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Probing the surfaces of soybean protoplasts and of germ tubes of the soybean pathogen *Phytophthora megasperma* f. sp. *glycinea* with lectins

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Abstract

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Of 11 lectins tested 5 were capable of agglutinating protoplasts from soybean hypocotyls demonstrating the presence of mannose, galactose and N-Acetyl-galactosamine as carbohydrate determinants on the outside of the soybean plasma membrane. There were no quantitative differences in agglutinability among the two pairs of near-isolines tested, Harosoy/Harosoy 63 and Williams/Williams 79, which differ only in resistance against certain races of the soybean pathogen *Phytophthora megasperma* f. sp. *glycinea* (*Pmg*). FITC-Con A attached to germ tubes and even stronger to germ tube tips of different races of *Pmg* and other phytophthoras (*P. cactorum, P. palmivora, P. infestans*) while no attachment was noticed for 3 other lectins (wheat germ agglutinin, peanut, and soybean lectin). Of 9 lectins testet wheat germ agglutinin alone inhibited germ tube growth of all 5 Phytophthora spp. tested (*P. cactorum, P. drechsleri, P. infestans, Pmg,* and *P. palmivora*), and *Pmg* was the most sensitive species being at least partially inhibited by the majority of the other lectins (*Ulex europaeus,* soybean, pea, and to a lesser degree lentil agglutinin).

Introduction

Cell surfaces may be important for host – pathogen interactions (Keen 1982, Daly 1984). When fungal pathogens invade higher plants they encounter several plant surfaces, e.g. cuticle, cell wall and plasma membrane. It is not known which if any of these encounters are essential for recognition and subsequent signal transduction. Since the interaction of the fungus with the host plasma membrane appeared to be a potentially promising site for specific interactions a study was initiated (Hohl and Balsiger 1986) to investigate early reactions between the fungal hypha and protoplasts of host origin. It was shown that certain carbohydrates were capable of preventing adhesion of soybean protoplasts to hyphae of the soybean pathogen *Phytophthora megasperma* f. sp. *glycinea* (*Pmg*). This suggested to us that lectins and their corresponding carbohydrate haptens

might play part in this early phase of host – pathogen interaction. Consequently we have investigated the surfaces of soybean protoplasts from near-isogenic cv. and of germ tubes and cysts of *Pmg* for the presence of lectins and lectin binding carbohydrates. This paper describes the results obtained on lectin binding of these surfaces. In addition the growth inhibiting action of lectins on germinated fungal cysts was also investigated.

Several lines of evidence implicate surface glycoproteins in pollen – stigma and host – pathogen recognition (Albersheim et al. 1975, Wade and Albersheim 1979, Keen 1982, Clarke et al. 1985), even thouth it is largely unknown how these compounds interact to produce signals leading to compatibility or incompatibility of the system. Keen and Legrand (1980) characterized surface glycoproteins from *Pmg* and studied their capacity for race specific phytoalexin elicitation. Yoshikawa et al. (1983) showed specific binding of a fungus-derived glyceollin elicitor by membrane preparations of soybean cells and implicated a protein or glycoprotein as receptor. Ziegler and Pontzen (1982) and Hermanns and Ziegler (1984) demonstrated specific inhibition of glucan elicited glyceollin accumulation in soybeans by an extracellular mannan-glycoprotein of *Pmg*. Furuichi et al. (1980) proposed a role for potato lectins in the binding of germ tubes of *Phytophthora infestans* to potato cell membranes and Nozue et al. (1980) were able to suppress hypersensitivity against the late blight fungus *Phytophthora infestans* with chitobiose, the sugar hapten of the potato lectin. Finally, Kogel et al. (1985) provide evidence that reagents with affinity for wheat plasma membrane galactoconjugates suppress the hypersensitive response in the wheat stem rust interaction.

Lectins have been studied as potential determinants of recognition in plant-microbe (for review see Callow 1977, 1983, Sequeira 1978, Pistole 1981, Reisert 1981) as well as animal cell systems (e.g. Sharon 1979, 1984). Particularly in the *Rhizobium* – legume system the direct participation of specific lectins has been strongly indicated (e.g. Dazzo and Gardiol 1984, Halverson and Stacey 1985). Nordbring-Hertz et al. (1981, 1982) have provided convincing evidence for the participation of lectins in the adhesion of nematodes to nematode trapping fungi. Gibson et al. (1982) found twice as much soybean agglutinin (SBA) in Pmg-resistant compared to susceptible soybean varieties. They further demonstrated the inhibitory action of these lectins against *Pmg*. However, in no case has the specificity of the host – fungus interaction been convincingly attributed to lectins.

Several reports deal with the complementary carbohydrate determinants (detected by e.g. lectin-binding or lectin-mediated agglutination) present on surfaces of plant protoplasts (Larkin 1978, Williamson et al. 1976, Williamson 1979, Chin and Scott 1979, Gruber et al. 1984). Holden and Strange (1984) report a lack of specific interaction of lectins with protoplasts from wheat near-isolines differing in their resistance to *Puccinia graminis* f. sp. *tritici*. However, to gain more precise information on the distribution of lectin-binding sites it is necessary to conduct quantitative studies (Fenton and Labavitch 1980) on lectin binding to protoplasts of plants differing in their resistance to fungal pathogens.

Materials and methods

Host plants. – Soybean [*Glycine max* (L.) Merr.] seedlings of cv. Williams, Williams 79, Harosoy and Harosoy 63 were grown for 5 to 6 d at 23–25 °C in the dark in vermiculite-containing plastic boxes. Barley seeds (*Hordeum vulgare* L.) cv. Gerbel were grown for 8–10 d in 12 cm pots,

at 25 °C during the day and 10 °C at night, with 70% relative humidity and a photoperiod of 12 h. The light source consisted of a PHILIPS HPLR 400 W lamp with a photon flux of 500–600 μmol photons/m². Potatoes (*Solanum tuberosum* L.) cv. Bintje were grown in pots in the greenhouse. The first 3 leaflets of 6 week old plants were used for isolation of protoplasts.

Growth of the pathogen and production of cysts. – The following strains of *Phytophthora* were used: *P. cactorum* (Leb. & Cohn) Schroeter S, *P. drechsleri* Tucker 103,*P. infestans* (Mont.) de Bary 515, *P. palmivora* (Butler) Butler P 113, and *P. megasperma* f. sp. *glycinea* Kuan and Erwin (*Pmg*), races 1 and 6 (the former pathogenic on Harosoy but not on Harosoy 63, the latter pathogenic on Williams but not on Williams 79). Stock cultures of *Pmg* were grown on Borlotti bean agar (30 g of Borlotti beans were autoclaved for 8 min in 0.5 L of distilled water and then filtered through nylon cloth. 15 g of Difco agar were added to the filtrate which was filled up with distilled water to make 1 L and subsequently reautoclaved), of the other phytophthoras on rye-glucose medium (rye 100 g, glucuse 5 g, Difco agar 15 g per L of medium) and transferred at 4-weekly intervals. Production of zoospores and cysts of *Pmg* was done according to Eye et al. (1978) using material pregrown on Lima bean agar which gives high yields of zoospores. The other *Phytophthora* species were grown on rye-glucose medium, flooded with 8 ml of deionized water and then cooled in a refrigerator for about 3 h. Encystment of the liberated zoospores was achieved by shaking the zoospore suspension for 1 min on a Vortex mixer.

Isolating protoplasts. – Hypocotyls were finely sliced (slices appr. 1 mm thick) and the tissue fragments transferred to charcoal-agar plates (agar 200 mg, gelatine 300 mg, activated charcoal 100 mg, in 10 ml of distilled water) flooded with 10 ml of the following isolation medium: 2% cellulase Onozuka R-10 (SERVA, Heidelberg, FRG) and 0.1% pectolyase Y-23 (SEISHIN Pharmaceutical Comp., Noda, Chiba, Japan) in a near-isotonic buffer composed of mannitol 0.4 M, $CaCl_2 \cdot 2H_2O$ 4 mM, MES 10 mM, and adjusted to pH 5.8 with NaOH. The material was incubated for 3 h at 28 °C on a shaker with 50 rpm. Following this the suspension was filtered through a 150 μm nylon mesh and the protoplast suspension further cleaned on a Ficoll step gradient (15 and 5% Ficoll, topped with the mannitol-buffer where the intact protoplasts collected after centrifugation at 180 g for 15 min). The purified protoplasts were washed once with the mannitol-buffer and then counted. Cell viability was assessed with 0.02% methylene blue (Berliner et al. 1978) and fluorescein diacetate (FDA) according to Widholm (1972). About 90% of freshly isolated protoplasts were viable.

For testing lectin-mediated agglutination the protoplasts were finally resuspended in a near-isotonic KCl-buffer (KCl 0.2 M, MES – morpholino ethanol sulfonic acid – 10 mM, and EDTA 2 mM, adjusted to pH 7.0 with 1 N NaOH). The KCl-buffer was designed to prevent unspecific clumping of protoplasts observed to occur in the mannitol-buffer. It is characterized by its high ionic strength, lack of added calcium ions and presence of EDTA. For one experiment the protoplast suspension (mannitol-buffered) was aged for 20 h in a refrigerator at appr. 8 °C.

The following lectins were obtained from SIGMA Corp. and used in the agglutination experiments: Concanavalin A no. L2631 (Con A) from *Canavalia ensiformis,* lentil agglutinin no. L5880 (LCA) from *Lens culinaris,* red kidney bean agglutinin V no. L8754 (PHA V) from *Phaseolus vulgaris,* garden pea agglutinin no. L5380 (PSA) from *Pisum sativum,* castor bean agglutinin I no. L8508 (RCA I) from *Ricinus communis,* peanut agglutinin no. 0881 (PNA) from *Arachis hypogaea,* soybean agglutinin VI no. L8004 (SBA VI) from *Glycine max,* wheat germ agglutinin no. L1005 (WGA) from *Triticum vulgaris,* poke weed agglutinin no. L9379 (PAA) from *Phytolacca americana,* winged pea agglutinin no. L9254 (TPA) from *Tetragonolobus purpureus,* gorse agglutinin I no. L5505 (UEA I) from *Ulex europaeus.*

Agglutination of protoplasts. – 1 drop each of protoplast suspension (50,000 protoplasts/ml in the near-isotonic KCl-buffer) and a lectin solution (1 mg/ml, in KCl-buffer) was mixed on a depression slide and agglutination scored at intervals up to 2 h according to the following scheme: – no agglutination, + 5–25%, + + 26–50%, + + + 51–75%, and + + + + 75–100% agglutinated protoplasts. For controls the agglutination test was carried out after preincubating the lectins in 10 mM of the appropriate sugar hapten: Methyl-α-D-mannopyranoside for Con A, LCA, and PSA; N-Acetyl-α-D-galactosamine (α-D-gal-NAc, galNAc) for RCA and SBA VI; β-D-galactose (β-D-gal, gal) for PNA; α-L-fucose (α-L-fuc, fuc) for UEA.

Inhibition of germ tube growth by lectins. – Cysts were germinated on coverslips in 24 well plastic tissue culture dishes in 0.5 ml of pea broth (75 g of commercial, freshly frozen garden peas were cooked in 250 ml of water and filtered. To this were added 10 g of sucrose, 1 g of asparagine, 0.25 g of $MgSO_4 \cdot 7H_2O$, and 0.5 g of KH_2PO_4 and the medium filled up to 1 L before being autoclaved) for 2 h (1 h for *P. infestans),* at 18 °C for *P. infestans*), at 30 °C for *Pmg* and *P. palmivora,* at 22 °C for the others. The coverslips were then rinsed with 0.01 mol of phosphate buffer at ph 5.8. 0.5 ml of the lectin solutions (500 µg/ml and 50 µg/ml respectively) were added to the germlings on the coverslips and the cultures incubated for another 2 h. The lectin solutions were then removed, the cultures were fixed with 2 drops of 4% paraformaldehyde and the lengths of the germ tubes determined. From each coverslip the lengths of 40 germ tubes were measured microscopically with the aid of a measuring eye piece and compared with those from a control grown in phosphate buffer only.

Lectin-mediated agglutination of germ tubes. – This was attempted with 4 h germlings of *Pmg* using the agglutination test described above, or by incubating the germ tubes with the lectins in Eppendorf tubes on a rotary shaker (50 rpm) followed by periodic microscopic checks up to 2 h.

FITC – lectin labelling of germ tubes. – Cysts of *P. cactorum, Pmg* races 1 and 6, *P. palmivora,* and *P. infestans* were pregrown for 4 h on coverslips in wells and washed with 0.01 M phosphate buffer of pH 6.8. 0.1 ml of FITC-labelled lectin solution (from SIGMA, 1 mg/ml) was added for a total of 30 min. The coverslips were washed three times with phosphate buffer and mounted upside down in a drop of glycerol – phosphate buffer (1 : 1) on a microscope slide. Fluorescence was observed on a ZEISS photomicroscope II equipped with epifluorescence. The following filter combination was used: Excitation wavelengths from 450–490 nm, barrier filter 520–560 nm.

Results

Agglutination of protoplasts with lectins. – Of the 11 lectins tested five agglutinated soybean protoplasts (table 1). Agglutination was noticeable as early as 15 min and maximal after 120 min. It was inhibited in the presence of the corresponding carbohydrate hapten. Mannose and galactose (including galNAc) binding lectins agglutinated soybean protoplasts. In protoplasts aged for 20 h agglutination by mannose-binding lectins was greatly reduced. Also abolished was the agglutinability of aged protoplasts by the peanut agglutinin which binds to galactose residues but not of castor bean and soybean agglutinins which also have gal or galNAc residues as haptens. In comparison potato protoplasts had a diminished affinity for mannose-binding lectins and did not agglutinate with the soybean lectin. Barley protoplasts had no affinity for mannose-binding lectins except for the Pisum lectin but agglutinated strongly with the fucose specific Ulex europaeus agglutinin.

A quantitative comparison of agglutination by end titer determinations revealed that soybean VI agglutinin SBA had the highest and the lentil agglutinin LCA the lowest activity (table 2). There were slight differences between Harosoy and Williams but no differences were observed among the near-isolines Harosoy and Harosoy 63 or Williams and Williams 79, respectively.

Lectin binding sites on germinating cysts of *Pmg.* – Attempts to demonstrate lectin binding by agglutination of cysts or germinated cysts failed. Yet it was possible to demonstrate lectin binding by using FITC-labelled lectins. The results are shown in table 3. Of four lectins tested Con A alone gave positive results and adhered strongly to the germ tubes and hyphae of all the test organisms (*P. cactorum, Pmg* races 1 and 6, *P. palmivora* and *P. infestans*). The tip exhibited stronger fluorescence than the main body of the germ tube.

Inhibition of germ tube growth by lectins. – Results are given in table 4. *Pmg* was the most sensitive of the phytophthoras tested and WGA was the only lectin inhibiting

Table 1. Agglutination (after 90 min) by lectins of potato, barley, and of fresh and aged (20 h) Harosoy protoplasts. Harosoy and Harosoy 63 (results not shown) gave identical results.

Lectin	Sugar hapten(s)	Agglutination of			
		soybean		potato	barley
		fresh	aged	fresh	fresh
Con A	α-D-man, α-D-glc	+ + +	–	+ +	–
LCA	α-D-man, α-D-glc	+ + +	–	–	–
PSA	α-D-man, α-D-glc	–	–	–	+ + + +
RCA I	D-galNAc, β-D-gal	+ + + +	+ + + +	+ + + +	+ + + +
PNA	β-D-gal(1-3)-D-galNAc	+ + + +	–	+ + + +	+ + + +
SBA VI	α-D-galNAc, β-D-galNAc	+ + + +	+ + + +	–	+ + + +
PHA V	β-D-gal-β-D-glcNAc-D-man	–	–	–	–
WGA	$(\beta\text{-D-glcNAc})_2$*	–	–	–	–
PAA	$(\text{D-glcNAc})_3$	–	–	–	–
TPA	α-L-fuc	–	–	–	–
UEA I	α-L-fuc	–	–	+	+ + + +

* chitobiose

Table 2. Minimum concentration of lectins capable of agglutinating protoplasts of soybean (cv. Williams W, Williams 79 W79, Harosoy H and Harosoy 63 H63) and of barley.

Lectin	MW	Sugar hapten**	Soybean cultivars				Barley
	kDa		W	W79	H	H63	
LCA	49	man	500.0*	500.0	125.0	125.0	na
Con A	102	man	125.0	125.0	250.0	250.0	na
SBA VI	110	galNAc	7.8	7.8	3.9	3.9	3.9
PNA	120	gal	31.2	31.2	62.5	62.5	125.0
RCA I	60	gal	31.2	31.2	31.2	31.2	7.8
UEA I	170	fuc	na	na	na	na	125.0

* µg lectin/ml, na no agglutination, ** see table 1 for more detailed list

Table 3. Binding of FITC-labelled lectins to 4 h germ tubes and germ tube tips of *Pmg* races 1 and 6 and other phytophthoras (see results). Except for WGA these lectins agglutinate soybean protoplasts

Lectin	Sugar hapten*	Germ tube	Germ tube tip
Con A	man	+	+ +
WGA	$(\text{glcNAc})_2$	–	–
PNA	gal	–	–
SBA	galNAc	–	–

* see table 1 for more detailed list

Table 4. Inhibition (as percentage of control) by lectins of germ tube growth of several species of *Phytophthora*

Lectin	Sugar hapten**	*Pi*		*Pd*		*Pp*		*Pc*		*Pmg*	
		500*	50	500	50	500	50	500	50	500	50
Con A	man	0	0	–	0	0	0	0	0	0	0
LCA	man	10	0	0	–	23	30	0	15	29	12
PSA	man	0	0	0	–	0	0	0	0	66	51
PNA	gal	0	0	0	–	0	0	13	0	0	0
RCA I	galNAc	–	–	0	–	15	0	0	0	–	–
SBA VI	galNAc	0	0	0	–	0	0	0	0	41	24
WGA	$(glcNAc)_2$	47	18	65	35	75	66	74	62	65	40
UEA I	fuc	18	0	0	–	0	0	0	0	57	52

Pi, P. infestans; Pd, P. drechsleri; Pp, P. palmivora; Pc, P. cactorum; Pmg, P. megasperma f. sp. *glycinea*
* lectin conc. in µg/ml, ** see table 1 for more detailed list
– not tested

all fungal species tested. It is also noteworthy that *Pmg* is the only species inhibited by the soybean agglutinin. In general though, inhibitory concentrations are high and there is only partial inhibition even at these high concentrations. Con A which bound to the fungal surface did not inhibit any of the species tested.

Discussion

It was possible to demonstrate the presence of mannose and galactose (and/or galNAc) residues on freshly prepared soybean protoplasts. Con A binding to soybean protoplasts has been demonstrated before (Williamson et al. 1975). The presence of mostly galactose and mannose moieties on the surface of plant protoplasts appears to be common (e.g. Larkin 1978, Fenton and Labavitch 1980, Gruber et al. 1984). Dissimilarities may also occur. In our test barley protoplasts agglutinated strongly with the fucose specific UEA while soybean did not.

No quantitative differences in lectin-mediated agglutination were dectected among the two pairs of near-isolines Harosoy (H and H63) and Williams (W and W79) respectively. Therefore, at present the differences in resistance observed among these isolines against *Pmg* (race 6 with W and W79 and race 1 with H and H63 respectively) cannot be attributed to any qualitative or quantitative differences in lectin binding of the host plasma membrane. It remains to be determined whether or not such differences exist at or in the host cell walls. Holden and Strange (1984) also report a lack of specific interaction of lectins with protoplasts from wheat near-isolines differing in their resistance to *Puccinia graminis* f. sp. *tritici.*

However, there were noticeable interspecific differences of lectin-binding. Fucose could only be detected at the cell surface of barley and to a minor degree of potato but not at all of soybean. Mannose and galactose carbohydrates were detected in all three species but different lectins bound protoplasts to varying degrees. Agglutination of soybean protoplasts by the peanut agglutinin, by the lentil agglutinin, and by Con A was

lost upon ageing of the protoplasts while that of castor bean agglutinin was not. This points to the possible existence of two different galactose-containing sites on the protoplast surface. Furthermore, while Con A and LCA, both mannose binding lectins were capable of agglutinating soybean protoplasts these two lectins did not agglutinate barley protoplasts which are agglutinated, however, by PSA, another mannose-binding lectin. Obviously the specificities of these lectins are not identical even though they have common affinities to major haptens. In addition their binding properties may be modulated by the environment in which the major hapten is located.

We did not observe proper lectin-mediated agglutination of germlings of *Pmg.* However, FITC-Con A positively stained germ tube surfaces with apparently more intense staining of the tip region indicating the presence of mannose and/or glucose haptens. Interestingly, WGA and some of the other lectins inhibiting germ tube growth apparently did not bind to the wall surfaces. In addition, the two different races of *Pmg* and the other phytophthoras tested gave very similar results indicating a lack of carbohydrate specificity on these surfaces. However, this does not preclude the possibility that more refined methods might reveal more subtle differences among the races and species.

The observation that good binding to germ tube or hyphal walls and especially to the tip region of *Phytophthora* spp. is only obtained with Con A has been made before on *Pmg* (Keen and Legrand 1980, Hermanns and Ziegler 1982) and other *Phytophthora* species (Sing and Bartnicki-Garcia 1975 on *P. palmivora,* Galun et al. 1976, and Barkai-Golan et al. 1978 on *P. citrophthora*). All races of *Pmg* tested bound FITC-Con A (Keen and Legrand 1980). Barkai-Golan et al. 1978 noted positive staining of *P. citrophthora* with FITC-Con A, moderate staining with FITC-SBA (galNAc, gal), and none with FITC-PNA (gal) and FITC-WGA (glcNAc), the latter having been reported before (Galun et al. 1976). Furthermore, Keen and Legrand (1980) found only glucose and mannose in the glycoproteins isolated and purified from cell walls of *Pmg.* Whether or not infection structures of *Phytophthora* display a more varied surface carbohydrate pattern remains to be determined. Mendgen et al. (1985) made a study of surface carbohydrates of *Puccinia coronata* and *Uromyces appendiculatus.* Using FITC-labelled lectins and glucanases they found different distribution patterns on germ tubes, appressoria and substomatal vesicles indicating pronounced variations in surface properties of these infection structures.

Of the lectins tested WGA proved to be the most toxic to all phytophthoras. The fact that it inhibited germ tube growth of all 5 test strains to similar degrees indicates that a lectin of this type could not be responsible for host specificity. WGA also inhibits growth of other fungi (Mirelman et al. 1975). *Pmg* was the most sensitive of the species tested. It is also partially inhibited by the soybean agglutinin as already noticed by Gibson et al. (1982), and it is the only species affected by this lectin. SBA could thus play some role in general resistance in this system, a notion supported by the observation of Gibson et al. (1982) that resistant soybean varieties contained twice as much soybean agglutinin (SBA) as susceptible ones.

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