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Cell-to-Cell Signaling in the Symbiotic Nitrogen-Fixing Bacterium *Rhizobium leguminosarum*: Autoinduction of a Stationary Phase and Rhizosphere-Expressed Genes

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The Sym plasmid pRL1JI encodes functions for the formation of nitrogen-fixing pea root nodules by *Rhizobium leguminosarum*. Some of the nodulation genes are involved in recognition of chemical signals produced by the plant root, and others are required for production of chemical signals recognized by the plant. pRL1JI also contains a regulatory gene, *rhiR*, that is homologous to *luxR*, the transcriptional activator of luminescence genes in *Vibrio fischeri*. LuxR requires a signal compound, an autoinducer, for its activity. We have identified an *R. leguminosarum* autoinducer that, together with RhiR, is required to activate both the rhizosphere-expressed *rhiABC* operon and a growth-inhibiting function encoded by pRL1JI. This intercellular signal is an *N*-acylated homoserine lactone structurally related to the *V. fischeri* and other autoinducers. These findings indicate a new level of intercellular communication in root nodule formation.

Population density-dependent gene activation by the LuxR-LuxI family of transcriptional regulators is common to a diverse group of gram-negative bacteria (9, 21). Known as quorum sensing, this regulatory mechanism involves the interaction of self-produced extracellular signal compounds, called autoinducers, with transcriptional activator proteins (9). Because an autoinducer normally accumulates above its required threshold concentration only after a sufficient cell density has been achieved, quorum sensing allows bacteria to take a census and regulate the expression of specific sets of genes when a critical cell density is reached. The conserved LuxR family of transcriptional activators involved in quorum sensing currently includes LuxR (which regulates luminescence in *Vibrio fischeri* [6]), LasR (which activates virulence functions in *Pseudomonas aeruginosa* [10]), and TraR (which regulates conjugation in *Agrobacterium tumefaciens* [8, 17]), among others.

The pea-nodulating biovar (bv. viciae) of the symbiotic, nitrogen-fixing bacterium *Rhizobium leguminosarum* contains a transcriptional activator, RhiR, that is a member of the LuxR family of transcription factors (2). RhiR activates an operon of rhizosphere-expressed genes (the *rhiABC* operon) and plays a role in root nodulation (2). The *rhiABC* operon is adjacent to *rhiR* on the Sym plasmid pRL1JI. We report here the identification of an autoinducer signal from *R. leguminosarum* that is required for activation of *rhiABC* by RhiR. This signal also triggers a RhiR-dependent induction of growth-inhibiting functions that can result in an early onset of stationary phase in cultures of *R. leguminosarum* cells containing pRL1JI.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *R. leguminosarum* 8401 is a streptomycin-resistant derivative of a bv. phaseoli isolate that has been cured of its Sym plasmid (14). Type strains of *R. leguminosarum* bv. phaseoli (ATCC 14482), bv. trifolii (ATCC 14480), and bv. viciae (ATCC 10004) were obtained from the American Type Culture Collection (Rockville, Md.). *Rhizobium meliloti* Rm1021 was provided by Graham Walker.

Plasmids used include the *R. leguminosarum* bv. viciae Sym plasmid pRL1JI (12) and pRL1JI*rhiR1*::Tn*5*, which is the same plasmid with an insertionally inactivated *rhiR* gene (3). The recombinant plasmid pIJ1089 contains the pRL1JI *rhiABCR* genes, as well as flanking *nif* and *nod* genes (4). pIJ1642 contains the same genes as pIJ1089, but with a Tn*3*::*lacZ* insertion in the *rhiA* gene (5). The *rhiA*::*lacZ* reporter plasmid pIJ1769 was constructed by cloning a 0.5-kb *Bam*HI fragment from pIJ1753 (2) into pIJ1363 (18). This plasmid was mobilized into *R. leguminosarum* by triparental mating with *Escherichia coli*, with pRK2013 as a helper plasmid (7).

Rhizobium cultures were maintained at 30°C on TY medium (1) with the addition of appropriate antibiotics. *E. coli* cultures were maintained on LB medium (19) at 37°C with antibiotics.

Extraction and purification of *R. leguminosarum* autoinducer. Extraction of culture fluid from late-exponential-phase cultures was with ethyl acetate as previously described (16). For large-scale isolation and purification of the autoinducer, cultures of *R. leguminosarum* ATCC 14482 were grown in YM medium (13) with a final concentration of 0.1% yeast extract. Purification of the autoinducer from ethyl acetate extracts of these cultures was by sequential extraction and preparative high-performance liquid chromatography (HPLC) as described previously (16) with two modifications. First, prior to HPLC the extracts were dried, dissolved in 50% methanol in water, and passed through a C_{18} BondElut column (Varian Sample Preparation Products, San Diego, Calif.) from which the autoinducer activity was eluted in 80% methanol. Second, the final step-gradient HPLC was from 50 to 65% methanol.

Autoinducer bioassays. *Pseudomonas* autoinducer bioassays were performed as previously described (16). For detection of *Rhizobium* autoinducer activity, cultures of *R. leguminosarum* 8401 cells containing *rhiA*::*lacZ* reporter plasmids were grown overnight in TY broth and reinoculated to an optical density (660 nm) of 0.002 in fresh medium. Individual samples of *Rhizobium* culture fluid extracts or synthetic *N*-acyl homoserine lactones to be tested were dried in sterile tubes before being redissolved in 2 ml of the bioassay culture suspension. All tubes were then shaken at 30°C for 16 h. Activation of the *rhiA* promoter was assessed by measuring β-galactosidase activity (15).

NMR and mass spectra. Proton nuclear magnetic resonance (NMR) spectroscopy was performed with a Varian Unity 500-MHz instrument. Chemical ionization mass spectrometry was performed on a Nermag R10-10C instrument with a desorption chemical ionization probe, with ammonia as the reagent gas. High-resolution fast atom bombardment was performed at the University of Nebraska Midwest Center for Mass Spectrometry.

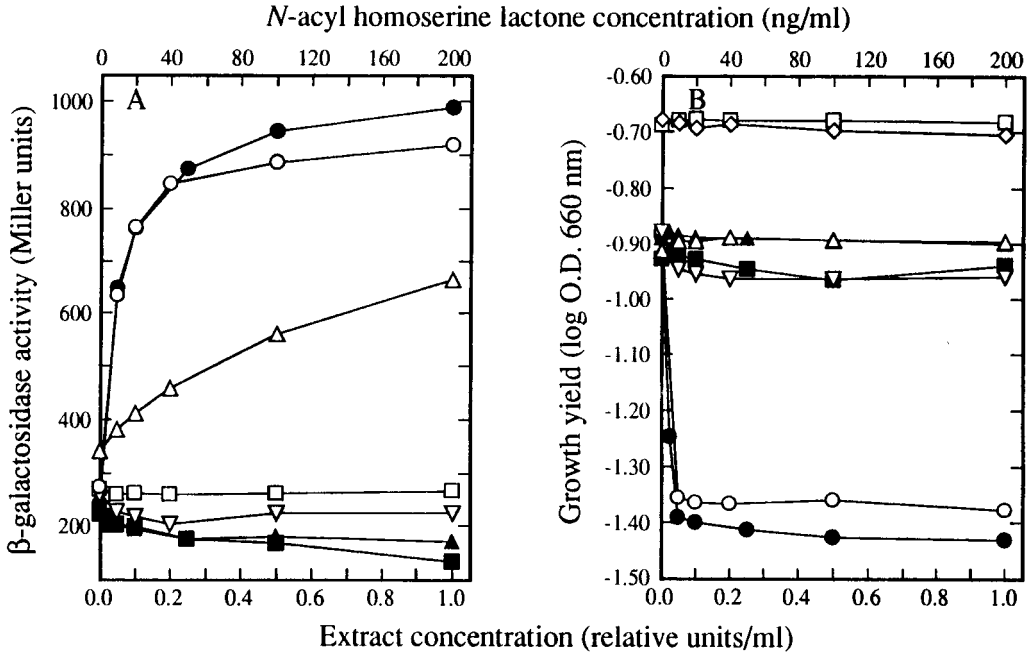

FIG. 1. Influence of culture fluid extracts and *N*-acylated homoserine lactones on *rhiA*::*lacZ* promoter activity (A) and growth of *R. leguminosarum* 8401 (B). Cultures were grown for 16 h as described in Materials and Methods. Open symbols, responses to an ethyl acetate extract of *R. leguminosarum* ATCC 14482 culture fluid. ○, cells containing the *rhiA*::*lacZ* reporter plasmid pIJ1769 and the Sym plasmid pRL1JI; □, cells containing only pIJ1769; ▽, cells containing pIJ1769 and pRL1JI*rhiR1*::Tn*5*; ◇, cells containing the *rhiABCR*-encoding plasmid pIJ1089; △, cells containing pIJ1642, which was derived from pIJ1089 by insertion of Tn*3*::*lacZ* in *rhiA*. Solid symbols, responses of cells containing pIJ1769 and pRL1JI to purified *R. leguminosarum* autoinducer (●), synthetic *P. aeruginosa* autoinducer [*N*-(3-oxododecanoyl)-L-homoserine lactone] (■), and synthetic *N*-(3-oxotetradecanoyl)-L-homoserine lactone (▲). Relative units of extract added indicate the corresponding amount of culture fluid extracted in milliliters. O.D., optical density.

RESULTS AND DISCUSSION

Identification of *Rhizobium* autoinducer activities. We initially discovered that each of the three biovars of *R. leguminosarum*, as well as the related species *R. meliloti*, produces extracellular factors that can substitute for the *P. aeruginosa* autoinducer in the LasR-dependent activation of the *lasB* promoter (16). Because at least limited cross-reactivity of different autoinducer molecules with transcriptional activators is possible (9, 11), this result suggested that the *Rhizobium* cultures were producing compounds related to, but not necessarily identical to, the *Pseudomonas* autoinducer, *N*-(3-oxododecanoyl)-L-homoserine lactone. To determine whether the activation of *rhiABC* by RhiR was influenced by these *Rhizobium* factors, we examined the effects of *R. leguminosarum* and *R. meliloti* culture fluid extracts on β-galactosidase activity in *R. leguminosarum* 8401 containing the Sym plasmid pRL1JI and pIJ1769, which contains a *rhiA*::*lacZ* translational fusion. Although it strongly activates the *P. aeruginosa lasB* gene with LasR, the *R. meliloti* extract had no effect on *rhiA* expression. The *R. leguminosarum* culture fluid extracts, however, not only activated the *rhiA* promoter but also inhibited the growth of cells containing pRL1JI (Fig. 1). As expected, in cells lacking pRL1JI (and thus lacking *rhiR*), the autoinducer signal did not activate the *rhiA* promoter. Significantly, the autoinducer did not inhibit the growth of these cells either (Fig. 1).

One interpretation of these results is that, in addition to activating *rhiABC* (2), RhiR is involved in activating genes that result in growth inhibition. This was confirmed with *R. leguminosarum* cells containing pRL1JI*rhiR1*::Tn*5*. Cells containing this plasmid and pIJ1769 did not activate the *rhiA* promoter, nor were they inhibited in their growth by *R. leguminosarum* culture fluid extracts (Fig. 1). The inhibition of growth and the activation of *rhiABC* are separate functions, however, because cells containing pIJ1089, which contains a fragment of pRL1JI that includes all of the known pRL1JI *rhi* genes (4), were not inhibited for growth by addition of *R. leguminosarum* culture fluid extracts (Fig. 1). Activation of the *rhiA* promoter still occurred in the absence of growth inhibition, as shown by the induction of β-galactosidase expression in cells containing pIJ1642, which has the *rhi*-containing fragment from pIJ1089 with a Tn*3*::*lacZ* insertion in the *rhiA* gene (5) (Fig. 1).

HPLC analysis of *Rhizobium* autoinducer activities. We thus had evidence that *R. leguminosarum* produces (i) an extracellular signal that can function with the *P. aeruginosa* LasR; (ii) a signal that, together with RhiR, can activate transcription of *rhiABC*; and (iii) a signal that, together with RhiR, can activate growth-inhibiting functions in *R. leguminosarum* containing the Sym plasmid pRL1JI. To determine whether a single compound might account for all three of these activities or whether there might be multiple signals, extracts of culture fluid from each of the three biovars of *R. leguminosarum* were analyzed by methanol-gradient reverse-phase HPLC (16) (Fig. 2).

All three *R. leguminosarum* extracts possessed all three autoinducer activities, and for all of the extracts, the activities were eluted in the same position in the methanol gradient. This suggested that one autoinducer was responsible for *rhiABC* activation and growth inhibition in *R. leguminosarum* and for LasR-dependent *P. aeruginosa lasB* expression. The *Pseudomonas* autoinducer activity from *R. meliloti* eluted in a different position from that of the *R. leguminosarum* activities (Fig. 2), suggesting that the autoinducer from *R. meliloti* is a distinct chemical species. Furthermore, although the *P. aeruginosa* LasR protein does not discriminate between them, these dif-

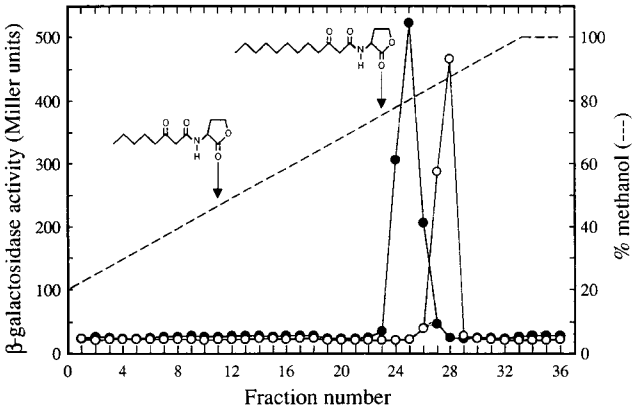

FIG. 2. HPLC analysis of *R. leguminosarum* bv. phaseoli (●) and *R. meliloti* (○) culture fluid extracts. Individual fractions were assayed for *P. aeruginosa* autoinducer activity. When assayed for activation of *rhiA* and growth inhibition of *R. leguminosarum* 8401(pRL1JI), the *R. leguminosarum* bv. phaseoli extract revealed a single peak of activity centered at fraction 25, while the *R. meliloti* extract showed no activity. Culture fluid extracts from *R. leguminosarum* bv. trifolii and bv. viciae gave results identical to those of bv. phaseoli. Arrows indicate the positions at which standards of *A. tumefaciens* autoinducer and *P. aeruginosa* autoinducer were eluted from the column (fractions 11 and 23, respectively), with the structure of each compound shown above the arrow.

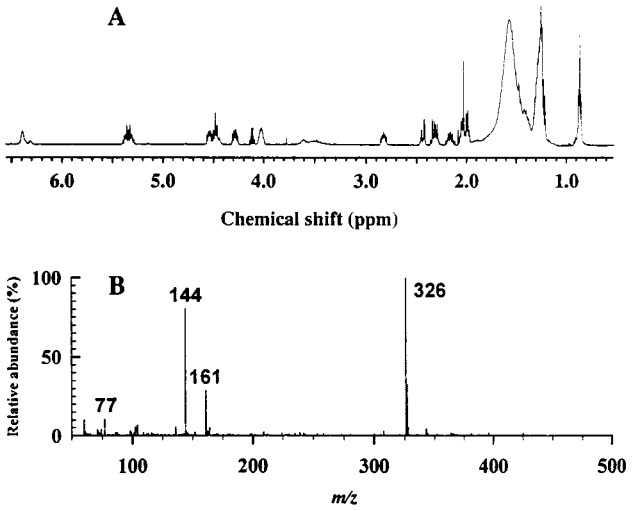

FIG. 3. High-resolution proton NMR and chemical ionization mass spectra of RLAI purified from *R. leguminosarum* culture fluid. (A) Proton NMR spectrum of RLAI in $CDCl_3$. δ_H 0.87 (t, CH_3), 1.23 (broad m, CH_2), 1.58 (broad s, H_2O), 2.05 (m, $CH_2CH{=}CHCH_2$), 2.17 (m, β-CH_2), 2.33 (m, CH_2CHOH), 2.45 (m, $CHOH$), 2.83 (m, β-CH_2), 3.51 (broad s, OH), 4.02 (broad m, $CHOHCH_2O$), 4.28 (m, γ-CH_2), 4.47 (m, γ-CH_2), 4.53 (m, α-CH_2), 5.35 (m, CH═CH), 6.39 (broad d, NH). (B) Chemical ionization mass spectrum of RLAI. The *m/z* of the $(M+1)^+$ was 326, consistent with the structure shown in Fig. 4.

ferent *Rhizobium* signals appear to be highly species specific in that the *R. meliloti* compound is not recognized by the *R. leguminosarum* RhiR protein.

Structural analysis of the *R. leguminosarum* autoinducer. The HPLC elution profile of the *R. leguminosarum* autoinducer and its ability to activate the *P. aeruginosa lasB* gene with LasR (Fig. 2) suggested that the compound was a related *N*-acyl homoserine lactone with a larger, more hydrophobic acyl side group than the *P. aeruginosa* autoinducer. We then purified and analyzed the *rhiABC*-activating compound from *R. leguminosarum*. Proton NMR confirmed that the compound was an *N*-acylated homoserine lactone similar in structure to the known autoinducers of other bacteria (Fig. 3A). We deduced that the purified compound possessed a hydroxyl group at the third carbon, a feature formerly unique to the *Vibrio harveyi* autoinducer [*N*-(3-hydroxybutanoyl)homoserine lactone], as well as a single unsaturated carbon-carbon bond within a 14-carbon acyl side chain. Although the exact position of this alkene group was not resolved, it was at least two methylene groups removed from the hydroxylated carbon and at least another two methylenes removed from the terminal methyl group. We will refer to this *R. leguminosarum* autoinducer as RLAI (Fig. 4).

The purified RLAI was analyzed by chemical ionization mass spectrometry, revealing a strong quasimolecular ion with an *m/z* of 326 (Fig. 3B). This was in agreement with the proposed structure (Fig. 4). High-resolution fast atom bombardment showed the *m/z* of the $(M + H)^+$ to be 326.2329. This corresponded to a chemical composition of $C_{18}H_{31}O_4N$, which is consistent with the structure shown in Fig. 4.

Thus, *R. leguminosarum* apparently produces a unique acyl homoserine lactone autoinducer that both activates the *rhiABC* promoter and inhibits growth of *R. leguminosarum* cells containing pRL1JI. RhiR shows a high degree of specificity for this molecule in that neither the *P. aeruginosa* autoinducer [*N*-(3-oxododecanoyl)homoserine lactone] nor *N*-(3-oxotetradecanoyl)homoserine lactone was active (Fig. 1). As mentioned previously, all three biovars of *R. leguminosarum* produced RLAI, while *R. meliloti* did not.

Interestingly, cultures of *R. leguminosarum* 8401 cells containing the Sym plasmid pRL1JI did not produce detectable amounts of RLAI activity, whereas the identical strain without any Sym plasmid did produce RLAI. This indicates that the genes required for RLAI synthesis do not reside on a Sym plasmid, and it presents a conundrum: cells require functions on pRL1JI to respond to autoinducer, but if they contain pRL1JI they do not produce the autoinducer. Because *R. leguminosarum* cells containing pRL1JI express high levels of the *rhiA* gene product in the pea root rhizosphere and in stationary-phase laboratory cultures (3), we believe that under some circumstances, either the Sym plasmid functions that block RLAI production are inactive or there is an additional RLAI-independent means for *rhiABC* activation. It should be pointed out that the production of autoinducer by the plant pathogen *A. tumefaciens* depends on the presence of a host plant-generated signal (8).

Autoinduction of stationary phase in *R. leguminosarum* by RLAI. The pRL1JI-associated phenotype of suppressed growth in response to an exogenous autoinducer is reminiscent of the activity of the *R. leguminosarum small* bacteriocin (22). The *small* bacteriocin is a low-molecular-weight compound produced by *R. leguminosarum* without pRL1JI, but not by cells containing pRL1JI. Conversely, this bacteriocin inhibits growth of *R. leguminosarum* cells with pRL1JI but not without it (22). The possibility exists that RLAI and the *small* bacteriocin are the same compound. We therefore examined more

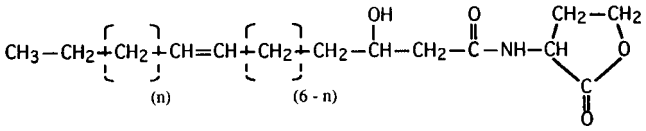

FIG. 4. Structure of the *R. leguminosarum* autoinducer (n = 1 to 5). The *N*-β-hydroxyacyl amide of homoserine lactone contains an unsaturated bond within its acyl chain, the exact position of which remains to be assigned.

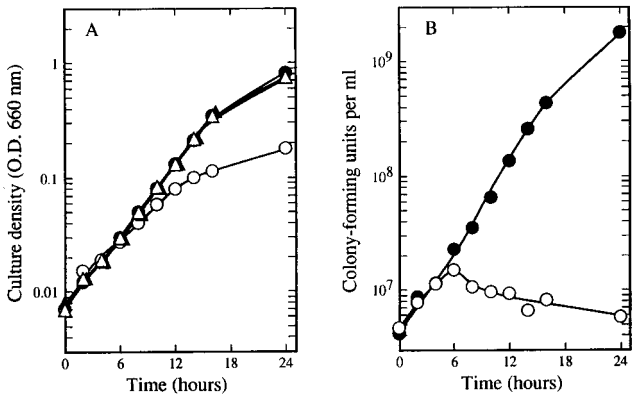

FIG. 5. Influence of an RLAI-containing extract on the growth and viability of Sym plasmid-containing *R. leguminosarum* 8401. (A) Culture growth as assessed by optical density (O.D.). (B) Culture growth and cell viability as assessed by plate counts of CFU on TY agar. Cultures of *R. leguminosarum* 8401 cells containing pRL1JI (○, ●) or pRL1JI*rhiR1*::Tn*5* (△, ▲) were grown in TY broth with (open symbols) and without (solid symbols) added RLAI extract. Two-tenths of a relative unit of RLAI extract (as defined in the legend to Fig. 1) was added per ml of culture in each case.

closely the effect of RLAI on the growth of cells containing pRL1JI (Fig. 5). The inhibition of growth by RLAI was not immediate; approximately two cell doublings occurred after the addition of RLAI before growth inhibition was detected. Growth inhibition required the presence of a functional *rhiR*, although the presence of *rhiR* and *rhiABC* alone was not sufficient for growth inhibition (Fig. 1 and 5A). Thus, there must be other genes on pRL1JI that are required for growth inhibition. Microscopic examination of cells from a culture of *R. leguminosarum* containing pRL1JI incubated for 16 h in the presence of RLAI revealed a morphology indistinguishable from that of cells grown in the absence of RLAI. Furthermore, although culture growth was inhibited by RLAI, there was no great loss of viability (Fig. 5B). In a related article that appears in this issue, Schripsema et al. (20) show a structure for the *small* bacteriocin that is consistent with the structure described here (Fig. 4).

Apparently RLAI and RhiR induce functions on pRL1JI other than *rhiABC*, and these other functions interfere with the growth of *R. leguminosarum*. The inhibition of growth is a novel autoinducer function. One interesting possibility is that the autoinducer limits the growth of *R. leguminosarum* bv. viciae during host root infection, thereby avoiding excessive bacterial growth that might induce a plant defense response. Further work is required to understand the significance of growth inhibition to *R. leguminosarum*, both in the rhizosphere and in the root nodule symbiosis.

Conclusions. In summary, we have discovered that *R. leguminosarum* produces a homoserine lactone autoinducer with a C-14 acyl side group containing a hydroxylated carbon in the 3 position and a single, unsaturated carbon-carbon bond (Fig. 4). The presence of the Sym plasmid pRL1JI blocks the production of the autoinducer and is required for a response to the autoinducer. The autoinducer activates expression of the rhizosphere-expressed *rhiABC* operon and additional growth-inhibiting functions carried on pRL1JI. These activities require the presence of a functional *rhiR* gene.

This initial report on the nature and function of an autoinducer controlling gene expression in *R. leguminosarum* raises a number of interesting questions. The genes required for the synthesis of the autoinducer are not on the Sym plasmid and remain to be identified. The mechanism by which autoinducer synthesis is inhibited by functions on pRL1JI remains to be determined. It remains unclear as to why the presence of the plasmid required for a response to the autoinducer interferes with the production of that autoinducer; this has not been observed in other quorum sensing systems (9). This report opens a new area of investigation: what role do intraspecific autoinducer signals play in the development of a root nodule? Research in this area should complement our present understanding of interspecific chemical signaling between legumes and their symbiotic nitrogen-fixing bacteria.

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