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Cooperative Action of Lipo-Chitin Nodulation Signals on the Induction of the Early Nodulin, ENOD2, in Soybean Roots

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Various lipo-chitin molecules were tested for their ability to induce the expression of the early nodulin, ENOD2, in *Glycine soja* roots. When inoculated separately onto *G. soja* roots, LCO-V (C18:1Δ11,Mefuc), LCO-V (C18:1Δ9,Mefuc), LCO-V (C16:0,Mefuc), and LCO-IV (C16:0) were unable to induce ENOD2 expression, even though these compounds had previously been shown to induce root hair curling, the formation of nodule-like primordia, and induction of the early nodulin, ENOD40. ENOD2 expression, however, was induced when any two of these molecules were inoculated in combination. Thus, the lipo-chitin nodulation signals appear to act cooperatively to induce ENOD2 expression. *B. japonicum* strains USDA110 and USDA135 and *B. elkanii* strain USDA61, all symbionts of soybean, were found to produce at least two distinct nod signals ([i.e., NodBj-V[C18:1,Mefuc] and NodBj-V[C16:0,Mefuc]). These two compounds were mixed in various ratios and tested for their ability to induce ENOD2 expression. The results indicate that the former compound must be present in equivalent or excess amount in order to obtain maximum ENOD2 expression. Additional nonspecific LCOs (e.g., LCO-IV[C16:2Δ2,9; SO_3]), incapable of inducing root hair curling or cortical cell division, were tested in combination with the four active LCOs listed above. It was found that any combination of one active LCO with a nonspecific LCO was sufficient to induce ENOD2 mRNA expression. The ENOD2 mRNA expression pattern detected by in situ hybridization closely resembled that found in bacterial-induced nodules with expression detected in cortical cells between primary and secondary meristems and around the vascular strands. These data demonstrate that the cooperative action of at least two LCO nodulation signals leads to a greater progression of nodule ontogeny as demonstrated by the expression of ENOD2, a marker gene for the differentiation of nodule parenchyma.

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Bacteria of the genera *Rhizobium, Bradyrhizobium,* and *Azorhizobium* possess the ability to infect the roots of leguminous plants resulting, in the formation of a novel organ, the nodule, in which the bacteria ultimately reside. The bacterial nodulation genes are essential for this infection process and are induced in response to plant excreted flavonoid compounds (reviewed in Peters and Verma 1990). It is now known that the *nod* genes encode enzymes that in turn synthesize a signal molecule active on the plant. These nodulation signals consist of a chitin oligomer (three to five residues) modified by a *N*-acyl group at the nonreducing terminus and by a variety of substituents on the terminal, reducing sugar (reviewed by Carlson et al. 1994). Application of these lipo-chitin oligosaccharides (LCOs) to susceptible plant roots results in a variety of physiological responses, among them the deformation of root hairs (i.e., HAD response) and induction of cortical cell division (i.e., NOI response) (Spaink 1994).

In the case of *B. japonicum*, symbiont of soybean, the primary nodulation signal has been identified as a chitin pentamer *N*-acylated with vaccenic acid and modified on the terminal, reducing sugar by 2-*O*-methylfucose [i.e., NodBj-V(C18:1,Mefuc); Sanjuan et al. 1992; Carlson et al. 1993). *B. japonicum* strain USDA110 has been reported to produce only this single nod signal. However, as shown in this study, this strain produces at least one additional minor LCO molecule. Other strains of *B. japonicum* have also been shown to produce a variety of substituted LCO molecules (Carlson et al. 1993). Likewise, various *Rhizobium* species have been shown to produce a wide diversity of LCO molecules (for reviews, see Carlson et al. 1994; Spaink 1994). The biological relevance of these mixtures of putative nodulation signals is presently unknown since single LCOs have been shown to induce nodule primordia formation (e.g., Truchet et al. 1991; Mergaert et al. 1993; Stokkermans and Peters 1994; Minami et al. 1996).

During the induction of nodule formation, specific plant genes are induced whose protein products are termed nodulins (for reviews see Nap and Bisseling 1990; Franssen et al. 1992). Nodulins are classified into early and late nodulins

based on the time of appearance of their transcripts during the nodule formation process. Of those nodulins known to be induced rapidly in soybean upon bacterial inoculation, ENOD40 (Kouchi and Hata 1993; Yang et al. 1993) and ENOD2 (Franssen et al. 1989; Van de Wiel et al. 1990) have been studied most extensively. ENOD40 has a unique characteristic in that the DNA sequence fails to predict a significant protein coding segment. Indeed, Crespi et al. (1994) have suggested that ENOD40 functions in planta as a cytoplasmic RNA to control phytohormone balance. The ENOD2 gene encodes a (hydroxy)proline-rich polypeptide that has been proposed to be a cell-wall protein, and the mRNA is exclusively localized in the inner cortex of nodules (Van de Wiel et al. 1990). Since both genes are highly conserved in many legume species and are rapidly induced during nodulation, their expression can be used as a sensitive tool to examine the signaling processes at work during nodule organogenesis.

Previously, we reported that ENOD40 expression could be rapidly induced in soybean roots by the addition of various LCO nodulation signals (Minami et al. 1996). An unexpected finding of this work was that a rapid, but transient, expression of ENOD40 mRNA could be induced by LCOs that lack the ability to induce a HAD or NOI response on soybean roots. However, those LCO molecules capable of inducing a HAD or NOI response elicited a prolonged expression of ENOD40. Thus, specificity for the *nod* signal is manifested not in the initial rapid induction of ENOD40 expression, but at a later step. To extend these structure/function studies of various LCO molecules, we tested the ability of various LCOs to induce ENOD2 expression. It was found that none of the individual LCO molecules could induce ENOD2 expression, even though some of these molecules could induce a nodule primordium. In this study, we explore this observation and show that a mixture of two or more LCO molecules is required to induce ENOD2 expression.

RESULTS

Expression of ENOD2 mRNA in *G. soja* roots inoculated with LCOs.

Previously, we examined the specificity of *nod* signal response by utilizing a variety of natural and chemically synthesized LCO molecules. These studies indicated that, of those compounds tested, only LCO-V (C18:1Δ11,Mefuc), LCO-V (C18:1Δ9,Mefuc), LCO-V (C16:0,Mefuc), and LCO-IV (C16:0) induced root hair curling and the formation of nodule-like primordia on *G. soja* roots when inoculated as single compounds (Stokkermans et al. 1995). The first of these compounds is identical in structure to the most abundant natural nod signal produced by *B. japonicum* strain USDA110 (Sanjuan et al. 1992, Carlson et al. 1993). More recently, we reexamined this question utilizing the expression of the early nodulin gene, ENOD40, as a bioassay to measure LCO activity (Minami et al. 1996). These results indicated that the same four compounds were the strongest inducers of ENOD40 expression. Therefore, it was expected that these same compounds would likely induce other early nodulin genes when inoculated onto soybean roots. However, when each of these compounds was tested, we were unable to detect transcripts of the early nodulin gene, ENOD2 (e.g., Fig. 1A).

Franssen et al. (1987) showed that ENOD2 was rapidly induced by inoculation of soybean with *Rhizobium fredii* USDA257 which forms nodule-like structures without intracellular bacteria. These data suggested the involvement of diffusible factors in the induction of ENOD2 expression. To identify possible factors, other than the nod signals previously tested, we tested crude butanol extracts of *B. japonicum* strain USDA110 cultures that had been induced for *nod* gene expression. These extracts are the starting material for purification of the lipo-chitin nodulation signals. As shown in Figure 1B, a butanol culture extract induced the accumulation of ENOD2 mRNA in *G. soja* roots 6 and 13 days after inoculation (lanes 3 and 4, respectively). Since the butanol extract is known to contain a mixture of lipo-chitin nodulation signals (Sanjuan et al. 1992; Carlson et al. 1993), these results suggested to us that such a mixture may be required for ENOD2 induction. Therefore, roots were inoculated with a mixture of the four chemically synthesized LCOs previously shown to induce nodule primordium formation on *G. soja* roots [i.e., LCO-V (C18:1Δ11,Mefuc), LCO-V (C18:1Δ9,Mefuc), LCO-V (C16:0,Mefuc), and LCO-IV (C16:0)]. As shown in Figure 1B, ENOD2 expression was induced by this mixture at 6 days but not 13 days postinoculation (lanes 1 and 2, respectively). From these results, we concluded that the induction of ENOD2 expression requires the cooperative action of more than one lipo-chitin nodulation signal. The use of chemically synthesized LCO molecules for these assays allows us to rule out the involvement of other possible contaminating factors.

Effect of mixtures of LCOs on the accumulation of ENOD2 mRNA.

To determine the minimum combination of LCOs required for ENOD2 induction, we decided to test all possible combinations of the four active LCO molecules. However, since LCO-V (C18:1Δ9, Mefuc) and LCO-V (C18:1Δ11,Mefuc) differ only in the position of the fatty acyl double bond and

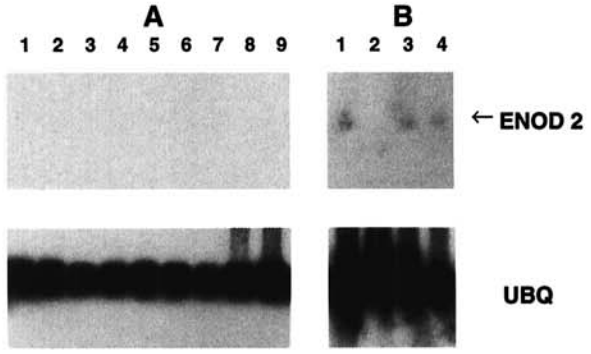


Fig. 1. The effect of LCO mixtures on ENOD2 expression **A,** *Glycine soja* roots were inoculated with a carboxymethylcellulose solution—lane 1, containing LCO-V (C18:1Δ11,Mefuc); lane 2 and 3, LCO-V (C16:0,Mefuc); lanes 4 and 5, LCO-V (C18:1Δ9,Mefuc); lane 6 and 7); LCO-IV (C16:0); lanes 8 and 9—and incubated for 6 days (lanes 1, 2, 4, 6, and 8) or 13 days (lanes 3, 5, 7, and 9). RNAs were isolated and processed as described in Methods. **B,** *G. soja* roots were inoculated with a mixture of LCO-V (C18:1Δ11,Mefuc), LCO-V (C18:1Δ9,Mefuc), LCO-V (C16:0,Mefuc) and LCO-IV (C16:0) (lanes 1 and 2) or a crude butanol extract of a *Bradyrhizobium japonicum* USDA110 culture induced for *nod* gene expression (lanes 3 and 4) and grown for 6 days (lane 1 and 3) or 13 days (lane 2 and 4). RNAs were isolated and processed as described in Methods. The cDNA generated by PCR using the appropriate gene-specific primers was hybridized to a ^{32}P-labeled probe for ENOD2 (upper panel) or ubiquitin (lower panel). Arrows indicate the signal from PCR products with expected size.

have been previously shown to have similar biological activity (Stokkermans et al. 1995), we decided to limit the number of samples to be tested by omitting LCO-V (C18:1Δ9,Mefuc) from the mixtures. As expected, a mixture of the three LCOs [i.e., LCO-V (C18:1Δ11, Mefuc), LCO-V (C16:0,Mefuc) and LCO-IV (C16:0)] gave strong induction of ENOD2 expression (Fig. 2, lane 2). Of these three LCOs, all pair-wise combinations were also active in inducing ENOD2 mRNA accumulation (lanes 3 to 5). In these experiments, no significant difference was detected with regard to the morphology of the nodule-like structures formed or in the efficiency of ENOD2 induction between the various LCO combinations (data not shown). Therefore, although a single LCO molecule is insufficient to induce ENOD2 expression, any combination of two LCOs active in inducing nodule primordia formation will lead to ENOD2 mRNA accumulation.

Lipo-chitin nodulation signals produced by *B. japonicum* strain USDA110.

Previously, Sanjuan et al. (1992) analyzed the nod signals produced by*B. japonicum* strain USDA110 and detected only one compound, NodBj-V(C18:1,Mefuc). Subsequently, Carlson et al. (1993) confirmed that strain USDA110 produced only one major nod signal, but found that another strain, USDA135, produced a mixture of lipo-chitin nodulation signals. Indeed, strain USDA135 was found to produce five separate compounds (i.e., NodBj-V[C18:1,Mefuc], NodBj-V[Ac,C18:1,Mefuc], NodBj-V(C16:0,Mefuc), NodBj-V(Ac,C16:0,Mefuc), and NodBj-V(C16:1,Mefuc). An even larger mixture of lipo-chitin molecules was identified from cultures of *B. elkanii* strain USDA61, also a symbiont of soybean (Carlson et al. 1993). The fact that more than one lipo-chitin molecule is necessary to induce ENOD2 expression provides a possible rationale for why *B. japonicum* and *B. elkanii* strains produce such mixtures. However, it was puzzling that strain USDA110 appeared to produce only a single nod signal.

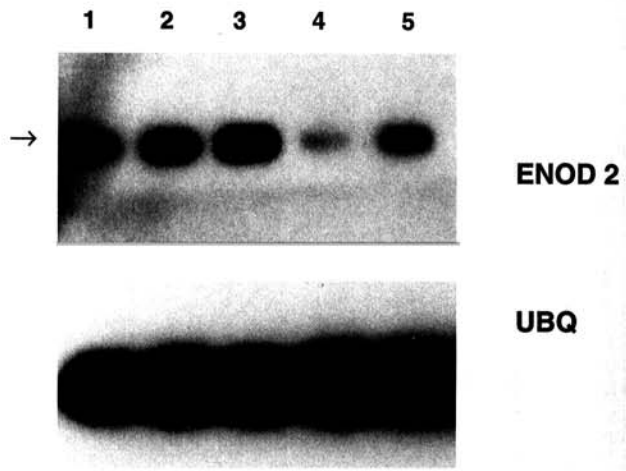


Fig. 2. Effect of pairwise mixtures of LCOs on the accumulation of ENOD2 mRNA in *Glycine soja* roots. LCO mixtures were inoculated onto *G. soja* roots and incubated for 6 days, followed by RNA isolation and PCR amplification as described in Methods. An arrow indicates the ENOD2 signal with the expected size. Lane 1, mixture of LCO-V (C18:1Δ11,Mefuc), LCO-V (C18:1Δ9,Mefuc), LCO-V (C16:0,Mefuc) and LCO-IV (C16:0); lane 2: the same mixture lacking LCO-V (C18:1 9, Mefuc); lane 3: mixture of LCO-V (C18:1 11, Mefuc) and LCO-V (C16:0,Mefuc); lane 4: mixture of LCO-V (C18:1Δ11,Mefuc) and LCO-IV (C16:0); lane 5: mixture of LCO-V (C16:0,Mefuc), and LCO-IV (C16:0). Lower panel shows the transcripts for ubiquitin.

To address this apparent difference, lipo-chitin molecules were purified from cultures of *B. japonicum* strain USDA110 induced for *nod* gene expression (Sanjuan et al. 1992; Carlson et al. 1993). In this case, five minor HPLC peaks were collected that eluted in a zone similar to the major nod signal [NodBj-V(C18:1;Mefuc)] previously reported. In each case, the peak fractions were analyzed by FAB-MS. Peak A (retention time = 21 min) was found to contain molecular species with pseudomolecular ions of m/z 1,418 and 1,460. The molecule of m/z 1,460 may be an acetylated version of the m/z 1,418 molecule. Peak B (retention time = 22.8 min) contained a molecule with a pseudomolecular ion of m/z 1,263. The structures of the molecules in peaks A and B are not known since the quantities and intensities of the molecular ions were insufficient for composition or MS-MS analyses. Thus, it cannot be stated with certainty that these molecules are indeed lipo-chitin nodulation signals. Peak C (retention time = 25.7 min) contained a molecule with a pseudomolecular ion of m/z 1,390 (and the sodium adduct at m/z 1,412) together with fragment ions of m/z 1009, 803, 600, and 400 (Fig. 3A). These data are consistent with those of the previously published nod signal, NodBj-V(C16:0,Mefuc), produced by *B. japonicum* strain USDA135 (Carlson et al. 1993). This structure is also shown in Figure 3A. The fatty acid analysis shown in Figure 3B confirms the presence of palmitic acid in this sample; vaccenic acid was not detected. The presence of this nod signal, NodBj-V(C16:0,Mefuc), was not previously reported for strain USDA110, but it is clearly a minor component. Analysis of peak D (retention time = 27.6 min) showed that it consists of the previously reported (Sanjuan et al. 1992) major nod signal from strain USDA110 (i.e., NodBj-V [C18:1Δ11,Mefuc]). Peak E (retention time = 28.9 min) contained molecular species with pseudomolecular ions of m/z 1,416 and 1,458. Insufficient amounts were obtained for further analyses. However, the compound of m/z 1,416 is most likely some residual NodBj-V(C18:1 11,Mefuc) and it is possible that the compound of m/z 1,458 is a small amount of its acetylated form, NodBj-V(Ac, C18:1Δ11,Mefuc). Peak F (retention time = 31 min) contained molecular species of m/z 1,416, 1,458, and 1,418. The first two are probably residual amounts of peaks D and E. The structure of the latter compound, i.e., of m/z 1,418, is not known. Again, there was an insufficient amount for further types of analyses. It is possible that this compound could be NodBj-V(C18:0,Mefuc). However, other structures may also be possible. In summary, these data show that, in addition to the major nod signal, NodBj-V(C18:1Δ11,Mefuc), strain USDA110 produces a minor amount of NodBj-V(C16:0,Mefuc), and possibly NodBj-V(Ac,C18:1,Mefuc). Minor amounts of several other unidentified structures are also produced.

Dosage effects of LCO combinations.

Since *B. japonicum* strains USDA135 and USDA110, as well as *B. elkanii* strain USDA61, produce nod signals identical in structure to LCO-V(C18:1,Mefuc) and LCO-V(C16:0,Mefuc), we decided to further explore the cooperativity between these two molecules. We estimate the ratio of these two molecules produced by *B. japonicum* USDA110 to

be approximately 10:1, respectively, based on relative HPLC peak area. Therefore, *G. soja* roots were inoculated with 100 ng of LCO-V C16:0,Mefuc): LCO-V (C18:1Δ11,Mefuc) mixtures at ratios of 1:1, 1:10, and 1:100. As shown in Figure 4 (lanes 3, 4, and 5), all such mixtures were capable of inducing the rapid accumulation of ENOD2 mRNA. However, when similar LCO-V (C16:0,Mefuc): LCO-V (C18:1Δ11,Mefuc) mixtures were tested at ratios of 10:1 and 100:1, the intensity of the ENOD2 hybridization signal significantly decreased (Fig. 4, lanes 1 and 2). These results support the idea that the minor nod signal components act cooperatively with the major nod signal, NodBj-V(C18:1Δ11,Mefuc), to induce ENOD2 expression. The data also suggest that the relative ratios of the minor and major nod signal components are important for biological activity.

Structural specificity of nod signal activity.

Previously, using ENOD40 expression as a bioassay for nod signal activity, we examined the structure/function relationships of various LCO molecules (Minami et al. 1996). To extend this analysis to ENOD2, we examined the expression of ENOD2 in roots treated with combinations of various LCO structural variants. For example, when plants were inoculated with a mixture of LCO-V (C18:1Δ11,Mefuc) and LCO-IV (C18:1Δ11,Mefuc), ENOD2 expression could be detected after PCR amplification of isolated mRNA (Fig. 5, lane 2). NodBj-V (C18:1Δ11) produced by the *B. japonicum nodZ* mutant strain NAD138 (Stacey et al. 1994), is unable to induce a HAD or NOI response when inoculated singly (Stokkermans et al. 1995), but is capable of inducing ENOD2 expression when inoculated with LCO-V (C18:1Δ11, Mefuc)

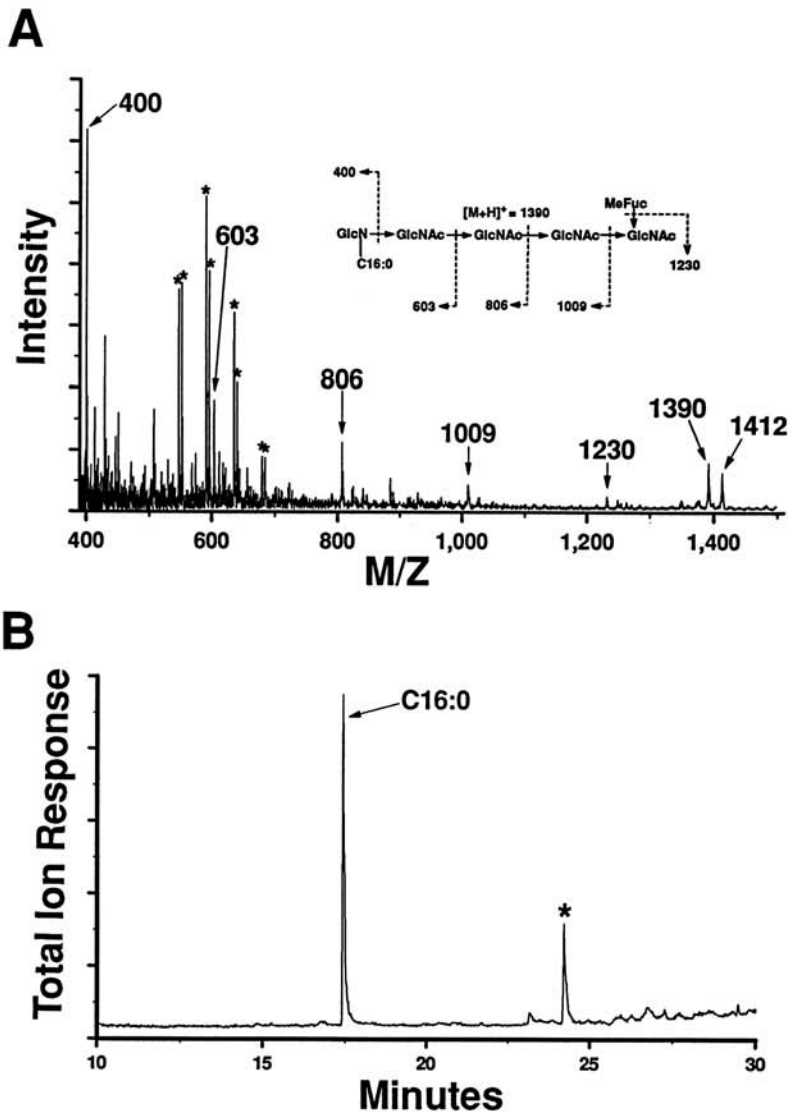


Fig. 3. A, A FAB-MS spectrum of HPLC purified peak C from induced cultures of *Bradyrhizobium japonicum* strain USDA110. The ions marked with * are due to a contaminant (polyethylene glycol) which has been frequently found in the n-butanol used during the extraction process. This spectrum shows the [M+H]+ and [M+Na]+ ions, 1,390 and 1,412, respectively, as well as the expected fragment ions for NodBj-V(C16:0,Mefuc). **B,** Fatty acid analysis of peak C shows only the presence of palmitic acid (C16:0). The peak marked with * is due to C18:0 which is a common contaminant that we have frequently observed in many fatty acid analyses of Nod factors, as well as of other glycolipids.

(Fig. 5, lane 1). Surprisingly, LCO-IV (C16:2, 2, 9, SO_3), a molecule identical in structure to the primary *R. meliloti* nod signal active on alfalfa (Lerouge et al. 1990), was also able to induce ENOD2 expression when co-inoculated with LCO-V (C18:1Δ11,Mefuc) (Fig. 5, lane 3). Both LCO-IV (C18:1Δ11,Mefuc) and LCO-IV (C16:2Δ2,9, SO_3) were able to induce ENOD2 expression when mixed with LCO-V (C16:0,Mefuc) (Fig. 5, lane 4 and 5, respectively).

An unexpected finding of our earlier studies is that unsubstituted chitin pentamer was capable of inducing the rapid accumulation of ENOD40 mRNA when inoculated onto *G. soja* roots (Minami et al. 1996). However, this expression disappeared within 6 days postinoculation. Considering the above results, we mixed chitin tetramer or pentamer with LCO-V (C18:1Δ11,Mefuc) and assayed for ENOD2 expression. As shown in Figure 5 (lanes 6 and 7, respectively) either the chitin tetramer or pentamer are capable of inducing ENOD2 expression when added in conjunction with a nod signal capable of inducing a HAD or NOI response [e.g., LCO-V (C18:1Δ11,Mefuc)].

These results suggest that any combination of an active LCO molecule (i.e., capable of inducing a HAD and NOI response) in conjunction with an additional active LCO, inactive LCO (i.e., incapable of inducing HAD or NOI), or chitin oligomer is sufficient for the induction of ENOD2 expression. In these experiments, the efficiency of formation of nodule-like structures was not significantly affected by the addition of the various LCO combinations (data not shown). None of the LCOs added singly is capable of inducing ENOD2 mRNA accumulation (data not shown).

Localization of ENOD2 mRNA by in situ hybridization.

Expression of ENOD2 mRNA in roots treated with various mixtures of LCOs and/or chitin oligomer was also demonstrated by in situ hybridization. At the early stages of nodule development induced by *B. japonicum* inoculation, ENOD2 mRNA were present most abundantly in the cortex around the connecting vascular strand (Fig. 6A), as first described by Van de Wiel et al. (1990). At much earlier stages, prior to complete connection of the vascular strands, the ENOD2 mRNA was detected in cortical cells behind the nodule primordia (data not shown). Figure 6B shows the localization of ENOD2 mRNA in the nodule-like structure elicited by crude butanol extracts of *B. japonicum* cultures induced for *nod* gene expression. The expression pattern seen closely resembles that found in bacterial-induced nodules. Figure 6C–E shows the localization of ENOD2 mRNA in roots treated with mixtures of LCO-V(C18:1Δ11,Mefuc)/ LCO-V (C16:0,Mefuc), LCO-V (C18:1Δ11,Mefuc)/ LCO-IV (C18:1Δ11,Mefuc), and LCO-V(C18:1Δ11,Mefuc)/chitin pentamer, respectively. ENOD2 mRNA was detected in the cortical cells between primary and secondary meristems, and around the vascular strands. These results demonstrate that the induction of ENOD2 in response to these LCO mixtures occurs with the same spatial distribution patterns as those seen during the development of bacterial-induced nodules. We were also able to detect ENOD2 expression in root sections treated with a mixture of LCO-V (C18:1Δ11,Mefuc)/ LCO-IV (C16:2Δ2,9, SO_3) and LCO-V(C18:1Δ11,Mefuc)/chitin tetramer, but the hybridization signals were rather weak in these cases (data not shown). However, consistent with previous hybridization results, root sections treated with LCO-V(C18:1Δ11,Mefuc) (Fig. 6F) or LCO-V (C16:0,Mefuc) (data not shown) alone exhibited no significant hybridization signal.

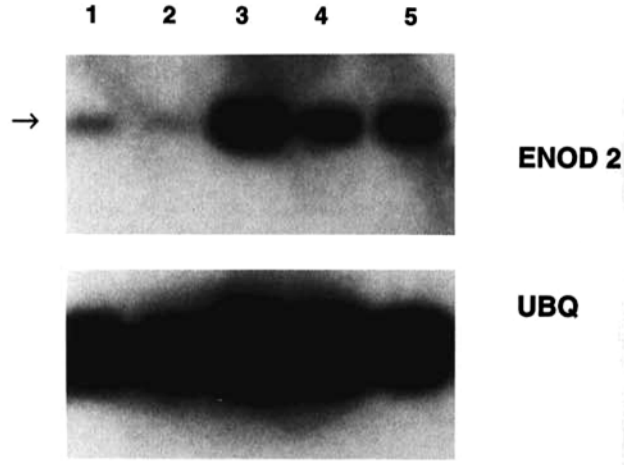


Fig. 4. Induction of ENOD2 in response to differing ratios of LCO-V (C18:1Δ11,Mefuc) to LCO-V (C16:0,Mefuc). Lane 1, 1:100; lane 2, 1:10; lane 3, 1:1; lane 4, 10:1; lane 5, 100:1. One hundred nanograms of a mixture of the two LCOs in the ratios indicated was inoculated onto *Glycine soja* roots and the plants were incubated for 6 days. RNAs were isolated and processed as described in Methods. An arrowhead indicates the amplified DNA fragments for ENOD2 of the expected size. Lower panel shows the transcripts for ubiquitin.

DISCUSSION

Single lipo-chitin nodulation signals purified from *Rhizobium meliloti, Azorhizobium caulinodans, B. elkanii,* and *B. japonicum* have been shown to induce nodule-like structures on alfalfa *(Medicago sativa,* Truchet et al. 1991), *Sesbania rostrata* (Mergaert et al. 1993) and soybean (*G. soja,* Stokkermans and Peters 1994; Minami et al. 1996), respectively. The ultrastructure of the nodule primordia formed in each case closely resembled that formed by bacterial inocula-

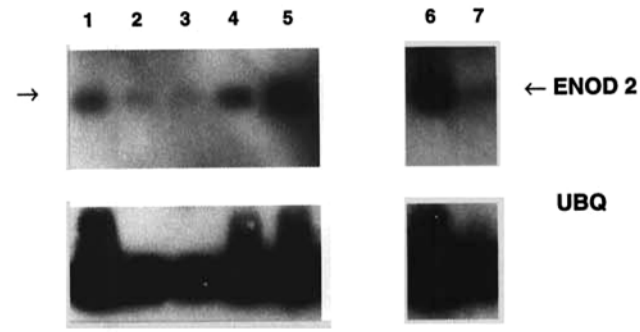


Fig. 5. Induction of ENOD2 by the mixtures of active and inactive LCOs, as well as chitin tetramer and pentamer. Plant roots were treated with the mixture of LCO-V (C18:1Δ11,Mefuc) and lane 1: NodBj-V (C18:1Δ11); lane 2: LCO-IV (C18:1Δ11, Mefuc); lane 3: LCO-IV (C16:2Δ2, 9, SO4); lane 6: chitin tetramer; or lane 7: chitin pentamer, respectively. Plant roots were inoculated with a mixture of LCO-V (C16:0,Mefuc) and Lane 4: LCO-IV (C18:1Δ11,Mefuc) or Lane 5: LCO-IV (C16:2Δ2, 9, SO_4), respectively, and incubated for 6 days. RNAs were isolated and processed as described in Methods. An arrowhead indicates the amplified DNA fragments for ENOD2 of expected size. Lower panel shows the transcripts for ubiquitin.

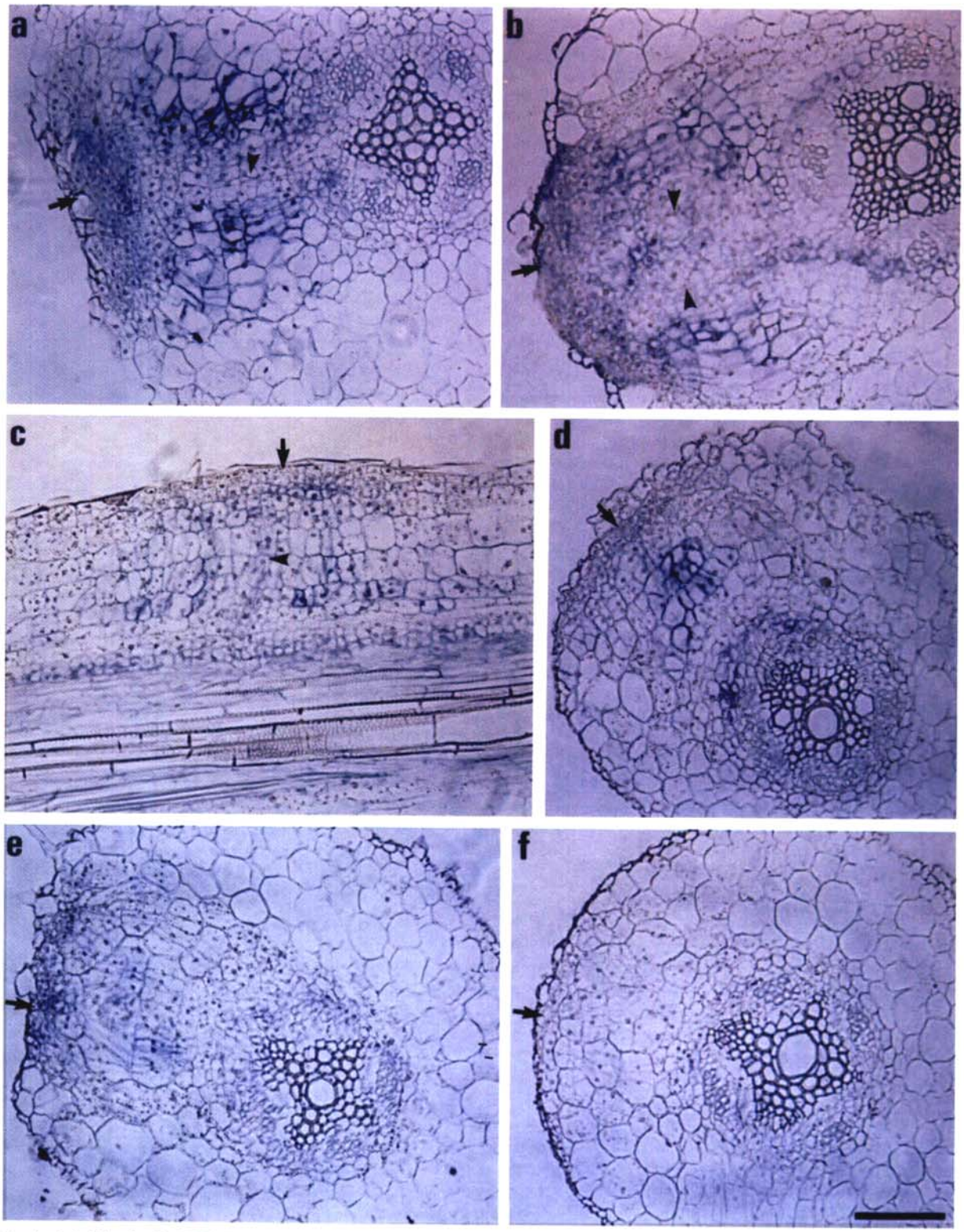


Fig. 6. In situ hybridization for ENOD2 expression in *Glycine soja* roots treated with mixtures of LCOs and/or chitin pentamer. Arrows and arrowheads indicate the nodule meristems and developing vascular strands, respectively. Hybridization signals are visible as purple or blue color development. All micrographs were taken at the same magnification (bar represents 100 μm). Hybridization with the sense probes gave no significant signal (data not shown). **A,** 7 days after inoculation with *Bradyrhizobium japonicum* strain USDA110. Hybridization signals appear at the cortical cells around the developing vascular strand; **B,** 13 days after treatment with a crude butanol extract of *B. japonicum* cultures induced for the expression of the *nod* genes; **C,** Longitudinal root section 7 days after treatment with a mixture of LCO-V(C18:1Δ11,Mefuc) and LCO-V(C16:0,Mefuc); **D,** 7 days after treatment with LCO-V(C18:1Δ11,Mefuc) and LCO-IV(C18:1Δ11,Mefuc); **E,** 7 days after treatment with LCO-V(C18:1Δ11,Mefuc) and chitin pentamer; **F,** negative control, root treated with LCO-V(C18:1Δ11,Mefuc) for 6 days. No hybridization signal is apparent.

tion. Since the early nodulins have been postulated to be involved in bacterial infection and/or nodule morphogenesis (Nap and Bisseling 1990), it is intriguing to investigate the relationships between nod signal inoculation and the induction of such early nodulin genes. Indeed, Journet et al. (1994) reported the rapid induction of ENOD12 expression when alfalfa roots were treated with the purified nod signal from *R. meliloti* (i.e., NodRm-IV [16:2Δ2,9, SO_3]). Likewise, Minami et al. (1996), Vijn et al. (1993, 1995), and Crespi et al. (1994) showed that ENOD40 expression could be induced in soybean, *Vicia sativa,* and alfalfa roots, respectively, treated with the appropriate lipo-chitin nodulation signal.

The induction of ENOD2 expression in soybean appears to require the combined action of at least two nod signals. One signal must be a lipo-chitin molecule with the ability to induce a HAD or NOI response when added singly to *G. soja* roots [e.g., LCO-V (C18:1Δ11,Mefuc), LCO-V (C18:1Δ9,Mefuc), LCO-V (C16:0,Mefuc) and LCO-IV (C16:0)]. However, there appears to be a wide latitude in the structural requirements for the second signal.

These results provide a possible explanation for the fact that rhizobia produce a mixture of lipo-chitin molecules. In the case of *B. japonicum* strain USDA135, we previously showed that five lipo-chitin molecules were produced (Carlson et al. 1993). *B. elkanii* strain USDA61 produces 10 structurally distinct substituted lipo-chitin nod signals. As shown above, *B. japonicum* strain USDA110 produces at least two additional nod signals besides the major NodBj-V(C18:1Δ11,Mefuc) nod signal. However, *B. japonicum* strain USDA110 has the potential to produce an even greater diversity of nod signals. For example, a *nolO* mutant of strain USDA110 was found to produce seven distinct substituted lipo-chitin molecules (Luka et al. 1993). Previously, we explained the production of these various lipo-chitin molecules based on a hypothesized function in host range determination and also as a consequence of the presence of various biosynthetic intermediates (Carlson et al. 1993; Luka et al. 1993). We can now offer an additional explanation in that structurally diverse nod signals appear to be a requirement for a full plant nodulation response, as exemplified by ENOD2 expression.

ENOD2 mRNA accumulation is exclusively induced in the nodule parenchyma (inner cortex) and is a good marker for the differentiation of parenchyma tissue during nodule development (Van de Wiel et al. 1990). Although inoculation of soybean roots with a single nod signal appears to be sufficient to trigger the nodule organogenesis program and the production of a nodule primordium, it does not induce ENOD2 expression. These data suggest that the differentiation of nodule parenchyma does not occur in those primordia induced by a single nod signal molecule. Clearly, a mixture of nod signals is required for the progression of nodule ontogeny to a stage in which nodule parenchyma differentiates, as evidenced by ENOD2 expression.

ENOD2 expression has been demonstrated in alfalfa and *S. rostrata* roots in response to the addition of cytokinin (Cooper and Long 1994; Dehio and deBruijn 1992). Furthermore, the application of auxin-transport inhibitors to alfalfa roots results in the expression of ENOD2 (Hirsch et al. 1989). Although similar studies of ENOD2 expression in soybean have not been done, it is very likely that the cooperative action of different nod signals results in an alteration of the phytohormone balance in the root cortical tissue. The action of two or more nod signals may lead to a stronger or more prolonged response than that obtained with a single molecule leading to a greater progression of nodule ontogeny.

An important feature of our earlier work (i.e., Minami et al. 1996) on ENOD40 expression was the demonstration that specific chemical substituents on the lipo-chitin nodulation signals, previously thought to be essential for biological activity (e.g., 2-*O*-methylfucose, Stacey et al. 1994), are not required for the rapid induction of some soybean early nodulin genes (i.e., ENOD40). However, these specific substituents are required for later events, thus providing evidence for our hypothesis that two distinct nod signal recognition events are involved. A major finding of the current work is that the expression of some early nodulin genes (i.e., ENOD2) requires the cooperative action of two, structurally distinct chitin signals, at least one of which must be an active lipo-chitin nodulation signal. We believe that these results provide the strongest evidence that at least two cellular recognition events are involved in nod signal action. The data argue that both recognition pathways need to be activated in order to induce ENOD2 expression. At present, the molecular mechanisms operable in these recognition pathways remain obscure.

In some respects our results are similar to those obtained by Ardourel et al. (1994) who suggested the involvement of two receptors in the recognition of nod signal by alfalfa. In this case, it was found that mutants of *R. meliloti* producing structurally distinct lipo-chitin nodulation signals elicited differing physiological responses. For example, unlike the wild-type *R. meliloti* strain, mutants defective in NodF and NodL function (involved in nod signal biosynthesis), were unable to induce infection thread formation and to penetrate into the host root. However, these mutants retained the ability to induce cell wall tip growth in trichoblasts (giving rise to root hairs) and were able to elicit the division of inner cortical cells. Thus, alfalfa roots appear to respond in different ways to *R. meliloti* strains producing structurally distinct lipo-chitin molecules. Ardourel et al. (1994) explained these results by way of a 'signaling receptor' responsible for the recognition of nonspecific signals leading to the induction of cell wall tip growth and cortical cell division. A more specific 'entry receptor' was required for recognition of specific nod signals leading to the initiation of infection thread formation, marked root hair curling, and the induction of a true nodule primordium. However, the model of Ardourel et al. (1994) differs in key steps from the data obtained with soybean. In soybean, the induction of cortical cell division and marked root hair curling show the same structural specificity with regard to nod signal recognition (Stokkermans et al. 1995). However, the induction of ENOD2 expression, that marks the progression of nodule ontogeny to a stage of primary differentiation of nodule parenchyma, requires two structurally distinct nod signals. Thus, it can now be established that full progression of the nodulation process involves the recognition of structurally diverse nod signal molecules.

Initial descriptions of *nod* gene induction in rhizobia in response to plant produced signal molecules (i.e., flavonoids) have now given way to more complex regulatory models involving multiple regulatory proteins (reviewed in Fisher and Long 1992; Kondorosi 1992; Stacey 1995). Similarly, the initial discovery of substituted lipo-chitin molecules was ex-

plained by simple models of receptor-signal recognition (reviewed in Denarie and Cullimore 1993; Schultze et al. 1994). However, such models only partially account for the presence of a large number of structurally distinct nod signals produced by a given rhizobial strain (e.g., Price et al. 1992; Schultze et al. 1992). This complexity is now compounded by the realization that plant recognition of these molecules involves at least two events with differing structural specificity. It is likely that further biological complexity will become apparent as researchers unravel the cellular signal transduction pathways involved in nod signal action.

METHODS

Nod signal nomenclature.

In this report, LCO refers to chemically synthesized lipochitin molecules. Nod signals purified from rhizobial cultures are given the designation Nod followed by the first letter of the genus and species (e.g., Nod*Bj* for a Nod signal purified from *B. japonicum*). The roman numeral following these designations indicates the number of *N*-acetylglucosamine residues (e.g., V indicates a pentamer). The specific fatty acid substituent (i.e., chain length, unsaturation, and double bond location ; e.g., C18:1Δ11) is given in parentheses. Finally, the presence of a 2-*O*-methylfucose residue is designated by 'Mefuc,' of sulfur by 'SO_3,' or of acetate by Ac.

Plant materials and inoculation of LCOs or chitin oligomer.

Glycine soja seeds (PI 468397) were surface sterilized and germinated for 2 days in the dark as described by Stokkermans and Peters (1994). The seedlings were transferred to a plastic growth pouch (Mega International, Minneapolis, MN) and kept in a growth chamber with a cycle of 16 h light at 26°C and 8 h dark at 20°C for 2 days. Roots were then spot-inoculated with 100 ng of a single LCO or mixtures as indicated (w/w, total amount 100 ng unless otherwise noted), the purified nod signal from *B. japonicum nodZ* mutant (i.e., NodBj-V[C18:1Δ11]; Stacey et al. 1994) or chitin oligomers suspended in 1% (w/v) carboxymethylcellulose (CMC) (Stokkermans and Peters 1994). As controls, seedlings were inoculated with *B. japonicum* strain USDA110 at 1×10^9 cells/plant or mock inoculated with a CMC solution lacking nod signal. Plants were then returned to the growth chamber and examined periodically. For isolation of RNA, the root was section just above and below the site of spot inoculation and the tissue frozen in liguid nitrogen until further analysis.

Table 1 shows the list of LCOs used in this study. All compounds were chemically synthesized (Ikeshita et al. 1994), except NodBj-V (C18:1Δ11) purified from a *B. japonicum nodZ* mutant strain (i.e., strain NAD138; Stacey et al. 1994). The NodBj-V (C18:1Δ11) compound was shown to be chemically pure by NMR and FAB-MS (Stacey et al. 1994).

Isolation of total RNA, reverse transcription, and polymerase chain reaction.

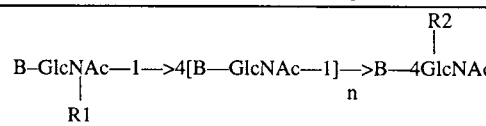
Total RNAs were isolated from nodules induced by bacterial inoculation, nodule primordia induced by LCO inoculation, root segments treated with chitin oligomers, or roots mock inoculated. Total RNA was isolated using the hot phenol method (Kohrer and Domdey 1991). For the preparation of RNA from nodules formed by bacterial infection, nodule primordia by LCOs, and root segments inoculated with chitin oligomer, at least 4, 8, and 8 plants were used, respectively. After treatment with RNAse-free DNAse I (Promega Co. Ltd), a 0.5-μg portion of total RNA was reverse-transcribed by M-MLV reverse transcriptase (United States Biochemical Co., Cleveland, OH) using oligo(dT)12-18 as a primer according to the manufacturer's manual. Products were recovered by ethanol precipitation after addition of 10 μg of glycogen (Behringer Mannheim, Indianapolis, IN), dissolved in 50 μl of sterile H_2O, and frozen until further analysis. An aliquot (5 μl) was utilized for polymerase chain reactions in the presence of 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 1 unit of Taq DNA polymerase (Promega Co., Madison, WI) for 30 cycles. PCR cycle conditions were as follows: 94°C, 1 min, 55°C, 1.5 min, 72°C 1.5 min, with 10 min of extension time at 72°C using primers at 0.5 μM specific to ENOD2 (forward; 5′-CGCTATCCACCATCCAACAA-3′, reverse; 5′-TAGAGTATTATACATAGGCA-3′; Franssen et al. 1989). As an internal control, PCR was also performed as described by Horvath et al. (1993) using primers identical in sequence to the soybean ubiquitin gene (forward; 5′-GGGTTTTAAGCTCGTTGT-3′, reverse; 5′-GGACACATTGAGTTCAAC-3′, Fortin et al. 1988). PCR products were separated by electrophoresis in 1.5% (w/v) agarose followed by blotting to a nylon membrane (Amersham Co., Arlington Heights, IL; Hybond-N). The ENOD2 (189 bp) and ubiquitin products (129 bp) were detected by hybridization with a ^{32}P-labeled 1.1-kbp *Hin*dIII fragment of genomic DNA for ENOD2 (Franssen et al. 1989) or cDNA fragment for ubiquitin amplified by PCR using the same primers followed by cloning into pCRII vector (Invitrogen Co., San Diego, CA). In the control experiments, RNA was treated in the same way without reverse transcriptase, and no significant signal was detected after hybridization (data not shown).

Chemical analysis of nod signals.

Mass spectra (MS) were obtained using a JEOL (Tokyo, Japan) SX/SX 102A tandem four-sector mass spectrometer, which was operated at 10 kV accelerating potential. Ions were produced by fast atom bombardment (FAB) with xenon using a JEOL FAB gun operating at 6 kV in a convential FAB ion source. Spectra acquired are averaged profile data of three scans as recorded by a JEOL complement data system. These spectra were acquired from 200 to 2,000 m/z at a rate that

Table 1. Natural and synthetic LCOs

LCO	R_1	R_2	n
NodBj-V(C18:1,MeFuc)	C18:1(11Z)	2-*O*-methylfucose	3
NodBj-V(C16:0,MeFuc)	C16:0	2-*O*-methylfucose	3
NodBj-V(C18:1	C18:1(11Z)	H	3
LCO-V (C18:1; MeFuc)	C18:1(11Z)	2-*O*-methylfucose	3
LCO-V (C18:1; MeFuc)	C18:1(9Z)	2-*O*-methylfucose	3
LCO-V (C16:0; MeFuc)	C16:0	2-*O*-methylfucose	3
LCO-1V (C16:0)	C16:0	H	2
LCO-V (C18:1; MeFuc)	C18:1(11Z)	2-*O*-methylfucose	2
LCO-V (C16:2Δ2,9;SO_3)	C18:1(11Z)	SO_3	2



would scan from m/z 0 to m/z 2,500 in 1 min. A filtering rate of 100 Hz and an approximate resolution of 1,000 were used in acquiring these spectra. The samples were dissolved in dimethyl sulfoxide and 1-ml aliquots were mixed with an equal volume of the FAB matrix, thioglycerol (TG), on the probe tip.

Fatty acid analysis was performed by hydrolyzing the sample in 0.5 ml of trifluoroacetic acid at 121°C for 3 h. The released fatty acids were extracted into chloroform and the chloroform was evaporated using a stream of air. The fatty acids were converted into their methyl esters by reacting in methanolic 1 M HCl at 80°C for 2 h. The solvent was evaporated with a stream of air and the fatty acid methyl esters were dissolved in hexane and analyzed by gas liquid chromatography (GLC)-MS using a capillary column from J & W Scientific.

In situ hybridization.

Root segments at various times after Nod signal inoculation were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in 10 mM sodium phosphate buffer (pH 7.0) and 100 mM NaCl at 4°C overnight. They were dehydrated through a graded ethanol series and embedded in Paraplast Plus. In situ hybridization was carried out as described previously (Kouchi and Hata 1993) with some modifications. The sections (8 to 10 μm thick) on slides were hybridized with digoxigenin-labeled RNA probes for ENOD2 at 52°C for more than 16 h. After hybridization, successive washing steps were as follows: twice at 4× SSC at room temperature for 10 min each, in RNase A (50 μg/ml) in STE (10 mM Tris-Cl, pH 7.5, 0.3 NaCl and 5 mM EDTA) at 37°C for 30 min, 3 times in STE at 37°C for 10 min each, once in 0.5× SSC at 55°C for 20 min, and twice in 0.5× SSC at room temperature for 10 min each. The sections were then processed for immunodetection of digoxigenin as described (Kouchi and Hata 1993). In every case, the sense probe of ENOD2 did not give a hybridization signal above background level (data not shown).

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