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Isolation of *Rhizobium meliloti nod* Gene Inducers from Alfalfa Rhizosphere Soil

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Methanolic extracts of alfalfa rhizosphere soil induce more *nod* gene transcription in *Rhizobium meliloti* than extracts of nonrhizosphere soil. Six peaks of *nod*-inducing activity were separated by high-performance liquid chromatography from rhizosphere soil extract, and one compound was identified by ^{1}H nuclear magnetic resonance, mass spectrometry, and UV-visible spectra as a formononetin-7-*O*-glycoside that activates both NodD1 and NodD2 proteins. The unanticipated presence of a glycoside in rhizosphere soil suggests either that large amounts of the glycoside were exuded by roots or that some glycosides are unexpectedly stable in soil.

The principle that plants use small molecules as signals to microbes was established clearly by data showing that some simple phenolics and flavonoids induce transcription of virulence (*vir*) genes in *Agrobacterium* spp. (27) and nodulation (*nod*) genes in *Rhizobium* (5, 20, 24) and *Bradyrhizobium* (12) spp. More complete descriptions of the many biologically active signals that several legumes release are now available (21, 23). Defining the diversity of *nod* gene inducers released by alfalfa has contributed unexpected new knowledge by identifying two betaines that activate the NodD2 but not the NodD1 regulatory protein in *Rhizobium meliloti* (22).

Although the capacity of legumes to release *nod* gene inducers under controlled laboratory conditions has been described, the reality of which signals are present in the natural soil ecosystem has not been explored. Numerous phenolics and flavonoids have been found in soil systems (26), but those studies usually isolated molecules by structural traits and not on the basis of biological activities. Searches for allelochemicals are exceptions to this generalization (e.g., isolation of medicarpin, an isoflavonoid inhibitor of seed germination [4]), but there are no studies that report extraction of rhizobial transcriptional activators from soil, the ecosystem in which they are assumed to function. Because the demonstrated presence of *nod* gene inducers in soil is crucial for establishing principles of rhizosphere ecology, this investigation analyzed soil extracts for the presence of compounds that induce transcription of a *nodC-lacZ* fusion in *R. meliloti*.

Bacteria. Four *R. meliloti* strains were used in these studies: the parent strain 1021 (17); 1021(pRmM57), which contains a *nodC-lacZ* fusion and the associated *nodD1* on pRmM57 (19); JM57(pRmJ30), which contains a *nodC-lacZ* fusion on pSym (JM57) (19) plus *nodD1* on multiple copies of pRmJ30 (10); and JM57(pRmM137), which contains *nodD2* on multiple copies of pRmM137 (6). Strains were donated by S. R. Long, Stanford University. Expression of *nodC-lacZ* was measured as β-galactosidase activity (18) by using previously described modifications (7). Activities of uninduced controls were subtracted.

Extraction and purification of *nod* inducers. Three types of soil samples were collected from four positions (replicate blocks) within a 10-m circle in a single field of 4-year-old Moapa 69 alfalfa at Davis, Calif. (i) Rhizosphere (≈1-cm thick) was defined as soil adhering to roots when the upper 20 cm of roots was removed from the soil and shaken. (ii) Nonrhizosphere soil was removed as cores (2-cm diameter by 40-cm long) from areas between plants in the alfalfa stand. (iii) Plant-free soil was collected as cores (2 by 40 cm) from soil that had been maintained bare for 4 years by cultivation. Every replicate for each type of soil sample contained at least 10 cores or plants. Core samples were separated into upper and lower 20-cm segments. Soil was a Zamora silt loam (fine-silty, mixed, thermic Mollic Haploxeralfs).

Soil was dried for 4 days at 95°C, stored at room temperature, and sieved through a 0.5-mm-pore-size screen to remove macroscopic organic matter. Samples were then extracted overnight at 4°C by shaking with 2 volumes of 50% methanol (MeOH) (1:1, MeOH-water). After the soil settled in the extraction solution, the supernatant was centrifuged, passed through 0.8-μm-pore-size polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.), and lyophilized. Dried samples were dissolved in 50% MeOH and centrifuged. Aliquots (100 μl) were injected into a Waters high-performance liquid chromatography (HPLC) system (Millipore Corp., Bedford, Mass.) and eluted at 0.5 ml/min in a 70-min linear gradient (0:99:1 to 99:0:1 [vol/vol/vol], MeOH-water-acetic acid) and isocratically from 70 to 90 min with MeOH-acetic acid (99:1, vol/vol). Eluting compounds were monitored for A_{max} at 230 to 400 nm with a Waters 990 photodiode array detector. Eluant fractions associated with absorbance peaks were collected and dried, and 20% of each peak was assayed for *nod* gene induction.

Larger-scale purifications were done with extracts obtained from 600 g of rhizosphere soil. Compounds separated on a semipreparative HPLC column under standard conditions were further purified by using appropriate methanolic gradients.

^{1}H nuclear magnetic resonance (NMR) measurements were recorded in [U-^{2}H]MeOH on a GN-300 Omega NMR

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TABLE 1. Transcription of a *nodC-lacZ* fusion in *R. meliloti* 1021(pRmM57) induced by methanolic extracts of soil taken from the top 20 cm of a 4-year-old alfalfa stand or an adjacent plant-free soil

Soil source	*nod*-inducing activity	
	β-Galactosidase (U/g of soil)	LE[a] (nmol/g of soil)
Plant-free soil	52	12.6
Alfalfa nonrhizosphere	145	35.4
Alfalfa rhizosphere	218	53.2
$LSD_{0.05}$[b]	44	10.6

[a] LE, luteolin equivalents.
[b] $LSD_{0.05}$, least significant difference at the 5% level.

spectrometer (General Electric Co., Fremont, Calif.). Fast-atom bombardment mass spectrometry (FAB-MS) data were collected with a ZAB-HS-2F MS (VG Analytical, Wythenshawe, United Kingdom) by using positive ionization (xenon, 8 keV, 1 mA) with a VG Dynamic FAB probe. FAB-MS measurements were made on samples dissolved in MeOH and injected with water-glycerol (95:5, vol/vol) at a flow rate of 5 μl/min. UV-visible spectra were recorded on a Lambda 6 dual-beam spectrophotometer (Perkin-Elmer, Norwalk, Conn.).

Occurrence of *nod* gene inducers in soil. Assays with *R. meliloti* 1021(pRmM57) showed that alfalfa rhizosphere contained 400% more *nod*-gene-inducing activity than plant-free soil (Table 1). The amount of *nod*-gene-inducing activity decreased with distance from the alfalfa rhizosphere, but soil that had not contained living alfalfa plants for at least 4 years still had detectable *nod*-gene-inducing activity. Rhizosphere extracts contained reproducibly higher levels of *nod*-gene-inducing activity than other soil samples in several experiments, but there were no significant differences in activity between the upper 20-cm and the lower 20-cm core segments in the nonrhizosphere samples.

HPLC analyses of rhizosphere extracts detected six fractions with *nod*-gene-inducing activity (Fig. 1A). Three of those eluant peaks contained compounds that activated both NodD1 and NodD2 proteins (Fig. 1B). Additional extractions of rhizosphere soil produced comparable results.

Structural traits of one rhizosphere compound. Repeated collections of the NodD1-NodD2 activator eluting at 66 min (Fig. 1) produced a small amount of material sufficient for ^1H NMR and MS analyses. The sample had a UV-visible spectrum, A_{max}, in MeOH at 203, 230, 249, 259, and 304 nm, similar to that of authentic formononetin-7-*O*-glucoside (15), and in the standard HPLC gradient it had the same retention time as that of a formononetin-7-*O*-(6″-*O*-malonylglycoside) isolated from the root exudate of alfalfa plants exposed to *R. meliloti* (3). Proton signals from the formononetin glycoside had the following resonances: δ_H ppm ([U-^2H]MeOH); 8.22 (1H, s, H-2), 8.14 (1H, d, J = 8.6 Hz, H-5), 7.47 (2H, d, J = 8.5 Hz, H-2′,6′), 7.24 (1H, s, H-8), 7.20 (1H, dd, J = 2.4, 8.6 Hz, H-6), 6.98 (2H, d, J = 8.6 Hz, H-3′,5′), 5.09 (1H, d, J = 7 Hz, H-1″), 3.90 (1H, d, J = 12 Hz, H-6″a), 3.81 (3H, s, OCH_3), 3.71 (1H, dd, J = 12, 7 Hz, H-6″b), 3.55 to 3.44 (4H, m, H-2″-5″). Those resonances were consistent with values measured in the same instrument for authentic formononetin-7-*O*-glucoside. FAB-MS analyses of the sample produced major ions at *m/z* values of 431 (relative intensity, 35) and 269 (relative intensity, 100), which were comparable to MH^+ values measured for authentic formononetin-7-*O*-glucoside and formononetin on the same instrument. There was no larger ion to support acylation of the sugar.

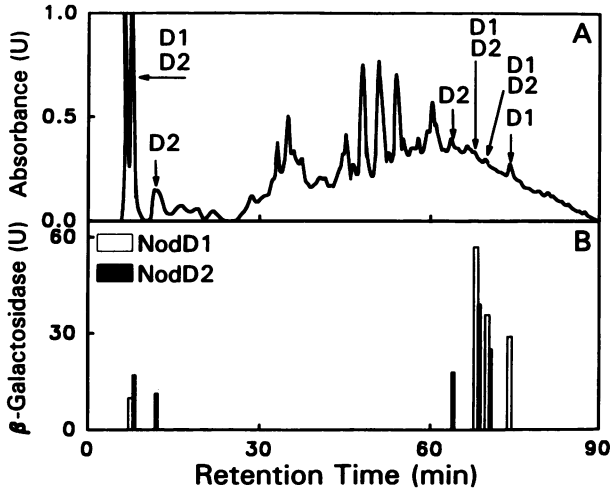

FIG. 1. HPLC characteristics and *nod*-inducing activity of natural products extracted from alfalfa rhizosphere soil. (A) A_{max} (240 to 400 nm) of eluant from 8 g of rhizosphere; (B) β-galactosidase activity induced from *nodC-lacZ* in *R. meliloti* strains containing extra copies of *nodD1* [JM57(pRmJ30)] or *nodD2* [JM57(pRmJ137)]. Luteolin (50 nM), a positive control for NodD1 activation, produced 24.5 ± 7.4 U of β-galactosidase activity, and trigonelline (10 μM), a positive control for NodD2 activation, produced 82.6 ± 3.4 U of β-galactosidase activity.

Activity of a rhizosphere compound. Assays that used the formononetin extinction coefficient (2) to measure concentration of the rhizosphere formononetin conjugate showed that the isoflavonoid induced significant transcription of *nodC-lacZ* fusions in the presence of extra copies of both *nodD1* and *nodD2* regulatory genes. Comparing the formononetin conjugate (100 nM) with luteolin (NodD1 activator) and trigonelline (NodD2 activator) controls in the same assay indicated that the soil compound was only 10% as active as luteolin but 200-fold more active than trigonelline

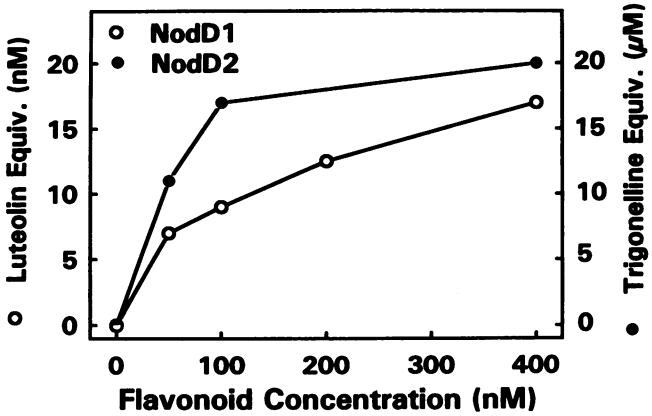

FIG. 2. Concentrations of a rhizosphere formononetin-7-*O*-glycoside required to produce *nod*-gene-inducing activities equivalent (Equiv.) to levels of β-galactosidase generated by luteolin or trigonelline controls. Compounds were assayed in *R. meliloti* strains containing a *nodC-lacZ* fusion with extra copies of *nodD1* [JM57(pRmJ30)] or *nodD2* [JM57(pRmM137)], which are induced by luteolin and trigonelline, respectively.

(Fig. 2). Because all of the purified sample was used in the assay whose results are shown in Fig. 2, further tests were not possible.

Data presented here offer the first evidence that inducers of rhizobial *nod* genes occur in a rhizosphere, the complex ecosystem in which they are postulated to effect nodule formation. Alfalfa rhizosphere contains more extractable *nod*-gene-inducing activity than soil further from the root (Table 1), but whether the compounds responsible for that activity are direct products of plant or microbial metabolism cannot be determined from this study. Although extracts of rhizosphere soil contained six separate peaks with *nod*-gene-inducing activity, HPLC retention traits and UV absorbance data indicated that no compounds were identical to the flavonoid and betaine *nod* gene inducers released from aseptically grown seedlings of the same alfalfa cultivar (21). Several soil compounds, however, had UV absorbance spectra similar to those measured for a *nod*-gene-inducing formononetin-7-*O*-(6″-malonylglycoside) found in root exudates of Moapa 69 alfalfa exposed to *R. meliloti* under controlled conditions (3).

The natural function of the *nod* gene inducers isolated in this study is not clear because the older tap roots from which the rhizosphere was collected were incapable of forming root nodules. Younger lateral roots in the same zone, however, may be infected by *R. meliloti* as a result of these compounds. It is interesting that assays for a single class of transcriptional regulators (i.e., *nod* gene inducers) in one microbe revealed so many active compounds in rhizosphere soil (Fig. 1). Because that activity declined significantly outside the rhizosphere (Table 1), these data are consistent with a role for root-associated compounds in the nodulation of legumes. The observations also suggest that defining how plants signal to soil microbes offers a new opportunity for understanding rhizosphere biology.

The limited amounts of active compounds that were isolated in this study did not permit a complete characterization of the *nod* gene inducers. One purified fraction contained a formononetin-7-*O*-glycoside that induced *nod* genes regulated by both NodD1 and NodD2 proteins (Fig. 2). It seems likely that the active compound in these samples was a derivative of the formononetin-7-*O*-glycoside because a natural formononetin-7-*O*-(6″-*O*-malonylglycoside), but not formononetin-7-*O*-glucoside, is an active *nod* gene inducer (3). Labile groups, such as malonyl, may have separated from the molecule during analyses (13). A second alternative is that the glycoside contains a sugar other than glucose which confers *nod*-gene-inducing activity on the formononetin. This second possibility is less likely because alfalfa synthesizes a number of isoflavonoid glucosides (11), and all formononetin conjugates isolated from roots of six other legumes were glucosides (13). Any acylation that may have been present on the sugar conjugated to the rhizosphere compound was not linked to C-6″ because the H-6″a and H-6″b proton signals were comparable to those observed in authentic formononetin-7-*O*-glucoside. The *nod* gene inducers in these soil samples may have included some of the formononetin conjugates noted previously in alfalfa root extracts (16). Those compounds may be exuded naturally in soil. It seems doubtful that compounds in Fig. 1 were extracted from root tissue, because small lateral roots and other organic debris were removed by sieving.

Flavonoid glycosides are found frequently in legumes (1, 11, 13, 14, 25), and they are exuded from roots under controlled conditions (3, 9). Whether the sugar on such molecules facilitates diffusion of the compound in soil is unknown. The ^{1}H NMR and FAB-MS data in this study, however, clearly support the unanticipated conclusion that a glycosidic microbial signal was present in alfalfa rhizosphere soil *R. meliloti* hydrolyzes luteolin-7-*O*-glucoside (8), and it generally is assumed that the typical carbon-limited microbes in soil use any carbon source, including glycosides, for energy (28). The fact that a glycoside was isolated suggests either that large amounts of the compound were exuded by roots or that this glycoside is unexpectedly stable in soil.

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