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Investigations into the occurrence of plant cell surface epitopes in exudate gums

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A panel of monoclonal antibodies, derived for the specific recognition of developmentally regulated epitopes of the plasma membranes and cell walls of plant cells, has been characterized in relation to binding activities with samples of exudate gums. The monoclonal antibodies are shown to recognize specifically structural features present in four exudate gums—arabic, karaya, ghatti and tragacanth. Dialysis of the gums and competitive inhibitor ELISAs indicated that the monoclonal antibodies recognize both high and low molecular weight components of each gum. The spatial relationships, and possible overlap, of the arabinogalactan epitopes on the most antigenic gum, gum arabic, have been explored in more detail by means of an epitope mapping technique.

INTRODUCTION

The derivation and use of monoclonal antibodies recognizing plant cell surface components has led to the identification of two classes of developmentally regulated cell surface glycoproteins. These are the plasma membrane-associated arabinogalactan proteins (AGPs) (Knox *et al.*, 1991) and the cell wall hydroxyproline-rich glycoproteins (HRGPs) (Smallwood *et al.*, 1994*a*). The patterns of the restricted occurrence of both sets of glycoprotein epitopes in plant structures closely reflect aspects of developmental plant anatomy.

Importantly, both sets of monoclonal antibodies recognize carbohydrate containing epitopes as determined by the sensitivity of binding to periodate treatment of antigens and, in certain cases (Knox, 1993; Yates & Knox, unpublished), the ability of monosaccharides to act as hapten inhibitors of antibody binding. It has proved difficult to determine the structure of the epitopes, and the precise structures recognized by these antibodies are yet to be determined.

During the derivation of monoclonal antibodies to components of plant tissues the reactivity of antibodies with gum arabic has been used as a diagnostic for certain classes of antibody, most notably anti-arabinogalactan (Knox *et al.*, 1989; Pennell *et al.*, 1989; Smallwood *et al.*, 1994*a*).

The exudate gums are extremely complex mixtures containing complex acidic polysaccharide and protein structures (Osman *et al.*, 1993). The origin and content of commercially available exudate gum samples are often largely unknown and any particular sample is likely to contain gums from different species (e.g. of *Acacias* in gum arabic). In addition, the exact composition of a gum sample derived from one particular species may vary with season of harvest or geographical location.

Gum arabic and gum mesquite contain antigenic carbohydrate structural features and have been used to raise antisera (Miskiel & Pazur, 1991). Monoclonal antibodies, with their capacity for the exquisite discrimination of related structures, offer the opportunity to provide precise probes for the antigenic features of complex gums (presumed to be oligosaccharide structural elements) and, most importantly, the capacity for the ultimate identification of those elements. In addition, antibodies recognizing defined structures contained within gums will also be useful for the analysis of structure–function relationships of gum components, for testing gum sample authenticity and for deriving taxonomic relationships between gum producing species.

This report provides initial immunochemical characteristics of the reactivity of the two groups of monoclonal antibodies (anti-AGP and anti-wall HRGP) with commercial samples of four exudate gums—arabic, ghatti, karaya and tragacanth. Competitive hapten/

antigen inhibition studies using enzyme-linked immunosorbent assays (ELISAs) demonstrate the presence of epitopes to many of these antibodies in both high and low molecular weight components from each gum. These studies point towards the exudate gums as a possible source of oligosaccharide fragments for the full structural characterization of the epitopes. One important question concerns the structural relationships of the related, but distinct, epitopes within the gums. This is addressed in the case of the arabinogalactan epitopes in the most strongly reacting gum, gum arabic, with the development of a method of epitope mapping involving the use of antibodies as competitive inhibitors of binding of a biotinylated antibody.

MATERIALS AND METHODS

Chemicals and materials

Gum arabic (G9752), gum karaya (G0503), gum ghatti (G0378), gum tragacanth (G1128) gum guar (G4129) and locust bean gum (G0753) were purchased from Sigma. All gums were used unpurified for the immunochemical assays. Gums were dissolved in distilled water or phosphate-buffered saline (PBS). *N*-Hydroxysuccinimide-DL-desthiobiotin ester and the detecting conjugates for anti-rat immunoglobulin IgG (whole molecule)-peroxidase and anti-biotin-peroxidase were obtained from Sigma. Nunc Immunosorb microtitre plates (GIBCO) were used for all ELISA assays.

Dialysed gum fractions were prepared by dialysis with a 3500 kDa cut off membrane (Spectra Medical Ind., Texas) against distilled water (3 × 1 litre). Solutions were reduced by rotary evaporation.

Monoclonal antibodies

The monoclonal antibodies used in this report have been characterized in relation to their binding to cell surface glycoproteins in cell-suspension cultures and root apices of carrot and rice. They were derived after immunization procedures involving complex cell or extracellular material. They fall into two groups. Group 1 (anti-AGP) monoclonal antibodies are MAC207 (Pennell *et al.*, 1989), JIM4 (Knox *et al.*, 1989), JIM13, JIM14, JIM15 and JIM16 (Knox *et al.*, 1991) and LM2 (Smallwood *et al.*, 1994*b*). Group 2 (anti-wall HRGP) are JIM11, JIM12 and JIM20 (Smallwood *et al.*, 1994*a*) and LM1 (Smallwood *et al.*, 1994*b*). The anti-pectin monoclonal antibodies JIM5 and JIM7 (Knox *et al.*, 1990) were used as controls. Abbreviated forms of the antibody names are used in some of the figures. All antibodies are rat monoclonal antibodies and were used unpurified from hybridoma supernatants unless otherwise stated.

ELISAs

Microtitre plates were coated in gum arabic to provide the immobilized antigen by incubation of the wells with 100 μl gum arabic at 1 mg/ml in PBS for 18 h at 4°C. The gum arabic solution was removed and the wells were then incubated with bovine serum albumin (BSA) (3% (w/v), 200 μl per well, 1 h, room temperature (RT)) in PBS to block any unbound sites on the plates. All antibody hybridoma supernatants were titrated against the gum arabic to determine the dilution that would provide 90% maximal binding. Anti-rat-immunoglobulin (whole molecule) linked to peroxidase (2000-fold dilution in 3% (w/v) BSA in PBS) was added to the plates (100 μl per well) and incubated (2 h, RT). The plates were washed thoroughly in tap water and excess water was removed. The peroxidase substrate (18 ml water, 2 ml, 1·0 M sodium acetate buffer pH 6·0, 200 μl of tetramethyl benzidene (10% (w/v) in dimethyl sulphoxide (DMSO)) and 20μl of hydrogen peroxide (6% (v/v)) was added to the plate (150 μl per well) and the reaction was allowed to develop. The reaction was stopped by the addition of 30 μl/well 2 M sulphuric acid, and the absorbance was determined at 450 nm. The selected dilution was used to provide the maximum sensitivity in the subsequent competitive assays.

Competitive inhibition ELISAs

The inhibitor (soluble antigen/hapten) in each case was titrated down the plate with a 5-fold dilution series (50 μl per well) and the antibodies (50 μl of each in 3% (w/v) BSA in PBS) were applied to give the selected 90% maximal binding in the absence of inhibitor. The plate was agitated immediately after the addition of the antibodies and incubated (2 h, RT). The plates were then thoroughly washed in tap water and residual water was removed. Antibody binding to the immobilized antigen was determined as described above.

Arabinogalactan epitope mapping with biotinylated antibodies

Biotinylation procedure

The anti-arabinogalactan monoclonal antibodies were purified from hybridoma supernatants by precipitation at 50% (w/v) saturated ammonium sulphate. The precipitate was pelleted by centrifugation (1000 *g*, 30 min) and dissolved in PBS and dialysed extensively against PBS, and the protein concentration was determined. The protein concentration in each case was adjusted to 1 mg/ml in PBS. The immunoglobulin proteins were biotinylated by mixing 1·0 mg of *N*-hydroxysuccinimide-DL-desthiobiotin ester (in 40 μl of DMSO) with 1·0 mg of antibody for 1·5 h, followed by dialysis against PBS to remove any unconjugated activated biotin.

Epitope mapping assay

A 5-fold dilution series of each biotinylated antibody (in 3% (w/v) BSA in PBS) was titrated on a plate coated with gum arabic and blocked with BSA. The plate was incubated (1·5 h, RT) followed by washes and the reporting second antibody (anti-biotin-peroxidase) was applied to the plate (100 μl per well in 3% (w/v) BSA in PBS) at a dilution dependent upon the batch and manufacturer's instructions and incubated (1·5 h, RT). The plates were developed with peroxidase substrate as described above. The lowest concentration of each biotinylated antibody which gave a signal at least twice that of the background was selected. Preliminary experiments (data not shown) had shown that, although higher concentrations of biotinylated antibodies could be employed, better sensitivity to inhibition could be achieved with these lower levels. The optimal dilutions of 1 mg/ml stocks of the biotinylated antibodies were as follows: 250-fold for all antibodies, except MAC207 (500-fold) and JIM13 (25 000-fold).

Microtitre plates were prepared with gum arabic as immobilized antigen as described above. In this case the nonbiotinylated antibodies (Ab A) were to be used as possible inhibitors of binding of the biotinylated antibodies (Ab B) by binding to the immobilized antigen and possible common epitopes. Each Ab A was applied to the plate in excess (5-fold dilution from hybridoma supernatant, in 3% (w/v) BSA in PBS, 50 μl per well) and incubated (1·5 h, RT). The biotinylated antibody was then added at the appropriate concentration (50 μl/well in 3% BSA in PBS). The plates were agitated to allow thorough mixing, and the binding was allowed to continue for 1·5 h. After the usual washing procedures anti-biotin-peroxidase and peroxidase substrate were supplied as described above. These epitope mapping assays were performed on six replicate plates and the absorbance values were determined three times for each plate and averaged. (See Fig. 3 for a diagram indicating how the epitope mapping results were determined.)

RESULTS AND DISCUSSION

Degree of inhibition of binding of each antibody by each intact gum

In initial experiments, anti-plant cell surface antibodies were titrated against six gums as immobilized antigens in ELISAs and were found to be significantly reactive with the four exudate gums, but not with the seed gums—gum guar or locust beam gum (data not shown). The ultimate aim of our investigations is the quantification and characterization of epitope components. This can be more readily done in competitive inhibitor ELISAs in which the antibody binding component is in the form of a soluble nonimmobilized antigen/hapten inhibitor of binding. This system of competitive inhibition assays also avoids the probable differential attachment or nonattachment of gum components to the plastic surface of the microtitre plates. It is difficult to know what proportion of coating antigen sticks to a plate, and the competitive ELISA allows precise comparision of inhibitory activities and quantification. All of the anti-glycoprotein antibodies showed some reaction with gum arabic and this gum was used as the immobilized antigen in all experiments. Figure 1 shows the reaction of 11 monoclonal antibodies with gum arabic as the immobilized antigen at 90% of maximal binding used for the competitive inhibitor experiments. The anti-pectin monoclonal antibodies JIM5 and JIM7 were unreactive with gum arabic (Fig. 1).

Using gum arabic as an immobilized antigen the soluble components of each of the four exudate gums were then used as inhibitors of antibody binding to gum arabic. This method is capable of providing a more accurate indication of antibody-reactive components in each gum. Figure 2 shows the capacity of the intact gums (= the total unpurified water soluble components) of arabic, ghatti, karaya and tragacanth to act as inhibitors of the binding of the 11 antibodies to gum arabic.

All four gums are inhibitory of all the antibodies tested in this assay—but to different extents. The anti-AG MAC207, JIM15 and LM2 appear as the most reactive towards all four gums. The anti-HRGP antibodies, as a group, were most reactive with gum ghatti and karaya. The binding of the anti-AG antibodies to the four gums provides a similar profile of relative reactivities in each case with the exception of the lower reactivities of JIM4 and JIM13 towards gum karaya and tragacanth. It is possible that the reactivity of a panel of monoclonal antibodies with gums in this way may provide distinctive 'fingerprints' of component epitopes that may be useful in gum characterization.

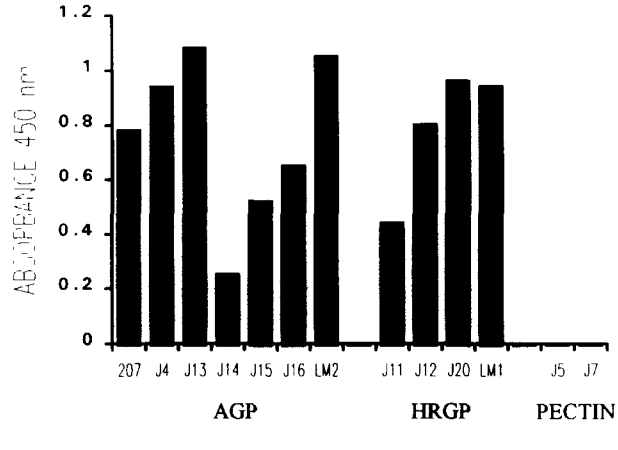

Fig. 1. Reactivity of monoclonal antibodies with gum arabic as an immobilized antigen as determined by ELISA. The antibody hybridoma supernatant was diluted in each case to provide 90% of maximal binding.

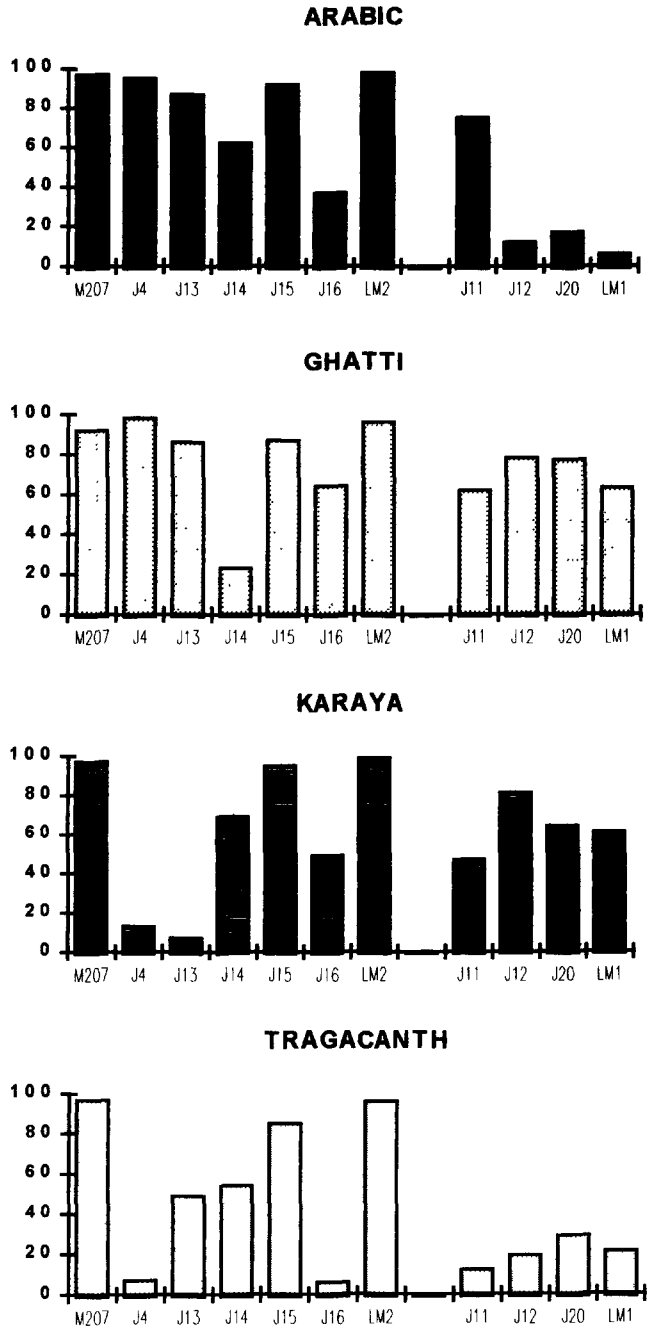

Fig. 2. Monoclonal antibody reactivity with exudate gums. Activity of intact exudate gums (at 1 mg/ml) as soluble competitive inhibitors of antibody binding. Results expressed as a percentage of maximum binding (in absence of soluble antigen) to immobilized gum arabic.

The differences in the patterns of reactivity between Fig. 1 (gum arabic as immobilized antigen) and Fig. 2(a) (gum arabic as soluble inhibitor antigen) may be explained by two possible factors. The first is that the competitive assay may detect components that did not attach to the plate in Fig. 1 experiments. Such components could be oligosaccharides, which would not adhere efficiently to the plastic surface of the plate wells. The possibility of the existence of small molecular weight components is addressed below. The second complicating factor is that the reactive components in the gum supplied as soluble inhibitor may bind to components in the immobilized gum arabic. If this were the case it would have the effect of lowering the percentage inhibition and thus the apparent reactivity of the 'intact gum'. In no case did a soluble gum in a competitive inhibition assay increase the antibody binding above that of the 'no inhibitor' control. The possibility of such gum–gum interactions was not addressed further in this study.

Dialysis of gums reveals low and high molecular weight gum fractions which are reactive with the antibodies

The inhibitor reactivity of the intact gum arabic did not always correlate closely with reactivities indicated by binding to gum arabic as immobilized gum. For example, JIM14 appeared to be more reactive than expected and the anti-wall HRGP antibodies JIM12, JIM20 and LM2 were less so (Fig. 2). This, as discussed above, indicated possible reactive soluble components that were contributing to reactivity, but were not immobilized on the microtitre plate. Dialysis of the four gums against distilled water was found to produce low molecular weight components that were in some instances, when supplied at an equivalent concentration, as reactive with the antibody as gum components that failed to pass through the 3500 cut-off dialysis membrane.

The reactivity of the two fractions of each gum are shown in Table 1. Great variation was observed, within the antibodies and the gums, in the <3500 fractions to inhibit antibody binding. The anti-arabinogalactan antibodies were more readily inhibited by the <3500 fractions although gum ghatti appears as an effective source of small molecular weight components capable of reaction with the anti-HRGP JIM12, JIM20 and LM1.

Epitope mapping—degree of binding of biotinylated antibody in presence of saturating levels of nonbiotinylated antibodies

In order to approach questions concerning the location of epitopes on the surface of the antigen, a method was devised which allowed the differential detection of a probing (biotinylated) antibody (Ab B), while excess of nonbiotinylated antibody (Ab A) was present as a potential blocker of the available epitopes. Thus, by combining, in turn, each biotinylated antibody with each nonbiotinylated antibody (and using anti-biotin-peroxidase for antibody detection) a picture of epitope relationship and possible overlap can be established.

The results of such an approach to mapping the

Table 1. Reactivity of gum fractions prepared by dialysis in competitive inhibition ELISAs

Gum	Antibody										
	Group 1							Group 2			
	MAC207	JIM4	JIM13	JIM14	JIM15	JIM16	LM2	JIM11	JIM12	JIM20	LM1
A i	36	27	21	90	66	6	22	1	10	6	9
A ii	100	100	99	100	94	100	100	100	97	98	100
G i	98	31	23	68	74	13	96	3	42	39	100
G ii	98	100	100	18	100	84	100	100	58	100	79
K i	58	1	12	50	39	100	6	0	1	6	19
K ii	100	86	86	100	100	100	100	100	67	48	74
T i	23	23	100	0	56	13	66	0	0	0	2
T ii	100	100	100	100	100	100	100	100	93	91	86

Results expressed as percentage inhibition relative to intact gum at equivalent concentration. For each gum i = < 3·5 kDa fraction and ii = > 3·5 kDa fraction.

Table 2. Epitope mapping of the arabinogalactan epitopes on gum arabic. The activity of a range of antibodies to bind to immobilized gum arabic and prevent the binding of a specific antibody (detected by biotinylation-detection system) expressed as percentage of inhibition

Ab B	(Ab A) Excess, nonbiotinylated antibodies								
	MAC207	JIM4	JIM13	JIM14	JIM15	JIM16	LM2	JIM12	LM1
MAC207	37	63	61	28	37	33	47	0	13
JIM4	100	88	78	32	77	34	78	0	0
JIM13	0	45	49	29	68	79	42	0	0
JIM14	100	100	95	59	92	87	83	0	0
JIM15	100	100	67	92	86	97	97	0	0
JIM16	100	93	88	89	87	87	83	0	0
LM2	100	100	100	100	95	86	90	0	0

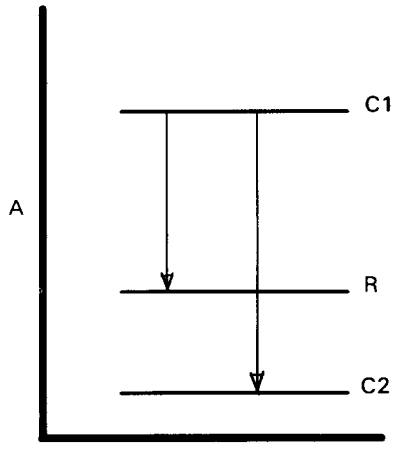

Fig. 3. Experimental design of the arabinogalactan epitope mapping experiment. Schematic representation of ELISA measurements and controls used for the calculation of the percentage inhibition of biotinylated antibodies of non-biotinylated antibodies. C1 = Binding of biotinylated antibody (Ab B) only, 0% inhibition. C2 = Unlabelled antibody (Ab A) only, 100% inhibition. R = Experimental reading (Ab A + Ab B). Results can be calculated from the reduction in signal by Ab A (C1–R) expressed as a percentage of maximum Ab B signal (C1–C2).

arabinogalactan epitopes in gum arabic, expressed as percentage inhibition of the binding of Ab B by excess Ab A (see Fig. 3), are shown in Table 2. The anti-AGP antibodies are all mostly strongly cross-reactive in this assay. The only case in which there was no reaction was MAC207 as an inhibitor of other anti-AGP antibodies. LM2 was the most readily inhibited antibody by a range of antibodies. Two anti-HRGP antibodies, JIM11 (not shown) and JIM12 were entirely ineffective as inhibitors, and LM1 was ineffective other than the low inhibition of MAC207 binding. The reasons why the antibodies are not always the most effective inhibitors of themselves is not clear. The close association of all the arabinogalactan epitopes may result from steric factors influencing the binding of the large IgM antibodies to complexed branched arabinogalactan structures. These results seem to indicate that the arabinogalactan epitopes are contained within the same macromolecular species within the complex gum. This supports the observations that different epitopes are carried on the same extracellular carrot AGP (Knox *et al.*, 1991). Other factors must also be considered here; for example, a bound antibody may cause conformational changes in adjacent structures. The

size of the antibody binding site and the epitopes are currently unknown. Recent work has indicated that antibodies can have binding sites which are filled by epitopes as small as trisaccharides (Cygler *et al.*, 1991; Vyas *et al.*, 1993). The results of the arabinogalactan epitope mapping procedure suggests a probable high degree of overlap or proximity amongst the AGP epitope structures although definitive conclusions must await a full structural characterization of these epitopes. Such studies are currently in progress.

CONCLUSIONS

This work has demonstrated the presence of epitopes recognized by anti-AGP and anti-wall HRGP monoclonal antibodies in commercial exudate gum samples. This supports structural analyses, indicating that domains characteristic of wall HRGPs occur in gum arabic along with AGP structures (Qi *et al.*, 1991). Epitopes were present in both a low molecular weight dialysate and a high molecular weight fraction. The former should prove a useful source of epitopes or epitope fragments for characterization in conjunction with hapten inhibitor studies, while the latter may provide additional fragments after suitable chemical degradative or enzymic treatments.

Monoclonal antibodies such as those described in this report are also extremely useful for the determination of the structural characteristics and the composition of exudate gums and structure–functional relationships of the structural elements occurring within complex polysaccharides and glycoproteins.

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