Lotus japonicus Forms Early Senescent Root Nodules with *Rhizobium etli*

Mari Banba,¹ Abu-Baker M. Siddique,² Hiroshi Kouchi,² Katsura Izui,¹ and Shingo Hata¹

¹Laboratory of Plant Physiology, Graduate School of Biostudies, Kyoto University, Sakyo-ku, Kyoto

606-8502, Japan; ²National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305-8602, Japan

Accepted 11 October 2000.

Mesorhizobium loti and *Rhizobium etli* are microsymbionts of the *Lotus* and *Phaseolus* spp., respectively, and secrete essentially the same Nod factors. *Lotus japonicus* efficiently formed root nodules with *R. etli* CE3, irrespective of the presence or absence of a flavonoid-independent transcription activator *nodD* gene. On a nitrogen-free medium, however, the host plant inoculated with *R. etli* showed a severe nitrogen deficiency symptom. Initially, the nodules formed with *R. etli* were pale pink and leghemoglobin mRNA was detectable at significant levels. Nevertheless, the nodules became greenish with time. Acetylene-reduction activity of nodules formed with *R. etli* was comparable with that formed by *M. loti* 3 weeks postinoculation, but thereafter it decreased rapidly. The nodules formed with *R. etli* contained much more starch granules than those formed with *M. loti*. *R. etli* developed into bacteroids in the *L. japonicus* nodules, although the density of bacteroids in the infected cells was lower than that in the nodules formed with *M. loti*. The nodules formed with *R. etli* were of the early senescence type, in that membrane structures were drastically disintegrated in the infected cells of the greenish nodules. Thus, *L. japonicus* started and then ceased a symbiotic relationship with *R. etli* at the final stage.

Additional keywords: nitrogen fixation, symbiosis.

The symbiosis between legumes and rhizobia is initiated by reciprocal signal exchange (Denarie et al. 1996; Long 1996; Spaik and Lugtenberg 1994). Flavonoid compounds released from a host plant activated bacterial NodD protein, a DNA-binding protein, and induced a number of *nod* genes, resulting in the secretion of Nod factors, lipo-chitin oligosaccharides. In turn, the Nod factors secreted from the bacteria elicit sequential responses of the host plant, including root hair curling, infection thread formation, and cortical cell division, which led to the formation of nodules. After entering the host cells in the nodules through endocytosis, the rhizobia differentiate into bacteroids and nitrogen fixation occurs.

The structure of the Nod factor of *Rhizobium etli* strain

CE3, a bacterium that forms nitrogen-fixing root nodules on the *Phaseolus* spp., was reported to be the same as that of *Mesorhizobium loti*, which forms symbiotic nodules on *Lotus* spp. (Cárdenas et al. 1995; López-Lara et al. 1995). Cárdenas et al. (1995) examined the nodule formation activity of *R. etli* CE3 on *Lotus corniculatus*, *Lotus caucasicus*, and *Lotus preslii*, and reported that the bacterium is able to form nodules on *L. corniculatus* and *L. caucasicus* only when it has a flavonoid-independent transcription activator (FITA) *nodD* gene. *R. etli* formed nodules on *L. preslii* roots, however, irrespective of the presence or absence of the FITA *nodD* gene (Cárdenas et al. 1995). In any case, it has not been examined whether the *Lotus* spp. nodules formed with *R. etli* CE3 are functional.

Lotus japonicus has attracted much attention as a model legume plant, mainly because it is diploid with a small genome, self-compatible, and rather easy to transform (Handberg and Stougaard 1992; Imaizumi-Anraku et al. 1997; Jiang and Gresshoff 1997). Unlike *Medicago truncatula*, another model legume that forms indeterminate nodules (Cook 1999), *L. japonicus* forms determinate nodules. The nodulation of both model plants should be studied extensively because the signal exchange pathways may be different in indeterminate and determinate nodule forming hosts (Roest et al. 1995). We used *L. japonicus* to investigate the mutual recognition between legumes and rhizobia. In the present study, we examined the nodule-formation activity of *L. japonicus* with *R. etli* CE3 and characterized the resulting nodules.

RESULTS

R. etli forms partly effective nodules on *L. japonicus* roots.

We inoculated *L. japonicus* roots with *R. etli* CE3 (Vasquez et al. 1991) and its derivative, CE3-604 (Cárdenas et al. 1995), which contains plasmid pMP604 harboring a hybrid FITA *nodD* gene that confers flavonoid-independent transcription activation (Diaz et al. 2000; Spaik et al. 1987) as well as *M. loti* strain Tono as a control. The plants were grown on vermiculite supplemented with a nitrogen-free medium, which is an efficient synthetic soil for the growth and nodulation of *L. japonicus* (Hussain et al. 1999). Nodules were formed on *L. japonicus* with CE3 (Fig. 1A and D) and CE3-604 (not shown), almost as efficiently as with *M. loti* (Fig. 1B and E). Thus, the nodule-formation activity of *L. japonicus* is similar to that of *L. preslii* (Cárdenas et al. 1995). The nodule primordia formed with *R. etli* CE3 and CE3-604 emerged at

Corresponding author: S. Hata; Telephone: +81 75 753 6141
Fax: +81 75 753 6470; E-mail: shing@kais.kyoto-u.ac.jp

Present address of A.-B. M. Siddique: Microscopy Centre, GPO Box 1600, Plant Industry, CSIRO, Canberra ACT 2601, Australia.

the same time as those formed with *M. loti*, approximately 7 days postinoculation. The nodule sizes were comparable up to 3 weeks postinoculation under these experimental conditions. Because *Lotus* and *Phaseolus* spp. are determinate nodule-forming plants, these results suggest that the *R. etli* infection process was normal in *L. japonicus* roots. Unexpectedly, when *L. japonicus* was grown for up to 4 weeks, we noticed that the growth of the plant shoots differed greatly with the inoculated bacteria (Fig. 1A to C). *M. loti* supported plant growth as expected, whereas the growth of the plants inoculated with CE3 or CE3-604 (not shown) was as poor as that of noninoculated plants. When the plants were grown on a medium containing 10 mM nitrate, their growth was good in the presence and

absence of CE3 (data not shown). Thus, the growth retardation seemed to simply be a result of a nitrogen deficiency in the *L. japonicus* plants. The supplying of such high concentrations of nitrate significantly inhibited nodule formation with CE3 or CE3-604 (data not shown), indicating that the nodulation of *L. japonicus* with *R. etli* is regulated by the nitrogen source similar to that of *M. loti*.

The bacterium recovered from surface-sterilized nodules formed with *R. etli* CE3 was resistant to streptomycin and piperacillin and efficiently formed nodules on *P. vulgaris* roots (data not shown), confirming that CE3 is indeed the inhabitant of the nodules.

Unless otherwise stated, we hereafter characterize nodules formed only with *R. etli* CE3 in comparison with *M. loti* because the FITA *nodD* gene of CE3-604 did not affect the nodule characteristics.

Outlook of the nodules.

After inoculation of *L. japonicus* with *R. etli* CE3 or *M. loti* and growth for 3 to 5 weeks on nitrogen-free vermiculite, we checked the number and color of the nodules. Three weeks after the inoculation of CE3, all nodules were pale pink (Fig. 1D, bottom nodule). Subsequently, 53 and 63% of the nodules became greenish (Fig. 1D, top nodule) at 4 and 5 weeks postinoculation, respectively. There was a tendency for the nodules formed with CE3 on the basal part of the roots to turn greenish earlier than those near the emerging root hairs. In contrast, all nodules formed with *M. loti* were pink even at 5 weeks postinoculation. In nitrogen-free vermiculite, the mature nodules formed with *M. loti* often became larger and deeper in color than those formed with CE3 (Fig. 1D and E).

In situ hybridization.

In situ hybridization with the *L. japonicus* leghemoglobin gene (GenBank accession no. AB008224) as a probe showed that leghemoglobin mRNA is detectable 2 weeks after *R. etli* CE3 is inoculated and is expressed at significant levels in *R. etli* CE3-infected cells of young, pink nodules (Fig. 2A).

Acetylene-reduction activity.

Because it was unknown whether the nodules formed with CE3 performed nitrogen fixation, we determined the acetylene-reduction activities of nodules formed in nitrogen-free vermiculite. Three weeks after the inoculation of *L. japonicus*, the nodules formed with CE3 showed comparable activity with that of nodules formed with *M. loti* (Fig. 3A and C). The total and specific activity, however, decreased rapidly 3 and 4 weeks postinoculation (Fig. 3A and C). The weight and number of nodules formed with CE3 became maximum approximately 5 weeks postinoculation (Fig. 3B). In contrast, the total activity of the nodules formed with *M. loti* continued to increase (Fig. 3A), as did the nodule weight (Fig. 3B), and the specific activity remained nearly constant (Fig. 3C).

Inspection of the insides of the nodules.

We examined the insides of the nodules by light microscopy (Fig. 4) and transmission electron microscopy (Fig. 5). The infection, release from the infection thread, and subsequent proliferation of *R. etli* CE3 appeared normal, although the nodules contained less bacteroid-infected cells than those formed with *M. loti* (Fig. 4A and C). In the nodules formed

Fig. 1. Nodule formation on *Lotus japonicus* roots. **A**, Plants were inoculated with *Rhizobium etli* CE3 or **B**, wild-type *Mesorhizobium loti* and grown in sterile vermiculite with nitrogen-free B&D medium for 5 weeks. **C**, Noninoculated control plant. **D**, Nodules formed with *R. etli* CE3 and **E**, *M. loti* 5 weeks postinoculation. Bar = **A** to **C**, 3 cm; **D** and **E**, 1 mm.

with CE3, however, there were much more starch granules in uninfected cells than in those formed with *M. loti* (Fig. 4A and C). This is probably because the nodules formed with CE3 cannot utilize photosynthate as an energy source for nitrogen fixation. In the infected cells, *R. etli* CE3 differentiated into bacteroids, although the number was lower than that of *M. loti* (Figs. 4B and D and 5A and C). Bacteroids of CE3 were occasionally branched (data not shown). Often there were more than two bacteroids of *M. loti* in a symbiosome (Fig. 5D), but this was rare in the case of *R. etli* (Fig. 5B). The nodules formed with CE3 appeared to be of the early-senescence type because greater amounts of disintegrated membrane structures were found in the infected cells in pale-pink nodules (Fig. 5B). The cytoplasmic structures in the infected cells of the greenish nodules formed with CE3 degraded remarkably, and deteriorated bacteroids were aggregated (Figs. 4E and F and 5E). Leghemoglobin mRNA was still detected in the outer regions of the infected zone, although it became undetectable in the greenish nodules (Fig. 2C). Thus, the disorganization seemed to be rather rapid and was initiated at the center of the infected zone. Because the uninfected cells were fully vacuolated, it is unclear whether they were undergoing degradation.

The bacteroids of CE3 contained greater amounts of poly-beta-hydroxybutyrate, which is thought to be an energy source for bacteroids, than those of *M. loti* (Fig. 5B and D). This seems to be a result of the inherent nature of CE3 rather than the inability of bacteroids to consume energy. The bacteroids of CE3 in the nodules on *Phaseolus vulgaris* also were filled with poly-beta-hydroxybutyrate (Fig. 5F).

The inside of the nodules formed with CE3-604 was very similar to that of the nodules formed with CE3 (data not shown).

The cytoplasmic disorganization of *R. etli*-infected nodule cells is not caused by nitrogen starvation.

To determine whether the disintegration of the membrane structures in CE3-infected nodule cells is a result of nitrogen starvation, we grew *L. japonicus* inoculated with *R. etli* CE3 on vermiculite containing 1 mM urea, which corresponds to the best nitrogen supplement concentration for optimal nodulation (Hussain et al. 1999). Plant growth was much better in the presence of urea than in its absence because the nodule numbers were similar to each other (data not shown). Nevertheless, an effect of urea on either acetylene-reduction activity (Fig. 3) or the disintegration of the nodule cells was not detected (data not shown). Therefore, it is unlikely that the disintegration of the membrane structures of CE3-infected cells results from the lack of a nitrogen supply.

DISCUSSION

It is well documented that Nod factors are major mediators of the host specificity of the infection and nodule initiation processes (Denarie et al. 1996; Long 1996; Spaink and Lugtenberg 1994; Spaink et al. 1991; Truchet et al. 1991). The putative Nod factor receptor is thought to be on the surface of root hairs (Felle et al. 1998; Goehart et al. 1999). It has been suggested that plant lectins, which also are located in root hairs, are involved in the initial recognition of rhizobial extracellular polysaccharides such as lipopolysaccharides and exopolysaccharides (Diaz et al. 1989; Eggleston et al. 1996;

Van Rhijin et al. 1998), but the mechanism remains unknown (Diaz et al. 2000). Notably, it has been suggested recently that a novel Nod-factor-binding lectin is involved in the initial recognition event (Etzler et al. 1999).

Here we revealed that *L. japonicus* is able to form nodules with *R. etli* CE3, irrespective of the presence or absence of the *FITA nodD* gene. In the nodules, CE3 performs nitrogen fixation, although its activity was much lower than that of *M. loti*. Because Nod factors of *R. etli* and *M. loti* are indistinguishable from each other (Cárdenas et al. 1995), it is reasonable to assume that *L. japonicus* accepts *R. etli* CE3 as a symbiont.

Within 2 to 3 weeks after infection with *R. etli* CE3, the nodule formation and bacteroid differentiation appear to be almost comparable with those infected with *M. loti*, as revealed on microscopic observation (Figs. 4 and 5). Thereafter, *L. japonicus* cannot continue the symbiotic relationship with CE3, and the nodules formed with CE3 exhibit early senescence such as disintegration of symbiosome membranes, deterioration and aggregation of bacteroids, and degradation of the cytoplasmic structures of the infected cells (Fig. 5). Consequently, the plants suffer a severe nitrogen deficiency. Thus, *R. etli* CE3 can establish a symbiotic association with *L. japonicus* transiently, in that it cannot develop and maintain the functional symbiotic relationship with *L. japonicus*.

It has been suggested that the specific interactions between host plants and rhizobia are important even after the establishment of symbiosis. These interactions seem to be distinct

Fig. 2. Leghemoglobin gene expression in *Lotus japonicus* nodules formed with *Rhizobium etli* CE3. **A**, Pink nodule section hybridized with a digoxigenin-labeled antisense leghemoglobin probe. **B**, Sense control of panel **A**. **C**, Sections of pale-pink (left) and greenish (right) nodules on the same root hybridized with the antisense probe. Bar = 300 μ m.

from those mediated by Nod factors. For example, *Rhizobium leguminosarum* bv. *trifolii*, transformed to produce the same Nod factor as that of *R. leguminosarum* bv. *viciae*, can cause the formation of nodules on vetch and pea, which do not undergo nodulation with the former biovar (Spaink et al. 1989). The nodules were ineffective (fix^-), although the bacteria apparently differentiated into bacteroids (De Maagd et al. 1994). The relationship among *L. japonicus*, *M. loti*, and *R. etli* CE3 appears to be similar to this example because the structure of the Nod factors produced by *R. etli* and *M. loti* are identical. There are other examples of unnatural combinations of host plants and rhizobia resulting in fix^- nodules, as reported by Young and Johnston (1989).

The identification of a number of plant mutants that form ineffective nodules with compatible rhizobia indicates the presence of plant genes that control functional symbiosis other than those involved in Nod-factor perception during infection and nodule initiation. Such mutants have been reported for alfalfa (Vance and Johnson 1983), pea (Kneen et al. 1990; Novak et al. 1995; Postma et al. 1990), cowpea (Purdum and Trese 1995), and *L. japonicus* (Imaizumi-Anraku et al. 1997; Schauser et al. 1998). These mutants form nodules of the fix^- phenotype with compatible rhizobia, which normally differentiate into bacteroids. A typical and most intensively studied example is pea mutant E135, which forms nodules with the regular microsymbiont *R. leguminosarum* bv. *viciae*, yet the nodules are completely ineffective even though nitrogenase

proteins are present in the bacteroids (Suganuma et al. 1998). These plant-conditioned, ineffective nodules are primarily of the early senescence type. Therefore, the genes responsible for the mutations also may be involved in the discrimination between *M. loti* and *R. etli* CE3.

The findings described here may lead to the identification of a possibly novel interaction(s) between legumes and rhizobia. After beginning nitrogen fixation with *R. etli* CE3, *L. japonicus* seems to recognize that CE3 is not a regular symbiont and, therefore, exhibits a kind of defense response that leads to the death of infected cells. The cytoplasmic disorganization of *R. etli*-infected cells is rather rapid (Fig. 2C). It is noteworthy that extracellular polysaccharide mutants of compatible rhizobia sometimes induce defense-like responses, even after differentiation into bacteroids (Niehaus et al. 1993, 1998; Parniske et al. 1994; Perotto et al. 1994). The structures of the Nod factors are again identical between the wild-type and mutant rhizobia. Interestingly, cytoplasmic disorganization also was observed in one case (Perotto et al. 1994). Likewise, it is possible that the difference in extracellular polysaccharides between *M. loti* and *R. etli* CE3 is recognized by *L. japonicus* cells. Although virtually nothing is known about this recognition, we speculate that isoforms of a lectinlike glycoprotein in symbiosomes might be the key (Dahiya et al. 1998). Further analyses of the structural and physiological characteristics of *R. etli*-induced nodules will provide greater insight into the specific interactions between host legumes and

Fig. 3. Acetylene reduction activity and fresh weight of *Lotus japonicus* nodules. Plants were grown in sterile vermiculite with nitrogen-free B&D medium (open symbols) or B&D medium containing 1 mM urea (closed symbols) for the indicated periods after inoculation with *Rhizobium etli* CE3 (circles) or *Mesorhizobium loti* (squares). Error bars indicate standard error. Data are the means for six plants. **A**, Total acetylene-reducing activity of the nodules. **B**, Fresh weight of nodules per plant. **C**, Specific acetylene-reducing activity of the nodules.

rhizobia, which might be responsible for the establishment of nitrogen-fixing symbiosis.

MATERIALS AND METHODS

Plant materials.

L. japonicus B-129 Gifu (originally obtained from J. Stougaard, University of Aarhus, Denmark) and *P. vulgaris* cv.

Honkintoki (Takii Seeds, Kyoto, Japan) were grown in sterile vermiculite with liquid B&D medium (Broughton and Dilworth 1971). *L. japonicus* seeds were scarified and sterilized with a sodium hypochlorite solution, rinsed with sterile water, and allowed to germinate under sterile conditions. Three seedlings were transferred to sterile double Magenta jars containing vermiculite in the upper jar supplied with B&D medium from the lower jar (Hussain et al. 1999). Then,

Fig. 4. Light micrographs of toluidine blue-stained nodule sections of *Lotus japonicus*. **A and B**, Pink nodule formed with *Rhizobium etli* CE3. **C and D**, Pink nodule formed with wild-type *Mesorhizobium loti*. **E and F**, Greenish nodule formed with *R. etli* CE3. Arrows indicate starch granules. ic: infected cell; uc: uninfected cell; vb: vascular bundle. Bars = 50 µm (**A**, **C**, and **E**) and 10 µm (**B**, **D**, and **F**).

5 ml of inoculant (6.5×10^9 cells per ml) per plant was added. Surface-sterilized *P. vulgaris* seeds were allowed to germinate on sterile vermiculite with B&D medium and 5 ml of inoculant (6.5×10^9 cells per ml) per plant was added. All plants were grown in a controlled-environment growth chamber with a 16-h day–8-h night cycle at 25°C with 60% humidity. The intensity of the fluorescent light was $260 \mu\text{E s}^{-1} \text{m}^{-2}$.

Bacterial strains.

R. etli CE3 (Vasquez et al. 1991) and its derivative, CE3-604, which contains plasmid pMP604 (Spaink et al. 1987), were gifts from H. Spaink and A. Wijffes (Leiden University,

The Netherlands). Wild-type Tono was isolated by M. Kawaguchi (University of Tokyo). Bacteria were grown at 28°C for 2 days in yeast extract–mannitol medium (Vincent 1970) with 20 μg of piperacillin per ml for *R. etli* CE3, 20 μg of piperacillin and 2 μg of tetracycline per ml for *R. etli* CE3-604, and without antibiotics for Tono. Antibiotics were added to the medium to avoid bacterial contamination. After growth, bacteria were harvested and resuspended in sterile water for inoculation.

Recovery of *R. etli* CE3 from the nodules.

Nodules formed with CE3 were harvested with *L. japonicus* roots at 5 weeks postinoculation, sterilized with diluted so-

Fig. 5. Transmission electron micrographs of bacteroids. **A and B**, *Rhizobium etli* CE3 in a pink nodule of *Lotus japonicus*. **C and D**, *Mesorhizobium loti* in a pink wild-type nodule. **E**, *R. etli* CE3 in a greenish nodule of *L. japonicus*. **F**, *R. etli* CE3 in a pink wild-type nodule of *Phaseolus vulgaris*. Short arrows indicate poly-beta-hydroxybutyrate. Arrowheads indicate peribacteroid membranes. Long arrows indicate disintegrated membranes. b: bacteroid; cw: cell wall; hc: host cytoplasm; is: intercellular space; s: starch granule. Bars = 2 μm (**A** and **C**), 0.5 μm (**B** and **D**), and 1 μm (**E** and **F**).

dium hypochlorite solution (1% available chlorine) and 0.02% Tween 20 for 5 min, washed three times with sterile water, and squashed with a sterile plastic pestle in 50 µl of sterile water in an Eppendorf tube. The resulting suspension was plated on yeast extract-mannitol agar medium, purified to single colonies, tested for streptomycin and piperacillin resistance. Then, nodulation of *P. vulgaris* was checked.

In situ hybridization.

In situ hybridization of leghemoglobin transcripts in *L. japonicus* nodules at 4 and 5 weeks postinoculation was carried out as described (Kouchi and Hata 1993). In brief, nodules were harvested at 4 and 5 weeks postinoculation and immediately fixed, dehydrated through a graded ethanol series, and embedded in paraffin. Microtome sections (10 µm thick) were hybridized with digoxigenin-labeled antisense and sense probes at 50°C for 16 h and successive washings were performed. The probes were synthesized from the following polymerase chain reaction (PCR) product after subcloning in pBluescriptII KS(−) (Stratagene, La Jolla, CA, U.S.A.). Nucleotides 38 to 403 of the *L. japonicus* leghemoglobin cDNA sequence (GenBank accession no. AB008224) were amplified from *L. japonicus* genomic DNA, which was isolated from leaves by the CTAB method, as described by Rogers and Bendich (1988). The resulting PCR product contained an intron of about 930 bp.

Acetylene reduction assay.

Each plant root with intact nodules was cut at the hypocotyl node and placed in a 20-ml glass vial in which 5% of the air was replaced with acetylene. After 1 and 30 min, 1 ml of samples were withdrawn and subjected to gas chromatography to determine ethylene production as described previously (Kouchi et al. 1989). The values at 30 min were subtracted from those at 1 min. After the assay, the nodules were harvested, counted, and weighed and the nodule color was checked.

Light and transmission electron microscopy.

L. japonicus and *P. vulgaris* were grown and allowed to undergo nodulation as described above. Nodule samples were processed for microscopic studies, according to Siddique and Bal (1992). Briefly, harvested nodules were prefixed immediately with 4% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 6.8), washed with the buffer, postfixed in 1% osmium tetroxide, dehydrated through an ethanol series, and infiltrated with and embedded in Spurr's resin (Spurr 1969). We used an Ultratome Nova microtome (LKB, Bromma, Sweden) to cut ultrathin sections that were poststained and viewed under a H-7100 electron microscope (Hitachi, Tokyo, Japan). For light microscopy, sections (1.5-µm thick) were cut and stained with 1% toluidine blue.

ACKNOWLEDGMENTS

We thank H. P. Spaink, A. H. M. Wijffes and M. Kawaguchi for providing the bacterial strains, S. Tajima, N. Suganuma, and H. P. Spaink for valuable discussions, and C. A. Asis Jr., H. Imaizumi-Anraku, and M. Shinozaki for technical advice. This work was supported by the Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency of Japan.

LITERATURE CITED

- Broughton, W. J., and Dilworth, M. J. 1971. Control of leghemoglobin synthesis in snake beans. *Biochem. J.* 125:1075-1080.
- Cárdenas, L., Dominguez, J., Quinto, C., López-Lara, I. M., Lugtenberg, B. J. J., Spaink, H. P., Rademaker, G. J., Haverkamp, J., and Thomas-Oates, J. E. 1995. Isolation, chemical structures and biological activity of the lipo-chitin oligosaccharide nodulation signals from *Rhizobium etli*. *Plant Mol. Biol.* 29:453-464.
- Cook, D. R. 1999. *Medicago truncatula*: A model in the making! *Curr. Opin. Plant Biol.* 2:301-304.
- Dahiya, P., Sherrier, D. J., Kardailsky, I. V., Borisov, A. Y., and Brewin, N. J. 1998. Symbiotic gene *Sym31* controls the presence of a lectin-like glycoprotein in the symbiosome compartment of nitrogen-fixing pea nodules. *Mol. Plant-Microbe Interact.* 11:915-923.
- De Maagd, R. A., Yang, W.-C., Goosen-de Roo, L., Mulders, I. H. M., Roest, H. P., Spaink, H. P., Bisseling, T., and Lugtenberg, B. J. J. 1994. Down-regulation of expression of the *Rhizobium leguminosarum* outer membrane protein gene *ropA* occurs abruptly in interzone II-III of pea nodules and can be uncoupled from *nif* gene activation. *Mol. Plant-Microbe Interact.* 7:276-281.
- Denarie, J., Debelle, F., and Prome, J. C. 1996. Rhizobium lipochitooligosaccharide nodulation factors: Signaling molecules mediating recognition and morphogenesis. *Annu. Rev. Biochem.* 65:503-535.
- Diaz, C. L., Melchers, L. S., Hooykaas, P. J. J., Lugtenberg, B. J. J., and Kijne, J. W. 1989. Root lectin as a determinant of host-plant specificity in the *Rhizobium*-legume symbiosis. *Nature* 338:579-581.
- Diaz, C. L., Spaink, H. P., and Kijne, J. W. 2000. Heterologous rhizobial lipochitin oligosaccharides and chitin oligomers induce cortical cell divisions in red clover roots, transformed with the pea lectin gene. *Mol. Plant-Microbe Interact.* 13:268-276.
- Eggleston, G., Huber, M. C., Liang, R., Karr, A. L., and Emerich, D. W. 1996. *Bradyrhizobium japonicum* mutants deficient in exo- and capsular polysaccharides cause delayed infection and nodule initiation. *Mol. Plant-Microbe Interact.* 9:419-423.
- Etzler, M. E., Kalsi, G., Ewing, N. N., Roberts, N. J., Day, R. B., and Murphy, J. B. 1999. A nod factor binding lectin with apyrase activity from legume roots. *Proc. Natl. Acad. Sci. USA* 96:5856-5861.
- Felle, H. H., Kondorosi, E., Kondorosi, A., and Schultze, M. 1998. The role of ion fluxes in nod factor signaling in *Medicago sativa*. *Plant J.* 13:455-463.
- Goedhart, J., Rohrig, H., Hink, M. A., Van Hoek, A., Visser, A. W. G., Bisseling, T., and Gadella, T. W. J., Jr. 1999. Nod factors integrate spontaneously in biomembranes and transfer to root hairs, but transbilayer flip-flop does not occur. *Biochemistry* 38:10898-10907.
- Handberg, K., and Stougaard, J. 1992. *Lotus japonicus*, an autogamous, diploid legume species for classical and molecular genetics. *Plant J.* 2:487-496.
- Hussain, A. K. M. A., Jiang, Q., Broughton, W. J., and Gresshoff, P. M. 1999. *Lotus japonicus* nodulates and fixes nitrogen with the broad host range *Rhizobium* sp. NGR234. *Plant Cell Physiol.* 40:894-899.
- Imaizumi-Anraku, H., Kawaguchi, M., Koiwa, H., Akao, S., and Syono, K. 1997. Two ineffective-nodulating mutants of *Lotus japonicus*: Different phenotypes caused by the blockage of endocytotic bacterial release and nodule maturation. *Plant Cell Physiol.* 38:871-881.
- Jiang, Q., and Gresshoff, P. M. 1997. Classical and molecular genetics of the model legume *Lotus japonicus*. *Mol. Plant-Microbe Interact.* 10:59-68.
- Kneen, B. E., LaRue, T. A., Hirsh, A. M., Smith, C. A., and Weeden, N. F. 1990. *sym 13*: A gene conditioning ineffective nodulation in *Pisum sativum*. *Plant Physiol.* 94:899-905.
- Kouchi, H., and Hata, S. 1993. Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. *Mol. Gen. Genet.* 238:106-119.
- Kouchi, H., Tsukamoto, M., and Tajima, S. 1989. Differential expression of nodule-specific (nodulin) genes in the infected, uninfected and cortical cells of soybean (*Glycine max*) root nodules. *J. Plant Physiol.* 135:608-617.
- Long, S. R. 1996. *Rhizobium* symbiosis: Nod factors in perspective. *Plant Cell* 8:1885-1898.
- López-Lara, I. M., Van den Berg, J. D. J., Thomas-Oates, J. E., Glushka, J., Lugtenberg, B. J. J., and Spaink, H. P. 1995. Structural identification of the lipo-chitin oligosaccharide nodulation signals of *Rhizo-*

- bium loti*. Mol. Microbiol. 15:627-638.
- Niehaus, K., Kapp, D., and Puhler, A. 1993. Plant defence and delayed infection of alfalfa pseudonodules induced by an exopolysaccharide (EPS I)-deficient *Rhizobium meliloti* mutant. Planta 190:415-425.
- Niehaus, K., Lagares, A., and Pühler, A. 1998. A *Sinorhizobium meliloti* lipopolysaccharide mutant induces effective nodules on the host plant *Medicago sativa* (alfalfa) but fails to establish a symbiosis with *Medicago truncatula*. Mol. Plant-Microbe Interact. 11:906-914.
- Novak, K., Pesina, K., Nevesarova, J., Skrdleta, V., Lisa, L., and Nasinec, V. 1995. Symbiotic tissue degradation pattern in the ineffective nodules of three nodulation mutants of pea (*Pisum sativum* L.). Ann. Bot. 76:303-313.
- Parniske, M., Schmidt, P. E., Kosch, K., and Müller, P. 1994. Plant defense responses of host plants with determinate nodules induced by EPS-defective *exoB* mutants of *Bradyrhizobium japonicum*. Mol. Plant-Microbe Interact. 7:631-638.
- Perotto, S., Brewin, N. J., and Kannenberg, E. L. 1994. Cytological evidence for a host defense response that reduces cell and tissue invasion in pea nodules by lipopolysaccharide-defective mutants of *Rhizobium leguminosarum* strain 3841. Mol. Plant-Microbe Interact. 7:99-112.
- Postma, J. G., Jager, D., Jacobsen, E., and Feenstra, W. J. 1990. Studies on a non-fixing mutant of pea (*Pisum sativum* L.). I. Phenotypical description and bacteroid activity. Plant Sci. 68:151-161.
- Purdom, D., and Trese, A. T. 1995. Morphological and molecular characteristics of host-conditioned ineffective root nodules in cowpea. Plant Physiol. 109:239-244.
- Roest, H. P., Goosen-de Roo, L., Wijffelman, C. A., De Maagd, R. A., and Lugtenberg, B. J. J. 1995. Outer membrane protein changes during bacteroid development are independent of nitrogen fixation and differ between indeterminate and determinant nodulating host plants of *Rhizobium leguminosarum*. Mol. Plant-Microbe Interact. 8:14-22.
- Rogers, S. O., and Bendich, A. J. 1988. Extraction of DNA from plant tissues. Pages A6/1-11 in: Plant Molecular Biology Manual. S. B. Gelvin, R. A. Schiperoort, and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Schauser, L., Handberg, K., Sandal, N., Stiller, J., Thykjaer, Pajuelo, E., Nielsen, A., and Stougaard, J. 1998. Symbiotic mutants deficient in nodule establishment identified after T-DNA transformation of *Lotus japonicus*. Mol. Gen. Genet. 259:414-423.
- Siddique, A.-B. M., and Bal, A. M. 1992. Morphological and biochemical changes in peanut nodules during photosynthate stress. Can. J. Microbiol. 38:526-533.
- Spaink, H. P., and Lugtenberg, B. J. J. 1994. Role of rhizobial lipo-chitin oligosaccharide signal molecules in root nodule organogenesis. Plant Mol. Biol. 26:1413-1422.
- Spaink, H. P., Okker, R. J. H., Wijffelman, C. A., Pees, E., and Lugtenberg, B. J. J. 1987. Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1J1. Plant Mol. Biol. 9:29-37.
- Spaink, H. P., Weinman, J., Djordjevic, M. A., Wijffelman, C. A., Okker, R. J. H., and Lugtenberg, B. J. J. 1989. Genetic analysis and cellular localization of the *Rhizobium* host-specificity-determining NodE protein. EMBO J. 8:2811-2818.
- 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. A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. Nature 354:125-130.
- Spurr, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31-43.
- Suganuma, N., Sonoda, N., Nakane, C., Hayashi, K., Hayashi, T., Tamaoki, M., and Kouchi, H. 1998. Bacteroids isolated from ineffective nodules of *Pisum sativum* mutant E135 (sym13) lack nitrogenase activity but contain the two protein components of nitrogenase. Plant Cell Physiol. 39:1093-1098.
- Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., De Billy, F., Prome, J.-C., and Denerie, J. 1991. Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature 351:670-673.
- Vance, C. P., and Johnson, L. E. B. 1983. Plant determined ineffective nodules in alfalfa (*Medicago sativa*): Structural and biochemical comparisons. Can. J. Bot. 61:93-106.
- Van Rhijn, P., Goldberg, R. B., and Hirsch, A. M. 1998. Lotus corniculatus nodulation specificity is changed by the presence of a soybean lectin gene. Plant Cell 10:1233-1249.
- Vasquez, M., Davalos, A., De las Penas, A., Sanchez, F., and Quinto, C. 1991. Novel organization of the common nodulation genes in *Rhizobium leguminosarum* bv. *phaseoli* strains. J. Bacteriol. 173:1250-1258.
- Vincent, J. M. 1970. A manual for the practical study of root nodule bacteria. IBP Handbook No. 15. Blackwell Scientific Publications, Oxford, U.K.
- Young, J. P. W., and Johnston, A. W. B. 1989. The evolution of specificity in the legume-*Rhizobium* symbiosis. Trends Ecol. Evol. 4:341-349.