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Localization of peanut (*Arachis hypogaea*) root lectin (PRA II) on root surface and its biological significance

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The glucose-specific peanut root lectin, PRA II, is localized on the surface of 7-day-old peanut seedling root and in root cortical parenchymatous cells. The lectin is eluted from intact roots upon washing with buffer containing glucose. Rabbit erythrocytes bind to the root surface and the cortical cells; the binding is inhibited by antibodies raised against PRA II, peanut-specific *Rhizobium* cells and by glucose. Lipopolysaccharides isolated from host-specific *Rhizobium* strain inhibit the haemagglutinating activity of PRA II and are precipitated by the lectin. Our results suggest that PRA II might be involved in recognition of *Rhizobium* by peanut roots.

Keywords: root lectin, peanut, lipopolysaccharides, symbiosis, *Rhizobium*

Introduction

Rhizobium infects, nodulates, and enters a nitrogen-fixing symbiosis with roots of various leguminous plants. The host-*Rhizobium* interaction that results in nodulation is very specific and the host-specificity is expressed prior to Rhizobium infection mostly through root hairs or through cracks that appear at the site of emergence of root hairs as in the peanut [1]. Legume root hairs are elongated epidermal root cells that differentiate from epidermis early in seedling ontogeny and occur in discrete regions of primary and secondary roots.

Lectins have been isolated from the vegetative parts of legumes, such as roots, leaves, barks, bulbs, and rhizomes in addition to seeds [2]. Root lectins of leguminous plants have gained importance, and are currently attracting attention as mediators of symbiosis between nitrogen fixing microorganisms, primarily rhizobia and their host plant.

Several lectins from roots of leguminous plants have been isolated and characterized [3–6]. To assign their role in the host-*Rhizobium* recognition process, it is a prerequisite to establish their presence on the root surface at the time of infection. The presence of a lectin on the root surface of *Trifolium repens* [7] and *Phaseolus vulgaris* [8] has been reported by other workers.

Our studies on lectin activities in developing peanut roots have been reported earlier. Purification and characterization of PRA II from root extracts of 7-day-old peanut seedlings were described recently [9, 10] from this laboratory. In the present report the involvement of PRA II in the host-*Rhizobium* recognition process is discussed.

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Materials and methods

Peanut seeds (*Arachis hypogaea* L, cv. NFG 7) and rhizobial strains (IGR 92, RCL 4, M 10, S 3) were obtained from the Indian Agricultural Research Institute, New Delhi, India. Sephadex G-50 and Sepharose 4B were from Pharmacia Fine Chemicals, Sweden. Coomassie brilliant blue R-250, acrylamide, *N*, *N'*-methylene bis acrylamide, ammonium persulfate, TEMED, 2-mercaptoethanol, silver nitrate, agarose and 3-deoxyoctulosonic acid (KDO) were from Sigma Chemical Co., USA. Protein molecular weight kits were from Bio-Rad. The bicinchoninic acid protein assay reagent kit was from Pierce, USA. Jensen's medium was purchased from Hi-media, India. All other reagents were of analytical grade.

Germination of seeds Peanut seeds were surface sterilized and grown in a nitrogen free Jensen's medium [9].

Erythrocyte binding to roots and cortical cells Roots from 1- to 7-day-old seedlings were thoroughly washed with distilled water and then with 10 mM Tris-HCl, pH 7.2, 0.15 M NaCl (TBS) containing 1.0 mM $MnCl_2$. The roots were then incubated with a rabbit erythrocyte (R-RBC) suspension (2%) in TBS containing 1.0 mM $MnCl_2$ in petri dishes at 30°C for 30 min. The roots were finally washed with TBS and observed under the phase contrast microscope and photographed. Binding of R-RBC to root cortical cells (hand cut sections) was also observed and photographed under the same conditions. To study the inhibition of R-RBC binding, peanut roots and their cross sections were preincubated with either 0.1 M glucose, 0.1 M cellobiose, or a suspension (2×10^8 cells per ml) of the host-specific *Rhizobium* (IGR 92) for 20 min at 30°C, washed

with TBS containing 1.0 mM $MnCl_2$ before incubating with R-RBC. Erythrocyte binding inhibition studies with the antiserum raised against the purified PRA II were carried out using a serum protein concentration of 19.0 mg ml^{-1}.

Elution of lectin from intact roots Intact roots of seedlings at different ages were thoroughly washed with distilled water and then with TBS. These were suspended in TBS containing 0.1 M glucose and 5.0 mM EDTA in a conical flask and allowed to shake in a New Brunswick Scientific environmental shaker (series 25D) at 30°C, 50 rpm for 2 h. The suspension was filtered through glass wool and dialysed against TBS containing 1.0 mM $MnCl_2$. This dialysate was designated as 'root washing'. Lectin activity in the root washings was assayed by haemagglutination.

Purification of root lectins Lectins from the root extract and the root washings of 7-day-old peanut seedlings were purified by affinity chromatography using Sephadex G-50 [9].

Agglutination assays Haemagglutination assays of the root washings and purified lectin were carried out using a 2% rabbit erythrocyte suspension (final concentration) in a total volume of 40 μl in TBS containing 1.0 mM $MnCl_2$ at 30°C (unless otherwise mentioned) for 20 min. Agglutination of *Rhizobium* cells was also investigated under the same conditions using freshly grown cells collected from mid-log phase at a 1×10^6 cells per ml concentration in the assay mixture. The haemagglutination titre is defined as the reciprocal of the highest dilution that gives complete agglutination of erythrocytes. One haemagglutinating unit (HU) is defined as the minimum amount of protein required for 100% agglutination of erythrocytes. Haemagglutination inhibition assays were performed under the same conditions except that the lectin was preincubated with sugar or LPS (lipopolysaccharide) at 30°C for 20 min; the concentration at which 50% inhibition of the agglutination occurred was taken as the minimum inhibitory concentration.

Effects of temperature, pH and metal ions The effects of temperature and pH on haemagglutinating activity of the root surface lectin and its divalent metal ion requirement were determined [9].

Rhizobium culture *Rhizobium* strains, specific to peanut (IGR 92) and non-specific [soybean (S 3), mung (M 10) and clover (RCL 4)] were grown as described [9]. Cells were harvested and used for agglutination and for LPS isolation, after washing with Tris-buffer saline.

Isolation of LPS from Rhizobium strains LPS from different *Rhizobium* strains were extracted by the hot water-phenol method [11]. LPS isolated from strain IGR 92 were fractionated by gel filtration chromatography, using a Sepharose 4B column (0.9 × 65 cm) equilibrated in TBS buffer containing 0.1% Triton X. Two ml fractions were collected at a flow rate of 16.0 ml h^{-1}.

The carbohydrate content of LPS isolated from different *Rhizobium* strains was estimated by the orcinol-sulfuric acid method [12] using glucose as standard; the 3-deoxyoctulosonic acid (KDO) content was determined by the thiobarbituric acid test [13], using commercially available KDO as standard.

Polyacrylamide gel electrophoresis (PAGE) Native (7.5%) and denaturing sodium dodecyl sulfate (SDS) polyacrylamide gel (10%) electrophoresis of proteins were performed according to the methods of Davis [14] and Laemmli [15], respectively, using Tris-glycine (pH 8.53) as electrode buffer. SDS-PAGE was carried in the same electrode buffer containing 0.1% SDS (w/v). The samples were dissolved in the sample buffer containing 2% SDS (w/v) in the presence or absence of 2.5% 2-mercaptoethanol (v/v) and heated at 100°C for 5 min before being loaded onto the gel. SDS-PAGE (7%) of LPS was carried out in the same electrode buffer and the gels were stained and destained following the procedure of Hitchcock and Brown [16].

Immunochemical methods Antisera against the root extract of 7-day-old seedlings and PRA II were raised in rabbits [9] and Ouchterlony immunodiffusion was carried out as described [17].

Agar diffusion gel The precipitin reaction of alkali treated (0.25 N NaOH at 56°C for 1 h) LPS with the root surface lectin was carried out in 0.7% (w/v) agar gels prepared in 0.1 M barbital buffer, pH 8.6 [18].

Determinations of carbohydrate and protein The total neutral sugar content of the lectin was determined by the phenol-sulfuric acid method [19] using D-glucose as standard. Protein was assayed by the bicinconinic acid assay [20] using bovine serum albumin as standard.

Results

Rabbit erythrocytes bind to peanut root hairs, preferentially at the junction of primary and secondary roots from where root hairs originate copiously (Fig. 1a). Binding of R-RBC to a certain population of parenchymatous cortical cells in cross-sections of roots was also observed (Fig. 2a). Binding of R-RBC to roots (Fig. 1b) and root cortical cells (Fig. 2b) was inhibited by the peanut-specific *Rhizobium* IGR 92 but not by non-specific *Rhizobium strains* i.e. soybean (S 3), clover (RCL 4) and mung (M 10). Similar inhibitions of R-RBC binding were also observed with glucose, cellobiose and the antibody raised against PRA II.

Washings of the intact hairy roots (4- to 7-day-old) agglutinate R-RBC and IGR 92 cells but not the non-specific *Rhizobium* cells such as, S 3, M 10, and RCL 4.

The lectin in the 7-day-old root washing binds to the Sephadex G-50 affinity matrix in the presence of Mn^{2+} ions

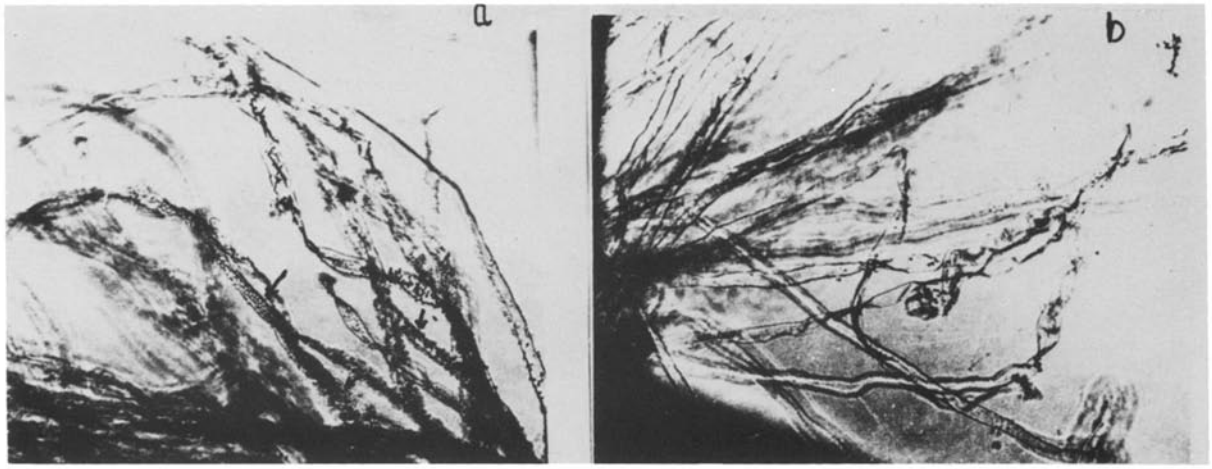

Figure 1. Rabbit erythrocyte binding to the intact roots of 7-day-old peanut seedling with and without preincubation with host-specific *Rhizobium* IGR 92. a) An abundance of bound erythrocytes is seen at the sites from where the root hairs emerge (×20). **b)** Inhibition of R-RBC binding to the root hairs after preincubation with peanut-specific *Rhizobium* strain IGR 92 as described under Materials and methods.

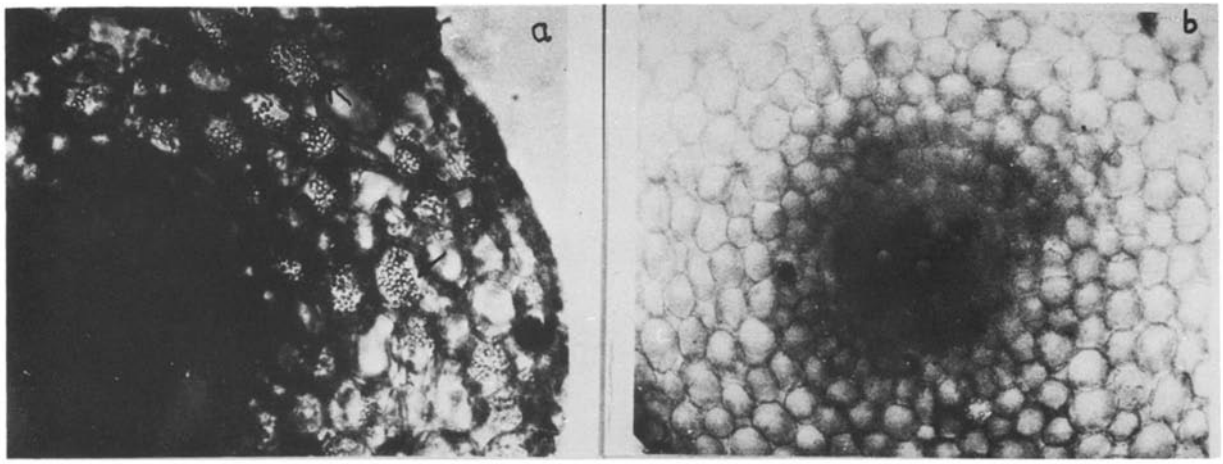

Figure 2. Cross-sections of the 7-day-old seedling roots incubated with R-RBC with and without preincubation with peanut-specific *Rhizobium* IGR 92. a) R-RBC binding to the root cortical parenchymatous cells. Binding of R-RBC is not observed in all the cortical cells (×40). **b)** inhibition of R-RBC binding to the parenchymatous cells of the cortex after preincubation with peanut-specific *Rhizobium* IGR 92 (×20) as described under Materials and methods.

and the bound lectin is eluted as a single protein peak with buffer containing glucose and EDTA (results not shown). More than 80% of the lectin from the root washing was recovered by affinity chromatography.

Electrophoretic mobilities of lectin preparations from 7-day-old washings and the extract were compared in 7.5% native-PAGE (Fig. 3) and 10% denaturing SDS-PAGE (Fig. 4). Both preparations migrate identically in native-PAGE as well as in SDS-PAGE. The subunit molecular weight was 33 000. The purified root surface lectin was also found to be a glycoprotein containing 10% carbohydrate.

The antiserum raised against PRA II reacted to give single precipitin arcs with both purified preparations of the lectin in Ouchterlony immunodiffusion gel (Fig. 5). This antiserum did not react with PRA I and PNA (Results not shown).

Both the lectin preparations agglutinated the peanut-specific *Rhizobium* strain IGR 92 but not the non-specific strains such as soybean (S 3), clover (RCL 4) and mung (M 10). The purified lectin preparations also showed identical divalent metal ions (Mn^{2+}) requirement, pH (6–8.5), and temperature (27–40°C) optima.

Lipopolysaccharides of IGR 92 separate into three fractions when fractionated by Sepharose 4B gel filtration chromatography. Fraction I containing 63% of the loaded LPS was eluted immediately after the void volume. The remainder was eluted in two separate fractions (results not shown). In denaturing SDS-PAGE (Fig. 6), Fraction I moved as a single band and gave single precipitin band with PRA II in agar diffusion gel as shown in Fig. 7. However, unfractionated LPS of *Rhizobium* strain IGR 92 also precipitated as a single band (results not

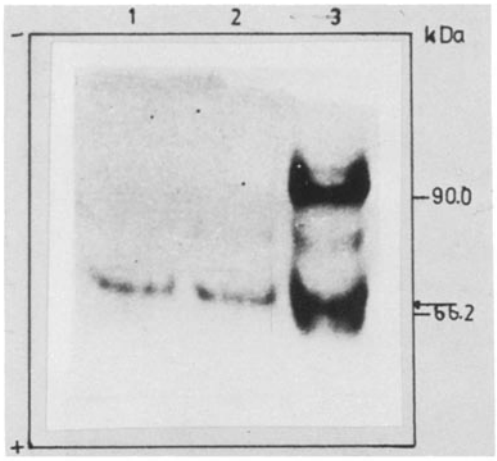

Figure 3. Native-PAGE of purified lectin preparations. Lane 1, lectin preparation from 7-day-old root washings (15.0 μg); lane 2, PRA II (18.5 μg); and lane 3, marker proteins (transferin, 90 kDa; bovine serum albumin, 66.2 kDa). Electrophoretic details are as given under Materials and methods.

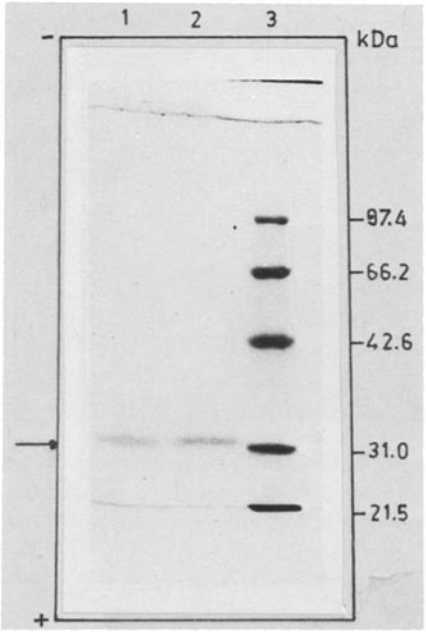

Figure 4. SDS-PAGE (10%) of PRA II and lectin purified from the root washings. Lane 1, lectin from the root washing (18.0 μg); lane 2, PRA II (20.0 μg); lane 3, molecular mass marker proteins (soybean trypsin inhibitor, 21.5 kDa; bovine carbonic anhydrase 31.0 kDa; hen egg white albumin, 42.6 kDa; bovine serum albumin, 66.2 kDa; phosphorylase b, 97.4 kDa). Conditions of electrophoresis are as given under Materials and methods.

shown). Lipopolysaccharides extracted from non-specific *Rhizobium* strains (S 3, RCL 4, M 10) were not precipitated by this root lectin. Peanut seed lectin (PNA) did not precipitate LPS isolated from specific or non-specific *Rhizobium* strains.

In Table 1 the effects of some sugars on the haemagglutinating activity of the root surface lectin are shown. The effects of

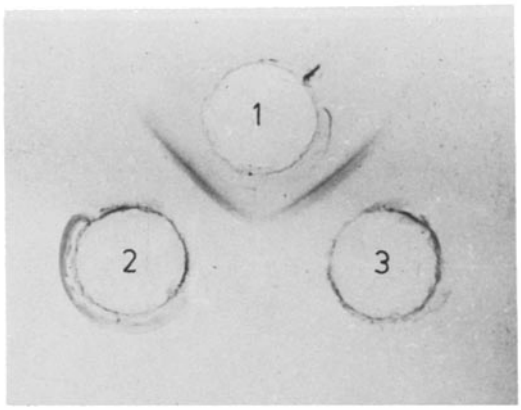

Figure 5. Immunodiffusion pattern of PRA II and lectin purified from root washings with the antiserum raised against PRA II. Well 1, antiserum; well 2, PRA II (13.5 μg); well 3, lectin from root washings (15.0 μg).

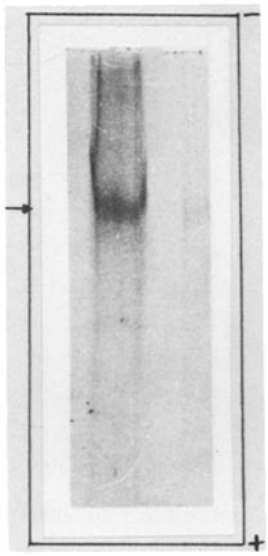

Figure 6. Mobility of *Rhizobium* IGR 92-LPS (16.0 μg) fraction I as single band in 7% SDS-PAGE. Electrophoresis was carried as described under Materials and methods.

other sugars [ref. 9] on this lectin were also found to be identical to that on PRA II. Glucose is the most potent monosaccharide inhibitor whereas cellobiose is the best disaccharide inhibitor of both root lectins. The inhibitory potency of fractionated (Fraction I) IGR 92-LPS is nearly 100-fold higher (Table 1) than glucose. The difference between inhibitory potencies of fractionated and unfractionated IGR 92-LPS is less than two-fold. Peanut seed lectin was not inhibited by these LPS fractions even at a concentration of 100 μg ml^{-1}. Cell wall LPS of non-specific *Rhizobium* strains (S 3, RCL 4, M 10) did not inhibit the haemagglutinating activity of these root lectins even at a 150-fold higher concentration than IGR 92-LPS.

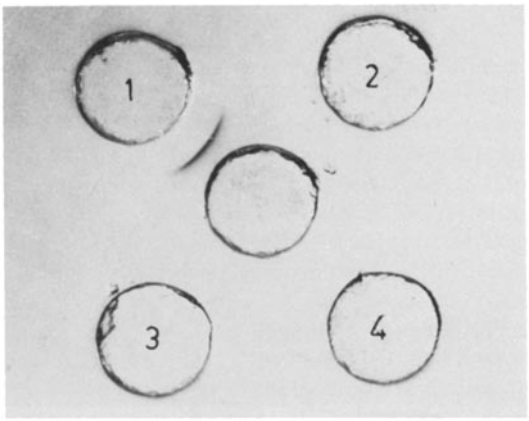

Figure 7. Double diffusion pattern of the lectin prepared from root washings with total LPS of host-specific and non-specific *Rhizobium* strains in 0.8% (w/v) agarose gel. Central well, lectin (11.5 μg); well 1, LPS from *Rhizobium* IGR 92 (3.2 μg); well 2, LPS from *Rhizobium* RCL 4 (3.8 μg); well 3, LPS from *Rhizobium* M 10 (3.6 μg); well 4, LPS from *Rhizobium* S 3 (3.4 μg). Experimental details are given in Materials and methods.

Table 1. Concentration of different sugars and LPS giving 50% inhibition of haemagglutinating activity of peanut root lectin purified from 7-day-old root washings

Inhibitor	*Concentration (mM) giving 50% inhibition of 4 HA units*	*Relative[a] inhibitory potency*
D-Glucose	0.19 (36 μg ml^{-1})	1.00
D-Mannose	0.38	0.50
D-Fructose	0.76	0.25
N-Acetyl-D-Glucosamine	25.00	<0.01
Cellobiose	0.38	0.50
Maltose	0.76	0.25
Sucrose	1.52	0.12
LPS from *Rhizobium* IGR 92	0.53 μg ml^{-1}	68.00
Sepharose 4B fraction I of LPS from *Rhizobium* IGR 92	0.39 μg ml^{-1}	92.00
LPS from *Rhizobium* S 3	170.00 μg ml^{-1}	0.21
LPS from *Rhizobium* M 10	183.00 μg ml^{-1}	0.19
LPS from *Rhizobium* RCL 4	97.00b μg ml^{-1}	

[a] The inhibitory potency of D-glucose is taken as reference and is arbitrarily fixed as 1.0.

[b] Non-inhibitory at the indicated concentration.

D-Gal and L-Fucose were found to be non-inhibitory at a final concentration of 100.0 mM.

LPS concentration is expressed in terms of their carbohydrate content as described in Methods and materials.

Discussion

Binding of R-RBC to the peanut root surface and root cortical cells, and inhibition of this binding by specific sugars such as glucose, cellobiose and also by peanut-specific *Rhizobium* indicate that the binding observed is specific. Elution of lectin (PRA II) from the intact root by glucose or cellobiose and inhibition of RBC binding to the root surface and root cortical cells by the antiserum raised against PRA II suggests that the lectin is present on the root surface and the binding is mediated through the lectin. Similar binding of human RBC to the roots of *Phaseolus vulgaris* L. [8] and elution of the lectin trifolin from the roots of *Trifolium repens* by 2-deoxy-glucose [21] were reported earlier. Agglutination of R-RBC and host-specific *Rhizobium* by lectin eluted from the intact legume roots suggest that the lectin is anchored to the root surface and is also available for interaction with the host-specific bacteria. Preferential binding of RBC at the sites of emerged root hairs might be due to the availability of more lectin that oozes out through the cracks from cortical cells.

The identical mobility of PRA II and root surface lectin in native-PAGE indicate their same surface charge and hydrodynamic properties. In SDS-PAGE the identical mobility of these lectins corresponds to the same subunit molecular weight of 33 000.

The formation of a single precipitin band by the purified root lectin with the antiserum raised against PRA II (Fig. 5) and non-reactivity of this antiserum with PNA and PRA I [22] indicate that both preparations are immunochemically identical and distinct from PRA I or PNA.

The carbohydrate-specificity of the lectin purified from 7-day-old root washings is identical to that of PRA II. Like PRA II this is also a novel lectin among glucose/mannose specific lectins [23–25] as the inhibitory potency of glucose is higher than mannose and it prefers β-linked sugars over α-linked. Thus these two root lectins of 7-day-old peanut seedlings are identical in all investigated respects but differ from other reported peanut lectins [26–28].

One species of LPS predominates in IGR 92 cells. The reactions of root lectin with peanut-specific and non-specific LPS (Table 1 and Fig. 7) indicate that lectin-LPS interaction is specific and *Rhizobium* binding to peanut root surface is mediated through this interaction. The true ligands for the root surface lectin are the oligosaccharides of the bacterial cell wall LPS but not the mono- or disaccharides. Recognition of carbohydrate ligands of cell wall LPS of specific *Rhizobium* by the host root lectin of *R. trifolii* [29], *R. japonicum* [30] and *R. leguminosarum* [31] has also been reported earlier. Host-*Rhizobium* recognition through lectin-carbohydrate interaction in the peanut may be the crucial step that is needed to start the two-way signalling between plant and bacterium for the coordinated expression of bacterial *nod* genes. These *nod* genes, in turn, encode enzymes involved in the synthesis of diffusible lipo-oligosaccharides, *nod* factors that determine the host specificity [32]. All these oligosaccharides are oligomers of *N*-acetylglucosamine, with different host-specific modifications.

Further work is necessary to identify the specific carbohydrate ligand on the bacterial surface that binds to this peanut root lectin. The involvement of the root lectin in host-

Rhizobiuim recognition is also evident from the nodulation in transgenic pea plants by clover-specific *Rhizobium* on introduction of the trifoliin gene in pea roots [33].

The appearance of root lectin, PRA II, with the emergence of root hairs in peanut seedlings indicate that this lectin may be one of the key factors in determining the host specificity, and it is expressed prior to *Rhizobium* infection.

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