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# A Family of Abundant Plasma Membrane-associated Glycoproteins Related to the Arabinogalactan Proteins Is Unique to Flowering Plants

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**Abstract.** We have identified a family of abundant peripheral plasma membrane glycoproteins that is unique to flowering plants. They are identified by a monoclonal antibody, MAC 207, that recognizes an epitope containing L-arabinose and D-glucuronic acid. Immunofluorescence and immunogold labeling studies locate the MAC 207 epitope to the outer surface of the plasma membrane both in protoplasts and in intact tissues. In some cells MAC 207 also binds to the vacuolar membrane, probably reflecting the movement of the plasma membrane glycoproteins in the endocytic pathway. The epitope recognized by MAC 207 is also pres-

ent on a distinct soluble proteoglycan secreted into the growth medium by carrot (*Daucus carota*) suspension culture cells. Biochemical evidence identifies this neutral proteoglycan as a member of the large class of arabinogalactan proteins (AGPs), and suggests a structural relationship between it and the plasma membrane glycoproteins. AGPs have the property of binding to  $\beta$ -glycans, and we therefore propose that one function of the AGP-related, plasma membrane-associated glycoproteins may be to act as cell surface attachment sites for cell wall matrix polysaccharides.

THE presence of a rigid cell wall makes the plant plasma membrane a relatively inaccessible structure. Quantitative data on protein composition (Kjellbom and Larsson, 1984) and protein topography (Grimes and Breidenbach, 1987) are beginning to facilitate comparative studies on plasma membranes, but knowledge of constitutive cell surface glycoproteins is still fragmentary. Information of the kind available for animal cell surface glycoproteins (Hynes, 1985) and proteoglycans (Hook et al., 1984) is entirely lacking.

Agglutination and fluorescence labeling of plant protoplasts by lectins has permitted the identification of terminal glycan residues on the outer face of the plasma membrane (Walko et al., 1987), presumably components of glycoproteins or glycolipids. The presence among these of members of the class of glycoproteins termed arabinogalactan proteins (AGPs)<sup>1</sup> (Fincher et al., 1983) has been surmised from protoplast agglutination with the  $\beta$ -glycosyl Yariv reagents (Larkin, 1977, 1978; Samson et al., 1983), and from the coincident migration during isoelectric focussing of hydroxyproline-rich membrane components and components with affinity for  $\beta$ -galactosyl Yariv reagent (Samson et al., 1983). However, since Yariv reagents do not interact with AGPs specifically (Jermyn, 1978), and hydroxyproline is the principal amino acid of other arabinosylated plant cell extracellular matrix glycoproteins such as extensin (Showalter and

Varner, 1989), it has not yet been possible to confirm that some AGPs are plasma membrane components. Moreover, few of the antisera and monoclonal antibodies that recognize flowering plant cell plasma membranes (Norman et al., 1986; Villaneuva et al., 1986; Grimes and Breidenbach, 1987; Lynes et al., 1987; Bradley et al., 1988; Meyer et al., 1988) have been biochemically characterized, and at present only identification of the plasma membrane H<sup>+</sup>-ATPase has been achieved immunologically (DuPont et al., 1988).

In this report we describe the characterization of a monoclonal antibody (MAC 207), originally prepared from immunizations with peribacteroid membrane of pea root nodules (Bradley et al., 1988) that recognizes a family of antigens associated with the plant cell plasma membrane. We show that MAC 207 also binds to an AGP secreted by carrot suspension culture cells and reason that the composition of the epitope and the structural specificity of the antibody can be used to define a specific family of AGP-related glycoproteins that are associated with the extracellular face of the plasma membrane. We suggest that this family of glycoproteins are plasma membrane components in somatic cells of many or all flowering plants, and that they may function as cell surface receptors for cell wall matrix molecules.

## Materials and Methods

### Plant Material

Plant material other than suspension cultures was collected locally.

1. *Abbreviation used in this paper:* AGP, arabinogalactan protein.

## Monoclonal Antibody

The monoclonal antibody MAC 207 is derived from the fusion described by Bradley et al. (1988), and in that study was indistinguishable from another antibody (MAC 209) derived from the same fusion. The antibody was used unpurified from hybridoma culture supernatant. MAC 207 is a rat immunoglobulin of class IgM.

## Culture of Suspension Cells and Preparation of Protoplasts

Suspension culture cells derived from roots of carrot (*Daucus carota*) were grown in continuous light at 25°C in Murashige and Skoog medium supplemented with 30 g l<sup>-1</sup> sucrose and 1 mg l<sup>-1</sup> 2,4-dichlorophenoxyacetic acid (2,4-D). Sugar beet (*Beta vulgaris*) suspension culture 68.3 (kindly supplied by De Danske Sukkerfabrikker, Copenhagen, Denmark) was grown in PG<sub>08</sub> medium (de Greef and Jacobs, 1979) supplemented with 0.2 mg l<sup>-1</sup> 2,4-D and 0.2 mg l<sup>-1</sup> benzylaminopurine. Cells were subcultured every 7 d and used for experimentation 5 or 6 d after subculture. Protoplasts were prepared from carrot cells by cell wall digestion with Driselase (Sigma Chemical Co. Ltd., Poole, England) and Cellulase (Onozuka R-10; Yakult Honsha Co. Ltd., Tokyo, Japan), both at 0.1% (wt/vol) for 18 h at 25°C in a protoplasting medium consisting of 0.45 M sorbitol, 10 mM CaCl<sub>2</sub>, 1 mM KNO<sub>3</sub>, 200 μM KH<sub>2</sub>PO<sub>4</sub>, 1 μM KI, and 0.1 μM CuSO<sub>4</sub> adjusted to pH 5.8. Sugar beet protoplasts were prepared by digestion with 0.5% (wt/vol) concentrations of Cellulase, Macerozyme R-10 (Yakult Honsha Co., Ltd.) and Rhozyme (Pollock & Poole, Ltd., Reading, England) prepared in PG<sub>08</sub> containing 0.5 M mannitol, 3.1 mM 2-(*N*-morpholino) ethanesulfonic acid, and 0.1% (wt/vol) BSA under the same ambient conditions. The progress of the digestion was monitored by staining with a freshly prepared solution (1 mg l<sup>-1</sup>) of Fluorescent Brightener 28 (Calcofluor White M2R; Sigma Chemical Co.). Protoplasts were separated from cell debris by filtration through muslin and passage through two 0.63 M sucrose cushions, and were maintained in protoplasting medium containing the appropriate osmoticum until use.

## Preparation of Microsomal Membrane Fraction

Carrot protoplasts, prepared from up to 500 ml of cell culture, were homogenized in 4 ml g<sup>-1</sup> fresh weight (of protoplasts) of 50 mM Tris-HCl pH 7.5 containing 0.25 M sucrose, 3 mM EDTA, 2.5 mM DTT, and 1 mM PMSF. Homogenization was performed on ice with 50 strokes of a Dounce glass-in-glass homogenizer (Jencons [Scientific] Ltd., Leighton Buzzard, England). The pellet collected by low speed centrifugation (5,000 g for 10 min at 4°C) was discarded and the supernatant further centrifuged at 100,000 g for 1 h at 4°C. This pellet was collected and resuspended in 5 mM potassium phosphate pH 7.8, containing 0.25 M sucrose, 2.5 mM DTT, and 1 mM PMSF. Electron microscopy confirmed the presence of microsomes within.

## Preparation of Conditioned Medium

Conditioned medium was prepared from carrot cell suspensions cultured for 5 d. The cells were removed by centrifugation and the conditioned medium filtered through No. 1 filter paper (Whatman Inc., Clifton, NJ). Ice-cold acetone was added to a final volume of 90% (vol/vol) and the solution mixed on ice for 20 min. The precipitate was collected, redissolved in PBS pH 7.4, and dialyzed extensively against PBS before analysis.

## PAGE and Western Blotting

One-dimensional 10% acrylamide slab gels were prepared according to Laemmli (1970). Two-dimensional gels were prepared following O'Farrell (1975). Gels were stained with Coomassie Brilliant Blue or ammoniacal silver, or were blotted onto nitrocellulose following standard procedures.

For immunoblotting, nitrocellulose was blocked with 2% (vol/vol) calf serum (Sigma Chemical Co.) and 0.2% (wt/vol) ovalbumin in PBS for at least 1 h, and reacted for 2 h with a 10–100-fold dilution of MAC 207 culture supernatant in the same solution. Bound antibody was located with a horseradish peroxidase-conjugated rabbit anti-rat IgG (H+L) antiserum (ICN Biomedicals Ltd., High Wycombe, England), using 4-chloro-1-naphthol in the developing solution. For affino blotting with Con A, nitrocellulose was blocked with 0.05% (vol/vol) polyoxyethylenesorbitan monolaurate (Tween 20) in Tris-buffered saline (TBS) pH 7.4 for 1 h, washed in TBS, and incubated for 24 h in TBS containing 1 mM CaCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, and 100 μg l<sup>-1</sup> horseradish peroxidase-conjugated Con A (Sigma Chemical Co.).

For blotting with Yaviv reagent, carrot conditioned medium was resolved electrophoretically and transferred to nitrocellulose as described above, blocked with 0.2% ovalbumin in PBS, and immersed for 15 min in the β-glucosyl Yaviv reagent, an artificial carbohydrate antigen bound by AGPs (Yaviv et al., 1965; Jermyn and Yeow, 1975). This was prepared by coupling diazotized *p*-aminophenyl-β-D-glucopyranoside (Sigma Chemical Co.) to phloroglucinol as described by Yaviv et al. (1962). The concentration of the Yaviv reagent was 2 g l<sup>-1</sup>. The nitrocellulose was washed in 1% (vol/vol) sodium chloride before observations were made.

## Isolation of MAC 207 Antigen

The MAC 207 antigen (identified by immunoblotting) was resolved from carrot-conditioned medium in a 2 × 40 × 110 mm 10% curtain gel, and its precise position identified by stained marker lanes with comparable protein loadings. Care was taken at this stage to discriminate between the MAC 207 antigen and the 56-kD band nearby. The acrylamide containing the MAC 207 antigen was cut from the gel and electroeluted for 5 h at 10 mA into 1 ml one-dimensional running buffer (250 mM Tris-base pH 8.3, 192 mM glycine, 10 g l<sup>-1</sup> SDS). The antigen was dialyzed extensively against distilled water to remove all traces of running buffer before lyophilization for chemical analysis.

## Chemical Analysis of MAC 207 Antigen

The protein content of the lyophilized proteoglycan was determined following Lowry et al. (1951). Hydroxyproline was measured by the technique of Kivirikko and Liesmaa (1959).

The content of neutral sugars was determined using D-galactose as the standard in a phenol-H<sub>2</sub>SO<sub>4</sub> assay. 5 ml of the diluted sample was added to 0.3 ml of 5% (wt/vol) aqueous phenol. After mixing, 2 ml of concentrated H<sub>2</sub>SO<sub>4</sub> was added rapidly and mixed. The absorbance at 484 nm was determined 30 min later. For carbohydrate analysis the lyophilized residue (~1 mg) was dissolved in 1 ml distilled water and an aliquot of 0.4 ml used for estimation of sugars as alditol acetates following Selvendran et al. (1979). The remainder was lyophilized again and subjected to methylation analysis during which the partially methylated alditol acetates (PMAA) were identified by GLC-MS as described by Ring and Selvendran (1978) and Selvendran and Stevens (1986). The PMAA were separated on a bonded phase OV-255 wide-bore capillary column (0.53 mm × 15 m) with 1 μm film thickness and carrier gas (He) at 8 cm<sup>3</sup> min<sup>-1</sup>. The temperature was 150°C for 5 min and 1°C per min to 220°C.

## Biochemical Analysis of MAC 207 Binding

To study the effects of protease and periodate and of various monosaccharides and glycoproteins (Table IV) on MAC 207 binding, a preparation of carrot microsomes containing 50 mg l<sup>-1</sup> protein was coated for 18 h onto microtitre plates. When the plates had been blocked by immersion in PBS containing 0.2% ovalbumin for 1 h, a 10-fold dilution (in the same buffer) of MAC 207 culture supernatant was applied and allowed to remain for 2 h. Bound antibody was visualized with a 2,000× dilution of the rabbit horseradish peroxidase-conjugated anti-rat Ig antiserum described for immunoblotting, but for ELISA tetramethylbenzidine was used in the developing solution. In certain instances the immobilized membranes were treated before addition of antibody with protease type XXV (Pronase E; Sigma Chemical Co.) at a concentration of 1 g l<sup>-1</sup> in 50 mM Tris-HCl pH 7.5, or 25 mM sodium metaperiodate in 50 mM sodium acetate buffer pH 4.3. In both cases, treatment was for 1 h at room temperature in the dark. The periodate reaction was stopped by incubation with 1% (wt/vol) glycine in PBS for 30 min. To determine inhibition, the monosaccharides and glycoproteins were titrated into coated microELISA plates before the addition of the 10-fold dilution of MAC 207. The concentration required to inhibit antibody binding by 50% was then determined.

## Detergent Partitioning of Carrot Microsomes

Triton X-114 partitioning of carrot microsomes was performed following Bordier (1981). Aqueous and (pooled) detergent phases were dialyzed against PBS, lyophilized, and reconstituted in 50 μl distilled water. 1-μl dots of these solutions were applied to nitrocellulose and reacted with MAC 207 and peroxidase-conjugated anti-rat Ig as described for immunoblotting.

## Immunoagglutination

Freshly prepared protoplasts of carrot or sugar beet were mixed (in the

**Table I. Distribution of Plasma Membrane MAC 207-binding Macromolecules among 18 Species of Autotrophic Plants**

| Species                                       | Plasma membrane MAC 207-binding macromolecules |
|---|--|
| <i>Chlamydomonas reinhardtii</i>              | —  |
| <i>Mnium hornum</i>                           | —  |
| <i>Equisetum arvense</i> (field horsetail)    | —  |
| <i>Pteridium aquilinum</i> (bracken)          | —  |
| <i>Taxus baccata</i> (English yew)            | —  |
| <i>Pinus sylvestris</i> (Scots pine)          | —  |
| <i>Beta vulgaris</i> (sugar beet)             | +  |
| <i>Papaver rhoeas</i> (common poppy)          | +  |
| <i>Halimione portulacoides</i> (sea purslane) | +  |
| <i>Cucurbita pepo</i> (zucchini)              | +  |
| <i>Brassica napus</i> (rape)                  | +  |
| <i>Pisum sativum</i> (pea)                    | +  |
| <i>Daucus carota</i> (carrot)                 | +  |
| <i>Nicotiana tabacum</i> (tobacco)            | +  |
| <i>Lonicera periclymenum</i> (honeysuckle)    | +  |
| <i>Allium cepa</i> (onion)                    | +  |
| <i>Hyacinthoides non-scriptus</i> (bluebell)  | +  |
| <i>Agave americana</i> (sisal hemp)           | +  |

—, absent; +, present.

proportion of  $10^5$  protoplasts per ml) with a 10-fold dilution of MAC 207 in 100 mM Tris-HCl pH 7.5 containing 0.45 M sorbitol (carrot) or 0.5 M mannitol (sugar beet) and 0.2% ovalbumin, and agitated for 10 min.

### Immunofluorescence Microscopy

For studies on living material  $\sim 10^5$  carrot or sugar beet protoplasts were pelleted at 60 g in Eppendorf tubes (Sarstedt Ltd., Leicester, England) and resuspended in MAC 207 culture supernatant as described for immunoaagglutination. The tubes were gently agitated for 40 min and washed by repeated centrifugation from Tris-HCl containing 0.45 M sorbitol (carrot) or 0.5 M mannitol (sugar beet).

Immunofluorescence was also performed upon frozen sections. Root tips of carrot, pea (*Pisum sativum*), and onion (*Allium cepa*) were excised and fixed for 18 h in 3.7% (vol/vol) fresh formaldehyde in 100 mM Pipes pH 6.94, 1 mM EGTA, 100  $\mu$ M  $MgSO_4$  (PEM), and infused for 3 d with a 0.5% (vol/vol) solution of formaldehyde containing 1.5 M sucrose. Transverse slices ( $\sim 1$  mm thick) were frozen onto stubs by immersion in liquid ethane cooled in liquid nitrogen to just above its freezing point. The stubs were fitted to an Ultracut E FC 4D cryo-ultramicrotome (Reichert-Jung Ltd., Slough, England) and 400 nm frozen sections cut at  $-111^\circ\text{C}$ . Sections were collected on small drops of 2 M sucrose and settled onto multiwell slides. A 10-fold dilution of MAC 207 culture supernatant (in PBS containing 0.2% ovalbumin) was applied to washed sections for at least 1 h at room temperature.

Bound rat immunoglobulins were detected in both instances by means of a goat anti-rat IgG (H+L) antiserum linked to fluorescein isothiocyanate (ICN Biomedicals Ltd.) applied at a 100-fold dilution in the appropriate buffer. Slides were examined with a Zeiss Photomicroscope III equipped with epifluorescence illumination. Control preparations were treated with an irrelevant hybridoma culture supernatant or hybridoma culture medium alone.

### Immunogold Electron Microscopy

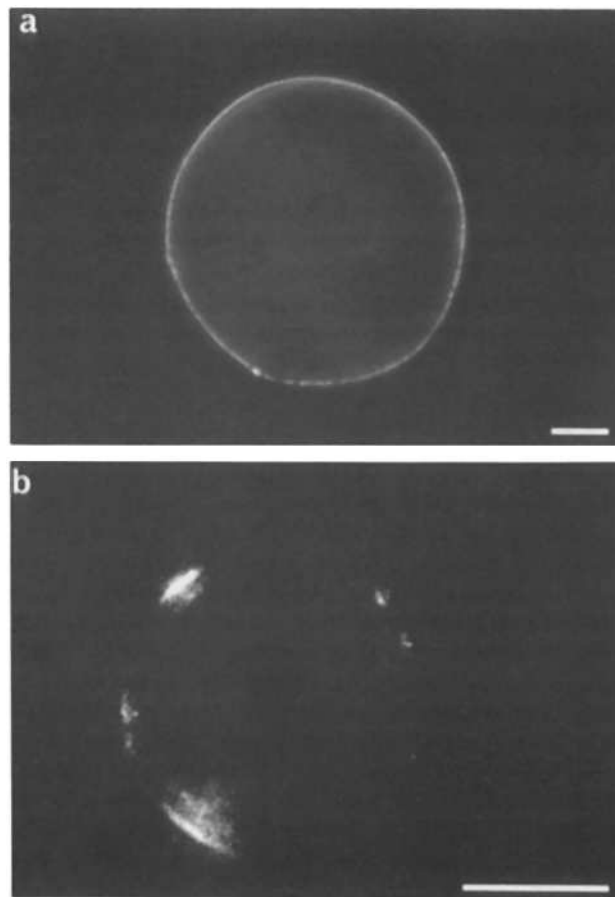
For ultrastructural labeling of frozen sectioned tissue, onion root tips were frozen in sucrose by liquid ethane as described for immunofluorescence. Sections  $\sim 100$  nm thick were cut at  $-111^\circ\text{C}$  and collected onto gold grids. For ultrastructural and topographical analysis of binding to low temperature resin-embedded sections, carrot suspension culture cells, vegetative tissues from 18 species (Table I) and mature sugar beet pollen grains were fixed in 2.5% (vol/vol) glutaraldehyde in PEM for 2–3 h in the dark. The suspension cells and pollen grains were washed and suspended in a small volume of 4% (wt/vol) low melting point agarose (Bethesda Research Laboratories,

Gaithersburg, MD) for ease of handling. Suspension cells and organized tissues were dehydrated and embedded in L. R. White resin (London Resin Co., Basingtoke, England) at sub-zero temperatures (Wells, 1985), with the exception that the 1:1 ethanol/resin mixture was cooled to only  $-20^\circ\text{C}$ . Sections were prepared for electron microscopy with an Ultracut ultramicrotome (Reichert-Jung Ltd.) and collected onto coated nickel grids.

Indirect immunogold labeling was performed by transferring grids between small drops of MAC 207 culture supernatant and a goat anti-rat IgG (H+L) antiserum conjugated to 10 nm gold particles (GARa G10; Janssen Pharmaceutica, Beerse, Belgium). Grids were blocked with TBS containing 10% (vol/vol) calf serum for 10 min, floated on culture supernatant for 18 h and, after washing, floated on the gold-conjugated antiserum (diluted 1:20 in the same buffer) for 1 h. Controls were as described for immunofluorescence microscopy. In some instances 100 mM concentrations of L-arabinose were mixed with the MAC 207 culture supernatant before application to sections of carrot suspension cells. All washings were performed with TBS and all incubations took place at  $4^\circ\text{C}$  in the dark. Grids were finally dried and stained with an aqueous solution of uranyl acetate and examined with a JEOL JEM-1200EX transmission electron microscope operating at 80 kV.

### Topographical Analysis of Antibody Binding

To determine the precise position of the MAC 207 epitope at the plasma membrane, high resolution immunogold electron micrographs were selected in which the section plane was perpendicular to the plasma membrane and in which the bilamellar leaflet could be accurately identified. These were photographically enlarged so that a distance of 1 mm on the print represented 15 nm on the section. A transparent overlay divided into 1 mm lanes was then used to ascribe each gold particle to a lane, taking into account all particles directly above the bilamellar leaflet and within 90 nm (6 mm on the print) of each surface. Since the gold particles were  $\sim 10$  nm in diameter it was possible to score each particle in one lane.



**Figure 1.** Immunofluorescence of living protoplasts prepared enzymically from suspension culture cells of sugar beet (a) and carrot (b). In the carrot protoplast the binding of the decavalent antibody to surface epitopes has led to patching. Bar, 10  $\mu$ m.

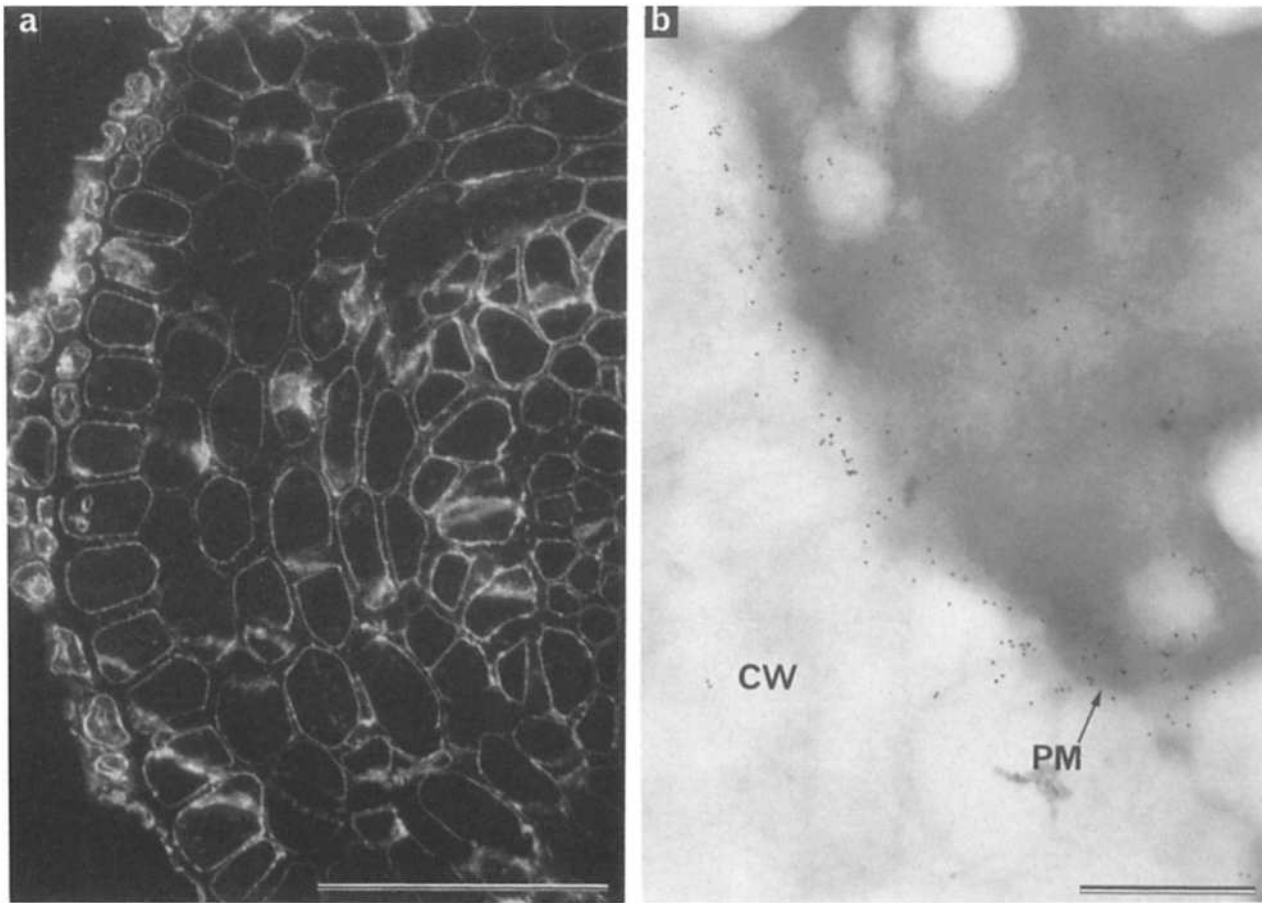


Figure 2. Frozen ultrathin sections of root cortex of carrot and onion prepared with MAC 207 for immunofluorescence and immunogold electron microscopy, respectively. CW, cell wall; PM, plasma membrane. Bars: (a) 50  $\mu\text{m}$ ; (b) 0.5  $\mu\text{m}$ .

## Results

### Binding of MAC 207 to Representatives of Three Classes of Autotrophic Plants

Taxonomic analysis by immunocytochemistry (Table I) revealed that MAC 207 bound to somatic cells of flowering plants and to vegetative cells of sugar beet pollen grains but not to cells of any class of cryptogams. Both dicotyledonous and monocotyledonous angiosperms possessed plasma membrane molecules containing the MAC 207 epitope, and it could be identified in eight families of the former (Papaveraceae [*Papaver*], Chenopodiaceae [*Beta*, *Halimione*], Cucurbitaceae [*Cucurbita*], Cruciferae [*Brassica*], Leguminosae [*Pisum*], Umbelliferae [*Daucus*], Solanaceae [*Nicotiana*], Caprifoliaceae [*Lonicera*]) and two families of the latter (Liliaceae [*Allium*, *Hyacinthoides*], Agavaceae [*Agave*]). Non-reactive species included an alga (*Chlamydomonas*), a moss (*Mnium*), two pteridophytes (*Equisetum*, *Pteridium*), and two conifers (*Taxus*, *Pinus*).

### Structural Analysis of MAC 207 Binding

MAC 207 agglutinated living protoplasts into clumps of up to 400 within 10 min of mixing. MAC 207 also labeled the surfaces of living carrot and sugar beet protoplasts (Fig. 1) and the cell surface in frozen sections of carrot (Fig. 2 a),

pea and onion root during immunofluorescence. Cell surface fluorescence of living protoplasts underwent a time-dependent "patching" in some instances (Fig. 1 b). Frozen section immunofluorescence often made apparent tissue-specific variation in the labeling of intracellular membranes. For example, in pea root tips tonoplasts were labeled in the cortical cells but not in the cells of the stele (Fig. 3). Immunofluorescence was not generated when MAC 207 culture supernatant was omitted or replaced with an irrelevant IgM antibody.

Frozen section immunogold electron microscopy of onion root confirmed that the plasma membrane was the main site of antibody binding (Fig. 2 b). Fine structural analysis of labeled resin sections revealed that in carrot suspension cells and tissues of all flowering plants the principal site of MAC 207 binding was the outer face of the plasma membrane (Fig. 4). In the somatic cells of some species there was little significant labeling of intracellular membranes (e.g., leaf mesophyll cells of sugar beet), but in some others there were occasional binding sites on local regions of the tonoplast (e.g., carrot suspension cells) or the membranes of small cytoplasmic vesicles (e.g., mesophyll cells of zucchini hypocotyl). Labeling the vegetative cell plasma membranes of sugar beet pollen was generally intense, but in all instances binding to the plasma membrane was accompanied by equally notable labeling of cytoplasmic vesicles. The only region of plasma membrane that was not labeled (in frozen ultrathin



Figure 3. Low power MAC 207 immunofluorescence micrograph of frozen ultrathin longitudinal section of part of a pea root tip. Although plasma membranes are labeled throughout, tonoplast membranes are labeled only in the cortex (C). S, stele; dotted line, approximate position of endodermis. Bar, 100  $\mu\text{m}$ .

sections) was that forming the lining of the plasmodesmata. There were no instances of labeling of other organelles or intracellular structures and control preparations were wholly negative. The presence of 100 mM arabinose in the MAC 207 culture supernatant completely abolished all binding to resin sections of carrot suspension cells.

In the most striking resin preparations the frequency of gold particles upon the plasma membrane was  $\sim 35$  per linear  $\mu\text{m}$ . Typically,  $>90\%$  of the gold particles associated with plasma membranes were at their outer surfaces at the interface with the cell wall (Fig. 4). The mean distance between the outer surface of the *Cucurbita* plasma membrane and the centres of the gold particles was 22.5 nm, and the modal value was 30 nm (Fig. 5).

#### **Characteristics of the MAC 207-reactive Plasma Membrane Macromolecules**

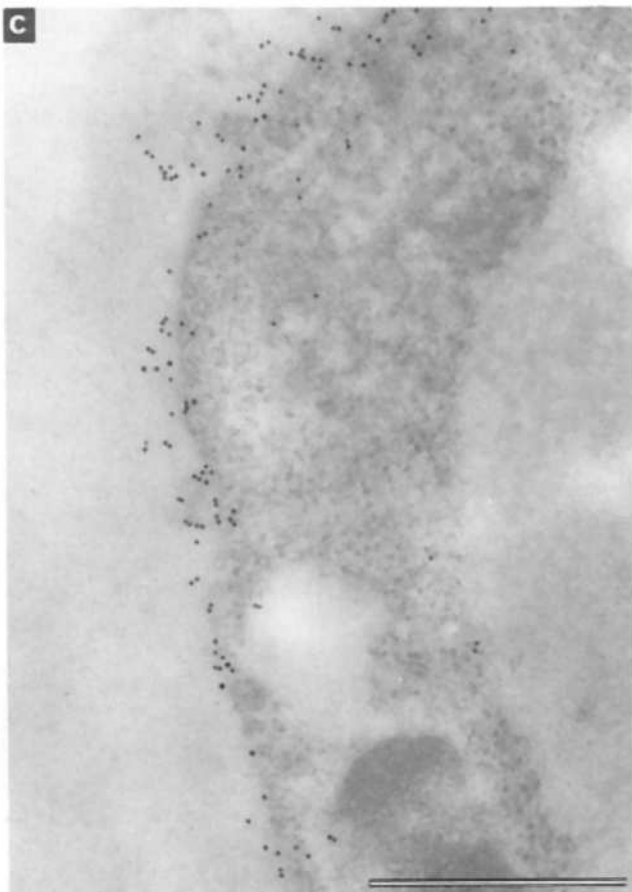
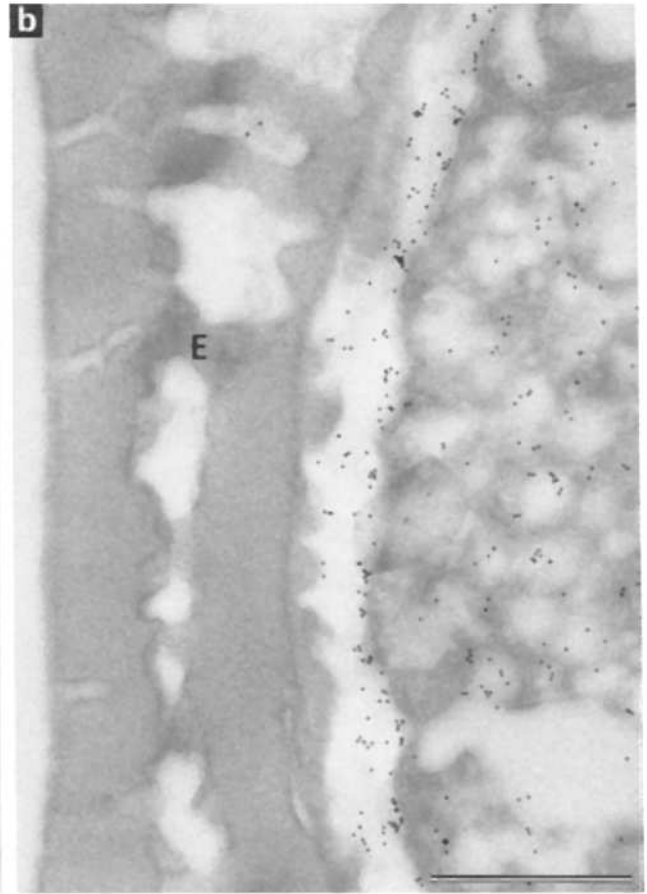
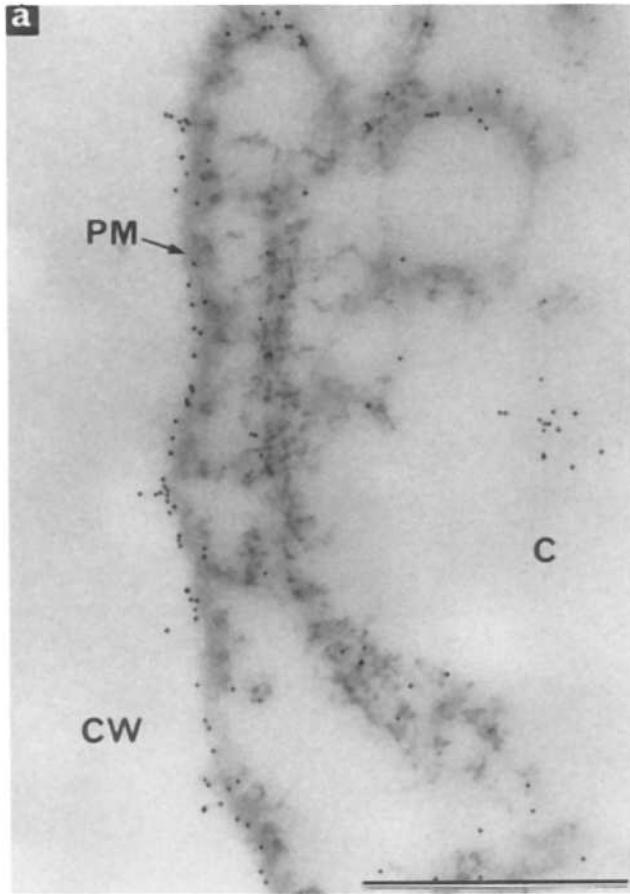
One-dimensional immunoblotting of carrot microsomes revealed that a discontinuous smear of  $\sim 15$  principal bands with  $M_r$  210,000–28,000 reacted with MAC 207. Blotting after two-dimensional separation indicated that 18 discrete spots with  $M_r$  66,000–13,200 (Fig. 6, a and c) bound the antibody. None of these spots reacted with Con A during

affinoblotting (Fig. 6 b). Since immunogold electron microscopy revealed that in carrot suspension culture cells the only significant MAC 207 binding site was the plasma membrane (Fig. 4 c), the MAC 207-reactive spots were judged to represent components of plasma membranes present in the microsome fraction. The isoelectric points and molecular masses of these macromolecules are listed in Table II. The spots numbered 4 (58.9 kD), 12 (39.7 kD), and 14 (35.5 kD) represented between them  $\sim 80\%$  of the MAC 207-binding activity (Fig. 6 a), and  $\sim 5\%$  of the total protein resolved from the microsome fraction.

Dot-blots of Triton X-114 partitioned carrot microsomes revealed that the MAC 207 epitope could be detected only in the aqueous phase.

#### **Characterization of the Soluble MAC 207-reactive Proteoglycan Secreted by Carrot Suspension Culture Cells**

MAC 207 was also found to bind to components of carrot suspension culture conditioned medium (in addition to plasma membrane glycoproteins). SDS-PAGE (Fig. 7 a) and subsequent immunoblot analysis (Fig. 7 b) of concentrated conditioned medium indicated that a polydisperse smear of



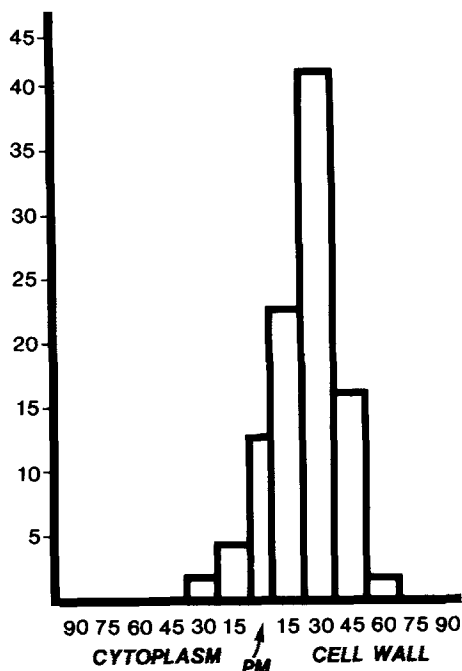


Figure 5. Distribution of 119 gold particles around the plasma membrane of a representative *Cucurbita* mesophyll cell. Numbers on vertical axis denote gold particles counted; numbers on horizontal axis, nanometers across transect.

material with an apparent  $M_r$  of 100,000–70,000 reacted with the antibody. The same component of the conditioned medium also stained red with  $\beta$ -glucosyl Yariv reagent (Fig. 7 c). Two-dimensional electrophoresis followed by silver staining revealed that the MAC 207-reactive material identified by one-dimensional immunoblotting focussed between pH 6.6 and 7.1 (Fig. 7 d).

Chemical analysis of the MAC 207-reactive material indicated that it was a proteoglycan containing 95% carbohydrate and 5% protein. Hydroxyproline accounted for 20% of the protein. Analysis of the glycan component is presented in Table III and shows the linkages of arabinose and galactose, the two most abundant sugars. The mole percentages were calculated after the removal of glucose (34.6% of the carbohydrate in the sample) which was lost during dialysis and therefore presumed not to be a covalently linked component of the proteoglycan. Only 43  $\mu$ g of uronic acids were present. This was insufficient for further analysis and hence uronic acids have been identified in toto. This analysis indicated that the neutral proteoglycan is an arabinogalactan protein (AGP).

#### Inhibition of MAC 207 Binding

Treatment of carrot microsomes with protease decreased MAC 207 binding by 58% (relative to controls) and periodate by 62%. Hapten inhibition with saccharides revealed that MAC 207 binding to carrot microsomes was effectively restricted by L-arabinose (presumably the furanoside isomer

since the 1-*O*-methyl- $\beta$ -L-arabinopyranoside was significantly less active) and D-glucuronic acid (Table IV). 200- $\mu$ M concentrations of D-galactose, 1-*O*-methyl- $\beta$ -D-galactopyranose, and 3-*O*- $\beta$ -D-galactopyranosyl-D-arabinose did not inhibit MAC 207 binding. Two glycoproteins were also potent inhibitors of MAC 207 binding to carrot microsomes: the *Acacia senegal* AGP gum arabic, and the secreted carrot proteoglycan recovered from carrot suspension culture conditioned medium (Table IV). Neither *Solanum tuberosum* lectin nor *Chlamydomonas reinhardtii* cell wall glycoprotein 2BII (Roberts et al., 1985, both arabinosylated hydroxyproline-rich glycoproteins) were effective inhibitors, however.

#### Discussion

##### Monoclonal Antibody MAC 207 Recognizes a Family of Abundant Glycoproteins That Are Associated with the Plant Plasma Membrane

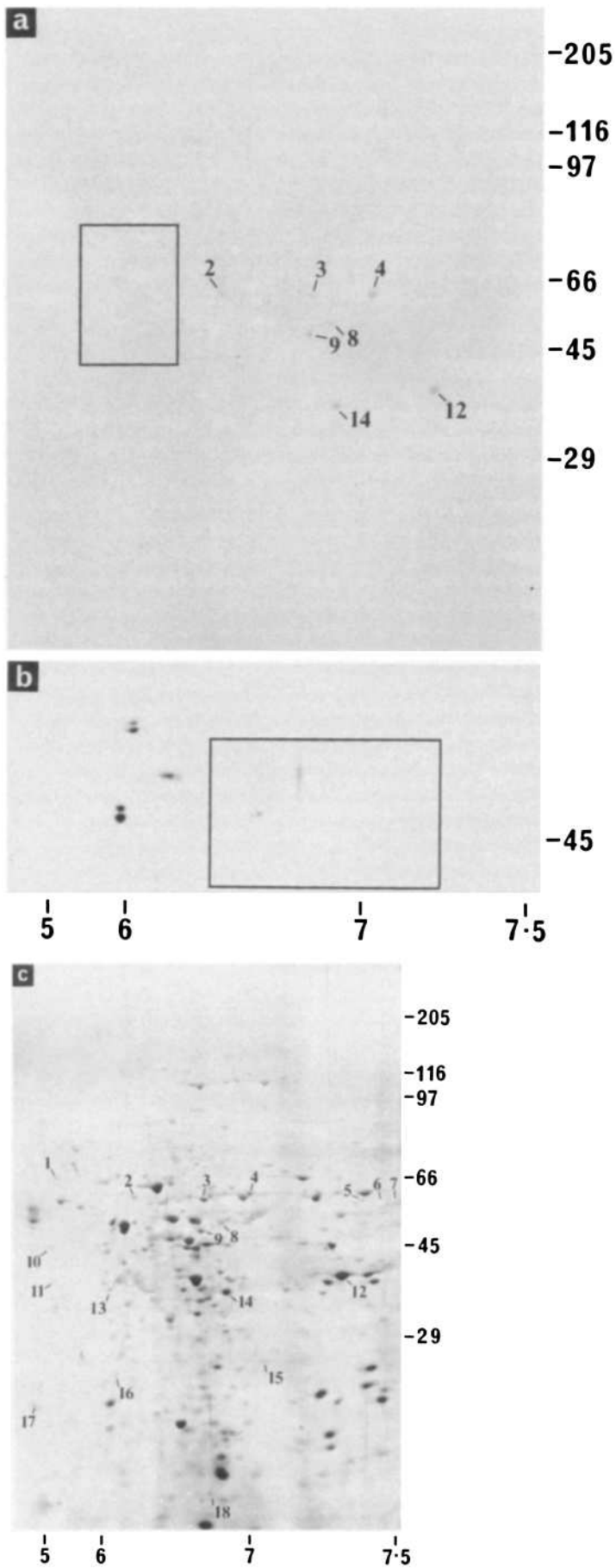
Immunoagglutination of suspension cell protoplasts, immunoblotting of carrot microsomes, and immunocytochemistry suggest that MAC 207 locates a family of abundant macromolecules that are associated with plant plasma membranes. Two-dimensional immunoblots of carrot microsomes reveal 18 plasma membrane-associated MAC 207-reactive spots, of which three ( $M_r$  58,900, 39,700, 33,500) represent  $\sim$ 80% of the total. Immunoagglutination and immunofluorescence (of whole protoplasts and of frozen sections) and immunogold labeling (of resin sections) confirm that the MAC 207-reactive antigens are located on the outer face of the plasma membrane. The topographical spread (from the mode) of gold particles binding to a section of *Cucurbita* plasma membrane conforms to a simple and symmetrical distribution, suggesting that the MAC 207 epitope is present at a more or less constant distance ( $\sim$ 22 nm) from the external face of the plasma membrane. This suggests that they could be either integral or peripheral membrane components. Movement of the MAC 207 antigen into the aqueous phase during detergent partitioning favors the notion that they are peripheral.

The extent to which other membranes in the cell (and in particular the tonoplast) are labeled by MAC 207 appears to reflect consistent differences between tissues, and is probably a measure of endocytic activity. It is known that material taken up by endocytosis in plant protoplasts ends up in the vacuole (Tanchak et al., 1984).

##### Plasma Membrane Macromolecules Recognized by MAC 207 Are Unique to Flowering Plants

Structural analysis of resin sections indicates that MAC 207 binds to the plasma membranes of all flowering plants examined, including both monocots and dicots. This distribution contrasts markedly with that of other related plant cell surface macromolecules that occur also in cryptogams (Clarke et al., 1979; Roberts et al., 1985).

Figure 4. High resolution MAC 207 immunogold electron micrographs. Cells from three dicots and one monocot are represented in the same orientation. The dicot examples are a mesophyll cell of *Cucurbita* hypocotyl (a), a vegetative cell of a *Beta* pollen grain (b), and a suspension culture cell of *Daucus* (c). The monocot cell is from leaf mesophyll of *Hyacinthoides* (d). Binding in each instance is principally at the plasma membrane (PM). C, cytoplasm; CW, cell wall; E, exine. Bar, 0.5  $\mu$ m.



*Figure 6.* Identification of MAC 207-reactive macromolecules of carrot microsomes by two-dimensional SDS-PAGE and blotting. Numbers refer to MAC 207-binding spots, ranked by molecular mass and listed in Table II. (a) MAC 207 immunoblot; (b) Con A affinity blot; (c) silver-stained polyacrylamide gel. The eight principal MAC 207-binding spots in *a* fail to react with Con A (square in *b*). Similarly, the Con A-binding macromolecules do not react with MAC 207, and are made visible in the (overdeveloped) immunoblot by slight negative staining (square in *a*). Numbers on vertical axes are relative molecular masses in kilodaltons; numbers on horizontal axes are pH.

Table II. Features of Carrot Microsome MAC 207-reactive Macromolecules

| No.      | pI               | $M_r$  |
|----------|------------------|--------|
|          | <i>pH</i>        |        |
| 1        | 5.55             | 66,000 |
| 2, 3, 4* | 6.25, 6.75, 7.00 | 58,900 |
| 5, 6, 7  | 7.25, 7.45, 7.50 | 57,400 |
| 8        | 6.85             | 52,500 |
| 9        | 6.70             | 47,800 |
| 10       | 5.20             | 44,700 |
| 11, 12*  | 5.15, 7.20       | 39,700 |
| 13, 14*  | 6.10, 6.85       | 35,500 |
| 15       | 7.00             | 24,500 |
| 16       | 6.05             | 24,000 |
| 17       | 4.90             | 19,800 |
| 18       | 6.80             | 13,200 |

\* Molecular species representing together ~80% of the MAC 207 reactivity.

### Saccharide Inhibition Suggests an L-Arabinose-containing Epitope

L-Arabinose and D-glucuronic acid inhibited the binding of MAC 207 to membrane microsomes. Of the known classes of arabinosylated glycoproteins only the AGPs contain significant amounts (up to 20% of the carbohydrate) of uronic acids (Clarke et al., 1979). Gum arabic, an extracellular AGP prepared from *Acacia senegal*, and the soluble carrot AGP described in this paper were very effective inhibitors of MAC 207 binding, also implying that the antibody binds AGP-related macromolecules. However, three other classes of arabinosylated hydroxyproline-rich glycoproteins occur in plants: the extensins (Showalter and Varner, 1989), *Solanum*

*tuberosum* lectin (Ashford et al., 1982), and the *Chlamydomonas reinhardtii* cell wall glycoproteins (Roberts et al., 1985). Of these, neither *Solanum* lectin nor *Chlamydomonas* glycoprotein 2BII cross-reacted with MAC 207 in ELISA, and can therefore be discounted as possible antibody binding sites. Purified extensins were not available for inhibition studies. Nevertheless, we believe it is unlikely that extensins contain the MAC 207 epitope for three reasons: extensins contain mono-, di-, tri-, and tetra-arabinosides linked without galactose directly to hydroxyproline (Lampert, 1967; Showalter and Varner, 1989), and not the arabino-3,6-galactan linkages characteristic of gum arabic and other AGPs (Fincher et al., 1983); the arabinosides of extensin contain only 1,2 and 1,3 linkages (Akiyama et al., 1980), and not the 1,5 linkages present in the soluble carrot AGP described in this paper; and recently extensins have been immunolocalized throughout the texture of the cell wall (Averyhart-Fullard et al., 1988; Stafstrom and Staehelin, 1988), and not at the interface with the plasma membrane as are the macromolecules discussed here. Taken together, these data indicate that MAC 207 binds to membrane glycoproteins related to AGPs and not to any other known family of arabinosylated macromolecules.

### Soluble, Secreted MAC 207-reactive Proteoglycan Is an Arabinogalactan Protein

The chemical composition and affinity for Yariv reagent of the MAC 207-reactive proteoglycan found in carrot suspension culture-conditioned medium indicates it is an arabinogalactan protein (Clarke et al., 1979; Fincher et al., 1983). A suggested partial structure of the soluble carrot AGP (determined from methylation analysis) is shown in Fig. 8. AGPs form a large class of plant glycoproteins and soluble

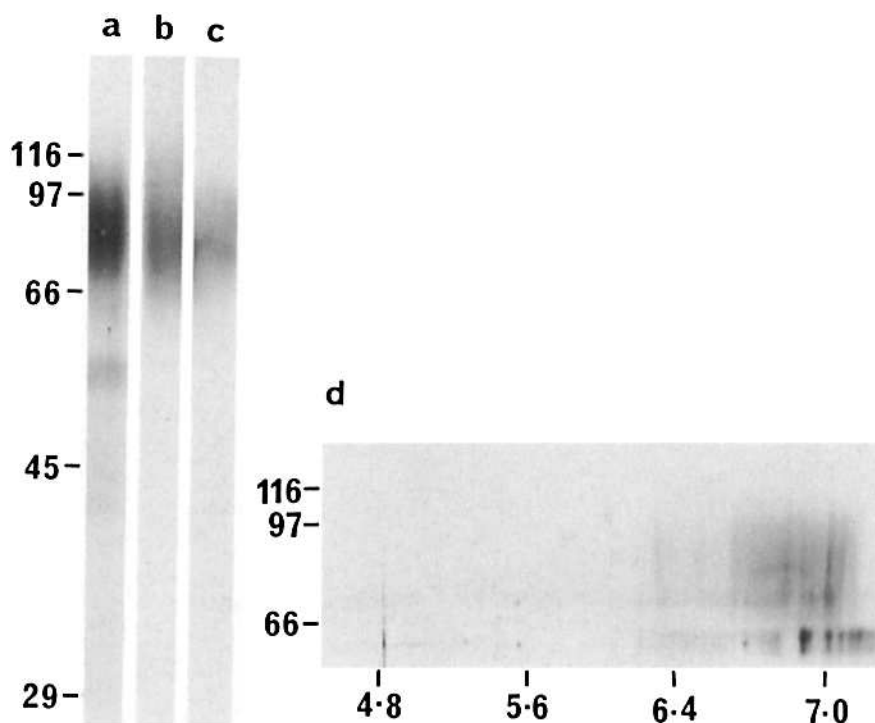


Figure 7. Coomassie Brilliant Blue stain (a) and corresponding MAC 207 immunoblot (b) and Yariv reagent stain (c) of carrot suspension culture-conditioned medium macromolecules. MAC 207 and Yariv reagent bind only to the  $M_r$  100,000-70,000 band. (d) Detail of silver-stained two-dimensional polyacrylamide gel of the carrot-conditioned medium reveals that the  $M_r$  100,000-70,000 band focusses at pH 6.6-7.1 and is relatively homogeneous in composition. The stained material below the 66-kD marker in a and d is not a component of the smear that reacts with MAC 207 in b. Numbers on vertical axes are relative molecular masses in kilodaltons; numbers on horizontal axis (in d) are pH.

**Table III. Sugar Composition and Linkage Analysis of the Soluble AGP Secreted by Carrot Suspension Cells**

| Sugar                      | Linkage | Mole percent |
|----------------------------|---------|--------------|
| Rha                        |         | 2            |
| Fuc                        |         | 4            |
| Ara                        |         | 48           |
| 2,3,5-Me <sub>3</sub> -Ara | 1-      | 29           |
| 2,3,-Me <sub>2</sub> -Ara  | 1,5-    | 21           |
| Xyl                        |         | 10           |
| Man                        |         | 7            |
| Gal                        |         | 29           |
| 2,3,4-Me <sub>3</sub> -Gal | 1,6-    | 12           |
| 2,3,6-Me <sub>3</sub> -Gal | 1,4-    | 13           |
| 2,4-Me <sub>2</sub> -Gal   | 1,3,6-  | 25           |

The Ara and Gal linkages are expressed as mole percentages of the linkages involving Ara and Gal, not of all linkages present in the AGP.

proteoglycans. They vary in composition (Cassab, 1986; van Holst and Clarke, 1986; Tsumuraya et al., 1988), and are found throughout the plant kingdom (Clarke et al., 1979). One characteristic of AGPs is their lectin-like ability to bind artificial carbohydrate antigens or Yariv reagents (Yariv et al., 1962). The presence of AGPs in the conditioned medium of suspension culture cells has been reported before (Anderson et al., 1977; Pope, 1977), and it is likely that soluble AGPs are also secreted into the apoplast of *Phaseolus* hypocotyls (Samson et al., 1983) and *Nicotiana* styles (Sedgley et al., 1985). The compositions of the slimes secreted by plant roots (Moody et al., 1988) also resemble the soluble AGP described in this paper.

#### **Analysis of the Soluble Proteoglycan Provides a Basis for Discriminating between Families of AGPs**

The finding that MAC 207 recognizes a soluble AGP supports the view (drawn from inhibition studies) that the MAC 207-reactive plasma membrane-associated glycoproteins form a specific family of this large class of plant macromolecules. It is noteworthy in this context that none of the membrane-bound arabinosylated glycoproteins bind Con A, confirming the absence from them of terminal glucosyl and mannosyl residues. Although it has been suggested before that AGPs may be plant plasma membrane components (Larkin, 1978; Samson et al., 1983), data in each instance have been based only upon observations involving Yariv reagents. It is known, however, that macromolecules other than AGPs bind Yariv reagents (Jermyn and Yeow, 1975), and therefore conclusions based solely upon Yariv reagents are open to doubt.

Our data therefore define for the first time (by their ability to bind MAC 207) a putative family of plasma membrane-associated arabinosylated glycoproteins. It seems that in both intact and wounded tissues (e.g., suspension culture cells) these macromolecules may coexist with soluble extracellular proteoglycans that form another distinct AGP family. The membrane-bound glycoproteins probably contain a lower proportion of carbohydrate than the soluble proteoglycan (as evidenced by their mobility during electrophoresis), but otherwise nothing is known of the difference between these two families chemically. In the absence of a clear functional definition of AGPs in general (Fincher et al., 1983), we sug-

gest that this large heterogeneous class of glycoproteins contains numerous overlapping families of related, arabinosylated macromolecules. Moreover, characterization of the soluble proteoglycan has allowed us to define a major group of plasma membrane-associated macromolecules immunologically, and in a way that could not be achieved by MAC 207 affinity separation of the membrane molecules per se. Until functions can be ascribed to some of these families of arabinosylated glycoproteins, they will remain classified under the unsatisfactory "AGP" umbrella.

#### **Biochemical and Structural Features of the Plasma Membrane MAC 207-reactive Glycoproteins Suggest Functional Parallels with Known Plasma Membrane Matrix-binding Macromolecules**

The conserved nature of the MAC 207 epitope and the possibly ubiquitous distribution among flowering plants of the macromolecules bearing it implies that the MAC 207-reactive plasma membrane macromolecules play a fundamental role at the cell surface. Our data suggest that this role may be to bind components of the extracellular matrix to the plasma membrane. The absence of the MAC 207-reactive glycoproteins from the membrane linings of the plasmodesmata and the rapid return of the antigens to the plasma membrane after enzymic digestion of cell surface polysaccharides imply that they are important cell surface components whose presence at the plasma membrane is a necessary antecedent to wall assembly. Moreover, the ability of the MAC 207-reactive component of conditioned medium to bind the  $\beta$ -glucosyl Yariv reagent suggests that the MAC 207-reactive macromolecules themselves may have lectin-like properties (Barondes, 1988). This would make the related plasma membrane macromolecules the functional equivalents of the well-characterized adhesive glycoproteins fibronectin and laminin that bind mammalian cells to connective tissues and basal laminae, respectively (McDonald, 1988). Discoidin I plays a comparable role in *Dictyostelium* (Springer et al., 1984). It is also an endogenous *N*-acetylgalactosamine-binding lectin with distinct cell-binding and carbohydrate-binding domains (Gabijs et al., 1985). Bridging macromolecules such as these may allow plant cells to detect radial tensions set up between plasma membrane and cell wall during turgor pressure changes. Comparable stresses and tissue tensions could also help determine division planes and organogenesis in plant tissues (Green, 1986). Although speculative, this hypothesis is open to experimentation.

**Table IV. Inhibition of MAC 207 Binding by Hapten Saccharides and Arabinosylated Hydroxyproline-rich Glycoproteins**

| Inhibitor                                | Concentration required for 50% inhibition of binding |
|--|--|
| L-Arabinose                              | 4 mM   |
| D-Glucuronic acid                        | 9 mM   |
| 1-O-methyl- $\beta$ -D-arabinopyranoside | 40 mM  |
| 1-O-methyl- $\beta$ -L-arabinopyranoside | 82 mM  |
| Gum arabic                               | 5 nM   |
| Carrot-secreted AGP                      | 10 nM  |

The molarity of gum arabic has been calculated assuming a mean  $M_r$   $1.5 \times 10^6$  (Clarke et al., 1979).

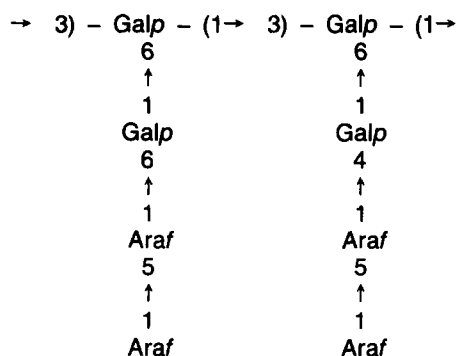


Figure 8. Suggested partial structure of the soluble AGP recovered from carrot suspension cell-conditioned medium.

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