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Phospholipids of *Rhizobium* Contain *nodE*-determined Highly Unsaturated Fatty Acid Moieties*

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In *Rhizobium leguminosarum* the *nodABC* and *nodFEL* operons are involved in the production of lipooligosaccharide signals, which mediate host specificity. A *nodE*-determined highly unsaturated C18:4 fatty acid (*trans*-2,*trans*-4,*trans*-6,*cis*-11-octadecatetraenoic acid) is essential for the ability of the signals to induce nodule primordia (Spaink, H. P., Sheeley, D. M., van Brussel, A. A. N., Glushka, J., York, W. S., Tak, T., Geiger, O., Kennedy, E. P., Reinhold, V. N., and Lugtenberg, B. J. J. (1991) *Nature* 354, 125–130) and preinfection thread structures (van Brussel, A. A. N., Bakhuizen, R., van Spronsen, P. C., Spaink, H. P., Tak, T., Lugtenberg, B. J. J., and Kijne, J. W. (1992) *Science* 257, 70–72) on the host plant *Vicia sativa*.

We presently focus on the question of how these lipooligosaccharide signals are synthesized in *Rhizobium*. Here we show that after the induction of the *nodFE* genes, even in the absence of the *nodABC* genes, the *trans*-2,*trans*-4,*trans*-6,*cis*-11-octadecatetraenoic acid, which has a characteristic absorbance maximum of 303 nm, is synthesized; this shows that the biosynthesis of the unusual fatty acid is not dependent on the synthesis of the lipooligosaccharides. This finding also suggests that the biosynthesis of the unusual fatty acid is completed before it is linked to the sugar backbone of the lipooligosaccharide. In an attempt to identify the lipid fraction with which the unusual C18:4 fatty acid is associated, we found that it is linked to the *sn*-2 position of the phospholipids. Even when lipooligosaccharide signals are produced in a wild type *Rhizobium* cell, a fraction of the unusual fatty acid is still bound to all major phospholipids. These findings offer interesting possibilities. 1) The phospholipids might be biosynthetic intermediates for the synthesis of lipooligosaccharide signals, and 2) phospholipids, containing *nodFE*-derived fatty acids, might have a signal function of their own.

Rhizobium bacteria interact with leguminous plants in a host-specific manner, thereby causing the formation of nitrogen-fixing root nodules. Plant flavonoids induce the *nod* genes, which are rhizobial genes essential for nodulation. The *nodE* gene in the *nodFEL* operon is the main factor in determining the difference in host-specific characteristics of *Rhizobium leguminosarum* biovars (bv.)[1] *viciae* and *trifolii* (1). In *R. leguminosarum* bv. *viciae* the *nodABC* and *nodFEL* operons are involved in the production of lipooligosaccharide signals, which mediate host specificity. A *nodL*-determined *O*-acetyl substituent and a *nodE*-determined highly unsaturated C18:4 fatty acid (*trans*-2,*trans*-4,*trans*-6,*cis*-11-octadecatetraenoic acid) are essential for the ability of the purified signal molecules to induce nodule primordia (2) and preinfection thread structures (3) on the host plant *Vicia sativa*.

We now focus on the question of how these lipooligosaccharide signals are synthesized in *Rhizobium*. In this study we report on the synthesis of the unusual *nodE*-derived fatty acyl residue. Interestingly, in *nodE* mutants of *R. leguminosarum* bv. *viciae* only non-mitogenic lipooligosaccharides, substituted with the most abundant rhizobial fatty acid, *cis*-vaccenic acid, are formed (2). This finding implies the possibility that during lipooligosaccharide biosynthesis, first *nodABCL*-dependent lipooligosaccharides might be synthesized, and only in a second set of events might NodE be involved in the conversion of such non-mitogenic, *nodABCL*-dependent lipooligosaccharides into host-specific, mitogenic *nodABCFEL*-dependent lipooligosaccharides, substituted with a *trans*-2,*trans*-4,*trans*-6,*cis*-11-octadecatetraenoic acid. However, *nodE* shows homology to β-ketoacylsynthases, *nodF* is homologous to acyl carrier proteins (4), and the NodF protein carries the 4′-phosphopantetheine prosthetic group (5) characteristic for acyl carrier proteins, suggesting that, as an alternative, NodE and NodF work together in the synthesis of a possibly novel fatty acyl residue. Only after completion of the synthesis of such a *nodFE*-dependent fatty acid would it be introduced into nascent lipooligosaccharides.

We therefore investigated whether we could find novel, NodFE-derived compounds formed *in vivo* in *Rhizobium* after induction with flavonoids. In this report we show that, of all the *nod* genes, *nodFE* alone is sufficient for the synthesis of the host-specific *trans*-2,*trans*-4,*trans*-6,*cis*-11-octadecatetraenoic acid. We also demonstrate that this *nodFE*-derived fatty acid is linked to all major phospholipids in *Rhizobium*.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—Strains and plasmids are listed in Table I. Broad host range plasmids were mobilized from *Escherichia coli* KMBL1164 to *R. leguminosarum* by using pRK2013 as a helper plasmid (12) as previously described (13).

Cultures of *R. leguminosarum* were grown on medium B (14) at 30 °C on a gyratory shaker. If strains harbored broad host range plasmids, 0.5 mg of streptomycin/ml was added to maintain IncQ plasmids, and 2 μg of tetracycline/ml was added to maintain IncP plasmids. For induction, naringenin (1.5 μM final concentration) was added at a cell density of 5 × 10^7/ml. Cells were usually harvested after three generations of further growth.

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[1] The abbreviations used are: bv., biovar; HPLC, high pressure liquid chromatography; PC, phosphatidylcholine; LPS, lipopolysaccharide.

TABLE I
Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Ref.
R. leguminosarum		
248	*R. leguminosarum* bv. *viciae* wild type	6
LPR5045	*R. leguminosarum* bv. *trifolii* RCR5, Rif^r, cured of Sym plasmid	7
RBL5560	LPR5045 with Sym plasmid pRL1JI	8
Plasmids		
pMP247	IncP carrying *nodDABCIJ*	9
pMP280	IncP carrying pr.*nodD-nodD* from *R. leguminosarum* bv. *viciae*	10
pMP604	IncP carrying flavonoid-independent transcription activation)-type *nodD604*	11
pMP1255	IncQ carrying *nodFE*	5

Extraction of Lipids—Lipids were extracted by a modified Bligh and Dyer procedure (15). Wet cell paste was made up to 1 volume with water, and 3.75 volumes of methanol/chloroform (2:1, v/v) were added to the suspension. The mixture was gently stirred for 1 h at room temperature. After centrifugation, the supernatant extract was decanted, and the pellet was reextracted with 4.75 volumes of methanol/chloroform/water (2:1:0.8, v/v) and centrifuged. To the combined supernatant extracts, 2.5 volumes each of chloroform and water were added, and the mixture was centrifuged. The lower chloroform phase was withdrawn and dried in a rotary evaporator. The lipid residue was immediately dissolved in methanol/chloroform (1:1, v/v) and was stored under nitrogen at −20 °C.

Two-dimensional Thin-layer Chromatographic Analysis of Phospholipids—Lipid extracts were separated using two-dimensional thin-layer chromatography (16) on Silica Gel 60 plates. Separation in the first dimension was performed with chloroform/methanol/28% ammonia (65:25:5, v/v). After drying, plates were developed in the second dimension with chloroform/acetone/methanol/acetic acid/water (30:40:10:10:5, v/v). Rapid detection of lipids was achieved by exposing dried plates to iodine vapor in which lipophilic material rapidly stains with a brown color.

Purification of Phospholipids—To obtain enough material to allow NMR spectroscopy on individual phospholipid species, 70 liters of rhizobial culture were grown to obtain 655 g, wet cells, of naringenin-induced *R. leguminosarum* LPR5045.pMP280.pMP1255. Bligh-Dyer extraction yielded about 4.5 g of total lipids.

DEAE-cellulose Chromatography—A crude separation of neutral and anionic phospholipids was achieved using chromatography on DEAE-cellulose (DE52, Whatman) (16). The sample was applied as a solution in 20 ml of chloroform (4.0 g of total lipids) to a 200-ml column of DEAE 52-cellulose in its acetate form, which had been equilibrated with chloroform. The column was then sequentially eluted with the following solvents: 5 volumes of chloroform, 9 volumes of chloroform/methanol (9:1, v/v), 5 volumes of chloroform/methanol (1:1, v/v), and 10 volumes of chloroform/methanol (4:1, v/v) containing 50 mM ammonium acetate. The individual fractions were brought to dryness in a rotary evaporator, and they were immediately dissolved in methanol/chloroform (1:1, v/v) and stored under nitrogen at −20 °C.

Normal Phase Silica Gel HPLC—All major phospholipid classes were separated by normal phase silica gel HPLC (17), either on an analytical Hypersil column (4.6 × 160 mm, Shandon) with a flow rate of 1 ml/min or on a Hyperprep 120 Silica 12U column (4.6 × 250 mm, Alltech Association, Inc.) with a flow rate of 2 ml/min. The mobile phase consisted of hexane/2-propanol/ethanol/25 mM potassium phosphate (pH 7.0)/acetic acid (485:376:100:56:0.275, v/v). Diode array spectroscopic detection was performed using an RSD 2140 optical unit (Pharmacia LKB Biotechnology Inc.).

Reverse Phase Silica Gel HPLC—The purified phospholipid classes were separated into molecular species on a Superpac Spherisorb ODS-2 column (4 × 250 mm, Pharmacia) (18). They were eluted with methanol/water/acetonitrile (90.5:7:2.5, v/v) containing 20 mM choline chloride at a flow rate of 0.5 ml/min.

Mass Spectrometry—Positive and negative ion mode mass spectra were obtained on the purified 303-nm-absorbing phospholipid assumed to be phosphatidylcholine. Samples redissolved in chloroform/methanol (1:1, v/v) were analyzed by loading 1–3 μl of sample solution into a matrix of monothioglycerol (positive ion mode) or *m*-nitrobenzyl alcohol (negative ion mode). Mass spectra were obtained using MS1 of a JMS-SX/SX102A tandem mass spectrometer (Jeol Ltd.) operated at 10 kV accelerating voltage. The fast atom bombardment gun was operated at 6 kV with an emission current of 10 mA, and xenon was used as the bombarding gas. Spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage used and were recorded and averaged using Jeol Complement software run on a Hewlett-Packard 9000 series data system. Tandem mass spectra were obtained from the same samples following collision-induced dissociation in the third field free region, using the same instrument in its 4-sector mode under similar conditions; helium was used as the collision gas at a pressure sufficient to reduce the parent ion to one-third of its original intensity.

^1H NMR Analysis—The phosphatidylcholine sample with an absorption maximum at 303 nm (E_{303} = 47/ml) was dissolved in 0.6 ml of $CD_3OD/CDCl_3$ (1:5, v/v). Spectra were acquired on a Bruker AMX600 spectrometer at 25 °C. Two-dimensional double quantum filtered-COSY experiments (19) were performed in phase-sensitive mode using the time proportional phase increment method (20).

RESULTS

Lipid Extracts Contain nodFE-dependent Metabolites—During our search for metabolites made by the action of the rhizobial *nod* gene products, we observed that in addition to lipooligosaccharides other, more hydrophobic metabolites with an absorbance maximum at 303 nm were synthesized in a *R. leguminosarum* strain (LPR5045), which had been cured of its Sym plasmid and contained pMP247 (*nodDABCIJ* on an IncP plasmid) and pMP1255 (*nodFE* on an IncQ plasmid). In our attempt to search for metabolites that are made by NodFE in the absence of the other *nod* genes, we used an *R. leguminosarum* strain (LPR5045), which contained pMP280 (*nodD* on an IncP plasmid) and pMP1255 (*nodFE* on an IncQ plasmid). Cultures were grown with or without the inducer naringenin, and lipids were extracted as described under "Experimental Procedures." They were separated by HPLC on silica gel and analyzed by diode array spectroscopy. The chromatogram of the lipids from the naringenin-induced situation (Fig. 1*B*) differed significantly from the one of a non-induced situation (Fig. 1*A*). In the *nodFE*-induced situation two major lipids and another four to five minor lipids were present, which showed a maximum in their absorption spectra at 303 nm. These absorptions were absent in the lipid extracts when *nodFE* was not induced. Because the mitogenic lipooligosaccharides from *R. leguminosarum* bv. *viciae* contain a host-specific *nodFE*-related fatty acid that shows a characteristic absorption maximum at 303 nm (2), we supposed that the same fatty acid was present in the lipids under investigation. Surprisingly, there was a second *nodFE*-dependent absorption maximum at 225 nm associated with the lipids, presumably due to another, less unsaturated, *nodFE*-dependent fatty acid. These novel lipids are clearly not lipooligosaccharides because lipooligosaccharides would not migrate at all under these chromatography conditions. In the strain LPR5045.pMP1255.pMP604, which contains a *nodD* gene that activates transcription in a flavonoid-independent way (10), 303- and 225-nm-absorbing lipids were synthesized (data not shown), showing that the flavonoid effect on the synthesis of *nodFE*-dependent fatty acids is exerted via NodD.

General Characterization of nodFE-dependent Lipids—Separation of lipid extracts from a *nodFE*-overproducing situation showed that 303- and 225-nm absorbances did not migrate as free fatty acids in a two-dimensional TLC but were associated with slower migrating iodine-stainable spots, which became intensely labeled when [^{32}P]phosphate had been added to the growth medium (data not shown). When phospholipid fractions, which had been separated on silica gel HPLC according to their head groups, were analyzed on two-dimensional TLC, the 303- and 225-nm absorbances comigrated with only one of the ^{32}P-containing spots (data not shown). These results strongly suggest that the 303- or 225-nm absorbances are associated with the individual phospholipid classes in *Rhizobium*, namely phosphatidylethanolamine, monomethylphosphati-

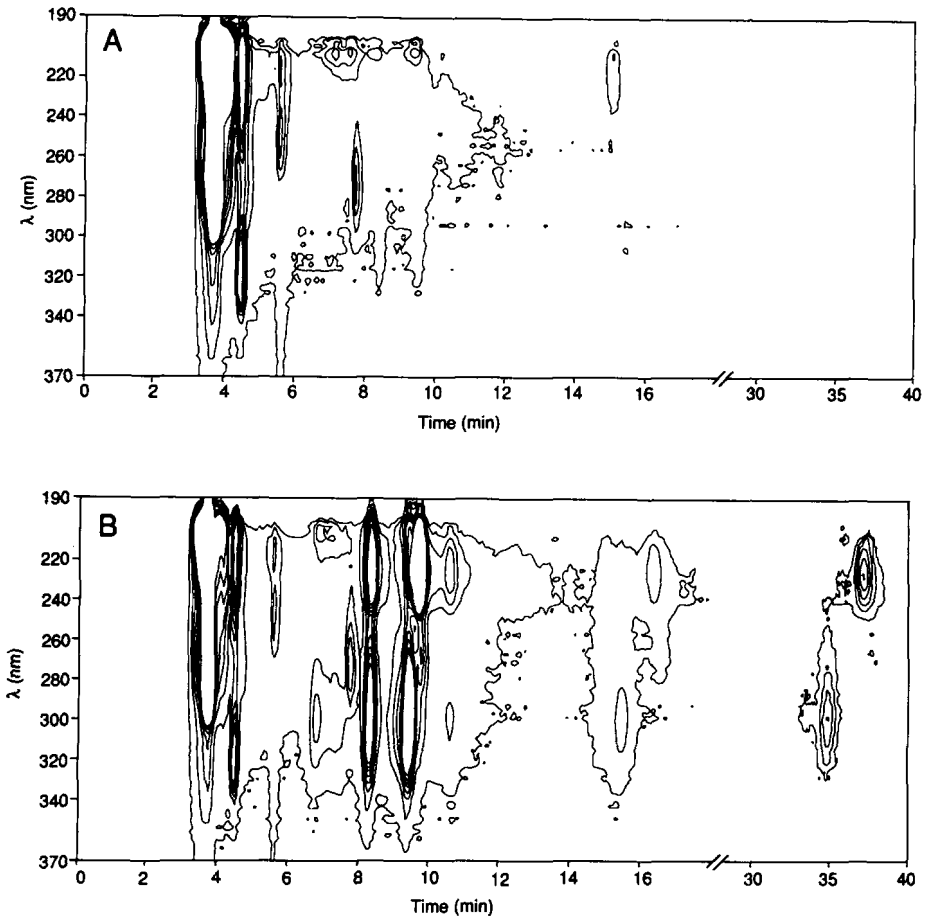

FIG. 1. **Fractionation of uninduced and *nodFE*-induced lipid extracts from *R. leguminosarum* LPR5045.pMP280.pMP1255.** Photodiode detection is shown of uninduced (*A*) and *nodFE*-induced (*B*) lipid extracts from *R. leguminosarum* LPR5045.pMP280.pMP1255 (obtained from about 4 × 10^9 cells) that had been separated by analytical HPLC on silica gel.

dylethanolamine, dimethylphosphatidylethanolamine, phosphatidylcholine (PC), phosphatidylglycerol, and cardiolipin.

Treatment of the 303- or 225-nm-absorbing individual phospholipid classes with 0.2 M NaOH in MeOH at room temperature led to rapid hydrolysis ($t_{1/2}$ = 4 min, in each case) as expected for ester bonds under these conditions (Fig. 2). Lipooligosaccharides, which have their fatty acyl residue attached through an amide bond, are not hydrolyzed under such mild conditions. During hydrolysis more polar 303- and 225-nm-absorbing intermediates, presumably lysophospholipids and phosphatidic acids, were formed, which disappeared again on prolonged hydrolysis (data not shown). As final 303- and 225-nm-absorbing products, we obtained only apolar compounds, the free fatty acids (Fig. 2*C*). The final products were stable at room temperature for at least 24 h under these conditions as judged from the maintenance of the 303- and 225-nm absorbance. These experiments suggest that the 303-nm-absorbing fatty acid can be prepared easily from *nodFE*-dependent phospholipids and might be of great value for future synthetic purposes.

Phospholipids in Wild Type R. leguminosarum Also Contain nodFE-dependent Fatty Acids—Our initial experiments to identify *nodFE*-related metabolites were performed with a *nodFE*-overproducing strain (LPR5045.pMP280.pMP1255). Acyltransferases involved in the assembly of phospholipids are known to be quite unselective with regard to the type of fatty acyl chain they incorporate into the phospholipids (21). Presumably the fatty acids found in the phospholipids reflect to a large extent what has been synthesized by the cell, and one might find it not too surprising to detect overproduced fatty acids not only in the lipooligosaccharides but in the phospholipids as well. We therefore investigated several wild type *R. leguminosarum* bv. *viciae* strains for the presence of similar *nodFE*-related phospholipids after induction of the *nod* genes to those we had found in a *nodFE*-overproducing strain of *R. leguminosarum*. In Fig. 3 the diode array spectra of lipids isolated from an uninduced (Fig. 3*A*) and an induced culture (Fig. 3*B*) of strain RBL5560 are compared. The spectra show that there are at least three flavonoid-inducible 303-nm-absorbing lipids that migrate at the same positions as the phospholipids from the *nodFE*-overproducing strain LPR5045.pMP280.pMP1255 and are clearly not lipooligosaccharides. Similar patterns of naringenin-inducible 303-nm-absorbing phospholipids were obtained with wild type strain 248 (data not shown).

Purification of 303-nm-absorbing Phospholipids—Bligh-Dyer extracts were chromatographed on DEAE-cellulose, which allowed separation of the neutral phospholipids (phosphatidylethanolamine, monomethylphosphatidylethanolamine, dimethylphosphatidylethanolamine, and phosphatidylcholine) from anionic phospholipids (phosphatidylglycerol and cardiolipin). The neutral phospholipid fraction eluted with chloroform/methanol (9:1, v/v), whereas the anionic phospholipids were detected in the chloroform/methanol (4:1, v/v) eluate containing 50 mM ammonium acetate. Neutral and anionic fractions were further separated by HPLC on silica gel, which allows a separation mainly according to the head groups. Under these conditions phosphatidylcholine, as part of the neutral fraction, is retained quite strongly on the column, elutes at a retention time of 34–40 min, and can therefore be totally separated from all other phospholipid classes. In a final purification step the 303-nm-absorbing PC was subjected to HPLC on reverse phase silica gel yielding a nearly homogenous preparation of 303-nm-absorbing PC, which allowed the determination of the precise covalent structure of this phospholipid species.

Chemical Structures—The purified 303-nm-absorbing phosphatidylcholine was analyzed using 2- and 4-sector mass spec-

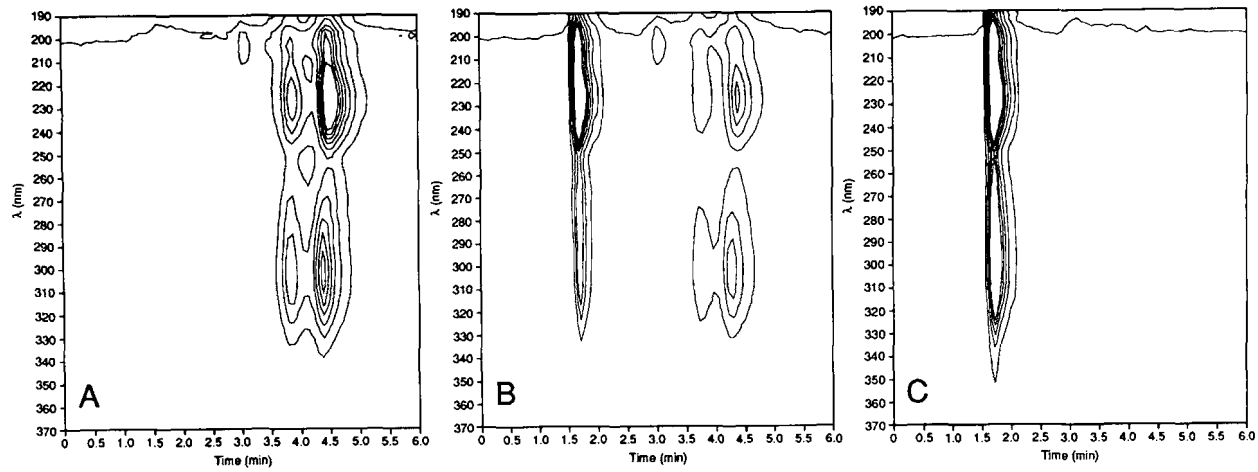

FIG. 2. **Time course of mild alkaline hydrolysis of 303- and 225-nm-absorbing phospholipids.** A mixture of 303- and 225-nm-absorbing phosphatidylethanolamine and phosphatidylglycerol was treated with mild alkali at 25 °C. Aliquots were neutralized at different time points and analyzed by analytical HPLC on silica gel after 0 (*A*), 3 (*B*), and 60 (*C*) min of treatment.

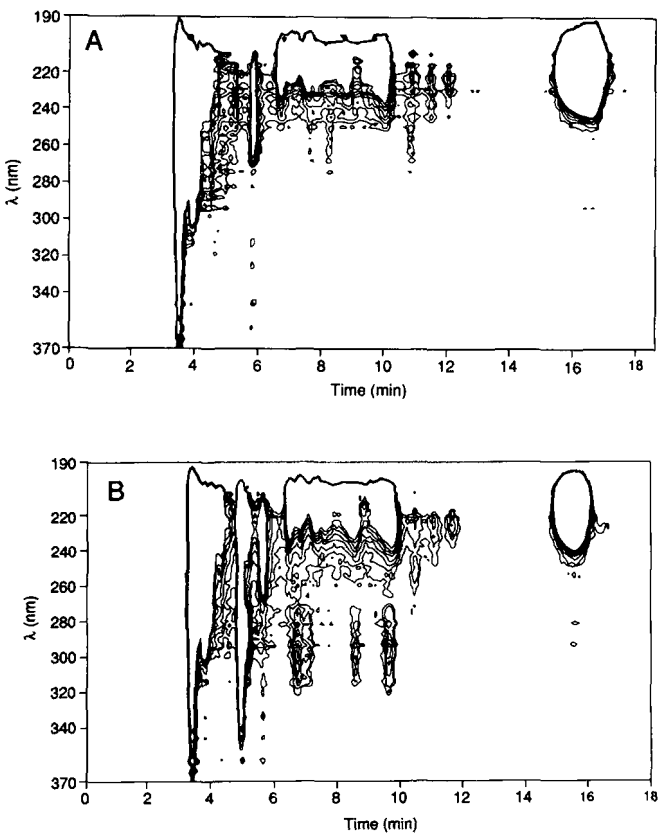

FIG. 3. **Comparison of phospholipid extracts of uninduced and *nod* gene-induced cells of wild type *R. leguminosarum* bv. *viciae* RBL5560.** Photodiode detection is shown of uninduced (*A*) and naringenin-induced (*B*) lipid extracts from *R. leguminosarum* RBL5560 (obtained from about 4 × 10^9 cells) that had been separated by analytical HPLC on silica gel.

trometric methods in both the positive and the negative ion modes. The positive ion fast atom bombardment-mass spectrum contained a very intense $[M + H]^+$ molecular ion at *m/z* 780, as expected from PC, which bears a fixed positive charge. A very minor $[M + Na]^+$ ion was observed at *m/z* 802, along with a series of cluster ions corresponding to adducts formed between both of the molecular species and one or more thioglycerol molecules. The formation of strong adducts between thioglycerol and species bearing unsaturated fatty acyl chains has been described (22), and in the present study this indicates that the phospholipid contains unsaturated fatty acid chain(s). The molecular ion corresponds to a phosphatidylcholine species that contains fatty acyl chains with a total of 36 carbon atoms and 5 double bonds.

Negative ion mass spectra were difficult to obtain, as might be expected from a molecule bearing a positively charged group, and were only obtainable when using a matrix of *m*-nitrobenzyl alcohol. A pseudomolecular ion was observed at *m/z* 778 corresponding to $[M - H]^-$. Fragment ions were observed at *m/z* 764 (corresponding to $[M - CH_3]^-$), *m/z* 719 $[M - HN(CH_3)_3]^-$, and *m/z* 693 $[M - CHCH_2N(CH_3)_3]^-$. The pseudomolecular species confirms the positive mode interpretation, while the fragment ions are those expected in the negative ion mode from a phosphatidylcholine (23).

To further confirm the presence of PC, the $[M + H]^+$ ion at *m/z* 780 was subjected to collision-induced dissociation tandem mass spectrometry in the positive ion mode. One major product ion was observed (Fig. 4*A*) at *m/z* 597, corresponding to the loss of choline phosphate $[M - PO_4CH_2CH_2N(CH_3)_3]^+$ as expected from PC, based on the low energy collision data previously published (24).

Collision-induced dissociation tandem mass spectrometry of the negative ion fragment at *m/z* 693 was carried out (Fig. 4*B*) to establish the fatty acids present and their positions of esterification. Two sets of product ions used for this purpose are those corresponding to the carboxylate anions, which allow the fatty acid chains to be identified (23), and those produced by the loss of each of the fatty acids from the precursor ion, the relative intensities of which allow the esterification positions of the fatty acyl chains to be determined. The ion formed by loss of the *sn*-2 fatty acid has been shown to be always more intense than that formed by the loss of the *sn*-1 group (25). Product ions were observed at *m/z* 275 and 281, corresponding to the carboxylate anions of a C18:4 and a C18:1 fatty acid. Two further product ions were observed at *m/z* 417 and 411, arising by loss of the C18:4 and the C18:1 carboxylate ions from the precursor, respectively. The more intense of the two is *m/z* 417, indicating that the *sn*-2 fatty acid is the C18:4.

The ^1H NMR spectra and two-dimensional, double quantum-filtered correlated spectroscopy of the 303-nm-absorbing PC (Fig. 5) showed coupling patterns as expected for acyl side chains containing double bonds. It revealed six signals between δ 5.7 and 7.2, which correspond to methine protons from positions 2 to 7 of the acyl chain, and show connectivity to the upfield signals of the methylene protons (δ 2.4–1.2) in the fatty acid chain. The 303-nm-absorbing PC contains the C18:4 fatty

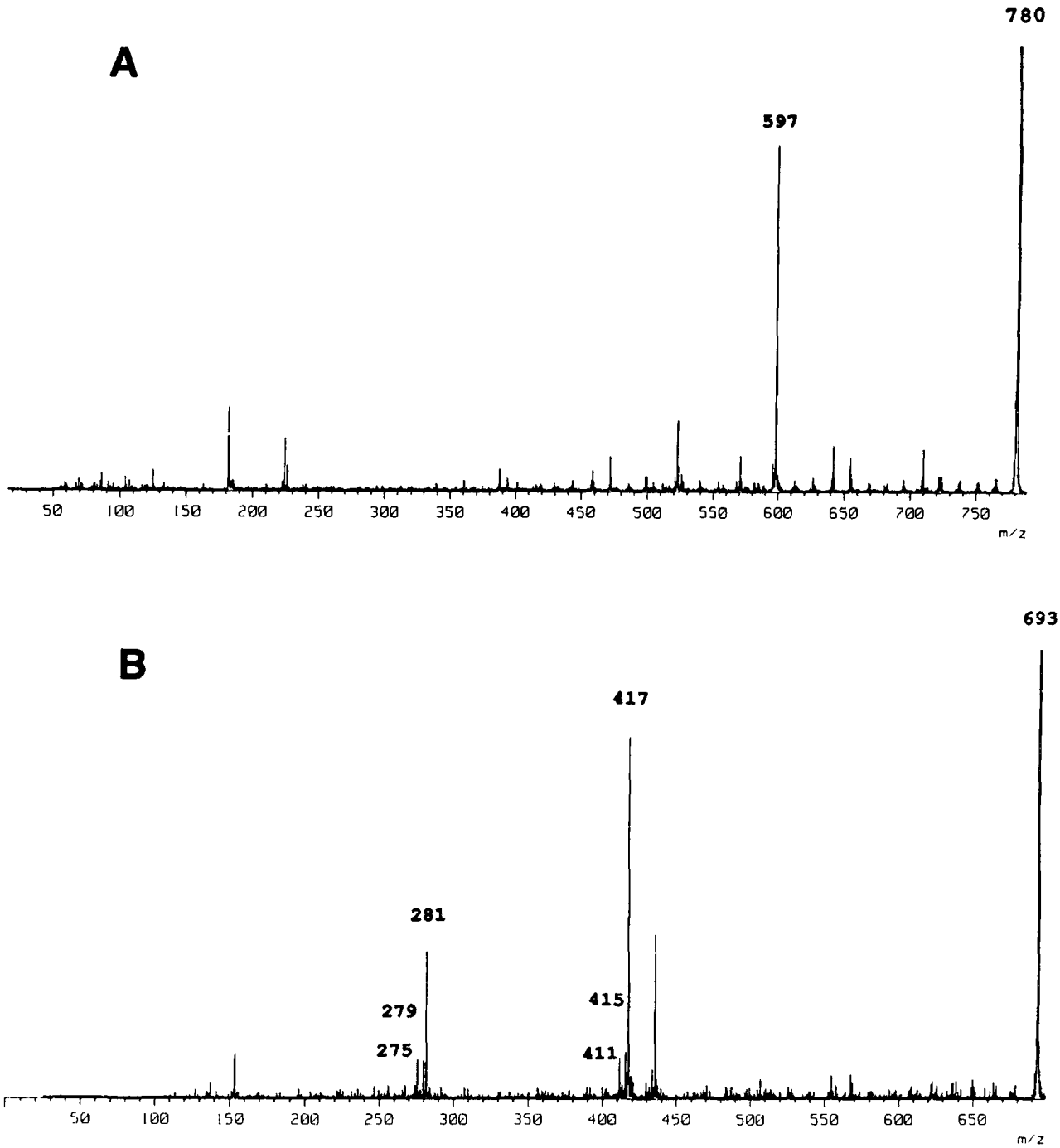

FIG. 4. **Mass spectrometry of 303-nm-absorbing PC.** Collision-induced dissociation tandem mass spectra are shown of the 303-nm-absorbing PC in the positive (*panel A*) and negative (*panel B*) ion modes.

acid where the three conjugated double bonds are all in trans (*E-E-E*) with the same chemical shifts as found for the acyl chain of mitogenic lipooligosaccharides from *R. leguminosarum* bv. *viciae* (2). In addition we find other compounds in our 303-nm-absorbing PC sample substituted with C18:4 acyl chains of a *Z-E-E*, *E-Z-E*, or *Z-Z-E* configuration for the three conjugated double bonds. Examination of the proton coupling constants (typically 15–16 Hz for *E* and 10.5–11.5 Hz for *Z*) in the one- and two-dimensional spectra provided the configuration of the conjugated double bonds.

From the mass spectrometric and the NMR spectroscopic studies we can conclude that the 303-nm-absorbing phosphatidylcholine has the primary chemical structure presented in Fig. 6.

Lipopolysaccharide Does Not Contain nodFE-derived, 303- or 225-nm-absorbing Fatty Acids—Lipopolysaccharide (LPS) was isolated from naringenin-induced or non-induced cells of LPR5045.pMP1255.pMP280 by the hot phenol method exactly as described earlier (26). The water phases were dialyzed and lyophilized. The LPS fractions were then treated with 1% acetic acid at 100 °C for 1 h to obtain relatively pure chloroform-extractable lipid A preparations. Gas chromatography analysis of aliquots, which had been treated with strong alkali to release amide-bound fatty acids, demonstrated the presence of 27-hydroxyoctacosanoic acid in our lipid A preparations (data not shown), which is a characteristic marker of rhizobial LPS. However, no *nodFE*-inducible fatty acid was detected when we compared the fatty acid compositions of lipid A preparations

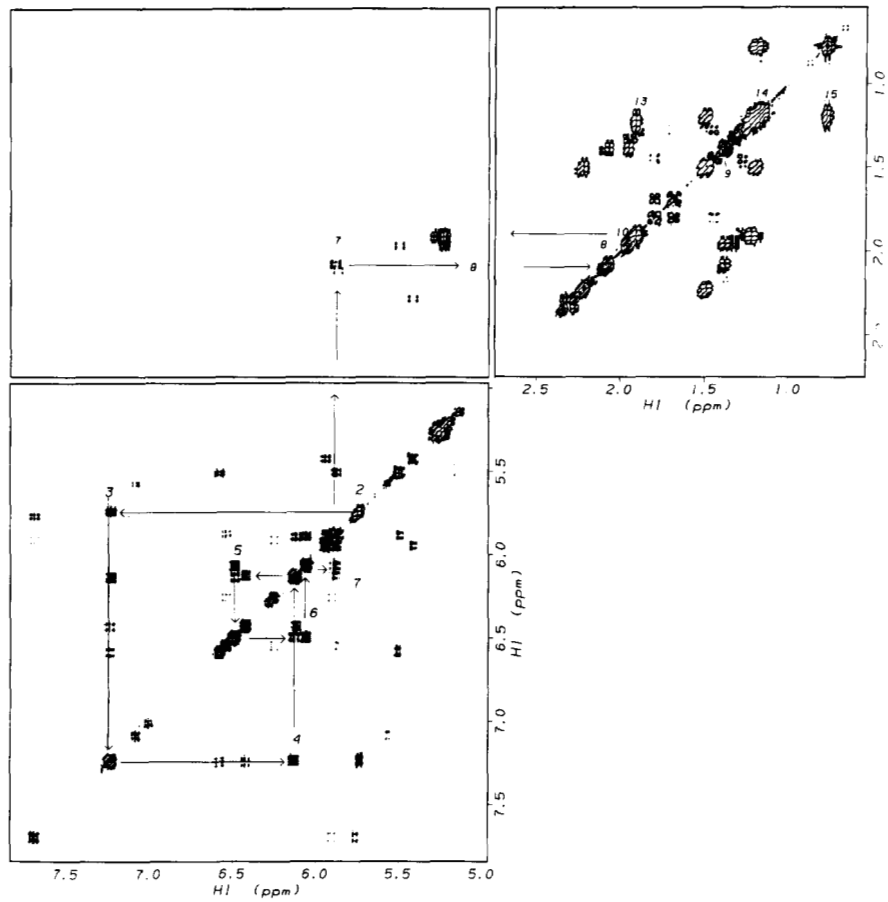

FIG. 5. **Regions from the 600-MHz double quantum filtered-COSY spectrum of the 303-nm-absorbing PC.** The connectivity indicated corresponds to the conjugated double bond system (*peaks* labeled *2–7*) of the all-trans compound and to the rest of the acyl chain (*peaks 8–15*). The other isomers can be traced in a similar fashion.

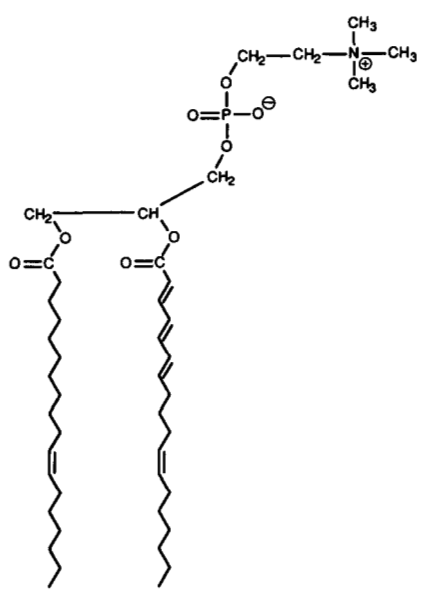

FIG. 6. **Structure of 303-nm-absorbing PC of *R. leguminosarum* bv. *viciae*.**

from a naringenin-induced and a non-induced situation (data not shown). We also analyzed such lipid A preparations with diode array spectroscopy after HPLC on silica gel and could neither detect a *nodFE*-dependent 225- nor a 303-nm absorbance (data not shown). Thus, we conclude that lipid A and, therefore, also LPS do not contain *nodFE*-derived 303- or 225-nm-absorbing fatty acids. This result shows that not all fatty acid-containing pools in *Rhizobium* incorporate *nodFE*-derived fatty acids.

DISCUSSION

The *nodFE* gene products are involved in the synthesis of the mitogenic lipooligosaccharides in *R. leguminosarum* bv. *viciae* (2). An important structural feature of those lipooligosaccharides is a *trans*-2,*trans*-4,*trans*-6,*cis*-11-octadecatetraenoic acid responsible for a 303-nm absorbance maximum, which is essential for their mitogenicity. In our attempt to understand the biosynthesis of rhizobial mitogenic lipooligosaccharides, we found that from the *nod* genes *nodFE* is sufficient to allow the synthesis of 303-nm-absorbing lipids. Purification and structural analysis of such lipids revealed that the *trans*-2,*trans*-4,*trans*-6,*cis*-11-octadecatetraenoic acid is linked to the *sn*-2 position of phospholipids.

As only NodFE is needed for the synthesis of the *trans*-2,*trans*-4,*trans*-6,*cis*-11-octadecatetraenoic acid, this shows that the common *nodABC* genes, essential for lipooligosaccharide synthesis, are not needed for this process. We therefore suggest that during lipooligosaccharide biosynthesis, first the unusual fatty acid is synthesized; only after this has been completed will it be linked to the sugar backbone of the nascent lipooligosaccharide.

If one considers that three *trans* double bonds have to be made at different positions of a fatty acid, it is evident that this cannot be achieved by the action of only two proteins (NodF and NodE). Other common household enzymes, which are involved in normal fatty acid synthesis in the cell, are also needed for the synthesis of such a polyunsaturated C18:4 fatty acid. The simplest mechanism for such a synthesis would be a variation of normal fatty acid biosynthesis using a condensing enzyme (β-ketoacylsynthase) that exhibits a different substrate specificity (NodE) and an acyl carrier protein (NodF) that specifies the substrates to be used. We suggest the model presented in Fig. 7 for the biochemical function of NodF and NodE. Importantly,

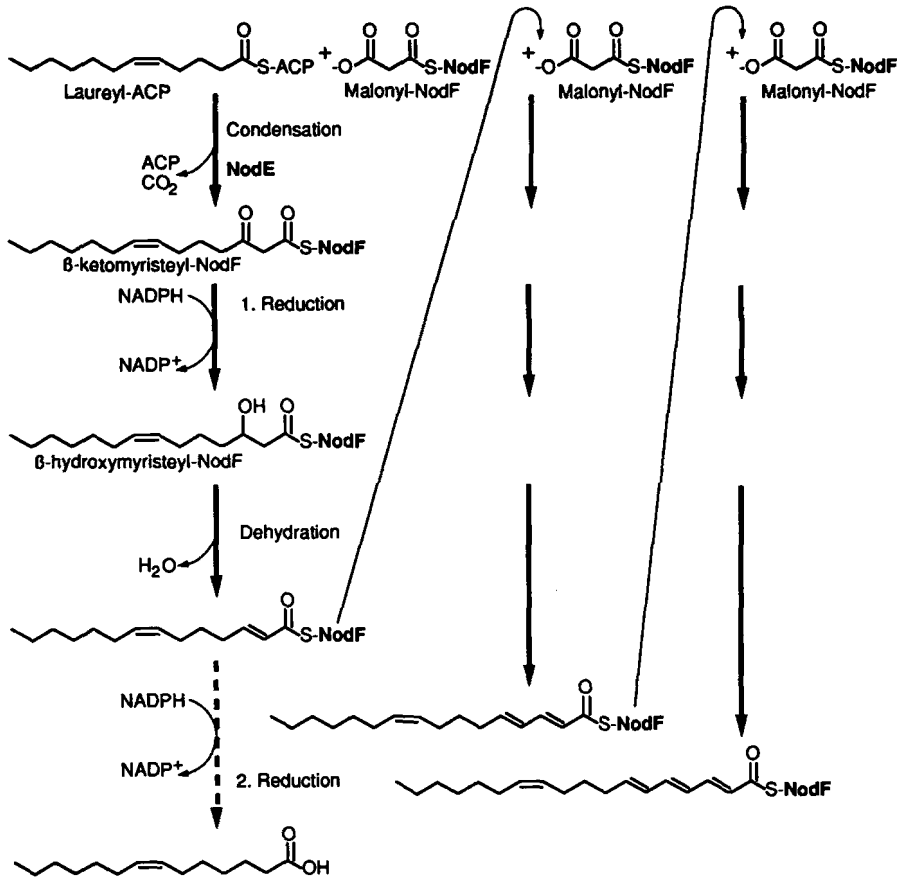

FIG. 7. **Model of biochemical function of NodF and NodE.**

in this model acyl residues linked to NodF would be protected and would not undergo the second reduction step.

Although there exists a fair number of investigations on the lipid composition of *Rhizobia* (27) and *Bradyrhizobia* (28), a search for *nod* gene-dependent lipids has so far not been successful (29). We found that the *trans*-2,*trans*-4,*trans*-6,*cis*-11-octadecatetraenoic acid, synthesized by the action of NodFE, is linked to all major phospholipid classes. Even when lipooligosaccharide signals are produced in a wild type *Rhizobium* cell, a fraction of the unusual fatty acid is still bound to all major phospholipids. One therefore cannot attribute this fact to the overproducing situation only. The fact that no *nodFE*-dependent fatty acids are present in LPS shows that these fatty acids are not just randomly assembled into all fatty acid-containing substances of the cell. Rather it seems that one type of *nodFE*-derived fatty acid, the 303-nm-absorbing *trans*-2,*trans*-4,*trans*-6,*cis*-11-octadecatetraenoic acid, is incorporated into the lipooligosaccharide signals, whereas more than one type, the 303- and 225-nm-absorbing ones, are incorporated into the phospholipids. At present we do not know whether these *nodFE*-dependent fatty acids remain stably associated with the phospholipids or whether these phospholipids are subject to turnover. Although research on phospholipid turnover in eukaryotes has led to the spectacular discoveries that polyunsaturated fatty acids, such as arachidonic acid, are converted to eicosanoid signals (*i.e.* prostaglandins, prostacyclins, thromboxanes, and leucotrienes) after their release from the *sn*-2 position of the phospholipids, no similar findings had been reported for bacteria. For the first time we report on bacterial phospholipids that are substituted with a polyunsaturated fatty acid at their *sn*-2 position. It is interesting to note that the final product of the rhizobial NodABCFEL proteins is a lipooligosaccharide that also functions as a signal on host plants. We therefore presently study whether there are conditions under which these *nodFE*-derived fatty acids are selectively released from the phospholipids. The dynamics of phospholipid turnover in bacteria is poorly understood, and only a few examples have been investigated in more detail. Transfer of the polar head groups from phospholipids occurs during biosynthesis of periplasmic glucans, the membrane-derived oligosaccharides in *E. coli* (30), or the cyclic glucans in the *Rhizobiaceae* (31, 32). Also, the acyl residues from phospholipids can be transferred, as is the case during the production of outer membrane lipoproteins. The acyl residue attached to Braun's lipoprotein seems to be mainly derived from the *sn*-1 position of phosphatidylethanolamine (33, 34). All systems of phospholipid turnover in bacteria known so far are therefore involved in synthesis of biologically important molecules leaving the cytoplasmic area of the cell. With this in mind it is tempting to speculate that the phospholipids of *Rhizobium* are biosynthetic intermediates for the synthesis of lipooligosaccharide signals. In such a case the transfer of the acyl residue could even occur on the outer surface of the cytoplasmic membrane. This possibility is currently being investigated.

Mitogenic lipooligosaccharides are only the first set of rhizobial signals required for establishing a stable *Rhizobium*-legume symbiosis. Other signals (exopolysaccharides, lipopolysaccharides, and cyclic glucans) are thought to be essential at other stages of the symbiotic development. We have identified *nod* gene-dependent rhizobial metabolites that are not lipooligosaccharides. These novel Nod metabolites are phospholipids, substituted at their *sn*-2 position with a *nodFE*-dependent fatty acid. We are presently studying the possibility that any of these novel phospholipids might function as an additional rhizobial signal in establishing a stable symbiosis.

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