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The diversity of lectin-detectable sugar residues on root hair tips of selected legumes correlates with the diversity of their host ranges for rhizobia

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Summary. Four legumes and two nonlegumes were investigated for the presence of sugars at the tips of their root hairs, using commercially available lectins which have specific affinities for certain sugars. It was found that while only one lectin (RCA-I, which binds to β-D-galactose) bound to narrow-host-range legumes and one nonlegume, five out of ten lectins tested bound to the root hair tips of the broad-host-range legume siratro (*Macroptilium atropurpureum*). None of the lectins tested bound to any part of *Arabidopsis* roots. Binding of lectins (and therefore the presence of sugars) only at the tips of growing root hairs has led us to deduce that the sugars are the glyco moiety of membrane-bound glycoproteins that are recycled at the base of the tip apical dome, along with excess plasma membrane that is known to be recycled there. As many kinds of signal transduction molecules are membrane-bound glycoproteins, we suggest that these sugars may be involved in early interactions with rhizobia, and that the broad-host-range legume siratro has more kinds of sugars to cope with the wide range of rhizobia it is able to accept for symbiotic interactions. As far as we know, this is the first report of multiple sugars at the same surface area of a tip-growing plant cell.

Keywords: Glycoproteins; Host specificity; Lectins; Legumes; Root hairs.

Introduction

The *Rhizobium*–legume symbiosis occurs in specific relationships between host and symbiont, and indeed most rhizobia taxonomy is based on host specificity towards legumes (Gallon and Chaplin 1987). *Rhizobium* strains either have broad or narrow host range. For example, *Rhizobium leguminosarum* bv. *trifolii* can only nodulate plants of genus *Trifolium* whereas *Rhizobium* sp. NGR234 nodulates over 70 legume genera and also the nonlegume *Parasponia* (Dénarié and Cullimore 1993, Geurts and Franssen 1996). This host specificity is known to be determined at a very early stage of plant–microbe interactions (Diaz et al. 1989).

Conversely, it is known that plants too have a broad or narrow range of rhizobia by which they can be infected. A legume with one of the broadest host ranges known is siratro (*Macroptilium atropurpureum*), which will accept and nodulate with many kinds of rhizobia (including NGR234), whereas narrow-host-range legumes such as alfalfa or *Trifolium* accept only a single strain of rhizobia.

In the early stages of infection between rhizobia and legume, an exchange of signals occurs, where plant-secreted flavonoids induce the expression of a nodulation (Nod) factor expressed by rhizobia (Lerouge et al. 1990, Dénarié and Cullimore 1993, Geurts and Franssen 1996). Nod factors are lipo-oligosaccharide molecules with various acyl chains and terminal sugars that decorate it and define specific interactions with specific legumes, and therefore are in part responsible for host specificity. However, the molecule which Nod factors and/or other molecules from rhizobia interact with at the plant surface (root hair surface) is still unknown, though it is assumed to be a signal transduction molecule because rhizobia or purified Nod factors are able to set off a cascade of developmental and morphological events in the root hair (Hirsch 1992, Kijne 1992, Ardourel et al. 1994). This initial interaction thus represents the "black box" of rhizobia-legume associations, and is under inten-

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sive study. It is also known that rhizobial polysaccharides may play a specific role in the infection process (see Rolfe et al. 1996).

We have used fluorophore-labelled (FITC) lectins to investigate the surface of legume root hairs to determine any surface sugar profile that may exist. A lectin is a glycoprotein, glycolipid, or protein of nonimmune origin, which is capable of specific recognition of, and reversible binding to, the carbohydrate moieties of complex polysaccharides (Werner 1992, Sumar et al. 1993). Lectins are also useful tools for detecting the surface carbohydrate profile of any cells, because they bind to a specific carbohydrate (hapten sugar). Lectin binding is sugar specific, and it is defined in terms of monosaccharides or simple oligosaccharides which are able to inhibit lectin-induced agglutination, precipitation, or aggregation (Diaz et al. 1989, Tsuigi 1993).

Our hypothesis is as follows. It is known that some kinds of signal transduction molecules are membrane-bound, usually glycoproteins, with a protein moiety bound to the membrane and a carbohydrate (glyco) moiety free at the external surface (see Alberts et al. 1994). If there are specific sugar molecules at the surface of the tips of legume root hairs, this may lead us to suspect that such molecules are involved in interaction with external molecules. The surface carbohydrates of both plants and bacteria are possible molecules that function in the recognition process and there is reported to be a range of sugars wide enough to provide sufficient diversity (Dixon and Wheeler 1986). Ridge and Rolfe (1986) reported an investigation of the carbohydrates present on the surface of the tropical legume siratro (*Macroptilium atropurpureum*) roots, using fluorescent isothiocyanate(FITC)-labeled lectins. They observed that the lectin from *Ricinus communis* (RCA-I), which specifically recognizes and binds to β-D-galactose, bound to the root hair tips of siratro, indicating the presence of the specific carbohydrate. They also observed that pre-incubation of siratro roots with RCA-I lectin inhibited infection and nodulation by *Rhizobium* sp. The possibility of β-D-galactose functioning as a target molecule for *Rhizobium* for siratro–*Rhizobium* sp. interaction was suggested from this experiment.

We report here that there is in fact a range of sugars at the tips of the broad-host-range legume siratro, but only a limited range on narrow-host-range legumes. When we count only those lectins that bind to sugars found just at the tips, then we have found only one lectin that binds to the tips of narrow-host-range legumes, and five to the tips of siratro. We believe this finding has important implications for host specificity.

Material and methods

Plant culture

Plant material

White clover (*Trifolium repens*), red clover (*T. pratense*), and siratro (*Macroptilium atropurpureum*) were obtained from Australia; alfalfa (*Medicago sativa*) was obtained from Shirayuki Seed Co. Ltd.; *Arabidopsis* was obtained from the Arabidopsis Genome Center at the Ohio State University, U.S.A.; and *Lafanus sativus* (daikon, or large white radish, a plant from the brassica family) was obtained from the Tohoku Seed Co., Japan.

Plant germination and growth

All plants were germinated and grown on solid Fahraeus (1957) media (F-media), prepared with deionized water and sterilized by autoclaving at 120 °C for 20 min.

Plant seed preparation

White clover, red clover, alfalfa, *Lafanus*, *Arabidopsis*. The seeds were treated with 99.5% ethanol for 10 min in an Erlenmeyer flask (Eppendorf microtubes for *Arabidopsis*) to remove the waxy coating on the surface of the seeds, and were then rinsed thoroughly with sterilized deionized water (SDW). The seeds were surface-sterilized with 6% sodium hypochlorite for 10 min, rinsed with SDW 5–6 times, left to soak in SDW for 10 min, and then placed on F-agar plates. Plates were wrapped in aluminium foil. The seeds were germinated in a 20 °C growth chamber for approximately 24 h in an upright position. After 24 h of germination, seedlings of a few millimeter in length were transferred aseptically onto a new F-agar plate, about 10 seeds per plate, wrapped in aluminium foil and placed upright in a 20 °C growth chamber for another 24 h. For *Arabidopsis* the plates were placed upright at 22 °C, in 18 h light and 6 h dark for 2–3 days.

Siratro. The seeds were treated with sulphuric acid for approximately 10 min in an Erlenmeyer flask and were rinsed with water several

Table 1. Lectins used

Lectin (abbreviation)	Hapten sugar
Arachis hypogaea (PNA)	β-Gal(1-3)GalNAc
Bandeiraea simplicifolia (BS-I)	α-D-galactose, methyl-α-D-galactopyranoside
B. simplicifolia (BS-II)	D-GlcNAc, α-D-glucose
Erythrina corallodendron (ECN)	N-acetylgalactosamine, methyl-α-D-galactopyranoside
Glycine max (SBA)	α-D-GalNAc, methyl-α-D-galactopyranoside
Maclura pomifera (MPA)	α-D-galactose, GalNAc
Limulus polyphemus (Limulin)	Fetuin, NeuNAc
Ricinus communis (RCA-I)	β-D-galactose
Triticum vulgaris (WGA)	N,N′,N′′-triacetylchitotriose
Vicia faba (VFA)	α-D-mannose

times. The seeds were then surface-sterilized with 6% sodium hypochlorite for 15 min and were washed 5–6 times with sterile distilled water (SDW). The seeds were left to soak in SDW for approximately 10 min and were then placed on an F-agar plate. The seeds were covered with 0.7% water agar to keep them in place. The rest of the procedures were the same as that of white clover described above except for an incubation temperature of 28 °C.

Lectin preparation

All lectins (Table 1) were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.) and were supplied labeled with fluorescein isothiocyanate (FITC).

All lectin solutions were prepared with phosphate-buffered saline (PBS). 12.25 ml of solution A (below), 12.75 ml of solution B, and 4.5 g of NaCl were mixed with distilled water (DW) to make 500 ml 0.01 M 0.9% NaCl solution at pH 6.8. The solution was stored at 4 °C.

Solution A, $Na_2HPO_4 \cdot 12H_2O$, 35.82 g per 500 ml DW;

Solution B, $NaH_2PO_4 \cdot 2H_2O$, 15.605 g per 500 ml DW.

Each lectin was diluted to 200 mg/ml stock with PBS, divided into

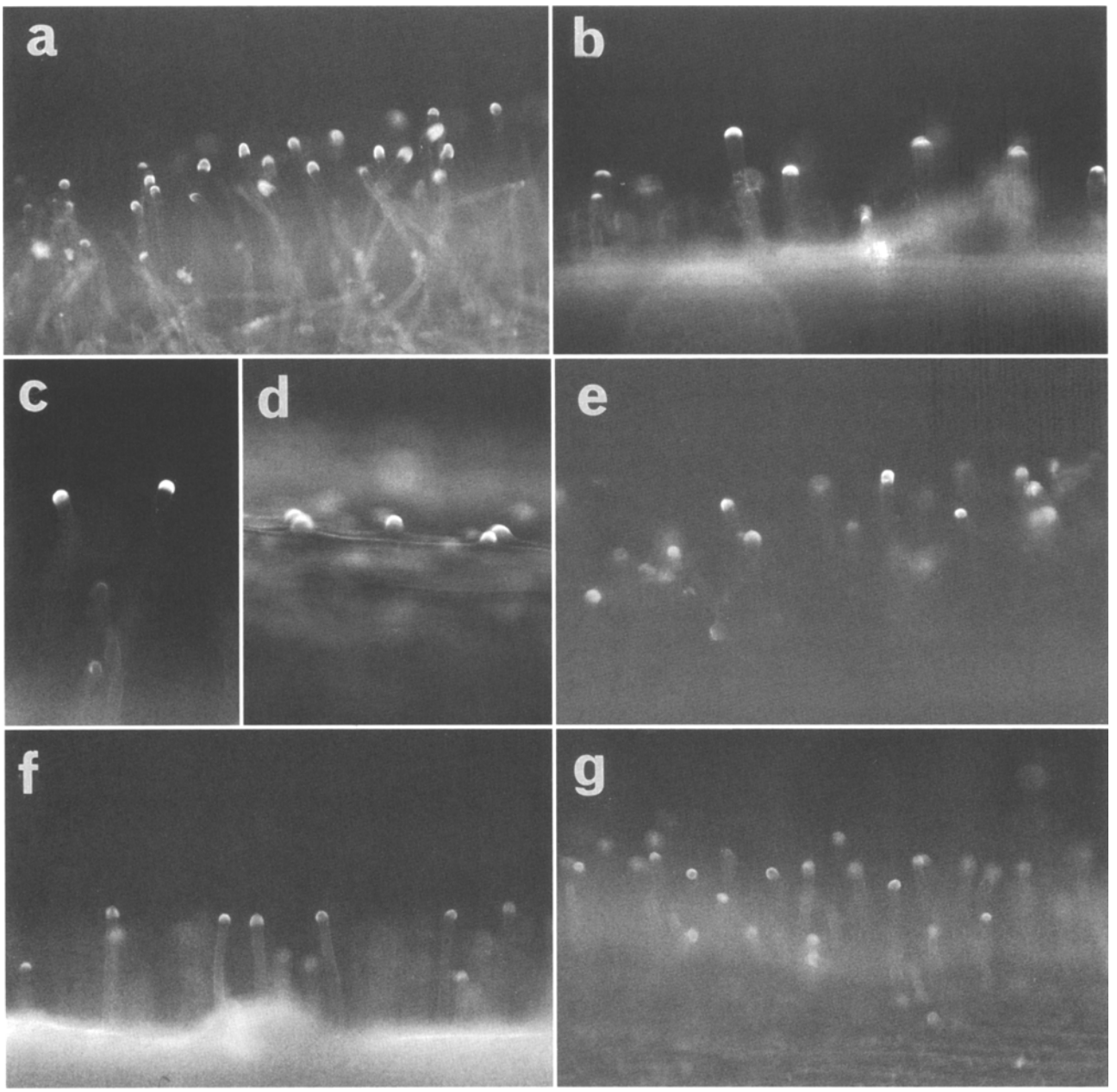

Fig. 1 a–g. Lectin binding to root hairs of siratro. Emerging (**d**) and actively growing hairs are shown. **a** and **c** Labelled with MPA; **b** and **d** labelled with RCA-1; **e** labelled with PNA; **f** labelled with VFA; **g** labelled with WGA. All hairs are approximately 10 μm in diameter

0.3 ml aliquots in Eppendorf tubes, and maintained at –30 °C, except RCA-I and WGA which were maintained at 4 °C.

Lectin-binding test

Each stock lectin was diluted to 12.0 μg/ml working solution by mixing stock solution with PBS in an Eppendorf micro test tube. Prepared plants were incubated in lectin solution for 1 h in the dark at room temperature (20–22 °C) and were then washed three times with PBS, 10 min each wash. Control experiments were: (i) incubation of each lectin with its hapten sugar (1 mM, 10 mM, and 20 mM concentrations were tested) before application to the plant tissue, (ii) incubation with a solution of FITC fluorophore only, (iii) incubation with an FITC-conjugated antibody against mouse IgG.

Fluorescence microscope observation

After lectin incubation, plants were mounted in PBS on a microscope slide. Materials were observed with a mercury vapor source and filters to provide excitation of FITC, using an Olympus Vanox-S AH-2RFL. Samples were photographed with Ektachrome 400 daylight diapositive film; these slides were subsequently copied onto black and white Ilford FP4-plus negative film and printed.

Results

When sample legume roots were incubated in FITC-labeled lectins for 1 h, the binding of lectins was observed as a bright fluorescence on the root hair tips of sample plants (Figs. 1 and 2). A summary of the results of the lectin-binding test with 10 kinds of lectins is shown in Table 2.

All control experiments resulted in no binding. Preincubation with lectins and their hapten sugars at various concentrations (1–20 mM) completely prevented binding of the lectins to the root hair tips (data not shown, as micrographs of negative results are completely black). Incubation with FITC alone or a mouse IgG-FITC antibody resulted in no binding.

With the exception of VFA on two plants, those lectins that bound to root hairs bound to the tip region only. Binding was observed for both mature and growing root hairs, including emerging root hairs (Fig. 1 d, siratro with RCA-1). For VFA on white clover (Fig. 2 e) and *Lafanus*, binding occurred both

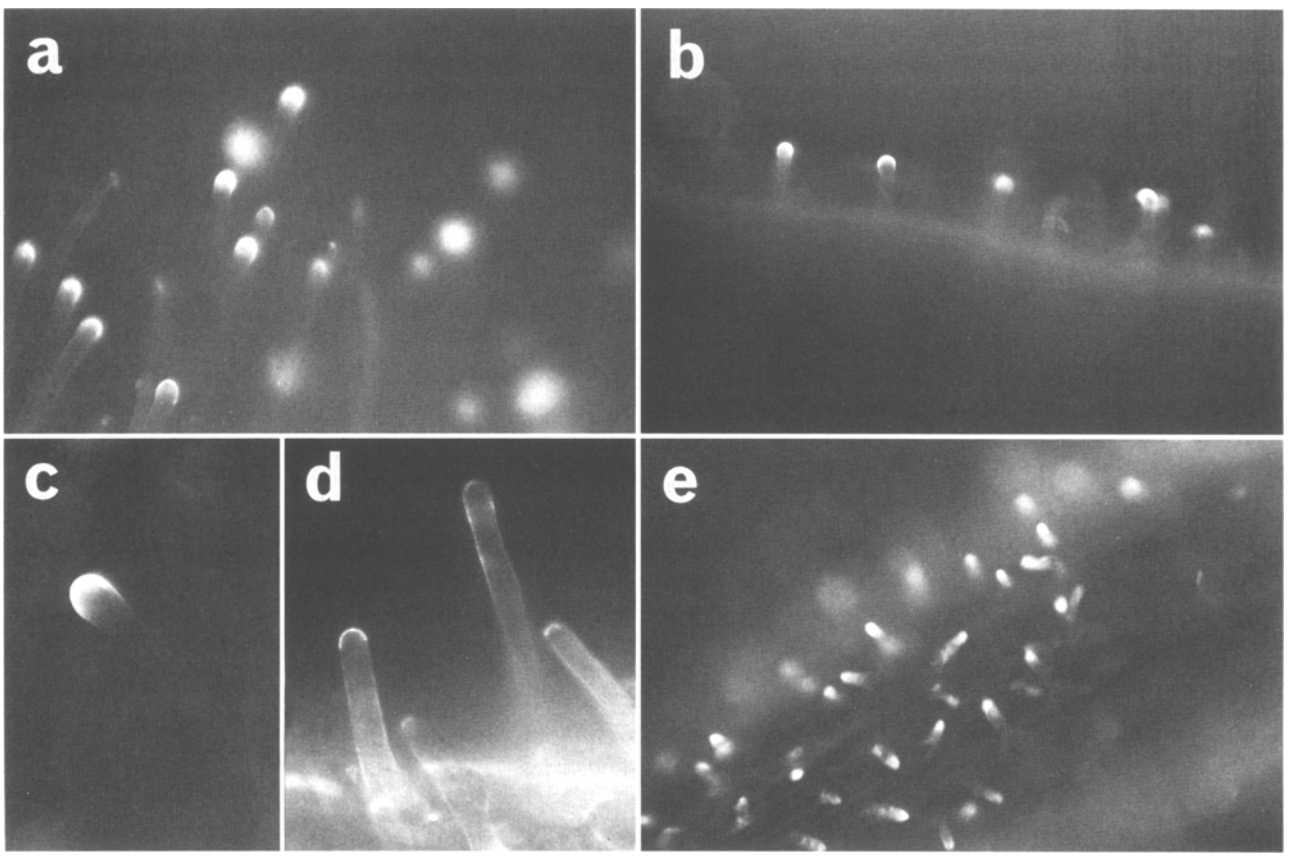

Fig. 2 a–e. Lectin binding to root hairs of various legumes and a nonlegume. **a** and **c** *Lafanus* labelled with RCA-1; **b** alfalfa labelled with RCA-1; **d** white clover labelled with RCA-1 (note the comparatively weaker labelling); **e** white clover labelled with VFA, note how the labelling persists down the tube of the hair compared to all the other illustrations

Table 2. Lectin binding to root hair tips

Plants	Lectins									
	SBA	BS-I	BS-II	ECN	PNA	MPA	RCA-I	VFA	WGA	Lim
Legumes										
white clover	–	–	–	–	–	–	+	+E	–	–
red clover	–	–	–	–	–	–	+	–	–	ND
alfalfa	–	–	–	–	–	–	+	–	–	–
siratro	–	–	–	–	+	+	+	+	+	–
Nonlegumes										
Lafanus	–	–	–	–	–	–	+	+E	–	–
Arabidopsis	–	–	–	–	–	–	–	–	–	–

+ Positive; – negative; *ND* not tested; *E* extended staining proximal to the tip

at the tip and along the tube of the hair, the strength of binding fading with distance from the tip of about 3–5 root hair diameters down the tube of the hair from the tip (up to 60 μm). However, VFA bound only to the root hair tips of siratro (Fig. 1 f). For all other legumes tested and *Lafanus*, root hair tip binding was only achieved by RCA-1. None of the ten lectins tested bound to any part of *Arabidopsis* roots.

While the intensity of binding varied with some legumes (patchy or weak for RCA on white clover, e.g., Fig. 2 d) positive binding was very clear compared to negative binding which showed no fluorescent binding at all. Doubling the concentration of RCA-1 on white clover did not increase binding, and we conclude that the presence of this lectin's hapten sugar β-D-galactose may be small on white clover compared to the other positive tested plants. The *Maclura pomifera* lectin (MPA) gave the brightest binding of all lectins tested at the 12 μg/ml concentration of working solution (Fig. 1 a, c). Halving the concentration of working solution for MPA on siratro made no difference to the brightness of binding, and we conclude that one or both of this lectin's hapten sugars (α-D-galactose, GalNAc) are prominently featured on this plant's root hair tips.

No other parts of the plant root showed any prominent labelling with the lectins tested. However, we did not use lectins with binding ability to fucose, known to be a prominent sugar secreted by the root cap.

Discussion

The results show that various kinds of lectins, all with specific affinities for different kinds of sugars, bind to the root hair tips of some legumes, and to no other parts of the root. In some cases this binding to root hair tips was found to be quite spectacular (as MPA on siratro, Fig. 1 a, c). Binding of RCA-1 to white clover was weak (Fig. 2 d) but still considered positive compared to negative controls.

The lectin RCA-I, which has specific affinity for β-D-galactose, bound to all wild-type legumes tested and a species of the brassica family, *Lafanus*. However, RCA-I did not bind to *Arabidopsis* root hair tips. Although the positive binding of lectins to root hair tips was confined to the apical dome, one lectin, VFA, bound to the tip as well as to the tube of the hair, the binding gradually decreasing to zero to a distance of about 3–5 root hair diameters down the tube of the hair from the tip (up to 60 μm). The implications of these results are discussed as follows.

Sugar molecules at the surface of the root hair apical dome

As far as we know, this is the first report of multiple sugars at the same surface area of a plant cell. The lectins may be binding to either a single large molecule with multiple sugar sites, or different molecules with single sugar sites, or variations thereof. The presence of sugars only at the apical dome of the root hair, which is a tip-growing cell, suggests that there is a transient expression of the sugars at the tip, otherwise they would continue to be present down the tube of the hair. Indeed such a phenomenon does happen, but only with the VFA lectin on white clover and *Lafanus*. There are some logical explanations for these results: It is possible that there is either an endocytotic recycling system in the apical dome for retrieving sugars, or the sugars are rapidly degraded at the base of the apical dome. We favour the former idea, because there is already known to be a large amount of clathrin at the base of the apical dome of

root hairs from freeze-substitution/electron microscopy evidence, and strong evidence for a clathrin-based recycling system for membrane at the base of the apical dome inside the cell (Ridge 1992, 1995). It is very likely therefore that these sugars are recycled to the apex of the dome by a clathrin-based system that recycles membrane. We strongly suggest that membrane-bound molecules, such as glycoproteins, would be recycled with the plasma membrane back into the endo-membrane system and back to the apex via tip growth. Sugars that are excreted or released into the cell wall matrix are much less likely to be recycled, and would be expected to be seen further down the tube of the hair rather than just at the apical dome. Indeed, in the case of the VFA-binding sugar, it appears that at least some of the molecule remains in the cell wall after being brought to the surface of the root hair tip, but as there is strong binding at the tip for this lectin, we consider that this molecule is also recycled at the base of the apical dome.

The RCA-I lectin bound to all wild-type legumes tested and also to a nonlegume (*Lafanus*) and we propose that there is a conserved expression of a basic sugar at the apical dome of many kinds of root hairs. As a nonlegume also showed RCA-I binding, it is possible that the RCA-I affinity sugar β-D-galactose may be a sugar expressed by many kinds of plants at their root hair tips.

Possible role of cell surface sugars in early interactions of rhizobia and legumes

The results presented here come as the first piece of hard evidence for receptors at the root hair tip. As a number of authors have mentioned (Hirsch 1992; Ridge 1992, 1995; Geurts and Franssen 1996) the root hair membrane is the first cell to encounter Nod factors, and that a receptor, if one exists, is likely to be present in this position. Plant root lectins have been suggested to be a Nod factor receptor, but they lack a transmembrane domain which is necessary for function as a receptor. If some of the sugars at the surface of root hair tips emanate from membrane-bound glycoproteins, then they may be possible receptor(s) for the onset of the symbiosis. Indeed, if one includes the argument above that these molecules are recycled, then it is very likely that they are part of a molecule that is embedded in the plasma membrane of the cell. Thus we speculate that rhizobia, Nod factors, or as yet unknown factors could bind directly to one or more of the sugars at the tip of the hair, setting off the transduction events necessary to start the symbiosis.

Of all the tested legumes, only siratro showed binding by various kinds of lectins. Five kinds of lectins, PNA, MPA, RCA-I, VFA, and WGA, bound to the root hair tips of siratro, implying the presence of various sugars at the surface as part of a single molecule or as multi-molecules. This may be related to the fact that siratro is infected by a broad range of rhizobia. All the other legumes tested are each infected by a single strain of rhizobia and these legumes only showed binding by RCA-I at the apical dome of the root hair. It is important that researchers in the field test their various experimental legumes (broad- and narrow-host-range plants; determinate and indeterminate nodules) for lectin-binding to confirm this point and expand the database.

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