Plant Cell Physiol. 47(8): 1102–1111 (2006)
doi:10.1093/pcp/pcj081, available online at www.pcp.oxfordjournals.org

RNAi Knock-Down of *ENOD40*s Leads to Significant Suppression of Nodule Formation in *Lotus japonicus*

Hirotaka Kumagai [1], Eri Kinoshita [2], Robert W. Ridge [2] and Hiroshi Kouchi [1,*]

[1] *National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba, Ibaraki, 305-8602 Japan*
[2] *International Christian University, 3-10-2, Osawa, Mitaka, Tokyo, 181-8585 Japan*

***ENOD40* is one of the most intriguing early nodulin genes that is known to be induced very early in response to interaction of legume plants with symbiotic *Rhizobium* bacteria, but its function in the nodulation process is still not known. *Lotus japonicus* has two *ENOD40* genes: *LjENOD40-1* is abundantly induced in very early stages of bacterial infection or Nod factor application, whereas *LjENOD40-2* is abundantly expressed only in mature nodules. We generated transgenic lines of *L. japonicus* with an RNAi (RNA interference) construct that expresses hairpin double-stranded RNA for *LjENOD40-1* to induce sequence-specific RNA silencing. In the transgenic plants, expression of both *LjENOD40-1* and *-2* was significantly reduced, and no accumulation of *ENOD40* transcripts was detected upon *Mesorhizobium loti* inoculation. The transgenic plants exhibited very poor nodulation (only 0–2 nodules per plant) and could not grow well without additional nitrogen supply. Analysis of segregation in the T_2 progeny indicated that the suppression of nodulation is perfectly linked with the presence of the transgene. Microscopic observation of the infection process using *lacZ*-labeled *M. loti*, together with expression analysis of infection-related nodulin genes, demonstrated that *ENOD40* knock-down did not inhibit the initiation of the bacterial infection process. In contrast, nodule primordium initiation and subsequent nodule development were significantly suppressed in the transgenic plants. These results clearly indicate that *ENOD40* is required for nodule initiation and subsequent organogenesis, but is not involved in early infection events.**

Keywords: Early nodulin — *Lotus japonicus* — Nodulation.

Abbreviations: CaMV, cauliflower mosaic virus; GUS, β-glucuronidase; ihpRNA, intron-containing hairpin RNA; ORF, open reading frame; RNAi, RNA interference; RT–PCR, reverse transcription–PCR; siRNA, short interfering RNA.

Introduction

Legume plants form root nodules in which symbiotic *Rhizobium* bacteria are able to fix atmospheric nitrogen. The legume nodulation process is accompanied by the activation of a unique set of nodule-specific genes termed 'nodulin genes' (Legocki and Verma 1980, van Kammen 1984). Early nodulin (*ENOD*) genes, that are expressed at very early stages of legume–*Rhizobium* interactions, have been thought to play crucial roles in the bacterial infection process and/or initiation of nodule organogenesis (Nap and Bisseling 1990). Among the *ENOD* genes, *ENDO40* is the most intriguing one, whose functions in the nodulation process are not yet resolved. *ENOD40* is expressed in the root pericycle a few hours after bacterial inoculation or application of purified Nod factors, and subsequently in dividing cortical cells in nodule primordia (Kouchi and Hata 1993, Yang et al. 1993, Minami et al. 1996). In the mature nodules, *ENOD40* expression is restricted to the pericycle surrounding the vascular bundles. Although the *ENOD40* nucleotide sequence is conserved well in many plant species, including legumes and various non-legumes, it lacks long open reading frames (ORFs), and only a small oligopeptide of 9–13 amino acids seems to be a possible translation product (Kouchi et al. 1999, Vleghels et al. 2003). Thus it has been proposed that *ENOD40* encodes a signaling peptide which controls growth and organ differentiation in plants (Vijn et al. 1995). However, there has been as yet no direct evidence of the presence of this small peptide in legume tissues. Alternatively, it has also been proposed that *ENOD40* functions as untranslated mRNA based on the tendency of its nucleotide sequence to form a very stable secondary structure (Crespi et al. 1994, Girard et al. 2003). Furthermore, recent evidence indicated that *ENOD40*s in some non-legumes do not contain the conserved small peptide ORF (Santi et al. 2003). It is, therefore, still not known whether *ENOD40* encodes a signaling peptide or acts as untranslated RNA.

On the basis of the spatial and temporal expression patterns during early stages of nodulation, *ENOD40* functions have been implicated in triggering cortical cell division leading to formation of nodule primordia (Mylona et al. 1995). This hypothesis was first evidenced by the fact that overexpression of *ENOD40* with cauliflower mosaic virus (CaMV) 35S promoter resulted in a significant increase in spontaneous cortical cell division in the roots of *Medicago truncatula* (Charon et al. 1997). The same group also reported that transgenic *M. truncatula* plants

* Corresponding author: E-mail, kouchih@nias.affrc.go.jp; Fax, +81-29-838-8347.

overexpressing *ENOD40* exhibited accelerated nodulation in response to *Sinorhizobium meliloti* inoculation, whereas their descendants with reduced *ENOD40* expression by co-suppression exhibited suppression of nodule formation (Charon et al. 1999). Although these previous results suggest that *ENOD40* plays a role in the initiation of nodule organogenesis, they are still not sufficiently convincing with regard to the involvement of *ENOD40* in the nodule initiation process because of the limited number of independent transgenic lines and relatively large fluctuations in the alteration of the nodulation phenotype in the transgenic plants. We believe, therefore, that it is essential to perform more extensive transgenic studies in order to elucidate the functions of *ENOD40* in the nodule formation process. Here we describe the nodulation performance of transgenic *Lotus japonicus* harboring the construct to generate double-stranded hairpin RNA for *ENOD40* that induces sequence-specific RNA interference (RNAi). We demonstrate that RNAi knock-down of *ENOD40*s leads to severe suppression of nodule primordium initiation but does not affect early bacterial infection events, thus clearly indicating that *ENOD40* is required for nodule primordium formation as well as for subsequent nodule organogenesis.

Results

Expression of two ENOD40s in Lotus japonicus

Lotus japonicus contains two distinct *ENOD40*s, *LjENOD40-1* and *-2*, the transcripts of which are both approximately 0.7 kb length (Flemetakis et al. 2000). Although their entire sequence homology is only 55%, they weakly cross-hybridize with each other even under high stringency conditions because of the presence of a number of nucleotide stretches that are highly conserved in both sequences. Therefore, we employed real-time reverse transcription–PCR (RT–PCR) with the primer sets specific to each sequence to detect their transcripts separately. Fig. 1 shows the temporal expression patterns of *ENOD40-1* and *-2*. *ENOD40-1* was induced significantly within 2 d of bacterial inoculation, whereas the expression of *ENOD40-2* was only slightly enhanced during the very early stage of nodulation. In nitrogen-fixing mature nodules (12 d after inoculation), both *ENOD40*s were abundantly expressed. These results correlate well with those previously described by Takeda et al. (2005). It has been well documented that legume *ENOD40* is induced very rapidly in response to purified Nod factors (Crespi et al. 1994, Minami et al. 1996, Flemetakis et al. 2000, Niwa et al. 2001). Thus, the temporal expression patterns of the two *Lotus ENOD40*s as shown in Fig. 1 suggest strongly that *LjENOD40-1*, but not *LjENOD40-2*, is predominant in responding to Nod factors at the early stages of nodule formation. Genomic Southern hybridization analysis with *ENOD40-1*- and *-2*-specific probes indicated that each is present as a single gene in the *L. japonicus* genome (data not shown).

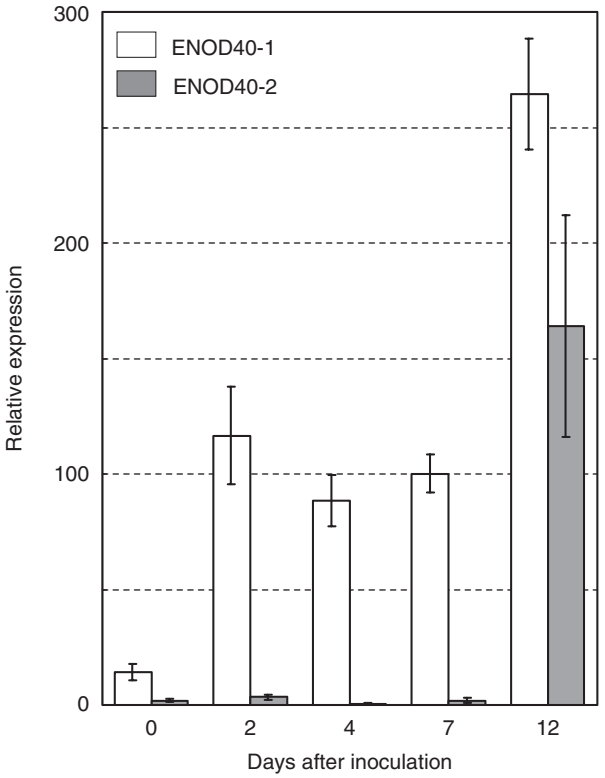

Fig. 1 Expression of *ENOD40-1* and *-2* during the early nodulation process. Seven-day-old *L. japonicus* seedlings were inoculated with *M. loti* MAFF303099. Root segments were harvested at the place where the nodulation was expected to occur, and nodulated root segments and nodules were harvested at 2 and 4 d, and 7 and 12 d after inoculation, respectively. Then the total RNA was prepared, reverse-transcribed and subjected to real-time PCR analysis. The data were normalized by adjusting the expression level of ubiquitin as a internal control to 1×10^4, and are represented as averages of triplicate RNA preparations.

Generation of ENOD40 RNAi transgenic plants

To make the *ENOD40* RNAi construct, we adopted a system to produce intron-containing self-complementary hairpin RNA (ihpRNA) that has been shown to induce highly efficient and effective RNA silencing (Wesley et al. 2001). The construct encoding ihpRNA for *ENOD40-1* under the control of the CaMV 35S promoter was transferred into *L. japonicus* B-129 'Gifu'. We obtained >20 independent transgenic plants, most of which exhibited more or less retarded nodulation. Five lines among them, namely D, F, H, N and R, and their T_2 descendants were chosen for further analyses. These transgenic lines were

sufficiently fertile, and the T_2 progeny stably inherited the transgene. Genomic Southern blot analysis of these transgenic lines indicated the presence of multiple transgene copies (Fig. 2).

Fig. 3 shows the estimation of the amounts of *ENOD40-1* and *-2* transcripts by real-time RT–PCR of total RNAs isolated from the transgenic lines of the T_2 progeny. Although the RNAi construct was designed for the *ENOD40-1* sequence, the levels of *ENOD40-1* and *-2* transcripts were both significantly reduced in the transgenic plants. This indicates that overexpression of ihpRNA for *ENOD40-1* by the CaMV 35S promoter also induced silencing of *ENOD40-2*, probably because there are several regions with high identity (>95% identical over a >30 nt stretch) between them, despite the fact that their overall homology is not very high. Basal expression levels of the two *ENOD40*s in uninfected roots of the transgenic lines ranged from 1 to 40% of those in the control roots, but more striking differences were detected after inoculation of *M. loti*. The transcript level of *ENOD40-1* showed a several-fold increase within 48 h of bacterial inoculation in the control plants, whereas no accumulation of *ENOD40-1* transcripts was detected in the RNAi transgenic lines (Fig. 3A). As a consequence, the levels of *ENOD40-1* transcripts ranged from 0.1 to 3% of that in the control roots. In the T_1 transgenic plants, *ENOD40-1* expression was relatively strongly suppressed compared with *ENOD40-2* (data not shown), but in their T_2 descendants, *ENOD40-2* appeared to be silenced much more strongly than *ENOD40-1* (Fig. 3A, B).

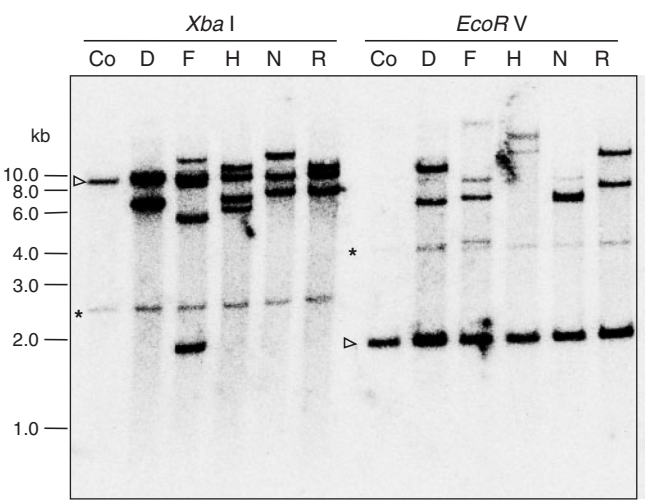

Fig. 2 Genomic Southern blot hybridization analyses of DNA isolated from wild-type 'Gifu' (Co) and RNAi transgenic lines of T_1 progeny (D, F, H, N and R) with a ^{32}P-labeled *ENOD40-1* cDNA probe. Open triangles indicate the bands from endogenous *ENOD40-1*. The faint bands marked with asterisks are from endogenous *ENOD40-2* that is weakly cross-hybridized with the *ENOD40-1* probe.

Accumulation of short interfering RNA (siRNA) is a hallmark of RNA silencing (Hamilton and Baulcombe 1999, Waterhouse et al. 2001). We performed Northern blot hybridization of low molecular weight RNA prepared from roots of T_1 transgenic plants with an in vitro transcribed ^{32}P-labeled *ENOD40-1* RNA probe, demonstrating the abundant accumulation of siRNA (~21–23 nt) in the transgenic lines (Fig. 4), thus showing ihpRNA-mediated *ENOD40* silencing.

Nodulation of ENOD40-suppressed plants

The phenotypic appearances of RNAi transgenic plants were examined in the T_1 progeny and their T_2 descendants. The transgenic plants exhibited very poor nodulation (only 0–2 nodules per plant vs. >15 nodules in control plants after 3 weeks of inoculation) and significantly

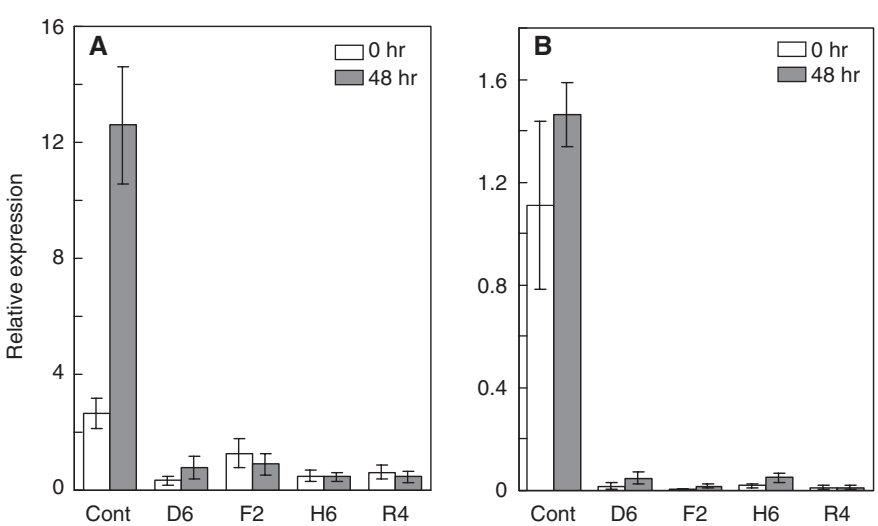

Fig. 3 Estimation of *ENOD40-1* (A) and *-2* (B) transcripts in the roots of the T_2 plants inoculated with *M. loti Tono* by real-time RT–PCR. The data are represented as averages from duplicate RNA preparations.

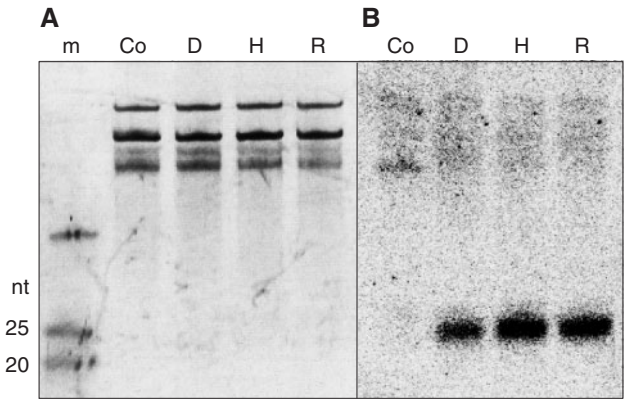

Fig. 4 Detection of short interfering (si) RNAs in T_1 transgenic lines (D, H and R). Low molecular weight RNAs were enriched, separated on a 15% denaturing polyacrylamide gel, blotted on a nylon membrane and hybridized with ^{32}P-labeled RNA probe for *ENOD40-1*. Gel images after staining with cyber-green (A) and an autoradiogram (B) are shown. DNA oligonucleotides were used as size markers (m). It should be noted that DNA oligonucleotides run about one base faster than oligo-RNA. The non-transgenic control 'Gifu' plants is designated Co.

retarded shoot and root growth when the plants were grown in nitrogen-free medium (Fig. 5A). The nodulation process of the T_2 plants during the first 16 d of *M. loti* inoculation is depicted in Fig. 5B. At the very early stages of nodulation (3 and 6 d after inoculation), initiation of nodule primordia was severely inhibited in the transgenic lines, resulting in subsequent poor nodule formation. Thus the significantly low nodule number in the transgenic plants is primarily due to the inhibition of nodule primordium initiation. However, the ratios of the number of nodules to that of nodule primordia formed on the transgenic plants were significantly lower than those in the control plants, suggesting that nodule development following the initiation of nodule primordia was also more or less inhibited in the RNAi transgenic plants. Indeed, we often found that a relatively high proportion of nodules formed on the transgenic plants remained as small bumps in some individual T_2 plants (data not shown). However, mature nodules that formed on the roots of transgenic plants appeared to be quite normal and retained nitrogen fixation activity, even though only one or two nodules were formed. We did not observe any apparent alterations in morphology of leaves, stems and roots of RNAi transgenic plants other than their nodulation phenotypes. Fig. 5C shows the effects of various concentrations of supplemented nitrogen in the culture medium on shoot growth. Growth of the transgenic plants recovered to levels comparable with those of wild-type controls when they were supplemented with >1.5 mM ammonium nitrate, indicating that reduced shoot growth of RNAi transgenic plants is primarily due to nitrogen deficiency from poor nodulation.

Since the transgenic plants contained multiple copies of the transgene (Fig. 2), most of the T_2 descendants showed no apparent segregation of low nodulation phenotype. However, some lines exhibited phenotypic segregation in the T_2 progeny. Among them, two T_2 lines, H1 and R3, were further analyzed to examine the correlation of their nodulation phenotypes with the presence of the transgene (Fig. 6). Twenty-two individuals of each line were analyzed for shoot length and nodule number 4 weeks after *M. loti* inoculation; the results show that suppression of nodulation is perfectly linked to the presence of the transgene.

Infection process in ENOD40-suppressed plants

To address the possible involvement of *ENOD40*s in the early infection process, we examined the formation of infection threads using *M. loti* that contains a constitutively expressing β-galactosidase (*lacZ*) gene. The results for T_2 representatives are shown in Fig. 7, and indicate that there is no significant difference in the number of infection threads between *ENOD40*-suppressed plants and control plants. Nevertheless, nodule formation was significantly suppressed in these transgenic lines compared with the control plants. The lack of involvement of *ENOD40* in the early infection events was further confirmed by assaying the expression of two infection-related early nodulin genes (Fig. 8). The *Nin* (nodule inception) gene has been shown to be required for infection thread formation as well as for the initiation of nodule primordia (Schauser et al. 1999). The *nin* mutants completely abolish the entry of symbiotic bacteria into root hair cells, and never form nodule primordia. *LjENOD16* is the homolog of *ENOD16* and/or *ENOD20* isolated from *M. truncatula*, which are predicted to function in cell wall reorganization during growth and/or differentiation of infection threads (Greene et al. 1998). Both of *Nin* and *LjENOD16* transcripts accumulated very rapidly in response to bacterial inoculation, and there was no reduction of their transcript levels in *ENOD40*-suppressed plants in comparison with the control plants. Rather, their expression appeared to be enhanced in the transgenic lines, though the data fluctuated considerably between each transgenic line. These results, together with microscopic observation of the formation of infection threads, indicate that *ENOD40* knock-down does not inhibit early infection events.

Discussion

RNA silencing, or post-transcriptional gene silencing (PTGS), is a powerful tool for the analysis of gene functions in plants. In this study, we show, for the first time, 'loss-of-function' analysis of legume *ENDO40* by means of RNA silencing mediated by transformation with ihpRNA.

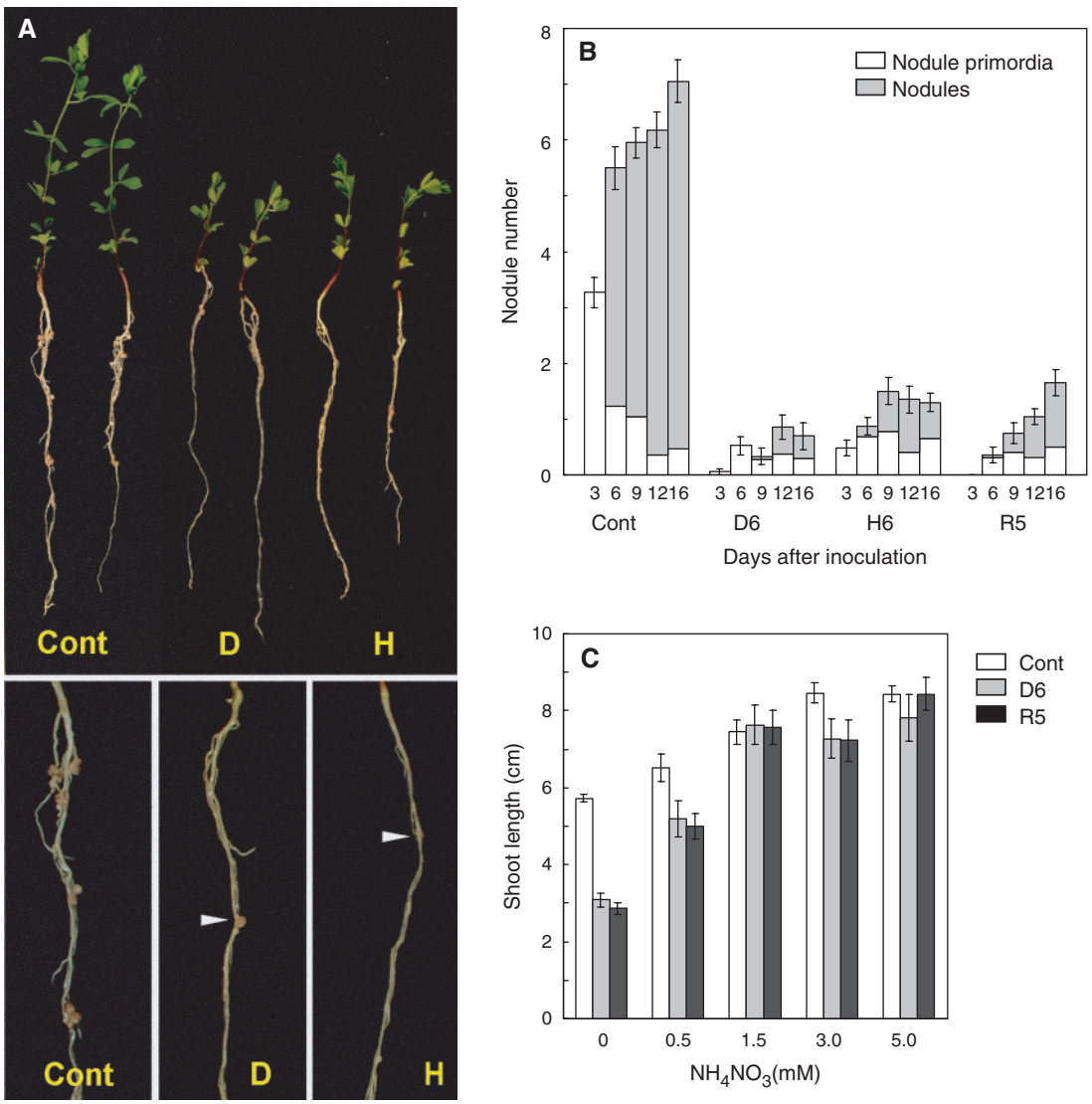

Fig. 5 Analysis of growth and nodulation of *ENOD40*-suppressed transgenic plants. (A) Two representatives of T_1 plants (lines D and H) compared with wild-type 'Gifu' plants (Cont). The plants were inoculated with *M. loti* MAFF303099 and grown under nitrogen-free conditions for 3 weeks. The RNAi transgenic plants exhibited very poor nodulation (only 0–2 nodules per plant, indicated by white arrowheads in the lower panel). (B) Nodulation profiles of representatives of the T_2 plants (D6, H6 and H5) compared with wild-type plants (Cont). Nodule primordia were counted as the dividing cell clusters in root cortex, which had not yet appeared on the root surface, whereas small bumps just emerged were counted as nodules. The data are represented as averages of 16–22 individual plants, and standard error bars are given for total numbers of nodules and nodule primordia. (C) Shoot growth of T_2 transgenic plants with supplemental nitrogen source. The plants were inoculated with *M. loti*, and then grown with or without supplying various concentrations of ammonium nitrate for 3 weeks. The data are given as averages of 16–22 individual plants.

Lotus japonicus, a model legume, contains two distinct *ENOD40*s, and we designed a transgenic construct encoding ihpRNA for *ENOD40-1*, which is postulated to function predominantly in the early stages of the nodule formation process (Fig. 1). Transformation of *Lotus* plants with this ihpRNA construct resulted in silencing of *ENOD40-1* as expected, but also induced significant reduction of *ENOD40-2* transcripts as well (Fig. 3). The region of *ENOD40-1* mRNA sequence used for the ihpRNA construct is 516 nt length, and covers 75% of the full-length mRNA. Although its overall homology to the corresponding region of *ENOD40-2* was only about 58%, it contains a number of clusters (>30 nt) that are highly homologous (>95% homology) to the *ENDO40-2* sequence. Therefore, it is likely that 21–23 nt siRNA produced by degradation of ihpRNA for *ENOD40-1* could

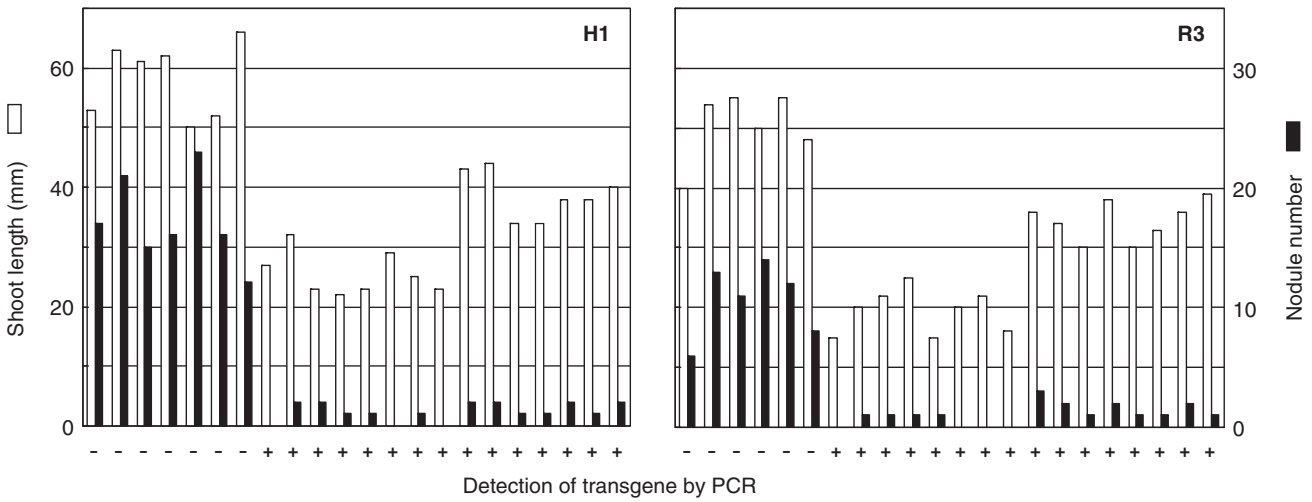

Fig. 6 Segregation of the low nodulation phenotype and transgene in T_2 generation. Two representatives of T_2 lines (H1 and R3) were inoculated with *M. loti* and grown in nitrogen-free medium for 3 weeks. Shoot length and nodule number were measured, and then the DNAs were prepared from nodulated roots of each individual plant, followed by detection of the transgene by PCR with a primer set for *ENOD40-1* (forward) and the pdk intron (reverse) sequences.

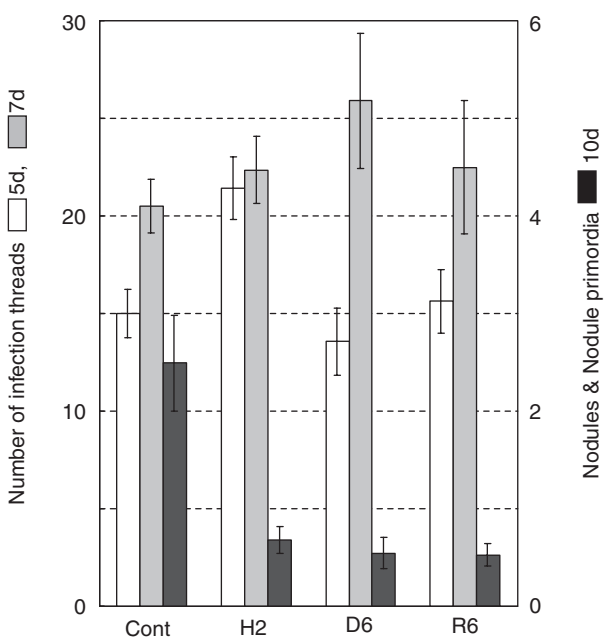

Fig. 7 Frequency of infection thread formation 5 and 7 d after *lacZ*-labeled *M. loti* inoculation. Only the infection threads clearly penetrated into root hair cells were counted. The total numbers of nodule and nodule primordia at 10 d after bacterial inoculation are also shown. The data are represented as averages of 15–24 plants.

secondarily trigger the breakdown of *ENOD40-2* mRNA with substantial efficacy.

Although RT–PCR assay does not allow us to compare precisely the levels of expression of different genes with different sets of primers, it is evident that *ENOD40-1* expression is more dominant in the nodulation process than *ENOD40-2* (Figs. 1, 3). *ENOD40-1* was induced very rapidly in response to bacterial inoculation, while no significant accumulation of *ENOD40-2* transcripts was detected in early stages of nodule initiation (Fig. 1). Thus it may be the case that silencing of *ENOD40-1*, but not of *ENOD40-2*, is primarily responsible for suppression of nodulation. Alternatively, silencing of both *ENOD40-1* and *-2* at the same time may be required for suppression of nodulation. Although *ENOD40-1* and *-2* sequences are considerably different from each other, and their temporal expression patterns are not completely identical, both *ENOD40*s have been shown to be induced in response to purified Nod factors (Flemetakis et al. 2000). In addition, in situ localization of *ENOD40-1* and *-2* transcripts in mature nodules was shown to be exactly the same by using specific probes made from short stretches of 3′ ends that show no homology between the two *ENOD40s* (data not shown; see also Takeda et al. 2005). Therefore, it is likely that the functions of the two *Lotus ENOD40*s are somewhat redundant, if not identical, in the nodulation process. Many legume species contain two or more *ENOD40*s. In soybean, alfalfa and *Sesbania* plants, the two *ENOD40*s are highly homologous, with >85% identity over their entire mRNA sequences (Kouchi and Hata 1993, Corich et al. 1998, Fang and Hirsch 1998). One exception is the case of *TrENOD40-3* recently isolated from white clover (*Trifolium repens*), that shares only 33% identity with *TrENOD40-1* and *-2* from the same species, although the latter two share 90% homology with each other (Varkonyi-Gasic and

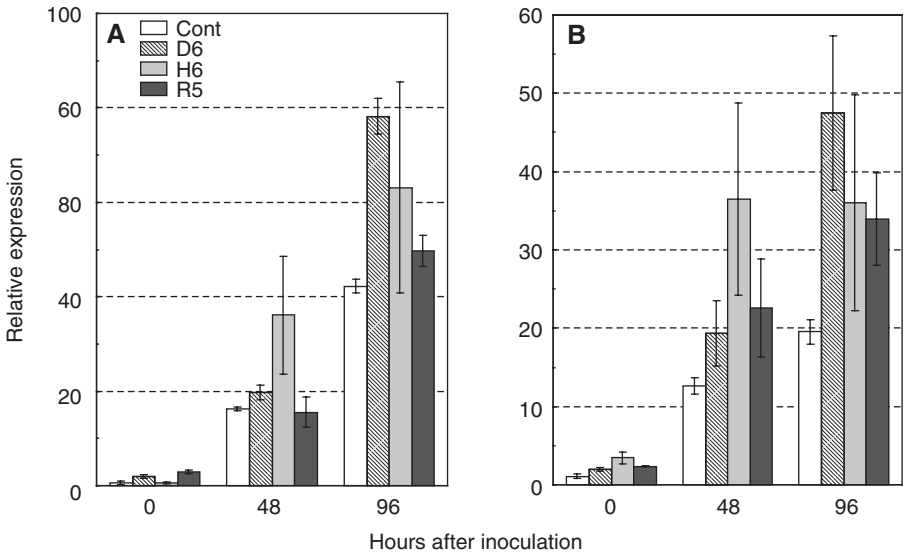

Fig. 8 Expression of two infection-related early nodulin genes, *Nin* (nodule inception) (A) and *ENOD16* (B) during the early infection process of the T_2 transgenic lines as assayed by real-time RT–PCR. The data are averages of duplicate RNA samples.

White 2002). These *ENOD40*s also share essentially the same temporal and spatial expression patterns, suggesting that their functions are more or less redundant. Taken together, it is very likely that simultaneous knock-down of two *ENOD40s* in *L. japonicus* caused strong inhibition of nodulation.

Our data indicate significant reduction of nodule primordium initiation by *ENOD40* knock-down (Fig. 5A, B), being consistent with the idea that the primary functions of *ENOD40* are in triggering the cortical cell division leading to nodule primordium formation (Crespi et al. 1994, Sousa et al. 2001). This idea is closely linked to the hypothesis that the small peptide potentially encoded by a short ORF in the box-1 that is well conserved in all legume *ENOD40*s identified so far, and/or the non-translated RNA (Sousa et al. 2001), are biologically active signal molecules that force cortical cells to enter into the cell division cycle (van de Sande et al. 1996, Sousa et al. 2001). However, there is no evidence yet to show the presence of the *ENOD40* peptides in legume tissues, though the first ATG of the short ORF is shown to act as a translation start in the roots as well as in in vitro translation (Sousa et al. 2001). Therefore, the mode of *ENOD40* action at the molecular level is still to be elucidated.

As an alternative to the above hypothesis, we have previously proposed that the primary functions of *ENOD40* are related to transport of photosynthates and/or other nutrients. This is based on the fact that the abundant expression of *ENOD40* in vascular bundles near to strong sink organs and/or tissues is the most prominent feature common for *ENOD40*s in all legumes and non-legumes investigated so far (Kouchi and Hata 1993, Kouchi et al. 1999). This hypothesis is not inconsistent with the possible involvement of *ENOD40* in triggering the initiation of nodule primordia, because a large influx of those photosynthates and nutrients could be a prerequisite for preparing cells to enter the cell division cycle, and furthermore the root adjacent to developing nodule primordia has to increase its sink strength to meet the demand of the microsymbionts for a carbon source (Santi et al. 2003). These issues will be further addressed by physiological analyses of the *ENOD40*-silenced plants in greater detail.

ENOD40 expression at the earliest stage of legume–*Rhizobium* interactions is not just restricted to the root pericycle opposite to the bacterial infection site, but it is also rapidly activated in root hair cells in response to bacterial inoculation, as well as to purified Nod factors, as shown by a transgenic study with a *ENOD40* promoter–β-glucuronidase (GUS) chimeric gene (H.K., unpublished results). In addition, *ENOD40*-overexpressing *M. truncatula* exhibited a considerable increase in the number of infection threads reaching the inner cortex (Charon et al. 1999). Therefore, it will be intriguing to investigate the possible involvement of *ENOD40* in the infection thread formation process. Our results (Fig. 7) indicate that *ENOD40* is not involved in controlling early infection events. This conclusion is further confirmed by the fact that temporal expression of *Nin* and *ENOD16* in early stages of nodulation, which are presumably involved in the bacterial entry into root hair cells and/or infection thread growth, is not reduced at all by *ENOD40* silencing (Fig. 8). Expression of these infection-related genes is independent or upstream of *ENOD40* activation, thus they serve as the earliest molecular markers of symbiotic interactions of legumes forming determinate nodules with compatible rhizobia.

It is well known that *ENOD40*s are expressed not only in symbiotic tissues but also in non-symbiotic tissues such as stems (Kouchi and Hata 1993, Varkonyi-Gasic and White 2002). The expression analyses of *ENOD40* orthologs in non-leguminous plant species suggest that *ENOD40* has a general role in plant development such as lateral root formation, flower development and vascular tissue development (Kouchi et al. 1999, Vleghels et al. 2003). However, we observed no apparent abnormality in the morphology of leaves, stems and roots, as well as flowering of *ENOD40*-suppressed plants. This might be interpreted by incompleteness of the *ENOD40* silencing in the transgenic plants. The levels of *ENOD40* expression in non-symbiotic organs were very low, and the extent of the RNAi knock-down for those low levels of the *ENOD40* transcripts did not appear very large (Fig. 1). In contrast, the induction of *ENOD40* expression upon *M. loti* infection was strongly suppressed in the transgenic plants, resulting in severe inhibition of nodule formation.

In conclusion, our results provide strong evidence that legume *ENOD40* plays essential roles in the initiation of nodule primordia, but not in the bacterial infection process. Further analysis of these *ENOD40*-silenced *L. japonicus* plants, mainly in terms of physiological aspects, is now in progress.

Materials and Methods

Construction of the RNAi plasmid

The gene constructs were made according to standard DNA manipulation protocols (Sambrook and Russel 2001). A cDNA fragment of *ENOD40* for the RNAi construct was amplified by PCR from *LjENOD40-1* cDNA with the primers, 5′-ATCTCGAGGATCCTCTGAACCAATCCATC-3′ (forward) and 5′-ATGGTACCATCGATACAAGAAGAGAAGGGAC-3′ (reverse), generating a 516 bp cDNA fragment that contains both box-1 and -2, which are well conserved in *ENOD40*s from various legumes and non-legumes. The amplification products were digested with *Xho*I–*Kpn*I and with *BamH*I–*Cla*I, and ligated into the pKANNIBAL plasmid vector (Wesley et al. 2001), in which the sense and antisense *ENOD40* RNA sequences were located in tandem with a pyruvate dehydrogenase kinase (pdk) intron between them, and this ihpRNA construct was placed behind the CaMV 35S promoter. Then the entire RNAi construct was subcloned as a *Not*I fragment into the binary vector pART27 and introduced into *Agrobacterium tumefaciens* strain AGL1 by the freeze–thaw procedure.

Transformation of Lotus japonicus

Transformation of *L. japonicus* was done according to the method described by Stiller et al. (1997) with some minor modifications. In brief, hypocotyls excised from *L. japonicus* B-129 'Gifu' seedlings were infected with *A. tumefaciens* strain AGL1 harboring the above-mentioned binary vector construct. Generated calli were screened for geneticin (G418) resistance and the regenerated plants were grown in vermiculite pots to harvest the T_1 seeds. More than 20 independent T_1 transgenic lines were generated, and plants with sufficient fertility were selected and propagated further.

Analysis of infection thread formation and nodulation phenotypes

Transgenic and wild-type 'Gifu' plants were grown in vermiculite pots as described previously (Kumagai and Kouchi 2003). The seedlings were inoculated with *M. loti* strain MAFF303099 that harbored the β-galactosidase (*lacZ*) gene as a constitutive marker (Tansengco et al. 2003). The roots were harvested at appropriate time intervals, fixed in 2.0% glutaraldehyde in 0.1 M Tris–HCl buffer (pH 7.0) for 2 h at room temperature, and then stained in 0.8 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal), 2.5 mM each of potassium ferrocyanide and potassium ferricyanide in the same buffer for 3–12 h at 37°C. After washing in 0.1 M Tris–HCl buffer, the root tissue was clarified in chloral hydrate (8 g) dissolved in 1 ml of glycerol and 2 ml of water, and observed under a microscope. For microscopic observation of nodule primordia, the roots from the plants inoculated with *M. loti* MAFF303099 were stained briefly in 0.025% safranin in 50% ethanol to visualize dividing cell clusters inside the root cortex. We used wild-type 'Gifu' plants as a positive control in these experiments, but the nodulation phenotype of the plants transformed with the vector containing GUS coding sequences instead of the *ENOD40* RNAi cassette was confirmed to be exactly the same as that of the wild-type plants.

Genomic PCR and Southern hybridization

Genomic DNA was isolated from trifoliate leaves or roots of the transgenic plants by the cetyltrimethylammonium bromide (CTAB) method as described by Li et al. (2001). The presence of the transgene was examined by PCR with a primer set specific to *ENOD40-1* and pdk intron sequences. The primer sequences were 5′-GAATCTGGTAACCATGAAGCTCTG-3′ (forward; *ENOD40-1*) and 5′-CTTCGTCTTACACATCACTTGTC-3′ (reverse; pdk intron). Genomic Southern blot hybridization was done by standard procedures (Sambrook and Russel 2001). Genomic DNA was digested with appropriate restriction enzymes to completion, subjected to 0.8% agarose gel electrophoresis, and transferred onto a nylon membrane (Hybond N Plus, Amersham, Tokyo, Japan). The membrane was hybridized with a ^{32}P-labeled *ENOD40-1* cDNA probe and processed as described previously (Kouchi and Hata 1993).

Real-time RT–PCR

Total RNA was isolated from uninfected and infected roots by an RNeasy Plant Mini kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions, followed by reverse transcription using Superscript II (Invitrogen K.K., Tokyo, Japan) with oligo(dT)$_{18}$ as a primer. The resultant cDNA was used as templates for real-time PCR with Lightcycler (Rosch Diagnostics, Tokyo, Japan) according to the standard procedures described in the manufacturer's manual, with 5′-GGAGGTATGCTCAA ACATTC-3′ (forward) and 5′-GTAACTTCTCAAGAGAAGA CC-3′ (reverse), 5′-CAAAACTCGTTATGTTGCGG-3′ (forward) and 5′-CACCTCAAAGGAAGAAGAACA-3′ (reverse), 5′-AAC TCACTGGAAACAGGTGCTTTC-3′ (forward) and 5′-CTATTG CGGAATGTATTAGCTAGA-3′ (reverse), and 5′-TCTGCTGT TAAGTGCGAAATAGTGG-3′ and 5′-GTATTGTTCCTGTAA GGACATCAC-3′ for *ENOD40-1*, *ENOD40-2*, *Nin* and *ENOD16*, respectively. Ubiquitin was used as an internal standard with PCR primers described by Flemetakis et al. (2000). All the expression data were normalized by adjusting the expression level of ubiquitin in each sample to 1×10^4.

Detection of small interferring (si) RNA

Total RNA was isolated from roots by Trizol (Invitrogen) according to the manufacturer's manual. Low molecular weight RNAs were enriched by precipitating high molecular weight RNAs in 10% (w/v) polyethylene glycol containing 0.5 M NaCl (Goto et al. 2003) and subjected to electrophoresis on a 15% denaturing polyacrylamide gel with 7 M urea (Sambrook and Russel 2001). After electrophoresis, the nucleic acids on the gel were electro-blotted onto a nylon membrane (Hybond NX, Amersham). The membrane was hybridized with an in vitro transcribed ^{32}P-labeled antisense RNA probe for *ENOD40-1* in 45% formaldehyde, 7% SDS, 5 × Denhardt's solution (1 × Denhardt's = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 0.3 M NaCl and 50 mM Na_2HPO_4 (pH 7.0) containing 0.1 mg ml^{-1} salmon sperm DNA at 40°C for > 16 h. Then the membranes were washed in 2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) containing 0.1% SDS at room temperature for 10 min and then for 15 min twice in the same solution at 50°C. The probe was pre-digested in 0.2 N sodium bicarbonate before hybridization.

Acknowledgments

The authors express their thanks to Peter M. Waterhouse of CSIRO, Australia, for providing pKANNIBAL and pART27 vectors, and M. Hayashi of Osaka University, Japan, for providing the *lacZ*-labeled *M. loti* strain. This work was supported by the Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

Charon, C., Johansson, C., Kondorosi, E., Kondorosi, A. and Crespi, M. (1997) *enod40* induces dedifferentiation and division of root cortical cells in legumes. *Proc. Natl Acad. Sci. USA*| 94: 8901–8906.

Charon, C., Sousa, C., Crespi, M. and Kondorosi, A. (1999) Alteration of enod40 expression modifies *Medicago truncatula* root nodule development induced by *Sinorhizobium meliloti*. *Plant Cell* 11: 1953–1965.

Corich, V., Goormachtig, S., Lievens, S., van Montagu, M. and Holsters, M. (1998) Patterns of ENOD40 gene expression in stem-borne nodules of *Sesbania rostrata*. *Plant Mol. Biol.* 37: 67–76.

Crespi, M., Jurkevitch, E., Poiret, M., Daubentoncarafa, Y., Petrovics, G., Kondorosi, E. and Kondorosi, A. (1994) *enod40*, a gene expressed during nodule organogenesis, codes for a non-translatable RNA involved in plant growth. *EMBO J.* 13: 5099–5112.

Fang, Y.W. 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.A. 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.

Goto, K., Kanazawa, A., Kusaba, M. and Masuta, C. (2003) A simple and rapid method to detect plant siRNAs using nonradioactive probes. *Plant Mol. Biol. Rep.* 21: 51–58.

Greene, E.A., Erard, M., Dedieu, A. and Barker, D.G. (1998) MtENOD16 and 20 are members of a family of phytocyanin-related early nodulins. *Plant Mol. Biol.* 36: 775–783.

Hamilton, A.J. and Baulcombe, D.C. (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 286: 950–952.

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.

Kumagai, H. and Kouchi, H. (2003) Gene silencing by expression of hairpin RNA in *Lotus japonicus* roots and root nodules. *Mol. Plant-Microbe Interact.* 16: 663–668.

Legocki, R.P. and Verma, D.P.S. (1980) Identification of 'nodule-specific' host proteins (nodulins) involved in the development of Rhizobium–legume symbiosis. *Cell* 20: 153–163.

Li, H., Luo, J., Hemphill, J.K., Wang, J.T. and Gould, J.H. (2001) A rapid and high yielding DNA miniprep for cotton (*Gossypium spp.*). *Plant Mol. Biol. Rep.* 19: 183a–183e.

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.

Nap, J.P. and Bisseling, T. (1990) Developmental biology of a plant–prokaryote symbiosis: the legume root nodule. *Science* 250: 948–954.

Niwa, S., Kawaguchi, M., Imaizumi-Anraku, H., Chechetka, S.A., Ishizaka, M., Ikuta, A. and Kouchi, H. (2001) Responses of a model legume *Lotus japonicus* to lipochitin oligosaccharide nodulation factors purified from *Mesorhizobium loti* JRL501. *Mol. Plant-Microbe Interact.* 14: 848–856.

Sambrook,, J. and Russel, D.W. (2001) edn. *Molecular Cloning: A Laboratory Manual* 3rd. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

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: 808–816.

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

Sousa, C., Johansson, C., Charon, C., Manyani, H., Sautter, C., Kondorosi, A. and Crespi, M. (2001) Translational and structural requirements of the early nodulin gene *enod40*, a short-open reading frame-containing RNA, for elicitation of a cell-specific growth response in the alfalfa root cortex. *Mol. Cell Biol.* 21: 354–366.

Stiller, J., Martirani, L., Tuppale, S., Chian, R., Chiurazzi, M. and Gresshoff, P. (1997) High frequency transformation and regeneration of transgenic plants in the model legume. *Lotus japonicus*. *J. Exp. Bot.* 48: 1357–1365.

Takeda, N., Okamoto, S., Hayashi, M. and Murooka, Y. (2005) Expression of *LjENOD40* genes in response to symbiotic and non-symbiotic signals: *LjENOD40-1* and *LjENOD40-2* are differentially regulated in *Lotus japonicus*. *Plant Cell Physiol.* 46: 1291–1298.

Tansengco, M.L., Hayashi, M., Kawaguchi, M., Imaizumi-Anraku, H. and Murooka, Y. (2003) *crinkle*, a novel symbiotic mutant that affects the infection thread growth and alters the root hair, trichome, and seed development in *Lotus japonicus*. *Plant Physiol.* 131: 1054–1063.

van de Sande, K., Pawlowski, K., Czaja, I., Wieneke, U., Schell, J., et al. (1996) Modification of phytohormone response by a peptide encoded by *ENOD40* of legumes and a nonlegume. *Science* 273: 370–373.

van Kammen, A. (1984) Suggested nomenclature for plant genes involved in nodulation and symbiosis. *Plant Mol. Biol. Rep.* 2: 43–45.

Varkonyi-Gasic, E. and White, D.W.R. (2002) The white clover *enod40* gene family. Expression patterns of two types of genes indicate a role in vascular function. *Plant Physiol.* 129: 1107–1118.

Vijn, I., Martinez-Abarca, F., Yang, W.C., Das Neves, L., van Brussel, A., van Kammen, A. and Bisseling, T. (1995) Early nodulin gene expression during Nod factor-induced processes in *Vicia sativa*. *Plant J.* 8: 111–119.

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.

Waterhouse, P.M., Wang, M.B. and Lough, T. (2001) Gene silencing as an adaptive defence against viruses. *Nature* 441: 834–842.

Wesley, S.V., Helliwell, C.A., Smith, N.A., Wang, M.B., Rouse, D.T., et al. (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J*. 27: 581–590.

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.

(Received May 28, 2006; Accepted June 23, 2006)