Aberrant Nodulation Response of *Vigna umbellata* to a *Bradyrhizobium japonicum* NodZ Mutant and Nodulation Signals

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The (*Brady*)*rhizobium* nodulation gene products synthesize lipo-chitin oligosaccharide (LCO) signal molecules that induce nodule primordia on legume roots. In spot inoculation assays with roots of *Vigna umbellata*, *Bradyrhizobium elkanii* LCO and chemically synthesized LCO induced aberrant nodule structures, similar to the activity of these LCOs on *Glycine soja* (soybean). LCOs containing a pentameric chitin backbone and a reducing-end 2-*O*-methyl fucosyl moiety were active on *V. umbellata*. In contrast, the synthetic LCO-IV(C_{16:0}), which has previously been shown to be active on *G. soja*, was inactive on *V. umbellata*. A *B. japonicum* NodZ mutant, which produces LCO without 2-*O*-methyl fucose at the reducing end, was able to induce nodule structures on both plants. Surprisingly, the individual, purified, LCO molecules produced by this mutant were incapable of inducing nodule formation on *V. umbellata* roots. However, when applied in combination, the LCOs produced by the NodZ mutant acted cooperatively to produce nodulelike structures on *V. umbellata* roots.

Species of *Rhizobium*, *Azorhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Bradyrhizobium* (collectively called rhizobia) enter into a mutualistic association with legumes in which the bacteria exchange reduced nitrogen for sources of carbon and energy. This association is initiated when plant flavonoids induce expression of the rhizobial nodulation genes (Peters et al. 1986; Kosslak et al. 1987; Banfalvi et al. 1988). In turn, the products of the nodulation genes synthesize a class of molecules known as Nod signals or lipo-chitin oligosaccharides (LCOs) that activate a developmental program in the host legume that leads to the formation of nodules in which nitrogen fixation occurs (Lerouge et al. 1990). The general structure of

LCO molecules consists of mono acylated oligomers of three to five β -1,4-linked, *N*-acetyl glucosamine units (chitin). Different strains of rhizobia produce LCOs with a wide variety of modifications to both terminal sugars (see Figure 1 for LCO structures). For example, *B. japonicum* produces a pentameric LCO substituted at the nonreducing end with a mono-unsaturated 18 carbon fatty acid chain and at the reducing end with a 2-*O*-methyl fucose (Sanjuan et al. 1992). Thus, this molecule is named NodBj-V(C_{18:1},Mefuc). It has been postulated that the chemical nature of Nod signals produced by different strains of rhizobia might control host range (for reviews, see Dénarié et al. 1996; Long 1996; Hanin et al. 1999; Cohn et al. 1998).

The induced nodule developmental program includes stimulation of root cortical cell division and dedifferentiation of cell types. On some plants (e.g., *Medicago sativa*), application of the correct LCO induces nodule structures that contain bifurcating vascular bundles and a mitotic center in the appropriate region of the root cortex (Truchet et al. 1991). However, on other plants, incomplete or aberrant nodule development has been observed in response to LCO addition. For example, nodule primordia did not develop past the primordium stage and vascular bundles were not observed on *Vicia sativa* roots treated with the appropriate Nod signal (Spaink et al. 1991). Similarly, on *Sesbania rostrata* Bremek. et Oberm., it was reported that LCO induced swellings at the base of secondary roots that, at a low frequency, developed into nodule structures (Mergaert et al. 1993). Nod signals produced by *Rhizobium* sp. strain NGR234 induced pseudonodules on *Macropitilium atropurpureum* and *Vigna unguiculata* that resembled bacterially induced nodules (Relić et al. 1993, 1994). On soybean roots, LCO induced incomplete nodule structures. However, the structures appeared to reach a more advanced stage of development than seen on other plants such as *V. sativa* or *S. rostrata* (Stokkermans et al. 1995; N. K. Peters, *personal communication*).

Despite the large number of reports on the chemical structures and biological activity of LCO, there are limited data on structure activity relationships of LCO on different legumes (Bec-Ferté et al. 1993; Carlson et al. 1993; Firmin et al. 1993; Lerouge et al. 1990; Mergaert et al. 1993; Poupot et al. 1993;

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Price et al. 1992; Roche et al. 1992; Sanjuan et al. 1992; Spaink et al. 1991; Stokkermans and Peters 1994, Relić et al. 1993, 1994). For example, application of different LCOs to *V. sativa* revealed that biological activity was dependent on the presence of a polyunsaturated acyl moiety and acetylation on the nonreducing end of the molecule (Spaink et al. 1991). Pentameric and tetrameric molecules showed similar activities. Efficient nodule initiation (NOI) on *M. sativa* was found to require the presence of a doubly unsaturated acyl moiety and sulfation of the terminal, reducing *N*-acetyl glucosamine (Truchet et al. 1991).

Induction of nodule primordia on soybean (*Glycine soja*) was found to be independent of the acyl substitution but required the presence of a reducing end 2-*O*-methyl-fucosyl moiety, depending on the length of the chitin backbone (Stokkermans et al. 1995). In this study, pentameric LCOs, without the 2-*O*-methyl-fucose modification, were found to be inactive. However, a tetrameric compound lacking this modification was able to induce cortical cell divisions (Stokkermans et al. 1995).

In an attempt to learn more about the structural requirements necessary for LCO activity on different legumes, we tested several hosts of *B. elkanii* with the spot inoculation assay. *V. umbellata* (rice bean) was found to respond well to the application of LCO. The structural requirements of LCO for NOI on this legume were found to be similar to the requirements for activity on *G. soja*. One major exception was that the synthetic tetramer LCO-IV(C_{16:0}), shown to be active on *G. soja*, did not induce a mitogenic response on *V. umbellata* roots (Stokkermans et al. 1995). Apparently, only pentameric LCOs with a 2-*O*-methyl fucose modification are active on rice bean. However, a *B. japonicum* NodZ mutant that does not produce any LCO with this modification was able to form nodules on this plant. Surprisingly, combinations of LCO produced by the mutant bacterium were able to elicit a morphological response. This phenomenon is similar to earlier studies

Fig. 1. Chemical structure of the Nod signal produced by *Rhizobium* spp. Various chemical modifications are present at the R-group positions on Nod signals produced by various rhizobial strains: e.g., *Bradyrhizobium* spp. produce a Nod signal with a 2-*O*-methyl fucose present at R₁ and a singly unsaturated 18 carbon fatty acyl chain at the R₅ position.

Table 1. Nodulation initiation (NOI) activity of lipo-chitin oligosaccharide (LCO) on *Glycine soja* and *Vigna umbellata* roots

LCO tested ^a	<i>G. soja</i>	<i>V. umbellata</i>
LCO-V(C _{18:1Δ11} ,Mefuc)	+	+
NodBj-V(C _{18:1Δ11})	—	—
LCO-V(C _{18:1Δ9} ,Mefuc)	+	+
LCO-V(C _{16:0} ,Mefuc)	+	+
LCO-IV(C _{18:1Δ11} ,Mefuc)	—	—
LCO-IV(C _{16:0})	+	—
NodBj-IV(C _{18:1Δ11}) + NodBj-V(Ac,C _{18:1Δ11})	+	—

^a LCO (100 ng per spot) added to 4-day-old roots. A (+) score was given if roots had macroscopic nodule structures within 12 days post inoculation. (—) indicates no nodule structures formed.

that showed induction of the soybean early nodulin gene ENOD2 requires the presence of two Nod signals (Minami et al. 1996a). We report here the LCO structural requirements for NOI on *V. umbellata* and a description of the aberrant nodule structures formed.

RESULTS

LCO structural requirements for NOI.

To assay the LCO structural requirements necessary to induce NOI on *V. umbellata*, a collection of different natural product and synthetic LCOs were spot inoculated onto *V. umbellata* roots. (Fig. 1 and Tables 1, 2). In 10 separate experiments, application of 100 ng of NodBj-V(C_{18:1},Mefuc) resulted in 60% or more of the plants showing a positive NOI response. All of the fractions of LCO produced by *B. elkanii* had NOI activity but, because many of these fractions contained mixtures of LCO, it was not possible to make firm conclusions about structure/function relationships. To get more detailed information about the structural requirements of LCO for NOI activity on *V. umbellata*, several synthetic LCOs were assayed with approximately 10 to 100 ng (10⁻¹¹ to 10⁻¹⁰ M) per spot (Tables 1, 2). No false positives were observed with the application of 1% carboxymethylcellulose alone, so any synthetic LCO inducing NOI is a true positive (data not shown). Of the synthetic LCOs tested, three were active;

Table 2. Nodulation initiation (NOI) activity of lipo-chitin oligosaccharide (LCO) on *Vigna umbellata*

LCO tested	NOI activity ^a
LCO-V(C _{18:1Δ11} ,Mefuc)	+
NodBe-V(C _{18:1Δ11} ,Mefuc)	+
NodBj-V(C _{18:1Δ11} ,Mefuc)	+
LCO-V(C _{18:1Δ9} ,Mefuc)	+
LCO-V(C _{18:1Δ11} ,Mefuc)	+
LCO-V(C _{16:0} ,Mefuc)	+
LCO-IV(C _{16:0})	—
LCO-IV(C _{18:1Δ11} ,Mefuc)	—
LCO-IV(C _{18:1Δ11} ,Mefuc,RGro)	—
LCO-IV(C _{18:1Δ11} ,Mefuc,SGro)	—
LCO-IV(C _{16:0} ,S)	—
LCO-IV(C _{16:2} ,Δ2,9,S)	—
NodBe-V(C _{16:0} ,Mefuc)	+
NodBe-V(Me,C _{18:1Δ11} ,Fuc,Gro)	+
NodBe-V(Ac,C _{18:1Δ11} ,Mefuc)	+
NodBe-IV(C _{18:1Δ11} ,Mefuc)	+
NodBe-IV(Cb,C _{18:1Δ11} ,Fuc,Gro)	+
NodBe-V(Cb,C _{18:1Δ11} ,NMe,MeFuc)	—
NodBe-IV(C _{18:1Δ11} ,NMe,MeFuc)	—
NodBe-IV(Cb,C _{18:1Δ11} ,NMe,Fuc,Gro)	—
NodBe-V(Ac,C _{18:1Δ11} ,Mefuc)	+
NodBe-V(Cb,Ac,C _{18:1Δ11} ,Mefuc)	+
NodBj-V(C _{16:0}) (= Peak 1 ^b)	—
NodBj-IV(C _{18:1} ,Gro)	—
NodBj-V(C _{18:1}) (= Peak 2)	—
NodBj-IV(C _{18:1}) (= Peak 3)	—
NodBj-V(Ac,C _{18:1}) (= Peak 4)	—
Mixture 1: All peaks NodBj-IV(C _{18:1} ,Gro)	+
NodBj-V(C _{18:1})	—
Mixture 2: NodBj-V(C _{18:1}) + Peak 2: NodBj-IV(C _{18:1} ,Gro)	+
NodBj-V(C _{18:1})	—
Mixture 3: NodBj-V(C _{18:1}) + Peak 3: NodBj-IV(C _{18:1})	+
V(Ac,C _{18:1})	—

^a LCO (100 ng) was spot inoculated onto 4-day-old roots. A (+) score was given if LCOs were able to induce macroscopically visible structures within 12 days post inoculation. (—) indicates no nodule structures formed. See Materials and Methods and Results.

^b High-pressure liquid chromatography (HPLC) peak fractions (see text).

LCO-V(C_{18:1Δ9},Mefuc), LCO-V(C_{18:1Δ11},Mefuc), and LCO-V(C_{16:0},Mefuc) (see Table 1). Tables 1 and 2 show NOI activity as either positive or negative. A positive NOI response included macroscopically visible nodule structures within 12 days post inoculation with LCO. Interestingly, LCO-IV(C_{16:0}) that is active on *G. soja* roots (Stokkermans et al. 1995; Minami et al. 1996a, 1996b) was not active on *V. umbellata* (Table 1). This compound was tested several times at varying concentrations up to 10⁻⁸ M with consistently negative results. In all experiments, a positive control of 100 ng (10⁻¹⁰ M) of NodBj-V(C_{18:1Δ11},Mefuc) was included. It was apparent from these data that only pentameric LCOs with a 2-*O*-methyl fucosyl moiety on the reducing end glucosamine were active on *V. umbellata*.

The activities of these synthetic LCOs are consistent with the findings that each of the active fractions of *Be*LCO contains some pentameric LCO with a methylfucose substitution. A detailed analysis of the structures of LCO produced by *B. elkanii* has been reported previously (Stokkermans et al. 1996).

LCO produced by NAD138.

A high-performance liquid chromatography (HPLC) elution profile of the LCO from the *B. japonicum* NodZ mutant NAD138 is shown in Figure 2A. The various Nod signals were distributed among four HPLC peaks. These four peaks contained five different structures that were identified by fast

Fig. 2. High-pressure liquid chromatogram of lipo-chitin oligosaccharides produced by the *Bradyrhizobium japonicum* NodZ mutant NAD138. Mass spectra—mass spectrometry data with molecular weight (M.W.) of each compound and a description of the chemical structure related to each peak.

atom bombardment–mass spectrometry (FAB-MS; spectra not shown), and by tandem mass spectra (MS)-MS of the various $[M+H]^+$ ions. The tandem MS-MS spectra are shown in Figure 2B–E. Peak 1 consisted exclusively of NodBj-V(C_{16:0}). Peak 2 contains approximately 85% of the total LCO and consists of a mixture of NodBj-IV(C_{18:1},Gro) and NodBj-V(C_{18:1}). If one assumes that the various LCOs ionize in the FAB source in an equivalent manner, then the relative intensities of their respective molecular ions would be indicative of their molar ratios. Based on that assumption, the molecules in peak 2 are present in a 1:1 ratio. Peak 3 consists of a mixture of NodBj-IV(C_{18:1}) and NodBj-V(Ac,C_{18:1}) in a 2.4:1.0 ratio. Peak 4 consists of a mixture of NodBj-V(Ac,C_{18:1}) and NodBj-V(C_{18:1}) in a 1:1 ratio. It is likely that peak 4 consists exclusively of NodBj-V(Ac,C_{18:1}) and that the detection of NodBj-V(C_{18:1}) is from the partial loss of the labile acetyl group subsequent to purification.

Multiple LCOs from NAD138 induce NOI on *V. umbellata* roots.

From the results of the NOI assay, it was assumed that a *B. japonicum* NodZ mutant, NAD138, would not be able to nodulate *V. umbellata*. This strain does not produce LCO with the 2-*O*-methylfucose on the reducing end (Stacey et al. 1994). Importantly, it was recently shown that the product of the *nodZ* gene is a fucosyl transferase (Quesada-Vincens et al. 1997; Quinto et al. 1997). To our surprise, NAD138 was able to nodulate *V. umbellata* with only a slight delay in initial nodule formation (data not shown). In a previous study, it was found that this same mutant was also capable of nodulating *G. max* and *G. soja* (Stacey et al. 1994). One explanation for this apparent paradox was that the *nodZ* mutant likely produced a nonfucosylated, tetrameric LCO. This idea arose from the knowledge that a synthetic LCO, LCO-IV(C_{16:0}), was shown to be capable of inducing an NOI response on *G. soja* roots (Stokkermans et al. 1996). A detailed analysis of LCO production by the mutant strain NAD138 revealed that the *nodZ* mutant produced a compound, NodBj-IV(C_{18:1}), that was postulated to be the factor necessary for *G. soja* nodulation. This natural product LCO was never applied to plants alone because it coelutes from a reverse phase HPLC column with another LCO (see above). However, as hypothesized, a peak that contains this tetramer was found to elicit a strong NOI response on *G. soja* roots (data not shown). Since neither the fatty acid chain length nor the degree of unsaturation affects mitogenic activity on this plant (Stokkermans et al. 1995), we conclude that the low abundance LCO NodBj-IV(C_{18:1}) is responsible for the ability of the NodZ mutant to nodulate *G. soja*. However, these data are not sufficient to explain how this mutant can nodulate *V. umbellata*, since neither the mixture containing NodBj-IV(C_{18:1}) nor the synthetic compound LCO-IV(C_{16:0}) had activity on this plant (Table 2).

In an attempt to explain how NAD138 can nodulate *V. umbellata*, all of the individual peak fractions collected from HPLC eluates were tested with the spot inoculation assay. As seen in Table 1, none of the individual peaks showed any NOI activity. However, when pooled together, LCOs produced by the NodZ mutant acted cooperatively to induce nodulelike structures.

From a previous study (Stacey et al. 1994), we have in our collection purified NodBj-V(C_{18:1}) that was analyzed by FAB-

MS analysis and determined to be a single molecule. The HPLC peaks obtained in this study were purified on a different reverse phase column, possibly explaining the coelution of LCO. The previously purified compound NodBj-V(C_{18:1}) was mixed with either peak 2 or 3. Peaks 2 and 3 contained mixtures of NodBj-IV(C_{18:1},Gro) + NodBj-V(C_{18:1}), and NodBj-IV(C_{18:1}) + NodBj-V(Ac,C_{18:1}), respectively. Neither of these peaks displayed any response when applied to *V. umbellata* roots alone. However, both peaks 2 and 3 were active when applied in combination with NodBj-V(C_{18:1}). Peak 4 likely contains only NodBj-V(C_{18:1}). Addition of this peak with the previously purified compound to *V. umbellata* roots displayed no NOI activity (data not shown). These data suggest that multiple LCOs with distinct structures are required for NOI activity on *V. umbellata*. It is possible that a combination of pentameric and tetrameric LCOs is required for biological activity on *V. umbellata* roots. The fact that peaks 2 and 3 alone did not have any activity is difficult to explain. It is possible that the concentration of total LCOs in these peaks was not sufficient to produce a response. It is also possible that the ratio of the different LCOs is critical to their ability to signal the plant. This phenomenon was seen in our earlier work, in which we showed that multiple LCOs, added in specific ratios, were required to initiate transcription of the soybean early nodulin gene ENOD2 (Minami et al. 1996a). In this study, we found that LCO-V(C_{18:1},Mefuc) had to be in a 1:1 ratio or higher with respect to LCO-V(C_{16:0},Mefuc) in order for the mixture to activate ENOD2 transcription in *G. soja* roots. Peak 2, the most abundant peak, was inoculated onto *V. umbellata* roots in several different experiments and no activity was ever detected, even when the total concentration of LCO was approximately 10⁻⁹ M per spot. These data argue against the possibility that NOI induction by LCO produced by the NodZ mutant is simply due to a concentration effect. Therefore, we conclude that the combined effect of multiple LCO molecules produced by the NodZ mutant is the most likely explanation as to how this mutant is able to nodulate *V. umbellata*.

LCOs induce abnormal nodule structures on *V. umbellata* roots.

To better determine the responsiveness of *V. umbellata* to LCO, roots were spot inoculated with various concentrations of NodBj-V(C_{18:1}, Mefuc), the most abundantly produced LCO from *B. japonicum*. This particular LCO induced nodulelike structures at 10 and 100 ng (10⁻¹¹ to 10⁻¹⁰ M) per spot, but did not induce any response when applied at lesser amounts. The major LCO produced by NAD138, NodBj-V(C_{18:1}), showed no response even when up to 10⁻⁸ M was applied to *V. umbellata* roots. The nodules that were induced by LCO were usually large, suggesting extensive nodule development. Additionally, several nodule structures were observed that contained emerging lateral roots (Fig. 3A and B). Similar lateral roots emerging from nodules were observed on *V. umbellata* roots that had been inoculated with the ineffective *B. elkanii* strain USDA61. Many large nodules induced with LCO attained an irregular surface, as observed under the stereomicroscope (Fig. 3B). The normal appearing nodules, large nodules with an irregular surface, and nodules with emerging lateral roots were analyzed microscopically, as seen in Figure 3C. *B. japonicum* USDA110 and *Rhizobium* sp. strain NGR234, which has a broad host range, were unable to infect *V. umbellata*, but were

able to induce nodule structures similar to those formed upon addition of LCO (data not shown). These results might be explained by the fact that growth pouches were used in our assay system to facilitate the visualization of nodule structures. It should be noted that NGR234 was recently reported to be capable of infecting *V. umbellata* when the plants were grown under different conditions (Pueppke and Broughton 1999).

From the sectioned LCO-induced nodules, it is evident that the nodule structures of *V. umbellata* do not resemble normal determinate nodule development in several respects. First, none of the LCO-induced nodule structures contain a clear mitotic center (Fig. 3C). Instead, the outer cortex of the nodule structure consists of smaller cells in which neither a clear mitotic center nor an epidermal layer can be distinguished. Second, a zone of large cells surrounds the vascular traces, and most of the peripheral cells in this zone contain densely

staining, thick cell walls. These cells resemble the cells that have been reported to contain calcium oxalate crystals in LCO-induced nodules on bean (Martinez et al. 1993; Sutherland and Sprent 1984). Third, some nodule structures contain multiple vascular bundles that often branch and that are positioned in a way that has not been observed in determinate nodules that contain rhizobia or in LCO-induced nodules of *G. soja* (Fig. 3C; Sen et al. 1986; Stokkermans and Peters 1994). Fourth, some of these vascular bundles give rise to lateral roots that emerge from the nodule structure (Fig. 3B). These nodule-lateral root hybrid structures may contain only one central vascular bundle. However, some also contain a vascular bundle that bifurcates in the nodule structure portion and comes together again to form a central vascular bundle in the lateral root portion, as seen with a structure induced with ineffective rhizobia (Fig. 3C).

Fig. 3. Micrographs of Nod factor-induced, nodulelike structures on *Vigna umbellata*. **A**, Whole mount nodule at emerged stage (ns). Bar = 1 mm. **B**, Whole mount nodule at emerging stage (ns) from which a lateral root (lr) has emerged. Bar = 1 mm. **C**, Cross section of an emerged nodule demonstrating formation of multiple vascular traces. Bar = 100 μ M. nb, nodular vascular bundle; tc, larger cells with thick cell wall in inner cortex of nodule structure; sc, small cells in outer cortex.

DISCUSSION

Several recent reports have established that both natural product and synthetic LCOs are capable of initiating nodule development on the roots of leguminous plants (for reviews, see Dénarié et al. 1996; Long et al. 1996; Cohn et al. 1998). It is clear that the structure of these molecules is critical for proper initiation of the morphogenic response, as specific chemical substitutions to the core structure have been proposed to play a role in controlling the host range of various *Rhizobium* spp. Importantly, it has been shown that application of the correct LCO to certain legumes results in varying degrees of development that depend on the plant inoculated (Truchet et al. 1991; Spaink et al. 1991; Mergaert et al. 1993; Stokkermans and Peters 1994). To further investigate the role of LCO in nodule development, we attempted to determine what effect the major LCO produced by *Bradyrhizobium* spp., namely NodBj-V(C_{18:1}, Me-fuc), would have on a variety of different legumes.

One of the tropical legumes studied, *V. umbellata*, displays a strong mitogenic response to LCO produced by *B. japonicum* and *B. elkanii*. The results of a detailed study of the LCO structural requirements for NOI on this plant closely resemble the results obtained from a previous study with *G. soja* as host plant (Stokkermans et al. 1995). The only difference noted was the fact that *V. umbellata* displayed no response to a synthetic compound, LCO-IV(C_{16:0}), previously shown to be active on *G. soja* (Stokkermans et al. 1995; Minami et al. 1996a, 1996b). From our results with the spot inoculation assays, we concluded that only rhizobia that produced pentameric LCO with a reducing end 2-*O*-methyl fucose would be able to nodulate *V. umbellata*. We were able to test this theory with the *B. japonicum* nodZ mutant strain NAD138, which does not produce any LCO with a 2-*O*-methyl fucosyl moiety (Stacey et al. 1994; Quesada-Vincens et al. 1997). We predicted that this mutant would not be able to nodulate *V. umbellata*. Surprisingly, this mutant was able to induce nodulelike structures on this plant.

Results from earlier work in our laboratory revealed the importance of the cooperative action of LCO molecules in the induction of the early nodulin gene ENOD2 (Minami et al. 1996b). These data stressed the requirement of rhizobia to produce multiple signals for the induction of plant genes involved in nodulation (Ardourel et al. 1994; Minami et al. 1996a). We have shown in this report that the action of multiple Nod signals might also act to extend the host range of rhizobia. Pentameric LCOs with 2-*O*-methyl fucose are sufficient to produce nodule structures on *V. umbellata*. However, the same developmental program can be induced via the action of multiple LCOs that do not have this reducing end modification. This might explain how the NodZ mutant is able to form nodule structures on *V. umbellata*. These data suggest that mixtures of Nod signals might be important determinants of host range for rhizobia.

The nodule structures produced by LCO or rhizobia on *V. umbellata* are very distinct from the nodule structures produced on *G. soja*. This was surprising with respect to the similarity of the LCO structural requirements for NOI induction. Several conclusions can be drawn from this observation. First, on *V. umbellata*, successfully infecting rhizobia are likely required for correct nodule development. These data suggest that additional signals not produced by the strains tested may be required for proper nodule development on *V. umbellata*. Second, the formation of lateral roots emerging out

of nodules indicates that, under certain conditions, the nodule developmental program can convert to the developmental program of a lateral root that contains a meristem. Interestingly, a similar alteration of nodule development has been observed on nodules formed on *G. max* roots transformed by *Agrobacterium rhizogenes* (Gresshoff 1993). In this case, it was proposed that these determinate nodules were altered by transformation to contain a persistent meristem.

In the aberrant nodule structures induced by LCO on *V. umbellata*, several independent vascular traces or bundles emerge from the root at the point of inoculation. In these vascular bundles there is no evidence of branching, rather each appears to be independently initiated from the root vascular bundle. Some pairs of these bundles resemble the vascular structures induced on *G. soja* (Stokkermans and Peters 1994). On *G. soja* these vascular traces had been interpreted as branching indicative of normal nodule development (Stokkermans and Peters 1994). However, in light of the structures produced on *V. umbellata*, it is reasonable to reexamine the structures on *G. soja*, recognizing that they likely represent independent vascular initiations. The data presented here suggest that additional signals, distinct from Nod signals, are necessary for proper nodule development.

MATERIALS AND METHODS

Plant material.

Seeds of *Lespedeza striata* (Thunb. ex J. A. Murray) Hook. & Arnott, *Crotalaria spectabilis* Roth, *Vigna aconitifolia* (Jacq.) Maréchal, *Vigna radiata* (L.) R. Wilcz., and *Vigna umbellata* (Thunb.) Ohwi & Ohashi were obtained from S. G. Pueppke, University of Missouri, Columbia, and similar *V. umbellata* seeds from The Bean Bag (Oakland, CA). *Glycine max* (L.) Merr. 'Williams', *V. aconitifolia*, *V. radiata*, and *V. umbellata* seeds were surface sterilized in 4% hypochlorite for 10 min, followed by 10 min in 0.01 N HCL. *L. striata* and *C. spectabilis* seeds were treated with concentrated sulfuric acid for 15 and 30 min, respectively. Seeds were washed thoroughly after each treatment with sterile, distilled H₂O and germinated in the dark for 2 days. Upon germination, the seedlings were aseptically transferred to growth pouches (Mega Int., Minneapolis) and allowed to grow in the dark until the roots were between 2 and 6 cm long (generally, 2 days of growth).

Rhizobial inoculation.

Bacteria were grown to log phase in Bergersen's minimal medium (Bergersen 1961) and diluted in sterile water to 10⁷ cells per ml. Ten milliliters of rhizobial inoculum was applied per plant grown in sterile vermiculite pots and 1 ml per plant for plants grown in growth pouches. Plants were grown in a growth chamber with a light/dark cycle of 16/8 h at 28/22°C with 70 to 80% relative humidity. Nodules were harvested between 12 and 18 days post inoculation.

Spot inoculation with LCOs.

LCOs from *B. elkanii* USDA61 and *B. japonicum* NAD138 were isolated as described in Carlson et al. 1993. The chemical structures of these LCO molecules were determined previously and are shown in Figure 1 (Carlson et al. 1993; Stacey et al. 1994; Stokkermans et al. 1996). Concentrations were determined with an extinction coefficient of 1,445 cm⁻¹M⁻¹ at

210 nm. To estimate the molar concentration of LCO in fractions that had been isolated as mixtures, the average molecular weight of the LCO molecules in the fraction was used. For example, the average molecular weight of LCO in mixture 1 is 1,366 g mol⁻¹ (Table 1 and Carlson et al. 1993). LCOs were diluted to 1 mg/ml in 1% carboxymethylcellulose (Sigma, St. Louis, MO) to increase the viscosity of the solution. For the mixtures used, the concentration of each peak was calculated and first resuspended in 50% vol/vol acetonitrile/H₂O. Equal volumes of each solution that contained a single peak were mixed in a separate vial with a 1 mg/ml solution of the major LCO, NodBj-V(C_{18:1}), produced by strain NAD138 and purified as described previously (Stacey et al. 1994). This solution was dried under nitrogen and resuspended to 1 mg/ml in 1% carboxymethylcellulose. The LCOs used in these experiments are the same LCO fractions used previously for structural analysis (i.e., Carlson et al. 1993; Stacey et al. 1994), excluding the LCO isolated in this work from NAD138. Synthetic LCOs were kindly provided by T. Ogawa (The Institute of Physical and Chemical Research, Wako, Japan). Spot inoculation of roots was performed as described (Stokkermans and Peters 1994).

Preparation of root tissues.

Roots were cleared for analysis following the method of Truchet et al. (1989). A 3-cm piece of treated root sample, including the inoculation site, was cut from the root and placed in 1% hypochlorite for 2 h. The roots were placed in 0.01% methylene blue for 1 h, and destained in 35% ethanol for 1 h and 70% ethanol for 10 to 16 h. The frequency of NOI was monitored by observation under the stereomicroscope at $\times 10$ to $\times 40$ magnifications. In most cases, roots were cleared and stained to identify true positives, but the nodulelike structures were large enough that this was not necessary.

For sectioning, roots were fixed at room temperature for 12 to 24 h with gentle agitation in 4% paraformaldehyde in 10 mM sodium phosphate buffer, pH 7.2 with 150 mM NaCl (PBS). The samples were rinsed in PBS and dehydrated with an ethanol series to 96% ethanol. Infiltration and embedding in JB-4 resin (Polysciences, Warrington, PA) were carried out according to the manufacturer's specifications. Alternatively, nodule tissue was fixed and sectioned as previously described by Roth and Stacey (1989).

Microscopy and photography.

Longitudinal serial sections of 8-mm thickness were cut and transferred onto drops of water on slides, heated to dryness, stained with 0.01% methylene blue for 30 min, and destained with 70% ethanol for 1 h. Sections were photographed with a Nikon FX-35WA camera together with a Nikon AFX-IIA photomicrographic system on a Zeiss Axioskop Microscope at $\times 100$ and $\times 200$ magnification.

Nod signal purification and analysis.

LCOs were purified from *Bradyrhizobium* strains as previously described (Sanjuan et al. 1992). HPLC analysis was performed on a Waters HPLC with a C18 reverse phase column. Millennium software (Waters) was used to analyze the data.

Mass spectrometry.

All mass spectra (MS) were obtained with an SX/SX 102A (JEOL, Tokyo) tandem four-sector mass spectrometer, which

was operated at 10 kV accelerating potential. Ions were produced by FAB with xenon, with a JEOL FAB gun operated at 6 kV in a conventional FAB ion source. Spectra acquired for the first MS 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 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 (a 10% valley) 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. The tandem MS analyses were performed on the monoisotopic mass ions. Collisional activation was performed in the third field free region, with helium as the collision gas. The helium pressure was sufficient to attenuate the primary ion beam by 75%. The collision cell was floated at 3 kV, providing a collision energy of 7 kV. Acquired tandem MS are averaged profiles of four scans as recorded by the JEOL complement data system.

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