereas a number of the hairy roots overexpressing *LjNin* showed GUS stain at the base of emerging lateral roots, extending to different degrees in the vascular tissue of the main root (Fig. 4A). Hairy roots of composite plants tested 10 days and 4 weeks after transfer to clay pots showed a background staining in uninoculated control plants in the vascular tissue at the base of emerging lateral roots (Fig. 4E). Although this expression pattern was comparable, to some extend, to what was contributed to an effect from overexpression of *LjNin* at the early time points (Fig. 4A), there still was a distinct difference in promoter activity in hairy roots containing the CaMV35S-*LjNin* construct; under the same growth conditions, the hairy roots overexpressing *LjNin* had a much-extended root vascular staining (Fig. 4B) compared with the control plants (Fig. 4E). In nodulated hairy roots, no effect was observed in the symbiotic expression pattern at 10 dpi and 4 wpi (compare Fig. 4C and F); only the root vascular staining was as described for the uninoculated plants.

DISCUSSION

The *Enod40* genes from various legumes have been studied intensively because they represent one of the nodulin genes

Table 5. Data summarizing the *GmEnod40* promoter activity, in response to overexpression of *LjNin* cDNA in hairy roots on plants of the pr*GmEnod40* reporter line *Lj3637.33-5b*[a]

	2 weeks, HRE	Uninoculated	R7A-inoculated	
Construct	**Root vasc.**	**Root vasc.**	**Symbiotic**	**Root vasc.**
LBA1334: no plasmid	0/12	23/44	25/44	6/44
prCaMV35S	0/24	12/44	35/42	8/42
prCaMV35S-*LjEnod40-1*	0/21	31/69	42/67	11/67
prCaMV35S-*LjEnod40-2*	0/15	9/22	17/22	6/22
prCaMV35S-*GmEnod40*	0/5	6/14	11/14	5/14
prCaMV35S-*LjNin*	13/24	8/44	33/42	0/42
Extended[b]				
prCaMV35S-*LjNin*	13/24	18/44	…	24/42

[a] Summary of data from composite plants with hairy roots induced by LBA1334 carrying the respective constructs: 2 weeks, HRE = plants screened 2 weeks after transformation and growth on hairy root emergence (HRE) medium; Uninoculated = uninoculated plants screened after 10 days and 4 weeks of growth in clay support; R7A-inoculated = plants inoculated with *Mesorhizobium loti* strain R7A and screened 10 days and 4 weeks post inoculation, growing in clay support; Root vasc. = root vasculature staining in main root, at the base of emerging lateral roots. Numbers give the ratio of composite plants having one or more hairy roots staining in the specified tissue out of the total number of composite plants.

[b] Extended = extended root vascular staining compared with controls.

that are induced early after rhizobial infection or Nod factor application. We have utilized *Enod40* promoters to establish a sensitive promoter–reporter gene system that greatly facilitates studies of symbiotic responses, particularly to Nod factors during the early stages of the symbiosis. We also have demonstrated the potential of this system by analyzing the effects of ectopically expressed *Enod40* and *Nin* genes, and our results suggest that NIN positively regulates *Enod40* gene expression, showing the applicability of these stable transgenic reporter lines in bioassays for advanced signal transduction studies in legumes.

Furthermore, we have studied the pattern of *Enod40* promoter activity in the model legume *L. japonicus* using both stable transformants and *A. rhizogenes* induced hairy roots and compared it with the symbiotic expression pattern of the endogenous gene by in situ mRNA hybridization analysis (Fig. 3). Analysis of stable transgenic plant lines carrying the heterologous *G. max Enod40* promoter fused to the *Gus* reporter gene showed that the *Enod40* promoter is activated within hours postinoculation with the compatible symbiont *M. loti* R7A or Nod factors thereof. The promoter activation seems very specific to symbiotically active compounds, as will be discussed below. The symbiotic activity of the *LjEnod40* promoters and *GmEnod40* promoter during nodulation of *L. japonicus* hairy roots is similar, indicating that the results obtained using the stable transgenic lines are not disturbed by the use of a heterologous reporter system. Surprisingly, the reverse orientation of the *LjEnod40-2* promoter also displayed symbiotic activity. The observation of reverse promoter activity of the *LjEnod40-2* promoter suggests it to be a bidirectional promoter. Bi-directional promoter activity recently has been shown to be rather common in animal systems; in a study by Trinklein and associates (2004), the distances between more than 23,000 genes in the human genome were analyzed and a major class of gene pairs was identified in which the genes were arranged head-to-head on opposite strands sharing a bidirectional promoter.

Comparison of *Enod40* expression in transgenes and in situ analysis.

Due to a difference in the occurrence of nonsymbiotic vascular staining in the stable transgenic plant lines, in situ mRNA hybridization analysis was performed on representative transgenic lines and wild-type *L. japonicus* plants and compared with *Gus* expression pattern in plants at a similar stage of symbiosis. The symbiotic pattern of *GmEnod40* promoter activity or GUS staining during nodulation (Fig. 2) was similar to the location of the *LjEnod40-1* transcripts in in situ hybridization analyses, whereas—within the level of sensitivity of in situ hybridization analysis—it seems that the nonsymbiotic vascular promoter activity, seen in some of the reporter lines, did not reflect the endogenous gene expression (Fig. 3).

Published in situ hybridization analyses of endogenous *LjEnod40-1* expression in young and mature nodules correlate with our data and show no signal in nonsymbiotic root vascular tissue, although reverse-transcriptase polymerase chain reaction (RT-PCR) performed on uninoculated root material showed a low expression of *LjEnod40-1* and *LjEnod40-2* (Flemetakis et al. 2000). Furthermore, the same *GmEnod40* promoter fragment, as used in these studies, previously was introduced into *V. hirsuta* by *A. rhizogenes* transformation and the promoter activity was observed only in symbiotic tissue in the resulting transgenic hairy roots (Roussis et al. 1995). Likewise, as described in the introduction, previous experiments analyzing *GmEnod40* promoter activity in *L. japonicus* showed only symbiotic promoter activity in whole plants (Martirani et al. 1999; Santi et al. 2003).

Deletion analysis has been reported for the *MsEnod40-1* promoter in alfalfa showing that a region of –231 bp was insufficient to obtain any promoter activity and that an approximately 600-bp region further upstream was essential and sufficient to obtain symbiotic promoter activity (Fang and Hirsch 1998). The results obtained in our promoter deletion analysis suggest that the –185-bp promoter fragment contains all the regulatory information needed for the tight symbiotic regulation of *GmEnod40* promoter in *L. japonicus*, as well as for the nonspecific root vascular activity observed. The different deletion constructs with *GmEnod40* promoter fragments of –894 to –185 bp all showed hairy roots with GUS staining in some nodules, as well as root vascular staining, with a frequency comparable between them but lower than for the –1.7-kb promoter construct (Table 4), which might indicate that the –1.7-kb to –894-bp promoter fragment contains a positive regulatory promoter element, enhancing the *GmEnod40* promoter responses in the transgenic *L. japonicus* plants. Considering the results from the double integration lines and the in situ RNA localization studies, indicating that the nonsymbiotic promoter activity observed in the single integration lines does not reflect the normal *Enod40* promoter activity, it can be speculated that a full *Enod40* promoter in its natural context also includes negative elements repressing a nonsymbiotic promoter activity. Our unpublished results of yeast one-hybrid screens with the 1.7-kb fragment of the *GmEnod40* promoter with a nodule cDNA library that did not yield positive clones also could be explained by the presence of negative regulatory elements in the *Enod40* promoter.

Effect of chitin oligosaccharides and Nod factor specificity.

When comparing the response of the two studied reporter lines, the symbiotically active *GmEnod40* promoter and the *LjNin* promoter line, to different symbiotically related compounds, it seems that the *LjNin* promoter is more responsive than the *GmEnod40* promoter in *L. japonicus*. Nod factors of the compatible symbiont *M. loti* R7A were needed in a 100-fold higher concentration to activate the *GmEnod40* promoter than for *LjNin* promoter activation. Furthermore, the *GmEnod40* promoter was activated only by the compatible symbiont or its Nod factors, whereas the *LjNin* reporter plants responded to a broader spectrum of compounds.

In *G. max*, the early induction of *Enod40* by chitopentaose and a synthetic Nod factor similar to the Nod factor of a nonsymbiont *Sinorhizobium meliloti* has been shown by the use of RT-PCR (Minami et al. 1996), although the same compounds failed to induce any cellular changes in *G. max*, such as root hair curling and cortical cell division. Although we cannot exclude that the lack of *Enod40* promoter activity in our transgenic *L. japonicus* reporter lines, in response to chitin and noncompatible Nod factors from *R. leguminosarum,* arose by technical limitations in terms of sensitivity, our bioassay results suggest that the early *Enod40* expression is regulated more stringently in *L. japonicus* than in *G. max*, possibly reflecting a difference in the regulation of nodulation between legume species.

The *LjNin* promoter responded to Nod factors from the noncompatible symbiont *R. leguminosarum* RBL5560, but a 1,000-fold higher concentration was needed to obtain a visual promoter response similar to Nod factors of *M. loti* R7A. Additionally, under certain conditions, the *LjNin* promoter responds weakly to nondecorated chitin tetraose and chitin pentaose in even higher concentrations (five orders of magnitude higher than for *M. loti* R7A). Similar weak promoter activity was observed when treating the plants with a mix of acetylated and fucosylated chitin tetraose and chitin pentaose. The plants responded to these compounds at concentrations 100-fold lower

than to nondecorated chitin molecules, supporting earlier experiments showing the importance of the acetyl-fucosyl decoration of *M. loti* Nod factors (Pacios-Bras et al. 2000).

The fact that the *Ljnin* promoter was more responsive than the *GmEnod40* promoter to Nod factors and related compounds, along with the indication that NIN positively regulates *Enod40*, suggest that *Nin* expression is activated upstream of *Enod40* in the Nod factor signaling cascade.

The concentration range of the different Nod factors which activate the *LjNin* promoter activity correlates with what was observed using the nodulin promoter *MtEnod12* in transgenic alfalfa *Gus* reporter lines (Journet et al. 1994). In the *MtEnod12* bioassay, application of Nod factors lacking the sulfate group, which is required for nodulation of alfalfa, induced cell-specific promoter response similar to that of sulfated Nod factors but reduced the Nod factor activity by 1,000-fold. No promoter response was observed when testing nondecorated chitotetraose at concentrations up to 10^{-6} M (Journet et al. 1994).

Responses to hormone treatments.

With the conditions used in our bioassays, we found that IAA or the synthetic cytokinin BAP alone had no effect on the *L. japonicus* pr*GmEnod40* and pr*LjNin* reporter lines either when using test conditions similar to those in the alfalfa experiments, where *MsEnod40* and *MsEnod12* promoter activities were induced by BAP (Bauer et al. 1996; Fang and Hirsch 1998), or at higher BAP concentrations, where induction of *Enod40* expression was reported for white clover (Mathesius et al. 2000). The difference in responses to BAP among *L. japonicus*, alfalfa, and white clover also was reflected by the fact that BAP can induce the formation of cortical cell division in alfalfa and white clover (Bauer et al. 1996; Mathesius et al. 2000), whereas this apparently is not the case in *L. japonicus* (Kawaguchi et al. 1996). We hypothesize that these discrepancies could demonstrate the differences in hormonal gene regulation between plants that form determinate and indeterminate nodules.

Recently, the cytokinin levels in *L. japonicus* roots were monitored during lateral root formation and nodulation using a cytokinin-responsive promoter *Gus*Aint reporter construct in hairy roots. During the initial cell divisions for lateral root formation, the promoter was not active, whereas the promoter was induced 48 hpi with *M. loti* in the symbiotically responding root hairs and in the nodule primordia. In additional experiments where a cytokinin oxidase was overexpressed in *L. japonicus* hairy roots, thereby reducing the cytokinin level in the roots, lateral root formation was increased and nodule formation decreased. These results indicate a positive role for cytokinin in nodule formation and an inhibitory effect on lateral root development in *L. japonicus* (Lohar et al. 2004).

Effect of NIN on *Enod40* expression.

To study the effect of nodulin genes on the *Enod40* promoter, we examined hairy roots on the stable transgenic pr*GmEnod40* reporter lines normally exhibiting only symbiotic promoter activity, in which ectopic expression of either *Enod40* genes or *LjNin* was driven by the CaMV35S double-enhancer promoter (Quaedvlieg et al. 1998).

Overexpression of the three different *Enod40* genes (*LjEnod40-1*, *LjEnod40-2,* and *GmEnod40*) did not result in any disturbance of the *GmEnod40* promoter activity compared with the controls (Table 5), indicating a lack of auto-regulation of *Enod40* expression. In contrast, overexpression of *LjNin* in such hairy roots altered the *Enod40* promoter activity by inducing or enhancing a nonsymbiotic root vascular expression, which was not found (at least not to the same extent) in control plants (Table 5; Fig. 4). The ability of ectopic *LjNin* expression to alter the *GmEnod40* promoter activity seem to suggest that NIN is positively regulating *Enod40*, possibly from an upstream position in the nodulation signal transduction pathways or in developmental processes. The pattern of the effect of NIN in the hairy roots suggests that additional NIN interacting partners are needed for NIN to act on the *Enod40* promoter and that such interaction partners are present in the root vascular tissue, because ectopic activity of the *GmEnod40* promoter is observed only in a subpart of the tissue where the CaMV35S promoter is active in *L. japonicus* (Quaedvlieg et al. 1998). Whereas the *LjNin* overexpression induced or enhanced nonsymbiotic promoter activity, it did not seem to affect the symbiotic *GmEnod40* promoter activity, possibly because NIN expression is not the limiting factor in tissue where the endogenous *LjNin* gene is already activated. The effects of overexpressing *LjNin* in hairy roots of the stable transgenic lines shows the usefulness of our promoter-reporter gene lines for analysis of the interplay between different nodulin genes. Therefore, future research can be directed at combinations of other genes and crosses with mutant plant lines.

MATERIALS AND METHODS

Plasmids and bacterial strains.

Constructs of the *GmEnod40-2* and *LjEnod40-1* promoters were fused to those of the bifunctional *Gus*Aint/*Gfp* reporter gene (Quaedvlieg et al. 1998), resulting in pMP3637 and pMP2875 (Fig. 1A and B). In all, 20 independent transgenic lines (T1) containing pr*GmEnod40-Gus*Aint/*Gfp* (Fig. 1A) were regenerated and analyzed by Southern hybridization using probes spanning the *NptII* and *Gus*Aint genes, respectively. We obtained single, double, and multiple T-DNA integration events in 13, 6, and 1 cases, respectively, and 15 of the independent transgenic lines were analyzed for GUS staining (Table 1). In 3 of the 6 double-integration lines, the T-DNAs were inserted right border to right border. In two of the latter lines as well as in one of the single integrations lines, GUS expression was not observed in any offspring of the T1 generation. All other lines did show at least symbiotic response in offspring of the T1 generation (Table 1).

Plasmid pbi/40 *GusA:intron* (Roussis et al. 1995) containing, the *Enod40-2* promoter of *G. max* (pr*Gmenod40*) fused to the *GusA*:intron reporter gene (*Gus*Aint) and the nopaline synthase (*nos*) terminator sequence (T*nos*), was provided by H. Franssen (Agricultural University, Wageningen, The Netherlands). A 3.9-kb *Eco*RI fragment from pbi/40 with pr*GmEnod40-Gus*Aint-T*nos* was cloned into the binary vector pPZP111 (Hajdukiewicz et al. 1994), resulting in pMP3636. The *Gus*Aint gene in pMP3636 was substituted for the bifunctional *Gus*Aint/*Gfp* reporter gene (Quaedvlieg et al. 1998) by swapping a *Sna*BI-*Kpn*I fragment from similarly digested pMP3632 (Pacios-Bras et al. 2003) into similarly digested pMP3636, resulting in pMP3637, pr*GmEnod40-Gus*Aint/*Gfp* (Fig. 1A), used in stable and transient transformation of *L. japonicus*.

A genomic clone containing a 2.3-kb *Eco*RI fragment including promoter and coding region of the *LjEnod40-1* gene (Flemetakis et al. 2000) was subcloned into pMP4606, a derivative of pBluescript II KS containing an *Nco*I site, and used as a template for amplification of a 1.7-kb *LjEnod40-1* promoter fragment (pr*LjEnod40-1*) using a T7 primer and an LjEnod40-P1 primer (5′-GAGCTCCATGGTTACCAGATTC-3′). The PCR product was digested and inserted in the *Nco*I, *Eco*RI sites of pBluescript SK yielding pMP2843. A *Nco*I-*Sac*II fragment from pMP2845 (Quaedvlieg et al. 1998) containing the bifunctional *Gfp/Gus*Aint reporter gene was transferred to pMP2843, thereby fusing the *LjEnod40-1* promoter

to the *Gfp/Gus*Aint gene, resulting in the plasmid p3.2L. Finally, a *Hin*dIII, *Acc*I fragment of p3.2L was transferred to *Hin*dIII, *Acc*I of the binary vector pMP2173 (Quaedvlieg et al. 1998), resulting in the binary vector pMP2875, pr*LjEnod40-1-Gfp/Gus*Aint (Fig. 1B), used in stable transformation of *L. japonicus*.

For the hairy root transformations of *L. japonicus*, an *Eco*RI fragment of p3.2L containing pr*LjEnod40-1-Gfp/Gus*Aint-Tnos was transferred to pPZP111, resulting in pMP7336, containing the same T-DNA as pMP2875 (Fig. 1B).

For the *LjEnod40-2* promoter, a genomic clone containing a 3.3-kb *Kpn*I-*Hin*dIII fragment, including promoter and coding region of the *LjEnod40-2* gene (Flemetakis et al. 2000), was subcloned into pMP4606 and used as a template for amplification of a 2.5-kb *LjEnod40-2* promoter fragment (pr*LjEnod40-2*) using an M13 Rev primer and an LjEnod40-P2 primer (5′-AATCCCATGGCTAACAGATTC-3′). The PCR product was digested and inserted into the *Nco*I, *Kpn*I sites of pBluescript II SK, yielding pMP2841. An *Nco*I-*Sac*II fragment from pMP2845 (Quaedvlieg et al. 1998) containing the bifunctional *Gfp/Gus*Aint reporter gene was transferred to pMP2841, thereby fusing the *LjEnod40-2* promoter to the bifunctional *Gfp/Gus*Aint reporter gene, resulting in the plasmid p6.1L.

For the hairy root transformations of *L. japonicus*, a *Kpn*I fragment of p6.1L containing the *LjEnod40-2* promoter fused to *Gfp/GusAint-Tnos* was transferred to pPZP111, resulting in pMP7335, pr*LjEnod40-2-Gfp/Gus*Aint (Fig. 1C).

A *Kpn*I fragment of p6.1L containing the *LjEnod40-2* promoter with the reporter gene was transferred to *Kpn*I of the vector pIC20H (Kieny et al. 1983), giving pMP2873. For the construction of the pr*LjEnod40-2*REV, the *LjEnod40-2* promoter was cut out from the pMP2873 plasmid with *Nco*I, *Cla*I, the vector and fragment were filled in with Klenow enzyme and religated to yield plasmids with a re-inserted *LjEnod40-2* promoter. One of the clones with the promoter in the reverse orientation with respect to the reporter genes was selected and a *Bam*HI, *Sal*I fragment was transferred to pPZP111 and digested with *Bam*HI and *Xho*I, resulting in pMP7340, pr*LjEnod40-2*REV-*Gfp/Gus*Aint-Tnos (Fig. 1D)

The *G. max Enod40-2* promoter deletions were made by PCR using as a template a 2.2-kb *Eco*RI genomic fragment (Sg 22, provided by H. Franssen) which was part of the accession X86442 (Roussis et al. 1995). To generate the appropriate fragments, the common 3′-end primer PR_{1751} (5′-GTTGTCCA TGGAGCTCTCTAC-3′) was combined with each of the 5′-end primers PR_{857} (5′-CAAACAGAGAATTCAAGCATG-3′), PR_{1127} (5′-CCTTTGTATTTGAATTCGATTAAGATA-3′), PR_{1377} (5′-GGGATCTCAAAACTGAATTCC-3′), and PR_{1566} (5′-GA GGCGAGTAGAATTCTCTTT-3′), resulting in each case in the respective truncated promoter region. The above primer sets introduced an *Eco*RI restriction site in the 5′ end and a *Nco*I in the 3′ end of the PCR products, which subsequently were cloned into pUC21 utilizing the compatible sites of the polylinker. According to the order that the primers are mentioned above, the following plasmids were generated in this way: pMP3995, pMP3994, pMP3993, and pMP3992 (Fig. 1E). The promoter deletions were subcloned from these plasmids as *Eco*RI*Nco*I fragments into pCAMBIA1301, thus resulting in plasmids pMP3983, pMP3985, pMP3982, and pMP3984, respectively. Finally, a 1.7-kb promoter fragment was generated in a similar manner with primers PR_{1751} and omp49 (standard M13 reverse); following digestion with *Eco*RI/*Nco*I, it was directly cloned into pCambia 1301, resulting in plasmid pMP3954.

The nodulin overexpression constructs (Fig. 1F) were made by cloning a CaMV35S promoter, double enhancer, and *nos* terminator (T*nos*), with *Sac*I, *Hin*dIII from the plasmid pMOG183 (Mogen International, Leiden, The Netherlands) into pPZP11. This resulted in the plasmid pMP7352, used as control in the hairy root transformations. The prCaMV35S-*Ljnin* construct, pMP7341, was obtained by cloning the full-length *Ljnin* cDNA (Schauser et al. 1999) from the plasmid LS7 into pMP7352 with *Bam*HI. To obtain the *Enod40* overexpression constructs, the pMOG183 and pPZP111 plasmids first were modified as follows. A polylinker, including *Bam*HI, *Not*I, *Eco*RI, and *Sal*I, was inserted in the *Bam*HI site of pMOG183 (inserted between the CaMV35S promoter and Tnos, destroying the *Bam*HI site at the 3′end of the polylinker). The pPZP111 plasmid was modified to destroy the *Eco*RI site at the left border; this was done by opening the plasmid with *Eco*RI, filling in with Klenow, and religating. The CaMV35S promoter-polylinker-Tnos was transferred to the modified pPZP111 plasmid with *Sac*I, *Hin*dIII, yielding pMP7344. The *GmEnod40* full-length cDNA was cloned into pMP7344 with *Bam*HI, *Sal*I from the plasmid gm40-2 (provided by H. Franssen), resulting in pMP7349, prCaMV35S-*Gmenod40*.

The two *L. japonicus Enod40* cDNAs were amplified by PCR using genomic clones (Flemetakis et al. 2000) as templates. The *LjEnod40-1* cDNA was amplified using the primers Ljenod40-1c5 (5′-GGAATCTCCTCTGAACCAATCC-3′) and Ljenod40-1c3 (5′-GGGACAGGAATGATAAGAGTC-3′). The *LjEnod40-2* cDNA was amplified using the primers Ljenod40-2c5 (5′-GCTTCCCAGAGAGCCATTTGG-3′) and Ljenod40-2c3 (5′-CAACCAATACACACATGAGAAAAGG-3′). The PCR products were cloned in the pCR-4-TOPO vector (Invitrogen) and transferred to pMP7344 as *Eco*RI fragments. The plasmids were checked by restriction digests to find the clones with a sense orientation of the cDNAs with respect to the CaMV35S promoter, resulting in the plasmids used for the overexpression experiments: pMP7346, prCaMV35S-*LjEnod40-1* and pMP7351, prCaMV35S-*LjEnod40-2*.

Binary plasmids were transferred to *A. tumefaciens* strain LBA4404 (Hoekema et al. 1984) or *A. rhizogenes* strain LBA1334 (Offringa et al. 1986) by electroporation (den Dulk-Ras and Hooykaas 1995) using selection on chloramphenicol (10 mg liter^{-1}) and kanamycin (50 mg liter^{-1}), respectively.

A. tumefaciens-mediated plant transformation.

Hypocotyl explants of *L. japonicus* (accession number B-129, "Gifu") were transformed using *A. tumefaciens* LBA4404 according to described procedures (Handberg and Stougaard 1992; Thykjaer et al. 1995) with minor modifications as described by Quaedvlieg and associates (1998).

A. rhizogenes-mediated plant transformation.

Hairy roots transformation was performed according to Díaz and associates (In press). In short, *L. japonicus* seed (wild type or transgenic) were germinated for 2 to 3 days at 28°C on solidified Jensen medium (Vincent 1970), upside down. Seedlings with roots of approximately 1 cm in length were mounted on transformation plates (Jensen medium containing 1.5 mM NO_3^-) and left to grow for 2 to 5 days at 20°C with 16 h of light and 8 h of darkness. Two days before transformation, the *A. rhizogenes* strains were streaked on selective plates. For the infection, *A. rhizogenes* (taken directly from the selective plates) was streaked on the hypocotyl approximately 2 to 3 mm below the point of insertion of the cotyledons. Hypocotyls were sectioned with a scalpel in the middle of the zone streaked with *Agrobacterium* spp. and the roots were removed. Plant and bacteria were cocultivated on the transformation plates for five days before transfer to hairy root emergence medium (HREM) (Schenk and Hildebrandt 1971) containing cefotaxime at 300 µg/ml. Plants containing hairy roots were either assayed for *Gus* expression or transferred to clay pots after 10 to 14 days of growth on HREM and analyzed two to

four weeks later. For transfer to clay pots, the bottom part of tissue culture boxes (RA60; ECOLINE BVBA, Zottegem, Belgium) were filled with clay pellets (2 to 4 mm in diameter; Hydro Jongkind BV, Alsmeer, Holland) and wetted with either Jensen medium for uninoculated plants or with 10× diluted *M. loti* R7A (grown for 2 days in YMB at 28°C) (Hooykaas et al. 1977) in Jensen medium for inoculated plants. Plants were transferred to the wet pots and watered with the same solutions, respectively, until liquid appeared in the bottom of the pot (total of 50 ml in the specified plant containers).

Histochemical analysis of transgenic tissue.

Promoter activity or *Gus* expression was determined histochemically by immersion of plant material in a solution containing 5-bromo-4-chloro-3-indoyl β-D-glucuronide (X-gluc) solution (BioSynth AG) at 1 mg/ml in 50 mM sodium phosphate buffer, pH 7.2, 0.1% Triton X-100, 10 mM EDTA, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$ at 37°C for 16 h; subsequently, the plant material was cleared in 70% EtOH prior to photography. GUS staining was examined with a Leica MZ12 stereomicroscope. Images were recorded with a Sony DKC5000 digital camera and processed with Adobe Photoshop 5 software.

Embedding of histochemically stained plant tissue.

GUS-stained tissues (nodules and roots) were separated from the rest of the plant and fixed in 10 mM sodium phosphate buffer, pH 7.2, containing 4% paraformaldehyde and 0.25% glutaraldehyde at 4°C overnight, slowly rotating. Serial ethanol dehydration was performed at room temperature at 70, 90, and 96% (twice) for 1 h at each step. Samples were embedded in Technovit 7100 resin as described in the protocol from Kulzer; then, 6-µm sections were made with a Leica microtome, dried onto glass slides at 60°C, mounted in Eukit (Kindler GmbH & Co., Freiburg, Germany), and examined in a Zeiss axioplan microscope prior to photography and image processing as described above.

In situ RNA localization studies.

L. japonicus (young) nodules were harvested 14 dpi with *M. loti* R7A and fixed in 10 mM sodium phosphate buffer, pH 7.2, containing 4% paraformaldehyde and 0.25% glutaraldehyde at 4°C overnight, slowly rotating. The fixed tissue was dehydrated through an ethanol series, embedded in paraffin sectioned with a Leica RM 2165 rotary microtome in 7-µm sections, and dried onto glass slides (Super Frost R plus, Menzel Glaser), as described by Yang and associates (1993). Antisense and sense RNA probes were transcribed from pTOPO-*ljEnod40-1* clones in opposite directions with the T7 RNA polymerase and labeled with digoxigenin (DIG)-11-rUTP (Roche), using the T7 transcription kit from Ambion, according to the manufacturer's protocol. The RNA probes were hybridized to the nodule sections and signals detected with anti-DIG antibodies conjugated with alkaline phosphatase (Kouchi and Hata 1993). Hybridization was performed at 42°C for 16 h before washing and signal detection, which was allowed to develop overnight, before the slides were rinsed, dehydrated, and mounted in Eukit, examined, photographed, and processed as described above.

Plant growth and treatment.

The bioassays were performed in Fahraeus slides (Fahraeus 1957), containing three plantlets per slide. *L. japonicus* seeds (wild type or transgenic) were germinated for 2 to 3 days at 28°C on solidified Jensen medium (Vincent 1970), upside down. Seedlings with roots of approximately 1 cm in length were introduced in a space of approximately 1 mm, and defined by a cover slip glued onto a microscope slide using four drops of silicon glue (Bhuvaneswari and Solheim 1985). The glass slide assembly was placed vertically in a glass slide holder containing Jensen medium. Plantlets were left to grow for 3 days at 21°C with 16 h of light. The lower part of the slide holder was covered in foil to shield the roots from light. The initial medium was replaced with Jensen medium containing the ethylene inhibitor L-α-(2-aminoethoxyvinyl) glycine (AVG) (Sigma, Zwijndrecht, The Netherlands) at 0.1 mg/liter and the different specified compounds (Table 3). BAP (Sigma), IAA, (Sigma), Mastoporan (Sigma), *N*-acetyl-glucosamine (GlcNAc) (Sigma), chitotetraose, and chitopentaose (COVI/V) (Seikagaku, Rockville, MD, U.S.A.) were applied from 1 mM stocks.

Fucosylation assay and Nod factor purification.

Chitotetraose and chitopentaose were fucosylated in vitro by purified NodZ protein, essentially as described by Quinto and associates (1997) in the following reaction mix: 0.3 mM $MgCl_2$, $MnCl_2$, $CaCl_2$, 1 mM ATP, 10 mM sodium phosphate buffer, pH 7.0, 0.05 mM chitin, 0.5 mM GDP-β-L-fucose (Sigma), and purified NodZ protein. NodZ protein was purified, resuspended, and used as described by Quinto and associates (1997).

Reaction products obtained from in vitro fucosylation assays were purified by high-pressure liquid chromatography using an isocratic elution of 75% acetonitrile in water on a nucleosil 120-7 NH_2 column (Macherey-Nagel, Düren, Germany). Non-fucosylated chitin compounds were run for comparison. The fucosylated, purified products, 10^{-4} M, were shown by ion trap mass spectrometry analysis (Pacios-Bras et al. 2002) to contain a fucosyl group.

Isolation and purification of Nod factors from *M. loti* strain R7A and *R. leguminosarum* strain RBL5560 were performed as described by López-Lara and associates (1995a and b, respectively). The purified Nod factor peaks were freeze dried and resuspended in 60% acetonitrile in water to 10^{-4} M stocks and applied as such to the Fahraeus slides.

O-acetylated chitin was produced in vitro by treatment of chitin oligosaccharides with the NodL protein using the methods described by Bloemberg and associates (1995).

ACKNOWLEDGMENTS

This work was funded by EU contract HPRN-CT-2000-00086 (Lotus) and by the Danish Agricultural and Veterinary Research Council grant no: 23-02-0027. We thank H. Franssen (Wageningen University) for providing the clone with the *Gmenod40*-2 promoter, E. Schrijnemaker (Leiden University) and F. Pedersen (Aarhus University) for plant culturing, and Y. van der Burgt and A. M. Deelder for assistance with mass spectrometry.

LITERATURE CITED

Bauer, P., Ratet, P., Crespi, M., Schultze, M., and Kondorosi. A. 1996. Nod factors and cytokinins induce similar cortical cell division, amyloplast deposition and MsEnod12A expression patterns in alfalfa roots. Plant J. 10:91-105.

Berck, S., Perret, X., Quesada-Vincens, D., Prome, J., Broughton, W. J., and Jabbouri, S. 1999. NolL of Rhizobium sp. strain NGR234 is required for O-acetyltransferase activity. J. Bacteriol. 181:957-964.

Bhuvaneswari, T. V., and Solheim, B. 1985. Root hair deformation in white clover *R. trifolii* symbiosis. Physiol. Plant Pathol. 63:25-34.

Bladergroen, M. R., and Spaink, H. P. 1998. Genes and signal molecules involved in the rhizobia-leguminoseae symbiosis. Curr. Opin. Plant Biol. 1:353-359.

Bloemberg, G. V., Lagas, R. M., van Leeuwen, S., Van der Marel, G. A., Van Boom, J. H., Lugtenberg, B. J., and Spaink, H. P. 1995. Substrate specificity and kinetic studies of nodulation protein NodL of *Rhizobium leguminosarum.* Biochemistry 34:12712-12720.

Campalans, A., Kondorosi, A., and Crespi, M. 2004. Enod40, a short open reading frame-containing mRNA, induces cytoplasmic localization of a nuclear RNA binding protein in *Medicago truncatula.* Plant Cell 16:1047-1059.

Compaan, B., Ruttink, T., Albrecht, C., Meeley, R., Bisseling, T., and Franssen, H. 2003. Identification and characterization of a *Zea mays* line carrying a transposon-tagged ENOD40. Biochim. Biophys. Acta 1629:84-91.

Denarie, J., Debelle, F., and Prome, J. C. 1996. Rhizobium lipo-chitooligosaccharide nodulation factors: signaling molecules mediating recognition and morphogenesis. Annu. Rev. Biochem. 65:503-535.

den Dulk-Ras, A., and Hooykaas, P. J. 1995. Electroporation of *Agrobacterium tumefaciens.* Methods Mol. Biol. 55:63-72.

D'Haeze, W., and Holsters, M. 2002. Nod factor structures, responses, and perception during initiation of nodule development. Glycobiology 12:79R-105R.

Díaz, C. L., Grønlund, M., Schlaman, H. R. M., and Spaink, H. P. Induction of hairy roots in *Lotus japonicus* for symbiotic gene expression studies. In: *Lotus japonicus* Handbook. A. J. Marquez, ed. Kluwer Academic Publishers, Dordrecht, The Netherlands. In press.

Engstrom, E. M., Ehrhardt, D. W., Mitra, R. M., and Long, S. R. 2002. Pharmacological analysis of nod factor-induced calcium spiking in *Medicago truncatula.* Evidence for the requirement of type IIA calcium pumps and phosphoinositide signaling. Plant Physiol. 128:1390-1401.

Fahraeus, G. 1957. The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J. Gen. Microbiol. 16:374-381.

Fang, Y., and Hirsch, A. M. 1998. Studying early nodulin gene ENOD40 expression and induction by nodulation factor and cytokinin in transgenic alfalfa. Plant Physiol. 116:53-68.

Flemetakis, E., Kavroulakis, N., Quaedvlieg, N. E. M., Spaink, H. P., Dimou, M., Roussis, A., and Katinakis, P. 2000. *Lotus japonicus* contains two distinct ENOD40 genes that are expressed in symbiotic, nonsymbiotic, and embryonic tissues. Mol. Plant-Microbe Interact. 13:987-994.

Girard, G., Roussis, A., Gultyaev, A. P., Pleij, C. W., and Spaink, H. P. 2003. Structural motifs in the RNA encoded by the early nodulation gene enod40 of soybean. Nucleic Acids Res. 31:5003-5015.

Handberg, K., and Stougaard, J. 1992. Lotus japonicus, an autogamous, diploid legume species for classical and molecular genetics. Plant J. 2:487-496.

Hajdukiewicz, P., Svab, Z.. and Maliga, P. 1994. The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol. Biol. 25:989-994.

Hirsch, A. M.. and Fang, Y. 1994. Plant hormones and nodulation: what's the connection? Plant Mol. Biol. 26:5-9.

Hoekema, A., Hooykaas, P. J.. and Schilperoort, R. A. 1984. Transfer of the octopine T-DNA segment to plant cells mediated by different types of *Agrobacterium* tumor- or root-inducing plasmids: generality of virulence systems. J. Bacteriol. 158:383-385.

Hooykaas, P. J., Klapwijik, P. M., Nuti, M. P., Schilperoort, R. A., and Rorsch, A. 1977. Transfer of the *Agrobacterium tumefaciens* Ti plasmid to avirulent agrobacteria and to rhizobia ex planta. J. Gen. Microbiol. 98:477-484.

Journet, E. P., Pichon, M., Dedieu, A., Debilly, B. F., Truchet, G., and Barker, D. G. 1994. *Rhizobium meliloti* Nod factors elicit cell-specific transcription of the ENOD12 gene in transgenic alfalfa. Plant J. 6:241-249.

Kawaguchi, M., Imaizumi-Anraku, H., Fukai, S., and Syono, K. 1996. Unusual branching in the seedlings of *Lotus japonicus*—gibberelins reveal the nitrogen-sensitive cell divisions within the pericycle on roots. Plant Cell Physiol. 37:461-470.

Kieny, M. P., Lathe, R., and Lecocq, J. P. 1983. New versatile cloning and sequencing vectors based on bacteriophage M13. Gene 26:91-99.

Kouchi, H., and Hata, S. 1993. Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. Mol. Gen. Genet. 238:106-119.

Kouchi, H., Takane, K., So, R. B., Ladha, J. K., and Reddy, P. M. 1999. Rice ENOD40: isolation and expression analysis in rice and transgenic soybean root nodules. Plant J. 18:121-129.

Larsen, K. 2003. Molecular cloning and characterization of a cDNA encoding a ryegrass (*Lolium perenne*) ENOD40 homologue. J. Plant Physiol. 160:675-687.

Lohar, D. P., Schaff, J. E., Laskey, J. G., Kieber, J. J., Bilyeu, K. D., and Bird, D. M. 2004. Cytokinins play opposite roles in lateral root formation, and nematode and Rhizobial symbioses. Plant J. 38:203-214.

López-Lara, I. M., van den Berg, J. D., Thomas-Oates, J. E., Glushka, J. Lugtenberg, B. J., and Spaink, H. P. 1995a. Structural identification of the lipo-chitin oligosaccharide nodulation signals of *Rhizobium loti.* Mol. Microbiol. 15:627-638.

López-Lara, I. M., van der Drift, K. M., van Brussel, A. A. N., Haverkamp, J., Lugtenberg, B. J., Thomas-Oates, J. E., and Spaink, H. P. 1995b. Induction of nodule primordia on *Phaseolus* and *Acacia* by lipo-chitin oligosaccharide nodulation signals from broad-host-range *Rhizobium* strain GRH2. Plant Mol. Biol. 29:465-477.

Madsen, E. B., Madsen, L. H., Radutoiu, S., Olbryt, M., Rakwalska, M., Szczyglowski, K., Sato, S., Kaneko, T., Tabata, S., Sandal, N., and Stougaard, J. 2003. A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. Nature 425:637-640.

Martirani, L., Stiller, J., Mirabella, R., Alfano, F., Lamberti, A., Radutoiu, S. E., Iaccarino, M., Gresshoff, P. M., and Chiurazzi, M. 1999. T-DNA tagging of nodulation- and root-related genes in *Lotus japonicus*: expression patterns and potential for promoter trapping and insertional mutagenesis. Mol. Plant-Microbe Interact. 12(4):275-284.

Mathesius, U., Charon, C., Rolfe, B. G., Kondorosi, A., and Crespi, M. 2000. Temporal and spatial order of events during the induction of cortical cell divisions in white clover by *Rhizobium leguminosarum* bv. *trifolii* inoculation or localized cytokinin addition. Mol. Plant-Microbe Interact. 13:617-628.

Matvienko, M., van de Sande, K., Pawlowski, K., Van Kammen, A., and Bisseling, T. 1996. *Nicotiana tabacum* SR1 contains two ENOD40 homologs. Pages 387-391 in: Biology of Plant-Microbe Interactions, Vol. 1. G. Stacey, B. Mullen, and P. M. Gresshoff, eds. International Society for Molecular Plant-Microbe Interactions. St. Paul, MN, U.S.A.

Minami, E., Kouchi, H., Cohn, J. R., Ogawa, T., and Stacey, G. 1996. Expression of the early nodulin, ENOD40, in soybean roots in response to various lipo-chitin signal molecules. Plant J. 10:23-32.

Mylona, P., Pawlowski, K., and Bisseling, T. 1995. Symbiotic nitrogen fixation. Plant Cell 7:869-885.

Offringa, I. A., Melchers, L. S., Regensbrug-Tuink, A. J. G., Constantino, P., Schilperoort, R. A., and Hooykaas, P. J. 1986. Complementation of *Agrobacterium tumefaciens* tumor-inducing aux mutants by genes from the TR-region of the Ri plasmid of *Agrobacterium rhizogenes.* Proc. Natl. Acad. Sci. U.S.A. 83:6935-6939.

Oldroyd, G. E., and Downie, J. A. 2004. Calcium, kinases and nodulation signaling in legumes. Nature Rev. Mol. Cell Biol. 5:566-576.

Pacios-Bras C., Jorda, M. A., Wijfjes, A. H., Harteveld, M., Stuurman, N., Thomas-Oates, J. E., and Spaink, H. P. 2000. *A Lotus japonicus* nodulation system based on heterologous expression of the fucosyl transferase NodZ and the acetyl transferase NoIL in *Rhizobium leguminosarum.* Mol. Plant-Microbe Interact. 13:475-479.

Pacios-Bras, C., van der Burgt, Y. E., Deelder, A. M., Vinuesa, P., Werner, D., and Spaink, H. P. 2002. Novel lipochitin oligosaccharide structures produced by *Rhizobium etli* KIM5s. Carbohydr. Res. 337:1193-1202.

Pacios-Bras, C., Schlaman, H. R. M., Boot, K., Admiraal, P., Langerak, J. M., Stougaard, J., and Spaink, H. P. 2003. Auxin distribution in *Lotus japonicus* during root nodule development. Plant Mol. Biol. 52:1169-1180.

Perret, X., Staehelin, C., and J. Broughton, W. 2000. Molecular basis of symbiotic promiscuity. Microbiol. Mol. Biol. Rev. 64:180-201.

Pingret, J. L., Journet, E. P., and Barker, D. G. 1998. Rhizobium nod factor signaling. Evidence for a g protein-mediated transduction mechanism. Plant Cell 10:659-672.

Quaedvlieg, N. E. M., Schlaman, H. R. M., Admiraal, P. C., Wijting, S. E., Stougaard, J., and Spaink, H. P. 1998. Fusions between green fluorescent protein and beta-glucuronidase as sensitive and vital bifunctional reporters in plants. Plant Mol. Biol. 38:861-873.

Quinto, C., Wijfjes, A. H., Bloemberg, G. V., Blok-Tip, L., López-Lara, I. M., Lugtenberg, B. J., Thomas-Oates, J. E., and Spaink, H. P. 1997. Bacterial nodulation protein NodZ is a chitin oligosaccharide fucosyltransferase which can also recognize related substrates of animal origin. Proc. Natl. Acad. Sci. U.S.A. 94:4336-4341.

Radutoiu, S., Madsen, L. H., Madsen, E. B., Felle, H. H., Umehara, Y., Grønlund, M., Sato, S., Nakamura, Y., Tabata, S., Sandal, N., and Stougaard, J. 2003. Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. Nature 425:585-592.

Roche, P., Lerouge, P., Ponthus, C., and Prome, J. C. 1991. Structural determination of bacterial nodulation factors involved in the *Rhizobium meliloti*–alfalfa symbiosis. J. Biol. Chem. 266:10933-10940.

Röhrig, H., Schmidt, J., Miklashevichs, E., Schell, J., and John, M. 2002. Soybean ENOD40 encodes two peptides that bind to sucrose synthase. Proc. Natl. Acad. Sci. U.S.A. 99:1915-1920.

Roussis, A., van de Sande, K., Papadopoulou, K., Drenth, J., Bisseling, T., Franssen, H., and Katinakis, P. 1995. Characterization of the soybean gene GmENOD40-2. J. Exp. Bot. 46:719-724.

Santi, C., von Groll, U., Ribeiro, A., Chiurazzi, M., Auguy, F., Bogusz, D., Franche, C., and Pawlowski, K. 2003. Comparison of nodule induction in legume and actinorhizal symbioses: the induction of actinorhizal nodules does not involve ENOD40. Mol. Plant-Microbe Interact. 16(9):808-16.

Schauser, L., Roussis, A, Stiller, J., and Stougaard, J. 1999. A plant regulator controlling development of symbiotic root nodules. Nature 402:191-195.

Schenk, R. U., and Hildebrandt A. C. 1971. Medium and techniques for induction and growth of monocotyledonous and dycotiledonous plant cell cultures. Can. J. Bot. 50:199-204.

Schlaman, H. R. M., Gisel, A. A., Quaedvlieg, N. E., Bloemberg, G. V., Lugtenberg, Kijne, J. W., Potrykus, I., Spaink, H. P., and Sautter, C. 1997. Chitin oligosaccharides can induce cortical cell division in roots of *Vicia sativa* when delivered by ballistic microtargeting. Development 124:4887-4895.

Schultze, M., and Kondorosi, A. 1998. Regulation of symbiotic root nodule development. Annu. Rev. Genet. 32:33-57.

Scott, D. B., Young, C. A., Collins-Emerson, J. M., Terzaghi, E. A., Rockman, E. S., Lewis, P. E., and Pankhurst, C. E. 1996. Novel and complex chromosomal arrangement of *Rhizobium loti* nodulation genes. Mol. Plant-Microbe Interact. 9:187-197.

Spaink, H. P. 1998. Flavonoids as regulators of plant development: new insights from studies of plant–rhizobia interactions. Pages 167-177 in: Flavonoids as Regulators of Plant Development: New Insights from Studies of Plant–Rhizobia Interactionsmical Signals and Plant–Microbe Interactions. Plenum Press, New York.

Spaink, H. P. 2000. Root nodulation and infection factors produced by rhizobial bacteria. Annu. Rev. Microbiol. 54:257-288.

Spaink, H. P., Okker, R. J., Wijffelman, C. A., Tak, T., Goosen-deRoo, L., Pees, E., van Brussel, A. A. N., and Lugtenberg, B. J. J. 1989. Symbiotic properties of rhizobia containing a flavonoid-independent hybrid *nodD* product. J. Bacteriol. 171:4045-4053.

Spaink, H. P., Sheeley, D. M., van Brussel, A. A., Glushka, J., York, W. S., Tak, T., Geiger, O., Kennedy, E. P., Reinhold, V. N., and Lugtenberg, B. J. 1991. A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. Nature 354:125-130.

Stougaard, J. 2000. Regulators and regulation of legume root nodule development. Plant Physiol. 124:531-540.

Thykjaer, T., Stiller, J., Handberg, K., Jones, J., and Stougaard, J. 1995. The maize transposable element Ac is mobile in the legume Lotus japonicus. Plant Mol. Biol. 27:981-993.

Trinklein, N. D., Aldred, S. F., Hartman, S. J., Schroeder, D. I., Otillar, R. P., and Myers, R. M. 2004. An abundance of bidirectional promoters in the human genome. Genome Res. 14(1):62-6.

van Spronsen, P. C., Grønlund, M., Pacios-Bras. C., Spaink, H. P., and Kijne, J. W. 2001. Cell biological changes of outer cortical root cells in early determinate nodulation. Mol. Plant-Microbe Interact. 14:839-847.

Vincent, J. M. 1970. A Manual for the Practical Study of Root-Nodule Bacteria. Blackwell Scientific Publications, Oxford, U.K.

Vleghels, I., Hontelez, J., Ribeiro, A., Fransz, P., Bisseling, T., and Franssen, H. 2003. Expression of ENOD40 during tomato plant development. Planta 218:42-49.

Yang, W. C., Katinakis, P., Hendriks, P., Smolders, A., de Vries, F., Spee, J., Van Kammen, A., Bisseling, T., and Franssen, H. 1993. Characterization of GmENOD40, a gene showing novel patterns of cell-specific expression during soybean nodule development. Plant J. 3:573-585.