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Arabinogalactan proteins at the cell surface of *Brassica* sperm and *Lilium* sperm and generative cells

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Abstract Antibodies to arabinogalactan proteins were tested for binding to sperm cells of *Brassica campestris* and to generative cells and sperm of *Lilium longiflorum*. Two monoclonal antibodies, JIM8 and JIM13, bound to *Brassica* sperm in pollen grains and pollen tubes and to isolated sperm. Sperm pairs retained within the vegetative cell inner plasma membrane fluoresced more brightly than single sperm, indicating that the vegetative cell inner plasma membrane that surrounds sperm pairs also contains arabinogalactan proteins. Isolated sperm pairs exhibited a uniform fluorescence while single sperm had patches of fluorescence. In *Lilium*, isolated generative cells and single sperm cells bound antibodies in a patchy pattern. Antibodies to arabinogalactan proteins may be useful in describing the overall shape of sperm cells and for identifying sperm among other cell types.

Key words Arabinogalactan proteins · *Brassica campestris* · Generative cell · JIM8 · JIM13 ·*Lilium longiflorum* · Sperm (plant)

Introduction

Fertilization in flowering plants, as in other organisms, involves fusion of egg and sperm plasma membranes. The mechanism of fusion, particularly the membrane components responsible, is unknown in plants. Two alternative hypotheses may explain egg-sperm fusion (Knox et al. 1988). Gametes may fuse because they have unique recognition molecules on their membranes which enable them to interact. This would parallel the situation in animal cells. Alternatively, they may fuse because they are the only cells without cell walls brought together in an environment that promotes fusion, a situation parallel to fusion of somatic protoplasts. The actual situation may include gamete-specific molecules plus non-specific membrane interactions. In tests of in vitro fusions, 80% of sperm-egg pairs fused, 2% of sperm-mesophyll protoplast pairs fused, and 18% of two-sperm pairs fused (Faure et al. 1994). Egg cells did not fuse with each other or with mesophyll protoplasts. These results suggest some specificity of gamete cell surfaces.

Antibodies have been raised to isolated sperm cells of *Plumbago zeylanica* (Pennell et al. 1987) and *Brassica campestris* (Hough et al. 1986). The antibodies were not characterized with regard to specific epitope, and none of the cell lines survive. Of 195 stable hybridomas to *P. zeylanica* sperm, 46 reacted exclusively with a sperm cell fraction rather than with pollen tube cytoplasm (Pennell et al. 1987). Further specificity to particular regions of sperm or to specific epitopes was not determined.

A monoclonal antibody raised to protoplasts from sugar beet suspension-culture cells reacted with surface regions of sperm cells in thin sections of pollen grains (Pennell et al. 1991). This antibody, JIM8, was specific to arabinogalactan protein (AGP), both membrane-bound and secreted. In chemically fixed thin sections of *B. napus* pollen, immunogold particles were found near the sperm plasma membrane, in the space between sperm and vegetative cell and near the vegetative cell inner plasma membrane (Pennell et al. 1987). In rapidly frozen and freeze-substituted pollen of *Arabidopsis thaliana*, immunogold particles bound to JIM8 were found in the intermembrane zone between sperm and vegetative cells (Van Aelst and Van Went 1992). While JIM8 is not specific to sperm cells, it is the first antibody that potentially recognizes molecular features of sperm cell membranes.

Other AGPs may be developmentally regulated (Knox et al. 1991). A monoclonal antibody, JIM13, recognized AGP2, a plasma membrane component. It was unreactive with root meristem cells but reacted with developing epidermis, root cap and pre-xylem tissues. Another monoclonal antibody, MAC207, recognized an arabinose-containing epitope in flower buds; however, this epitope was specifically not expressed by meiocytes and tapeta, nor by generative cells and sperm (Pennell and Roberts 1990).

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In pollen grains and pollen tubes, the inner membrane of the vegetative cell is closely appressed to the surface of sperm or generative cells (Southworth 1992). This prevents precise localization of antibody binding by immunogold labelling of thin sections. We tested binding of JIM8 to isolated sperm cells to determine whether antibody binding persists on free sperm cells that have been washed. This would distinguish binding between soluble, secreted AGPs and membrane-bound AGPs. In addition, we tested binding of JIM13, a second AGP antibody, and MAC207, an antibody that did not bind to sperm cell surfaces (Van Aelst and Van Went 1992). Monoclonal antibodies JIM8, JIM13 and MAC207 were tested for binding to isolated sperm cells, sperm cells in pollen tubes and leaf protoplasts of *B. campestris*, and generative cells and sperm of *Lilium longiflorum*.

Materials and methods

Plant material

Brassica campestris L. (syn., *B. rapa* L., Wisconsin Fast Plants) plants were grown in a greenhouse. Pollen was collected daily, dried overnight at 4°C over NaOH pellets in an airtight dish and stored at –14°C for up to 6 months. *Lilium longiflorum* L. cultivar Ace was grown in the greenhouse. Bulbs were a gift from the Oregon Lily Bulb Growers Cooperative. Anthers were removed from buds collected the day before opening, air dried aseptically for 2–3 days and stored for up to 1 year at –14°C.

Pollen germination

Brassica campestris pollen was germinated in media containing 1.3 mM $Ca(NO_3)2$, 1.0 mM KNO_3, 0.8 mM $MgSO_4$ and 1.6 mM H_3BO_3 (Brewbaker and Kwack 1963) with 0.6 M sucrose, and buffered to pH 8.5 with 20 mM TRIS for sperm isolation or brought to pH 8.5 with NH_4OH for extended pollen tube growth and antibody incubation within the pollen tube. Pollen (10 mg) was floated on 4 ml medium in 5-cm glass petri dishes on a reciprocal shaker at 21°C for 2 h on sperm isolation medium or 3 h on pollen tube growth medium. Over 50% of *B. campestris* pollen germinated after 2 h on sperm isolation medium. After 3 h incubation on pollen tube growth medium, *B. campestris* sperm had moved into pollen tubes.

Lilium longiflorum pollen was germinated in medium containing 1.3 mM $Ca(NO_3)_2$, 1.0 mM KNO_3, 0.16 mM H_3BO_3 and 0.3 M sucrose (Dickinson 1965). Aseptic lily anthers were floated, four per flask on 25 ml medium, on a reciprocal shaker at 30°C for 6 h and 21°C thereafter. After 6 h incubation in Dickinson's medium, *L. longiflorum* generative cells had moved into pollen tubes.

Brassica sperm isolation

Sperm cells were isolated by hypo-osmotic shock. Osmoticum was reduced by adding 1.5 ml H_2O followed in 45 sec by one plunge of the pestle in a 10-ml Teflon glass tissue grinder. Osmoticum was immediately returned to 0.6 M by addition of 1.5 ml of 1.2 M sucrose. Sperm were filtered through a 10-μm nylon screen and centrifuged at 300 *g* for 10 min. Sperm concentration was determined by staining with fluorescein diacetate (FDA) and with 4′,6-diamidino-2-phenylindole (DAPI, 0.01 mg/ml) and counting on a hemacytometer. FDA (0.2 g/ml) was dissolved in acetone and stored at –14°C. A working solution was prepared by adding 2 μl of the stock to 100 μl of phosphate-buffered saline (PBS); 1 μl of working solution was used to stain 10 μl of cell suspension. Because of the sensitivity of *Brassica* sperm to osmotic shock, double-strength (×2) PBS was used.

The pellet containing $1–2\times10^5$ sperm was resuspended in sperm isolation medium with 10% normal goat serum. Some samples were fixed for 15 min in freshly prepared 4% paraformaldehyde in ×2 PBS (Harlow and Lane 1988) and rinsed twice in ×2 PBS before resuspending in 10% normal goat serum in ×2 PBS.

Lilium generative and sperm cell isolation

Generative and sperm cells of *Lilium* were isolated after 6 h (generative cells) or 30 h (generative cells and sperm) and after 54 h or 72 h (sperm) of germination (Southworth et al. 1992). Osmotic shock by addition of an equal volume of H_2O (25 ml) was followed by one plunge of the pestle of a 25-ml glass-Teflon tissue grinder (6 h incubation) or by shaking on ice for 30 min (30–72 h incubation). Osmoticum was returned to 0.3 M sucrose by addition of 12.5 ml of 1.2 M sucrose. Generative cells and sperm were filtered through a 62 μm nylon screen to remove pollen and pelleted at 300 *g* for 10 min. Cells were fixed in 4% paraformaldehyde as with *B. campestris* but in ×1 PBS.

Permeablization of pollen tubes to antibodies

Pollen tubes were fixed for 30 min in 4% paraformaldehyde in ×2 PBS and rinsed three times. Pollen tubes from 1–5 mg pollen were incubated in 500 units of recombinant yeast lytic enzyme, B-1,3-glucanase (ICN Biochemicals, Costa Mesa, Calif.) for 30–60 min on a rotator (Southworth and Jernstedt 1995). Tube wall digestion was monitored by microscopic observation. Pollen was rinsed once in ×2 PBS, resuspended in methanol at –14°C for 10 min, and rinsed with PBS and with 10% normal goat serum (NGS) in PBS.

Antibody binding

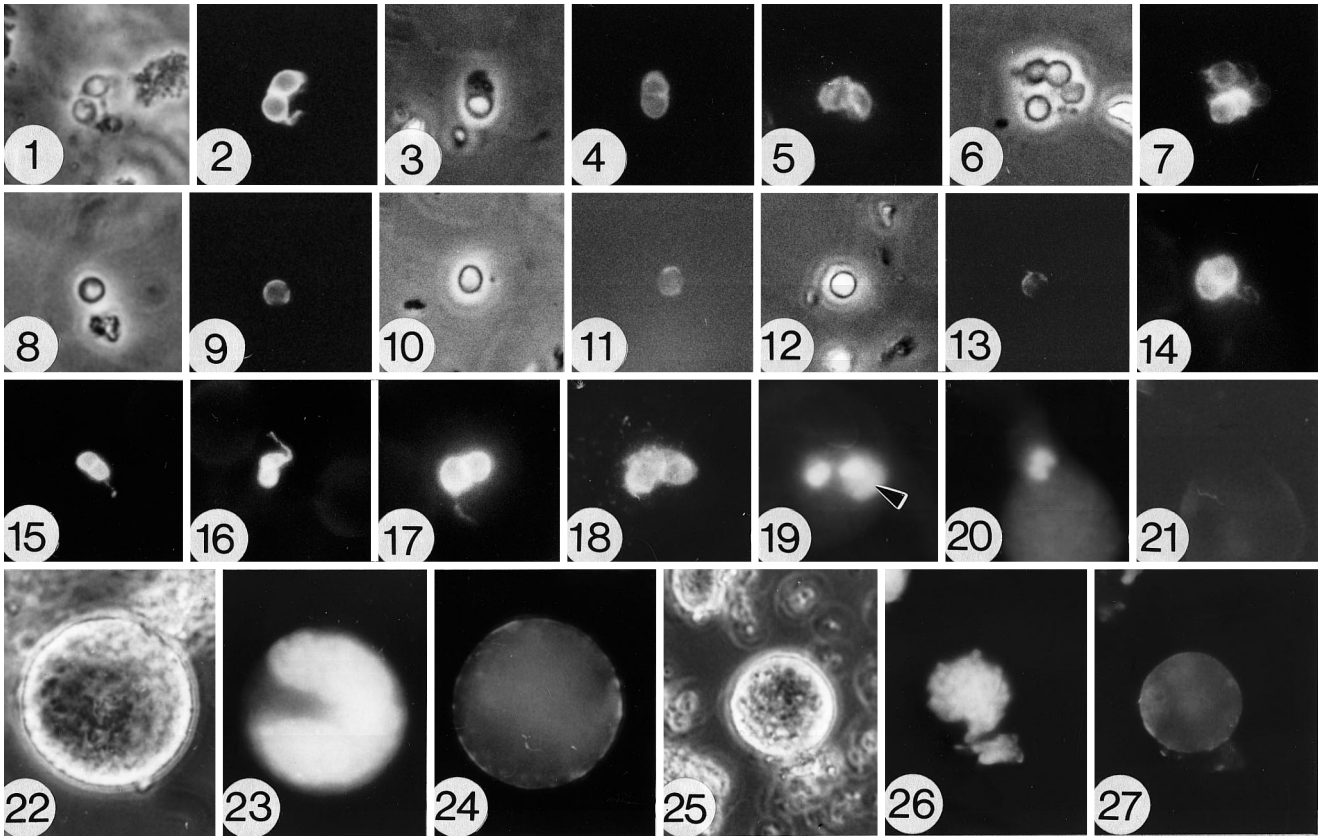
Monoclonal rat antibodies JIM8, JIM13 and MAC207 were obtained from Dr. Keith Roberts, (John Innes Institute, Norwich NR4 7UH, UK). Bound antibodies were detected with fluorescein isothiocyanate-(FITC) labeled goat anti-rat IgG (H+L) (Southern Biotechnology, Birmingham, Ala.). Hybridoma supernatant (50 μl) was added to $1–5\times10^4$ cells and incubated for 45 min at 21°C for isolated sperm and generative cells or overnight at 4°C for pollen and pollen tubes. Sperm and generative cells were washed with 10% NGS and pelleted at 300 *g*. Cells were incubated for 45 min at 21°C in secondary antibody (FITC-labeled goat anti-rat IgG). Pollen grains and pollen tubes were incubated for 3 h at 21°C. Cells were washed and pelleted as before, stained with DAPI and observed on a UV-equipped Leitz Ortholux II microscope. *Brassica* sperm were tested fixed and unfixed, but *Brassica* pollen tubes and *Lilium* sperm and generative cells were fixed. Controls lacked first antibody. MAC207 constituted a negative control.

Results

Antibody binding to *Brassica* sperm

Sperm cells in pollen grains and pollen tubes and isolated sperm cells bound JIM8 and JIM13 in similar patterns, but did not bind MAC207 (Figs. 1–27). Fixation did not alter antibody binding on isolated sperm.

Several patterns of JIM13 binding were observed on isolated sperm. Where sperm cell pairs remained together through the isolation procedure, the entire sperm surface,



Figs. 1–21 *Brassica campestris* sperm cells: **1–13** Sperm cells labeled with JIM13 antibody. **1, 2** Phase contrast and UV excitation of fluorescein isothiocyanate (FITC). Isolated sperm cell pair with labeled extension. **3, 4** Phase contrast, UV excitation of FITC. Isolated sperm cell pair without extension. **5** UV excitation of FITC. Sperm cell pair in pollen tube. **6, 7** Phase contrast and UV excitation of FITC. Isolated sperm cells clumped. **8–13** Pairs of phase contrast and UV images of isolated sperm cells with uneven FITC labeling around perimeter of cell. **8, 9** Sperm with a relatively large unlabeled cap. **10, 11** Sperm with two zones lacking antibody binding. **12, 13** Sperm cell with relatively large unlabeled cap. **14–17** UV excitation of FITC bound to JIM8 antibody on sperm cell pairs in fixed pollen grains. Fluorescence surrounds both sperm cells and cellular extensions that link sperm cells to vegetative nuclei. **14** Extension coiled. **15, 16** Extensions brighter at tip. **17** Curved extension without bright tip. **18, 19** FITC bound to JIM8 antibody and DAPI-labeled sperm cell pair in pollen tube. Vegetative nucleus (*arrowhead*) fluorescing with DAPI in 19 is not labeled with JIM8. **20, 21** UV excitation of DAPI and FITC to MAC207 antibody. Sperm nuclei fluoresce with DAPI, but sperm surfaces are unlabeled with MAC207. **22–24** *Lilium* generative cells isolated from pollen tubes after 6 h incubation. **22** Phase contrast. **23** UV excitation of DAPI. Nucleus in prophase. **24** UV excitation of FITC. JIM8 antibody bound to surface of generative cell. **25–27** *Lilium* sperm cells isolated from pollen tubes after 30 h incubation. **25** Phase contrast. **26** UV excitation of DAPI. Nucleus in late telophase. **27** UV excitation of FITC. JIM13 antibody bound to surface of generative cell. ×1000

including the cellular extension and contact zone between sperm cells, fluoresced uniformly (Figs. 1, 2). Often no cellular extension remained (Figs. 3, 4). In the pollen tube, sperm cells were more separated, and fluorescence was similar to that of isolated sperm pairs (Fig. 5).

Single isolated sperm cells bound JIM13 in a patchy pattern over the cell surface (Figs. 8–13). Fluorescence of single sperm was less than that of sperm pairs. Sperm cells were spheroidal and no structural evidence of sperm attachments or cellular extensions remained (Figs. 8, 10, 12). Some parts of the cell surface did not bind antibodies (Figs. 9, 11, 13). Surface zones without fluorescence formed a cap (Figs. 9, 13). Some isolated sperm formed clumps in presence of JIM13.

The pattern of JIM8 binding to isolated sperm was similar to that of JIM13 binding. JIM8 bound to the surface of paired sperm cells in pollen grains (Figs. 14–17). Antibodies fluoresced in the contact zone between sperm cells and over the entire surface. The cellular extension associated with the vegetative nucleus also fluoresced, sometimes with a bright area at the tip distal to the sperm pair (Figs. 15, 16). The pattern continued on sperm cells within pollen tubes (Figs. 18. 19). No fluorescence was associated with the vegetative nucleus. No fluorescence was observed at the pollen plasma membrane.

Antibody binding to *Lilium* generative cells and sperm

Isolated *Lilium* generative cells in early prophase from 6 h- and 30 h-pollen tubes and sperm cells from 30 h- and 72 h-pollen tubes showed surface staining from JIM8 and JIM13 in patches on the cell surface (Figs. 22–27). Nuclei and other cytoplasmic components of generative and sperm cells were not stained with antibodies. The

surface pattern was similar for generative cells and sperm and did not correlate with location of the nucleus. Sperm pairs were not found in isolated sperm preparations in *Lilium*.

Discussion

Persistence of antibody binding through washes necessary for immunolocalization procedures provided evidence that AGP epitopes are membrane components of sperm and generative cells and not in an extracellular matrix or cell wall compartment. AGPs from extracellular matrices were highly soluble, enabling them to be purified and analyzed (Showalter 1993). AGPs from plasma membranes occurred during reproductive development (Pennell and Roberts 1990; Pennell et al. 1991; Pennell 1992). The presence of AGPs on sperm cell surfaces did not indicate the existence of a cell wall around sperm.

The relatively greater brightness of sperm pairs than single isolated sperm suggested that AGP epitopes are also components of the vegetative inner membrane that surrounds sperm pairs in pollen grains and pollen tubes. The presence of two membranes, both fluorescing, would be responsible for the greater brightness.

Similarity of antibody binding to sperm in pollen grains and pollen tubes and to free sperm indicated that, within this limited developmental time frame, these epitopes do not change. Although morphology of sperm changed when released, surface properties were retained.

Patchiness of fluorescent antibody distribution indicated a non-uniform cell surface, at least in isolated sperm. Freeze-fracture of isolated sperm of *P. zeylanica* yielded fracture faces with patches of hexagonal arrays, also indicating specialized domains (Southworth et al., unpublished). Isolated *Brassica* sperm retained a circular raised area corresponding in size to a narrow bridge at the site of contact between two sperm (Taylor et al. 1991).

In serial thin sections, *Brassica* sperm have been shown to be dimorphic in size and organelle content (McConchie et al. 1987). The sperm with the extension that associates with the vegetative nucleus are slightly larger and contain more mitochondria. Binding of AGP antibodies indicated no major dimorphism of sperm surfaces with respect to these epitopes.

Serial reconstruction of the *B. campestris* male germ unit, the two sperm cells plus the associated vegetative nucleus, showed that the extension of the larger sperm was entwined through embayments in the vegetative nucleus (McConchie et al. 1985, 1987). The sperm was always separated from the nucleus by the vegetative cell inner membrane. At the tip of the extension was an expanded region or branched end similar to that seen with JIM8 and JIM13 antibodies to sperm in pollen grains.

These are the first specific epitopes recognized on plant sperm cell surfaces. Antibodies to AGPs may be particularly useful in identifying sperm cells in cell pellets or in tissue sections. They are useful in describing the overall shape of sperm cells.

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