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RESEARCH ARTICLE

Lotus corniculatus Nodulation Specificity Is Changed by the Presence of a Soybean Lectin Gene

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Plant lectins have been implicated as playing an important role in mediating recognition and specificity in the *Rhizobium*–legume nitrogen-fixing symbiosis. To test this hypothesis, we introduced the soybean lectin gene *Le1* either behind its own promoter or behind the cauliflower mosaic virus 35S promoter into *Lotus corniculatus*, which is nodulated by *R. loti.* We found that nodulelike outgrowths developed on transgenic *L. corniculatus* plant roots in response to *Bradyrhizobium japonicum*, which nodulates soybean and not *Lotus* spp. Soybean lectin was properly targeted to *L. corniculatus* root hairs, and although infection threads formed, they aborted in epidermal or hypodermal cells. Mutation of the lectin sugar binding site abolished infection thread formation and nodulation. Incubation of bradyrhizobia in the nodulation (*nod*) gene–inducing flavonoid genistein increased the number of nodulelike outgrowths on transgenic *L. corniculatus* roots. Studies of bacterial mutants, however, suggest that a component of the exopolysaccharide surface of *B. japonicum*, rather than Nod factor, is required for extension of host range to the transgenic *L. corniculatus* plants.

INTRODUCTION

The symbiotic interaction between rhizobia and legume roots is characterized by a high degree of specificity. For example, *Lotus* spp are nodulated by *Rhizobium loti* and soybean by *Bradyrhizobium japonicum.* The reciprocal interaction does not occur. Nod factor, a substituted lipochitooligosaccharide synthesized as a result of the expression of rhizobial *nod* genes, is a key factor in the initiation of nodule development. Low concentrations of purified Nod factor molecules elicit many of the responses observed during the early stages of nodule formation in a host-specific manner (Truchet et al., 1991). These responses include root hair deformation and curling, the initiation of cell division, and, in some hosts, the triggering of an organogenic program that leads to nodule initiation (see Hirsch, 1992). Rhizobial *nod* gene expression is mediated by the transcriptional activator NodD, a regulator of the LysR type, which interacts with plant-secreted flavonoids and nonflavonoid inducers, such as trigonelline and stachydrine (Phillips et al., 1994). These interactions also can be highly specific (see van Rhijn and Vanderleyden, 1995; Dénarié et al., 1996; Long, 1996).

Plant lectins have also been proposed to be involved in host–rhizobia recognition (Hamblin and Kent, 1973; Bohlool and Schmidt, 1974; Dazzo and Hubbell, 1975). Legume lectins, which are localized to root hairs, have been hypothesized to bind specifically to a saccharide moiety on the surface of a compatible *Rhizobium*, thereby allowing the two symbionts to recognize each other. Several studies using mutant rhizobia and transgenic plants have supported the lectin recognition hypothesis (Halverson and Stacey, 1985, 1986; Díaz et al., 1989; Kijne et al., 1994; van Eijsden et al., 1995). These experiments implicated lectin as playing an important role in both rhizobial attachment and infection thread formation.

Halverson and Stacey (1985, 1986) showed that a *B. japonicum* nodulation-defective mutant, which was capable of attaching to root hairs and stimulating their curling, could be restored to normal nodulation capability by pretreatment with soybean lectin. Díaz et al. (1989) later demonstrated that transgenic clover plants carrying a pea lectin gene are nodulated (at low efficiency) by *R. leguminosarum* bv *viciae*, which normally does not nodulate clover. Finally, site-directed mutagenesis studies demonstrated that the carbohydrate binding domain of the pea lectin was responsible for the change in host specificity (Kijne et al., 1994; van Eijsden et al., 1995).

Because of the close relationship between the two rhizobial biovars that nodulate pea and clover (*R. l.* bv *viciae* and *R. l.* bv *trifolii*, respectively), it is difficult to extrapolate the pea lectin results to other legume–*Rhizobium* interactions.

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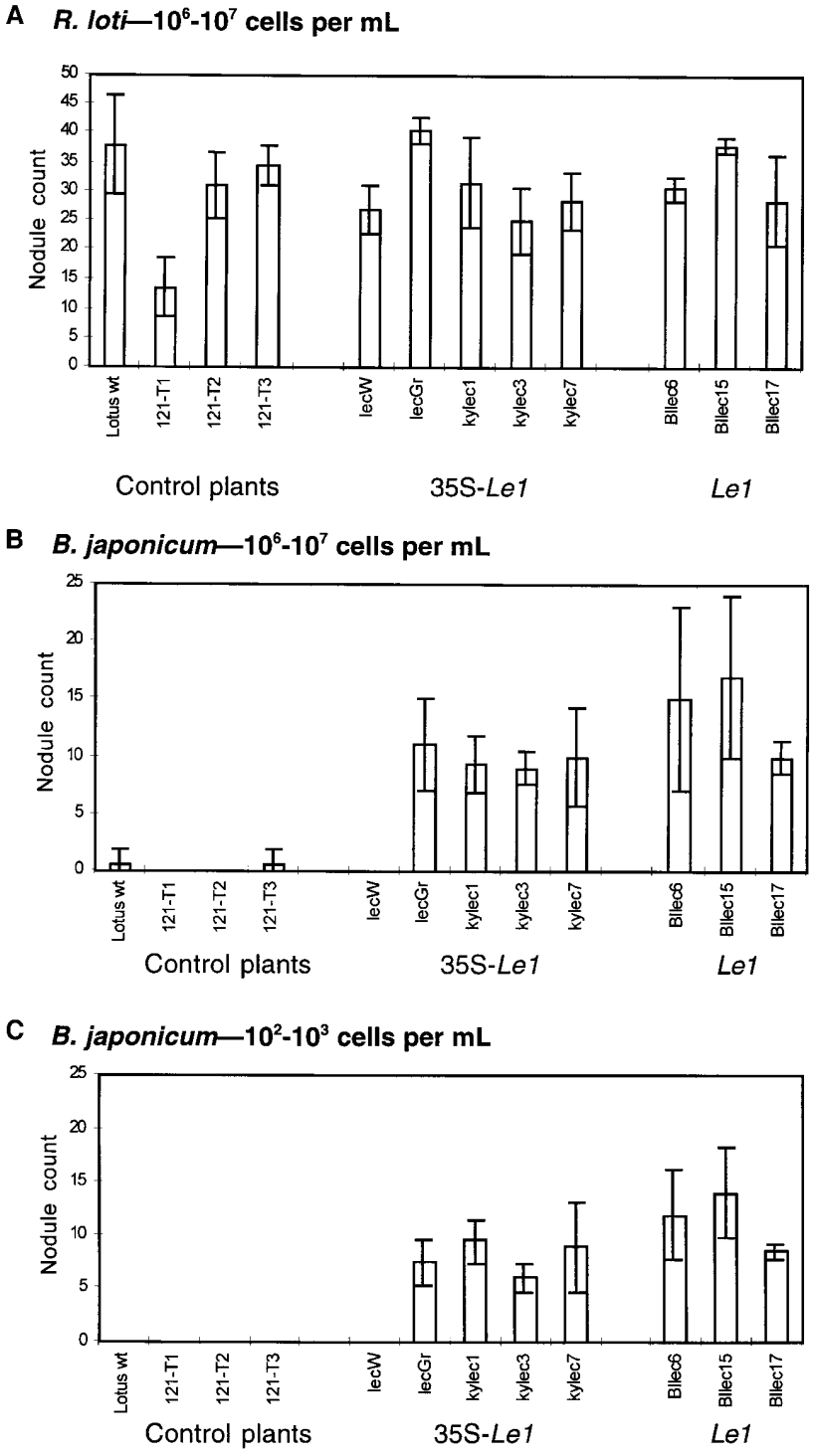

Figure 1. Nodulation Characteristics of Control and Transgenic *L. corniculatus* Plants when Inoculated with Different Rhizobial Strains.

The number of nodules per plant was scored 2 months after inoculation. The nodule numbers given are the mean with the standard deviation of at least three replicates. Protuberances were scored as nodules if, after clearing, cortical cell divisions could be detected in the inoculated root. wt, wild type.

(A) Inoculation with 10^6 to 10^7 cells per mL of *R. loti*.

(B) Inoculation with 10^6 to 10^7 cells per mL of *B. japonicum*.

(C) Inoculation with 10^2 to 10^3 cells per mL of *B. japonicum*.

Unlike more distantly related rhizobia, *R. l.* bv *viciae* bacteria deform clover root hairs, although nodules are not induced (Yao and Vincent, 1969). If lectin is important for recognition of the host by the bacterium, then it should be a universal determinant in *Rhizobium*–legume interactions.

In this study, we present results showing that soybean-specific *B. japonicum* can induce nodules on the roots of transgenic *L. corniculatus* containing the soybean lectin gene *Le1. Le1* was chosen because it is expressed in roots and because the soybean lectin used in the experiments of Halverson and Stacey (1985, 1986) is the product of the *Le1* gene. *Le2* appears to be a pseudogene (Goldberg et al., 1983). The nodules formed on the transgenic *Le1* roots resembled those induced by *R. loti* on roots of nontransgenic *L. corniculatus* in that nodule initiation occurred in the root outer cortex and infection threads were formed. In contrast, *B. japonicum* inoculation did not give rise to nodules on transgenic *L. corniculatus* roots containing a mutated soybean lectin gene with an altered carbohydrate binding domain. Inoculating the transgenic *L. corniculatus* roots containing a wild-type lectin with *B. japonicum* grown in genistein, the isoflavonoid inducer of *B. japonicum nod* genes, significantly increased the number of nodules. The soybean lectin is not the target for Nod factor–type molecules, however, because purified fractions of *B. japonicum* Nod factor, as well as synthetic Nod factors, induce nodules on both transgenic and nontransgenic plants. Together, these results show that soybean lectin can change the host specificity of *L. corniculatus* plants in a Nod factor–independent way. We postulate that a component of bradyrhizobial exopolysaccharide, rather than Nod factor, may be the ligand that interacts with the soybean lectin.

RESULTS

We introduced the complete soybean *Le1* lectin gene (Goldberg et al., 1983) into *L. corniculatus* and designated these as *Le1* plants. The *Le1* gene contained the lectin coding sequence plus 3 and 1.5 kb of 5′ and 3′ flanking sequences, respectively. Transgenic *L. corniculatus* plants carrying a cauliflower mosaic virus 35S–*Le1* chimeric gene (designated 35S–*Le1* plants) were constructed previously (Hirsch et al., 1995). Several independent *Le1* and 35S–*Le1* transgenic lines were inoculated with a wild-type *R. loti* strain to verify that the transformation process did not alter normal nodulation capability. As shown in Figure 1A, the number of nodules formed on the transgenic plants did not differ significantly from the number formed on nontransgenic plants or vector control plants, except for vector control line 121-T1, which developed significantly fewer nodules (Figure 1A). These data indicate that both sets of transgenic plants containing the *Le1* gene were normal in their responses to *R. loti.*

Soybean Lectin Accumulates in Transgenic *L. corniculatus* Root Tips

We used immunofluorescence to localize the soybean lectin protein to the *L. corniculatus* root tip region. No cross-reactivity with the anti-soybean lectin antibody was detectable in the roots of vector control plants, as shown in Figure 2A. In addition, Figure 2B shows that transgenic plant root hairs that were no longer elongating lacked anti-soybean lectin cross-reacting material. By contrast, Figures 2C and 2D show that root hairs of 35S–*Le1* (Figure 2C) and *Le1* (Figure 2D) plants were strongly labeled at the tips. Close inspection of both types of transgenic *L. corniculatus* plants indicated that the cross-reactive material accumulated more to the top of the root hairs of *Le1* plants compared with the 35S–*Le1* plants. Together, these results show that both the *Le1* and 35S–*Le1* genes are active within *L. corniculatus* roots and that the soybean lectin is correctly processed and targeted to root hair tips.

We examined extracts and exudates of the roots of the transgenic plants by using the antibody raised against the soybean lectin protein to determine whether the *Le1* gene product (Figure 3, lane 1) was present. As shown in Figure 3, lane 3, a cross-reacting protein similar in size to the Le1 protein was detected in extracts of 35S–*Le1* plants but not in extracts derived from *Le1* plants (Figure 3, lane 4). By contrast, soybean lectin cross-reacting material was detected in both 35S–*Le1* and *Le1* root exudates (Figure 3, lanes 6 and 7) but was not detected in root extracts and exudates of vector control plants (Figure 3, lanes 2 and 5). These data indicate that the soybean lectin protein is synthesized within transgenic *L. corniculatus* roots and then secreted into the extracellular environment.

Soybean Lectin Enables *B. japonicum* to Trigger Nodule Formation on *L. corniculatus* Plants

Transgenic *L. corniculatus* plants were inoculated with *B. japonicum* strain USDA110 at 10^6 to 10^7 cells per mL (Figure 1B) and 10^2 to 10^3 cells per mL (Figure 1C). The higher inoculum concentration induced an average of 10 nodules per plant for the 35S–*Le1* transgenic plants and 14 nodules per plant for the *Le1* plants, with the exception of the *Le1* line BIlec17, which developed $<$10 nodules per plant (Figure 1B). In contrast, lower inoculum levels reduced the nodule number on all of the transgenic plants by $\sim$20 to 30% (Figure 1C). Neither the vector controls nor the 35S–*Le1* line lecW, which expresses a truncated soybean lectin protein (Hirsch et al., 1995), was nodulated above background levels with either high or low inoculum densities (Figures 1B and 1C). Together, these data indicate that *B. japonicum* can induce nodules on transgenic *L. corniculatus* plants expressing the soybean lectin gene.

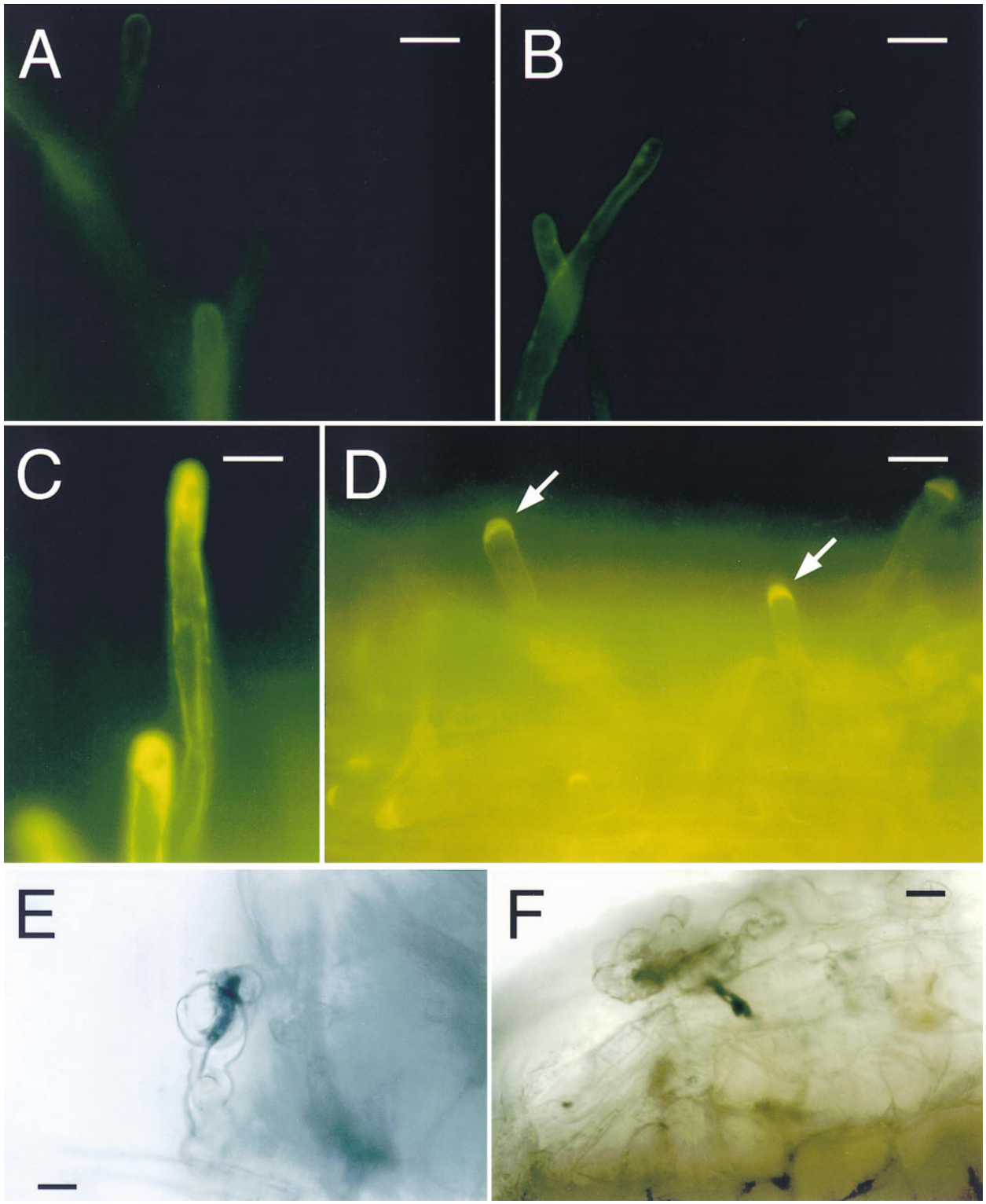

Figure 2. Characteristics of Transgenic *L. corniculatus* 35S–*Le1* and *Le1* Plants.

(A) Soybean lectin localization on the root hairs of transgenic *L. corniculatus* roots by using indirect immunofluorescence, as described in Methods, is shown. Only background fluorescence can be detected in the root hairs of plants that contain the vector only. Bar = 25 μm.

B. japonicum Induces "True" Nodules on Transgenic *L. corniculatus* Plants

Figure 4 shows that the nodules induced on the *Le1* plants are true nodules (Figure 4A) and not modified lateral roots (Figure 4B). Extensive cell divisions occurred in both the outer and inner cortical cells in a pattern that is identical to that of nodule development in wild-type *L. corniculatus* plants inoculated with *R. loti* (data not shown). Tissues that typically are found in *Lotus* spp nodules were observed in the transgenic nodules, including a nodule cortex with flavolan-containing cells (Pankhurst et al., 1979), an endodermis, and a well-developed central region (Figure 4C). Central cells of nodules from transgenic plants were filled with amyloplasts rather than bacteria and resembled the ineffective nodules formed by the *R. loti* strain NZP2213 on *L. pedunculatus* (Pankhurst et al., 1979). Transgenic *L. corniculatus* plants containing the 35S–*Le1* gene produced nodules that were smaller and less developed than those containing the *Le1* gene (cf. Figures 4D and 4E with Figures 4A and 4C). However, the tissue layers present in the nodules of both transgenic plants were indistinguishable. These data show that *B. japonicum* can induce *R. loti*–like true nodules on transgenic *L. corniculatus* roots carrying the soybean lectin gene but that the nodules do not harbor bacteria.

Rhizobial *nod* Genes Are Required to Induce Nodules on Transgenic *L. corniculatus* Plants

Figures 5A and 5B show that when we inoculated transgenic *L. corniculatus* plants with a *B. japonicum nodB* mutant (AN279) carrying an *R. loti nod* gene plasmid (pN366), 10 times more nodules per plant were formed compared with plants inoculated with wild-type *B. japonicum* (Figure 1B). The vector control plants were also nodulated to a limited extent in response to 10^6 to 10^7 cells per mL of *B. japonicum* AN279 (pN366). In contrast, no nodules were formed when *B. japonicum nodA* or *nodB* mutants were inoculated on transgenic roots (data not shown). In addition, inoculation with a *B. japonicum nodZ* mutant induced neither root hair curling nor cortical cell divisions (data not shown). The Nod factor produced by a *nodZ* mutant (NAD138) is characterized by the lack of the 2-*O*-methylfucose on the reducing end of the molecule (Stacey et al., 1994). These results demonstrate that functional *nod* genes are required for *B. japonicum*–induced nodule formation on both sets of transgenic *L. corniculatus* plants carrying *Le1*.

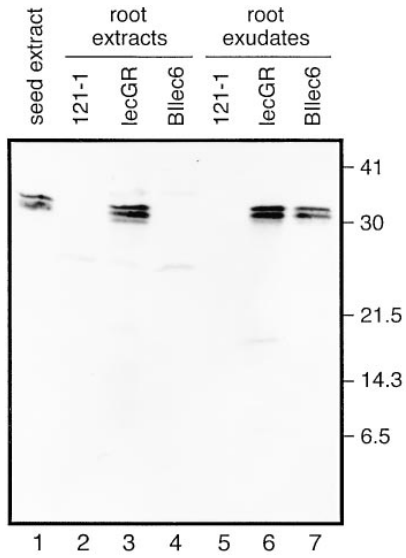

Figure 3. Immunodetection of Soybean Lectin in Root Extracts and Exudates of Transgenic *L. corniculatus* Plants.

Roots of vector control and transgenic *L. corniculatus* plants were incubated in water. After 24 hr, the exudates were collected and concentrated 200-fold. Both the root extracts (10 μg) and the root exudates (25 μL) were loaded onto an SDS–polyacrylamide gel and analyzed by immunoblotting using the antibody against soybean lectin. Lane 1 contains soybean seed extract (2 μg); lane 2, extract (10 μg) of *L. corniculatus* vector control (line 121-1) roots; lane 3, extract of *L. corniculatus* 35S–*Le1* (line lecGR) roots; lane 4, extract of *L. corniculatus Le1* (line BIlec6) roots; lane 5, exudate (25 μL) from *L. corniculatus* vector control roots; lane 6, exudate from *L. corniculatus* 35S–*Le1* roots; and lane 7, exudate from *L. corniculatus Le1* roots. Molecular mass markers in kilodaltons are indicated at right.

Figure 2. (continued).

(B) When an antibody raised to soybean lectin was used, only background fluorescence was visible in mature, no-longer-elongating root hairs of a *L. corniculatus* 35S–*Le1* (line lecGR) plant. Bar = 25 μm.

(C) When an anti-soybean lectin antibody was used, lectin was visible at the tips of young root hairs of a *L. corniculatus* 35S–*Le1* (line lecGR) root. Bar = 25 μm.

(D) Using an anti-soybean lectin antibody, we localized lectin to the very tips (arrows) of emerging root hairs of a *L. corniculatus Le1* (line BIlec6) root. Bar = 25 μm.

(E) Histochemical localization of β-galactosidase activity linked to AN279 (pN366) in an infection thread found in a root hair of a cleared *L. corniculatus Le1* (line BIec6) nodule 8 weeks after inoculation is shown. The infection thread aborted in the root hair. Bar = 30 μm.

(F) Histochemical localization of β-galactosidase activity linked to AN279 (pN366) in an infection thread found in a root hair of a cleared *L. corniculatus* 35S–*Le1* (line lecGR) nodule 8 weeks after inoculation is shown. The infection thread aborted in the hypodermal cell. Bar = 30 μm.

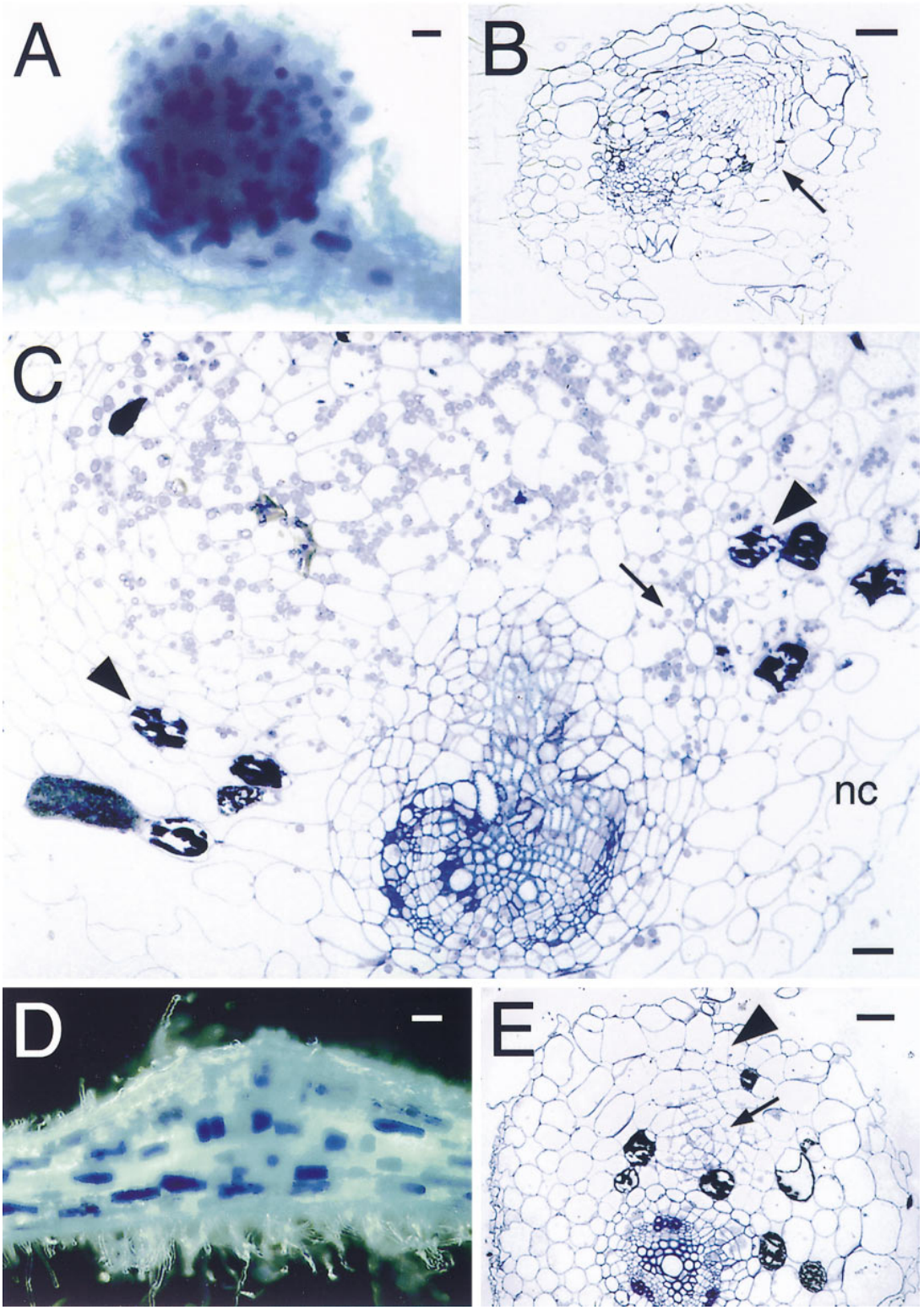

Figure 4. Characteristics of Transgenic *L. corniculatus* 35S–*Le1* and *Le1* Plants.

Because *B. japonicum* AN279 (pN366) contains a *lacZ* gene linked to *nodB*, we could monitor the progression of bacterial invasion in transgenic roots after inoculation. As shown in Figures 2E and 2F, aborted infection threads were found in the root hairs of both *Le1*-containing plants (Figure 2E) and 35S–*Le1*—containing plants (Figure 2F), whereas no infection threads were detected in the root hairs of control plants (data not shown). The transgenic nodules remained uninfected because the infection threads did not penetrate beyond the initially invaded root hair or adjacent hypodermal cell (Figures 2E and 2F). On rare occasions, we observed bradyrhizobia internally within the nodules of transgenic plants, and then only in intercellular spaces or in senescent host cells (data not shown). Coinoculation of *B. japonicum* AN279 (pN366) with an *R. loti* Nod$^-$ mutant did not rescue *B. japonicum*'s inability to colonize the nodules of the transgenic *L. corniculatus* plants. Together, these data show that Nod$^+$ bacteria, but not Nod$^-$ bacteria, can induce nodules on the roots of the transgenic *L. corniculatus* plants and that infection threads only penetrate these nodules to a limited extent.

A Modified Soybean Lectin Protein Does Not Bind *B. japonicum* Cells

We introduced a mutant 35S–*Le1* gene into *L. corniculatus* plants to determine whether soybean lectin was responsible for the nodules induced by *B. japonicum.* The *Le1* gene was mutated by changing the codon for Asn-130 (within the sugar binding site) into one specifying an aspartic acid residue. Twenty transgenic *L. corniculatus* plants containing the mutated *Le1* gene were regenerated and designated as 35S–*Le1*mut. Analysis of root protein extracts showed that lectin was produced at comparable levels in all 35S–*Le1*mut transgenic lines (data not shown). The mutant lectin protein, however, migrated more slowly in a gel as compared with the wild-type lectin protein produced by the transgenic *Le1*- and 35S–*Le1*—containing plants (data not shown). Presumably, the slower migration was due to substitution of a neutral amino acid for an acidic one (van Eijsden et al., 1992; Mirkov and Chrispeels, 1993). We used an *N*-acetyl-D-galactosamine affinity column to show that the mutant lectin did not bind carbohydrate (data not shown). In addition, microscopic examination of 35S–*Le1*mut root hairs showed that the mutated lectin was targeted to the root hair tips, as occurred in the 35S–*Le1*— and *Le1*-containing plants (data not shown; Figure 2).

Extracts from 35S–*Le1* and 35S–*Le1*mut roots were tested for binding to *B. japonicum.* Figure 6 (immunoblot 1) shows that when *B. japonicum* was incubated with extracts from 35S–*Le1* roots, lectin within the extracts bound to the bacterial cells. Adding 10 mM galactose to a mixture of *B. japonicum* USDA110 cells and 35S–*Le1* root extract decreased the amount of lectin bound to the bacterial cells (Figure 6, immunoblot 2). When cells of *B. japonicum* USDA31, which produces very little soybean lectin binding material (Bohlool and Schmidt, 1974), were incubated with 35S–*Le1* root extracts, barely any cross-reacting product was detected (Figure 6, immunoblot 1). Similarly, root extracts from the 35S–*Le1*mut plants did not bind to the *B. japonicum* USDA110 cells (Figure 6, immunoblot 3). Taken together, these data show that although mutant lectin protein is produced and targeted to the root hairs, it does not bind *B. japonicum* USDA110 cells.

B. japonicum Does Not Induce Nodules on *L. corniculatus* Plants Containing the Mutated Soybean Lectin Gene

Transgenic *L. corniculatus* plants containing the mutated lectin gene were inoculated with bacteria to determine whether they remained susceptible to *B. japonicum.* Figure 7A shows that 35S–*Le1*—containing plants produced an average of 30 nodules per plant in response to wild-type *R. loti*, demonstrating that the mutant lectin protein did not affect normal nodulation. Figures 7B and 7D show that a limited number of protuberances formed on the 35S–*Le1*mut roots in response to either *B. japonicum* USDA 110 (Figure 7B) or *B. japonicum* AN279 containing pN366 (Figure 7D). These protuberances were smaller than the nodules formed on the 35S–*Le1* plants. Moreover, the number of protuberances formed was the same as that obtained with both nontransgenic (data not shown) and vector control plants (Figures 7B and 7D) and was significantly less than that obtained with 35S–*Le1* roots (Figures 7B and 7D). Together, these data indicate that *B. japonicum* does not induce

Figure 4. (continued).

(A) Nodule from a cleared *L. corniculatus Le1* (line BIlec15) root at 8 weeks after inoculation with *B. japonicum* USDA110. The dark blue cells contain flavolans. Bar = 100 μm.

(B) Transverse section of an uninoculated *L. corniculatus* root showing an emerging lateral root (arrow). Bar = 50 μm.

(C) Section through a nodule formed on a *L. corniculatus Le1* (line BIlec15) root seen in transverse section. Flavolans are contained within some cells (arrowheads) of the nodule cortex (nc). The central cells of the nodule are filled with amyloplasts (arrow). Bar = 25 μm.

(D) Nodule from a cleared *L. corniculatus* 35S–*Le1* (line lecGR) root. The dark blue cells contain flavolans. Bar = 100 μm.

(E) Transverse section through a *L. corniculatus* 35S–*Le1* (line lecGR) root. Cortical cell divisions are not as extensive in this root, but they occur in both the outer (arrowhead) and inner (arrow) cortex. Bar = 50 μm.

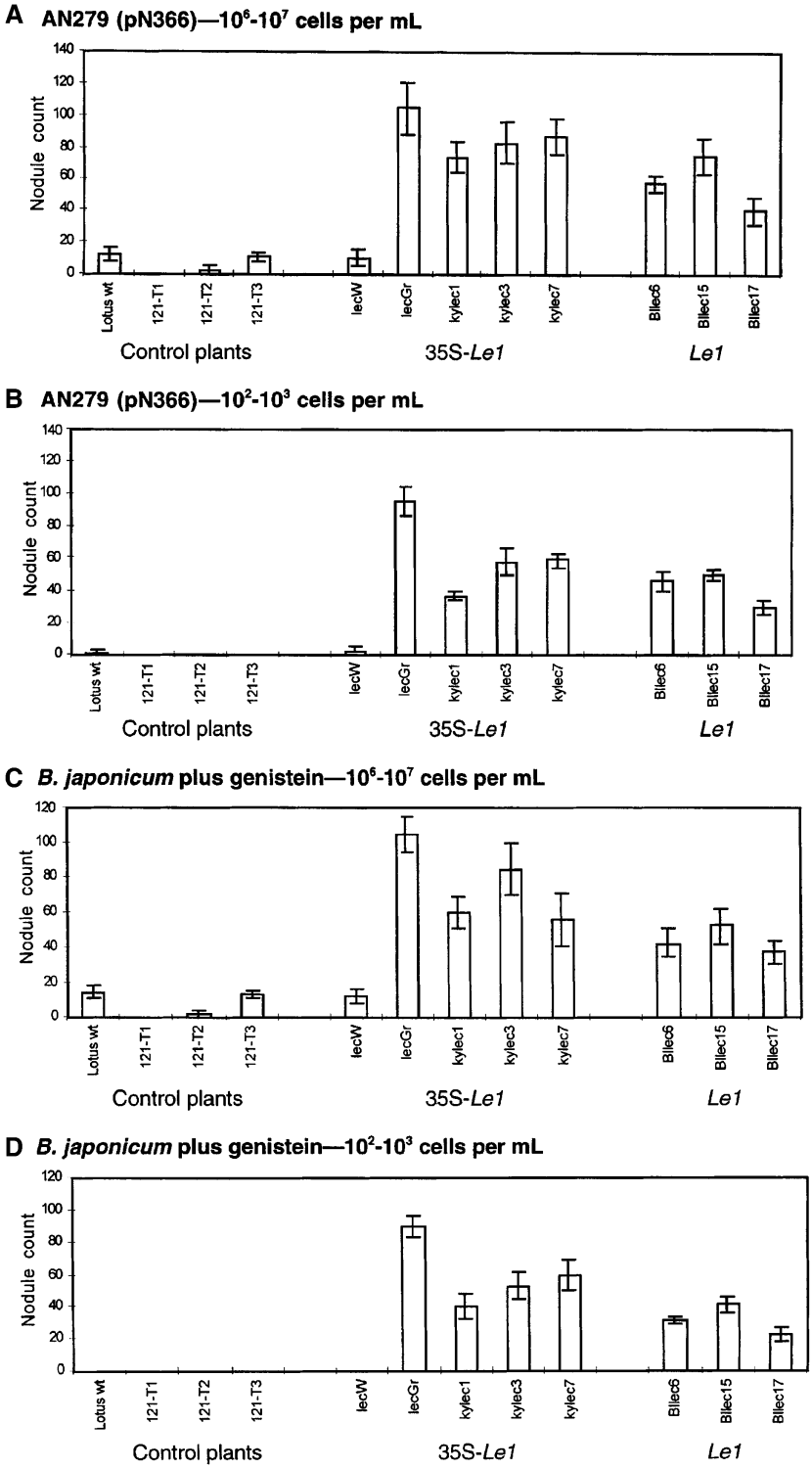

Figure 5. Nodulation Characteristics of Control and Transgenic *L. corniculatus* Plants When Inoculated with Different Bradyrhizobia.

(A) Inoculation with 10^6 to 10^7 cells per mL of the *B. japonicum nodB* mutant carrying a plasmid with the *R. loti* nodulation genes.
(B) Inoculation with 10^2 to 10^3 cells per mL of the *B. japonicum nodB* mutant carrying a plasmid with the *R. loti* nodulation genes.
(C) Inoculation with 10^6 to 10^7 cells per mL of *B. japonicum* preinduced with genistein.
(D) Inoculation with 10^2 to 10^3 cells per mL of *B. japonicum* preinduced with genistein.
wt, wild type.

nodules above background levels on *L. corniculatus* plants containing the mutant *Le1* gene.

Flavonoids Increase Nodulation of Transgenic *L. corniculatus* Plants by *B. japonicum*

We grew *B. japonicum* with either genistein, a soybean flavonoid, or root exudates from a lectinless *Le1*$^-$ soybean variety (Goldberg et al., 1983) before inoculation of transgenic *L. corniculatus* plants. Genistein is a soybean flavonoid that induces the *B. japonicum nod* genes responsible for the synthesis of Nod factor (Kosslak et al., 1987). Figures 5C and 5D show that transgenic 35S–*Le1* and *Le1* roots that were inoculated with genistein-grown bradyrhizobia developed four times and two times the number of nodules, respectively, compared with the number elicited by uninduced bacteria (cf. Figures 5C and 5D with Figures 1B and 1C). Root exudate–incubated bradyrhizobia induced similar numbers of nodules (data not shown). By contrast, transgenic *L. corniculatus* plants with the mutated lectin gene developed the same number of nodules as did control roots in response to 10^6 to 10^7 cells per mL of *B. japonicum* grown with genistein. This number was significantly less than that produced on 35S–*Le1* roots (Figure 7C). Together, these data show that genistein treatment and its effects on Nod factor production enhance the nodulation efficiency of *B. japonicum* on both sets of transgenic *L. corniculatus* plants carrying the wild-type *Le1* gene.

Soybean Lectin Does Not Appear to Interact with Nod Factor

We used purified Nod factors to test whether the soybean lectin was interacting with Nod factor directly. Mixtures of lipochitooligosaccharide (LCO) molecules that coeluted from an HPLC column were spot-inoculated onto roots of wild-type *L. corniculatus* plants, vector control transgenic plants, and both sets of *Le1*-containing transgenic plants. As summarized in Table 1, each of the natural Nod factor fractions induced macroscopically visible nodules on all of the different lines of *L. corniculatus* plants, including the nontransgenic control lines. In contrast, nodules did not develop on uninoculated roots or on roots that were mock-inoculated with carboxymethylcellulose (Table 1). When the different plants were compared, there were no statistical differences in the percentages of roots forming nodules except for vector control line 121-2, which produced significantly fewer nodules overall (see Figure 1A).

We also tested Nod factors produced by a *nodZ* mutant of *B. japonicum* (NAD138) as well as synthetic LCOs (Stokkermans and Peters, 1994; Stokkermans et al., 1995). The synthetic Nod factors exhibited a number of other chemical modifications (Table 1). All of the transgenic *L. corniculatus* lines and the control plant lines were unresponsive to these Nod factors (Table 1). In addition, the transgenic plants

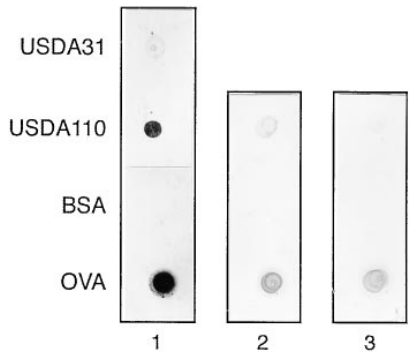

Figure 6. Binding of *B. japonicum* Strains to Root Extracts Containing Soybean Lectin or Altered Lectin after Transfer to Nitrocellulose.

Binding was detected by using an antibody to soybean seed lectin. USDA31 is *B. elkanii* USDA31 grown in PSY medium; USDA110, *B. japonicum* USDA110 grown in PSY medium; BSA, negative control; OVA, ovalbumin (positive control). Immunoblot 1 shows binding of soybean lectin in root extracts of *L. corniculatus* 35S–*Le1* (line lecGR) to USDA110 cells. USDA31 binds soybean lectin poorly. Immunoblot 2 shows decreased binding of lectin to USDA110 cells when 100 mM galactose was added. Immunoblot 3 illustrates no binding of extracts from 35S–*Le1*mut (line Mut8) roots to USDA110 cells.

with the mutated lectin gene responded to the different fractions of purified Nod factors in the same way as did the transgenic plants carrying the wild-type lectin (Table 2). These results indicate that *L. corniculatus* roots respond to bradyrhizobial LCO molecules and suggest that the presence of the *Le1* gene does not directly influence nodule primordia formation in response to purified Nod factor fractions.

A Component of the *B. japonicum* Exopolysaccharide Is the Ligand for Soybean Lectin

To determine what role lectin plays in nodule initiation, we tested whether rhizobia that are unable to bind lectin protein can induce nodules on the roots of the transgenic *L. corniculatus.* We obtained or constructed *Bradyrhizobium* and *Rhizobium* strains that produce *Bradyrhizobium* Nod factors but do not bind to soybean seed lectin. These strains included *B. elkanii* USDA31, *B. japonicum* USDA110 Δp22, and a *R. l.* bv *viciae* strain carrying the *B. japonicum nod* genes (pR32). These bacterial strains were grown with the appropriate flavonoid (genistein for *B. japonicum* and naringenin for *R. l.* bv *viciae*) before inoculation to ensure *nod* gene induction. As shown in Figure 8, no nodules above background were detected on any of the *L. corniculatus* transgenic plants when inoculated at both low and high densities with the bacterial strains that do not bind soybean lectin. In contrast, the transgenic *L. corniculatus* plants were nodulated only in response to wild-type *B. japonicum*

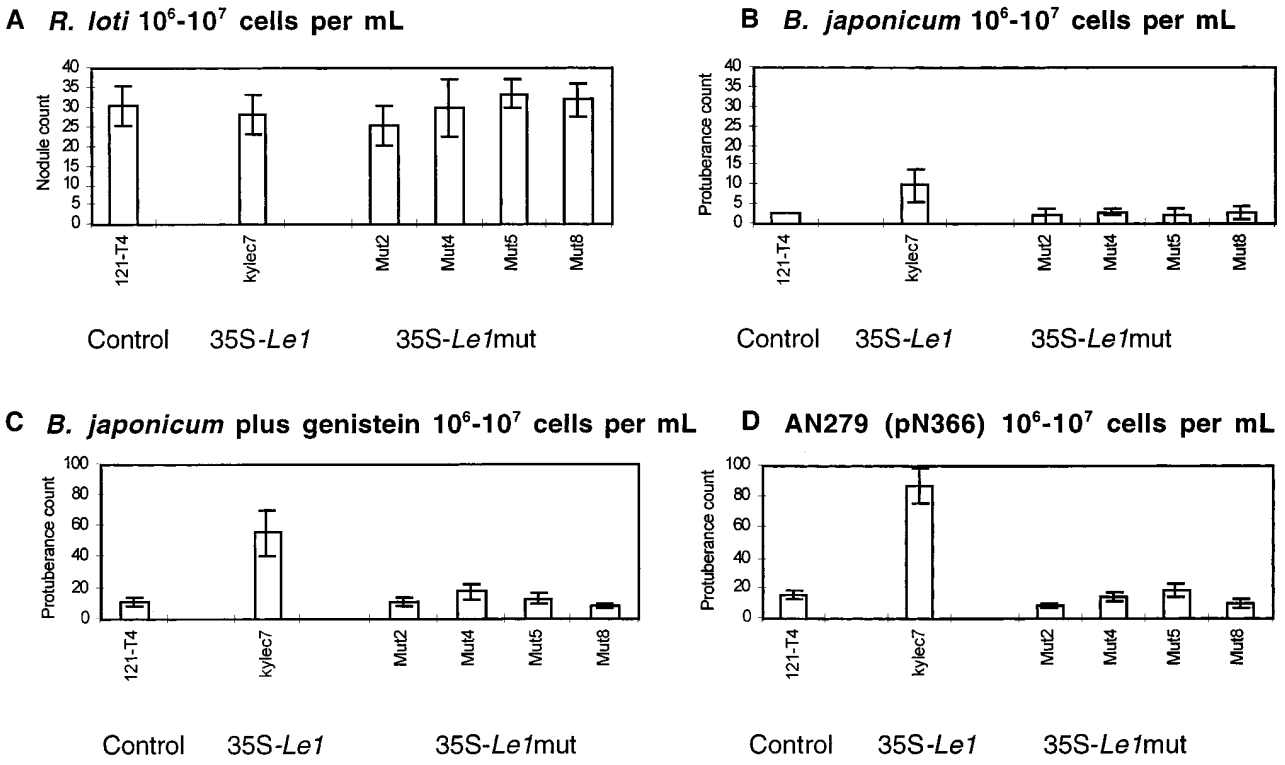

Figure 7. Nodulation Characteristics of Different Transgenic *L. corniculatus* Lines Containing the Mutated Soybean Lectin (Lines Mut2, Mut4, Mut5, and Mut8) Compared with Control and Transgenic 35S–*Le1* Plants after Inoculation with Different Rhizobial Strains.

Nodulation was scored 2 months after inoculation. The numbers given are the means of at least three replicates with the standard deviation. Protuberances were scored as positive if, after clearing the roots, cortical cell divisions could be detected.

(A) Inoculation with 10^6 to 10^7 cells per mL of *R. loti.*

(B) Inoculation with 10^6 to 10^7 cells per mL of *B. japonicum.*

(C) Inoculation with 10^6 to 10^7 cells per mL of *B. japonicum* preinduced with genistein.

(D) Inoculation with 10^6 to 10^7 cells per mL of the *B. japonicum nodB* mutant carrying a plasmid with the *R. loti* nodulation genes, AN279 (pN366).

USDA110 (Figure 8A). In addition, none of the strains elicited nodule formation on transgenic plants carrying the mutated soybean lectin (Figure 8A).

We counted the number of bacteria firmly attached to roots of the different *L. corniculatus* plants. Figure 8B shows that bacterial strains unable to bind soybean lectin did not attach above background levels to the roots of any transgenic *L. corniculatus* line. Only wild-type *B. japonicum* USDA110 attached. In addition, the loss of the carbohydrate binding site in the mutated lectin resulted in a severe reduction to background levels of attachment of wild-type *B. japonicum* USDA110 (Figure 8B). Thus, the increased amount of attachment of *B. japonicum* USDA110 to the plant roots was correlated with the presence of a functional *Le1* lectin gene in the transgenic *L. corniculatus* roots. Because *B. japonicum* USDA110 Δp22 differs from *B. japonicum* USDA110 by the absence of the *exoB* gene, exopolysaccharide must be involved in both the attachment and nodulation of transgenic *L. corniculatus* roots by wild-type bradyrhizobia. Taken together, these data indicate that a component of *B. japonicum* exopolysaccharide is the ligand for soybean lectin.

DISCUSSION

We have shown that the soybean lectin gene, driven by either the 35S gene promoter or its own promoter, is expressed within *L. corniculatus* roots and that lectin is localized to the root hair tips. This is consistent with our previous experiments with the soybean *Le1* gene showing that it is active at high levels during seed development and at low levels within soybean and transgenic tobacco roots (Okamuro et al., 1986). The expression of the soybean *Le1* gene at the root hair tips permitted *L. corniculatus* roots to

be nodulated by a non-host bacterial strain, *B. japonicum* USDA110, which nodulates soybean. By contrast, no nodules above background were produced when *L. corniculatus* plants carrying a mutant *Le1* gene were inoculated with *B. japonicum.* Nodules elicited by *B. japonicum* on both sets of *L. corniculatus* transgenic plants were devoid of bacteria. However, limited infection thread development did occur, and the nodules had anatomical features characteristic of those normally induced on *L. corniculatus* roots by *R. loti.* We conclude from these data that soybean lectin is involved in rhizobia–legume recognition.

Our results show that the introduction of a soybean lectin gene can extend the host range of *B. japonicum* to *L. corniculatus*, which is in a different clade than soybean (Doyle et al., 1997). Previously, Díaz et al. (1989) showed that pea lectin introduced into white clover roots stimulated nodulation by the heterologous symbiont *R. l.* bv *viciae.* However, white clover and pea are nodulated by rhizobia that are closely related to one another, whereas the soybean symbiont *B. japonicum* and the *Lotus* spp symbiont *R. loti* are in two very distinct genera of alpha-proteobacteria (Young, 1992). Collectively, our results and those of Díaz et al. (1989) indicate that lectins are involved in controlling the specificity of the *Rhizobium*–legume interaction.

A mutation in the carbohydrate binding site of *Le1* eliminated the response of transgenic *L. corniculatus* roots to *B. japonicum* USDA110 because the altered lectin is no longer capable of binding the heterologous bacteria (see Figure 8). These data strongly suggest that the lectin binding site is important for bacterial attachment to the root hair surface and the nodulation responses that follow. The cell surface component(s) to which the lectin binds is not yet known; however, exopolysaccharide involvement is suggested by the absence of nodulation and attachment by the *B. japonicum exoB* mutant.

Our results show that nodulation is enhanced when *B. japonicum* USDA110 cells are incubated with genistein, an inducer for *B. japonicum nod* genes. Nodulation did not occur when the transgenic *L. corniculatus* plants were inoculated with *B. japonicum nod* gene mutants. One explanation for these findings is that Nod factor is a ligand for soybean seed lectin. However, we found that treatment of uninoculated roots with purified Nod factor fractions and synthetic LCO molecules induced nodules on transgenic, nontransgenic, and vector control plants. These experiments indicate that Nod factor does not appear to interact with soybean lectin. Rather, the enhancement of nodulation brought about by genistein-induced *B. japonicum* on the *Le1*-containing transgenic lines is most likely a result of the accumulation of Nod factor brought about by the increased attachment and aggregation of the heterologous rhizobia at the infection site. *B. japonicum* Nod factors overlap to some extent with *R. loti* factors (see Sanjuan et al., 1992; Carlson et al., 1993; López-Lara et al., 1995); however, at physiological concentrations,

Table 1. Summary[a] of Nodule Initiation Activity of Natural and Synthetic LCOs on *L. corniculatus* Transgenic Plants

			L. corniculatus Vector Control		*L. corniculatus* 35S–*Le1*		*L. corniculatus Le1*	
LCO	Composition[b]	Wild Type	121-1	121-3	KYlec7	lecGR	BIlec6	BIlec15
Control	Carboxymethylcellulose	0 (5)	0 (3)	0 (2)	0 (3)	0 (6)	0 (4)	0 (6)
Fraction 1 of USDA61[c]	NodBe-V (C18:1, MeFuc)	9 (13)	1 (12)	3 (5)	4 (6)	17 (21)	3 (6)	5 (7)
Fraction 2 of USDA61	NodBe-V (Ac, C18:1, MeFuc) NodBe-IV (C18:1, Fuc, Gro)	15 (18)	2 (23)	9 (12)	3 (5)	19 (22)	5 (8)	6 (8)
Fraction 3 of USDA61	NodBe-IV (C18:1, MeFuc) NodBe-IV (Cb, C18:1, MeFuc) NodBe-IV (Cb, C18:1, Fuc, Gro) NodBe-IV (Cb, C18:1, NMe, MeFuc) NodBe-IV (C18:1, NMe, MeFuc) NodBe-IV (Cb, C18:1, NMe, Fuc, Gro)	10 (15)	2 (19)	5 (8)	3 (6)	15 (24)	7 (10)	5 (9)
LCO of NAD138	NodBj-V (C18:1)	0 (10)	—[d]	—	—	0 (9)	0 (7)	0 (5)
LCO-3026	LCO-IV (C18:1, Fuc, R-Gro)	0 (8)	—	—	—	0 (10)	0 (8)	—
LCO-1098[e]	LCO-IV (C16:0)	0 (9)	—	—	—	0 (8)	0 (6)	—
LCO-2086	LCO-IV (C18:1, MeFuc)	0 (12)	—	—	—	0 (12)	0 (7)	—
LCO-3027	LCO-IV (C18:1, Fuc, S-Gro)	0 (13)	—	—	—	0 (13)	0 (6)	—

[a] The number of sites of cortical cell division after application of 100 ng of test material per spot inoculation. Numbers in parentheses are the total number of roots assayed.

[b] Ac, acetyl; Cb, carbamoyl; Fuc, fucose; Gro, glycerol; Me, methyl; NMe, N-methyl; R-Gro and S-Gro, R-glycerol and S-glycerol; CX:Y, length of fatty acid:number of desaturations.

[c] Fraction 1 of USDA61 is structurally identical to the major fraction produced by *B. japonicum* USDA110.

[d] Not determined.

[e] After applying LCO-1098, all of the roots stopped growing, so no cortical cell divisions could be detected.

they do not induce nodules on *L. corniculatus.* The release of soybean lectin into the extracellular environment and its localization to root tips are likely to augment the aggregation and attachment of *B. japonicum* to the roots of the *L. corniculatus* transgenic plants. We suggest that the resulting increase in the concentration of Nod factor, even a heterologous one, induces nodule initiation.

Although our results strongly suggest that lectin is involved in the extension of *B. japonicum*'s host range to include the transgenic *L. corniculatus* plants, the inability of the rhizobia to enter nodule cells and differentiate into nitrogen-fixing bacteroids indicates that factors critical for invasion are either missing or not properly expressed in the transgenic hosts, or both. For example, soybean cultivars lacking seed lectin nodulate normally (Pull et al., 1978; Goldberg et al., 1983), arguing for the presence of a Nod factor binding protein/receptor distinct from the *Le1* gene product. One possible candidate for such a receptor is a soybean homolog of the lectin, DB46 from *Dolichos biflorus*, which may be a Nod factor binding protein (Etzler and Murphy, 1996). Other Nod factor binding proteins have been identified (Bono et al., 1995, 1996). However, it is not known whether these proteins are lectins.

DNA gel blot hybridization and S1 nuclease protection experiments failed to identify any functional lectin genes in soybean related to the *Le1* gene except the *Le2* gene, which may be a pseudogene (Goldberg et al., 1983; Okamuro et al., 1986). These experiments would not have detected *Le1*-related genes that are >60% divergent at the nucleotide level, however. Evidence from a number of laboratories indicates that legume roots release lectins into the rhizosphere and that these root lectins are either very similar or identical to the well-characterized seed lectins (Gietl and Zeigler, 1979; Gade et al., 1981; Kato et al., 1981; Díaz et al., 1986; Vodkin and Raikhel, 1986; Kjemtrup et al., 1995). For example, we showed previously that the same *Le1* lectin gene is active in both seeds and roots of soybean and transgenic tobacco plants (Okamuro et al., 1986). Although a 45-kD lectin specific for 4-*O*-methylglucuronic acid is present in soybean roots, the gene for this lectin has not been identified (Rutherford et al., 1986). A protein in extracts of both *Le1*$^+$ and *Le1*$^-$ soybean plants, which cross-reacts with an anti-soybean seed lectin antibody and which was proposed earlier to be a root lectin (Vodkin and Raikhel, 1986), is not a likely candidate. Our work indicates that this cross-reactive protein is similar to the acidic ribosomal protein P0 (Wycoff et al., 1997). Nevertheless, root exudate from *Le1*$^-$ soybeans reverses the delayed nodulation phenotype of *B. japonicum* HS111 in the same way that soybean seed lectin does (Halverson and Stacey, 1985), implicating the presence of a protein, presumably a lectinlike protein, in the exudate.

Our data support the idea that lectins are important for host recognition. In addition, they suggest a possible means of mediating host specificity by interacting with an exopolysaccharide component of the rhizobial cell surface. Lectin secretion and targeting to the root hair tips may result in the aggregation and attachment of a sufficient number of rhizobia so that a threshold level of Nod factor can interact with a receptor in a signal transduction pathway leading to nodule formation. In addition, lectins are involved in rhizobial entry into root hair cells via infection threads (see references in Kijne, 1997). Lectins are found in the root hairs, accumulate in the susceptible zone of the root, and facilitate infection thread formation by heterologous bacteria in root hairs of transgenic plants (see Figure 2). Thus, lectin proteins are expressed at the correct time and in the correct place to maximize the plant's responses to rhizobial bacteria. Nevertheless, the precise events and genes necessary to induce effective nodules on legume roots remain to be identified.

Table 2. Summary[a] of Nodule Initiation Activity of Natural and Synthetic LCOs on Transgenic *L. corniculatus* Plants Containing a Mutated Soybean Lectin Gene

LCO	Composition[b]	Mut2	Mut4
Control	Carboxymethylcellulose	0 (3)	0 (4)
Fraction 1 of USDA61[c]	NodBe-V (C18:1, MeFuc)	3 (5)	3 (5)
Fraction 2 of USDA61	NodBe-V (Ac, C18:1, MeFuc) NodBe-IV (C18:1, Fuc, Gro)	4 (6)	6 (7)
Fraction 3 of USDA61	NodBe-IV (C18:1, MeFuc) NodBe-IV (Cb, C18:1, MeFuc) NodBe-IV (Cb, C18:1, Fuc, Gro) NodBe-IV (Cb, C18:1, NMe, MeFuc) NodBe-IV (C18:1, NMe, MeFuc) NodBe-IV (Cb, C18:1, NMe, Fuc, Gro)	2 (5)	2 (4)
LCO of NAD138	NodBj-V (C18:1)	0 (5)	0 (7)

[a] The number of sites of cortical cell division after application of 100 ng of test material per spot inoculation. Numbers in parentheses are the total number of roots assayed.
[b] Ac, acetyl; Cb, carbamoyl; Fuc, fucose; Gro, glycerol; Me, methyl; NMe, N-methyl; CX:Y, length of fatty acid:number of desaturations.
[c] Fraction 1 of USDA61 is structurally identical to the major fraction produced by *B. japonicum* USDA110.

METHODS

Bacterial Strains and Plasmids

The *Rhizobium loti* strain used in this study was the wild-type NZP2037 (Scott et al., 1985). In addition to wild-type *Bradyrhizobium japonicum* USDA110 (Kuykendall and Elkan, 1976), the following USDA110 mutants were used: AN279 (*nodB*::Tn*5lacZ*; Sanjuan et al., 1992), NAD138 (*nodZ*::Tn*5*; Nieuwkoop et al., 1987), BjnodA (*nodA*::Tn*5*; Lamb and Hennecke, 1986), and the *exoB* mutant

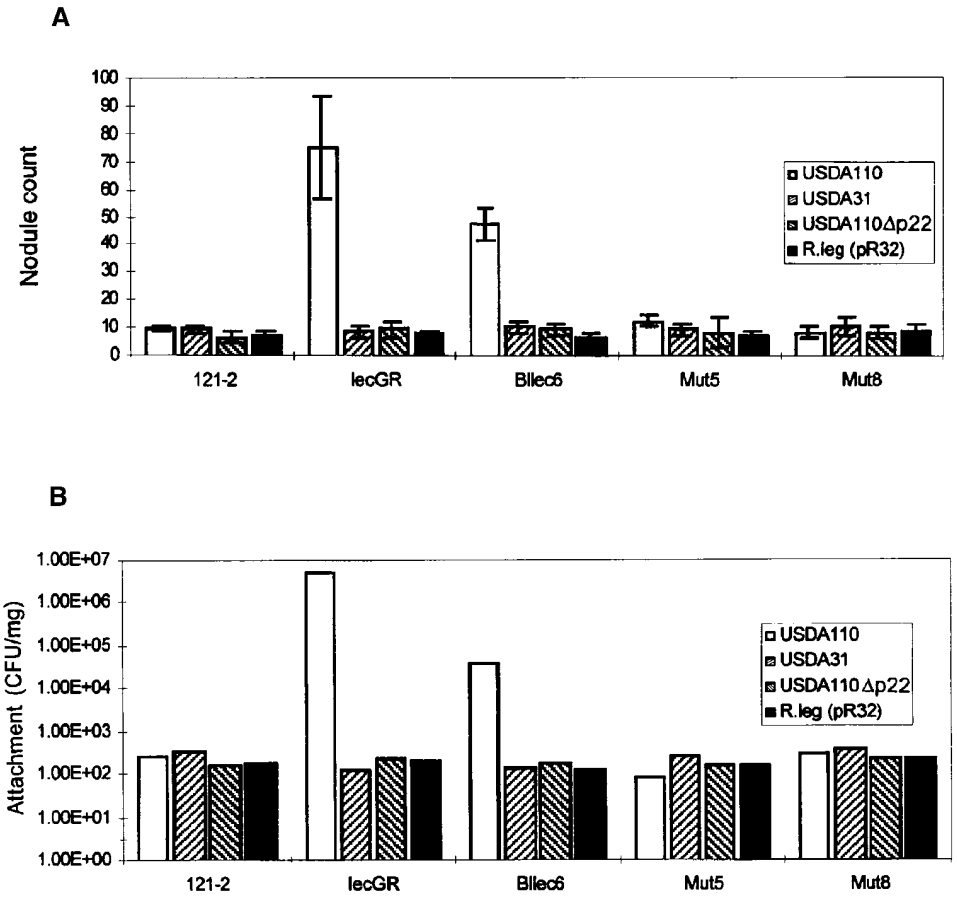

Figure 8. Nodulation and Attachment Characteristics of Several Rhizobial Strains to Various Transgenic *L. corniculatus* Lines.

(A) From each transgenic line, six independent plants were inoculated with bradyrhizobia (USDA110, USDA31, or USDA110 Δp22) that had been preinduced with genistein or with *R. leguminosarum* (pR32) that was grown with naringenin. Plants were harvested after 2 months. The number of protuberances formed from three plants was counted, and the average plus or minus the standard deviation is represented. Vector control, line 121-2; *L. corniculatus* 35S–*Le1*, line lecGR; *L. corniculatus Le1*, line Bllec6; *L. corniculatus* 35S–*Le1*mut, lines Mut5 and Mut8.
(B) From the other three plants, the bacteria were isolated from the roots, as described in Methods. Attachment is represented as the amount of colony-forming units per root dry weight (CFU/mg). The values shown are the means of three replicates. To enhance the readability of the graph, the standard deviations are not shown; they were within 10% of each mean. Transgenic lines are as given in **(A)**.

(USDA110 Δp22; Parniske et al., 1993). AN279 (pN366) was constructed by a triparental mating; pN366 is a pLAFR1 cosmid clone containing *R. loti nod* genes and was a generous gift of R.B. Scott (Massey University, Palmerston North, New Zealand). *B. elkanii* USDA31 (Kuykendall et al., 1988) and the wild-type *R. l.* bv *viciae* strain (Rl128C53) were also used in this study. The plasmid pR32, a pLAFR1 cosmid clone containing *B. japonicum* USDA110 *nod* genes (Russell et al., 1985), was mated into wild-type *R. l.* bv *viciae* to produce Rl128C53 (pR32).

Bradyrhizobium and *Rhizobium* strains were grown at 30°C in PSY medium (Regensburger and Hennecke, 1983) or in TY medium (Beringer, 1974), respectively. *Escherichia coli* strains were routinely grown at 37°C in Luria-Bertani medium (Miller, 1972) supplemented with the appropriate antibiotics in the following concentrations: ampicillin, 100 μg/mL; kanamycin, 25 μg/mL; and tetracycline, 10 μg/mL. For selections after mating with *Bradyrhizobium* strains, antibiotics were used as follows: chloramphenicol, 40 μg/mL; kanamycin, 100 μg/mL; and tetracycline, 100 μg/mL.

Construction of Binary Vectors Expressing the Soybean Lectin Gene

A 1-kb NsiI fragment of a soybean lectin genomic clone, designated as LeBgl5.6 (Goldberg et al., 1983; Vodkin et al., 1983; Okamuro et al., 1986), was recloned into the PstI site of pBlueScript II KS+ (Stratagene, La Jolla, CA) to yield pLec1.1. This plasmid was cut with XhoI and XbaI, both present in the polylinker and subcloned into the binary vector pKYLX71 (Schardl et al., 1987), placing the coding region of the soybean lectin gene under the control of the cauliflower

mosaic virus 35S promoter. The new plasmid was designated pKYlec and was introduced into *Lotus corniculatus* (see below).

A second construct was made in which the expression of the soybean lectin gene was driven from its own promoter. The soybean lectin sequence was introduced into the binary vector pBI101 (Jefferson, 1987) by replacing a SalI-EcoRI *uidA* gene of pBI101 with the 5.6-kb soybean genomic fragment of LeBgl5.6 (Goldberg et al., 1983; Okamuro et al., 1986), giving pBllec. This 5.6-kb fragment contains the coding region of the soybean lectin gene plus 3 kb of 5′ flanking DNA and 1.5 kb of 3′ flanking DNA; it has been shown to confer maximal seed expression in transgenic tobacco (Okamuro et al., 1986; Lindstrøm et al., 1990).

Site-Directed Mutagenesis of the Soybean *Le1* Lectin Gene

A mutation in the *Le1* gene that changed the codon for Asp-130 into one for aspartic acid was introduced by using the polymerase chain reaction with the mutagenic oligonucleotide primers 5′-CGCTAAGCTTCACCAATTGACACTAAGCC-3′ and 5′-GAAAGGCCCTGAGAACCCTAGGTGGT-3′. The amplified HindIII-BamHI fragment was cloned in pUC19 by using the BamHI and HindIII restriction sites and sequenced using Sequenase version 2.0 enzyme (U.S. Biochemical). The mutated soybean lectin gene was placed in the binary vector pKYLX71 (Schardl et al., 1987), producing pKYlecmut that was used to transform *L. corniculatus* plants (see below). The coding sequence of the mutated *Le1* gene (*Le1*mut) was under the control of the cauliflower mosaic virus 35S promoter.

Transformation of *L. corniculatus*

After electroporation of the transforming plasmid into *Agrobacterium rhizogenes* A4TC24, the resulting bacteria were used to infect young *L. corniculatus* seedlings (Hirsch et al., 1995). Transgenic seedlings that carried either pKYlec or pBllec were given individual numbers. Lines lecW and lecGR had been constructed previously (Hirsch et al., 1995). All of the transgenic plants were grown in a growth cabinet (Conviron Controlled Environments, Winnipeg, Canada) with a 16-hr-light and 8-hr-dark photoperiod and a 23°C day and 20°C night thermal period. Ten independent lines were selected from the kanamycin-resistant plants by the following criteria: (1) plants that exhibited normal shoot and root formation; (2) plants that formed normal nitrogen-fixing root nodules within 4 weeks after inoculation with *R. loti* NPZ2037; and (3) *Le1*-containing *L. corniculatus* plants whose NsiI-digested DNA produced the correct hybridization band with the *Le1* gene as a probe or 35S–*Le1*—containing plants that produced a detectable amount of cross-reacting material with the soybean lectin antibody (data not shown; Hirsch et al., 1995).

Plant Inoculations

Cuttings were made of plants that were 4 to 6 weeks old. After the transgenic plants had been rooted in half-strength Murashige and Skoog medium (Szabados et al., 1990), the plants were transferred to double Magenta (Magenta Corporation, Chicago, IL) jars containing a mixture of 2:1 vermiculite/perlite saturated with Jensen's medium minus N (Vincent, 1970). They were then flood-inoculated with high (10^6 to 10^7 cells per mL) or low (10^2 to 10^3 cells per mL) concentrations of bacteria grown in PSY medium and washed with PBS (0.88% [w/v] NaCl, 2.9 mM KH_2PO_4, and 7.1 mM K_2HPO_4, pH 7.2). When the bacteria were preinduced, the medium was supplemented with genistein (10 μM) or with soybean root exudates, as indicated below. When spot inoculations were used, transgenic or wild-type *L. corniculatus* plants were transferred to Petri dishes containing Jensen's agar minus nitrogen (Vincent, 1970) and spot-inoculated with Nod factors or with carboxymethylcellulose, following the procedure of Dudley et al. (1987) and Stokkermans et al. (1995). The Nod factor fractions and synthetic lipochitooligosaccharides (LCOs) were a generous gift from N. Kent Peters (Norwegian Agricultural University, As, Norway). For every four plants, one was left uninoculated to check for contamination.

Preparation of *B. japonicum* Inoculum Induced by Soybean Root Exudates

Soybean cultivar Sooty seeds (*Le1*$^-$; a soybean cultivar containing a nonexpressed mutant *Le1* gene; Goldberg et al., 1983) were surface-sterilized for 15 min in 30% H_2O_2 and then rinsed and soaked in water for 3 hr. The seeds were germinated on agar plates containing Jensen's medium (Vincent, 1970) at 20°C in the dark. After 72 hr, seedlings that were free of microbial contamination were transferred to 50-mL conical tubes filled with Jensen's medium. The seedlings were grown for 2 weeks before the exudates were collected. For preparation of inoculum, ~10^5 *B. japonicum* cells per mL were grown for 8 hr at 30°C in 25 mL of filtered soybean root exudates and were used as inoculum for the transgenic *L. corniculatus* plants.

Microscopic Analysis

Roots inoculated with various rhizobial strains or spot-inoculated with Nod factors were examined under an Axiophot microscope (Carl Zeiss Inc., Thornwood, NY) for root hair deformation, root hair curling, and cortical cell divisions. Eight weeks after inoculation, plants were harvested, and some root segments were cleared and stained following the procedure of Stokkermans et al. (1995). Other segments were fixed and stained overnight for β-galactosidase activity, according to Boivin et al. (1990). For embedding in Spurr's plastic resin, the sites where Nod factor had been applied or specific loci of cortical cell divisions were fixed in glutaraldehyde–paraformaldehyde and embedded in resin, as described previously by Yang et al. (1992). Sections were cut at 1-μm thickness and then stained with toluidine blue (Yang et al., 1992). Photographs were taken with Kodak Ektachrome 160 film (Eastman Kodak Co., Rochester, NY), and composites were made on the computer with Photoshop software (Adobe Systems, Inc., San Jose, CA).

Protein Gel Blot Analysis and Isolation of Secreted Proteins from Transgenic *L. corniculatus* Roots

Protein gel blot analysis was performed as described by Hirsch et al. (1995). Transgenic plants were grown in 50-mL plastic tubes filled with Jensen's medium. Root exudates were isolated according to the procedure of Kjemtrup et al. (1995) with the following modifications. When roots grew to ~5 cm long, the plants were transferred to 15-mL plastic tubes filled with water containing a mixture of protease inhibitors (final concentration of 1 mg/mL leupeptin, aprotinin, and pepstatin A) and left overnight in the growth chamber. Root exudates

were isolated after 24 hr of incubation and concentrated with Centricon concentrators (Amicon, Danvers, MA).

Localization of the Soybean Lectin Protein by Indirect Immunofluorescence

After the transgenic plants were rooted in half-strength Murashige and Skoog medium (Szabados et al., 1990), they were transferred to Petri dishes containing Jensen's agar (1%) minus nitrogen. The agar was partially covered with filter paper, thus allowing the roots to grow over the paper. After the roots were grown to a length of 4 cm, they were cut off and used for immunolocalization of lectin, according to Díaz et al. (1986), using the rabbit soybean seed lectin antibody (Sigma) as a primary antibody and a fluorescein isothiocyanate–labeled goat anti–rabbit antibody as a secondary antibody (Sigma).

Isolation of the Soybean Lectin Protein by Affinity Chromatography

Approximately 5 g of fresh root tissue from rooted cuttings was ground with a mortar and pestle in 4 mL of extraction buffer (PBS with 10 mM β-mercaptoethanol). After centrifugation to remove cell debris, the root extracts were concentrated with Centricon concentrators. Two hundred micrograms of the concentrated root extract was adjusted to a volume of 150 μL and applied to a 1-mL *N*-acetyl-D-galactosamine agarose gel (Pierce Chemical Co., Rockford, IL). The gel was washed with saline (0.9% NaCl) until the absorption at A_{280} of the effluent reached zero. The column was eluted with saline containing 100 mM galactose. Both the unbound and bound fractions were concentrated to the original volume of 150 μL and subjected to SDS-PAGE. The proteins were transferred to nitrocellulose for immunoblot analysis with the soybean seed lectin antibody, as described earlier (Hirsch et al., 1995).

Binding and Attachment Assays

When binding of the different strains of rhizobia to fluorescein isothiocyanate–labeled soybean seed lectin (Sigma) was measured, bacteria were grown in synthetic media, as described by Bhuvaneswari et al. (1977), and the assays were performed according to Bhuvaneswari and Bauer (1978).

The amount of bacteria attached to the roots was measured by collecting the roots aseptically in 50 mL of PBS and washing them five times in PBS to eliminate loosely attached cells. The roots were then vigorously shaken for 4 hr at 4°C to resuspend the bacteria attached to the roots. For each sample, the cell density was measured by plate counting.

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