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Host Recognition in the *Rhizobium*-Soybean Symbiosis[1]

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ABSTRACT

Polar binding of *Rhizobium japonicum* to roots and root hairs of *Glycine soja* (L.) Sieb. and Zucc. is specifically inhibited by D-galactose and *N*-acetyl-D-galactosamine, haptens of *Glycine max* seed lectin. A protein, immunologically cross-reactive with the *G. max* seed lectin, is present in *G. soja* seed extracts. Peptide mapping of the purified *G. max* and *G. soja* lectins indicates that the two are similar in structure. Soybean lectin can be localized on the surface of both *G. max* and *G. soja* roots by indirect immunolatex techniques. These observations indicate that the *Rhizobium*-binding lectin, previously isolated from seeds, also is present on the root surface—the site of the initial steps in the infection. This lectin is capable of binding *Rhizobium japonicum* to the root.

Symbiotic nitrogen fixation is a complex process involving physiological and biochemical properties of both the bacterium and the host plant. The first step in this process involves the selective attachment and penetration of the plant by the bacterium. Lectins, proteins that bind carbohydrates, have been implicated as important determinants in the specificity of this first step (2, 3, 10, 15, 32). In the soybean system, a 120,000 dalton glycoprotein with specificity for D-galactose and *N*-acetyl-D-galactosamine binds specifically to infective strains of *Rhizobium japonicum* (2, 3, 19-21). This lectin is found in seeds, leaves, stems, and roots of several varieties of *Glycine max* (23, 26), although it appears to be absent or in very low quantity in a few varieties (26, 29). Even with all of the evidence indicating that the lectin specifically binds to *R. japonicum*, there has been little direct evidence showing that this lectin actually binds the bacteria to the root. In only one legume system, that of white clover, has direct evidence been presented that a lectin binds the bacterium to the root (9, 10, 12). In that case, the lectin was localized at the site of infection. A hapten of the clover lectin, 2-deoxyglucose, specifically prevents binding of *Rhizobium trifolii* to clover roots (9, 11). Similar studies to those performed with clover have not been done with soybeans chiefly because of the difficulty of adapting light microscopic studies to a large plant.

Here, we overcame the difficulties of using a large plant such as *G. max* by using *Glycine soja*, a small-seeded soybean. The nodulation specificities of *G. max* and *G. soja* are very similar (6). Hymowitz (18) suggested, on cytogenetic evidence, that the two be considered the same species. Therefore, we propose the use of *G. soja* as a model system to study infection by *R. japonicum* microscopically. The binding of capsular polysaccharides isolated from *R. japonicum* to the root hairs of *G. soja* has already been demonstrated (17). We extend these studies by showing that the binding of *R. japonicum* cells to *G. soja* roots can be followed easily by light microscopy and that D-galactose and *N*-acetyl-D-galactosamine will specifically prevent this binding. Furthermore, the *G. soja* and *G. max* lectin can be localized on the root surface. The lectin isolated from seeds of *G. soja* is very similar to that isolated from *G. max*. This explains the binding similarities of the two plants.

MATERIALS AND METHODS

Organisms and Plant Material. *R. japonicum* 3I1b110, *Rhizobium meliloti* 102F51, *Rhizobium lupini* 96A6 and 96B8, and *R.* "lotus" 95E8 were generous gifts of J. C. Burton, The Nitragin Co., Milwaukee, Wis. *G. max* var. Corsoy and *G. soja* were used for infection and binding studies. Initial seed supplies of *G. soja* were kindly supplied by Paul Bishop, North Carolina State University. *G. max* seeds were obtained from the Agronomy Department, University of Wisconsin.

The *Rhizobium* inoculum was grown in yeast extract-mannitol broth (30) at 30 C. The plant nutrient solution used (W. Leps, unpublished results) was as follows: 2 mM $CaSO_4 \cdot 2H_2O$; 5 mM K_2SO_4; 5 mM $NaH_2PO_4 \cdot H_2O$; 40 μM ethylene diaminetetraacetic acid ferric salt; 0.5 μM $CoCl_2 \cdot 6H_2O$; 0.5 mM $MgSO_4 \cdot 7H_2O$; and 1 ml/l micronutrient solution (30). The pH of the PNS was adjusted to 7.0 with NaOH after autoclaving. Plants were grown at light intensity of 300 $\mu E/m^2 \cdot s$ with an 18-h photoperiod at 23-25C.

Assay for Binding of Bacteria to Roots. *G. soja* seeds were sterilized by swirling them for 10 min in Clorox (The Clorox Co., Oakland, Calif.). The seeds were rinsed with sterile distilled H_2O and scarified by soaking them in concentrated H_2SO_4 for 5 min. The seeds were rinsed with sterile H_2O and soaked for an additional 10 min in 0.01 N HCl. After the HCl treatment, seeds were rinsed repeatedly with sterile H_2O and germinated on sterile moist filter paper for 2 days.

R. japonicum binding to *G. soja* roots was examined by using the slide-culture method of Fahraeus (13). The slide assemblies were modified slightly by raising the coverslip approximately 1-2 mm on a cushion of silicon cement (Dow Corning Co., Midland, Mich.). This provides ample room for root growth and is within the working distance of microscopic observation.

Initial binding studies were performed by inoculating each plant-slide assembly with approximately 5×10^8 cells of *R. japonicum* 3I1b110. Plants were then incubated for 4 days in sterile, capped beakers containing 35 ml of PNS[2]. At the end of incubation, the plant-slide assemblies were removed and the plants rinsed with 20 ml of PNS. The plants were then examined using phase contrast or dark field optics at magnifications of 200 and 400.

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[2] Abbreviations: PNS: plant nutrient solution; PBS: phosphate-buffered saline; SBA: soybean hemagglutinin.

For short-term binding studies, uninoculated plants were grown in slide assemblies as above for 4 days. At the end of this period, the slides were removed and the plants were rinsed with 20 ml of PNS. A *Rhizobium* inoculum (approximately 1×10^9 cells) was then placed beneath the coverslip in contact with the root. The inoculated plants were then incubated at room temperature for 1 h. After this short incubation, the plants were again rinsed with 20 ml of PNS and examined.

The *Rhizobium* inoculum for binding studies was prepared from 4-day cultures grown in yeast extract-mannitol broth. Previous studies (2) have shown that 4-day cultures of *R. japonicum* 3I1b110 have optimal lectin-binding potential. Cells were washed with PNS and resuspended in PNS to give a cell concentration of approximately 1×10^9 cells/ml. Cell concentration was determined by comparing culture optical density to a standard curve based on plate counts. When sugars were used in the binding assays, they were prepared immediately before use, filter sterilized, and added to the bacterial inoculum immediately prior to inoculation of the plant.

After light-microscopic observations, portions of roots were sectioned into 1-cm segments and fixed overnight in 2.5% glutaraldehyde-25 mM K-phosphate (pH 6.8) at room temperature. The segments were rinsed twice with the phosphate buffer and dehydrated in a graded series of ethanol. The tissues were then critical-point dried (Denton, DCP-1) and sputter coated with gold-palladium (Denton, DV-502). Observations were made with a Jelco JSM-U3 scanning electron microscope at 20 kv.

Detection of *G. soja* Lectin. Antibody against purified SBA was a generous gift from Dr. Kenneth Keegstra (University of Wisconsin, Madison) or purchased from E-Y Laboratories, San Mateo, Cal. Ouchterlony double diffusion tests were performed in 0.7% agarose in 50 mM sodium barbital buffer, pH 8.2, containing 0.2% NaCl. *G. max* and *G. soja* seed extracts were prepared as described by Dazzo *et al.* (12).

Immunolatex beads were prepared essentially as described by Bracke and Markovetz (5). Lytron 5251 (0.25 μm diameter, Monsanto Polymers and Petrochemicals Co., St. Louis, Mo.) was dialyzed exhaustively against carbonate buffer (pH 9.2, 0.2 M Na_2CO_3-$NaHCO_3$). One ml of goat anti-rabbit IgG (2 mg/ml) was added to 0.99 ml of carbonate buffer (pH 9.2) and followed by 0.01 ml (10 mg/ml) of the latex suspension. The suspension was incubated at 30 C for 2 h with occasional agitation. The latex beads were pelleted at 7,500*g* for 10 min. After washing twice with 5 ml of PBS (50 mM K-phosphate, pH 6.8, with 150 mM NaCl), the beads were resuspended in 2 ml PBS and were used immediately.

For localization of the lectin on the surface of the root, *G. soja* seeds were germinated and grown for 4 days as described previously. The seedlings were then washed with PBS and placed in 1 ml of either anti-SBA or preimmune sera. The plants were incubated 1 h at room temperature and then washed with 20 ml PBS. The plants were then placed in 0.5 ml of a suspension of immunolatex beads and incubated for 1 h at room temperature. At the end of this period, the plants were washed exhaustively with PBS and prepared for scanning electron microscopy.

Immunolatex beads were also used to detect binding of the *G. soja* lectin to *R. japonicum* cells. For these studies, 4-day cultures of *R. japonicum* 3I1b110 in yeast-extract media were divided into aliquots containing approximately 1×10^9 cells. The cells were then pelleted, washed in PBS, washed again in PBS with or without sugar haptens, and resuspended in 50 μl of PBS containing the respective sugar haptens. At this time, 100 μl of a solution of purified *G. soja* lectin (1.3 mg/ml) was added and the tubes incubated at 30 C for 30 min. After this period, 5 ml of PBS containing bovine γ-globulins (5 mg/ml, Sigma Chemical Co.), was added and incubated 5 min at 30 C. The cells were pelleted and then incubated in 150 μl of anti-SBA antisera for 30 min at 30 C. This mixture was then diluted with 5 ml of PBS and the cells pelleted. At this time, the cells were resuspended in 150 μl of immunolatex suspension and incubated for 60 min at 30 C with occasional stirring. After the incubation period, the mixture was diluted with 5 ml of PBS and filtered through a 0.45 μm Nucleopore (Bio-Rad Laboratories, Richmond, CA) membrane. The filter was then washed with an additional 5 ml of PBS and prepared for scanning electron microscopy.

Peptide Mapping of *G. max* and *G. Soja* Lectin. *G. Soja* lectin was purified from defatted seed powder as described (1). *G. max* lectin was purified by an identical procedure or purchased from E-Y Laboratories, San Mateo, Calif. Peptide mapping was done by this procedure of Cleveland *et al.* (7). *Streptomyces griseus* protease and subtilisin (Sigma Chemical Co.) were used.

RESULTS

Slight modification of the original Fahraeus slide technique (13) for use with *G. soja* allowed good observation of *R. japonicum* binding. Binding could be seen easily under the light microscope 1 h after inoculation. Although more prevalent at the root-hair tip, cells of *R. japonicum* 3I1b110 bound to the entire root-hair surface (Fig. 1). *Rhizobium* cells could also be found bound to the epidermal cells. As controls, *R. meliloti* and strains of *R. lupini* and *R.* "lotus" were tested for their ability to bind to *G. soja* roots. When any of these strains are used to inoculate *G. soja* and the plants incubated for 1 h to 4 days, no bacteria bound to the roots (Fig. 1). Indeed, the roots appeared similar to that of an uninoculated plant. These strains do not nodulate *G. max* or *G. soja.*

To investigate the binding of *R. japonicum* to *G. soja* roots more closely, sections of the root were examined using scanning electron microscopy. Binding of *R. japonicum* to the root occurred in a polar, end-on fashion (Fig. 2). This polar attachment was seen on both *G. soja* and *G. max* root hairs and epidermal cells.

Inhibition of Bacterial Adsorption to Roots by Haptens of Soybean Lectin. The haptens, D-galactose and *N*-acetyl-D-galactosamine, are inhibitors of SBA binding to *R. japonicum* cells (2). These sugars also inhibit the binding of *R. japonicum* 3I1b110 to *G. soja* roots (Fig. 3). This effect was not observed in the presence of D-glucose and *N*-acetyl-D-glucosamine (Fig. 3).

The concentration of haptens (30 mM *N*-acetyl-D-galactosamine and 60 mM D-galactose) used in these experiments are not excessive and do not appear to affect the plant during the short incubation periods used. These levels of haptens are similar to those used previously to show hapten reversibility of clover lectin (9) and soybean lectin (2) binding.

***G. soja* Lectin.** The similarity of binding of *R. japonicum* to *G. soja* and *G. max* led us to examine *G. soja* seed extracts for a protein similar to that of *G. max* seed lectin. Immunological cross-reactivity was screened by Ouchterlony double diffusion against preimmune sera and sera prepared against pure SBA. A protein in *G. soja* extracts was detected that cross-reacted with anti-SBA and showed a line of identity with SBA in *G. max* seed extracts (Fig. 4). No line of precipitation was seen when control sera was substituted (Fig. 4).

To compare more directly the *G. max* and *G. soja* seed lectins, both were purified by affinity chromatography (1). The two purified proteins were then compared by peptide mapping. Treatment with *S. griseus* protease (data not shown) and subtilisin (Fig. 5) at various concentrations yielded similar digestion patterns for both proteins. In the case of the *G. soja* and *G. max* seed lectins, similar function is reflected in similar structure.

The similarity of structure between the *G. max* and *G. soja* lectins suggested the use of immunological techniques to localize physically the lectin on the surface of the root. Unfortunately, immunofluorescence could not be used since the yellow-green background fluorescence of the root makes the use of fluorescein dyes impractical and rhodamine fluorescence is very weak. There-

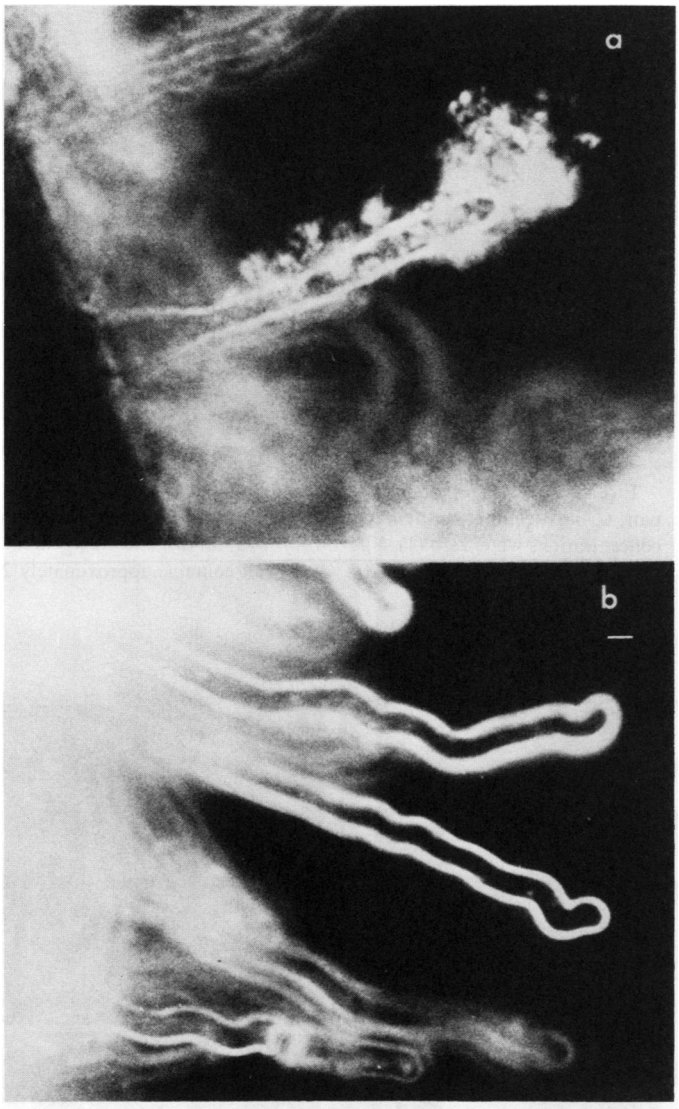

FIG. 1. Phase contrast micrographs showing adsorption of *R. japonicum* 311b110 to *G. soja* roots. Plants were grown as described and incubated for 1 h with *R. japonicum* cells (a). Other fast-growing or slow-growing *Rhizobium* strains such as *R. lupini* 96B8 (b) do not bind after 4 days of incubation. Bar represents 2 μm.

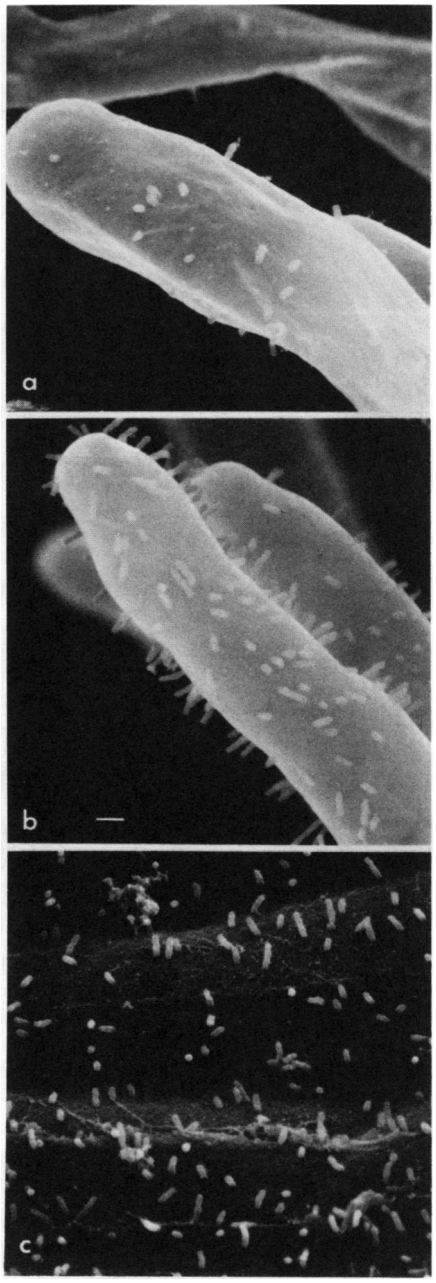

FIG. 2. Scanning-electron micrographs of *R. japonicum* 311b110 binding in a polar fashion to *G. max* root hairs (a), *G. soja* root hairs (b), and *G. soja* epidermal cells (c). Bar represents 2 μm.

fore, we utilized indirect immunolatex techniques for lectin localization (Fig. 6). The latex beads bound uniformly over the surface of the root hairs and epidermal cells of both *G. soja* and *G. max* (Fig. 6). Roots treated with preimmune sera had no latex beads bound (Fig. 6). Counts made from scanning electron micrographs of bacteria bound to the plant surface indicate that 1–3 bacteria/μm^2 of root surface are bound to the root hairs. Binding to the epidermal cell surface is somewhat less (0.7 bacteria/μm^2 of surface). Similar estimates of the density of latex beads bound to the root hair give a value of approximately 1 binding site/μm^2 of root surface. These are estimates obtained by direct counts on scanning electron micrographs and no attempt was made to correct for the cylindrical shape of the root hair during surface area calculations. Nonetheless, the beads occupy a significant proportion of the total lectin binding sites. These results suggest that the concentration of lectin molecules at the root surface may not be large; thus, isolation of significant quantities of lectin from the roots may be very difficult.

Immunolatex beads also were used to examine the binding of *G. soja* lectin to *R. japonicum* cells. The binding of *G. soja* lectin to *R. japonicum* cells is hapten reversible (Fig. 7). On close observation, the immunolatex beads bound predominantly to one end of the bacterial cell (Fig. 7).

DISCUSSION

The specificity of the *Rhizobium*-legume symbiosis is demonstrated by the lack of infection by soil microorganisms other than rhizobia and by the host-range specificity of *Rhizobium* species. This specificity implies that a process of cell recognition occurs between the bacterium and its host. Lectins have been proposed to be the primary determinants in *Rhizobium*-legume recognition (2, 3, 10, 15, 32). By this hypothesis, host-plant lectins would bind to carbohydrate receptors on the *Rhizobium* cell surface and, thereby, specifically bind the bacteria to the root. Evidence in support of this hypothesis comes mainly from studies performed

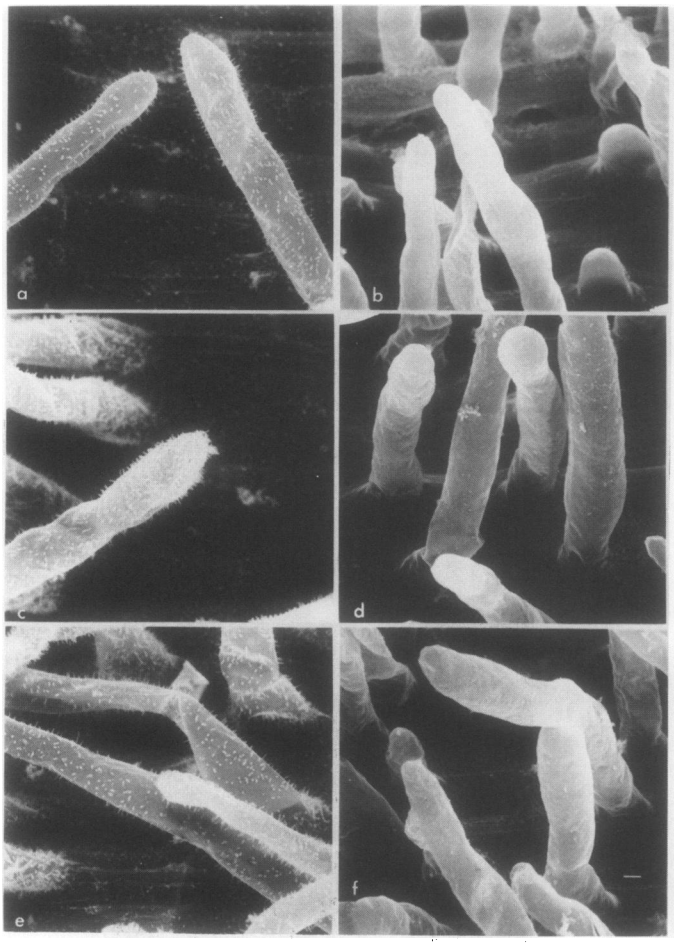

FIG. 3. Hapten inhibition of *R. japonicum* 3I1b110 adsorption to *G. soja* roots. Plants were uninoculated (b), inoculated (a), and inoculated in the presence of 30 mM *N*-acetyl-D-glucosamine (c), 30 mM *N*-acetyl-D-galactosamine (d), 60 mM D-glucose (e), or 60 mM D-galactose (f). After inoculation, plants were incubated for 1 h at room temperature and examined. Bar represents 5 μm.

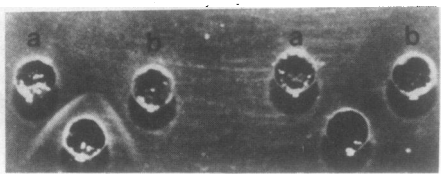

FIG. 4. Immunological cross-reactivity between *G. soja* seed lectin (a) and *G. max* seed lectin (b). The left center well contains anti-*G. max* seed lectin and the right center well contains preimmune serum.

with isolated lectin and free-living bacteria or isolated receptors. If better understanding of the role of lectins in the infection process is to be achieved, one must eventually study the process at the physiological site of infection, the root, and face the complexities that will accompany this approach.

The use of *G. soja* as a tool to investigate *R. japonicum* infection was first suggested by Brockwell and co-workers (6). They were able to demonstrate that *G. soja* and *G. max* were similar symbiotically when infected with a wide variety of *Rhizobium* strains. In these studies, we exploit the fact that *G. soja* is a smaller plant than *G. max* and can be adapted to light microscopic studies. Binding of *R. japonicum* to *G. soja* can be seen easily by phase contrast microscopy. This should make possible a wide variety of experiments which, until now, have been difficult to perform on

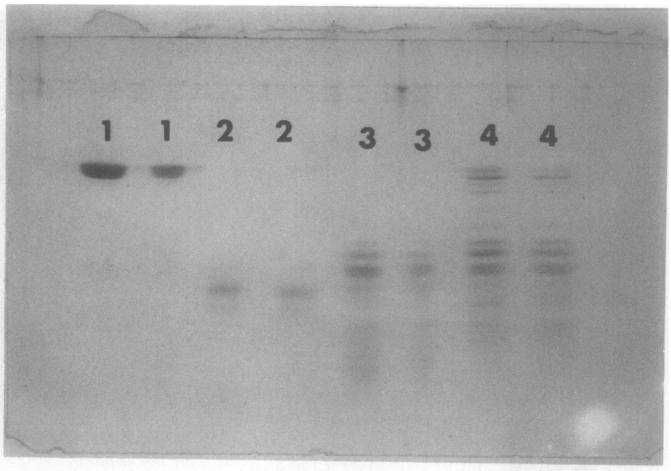

FIG. 5. Peptide map of purified *G. max* and *G. soja* seed lectin. In each pair, *G. max* lectin is on the left and *G. soja* lectin on the right. Subtilisin concentrations were: zero (1), 5 μg/ml (2), 1 μg/ml (3), and 0.2 μg/ml (4). Digestion was for 30 min at 37 C. Each well contains approximately 25 μg of protein.

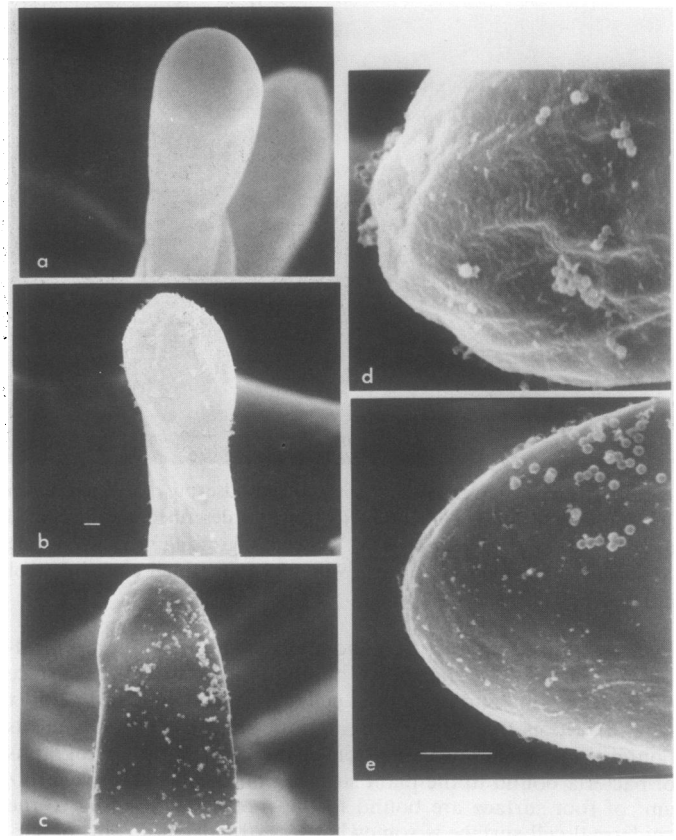

FIG. 6. Indirect immunolatex localization of lectin on the surface of *G. max* (b, d) root hairs and *G. soja* (c, e) root hairs. Root treated with preimmune serum in place of anti-SBA had no beads bound (a). Bar represents 2 μm in (a, b, c) and 2 μm in (d, e).

soybeans.

The importance of *Rhizobium* end-on attachment to the infection process is unclear. Polar attachment of bacteria to the roots of their respective hosts has been reported for *R. japonicum* (27), *R. trifolii* (11, 22, 23, 28), and *R. meliloti* (8). Attachment in this manner may reflect a polar arrangement on the bacterium of receptor sites involved in host recognition. The results of Bohlool

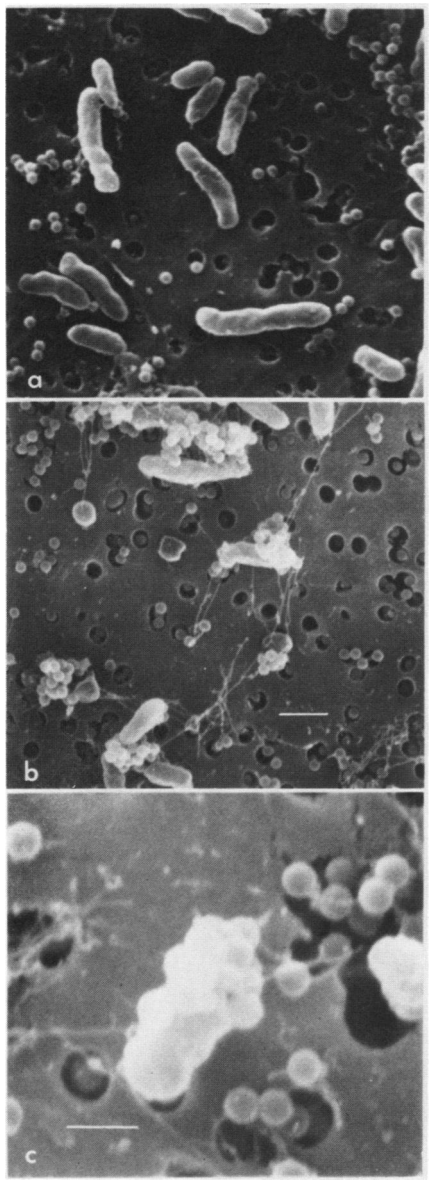

FIG. 7. Indirect immunolatex localization of lectin binding sites on cells of *R. japonicum* 3I1b110 (b, c). Cells treated with preimmune serum in place of anti-SBA had no beads bound (data not shown). Cells treated with 15 mM *N*-acetyl-D-galactosamine (a) had no beads bound while cells treated with 15 mM *N*-acetyl-D-glucosamine showed good binding (b). Polar binding of the beads can be seen clearly (c). Bar represents 1 μm in (a, b) and 0.5 μm in (c).

and Schmidt's (4) studies with immunofluorescence indicate that certain *R. japonicum* strains bind lectin predominantly in a polar fashion. Our results using immunolatex beads also indicate a polar arrangement of lectin binding sites on the *Rhizobium* cell.

The specific inhibition of *R. japonicum* binding to *G. soja* roots by the haptens of the soybean lectin (*i.e.* D-galactose and *N*-acetyl-D-galactosamine) strongly supports the role of lectin in the initial steps of infection.

This similarity in specificity between the two plant species supports the use of *G. soja* as a model system to study infection.

G. soja seeds contain a protein that is immunologically cross-reactive with the protein purified from *G. max* seeds. This cross-reactive material is a lectin because it bound to *R. japonicum* cells and is hapten reversible. However, immunological cross-reactivity cannot be taken as evidence that the two proteins are identical because Hankins and co-workers (16) recently demonstrated that lectins obtained from diverse sources having the same sugar specificity cross-react immunologically. Unfortunately, in that study the lectins were not compared further. Lectins of identical hapten specificity have similar amino acid compositions (14). Therefore, in the case of lectins, similar function may reflect similar structure. This is the case in *G. max* and *G. soja* because the two proteins yield identical peptide maps. *G. soja* has been proposed to be the ancestor of modern soybean varieties (24, 25). If the lectin-mediated symbiosis were important to the survival of the plant, then one might expect such a protein to be conserved during evolution. The similarity of the *G. soja* and *G. max* lectins supports this.

Localization of the soybean lectin to the surface of the root and the demonstration that *Rhizobium* binding was hapten-inhibited substantiates the proposed role of lectins in the initial steps of the symbiosis. Proof for the role of lectin in *Rhizobium* infection must await the isolation and reversion of nonnodulating, nonlectin binding mutants. *G. soja* should be useful in screening the potential of such mutants to bind to soybean roots. The convenience of using *G. soja* should stimulate other work on the role of lectin and other factors in rhizobial infection of soybeans. In this manner, studies presently performed only on the small-seeded legumes can be expanded to this agriculturally significant plant.

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