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A Peanut Nodule Lectin in Infected Cells and in Vacuoles and the Extracellular Matrix of Nodule Parenchyma[1]

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Root nodules on peanut (*Arachis hypogaea* L.) accumulate a galactose/lactose-binding lectin that is similar, but not identical, to the major seed lectin in peanut. The function of the peanut nodule lectin (PNL) is not known. In the current study, we have investigated the location of lectin in the nodule using immunogold labeling and enzyme-linked immunosorbant assays (ELISA). Lectin was most abundant in the nodule parenchyma, where it accumulated in vacuoles, suggesting a possible role as a vegetative storage protein. Lectin was also detected in the extracellular matrix in the nodule parenchyma, a location that corresponds to the tissue layer forming a barrier to oxygen diffusion. The potential for interactions between PNL and other cell wall components, including a previously described high-molecular weight glycoprotein that co-localizes with PNL, is discussed. Within infected cells, lectin was not detectable by immunogold labeling within the cytoplasm, but light labeling was suggestive of lectin localization within the symbiosome lumen. Analysis of fractionated symbiosomes by the more sensitive ELISA technique confirmed that lectin was present within the symbiosome, but was not bound to bacteroids. Our results indicate that PNL probably plays several roles in this nitrogen-fixing symbiosis.

Lectins are carbohydrate-binding proteins, other than enzymes or immunoglobulins, that bind reversibly to sugar moieties on glycoproteins, glycolipids, and polysaccharides (Barondes, 1988). Seed lectins are of widespread occurrence and are especially well known from leguminous plants, where they accumulate in protein bodies derived from vacuoles (Chrispeels and Raikhel, 1991). Vegetative plant organs may also contain lectins, which may be sequestered in vacuoles or secreted to the cell surface (Chrispeels and Raikhel, 1991). Multiple functions have been suggested for lectins in plants, including acting as a seed or vegetative storage protein, serving in defense against insect and fungal attack, agglutinating bacteria, and cross-bridging components in the extracellular matrix (Etzler et al., 1984; Etzler, 1985; Chrispeels and Raikhel, 1991).

Lectins have been detected on the surface of root hairs in the infectible zone of some legume roots, as has been most extensively characterized in pea (*Pisum sativum* L.; reviewed by Kijne, 1992). Secreted lectin is thought to enhance agglutination of potential symbionts in the family Rhizobiaceae on infectible root hairs (Kijne et al., 1988). In most important crop legumes, rhizobia invade the root via an infection structure called an infection thread. Lectin appears to play a role in determining the host-specificity of infection thread formation (Diaz et al., 1989). The rhizobial component bearing the lectin hapten has been suggested variously to be the surface constituents acidic polysaccharide or lipopolysaccharide (as reviewed by Kijne, 1992) or the lipo-oligosaccharide Nod factor synthesized by *nod* gene products of *(Brady)rhizobium* (Hirsch, 1992).

In peanut (*Arachis hypogaea* L.), two lectins have been found to accumulate in mature nodules, unlike other legumes that have been investigated that lack lectins in their nodules (Kishinevsky et al., 1988; Law et al., 1988). One of the two peanut nodule lectins, PNL, which binds Gal or lactose preferentially, resembles the major seed lectin (PSL) in amino acid composition and sugar specificity (Kishinevsky et al., 1988; Law et al., 1988). PNL also exhibits some differences from PSL. The nodule lectin is approximately 1 kD larger than PSL (Law et al., 1988; this study) and is a glycoprotein, whereas PSL is not glycosylated (Law et al., 1988). Furthermore, the N-terminal sequences of the isolated PNL and PSL agree in only 4 of 10 amino acids, and the nodule lectin contains a major isoform not found in the seed, suggesting that these two proteins may be products of different genes (Law et al., 1991). Whether the Gal-binding lectin in nodules constitutes a nodule-specific protein, or nodulin, is not clear because other tissues also contain low concentrations of an immunorelated lectin, based on ELISA (Law et al., 1990).

In peanut, *Bradyrhizobium* first invades the root intercellularly, via the break in the epidermis that occurs at the point of emergence of a lateral root; the bacteria are later taken up into cells without the formation of infection threads (Chandler, 1978). Since peanut is not infected via root hairs, nor does it form infection threads, possible symbiotic roles for the Gal-specific PNL are obscure. Furthermore, the time course of accumulation of PNL indicates that it is not involved in the establishment of the symbiosis (Kishinevsky et al.,

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Abbreviations: PNL, galactose/lactose-specific peanut nodule lectin; PSL, peanut seed lectin.

1988; Law et al., 1990). To discern a possible role for the Gal-specific lectin during later symbiotic interactions, we have used immunogold labeling and direct ELISA assays of nodule fractions to localize the protein in peanut nodules. While this work was in progress, a similar study was published by Law and van Tonder (1992). Our results support the major finding of the above study in that PNL is abundant in vacuoles of nodule parenchyma cells. In addition, our study has revealed the presence of PNL in two additional locations: the extracellular matrix of cells in the uninfected nodule parenchyma, the zone thought to form a barrier to oxygen diffusion (Witty et al., 1986), and within the symbiosome. Thus, the current study indicates a more complex role for Gal-specific lectin in peanut nodules than has been suggested previously.

MATERIALS AND METHODS

Growth and Nodulation of Plants

Captan-treated seeds of peanut (*Arachis hypogaea* L. var Virginia, cv NC7 and NC9) were surface sterilized in 70% ethanol, followed by full-strength commercial bleach, and allowed to germinate on moist filter paper for 3 d. The seedlings were transferred to 1-L plastic pots each containing 900 g of washed white silica sand and placed in a glasshouse under ambient sunlight conditions. Additional seedlings were transplanted to a vermiculite/perlite mixture and grown in a Conviron growth chamber at 21°C, with an RH of 90% and a 17.5-h day/6.5-h night cycle. Both groups of seedlings were inoculated at the time of transplant with *Bradyrhizobium* sp. (*Arachis*), strain 2209A (Lobel and Schiffmann, 1975), that had been grown in yeast-mannitol broth (Vincent, 1970).

Gel Electrophoresis and Western Blotting

Peanut nodules from greenhouse-grown plants were harvested 32 d after inoculation and ground in cold (4°C) extraction buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM DTT, 10 mM sodium ascorbate, plus 50 mg/mL polyvinylpolypyrrolidone). The extract was filtered through Miracloth and centrifuged for 2 min at 10,000*g*. Proteins in the supernatant were precipitated overnight with 80% ethanol at −20°C. The precipitated proteins were resuspended in Laemmli sample buffer (Laemmli, 1970) and boiled for 1 min. Seed proteins from dry seeds of the same cultivar were prepared similarly. Isolated seed lectin (peanut agglutinin purchased from Sigma) was also dissolved in sample buffer prior to running on gels. Samples were run on SDS-PAGE gels and transferred to nitrocellulose as previously described (VandenBosch et al., 1989), except that the transfer buffer contained 48 mM Tris, 39 mM Gly hydrochloride, 1.3 mM SDS, and 20% methanol. Immunolabeling of proteins on blots was also as previously described (VandenBosch et al., 1989), using anti-PSL IgG at a concentration of 2 μg/mL as the primary antibody (Kishinevsky et al., 1988). For a negative control, anti-PSL IgGs were incubated with a 20-fold excess (w/w) of isolated peanut agglutinin (Sigma) to preadsorb IgGs that were specifically reactive with PSL epitopes from the antibody solution. To detect extracellular matrix glycoprotein among proteins in nodule extracts, immunoblots were probed with a MAC 265, a previously characterized rat monoclonal antibody (VandenBosch et al., 1989).

Detection of PNL in Nodule Fractions by ELISA

Nodules were harvested from greenhouse-grown plants 6 weeks after inoculation and were homogenized in cold homogenization buffer (350 mM mannitol, 5 mM DTT, 20 mM sodium ascorbate, 1% BSA, 1% PVP 40, and 20 mM Mes-KOH, pH 7.0). A sample of the nodule extract was saved for analysis of PNL content in the total nodule proteins. Isolation of symbiosomes from peanut nodules was done on a Percoll step gradient according to Day et al. (1989). Nodule soluble proteins, which did not enter the Percoll, were collected from the top of the gradient. Symbiosomes were collected from the 30/60 interface. Osmotic shock was used to break the symbiosome membrane as follows: isolated symbiosomes were resuspended in wash buffer without mannitol and vortexed vigorously. *Bradyrhizobium* bacteroids were pelleted by centrifuging at 10,000*g* for 2 min. The supernatant, containing membrane fragments and soluble proteins, was designated the symbiosome lumen fraction. Bacteroids were washed twice by resuspending in wash buffer minus mannitol, followed by centrifugation. Protein concentrations in the various fractions were estimated using Bradford assays, and PNL content was assessed by a direct double-antibody sandwich ELISA using anti-PSL antibodies as previously described (Kishinevsky et al., 1988). A dilution series of isolated PSL (200–1.5 ng/mL) was used to generate a standard curve. PSL controls and test extracts were included on the same plates using identical conditions.

Immunogold Labeling

Peanut nodules were harvested from Conviron-grown plants 6 weeks after inoculation. Nodule slices were fixed in 2.5% glutaraldehyde in 100 mM potassium phosphate buffer (pH 7.0), dehydrated in an ethanol series, and embedded in LR White using heat (60°C) to effect polymerization. Peanut seeds were allowed to imbibe for 1 h in distilled water before fixation. Pieces of cotyledons, 1 mm in diameter, were then fixed and embedded as described for nodules. Silver- to silver-gold-colored sections were collected on uncoated nickel grids.

Sections on grids were incubated in blocking buffer (1% BSA [fraction V], 0.02% [w/v] sodium azide, and 0.05% [v/v] Tween 20 in Tris-buffered saline, containing 20 mM Tris-HCl, pH 7.4, and 150 mM NaCl). Grids were then transferred to anti-PSL IgG diluted to a concentration of 2.5 to 5 μg/mL in blocking buffer. After a 1-h incubation in primary antibody, grids were rinsed in Tris-buffered saline and placed into goat anti-rabbit antibody/gold conjugate (Auroprobe EM GAR10 or GAR15, Amersham) diluted 1:10 (v/v) in blocking buffer. Gold particle size was 10 nm unless otherwise noted. After incubation in the gold conjugate for 1 h, grids were rinsed in distilled water and poststained in 2% aqueous uranyl acetate for 5 min, followed by Reynolds' lead citrate (Reynolds, 1963) for 0.5 min. Sections were viewed in a Zeiss 10C transmission electron microscope operated at 60 or 80 kV.

For double labeling of PNL and matrix glycoprotein on the same specimen, labeling of PNL was first carried out as described above. Second, sections on grids were incubated in the monoclonal antibody MAC 265 appropriately diluted in blocking buffer, followed by goat anti-rat secondary antibody/5 nm gold conjugate (Auroprobe EM GARa 5, Amersham) diluted 1:10 (v/v) in blocking buffer.

As a negative control, the preadsorbed antibody solution, described above, was substituted as a primary antibody. Alternatively, primary antibody was omitted before incubation of sections in both secondary antibody/gold conjugates. As a control for nonspecific labeling, mild periodate oxidation was used to destroy glycan epitopes on cut sections prior to immunolabeling, as described by Woodward et al. (1985) and modified by Jones and Herman (1993).

For light microscopic detection of epitopes in tissue, sections 0.5 μm thick were collected on gelatin-coated slides. Sections were probed with anti-PSL or MAC 265, followed by the appropriate secondary antibody/gold conjugate (Auroprobe LM GAR or GARa, Amersham) and silver enhancement, following established protocols (VandenBosch, 1992).

RESULTS

Evaluation of Anti-PSL Antibody

On western blots, total seed protein showed immunoreactive bands similar to those of the purchased, isolated peanut agglutinin (Fig. 1A). Among nodule proteins, a single band was recognized, and this had a slightly higher M_r than did the seed lectin (Fig. 1A). Molecular masses for the major immunoreactive band in seeds and for the reactive band in nodules were calculated to be 30.5 and 31.7 kD, respectively, similar to previously reported values for purified seed (30.7 kD) and nodule (31.3 kD) lectins from peanut (Law et al., 1988). Several additional bands between 16 and 20 kD were detected among the seed proteins and in the pure peanut agglutinin preparation. These lower molecular mass bands are presumed to be proteolytic cleavage products of the peanut seed lectin. On blots that were probed with primary antibody that was preadsorbed with pure peanut agglutinin no bands among seed or nodule proteins were recognized (data not shown).

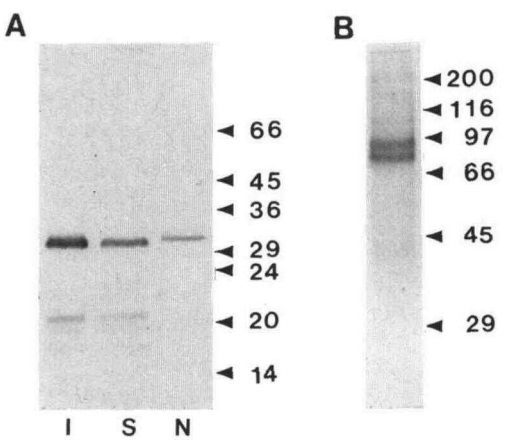

Figure 1. Characterization of anti-PSL and MAC 265 antibodies on western blots. A, Reactivity of anti-PSL with isolated PSL (I), total seed protein (S), and total nodule protein (N). Protein loading was as follows: lane I contained 1 μg, lane S contained 5 μg, and lane N contained 10 μg. B, Reactivity of the monoclonal antibody MAC 265 with 5 μg of total nodule protein.

Immunolocalization Controls

To ascertain the sensitivity and specificity of the antibody in immunogold assays, anti-PSL was applied to sections of peanut seed as a positive control. Numerous gold particles bound specifically to protein bodies as was expected, demonstrating that PSL was a sensitive probe for PSL under these fixation and embedding conditions (Fig. 2A). Preadsorbed antibody applied to sections of seeds and nodules resulted in no specific labeling; a view of nodule tissue immunolabeled with preadsorbed primary antibody is depicted in Figure 2B. Likewise, omission of primary antibody gave no detectable signal on either seed or nodule tissue (data not shown). Periodate oxidation of sections, carried out prior to immunolabeling, did not affect the localization pattern of PNL (data not shown).

Immunolocalization of PNL in Uninfected Tissues

In describing the anatomy of the peanut nodule, we have followed the terminology previously outlined for pea and soybean nodules (van de Wiel et al., 1990). The structural organization of a peanut nodule is shown in Figure 3. The central infected zone is surrounded by several layers of uninfected tissue, including a boundary layer adjacent to the infected zone, a multi-layered nodule parenchyma, and an outer cortex. The central infected zone contains few uninfected, interstitial cells, except those that are organized into rays that are often seen crossing the central zone. The rays commonly join the boundary layer opposite a vascular bundle. The structure of ray cells and boundary layer cells is similar and is characterized by abundant amyloplasts with large starch grains. The network of vascular bundles is contained within the nodule parenchyma, and although each vascular bundle has its own endodermis, a continuous nodule endodermis is not in evidence. Because the nodule endodermis is usually considered the dividing layer between the nodule parenchyma and the outer cortex (van de Wiel et al., 1990), a clear demarcation between these two tissues is not discernible in peanut nodules.

Among the uninfected tissues of the nodule, PNL was most abundant in vacuoles of the nodule parenchyma (Fig. 4). Within the largely electron-lucent vacuoles, lectin was abundant in protein aggregates in association with the tonoplast or free within the vacuolar sap. Cells in the boundary layer and in the uninfected rays contained smaller vacuolar protein aggregates, which were lightly labeled by immunogold labeling or not at all (not shown). In addition to lectin targeted to the vacuole, extracellular lectin was found in a discrete region between the boundary layer and the nodule parenchyma (Fig. 4). The secreted lectin did not become integrated into the primary wall, but was a component of the extracellular matrix that filled the intercellular spaces at this location.

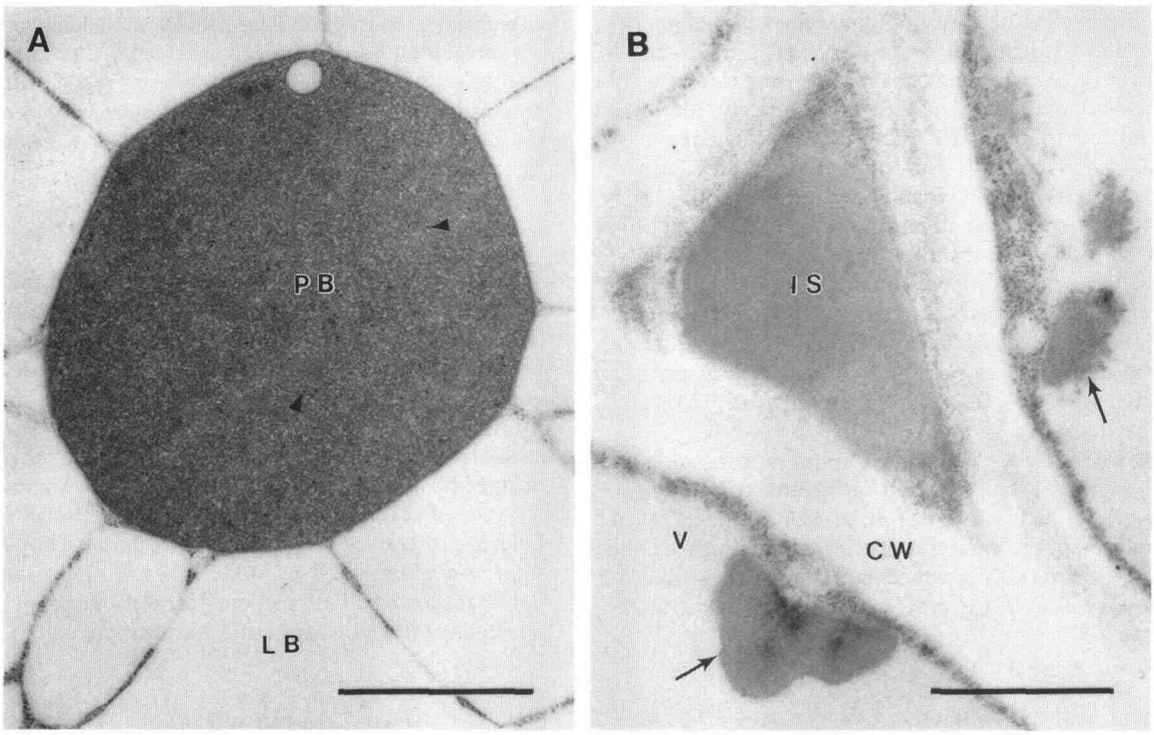

Figure 2. Positive and negative controls for specificity of anti-PNL. A, Positive control. Gold particles (10 nm, indicated by arrowheads) bound to sections indicate that anti-PNL specifically labels protein storage bodies (PB) in seeds. LB, Lipid body. 26,000× (bar = 1 μm). B, Negative control. Anti-PNL antiserum, depleted of specific antibodies by incubation with excess seed lectin, does not label vacuolar protein deposits (arrows) or protein in intercellular spaces (IS) of the nodule parenchyma. CW, Cell wall; V, vacuole. 24,000× (bar = 1 μm).

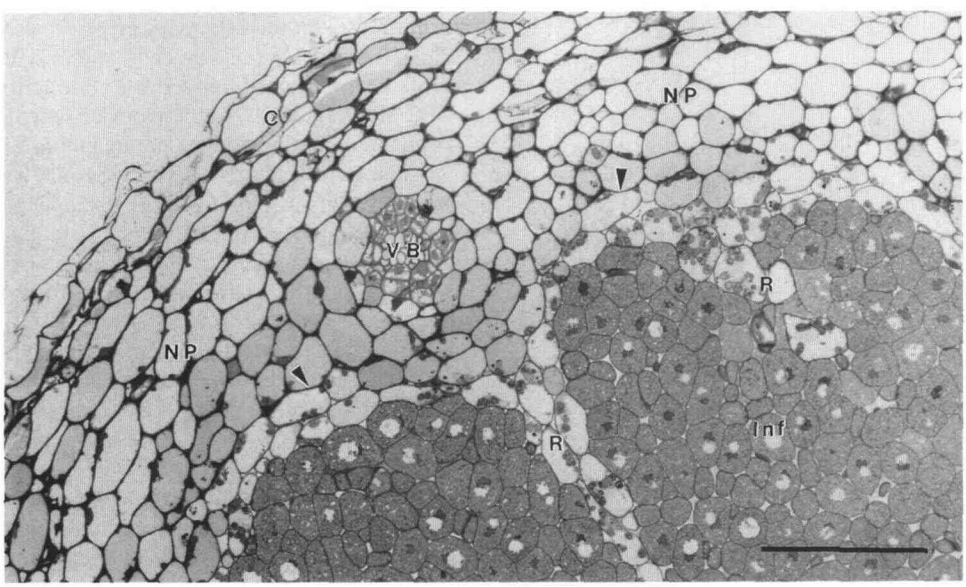

Figure 3. Light microscopic view of nodule anatomy. The micrograph depicts the periphery of a nodule cross-section, showing the cortex (C), the nodule parenchyma (NP), a vascular bundle (VB), the infected zone (Inf), and the boundary layer (indicated by arrowheads), which is continuous with two rays of uninfected, interstitial cells (R). 430× (bar = 50 μm).

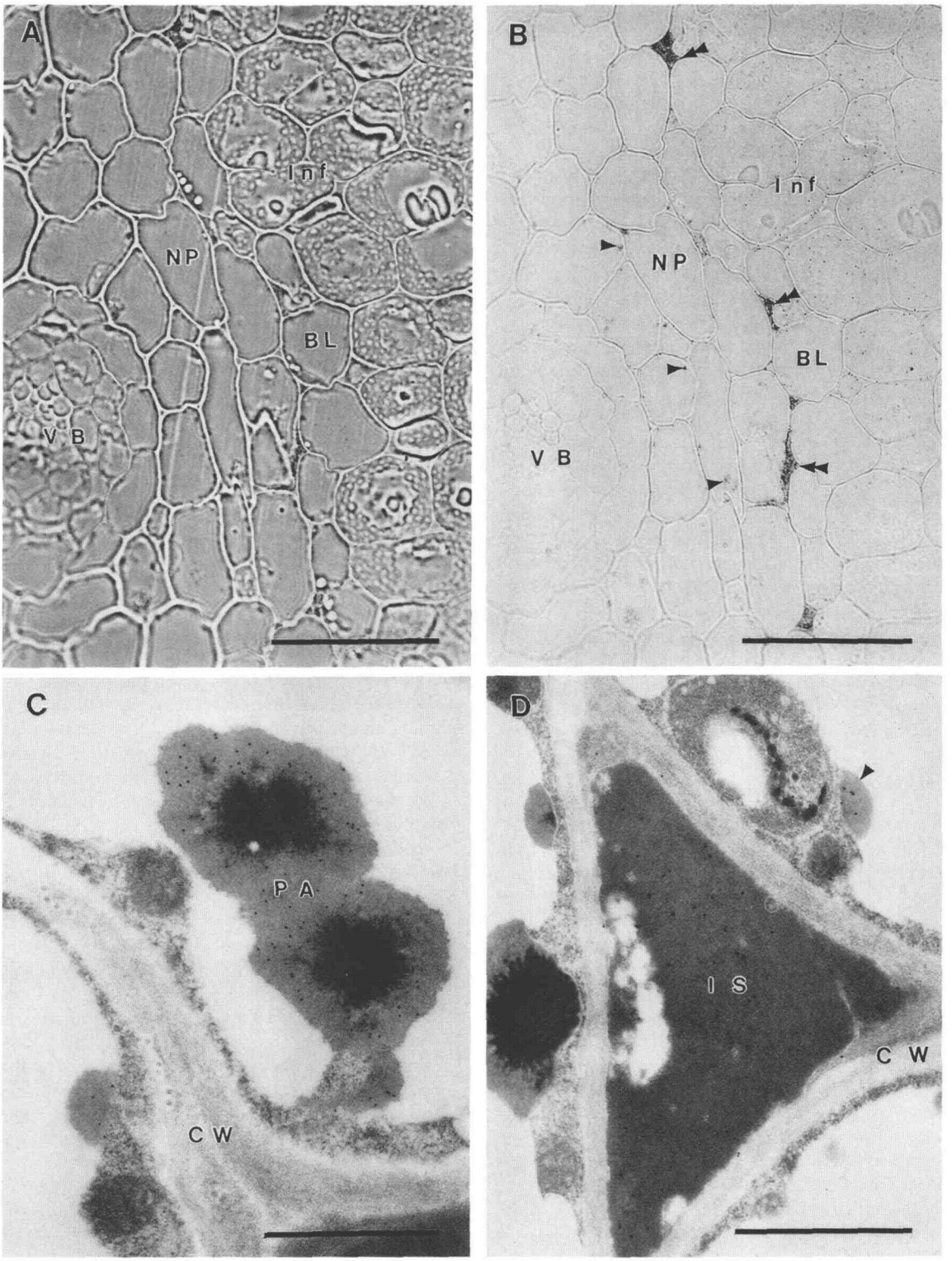

Figure 4. Labeling of PNL in the nodule parenchyma. A and B, Light microscopic localization of PNL. 570× (bars = 50 μm). A, Phase-contrast micrograph depicting a vascular bundle (VB), the nodule parenchyma (NP), the boundary layer (BL), and the infected zone (Inf). B, Bright-field micrograph of the same section, showing immunogold-silver staining of lectin in the nodule parenchyma. Single arrowheads indicate lectin in vacuolar aggregates and double arrowheads indicate labeling of lectin in intercellular spaces proximal to the boundary zone. C and D, Electron microscopic localization of PNL. 27,000× (bars = 1 μm). C, Immunogold labeling of PNL in a vacuolar protein aggregate (PA) in the nodule parenchyma. CW, Cell wall. D, Immunogold labeling of secreted PNL in an intercellular space (IS) in the nodule inner cortex. Arrowhead indicates a small protein aggregate in the vacuole of an adjacent cell.

The occurrence of secreted lectin just outside the boundary layer was reminiscent of the distribution of a high-mol wt glycoprotein found previously in the intercellular spaces of the nodule parenchyma in pea and soybean (VandenBosch et al., 1989). Occlusion of these intercellular spaces by secreted proteins is theorized to contribute to the barrier to oxygen diffusion that occurs in the nodule parenchyma (James et al., 1991). The occurrence of the high-mol wt glycoprotein in pea nodules was first revealed by three monoclonal antibodies that specifically recognize carbohydrate epitopes on this extracellular matrix glycoprotein (VandenBosch et al., 1989). One of these, the monoclonal antibody MAC 265, was used to probe isolated peanut nodule proteins to determine whether a similar extracellular matrix glycoprotein occurred in peanuts. On western blots, one or more MAC 265-reactive proteins were detected in a molecular mass range of approximately 80 to 95 kD (Fig. 1B).

Immunocytochemistry was employed to discern whether the MAC 265-reactive protein was distributed similarly to PNL. Light microscopic immunolabeling with MAC 265 revealed that the immunoreactive glycoproteins are widely distributed in intercellular spaces in the nodule parenchyma (Fig. 5A). Double-labeling experiments, in which the MAC 265 glycoprotein(s) and PNL were localized with different-sized gold particles, indicated that both types of proteins are found together in intercellular spaces in the nodule parenchyma adjacent to the boundary layer (Fig. 5C). PNL was again found to be absent from intercellular spaces more than two cells distant from the boundary layer, although MAC 265-reactive glycoprotein was a major component of the extracellular matrix in these same spaces (Fig. 5B).

Immunodetection of PNL in Infected Cells

Following immunogold labeling with anti-PSL antibodies, gold label was less abundant over the infected region than was seen over the nodule parenchyma. Vacuolar protein accumulations were not found within the central vacuole of infected cells, and gold particles were seen infrequently over vacuolar contents. When label was present over infected cell vacuoles, it was associated with membrane profiles within the vacuole (Fig. 6, C and D).

Label was occasionally detected in association with symbiosomes (formerly called peribacteroid units), the plant membrane-enclosed *Bradyrhizobium* bacteroids that resemble organelles (Roth and Stacey, 1989). In peanut nodules, symbiosomes each contain a single, round bacteroid, the mature, differentiated nitrogen-fixing stage of the bacteria. When present, label was associated with electron-dense bodies within the symbiosome lumen (Fig. 6, A and B) or with the symbiosome membrane (Fig. 6C). However, labeling of the symbiosomes was inconsistent and, although suggestive, was not considered to be clear and convincing evidence of the presence of PNL within the symbiosome.

To investigate further the possible presence of lectin within the symbiosomes, nodules were fractionated, and the fractions were probed with a direct ELISA. The results from such an experiment are given in Table I. PNL was detectable in isolated symbiosomes in dilutions down to a total protein concentration of 62 μg/mL. Following osmotic lysis of the symbiosomes, immunoreactive material was found in the supernatant (containing symbiosome membranes and soluble lumenal proteins) at the same dilution. Immunoreactive material was not detectable in association with bacteroids in any assay.

DISCUSSION

Peanut shows distinctly different patterns of lectin accumulation during symbiotic interactions with homologous strains of (brady)rhizobia than do other legumes. For example, studies on pea indicate that the lectin protein is present in roots in very low amounts (Diaz et al., 1984) and that expression is not transcriptionally activated in response to homologous rhizobia (Buffard et al., 1988). By contrast, PNL, a novel lectin related to the Gal/lactose-specific seed lectin, accumulates in peanut nodules during nodule development (Kishinevsky et al., 1988; Law et al., 1988).

To address a possible function for PNL, we have used immunogold labeling and ELISA to investigate the distribution of the protein within nodules at the time of maximum accumulation of the lectin (6 weeks after inoculation). The antibody used was generated against purified PSL and was found to react with one band on a one-dimensional western blot of nodule proteins. The molecular mass of the immunoreactive protein (31.7 kD) corresponded closely to that previously determined for isolated PNL (31.3 kD; Law et al., 1988). Because PSL, the protein used for producing the antibody, is not a glycoprotein, and because western blots showed a single immunoreactive protein in nodules, it is unlikely that the antibody would cross-react nonspecifically with other proteins via carbohydrate side chains. The persistence of the epitope after periodate oxidation also suggests that the epitope is not a carbohydrate. Furthermore, primary antibody that had been preadsorbed with lectin did not label sections. Therefore, we maintain that the observed results reflect the actual distribution of the Gal-specific lectin within nodules.

Lectin was revealed in several locations within the nodule, in both uninfected tissue (the nodule parenchyma) and infected tissue. The most abundant label occurred in the nodule parenchyma, where the protein was found sequestered in vacuoles and secreted to the extracellular matrix. In the large central vacuole of nodule parenchyma cells, PNL is found principally in protein aggregates associated with the tonoplast. The vacuolar localization of PNL, also seen by Law and van Tonder (1992), resembles the compartmentalization of PSL in protein bodies. The occurrence of seed lectins in protein bodies derived from vacuoles has been extensively documented for many species of legumes (Etzler, 1985). The transient nature of PNL accumulation in nodules implies that the lectin may function as a storage protein within nodules, as has also been suggested by Law and van Tonder (1992). Vegetative lectin has also been found to be targeted to protein storage vacuoles in leaves and bark of the leguminous tree *Sophora japonica* (Herman et al., 1988; Baba et al., 1991), and the circa annual pattern of accumulation and depletion of the bark lectin suggests that it may function as a storage protein (Baba et al., 1991). The relative abundance of lectin within peanut nodules decreases concomitantly with its accumula-

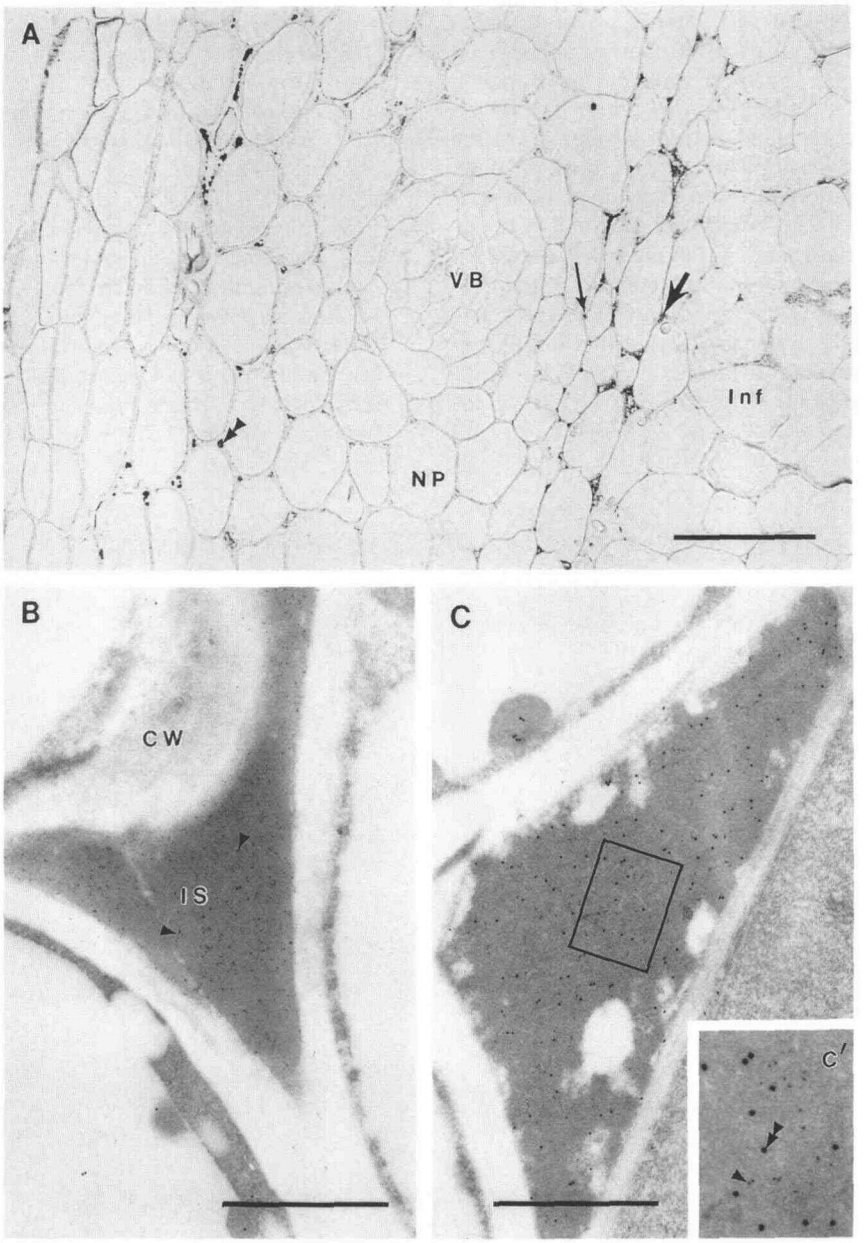

Figure 5. Localization of matrix glycoprotein in the nodule parenchyma. A, Immunogold labeling with the monoclonal antibody MAC 265, as seen with bright-field optics. Labeled protein is visible in intercellular spaces in the outer (double arrowhead) and inner (arrows) regions of peanut nodule parenchyma. The thin arrow indicates the cell layer depicted below in B, and the thick arrow indicates the cell layer that is proximal to the boundary layer and is depicted below in C. VB, Vascular bundles; NP, nodule parenchyma; Inf, infected zone. 370× (bar = 50 μm). B and C, Electron micrographs of double labeling of PNL and matrix glycoprotein in intercellular spaces in the cortex. Single arrowheads indicate 5-nm gold particles marking the location of matrix glycoprotein, and double arrowheads indicate 15-nm gold particles marking PNL. 21,000× (bars = 1 μm). B, An intercellular space (IS) in the nodule parenchyma, distal to the boundary layer, contains matrix glycoprotein but not PNL. C, PNL and matrix glycoprotein co-localize in an intercellular space proximal to the boundary layer. Inset C′ shows a magnified view of the boxed area in C. 52,000×.

tion in developing pods (Law et al., 1990), suggesting that stored protein may be mobilized from nodules during seed development. Verification of a storage protein role for PNL must await experimental confirmation by removal of developing pods, as has been demonstrated for vegetative storage protein in soybean leaves (Spilatro and Anderson, 1989; Staswick, 1989). If PNL does function as a storage protein, it would be expected that lectin concentrations would be maintained in older nodules after removal of alternate sinks.

Little or no vacuolar lectin was found to accumulate in the uninfected interstitial cells in the central infected zone. It has been frequently, though erroneously, stated that nodules of peanut and related species lack interstitial cells (e.g. Sprent and Raven, 1992). In peanut, interstitial cells are less abundant than in most legume nodules and are largely restricted to radially arranged files, or rays, that cross the infected zone. The presence of these rays in peanut and the closely related genus *Stylosanthes* was noted by Chandler and co-workers, who suggested that the infected regions divided by the rays are derived from separate infections (Chandler, 1978; Chandler et al., 1982); this derivation has not been demonstrated, however.

Peanut transports recently fixed nitrogen in the forms of both amides and ureides (Schubert, 1986). Ultrastructural and cytochemical evidence from peanut suggests that the ureides are synthesized in the interstitial cells (Tandon, 1990), as has been seen in soybean (Newcomb and Tandon, 1981; VandenBosch and Newcomb, 1986). Based on the alignment of uninfected cells into rays and the distribution of plasmodesmata within ray cells, Selker and Newcomb (Selker and

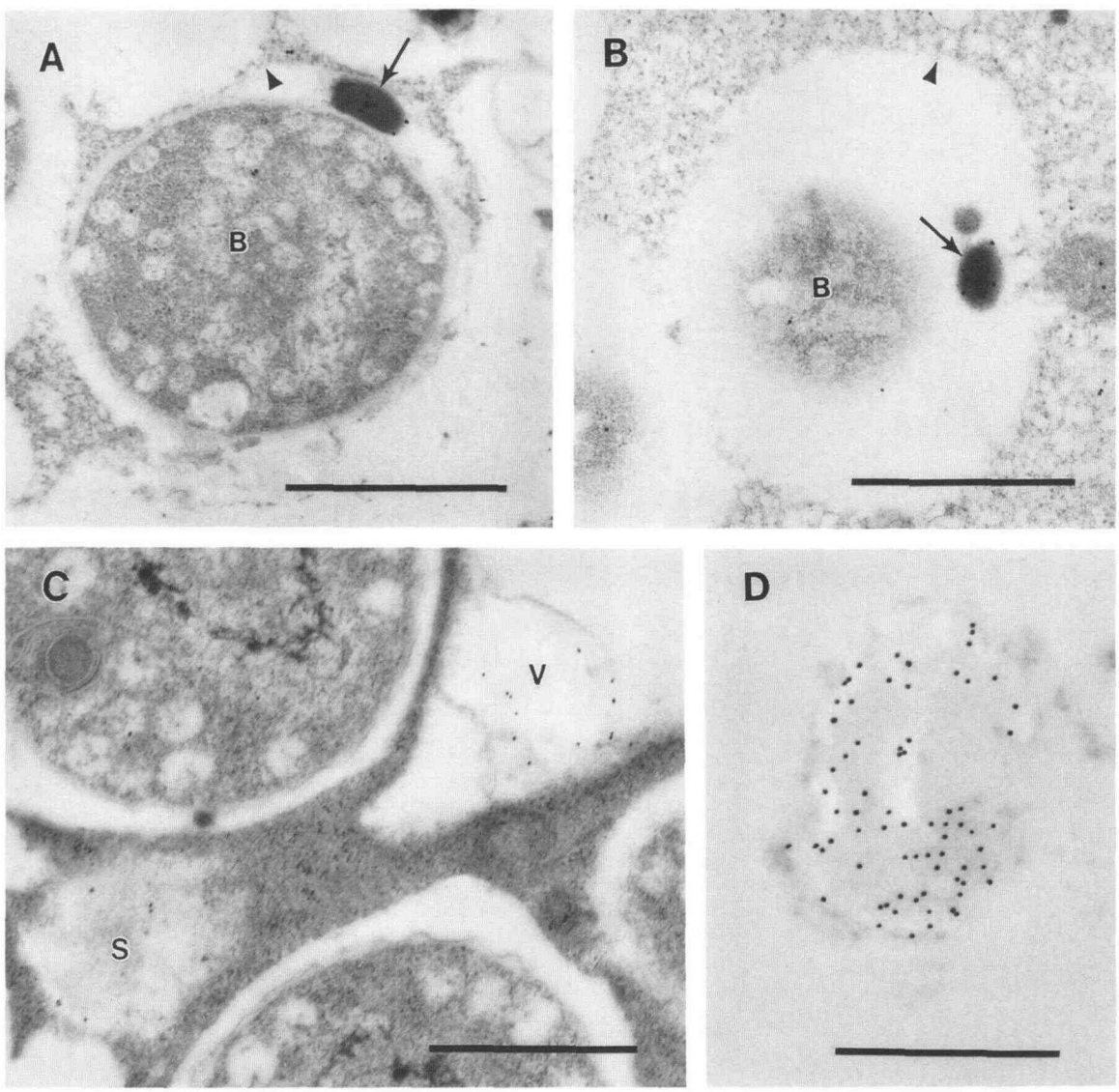

Figure 6. Immunogold labeling with anti-PSL in infected cells of nodules. 30,000× (bars = 1 μm). A and B, Examples of immunogold labeling on electron-dense bodies (arrows) within two symbiosomes. Arrowheads indicate the symbiosome membrane. B, *Bradyrhizobium* bacteroid. C and D, Membrane profiles within vacuoles (V) with bound gold particles. S, Symbiosome (tangential section).

Table I. *Lectin content in fractionated peanut nodules as determined by double-antibody sandwich ELISA*

Concentration of anti-PSL IgG = 3 μg/mL. Enzyme conjugate concentration = 1:250. Each value is a mean of two replicates.

Fraction	Lectin Concentration				
	Total protein (μg/mL) in extract				
	500	250	125	62	31
	ng/mL				
Total nodule homogenate	–[a]	14.1	12.0	8.9	4.2
Soluble protein	–	12.3	6.9	6.9	4.1
Total symbiosome	39.8	23.4	7.0	4.0	0[b]
Symbiosome lumen	17.0	7.1	3.7	2.9	0
Bacteroids	0	0	0	0	0

[a] –, Not tested. [b] 0, Not detectable (<1 ng/mL).

Newcomb, 1985; Selker, 1988) proposed that uninfected cells in soybean are key elements in transporting fixed nitrogen from the infected zone to the peripheral nodule vascular bundles. We hypothesize that two types of uninfected cells together play an important role in the nitrogen economy of peanut nodules: the interstitial ray cells may participate in the synthesis of ureides and amides and the transport of recently fixed nitrogen from the infected zone to the nodule parenchyma cells. The nodule parenchyma cells may then store nitrogen as protein before exporting it to the shoot.

Within cells infected by *Bradyrhizobium*, immunogold labeling suggested that little PNL occurs in vacuoles, unlike results reported by Law and van Tonder (1992). What label occurs appears to be associated with membrane profiles rather than amorphous protein aggregates. We suggest that lectin deposition in these vacuoles is not the result of conventional protein targeting via the endomembrane system, but is the result of autophagic destruction of symbiosomes. Herman (1994) has recently reviewed similar examples of sequestration and disposal of plant organelles via autophagy. Alternatively, membrane-associated lectin may be the result of cellular disruption due to inadequate fixation of these cells, although the overall appearance of the tissue was good.

Also within infected cells, immunogold labeling results were suggestive of lectin within symbiosomes, especially associated with electron-dense bodies in the symbiosome lumen. Compartmentalization of lectin within symbiosomes was confirmed by ELISA assays. Symbiosomes resemble vacuoles in biogenesis and in function. For example, nodulin-26 is a major integral membrane protein found in symbiosome membranes (Fortin et al., 1987; Weaver et al., 1991) that bears significant homology to the tonoplast intrinsic protein from *Phaseolus* protein storage vacuoles (Johnson et al., 1990). Both tonoplast intrinsic protein and nodulin-26 are believed to have transport functions (Johnson et al., 1990; Ouyang et al., 1991). Moreover, both symbiosome and vacuolar proteins are derived from the cell's endomembrane system (Brewin, 1991; Melroy and Herman, 1991; Chrispeels and Raikhel, 1992). Like vacuoles, symbiosomes can have lytic activity, and they have been compared with protein storage vacuoles and lysosomal compartments (Mellor, 1989; Brewin, 1991). Plant cells may contain two or more types of vacuole that differ in function (Griffing and Fowke, 1985, and refs. therein), so it is not surprising that lectin would accumulate in symbiosomes but not within the central vacuole of infected cells.

In addition to intracellular localization in the nodule parenchyma and infected cells, PNL was detected in intercellular spaces between the nodule parenchyma and the boundary layer. Intercellular spaces in this layer and throughout the nodule parenchyma were also found to contain a higher-mol wt glycoprotein, similar to that previously demonstrated for pea and soybean nodules, as revealed by immunolabeling with the monoclonal antibody MAC 265 (VandenBosch et al., 1989; James et al., 1991; Rae et al., 1991). The glycan composition of the nodule glycoprotein recognized by MAC 265 has not been determined, although a cross-reactive glycoprotein from *Phaseolus* bean contains Ara and Gal as its major sugars (Millar et al., 1992). Therefore, the glycoprotein recognized by MAC 265 may bear the hapten of this Gal-specific lectin.

The nodule parenchyma constitutes a variable barrier to oxygen diffusion, as demonstrated by oxygen microelectrode measurements (Tjepkema and Yokum, 1974; Witty et al., 1986). The nature of this oxygen barrier is considered to be water-filled intercellular spaces in the nodule parenchyma, which would provide 10^4 times the resistance to gas diffusion as would air-filled spaces (Witty et al., 1986). Extracellular matrix proteins in this cell layer are thought to contribute to the barrier by occluding air spaces and limiting cell expansion (van de Wiel et al., 1990; Brewin 1991; James et al., 1991; Rae et al., 1991). Extracellular PNL could serve to increase cell adhesion in this layer by agglutinating cell wall components.

This study constitutes the first report, to our knowledge, of electron microscopic visualization of an extracellular lectin in legumes. Analysis of root exudates and immunolocalization studies at the light microscopic level have indicated the presence of vegetative lectins on root surfaces of several species of legumes (e.g. Gade et al., 1983; Sherwood et al., 1984; Diaz et al., 1986) and within cell walls of stems and leaves in *Dolichos biflorus* (Etzler et al., 1984). However, Gade et al. (1983) presented evidence that extracellular lectin that could be eluted from roots of soybean seedlings was being mobilized from an intracellular location, and Vodkin and Raikhel (1986) demonstrated by immunofluorescence that the bulk of soybean root lectin was contained within epidermal cell organelles thought to be vacuoles. Also, more recent work employing immunoelectron microscopy has indicated that the vegetative *Dolichos* lectin is found within vacuoles, and that the earlier extracellular localization was in error, probably due to nonspecificity of the antiserum used for the original study (Bunker and Etzler, 1994). Thus, the occurrence of a secreted form of a phytohemagglutinin-type lectin has been called into question. In the current study, the data suggest that the extracellular location of lectin is authentic because it occurs in a specific cell layer known to be secreting extracellular matrix proteins. Furthermore, the specificity with which PNL is recognized on western blots indicates that PNL is likely to be the only protein detected by immunoelectron microscopy.

Targeting of proteins to plant vacuoles has been reviewed

recently (Chrispeels and Raikhel, 1992; Vitale and Chrispeels, 1992; Nakamura and Matsuoka, 1993). To enter the secretory pathway, proteins require a signal sequence, which is cleaved as the protein enters the ER. Targeting to the vacuole requires additional positive sorting information at the N or C terminus of plant vacuolar proteins; site-specific alteration or deletion of the vacuolar targeting sequence results in secretion of the protein.

Certain plant hydrolases have been found to exist in both vacuolar and secreted isoforms produced by separate genes. Among enzymes that have a dual localization, the secreted isoforms have been found to lack the vacuolar targeting information (Chrispeels and Raikhel, 1992; Vitale and Chrispeels, 1992). PNL is composed of one major and several minor isoforms (Law et al., 1991); it is possible that one of the minor isoforms represents the secreted form of PNL. These isoforms may represent different gene products. Alternative explanations would involve tissue-specific processing of transcripts from a single gene or masking of the vacuolar targeting sequence, resulting in differential targeting.

Although the accumulation of lectin within peanut nodules appears unique among legumes studied to date, involvement of lectins in mediating early symbiotic interactions between *(Brady)rhizobium* and legume roots has been theorized for more than two decades. This topic has been extensively reviewed (e.g. Dazzo and Truchet, 1983; Kijne, 1992). Lectins may increase the numbers of invasive rhizobia at infectible sites by agglutinating the bacteria via cell surface haptens (e.g. Kijne et al., 1988). Root lectins have also been touted as a possible factor in determining the host-specificity of infection by homologous rhizobia (Bohlool and Schmidt, 1974). The best evidence for involvement of lectin in regulating specificity of the interaction comes from experiments with pea lectin. *Rhizobium leguminosarum* bv *viciae* is the symbiont of pea and cannot normally infect clover. However, expression of pea seed lectin in transgenic clover enables *R. leguminosarum* bv *viciae* to infect and induce nodules on clover hairy roots at a low frequency (Diaz et al., 1989). This result indicates a possible role for pea lectin in determining the host-specificity of infection by *R. leguminosarum* bv *viceae*. To date, analogous experiments for other leguminous hosts have not been published.

Although the current study investigated only mature nodules, the discovery that lectin is secreted within peanut nodules prompts speculation that secreted lectin might also mediate early interactions between *Bradyrhizobium* and peanut roots. However, published studies disagree on the ability of PSL to bind cultured *Bradyrhizobium* strains that nodulate peanut (Bhagwat and Thomas, 1980; Pueppke et al., 1980). PNL, with the same sugar-binding specificity as the seed lectin, has not been found to bind cultured bacteria (Law et al., 1990) or *Bradyrhizobium* bacteroids (Law et al., 1990; this study). Furthermore, PNL is first detectable 2 weeks after inoculation (Kishinevsky et al., 1988; Law et al., 1990), well after infection and the onset of nodule development have taken place (Chandler, 1978). Therefore, the sum of the data from the current study and previous work indicates that evidence is lacking thus far to support a role for Gal-specific lectin in mediating early symbiotic interactions in peanut.

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