The Plant Cell, Vol. 3, 1317–1326, December 1991

Developmental Regulation of a Plasma Membrane Arabinogalactan Protein Epitope in Oilseed Rape Flowers

Roger I. Pennell,[a,b,1] Luise Janniche,[a] Per Kjellbom,[c] Graham N. Scofield,[a] Janet M. Peart,[a] and Keith Roberts[a]

[a] Department of Cell Biology, John Innes Institute, Colney Lane, Norwich NR4 7UH, United Kingdom
[b] Department of Biology, University College London, Gower Street, London WC1E 6BT, United Kingdom
[c] Department of Plant Biochemistry, University of Lund, P.O. Box 124, S-221 00 Lund, Sweden

We have identified and characterized the temporal and spatial regulation of a plasma membrane arabinogalactan protein epitope during development of the aerial parts of oilseed rape using the monoclonal antibody JIM8. The JIM8 epitope is expressed by the first cells of the embryo and by certain cells in the sexual organs of flowers. During embryogenesis, the JIM8 epitope ceases to be expressed by the embryo proper but is still found in the suspensor. During differentiation of the stamens and carpels, expression of the JIM8 epitope progresses from one cell type to another, ultimately specifying the endothecium and sperm cells, the nucellar epidermis, synergid cells, and the egg cell. This complex temporal sequence demonstrates rapid turnover of the JIM8 epitope. There is no direct evidence for any cell-inductive process in plant development. However, if cell-cell interactions exist in plants and participate in flower development, the JIM8 epitope may be a marker for one set of them.

INTRODUCTION

Plants develop from simple embryos and become complex only when cells differentiate from other cells and develop into tissues. In angiosperms, the different organ systems vary in complexity, with the root being the simplest and the flower the most elaborate, and a proportionate number of cell differentiation events are involved with their development. Cell differentiation is thus easiest to observe in the cell lineages that converge on root apical meristems. Shoot apices are more complex because they give rise to lateral appendages (leaves) and to an axis that is segmented in relation to them. Flower development also requires that different tissues, lateral appendages (floral organs), and segmental tissue patterns are formed. But in addition, flower development requires that germ cells are determined from somatic cells, the sporophytic phase of the life cycle gives way to the gametophytic phase, and pairs of gametes are formed with different developmental functions (Walbot, 1985; Goldberg, 1988; Bell, 1989; Sussex, 1989).

Although they are complex, flowers are now more useful than other plant organ systems for studying many aspects of plant development. This is because they develop in sequential fashion, with one tissue giving rise to another, and it to another, in strict order (Esau, 1977), and also because several well-characterized floral mutants allow this process to be analyzed by molecular genetic techniques (Coen, 1991; Drews et al., 1991). One of the developmental transitions in the sequence of events that accompany pea flower development—the determination of germ cells from somatic cells—is marked by the loss of an arabinose-containing epitope from a family of a plasma membrane arabinogalactan proteins (AGPs [Pennell and Roberts, 1990]). This epitope becomes detectable again only after fertilization has taken place and the embryo has attained the heart stage (Pennell and Roberts, 1990). This and several other lines of evidence (Knox et al., 1989, 1991) suggest that plasma membrane AGPs participate in the local control of floral histogenesis and differentiation.

In this paper, we describe another plasma membrane AGP epitope, identified by the monoclonal antibody (MAb) JIM8, that is characteristic of the gametes and certain other sexual and somatic cells in anthers and ovules of oilseed rape, as well as the embryo that results from gametic fusion. Developmental studies with oilseed rape demonstrate that expression of the JIM8 epitope is labile and is temporally and spatially regulated in a complex manner during the short period leading to fertilization. Although no direct evidence exists for cell-inductive

[1] To whom correspondence should be addressed at Department of Biology, University College London, Gower Street, London WC1E 6BT, United Kingdom.

processes during the differentiation of angiosperm gametes, the developmental regulation of the JIM8 epitope may reflect a sequence of cell-cell interactions that occurs during gametogenesis.

RESULTS

Derivation of the JIM8 Hybridoma

As shown in Table 1, we generated almost 400 stable hybridomas from our fusion. Of these, 99.5% secreted rat immunoglobulins, 12% secreted immunoglobulins that bound to sugar beet membranes, and 5% reacted with gum arabic at least as strongly as MAb MAC 207. Hybridoma supernatants in this 5% were screened by immunofluorescence on frozen sections of carrot suspension cultures. The immunoglobulin in only one supernatant recognized plasma membranes in carrot suspension cultures; after cloning, the hybridoma secreting this immunoglobulin was termed JIM8. Although the coimmunization was used in an attempt to raise an MAb against a developmentally regulated AGP (as was done for stage-specific cell surface antigens in Dictyostelium [Barclay and Smith, 1986]), we do not know to what extent it was effective.

Structural Analysis of JIM8 Binding

Immunoagglutination and immunofluorescence of carrot protoplasts with MAC 207, as shown in Figures 1A and 1B, and with JIM8, as shown in Figures 1C and 1D, gave rise to many small clusters of up to 10 protoplasts and to plasma membrane fluorescence. A control MAb raised against an antigen not present in plants neither agglutinated nor made fluorescent carrot protoplasts. The distribution of the MAC 207 epitope was uniform (Figure 1B), but that of the JIM8 epitope was conspicuously punctate (Figure 1D). In the immunofluorescence assay, >95% of the protoplasts prepared from the carrot suspension culture bound both MAC 207 and JIM8. These findings indicated that JIM8 recognizes a plasma membrane epitope in protoplasts, but to confirm this for intact cells we performed immunogold electron microscopy on resin sections of carrot suspension culture cells, as well as on two representative cell types from oilseed rape flowers, the endothecium and sperm cells. Figure 1E shows some of these data and demonstrates that the JIM8 epitope was at the outer plasma membrane face.

Table 1. Screening Data from This Study

Screen	Number
Stable hybridomas surviving culture in hypoxanthine/aminopterin/thymidine medium	382
Screen 1: supernatants binding gum arabic > MAC 207[a]	6
Screen 2: supernatants binding carrot protoplasts	2

[a] MAC 207 binding to gum arabic = 1.0 ELISA unit; JIM8 binding to gum arabic = 2.3 ELISA units.

Spatial Regulation of the JIM8 Epitope in Aerial Parts of Oilseed Rape

Immunocytochemistry also revealed differences in the expression of the JIM8 epitope in oilseed rape plants. Detailed immunofluorescence examination of all aerial parts of oilseed rape plants demonstrated that the JIM8 epitope could be detected only in mature anthers and ovules, as shown in Figures 2 and 3, and, transiently, in xylem vessels after wall thickenings had been deposited but before lignification had begun (data not shown). In mature anthers, the JIM8 epitope was at the plasma membrane of both of the sperm cells in each pollen grain (topographical analysis of the JIM8 epitope by immunogold electron microscopy suggested that the JIM8 epitope was absent from the inner vegetative cell plasma membrane) and of the endothecium cells (Figures 2E and 2G). In mature ovules, the JIM8 epitope was at the plasma membrane of the egg cell, both synergids, and most of the cells in the nucellar epidermis, as well as throughout the filiform apparatus, as shown in Figure 3A. In flowers in which fertilization had occurred, the JIM8 epitope was also at the plasma membranes of the embryo, as shown in Figure 4A. A control MAb raised against an antigen not present in plants did not give rise to immunofluorescence.

Developmental Regulation of the JIM8 Epitope in Oilseed Rape Flowers

Because the JIM8 epitope was present in a nonobvious pattern in certain tissues and cells in mature oilseed rape flowers, we undertook a developmental study to understand the origin of this pattern, as shown in Figures 2A to 2F, 3A and 3B, 4A to 4D, and 5. Analysis by immunofluorescence of developing oilseed rape flowers then revealed that the JIM8 epitope could not be detected in any part of either the young stamen or carpel primordia (Figure 2A). The first detectable expression of the plasma membrane JIM8 epitope, at approximately the stage when meiosis was ending in the microspore mother cells, was in the tapetum and in the microspore mother cells (Figure 2B). Shortly afterward, at the stage when the developing male gametophytes contained two cells, the JIM8 epitope was present in the middle layer in the anther wall and, far less

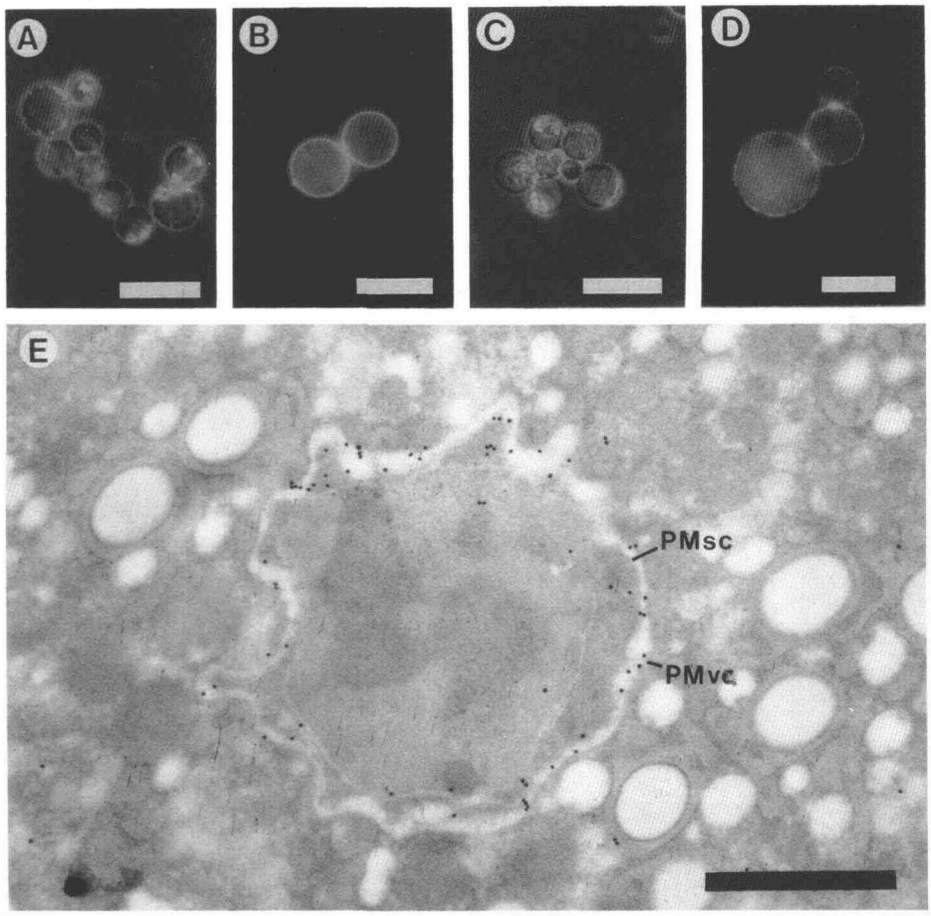

Figure 1. Cellular Characterization of JIM8 Binding.

(A) and **(C)** Immunoagglutination of carrot suspension cell protoplasts by MAC 207 and JIM8, respectively.
(B) and **(D)** Immunofluorescence of carrot suspension cell protoplasts with MAC 207 and JIM8, respectively.
(E) JIM8 immunogold micrograph of an oilseed rape sperm cell. Cell structures are given the following abbreviations: PMsc, sperm cell plasma membrane; PMvc, inner vegetative cell plasma membrane.
Bars for **(A)** through **(D)** = 50 μm. The bar for **(E)** = 1 μm.

abundantly, in the vegetative cell (Figure 2D). At this stage, the tapetum did not contain the JIM8 epitope and the microspore mother cells were no longer intact (Figure 2D). Finally, at the stage when the male gametophytes contained three cells, three anther cell types contained the JIM8 epitope: the endothecium (the next cell layer out from the middle layer, and multiseriate at its inner face) and both of the sperm cells in the pollen grains (Figures 2E and 2G). By this time, the middle layer in the anther wall did not contain the JIM8 epitope and was crushed (Figures 2E and 2G). This expression pattern persisted until anthesis.

Flowers in which anthesis was occurring contained immature ovules so that, at pollination, the embryo sacs contained two or four nuclei. It was at this stage that the plasma membrane JIM8 epitope was first detected in developing ovules. It was first in the nucellar epidermis close to the micropylar end of the ovule. Figure 3 shows that in mature ovules, the staining of the nucellar epidermis had extended to the chalazal end, and the egg cell and both synergids had also acquired the JIM8 epitope. In the synergids, small spots of cytoplasmic JIM8 epitope were found by immunogold electron microscopy to be associated with actively secreting Golgi stacks, apparently contributing to the filiform apparatus (Figure 3A).

Although zygotes were not discovered in any of the more than 50 embryo sacs labeled with JIM8, Figure 4 shows that embryos comprising between eight and several

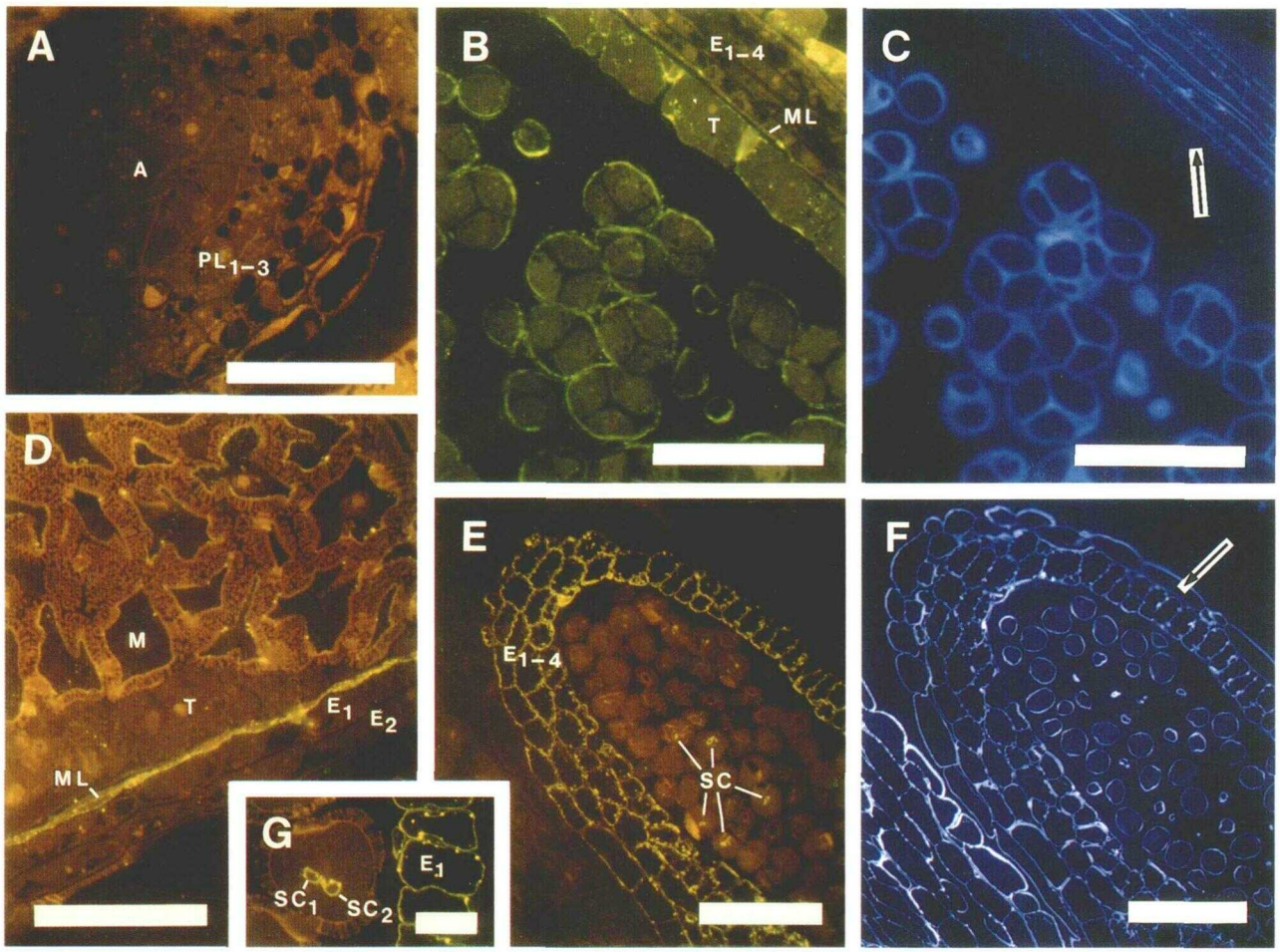

Figure 2. Temporal and Spatial Expression of the Plasma Membrane JIM8 Epitope in Developing Oilseed Rape Anthers.

(A) Part of a young anther primordium before first expression of the JIM8 epitope.
(B) Immunofluorescence of part of a developing anther at the end of meiosis.
(C) *Image shown in* **(B)** *stained with aniline blue.*
(D) Immunofluorescence of developing anther at the free microspore stage. Probaculae are present at the cell surface.
(E) Immunofluorescence of a mature anther containing tricellular male gametophytes.
(F) Image shown in **(E)** stained with aniline blue.
(G) Enlargement of pollen grain immunofluorescence.
Cell layers and cells are given the following abbreviations: A, archaesporium; E1 to E4, endothecium cell layers; M, microspore; ML, middle layer; PL1 to PL3, parietal cell layers; SC, SC1, and SC2, sperm cells; T, tapetum. In **(C)**, the arrow shows the position of the tapetum cells. In **(F)**, the arrow shows the position of the epidermis from which the JIM8 epitope is absent. Bars for **(A)** through **(F)** = 100 μm. The bar for **(G)** = 10 μm.

hundred cells were observed. Eight-celled embryos contained a two-celled embryo proper and a suspensor of six cells; in these embryos, all eight cells bound the JIM8 immunoglobulin (Figures 4A and 4B). In globular-stage embryos containing several hundred cells and a distinct protoderm, the embryo proper had lost the JIM8 epitope, even though it was still present in every cell of the suspensor (Figures 4C and 4D). In the young embryos, some JIM8 epitope was also associated with Golgi vesicles (Figure 4A).

This complex developmental timetable, in which a series of at least 10 cell types sequentially express and then lose the JIM8 epitope, is summarized in Figure 5.

Biochemical and Inhibition Analysis of the JIM8 Antigen

Immunoblotting of aqueous phase from Triton X-114-partitioned sugar beet leaf plasma membranes revealed that

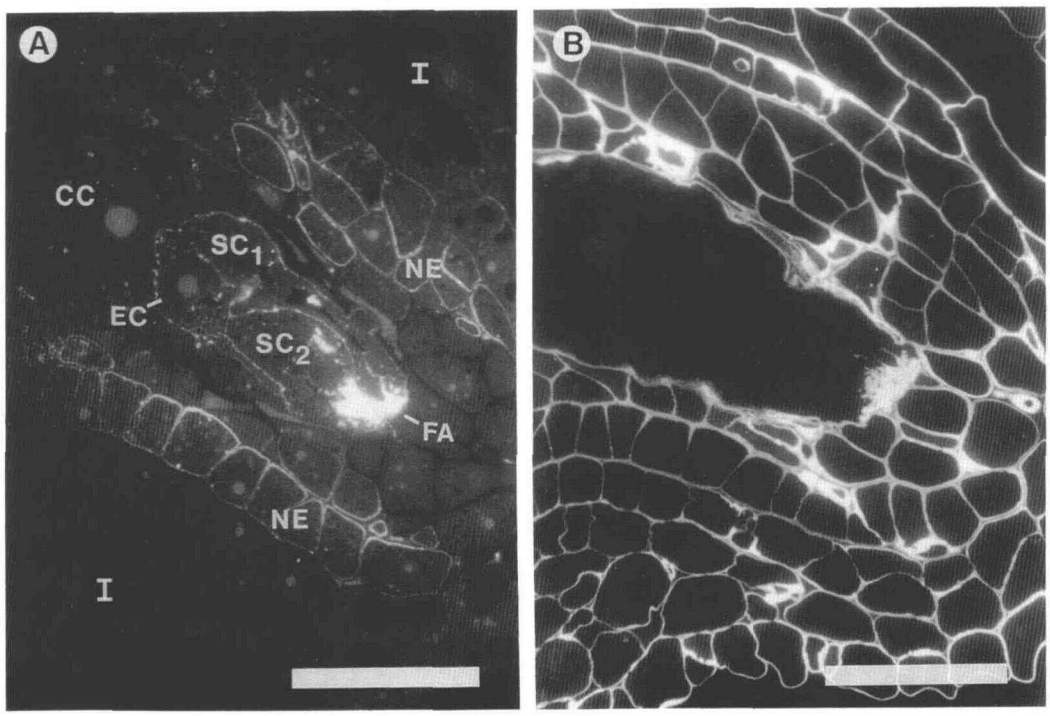

Figure 3. Spatial Expression of the JIM8 Epitope in Mature Oilseed Rape Ovules.

(A) JIM8 immunofluorescence.
(B) Image equivalent to that shown in **(A)** stained with aniline blue.
Cell layers, cells, and cell structures are given the following abbreviations: CC, central cell; EC, egg cell; FA, filiform apparatus; I, integument; NE, nucellar epidermis; SC1 and SC2, synergid cells. Bars in **(A)** and **(B)** = 100 μm.

MAC 207 reacted with four bands with approximate M_r values of 160,000, 104,000, 84,000, and 68,000, and that three of these bands (160,000, 84,000, and 68,000) also bound JIM8, as shown in Figure 6. Evidence from several blots demonstrated that the M_r 104,000 AGP species did not contain the JIM8 epitope. All JIM8 reactivity was vulnerable to oxidation by sodium metaperiodate but not to proteolysis. As shown in Table 2, only certain polysaccharides and glycoproteins inhibited JIM8 binding to gum arabic in an ELISA assay; many hapten monosaccharides and hapten disaccharides were tested but none was effective. The AGP gum arabic and the galacturonorhamnan gum karaya were equally potent inhibitors, reducing JIM8 binding by 50% (I_{50}) in subnanomolar concentrations. Two rhamnogalacturonans, RGI and RGII, were effective to a different extent: the I_{50} for RGI was approximately 50 nM (100 times less potent than gum arabic), and for RGII it was approximately 5 μM (100 times less potent than RGI). Inhibition by the galactomannan locust bean gum was equivalent to RGII. The secreted carrot AGP2 was active at >10 μM concentrations and, thus, only barely effective in the assay.

DISCUSSION

The MAb JIM8 Recognizes Plasma Membrane-Associated AGP

We resolved the identity of the JIM8 antigen by comparative immunoblotting of sugar beet leaf plasma membranes with MAbs MAC 207 and JIM8. MAC 207 is known to recognize an arabinose-containing AGP epitope and to be specific for AGPs (Pennell et al., 1989), so the MAC 207 blot in Figure 6 shows that sugar beet leaf plasma membranes contain four principle AGPs that partition with the aqueous phase during Triton X-114 separations. The JIM8 blot next to it thus demonstrates that three of these AGPs (M_r 160,000, 84,000, and 68,000) also express the JIM8 epitope. Because JIM8 does not bind to any other plasma membrane component, we conclude that the plasma membrane JIM8 antigens are all AGPs.

Sugar beet suspension cultures are recalcitrant in somatic embryogenesis and sugar beet flowers are small and complex, so we used carrot, a model somatic embryogenesis system (Nomura and Komamine, 1986), for

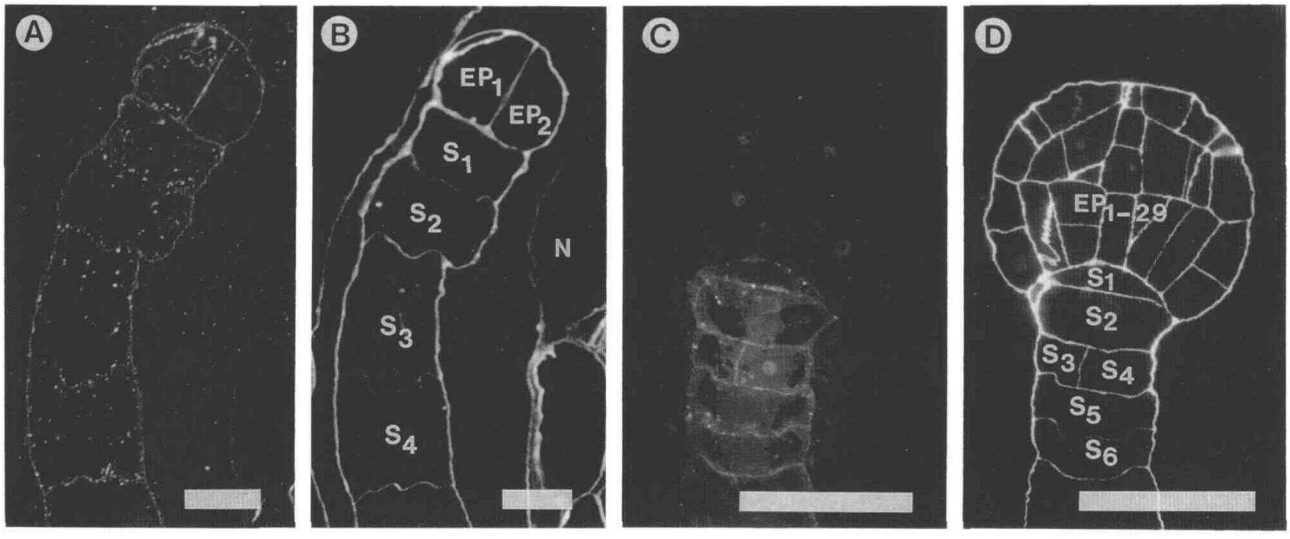

Figure 4. Temporal and Spatial Expression of the JIM8 Epitope at Two Stages of Oilseed Rape Embryo Development.

(A) Embryo proper containing two cells.
(B) Image shown in **(A)** stained with aniline blue.
(C) Globular stage embryo.
(D) Image shown in **(C)** stained with aniline blue.
Cells are given the following abbreviations: EP1 to EP29, embryo proper cells; N, inner nucellus cell layer; S1 to S6, suspensor cells. Numbers of suspensor cells do not imply cell lineage. Bars in **(A)** and **(B)** = 10 μm. Bars in **(C)** and **(D)** = 100 μm.

structural characterization of the plasma membrane JIM8 epitope and oilseed rape, which has big flowers, for developmental characterization. The results from our protoplast immunoagglutination and immunofluorescence experiments (together with the results from our immunoblotting) demonstrated that the JIM8 epitope was part of a peripheral plasma membrane antigen. Immunofluorescence and immunogold electron microscopy of cell sections confirmed this for intact carrot suspension culture cells, for oilseed rape endothecium cells (data not shown), and, as far as possible without recourse to isolations (Russell, 1991), for oilseed rape sperm cells. Electron microscopy also showed that the JIM8 epitope was associated with Golgi-derived vesicles in synergid cells and certain embryo cells.

Concerning the JIM8 epitope itself, our hapten inhibitions have failed to resolve its structure. However, because only galactose is common to both gum arabic and gum karaya (Anderson et al., 1967; Aspinall, 1969), their equivalent inhibitions suggest that the JIM8 epitope contains one or more galactose residues. The greater inhibition efficiency of the galactose-rich RGI in relation to the heterogeneous RGII (Fry, 1988) supports this view. The 3- and 6-linked galactose residues present in the side chains of gum arabic (Anderson et al., 1967) and gum karaya (Aspinall, 1969) are also present in other cell surface carbohydrates, however, indicating that the structural specificity of JIM8 is

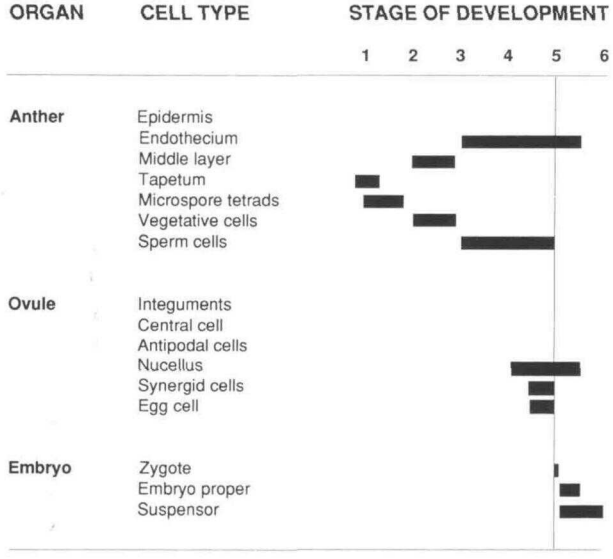

Figure 5. Schematic Developmental Regulation of Oilseed Rape Flower Plasma Membrane JIM8 Epitope.

Stage 1 shows microspore tetrads; stage 2, free microspores; stage 3, mature pollen; stage 4, anthesis; stage 5, fertilization; stage 6, globular stage embryo.

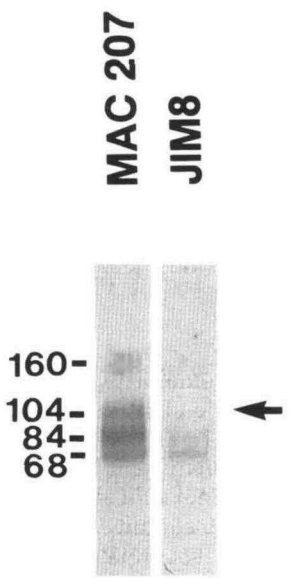

Figure 6. Protein Gel Immunoblots of Plasma Membrane JIM8 Antigen.

The two lanes contain 10 μg of the hydrophilic (peripheral) component of sugar beet leaf plasma membranes. The MAbs used for the immunoblots are designated by MAC 207 and JIM8. The numbers on the vertical axis denote approximate $M_r \times 10^3$. The arrow indicates the position of the AGP that does not contain the JIM8 epitope.

based not on an isomeric form of galactose alone but on galactose linked to arabinose, rhamnose, or the 4-*O*-methyl derivative of glucuronic acid, which are gum arabic constituents (Anderson et al., 1967).

Expression Patterns of the JIM8 Epitope

In our analysis of developing oilseed rape flowers, we were able to identify 12 cell types (scoring the sperm cells separately because they have different developmental fates in the embryo sac [Russell, 1985], as well as the cells of the suspensor and embryo proper) that at one stage or another consistently reacted with the JIM8 immunoglobulin. The zygote is probably the 13th. Prelignified xylem vessels are the 14th and only other cell type in which we have been able to observe the JIM8 epitope. Although the middle layer, endothecium, and embryo cells are somatic in origin, all the JIM8-reactive cells except the xylem vessels are associated with the process of gametogenesis and sexual reproduction. This pattern suggests a connection with class C floral homeotic genes (Coen and Meyerowitz, 1991), of which one, the Arabidopsis gene *AGAMOUS*, is expressed during late flower development in only certain floral cells. In the anther, these compose the endothecium, and in the ovule, they form the nucellus (Bowman et al., 1991). The presence of the JIM8 epitope in the xylem vessels appears be related to patterning of the vasculature (Knox et al., 1991).

When the floral expression pattern was mapped on to a time axis, the first cells to become reactive were approximately midway between the surface and center of the anther and ovule. Subsequently, the JIM8 epitope appeared in the adjacent cells and then in the cells adjacent to them, so that JIM8 reactivity spread radially toward the anther and ovule surface and toward the center. At each stage, the JIM8 epitope disappeared from the preceding cell type at approximately the same time or shortly thereafter.

In embryogenic carrot suspension cultures, almost all protoplasts contained the plasma membrane JIM8 epitope; those that were nonreactive probably were from dead cells or cell clusters. Thus, although the plasma membrane JIM8 epitope specifies certain cells and tissues in plants, it does not do this in embryogenic suspension cultures, even though the single cells in such cultures have different developmental fates (Nomura and Komamine, 1986). The unregulated expression of other plasma membrane AGP epitopes during the transition to suspension culture growth has also been reported (Knox et al., 1989, 1991). The coassembly of plasma membrane AGP epitopes that are developmentally regulated during tissue differentiation in cells growing in suspension cultures therefore implies that it is the temporal and spatial modulation of AGP epitopes that forms the basis of AGP function.

Table 2. Inhibition of JIM8 Binding by Polysaccharides and Arabinosylated Hydroxyproline-Rich Glycoproteins

Inhibiting Compound	I_{50}[a]
Gum karaya tree gum	420 pM[b]
Gum arabic tree gum	560 pM[c]
Sycamore RGI	56 nM[d]
Locust bean gum	3 mM[e]
Sycamore RGII	3 mM[f]
Carrot AGP2	12 mM[g]

[a] I_{50} was the concentration of inhibitor required to reduce binding of JIM8 to the antigen gum arabic by 50%.
[b] Calculated assuming an M_r of 9.5×10^6 (Sigma Chemical Co. catalog).
[c] Calculated assuming an M_r of 2.5×10^5 (Sigma catalog).
[d] Calculated assuming an M_r of 3.2×10^5 (assuming degree of polymerization of 1000 [Fry, 1988]).
[e] Calculated assuming an M_r of 3.1×10^5 (Sigma catalog).
[f] Calculated assuming an M_r of 1.1×10^5 (assuming degree of polymerization of 60 [Fry, 1988]).
[g] Calculated assuming an M_r of 8.5×10^5 (Pennell et al., 1989).

Flower Differentiation Is Accompanied by Complex Modulations of Plasma Membrane AGPs

In flowers, two kinds of plasma membrane AGPs can be identified: those like the MAC 207 AGP that define large cell collectives containing several tissues and those like the JIM8 AGP that specify positionally defined single tissues or single cell types. In carrot root apices, the JIM4 epitope identifies another kind of plasma membrane AGP that defines a small, position-specific cell collective (Knox et al., 1989). Evidently, expression of these types of AGPs is controlled precisely; the temporal and spatial expression programs are such that different AGP epitopes are expressed at different times or at the same time with different amounts of overlap. Thus, in mature flowers, the MAC 207 epitope specifies somatic cells (Pennell and Roberts, 1990), the JIM8 epitope certain sexual cells and certain somatic cells that surround them, and the combination of the MAC 207 and JIM8 epitopes only the endothecium.

Currently, there is little information on the chemical basis of AGP modulations during plant development. In tobacco suspension culture cells, a family of plasma membrane AGPs is elaborated by differential glycosylation from a single 50-kD protein (Norman et al., 1990). Our report describes that at least some AGP epitopes are labile, being expressed in and then lost from plasma membranes, and that in sugar beet leaves, the JIM8-reactive AGPs are a subset of those that bind MAC 207. Other blots demonstrate that other AGP epitopes are present in other AGP subsets (R.I. Pennell, unpublished observations). These findings indicate that AGP modulations are unlikely to be controlled transcriptionally or translationally, but depend on changes in AGP glycosylations that require the addition, loss, or isomerization of separate sugars. The biochemical analyses that are required to resolve this point are in progress.

Contiguous Cell Types on Radial Axes Express the JIM8 Epitope

The JIM8 epitope expression pattern we describe in this paper has two characteristics. First, it follows a complicated temporal sequence involving only contiguous cells, and second, it involves, at least transiently, many cell types. We do not know what this pattern defines. Although cell-inductive pathways have not been demonstrated in plants, the radial changes in the JIM8 epitope are consistent with a pathway of this kind. If present in oilseed rape flowers, and if identified by the JIM8 epitope, such a pathway begins with the tapetum in the anther and the nucellus in the ovule. In the anther, the pathway would first involve the microspore tetrads and the middle layer, next the vegetative cells in the pollen grains and the endothecium, and finally the sperm cells. In the ovule, the interaction would spread to the egg and synergid cells. At each stage of this model, the "inducing" cells lose the JIM8 epitope, giving rise to the patterns of JIM8 epitope expression characteristic of mature oilseed rape flowers. However, because certain cells, such as those of the epidermis, never express the JIM8 epitope, developing plant cells may differ in their competence to respond to the presumptive signal. This is suggested more clearly in Arabidopsis root apices, where an equivalent temporal pattern of plasma membrane AGP epitope expression follows an invisible boundary (K. Roberts, unpublished observations).

Plasma Membrane AGP Function

Plasma membrane AGPs are lectins (Pennell et al., 1989) whose ligands are thought to lie in the cell wall (Roberts, 1990). It is noteworthy that the cells that contain the plasma membrane JIM8 epitope also develop altered cell wall composition. For example, the tapetum cells lose their walls and disintegrate, the tetrads develop thickenings of callose, the cell walls are sloughed from the sperm cells during insemination of the embryo sac, and the xylem vessels become lignified. In this respect, angiosperm plasma membrane AGPs resemble mammalian substrate adhesion molecules (Edelman, 1986). However, the presence of the JIM8 epitope in the paired sperm cells and in the egg cell, but not in the central cell (which fuses with one sperm cell and gives rise to the endosperm), may suggest a role for plasma membrane AGPs in gametic recognition (Russell, 1985).

METHODS

Plant Material

The sugar beet (*Beta vulgaris*) suspension culture used for our immunization was derived from seedling explants. The carrot (*Daucus carota*) suspension cultures L1, used for production of our coimmunizing antiserum, and L2, used for purification of the extracellular AGP2, are described elsewhere (Knox et al., 1991). The carrot suspension culture used for the immunoagglutination, immunofluorescence, and immunogold electron microscopy was also derived from seedling explants. The sugar beet plants used for plasma membrane preparation were cultivar Hilma. The oilseed rape (*Brassica napus*) plants were cultivar Hanna. The carrot suspension cultures L1 and L2 were nonembryogenic, but the sugar beet suspension culture and the carrot suspension culture used for immunofluorescence displayed high embryogenic potential.

Suspension Culture Cells and Protoplast Preparation

We used PGo$_B$ medium (de Greef and Jacobs, 1979) supplemented with 2% (w/v) sucrose, 2 μM 2,4-D, and 1 μM benzylaminopurine to grow the sugar beet suspension culture, and B5

medium (Gamborg et al., 1970) supplemented with 2% (w/v) sucrose and 2 μM 2,4-D for the carrot suspension culture. Suspension culture cell protoplasts were prepared for immunization with Macerozyme R10 (Kinki Yakult, Nishinomiya, Japan), Rhozyme (Pollock and Poole Co., Reading, United Kingdom), and cellulase R10 (Yakult Honsha Co., Nishinomiya, Japan). Suspension culture cell protoplasts were prepared for immunoagglutination and immunofluorescence with Macerozyme and cellulase as described elsewhere (Pennell et al., 1989). All growth media were at pH 5.8 to 6.0. All suspension cultures were subcultured by volume fortnightly, and cells were used in experiments 7 days after subculture.

Preparation and Detergent Partitioning of Sugar Beet Plasma Membranes

Plasma membranes were prepared from sugar beet leaves by subjecting a preparation of microsomes to phase partitioning in a mixture of Dextran T500 (Pharmacia LKB Ltd., Uppsala, Sweden) and polyethylene glycol 3350 (Union Carbide) as described elsewhere (Kjellbom and Larsson, 1984). Hydrophobic and hydrophilic plasma membrane proteins were then separated by partitioning with Triton X-114 and water (Kjellbom et al., 1989), with the hydrophobic species entering the detergent phase and the hydrophilic entering the water phase.

MAbs

For MAb production, a 6-week-old female LOU/c rat was immunized intraperitoneally with 5×10^5 sugar beet protoplasts in 300 μL of sterile PBS to which had been added 250 μL of a rat antiserum prepared from an earlier immunization with carrot L1 protoplasts as described by Knox et al. (1989). Booster injections were performed with a preparation of 1.7×10^5 sugar beet protoplasts mixed with 150 μL of the rat antiserum on days 21 and 154. Splenectomy was performed on day 157. Fusion, selection in hypoxanthine/aminopterin/thymidine medium, and hybridoma culture were as described elsewhere (Galfrè and Milstein, 1981), using the myeloma IR983F (Bazin, 1982). Screening of hybridoma supernatants was by ELISA against gum arabic (Sigma) and frozen section immunofluorescence of carrot suspension cultures. The Ouchterlony assay revealed that JIM8 was an IgG2c.

MAC 207 is an MAb directed against a family of plasma membrane AGPs (Bradley et al., 1988; Pennell et al., 1989). For a negative control, we used a rat MAb raised against an antigen not present in plant cells.

Biochemical Analysis of JIM8 Antigen

Our SDS-PAGE of extrinsic components of sugar beet plasma membranes was performed by resolving 10 μg of protein in 8% to 18% gradient gels (Pharmacia LKB Ltd., Milton Keynes, United Kingdom). For immunoblotting, nitrocellulose blots were dried, washed with Tris-buffered saline, blocked with 2% (v/v) calf serum (Sigma) or 2% (w/v) dried milk, and incubated in the same blocking solution containing 1% (v/v) of JIM8 or MAC 207 hybridoma culture supernatant. After washing, horseradish peroxidase-conjugated and alkaline phosphatase-conjugated anti-rat immunoglobulin antisera (Sigma) were used to localize bound MAb.

Binding inhibition assay with glycoproteins and polysaccharides was performed by ELISA using the AGP gum arabic as the antigen, as described elsewhere (Pennell et al., 1989). Gum arabic was used because subnanomolar concentrations of it could be detected by JIM8. The inhibiting compounds were gum karaya (from *Sterculia urens*), gum arabic (from *Acacia senegal*), RGI and RGII (from *Acer psedoplatanus*), locust bean gum (from *Ceratonia siliqua*), and AGP2 (from *D. carota*). The gums were from Sigma, but the RGI and RGII were kindly supplied by Peter Albersheim and Alan Darvill (University of Georgia, Athens), and the carrot AGP2 was purified from carrot suspension culture L2-conditioned growth medium by electroelution from polyacrylamide gels (Pennell et al., 1989).

Immunoagglutination

For protoplast immunoagglutination, carrot protoplasts were resuspended in enzyme-free protoplasting medium, and JIM8 and MAC 207 culture supernatants were titrated into the mixture in the range of 0.2% to 10%. The protoplasts were agitated for 20 min and scored for agglutination by eye and with phase-contrast microscopy. An MAb against an antigen not present in plants was used in control experiments.

Fluorescence and Immunofluorescence Microscopy

Aniline Blue Fluorochrome (Biosupplies Australia Pty. Ltd., Parkville, Australia) was used as an aqueous 0.0001% solution to stain cell walls in resin sections. This fluorochrome stains β-(1,3)-linked glucans. Immunofluorescence of carrot suspension culture cell protoplasts was as described elsewhere (Pennell et al., 1989), using 2% solutions of JIM8 and MAC 207 culture supernatants. Immunofluorescence was performed on 0.5-μm-thick sections of oilseed rape floral organs cut from LR White resin (London Resin Co., Basingstoke, United Kingdom), also as described elsewhere (Pennell et al., 1989), using 2% solutions of JIM8 culture supernatant. An MAb against an antigen not present in plants was used in control experiments. A Zeiss Photomicroscope III was used for all photography.

Immunogold Electron Microscopy

High-resolution localization of the JIM8 antigen in carrot suspension culture cells and in oilseed rape anthers was performed on ultrathin sections prepared after low-temperature embedding in LR White resin as described elsewhere (Pennell et al., 1989), and sections were viewed with a Jeol JEM-1200 EX electron microscope operating at 80 kV.

ACKNOWLEDGMENTS

We thank Hilleshog AB (Lund, Sweden) for the sugar beet plants and Peter Albersheim and Alan Darvill (University of Georgia, Athens) for the rhamnogalacturonans. We also thank Paul Knox,

Jeremy Hyams, and Peter Bell for comments on the manuscript. The research was supported by awards from the European Economic Community, Danisco A/S (Copenhagen, Denmark), the Akademiet for de Tekniske Videnskaber (Lyngby, Denmark), the Swedish Council for Forestry and Agriculture Research, and the Royal Society of London.

Received August 9, 1991; accepted October 14, 1991.

REFERENCES

Anderson, D.M.W., Hirst, E., and Stoddart, J.F. (1967). Structural studies on uronic acid materials. 27. Some structural features of *Acacia senegal* gum (gum arabic). J. Chem. Soc. Section C, 1476–1486.

Aspinall, G.O. (1969). Gums and mucilages. Adv. Carbohydr. Chem. Biochem. **24,** 333–379.

Barclay, S.L., and Smith, A.M. (1986). Rapid isolation of monoclonal antibodies specific for cell surface differentiation antigens. Proc. Natl. Acad. Sci. USA **83,** 4336–4340.

Bazin, H. (1982). Production of rat monoclonal antibodies with the LOU rat non-secreting IR983F myeloma cell line. Prot. Biol. Fluids **29,** 615–618.

Bell, P.R. (1989). The alternation of generations. Adv. Bot. Res. **16,** 55–93.

Bowman, J.L., Drews, G.N., and Meyerowitz, E.M. (1991). Expression of the Arabidopsis floral homeotic gene *AGAMOUS* is restricted to specific cell types late in flower development. Plant Cell **3,** 749–758.

Bradley, D.J., Wood, E.A., Larkins, A.P., Galfrè, G., Butcher, G.W., and Brewin, N.J. (1988). Isolation of monoclonal antibodies reacting with peribacteroid membranes and other components of pea root nodules containing *Rhizobium leguminosarum*. Planta **173,** 149–160.

Coen, E.S. (1991). The role of homeotic genes in flower development and evolution. Annu. Rev. Plant Physiol. Plant Mol. Biol. **42,** 241–279.

Coen, E.S., and Meyerowitz, E.M. (1991). The war of the whorls: Genetic interactions controlling flower development. Nature **353,** 31–37.

de Greef, W., and Jacobs, M. (1979). In vitro culture of the sugar beet: Description of a cell line with high regeneration capacity. Plant Sci. Lett. **17,** 55–61.

Drews, G.N., Bowman, J.L., and Meyerowitz, E.M. (1991). Negative regulation of the Arabidopsis homeotic gene *AGAMOUS* by the *APETALA2* product. Cell **65,** 991–1002.

Edelman, G.M. (1986). Cell adhesion molecules in the regulation of animal form and tissue pattern. Annu. Rev. Cell Biol. **2,** 81–116.

Esau, K. (1977). The flower: Reproductive cycle. In Anatomy of Seed Plants, 2nd ed. (New York: Wiley), pp. 403–407.

Fry, S.C. (1988). Wall polymers: Chemical characterisation. In The Growing Plant Cell Wall: Chemical and Metabolic Analysis. (London: Longman), pp. 102–188.

Galfrè, G., and Milstein, C. (1981). Preparation of monoclonal antibodies. Methods Enzymol. **73,** 3–46.

Gamborg, O.L. (1970). The effects of amino acids and ammonium on the growth of plant cells in suspension culture. Plant Physiol. **45,** 372–375.

Goldberg, R.B. (1988). Plants: Novel developmental processes. Science **240,** 1460–1467.

Kjellbom, P., and Larsson, C. (1984). Preparation and polypeptide composition of chlorophyll-free plasma membranes from leaves of light-grown spinach and barley. Physiol. Plant. **62,** 501–509.

Kjellbom, P., Larsson, C., Rochester, C.P., and Andersson, B. (1989). Integral and peripheral proteins of the spinach leaf plasma membrane. Plant Physiol. Biochem. **27,** 169–174.

Knox, J.P., Day, S., and Roberts, K. (1989). A set of cell surface glycoproteins forms an early marker of cell position, but not cell type, in the root apical meristem of *Daucus carota* L. Development **106,** 47–56.

Knox, J.P., Linstead, P.J., Peart, J., Cooper, C., and Roberts, K. (1991). Developmentally regulated epitopes of cell surface arabinogalactan proteins and their relation to root tissue pattern formation. Plant J. **1,** 317–326.

Nomura, K., and Komamine, A. (1986). Molecular mechanisms of somatic embryogenesis. Oxf. Surv. Plant Mol. Cell. Biol. **3,** 456–466.

Norman, P.M., Kjellbom, P., Bradley, D.J., Hahn, M.G., and Lamb, C.J. (1990). Immunoaffinity purification and biochemical characterization of plasma membrane arabino-galactan-rich glycoproteins of *Nicotiana glutinosa*. Planta **181,** 365–373.

Pennell, R.I., and Roberts, K. (1990). Sexual development in the pea is presaged by altered expression of arabinogalactan protein. Nature **344,** 547–549.

Pennell, R.I., Knox, J.P., Scofield, G.N., Selvendran, R.R., and Roberts, K. (1989). A family of abundant plasma membrane-associated glycoproteins related to the arabinogalactan proteins is unique to flowering plants. J. Cell Biol. **108,** 1967–1977.

Roberts, K. (1990). Structures at the plant cell surface. Curr. Opin. Cell Biol. **2,** 920–928.

Russell, S.D. (1985). Preferential fertilization in *Plumbago*: Ultrastructural evidence for gamete-level recognition in an angiosperm. Proc. Natl. Acad. Sci. USA **82,** 6129–6132.

Russell, S.D. (1991). Isolation and characterization of sperm cells in flowering plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **42,** 189–204.

Sussex, I.M. (1989). Developmental programming of the shoot apex. Cell **56,** 225–229.

Walbot, V. (1985). On the life strategies of plants and animals. Trends Genet. **1,** 165–169.