Identification of a Transitional Cell State in the Developmental Pathway to Carrot Somatic Embryogenesis

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Abstract. We have located a novel carbohydrate epitope in the cell walls of certain single cells in embryogenic, but not in non-embryogenic, suspension cultures of carrot. Expression of this epitope, recognized by the mAb JIM8, is regulated during initiation, proliferation, and prolonged growth of suspension cultures such that changes in the abundance of JIM8-reactive cells always precede equivalent changes in embryogenic potential. Therefore, a direct correlation exists between the presence of the JIM8-reactive cell wall epitope and somatic embryo formation. The JIM8-reactive cell wall epitope is expressed in the cell walls of three types of single cells and one type of cell cluster. One of the single cell types seems able to follow one of two phytohormone-controlled developmental pathways, either a cell elongation pathway that eventually leads to cell death, or a cell division pathway that gives rise to proembryogenic masses. We demonstrate that all JIM8-reactive cell types in embryogenic carrot suspension cultures are developmentally related, and that the switch by one of them to somatic embryogenesis is accompanied by the immediate dissipation of the JIM8-reactive cell wall epitope. The cell wall carbohydrate epitope recognized by JIM8 therefore represents a cell wall marker for a very early transitional cell state in the developmental pathway to carrot somatic embryogenesis.

Somatic cells of many plant species can be cultured and induced to form embryos that are able to develop into mature plants. This process, termed somatic embryogenesis, was originally described in carrot (Steward et al., 1958*a*). With the exception of the suspensor, carrot somatic embryos at different stages of development (termed globular, heart, torpedo, and cotyledonary stages) are structurally similar to their zygotic counterparts (Halperin, 1964; Steeves and Sussex, 1989), and because several gene expression and protein synthesis programs are also identical in the two systems (Crouch, 1982; Choi et al., 1987; Franz et al., 1989; Perez-Grau and Goldberg, 1989; Sterk et al., 1991), it has been postulated that similar molecular mechanisms drive plant embryogenesis both in vivo and in vitro. The ease with which somatic embryos are obtained therefore recommends their use for both the structural (Schiavone and Racusen, 1990; 1991) and biochemical (de Vries et al., 1988*a*; Komamine et al., 1990; Cordewener et al., 1991; Dudits et al., 1991; de Jong et al., 1992) analysis of plant development.

Explants of all parts of a carrot plant can be induced to establish embryogenic suspension cultures. These cultures contain a heterogeneous but stereotyped array of several different types of single cells and cell clusters (Steward et al., 1958*b*; Backs-Hüsemann and Reinert, 1970; Nomura and Komamine, 1985), all of which proliferate and persist for the duration of the embryogenic potential. Somatic embryos usually form, after appropriate manipulation of cell density and of exogenous growth regulators, from specific surface cells in a particular type of cell cluster termed a proembryogenic mass, which only occur in embryogenic suspension cultures (Halperin and Wetherell, 1965). Different manipulations can induce small single cells termed type 1 cells to form somatic embryos (Nomura and Komamine, 1985), but these require a pre-culturing period during which they probably first give rise to small proembryogenic masses (Komamine et al., 1990).

Because proembryogenic masses develop from single cells (Backs-Hüsemann and Reinert, 1970; Nomura and Komamine, 1986; van Engelen and de Vries, 1992), it is essential to obtain reliable molecular markers with which the precursor single cell types can be identified and obtained. We have investigated the possibility that cells in the developmental pathway to carrot somatic embryogenesis express specific cell wall surface molecules, that can be localized with mAbs, during the transitional period when cells derived from tissue explants acquire embryogenic potential and develop into proembryogenic masses. In plant tissues, such epitopes are

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formed by the progressive modification of plasma membrane-associated arabinogalactan proteins (AGPs)[1] in response to inductive signals (Pennell and Roberts, 1990; Knox et al., 1991; Pennell et al., 1991). Because the epitopes involved in these modifications are glycans, we have looked for similar epitopes in the cell walls of carrot cells present specifically in embryogenic suspension cultures. One such epitope, recognized by the mAb JIM8 (Pennell et al., 1991) is described in this paper, and we discuss its developmental regulation and its significance for carrot somatic embryogenesis and plant development generally.

Materials and Methods

Carrot Suspension Cultures

The carrot (*Daucus carota* L.) cultivar Trophy was used to establish the wild-type suspension culture (X and FG) 10 (de Vries et al., 1988*a*), the cultivar Early Nantes was used for the wild-type cultures S6 (Pennell et al., 1991) and L1 (Lloyd et al., 1979), and the cultivar San Valery was used to establish the wild-type culture A^+ and the temperature-sensitive mutant culture ts11 (LoSchiavo et al., 1985). The suspension cultures were initiated and maintained in the following way: 50 carrot seeds were surface sterilized with ethanol and bleach, and germinated on sterile agar containing B5 culture medium (Gamborg, 1970) and 2% (vol/vol) sucrose. Hypocotyl segments were cut into 5-mm pieces and aseptically transferred to 10 ml of B5 growth medium supplemented with 2% sucrose and 2 μM 2,4-D, pH 6.0, and the released cells were allowed to proliferate in this medium. The suspension cultures formed in this way were sub-cultured biweekly and sampled 7 d after sub-culture for immunofluorescence. To promote the development of somatic embryos, the 50–125-μm size fraction present in suspension cultures, containing the proembryogenic masses, was transferred to hormone-free B5 growth medium. Somatic embryos developed in this medium, and gave rise to embryo cultures. For experiments with single cells, the <30-μm size fraction was transferred to B5 culture medium supplemented with 2% (wt/vol) sucrose and 0.05 μM 2,4-D, 1 μM zeatin, and 200 mM mannitol according to Nomura and Komamine (1985), so that the cell density was 2×10^4 cells ml^{-1}. The ts11 cultures required special handling, as described in LoSchiavo et al. (1985, 1990).

mAbs

The mAb JIM8 was originally developed from an immunization with sugar beet protoplasts (Pennell et al., 1991). JIM8 recognizes a carbohydrate epitope present in three plasma membrane arabinogalactan proteins in sugar beet leaves (Pennell et al., 1991) and an extracellular AGP secreted by carrot suspension cultures (Knox et al., 1991). The mAb JIM5 was developed from an immunization with carrot protoplasts, and recognizes unesterified homogalacturonan (Knox et al., 1990).

1. *Abbreviations used in this paper*: AGP, arabinogalactan protein; GRP, glycine-rich protein; HRGP, hydroxyproline-rich protein; PAS, periodic acid-Schiffs reagent; PEM, proembryogenic mass; PRP, proline-rich protein; RG, rhamnogalacturonan.

Table I. Wild-type and Mutant Carrot Suspension Culture Presence of JIM8-reactive Cell Wall Epitope

Suspension culture	Relative embryogenic potential	Percent of JIM8-reactive single cells
A^{+}*	100	16
X10‡	100	16
S6§	100	16
FG10‡	0	0
L1‖	0	0
ts11*	<1	0
ts11R**	20	2

* LoSchiavo et al., 1985; ‡ de Vries et al., 1988*a*; § Pennell et al., 1991; ‖ Lloyd et al., 1979; ** LoSchiavo et al., 1990.

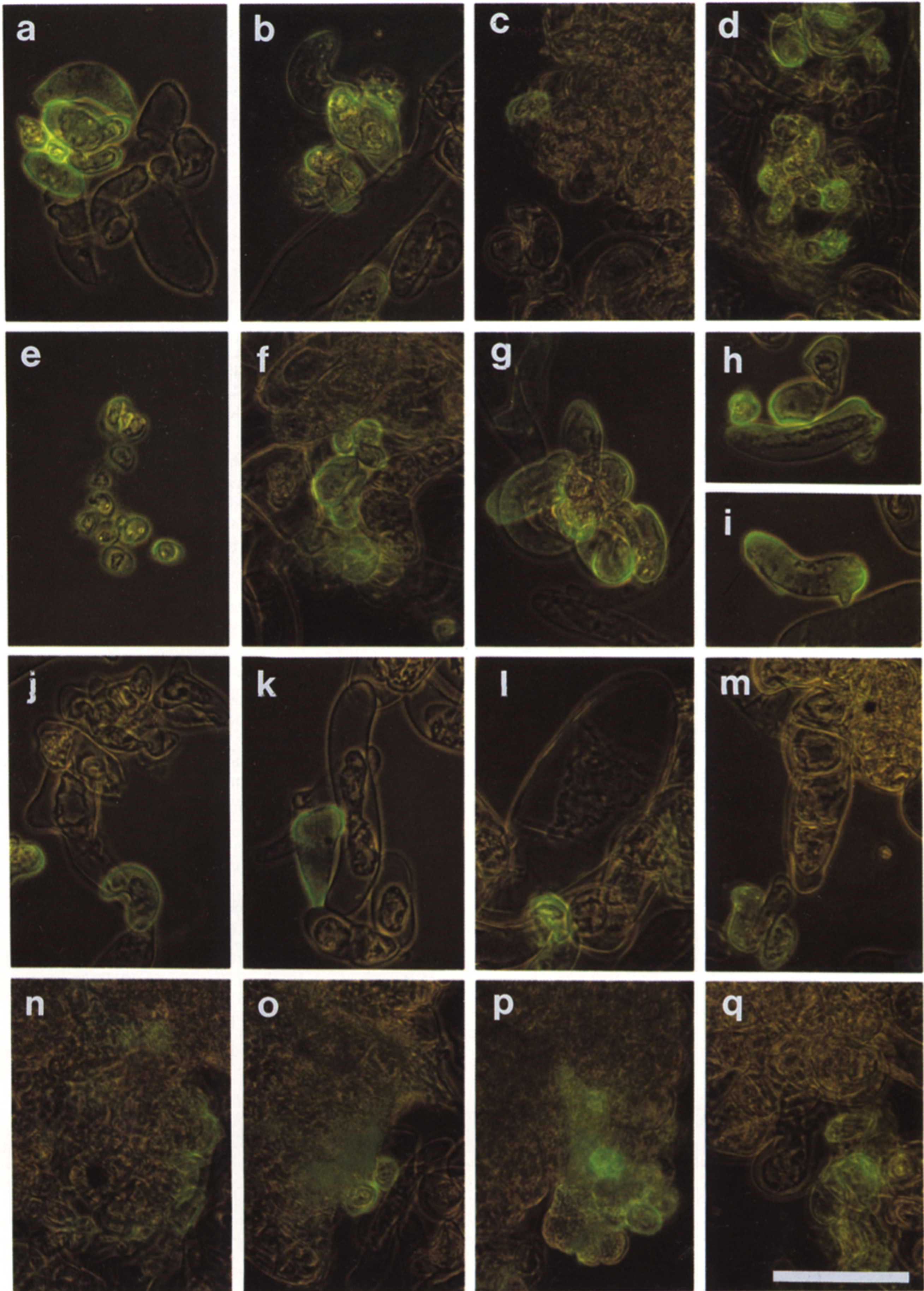
Extraction and Analysis of the JIM8 Cell Wall Antigen

Cell wall JIM8-reactive antigen was extracted from embryogenic suspension culture cells by taking the cultures through an eight-step extraction procedure (modified from Redgwell and Selvendran, 1986). The solvent extractions were with: (*a*) distilled H_2O; (*b*) 50 mM EDTA pH 7.15; (*c*) 50 mM EDTA pH 7.15; (*d*) 50 mM $NaCO_3$; (*e*) 50 mM $NaCO_3$; (*f*) 1 M KOH; (*g*) 1 M KOH; and (*h*) 4 M KOH. Stages *a*, *b*, *d*, and *f* lasted for 2 h and stages *c*, *e*, *g*, and *h* lasted for 18 h. The extractions used 40 ml of pelleted suspension culture in 25 ml of solvent at 20°C, except for *d* which was at 4°C. The extracts were filtered, dialyzed against distilled H_2O, lyophilized, and taken up in 1 ml of distilled H_2O for dot blotting and protein gel electrophoresis. Periodic acid-Schiffs reagent (PAS) staining and dot immunoblotting with JIM8 and JIM5 were performed following standard techniques.

Immersion Immunofluorescence

Labeling of the JIM8 cell wall epitope was performed on whole, unextracted cell, suspension and embryo cultures by transferring 1 ml of a culture to 10 ml of PBS (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.2) containing 2% (vol/vol) calf serum (Sigma Chemical Co., Poole, England), and 2% (vol/vol) of JIM8 hybridoma culture supernatant. The cells were washed in PBS, and bound JIM8 was localized by resuspending them in 2 ml of the PBS/calf serum solution, this time containing 2% (vol/vol) of a rabbit anti-rat IgG antiserum conjugated to FITC (Sigma Chemical Co.). Both labeling steps were for 1 h at 20°C. The cells were collected by centrifuging for 5 min at 100 *g*. Control labelings were performed with JIM5 and a mAb against an antigen not present in plants. Immunofluorescence was observed with a Zeiss Photomicroscope 3 (Carl Zeiss, Oberkochen, Germany) or a Nikon Labophot (Nikon Inc., Garden City, NY) using epifluorescence and a low level of phase contrast.

Figure 1. Cell specificity of the JIM8-reactive cell wall epitope in embryogenic suspension cultures, visualized by immunofluorescence and supplementary low-level phase contrast on whole, unfixed cells. Row 1 shows general features of embryogenic suspension cultures, row 2 shows detail of reactive single cell classes, row 3 shows detail of unreactive single cell classes, and row 4 shows detail of reactive cell clusters. (*a*) Group of 12 single cells, of which seven contain the JIM8 cell wall epitope and five do not; (*b*) Differential fluorescence intensity, apparently representing variation in abundance of the JIM8-reactive cell wall epitope; (*c*) single JIM8-reactive single cell; (*d*) two groups of JIM8-reactive single cells. (*e*) Spherical single cells, apparently type 1 cells; (*f*) cells of the class of oblate single cells, apparently type 2 cells; (*g*) cells of the class of oval single cells, apparently slightly larger type 2 cells; (*h*) elongated single cell with hemispherical disposition of the JIM8 cell wall epitope, apparently type 3 cells; (*i*) elongated single cell with polar disposition of the JIM8 cell wall epitope, also apparently type 3 cells. (*j*) Examples of the smallest unreactive cell type, type 4 cells; (*k*) example of the elongated unreactive cell type, a type 5 cell; (*l*) example of the ballooned unreactive cell type, a type 6 cell; (*m*) example of the filamentous unreactive cell type (in *j*, *k*, and *l* a single JIM8-reactive cell is included in the lower part of the picture to emphasize the labeling differences between the cells). (*n*) Weak localization of the JIM8 cell wall epitope at the periphery of a cell cluster, not distinctly associated with specific cells; (*o*) strong localization of the JIM8 cell wall epitope in two cells at the periphery of a cell cluster adjacent to a region of weak localization; (*p*) group of ~10 JIM8-reactive cells at the periphery of a cell cluster adjacent to area of weak localization; (*q*) group of JIM8-reactive cells close to, and apparently liberated from, the periphery of a cell cluster. We suggest that a group of JIM8-reactive cells such as those in *o*–*q* enter the single cell population as type 1 cells. Bar, 25 μm.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Frozen Section Fluorescence and Immunofluorescence

Fluorescence and immunofluorescence microscopy were performed on cryostat sections of embryos as described for shoot apices in Pennell and Roberts (1990) using a Zeiss photomicroscope 3. Staining of nuclei was with DAPI at 0.0001% (wt/vol) in PBS.

Immunogold Electron Microscopy

Immunogold EM was as described in Pennell et al. (1989) using the resin L. R. White (London Resin Co., Basingstoke, England) and 15-nm gold particles (Janssen Pharmaceutica, Beerse, Belgium). The transmission electron microscope was a JEM-1200 EX (JEOL USA, Peabody, MA).

Results

Expression Patterns of the JIM8 Cell Wall Epitope in Suspension Cultures

To label the surfaces of whole, unfixed cells in carrot suspension and embryo cultures, and to avoid labeling of plasma membranes, we performed immunofluorescence by immersing samples of cultures directly in solutions of antibodies. For suspension cultures, this technique revealed that the epitope recognized by the mAb JIM8 was present on the cell wall surface of cells only present in embryogenic suspension cultures (Table I) and in embryo cultures.

In the suspension cultures, as shown in Figs. 1 and 2, the JIM8-reactive cell wall epitope was present in single cells (Fig. 1 *a–m*) and much less frequently in some cells at the surfaces of some cell clusters (Fig. 1, *n–q*, and Fig. 2). The difference in immunofluorescence between the JIM8-reactive and unreactive cells was always quite clear (Fig. 1 *a*), but some reactive cells bound more of JIM8 per unit area (as evidenced by fluorescence intensity) than others (Fig. 1 *b*). Both free-floating single cells (Fig. 1 *c*) and loose groups of up to 10 cells also bound JIM8 (Fig. 1 *d*). The different kinds of JIM8-reactive single cells were categorized, after examining several thousand such cells from different embryogenic suspension cultures, as follows (Fig. 1, *e–i*): there was one spherical kind (Fig. 1 *e*), one oblate (Fig. 1 *f*) or oval (Fig. 1 *g*), and one elongated, which often contained only patches of the JIM8 epitope in the cell wall in one cell hemisphere (Fig. 1 *h*) or at one or both cell poles (Fig. 1 *i*). More than 90% of the small (<30 μm) single cells were JIM8 reactive, so that, in fully embryogenic suspension cultures they represented approximately 16% of the total (Table II). The unreactive single cells were also categorized (Fig. 1, *j–m*): one type contained lobed cells (Fig. 1 *j*), one contained cells which were much elongated (Fig. 1 *k*), and one contained cells, the biggest anywhere in the suspension cultures, that were balloonlike (Fig. 1 *l*). In fully embryogenic suspension cultures, the JIM8-unreactive cells represented ~84% of the total (Table III). Cell filaments, containing between three and eight cells, were the only other cells in the suspension cultures that were not components of cell clusters, and they also failed to react with JIM8 (Fig. 1 *m*).

For the cell clusters (Fig. 1, *n–q*, and Fig. 2), we distinguished two types. The first, which encompassed the proembryogenic masses, contained between 25 and 250 small, polyhedral cells, within small groups of which the JIM8-reactive cell wall epitope was regularly present. It was diffuse in some clusters (Fig. 1 *n*), but clearly a cell wall component in others (Fig. 1, *o* and *p*), from which JIM8-reactive cells were sometimes becoming detached (Fig. 1 *q*). The second cell cluster type was composed of up to 400 large, rounded cells that did not react with JIM8.

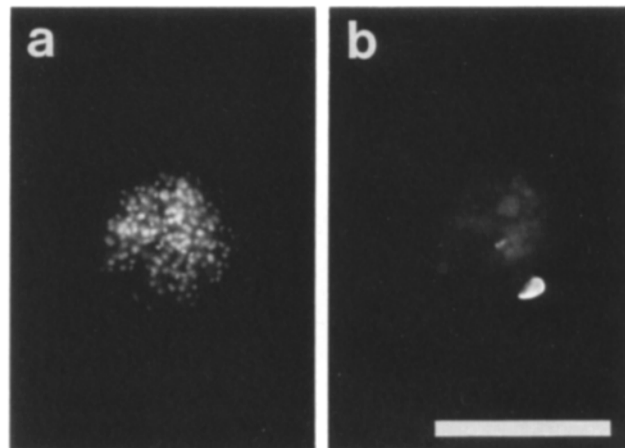


Figure 2. An intact proembryogenic mass, a form of cell cluster which occurs only in embryogenic suspension cultures, containing a single JIM8-reactive cell thought to have originated from the cell division that cut off the proembryogenic mass initial. (*a*) DAPI stain. (*b*) JIM8 immunofluorescence. Bar, 100 μm.

Unlike the epitope recognized by JIM8, that recognized by the mAb JIM5 was present in the cell walls of all the single cells except some of the very smallest, as well as those at the surfaces of the cell clusters (data not shown). A mAb raised against an antigen not present in plants did not bind anywhere (data not shown).

Expression Patterns of the JIM8 Cell Wall Epitope in Embryo Cultures

In the embryo cultures, as shown in Fig. 3, the immunolabelings demonstrated that some JIM8-reactive single cells were present as in the suspension cultures, but that the somatic embryos themselves were unreactive (Fig. 3, *a–h*). However, cells sometimes developed on the surfaces of heart and

Table II. Features of JIM8-reactive Cells

Shape	Size	Epitope distribution	Percent in culture	Cell type*
	(μm)			
Spherical	12 × 12	Entire	1	1
Oblate	12 × 15	Entire	7	2‡
Oval	15 × 20	Entire	5	2
Elongated	15 × 35	Patched	3	3

* After Nomura and Komamine, 1985.
‡ Includes thick-walled cells.

Table III. Features of JIM8-unreactive Cells

Shape	Size	Epitope	Percent in culture	Cell type
	(μm)			
Lobed	20 × 35	Absent	50	4
Much elongated	20 × 100	Absent	20	5
Ballooned	50 × 120	Absent	14	6

torpedo stage somatic embryos in auxin-containing medium, and the JIM8-reactive cell wall epitope was clearly present in these (Fig. 3, *g* and *h*). To ensure that an embryo cuticle was not preventing JIM8 from binding to the cell walls of the embryo, we repeated these labelings on sectioned embryos. The epitope recognized by JIM8 could not be detected in cell walls in longitudinal sections of somatic embryos at any stage of development, other than in occasional cells that remained adhering to the embryos from the time of proembryogenic mass induction (Fig. 3 *n*) or in embryo callus cells. However, it was detected in the plasma membrane AGPs in differentiating ground tissues (Fig. 3, *n–p*) (Pennell et al., 1991).

Cell Wall Composition

The electron microscope immunogold labeling shown in Fig. 4 demonstrates that the JIM8-reactive epitope was present throughout the cell walls of the JIM8-reactive cells identified by immersion labeling (Fig. 4 *a*) and at the outer face of the plasma membrane (Fig. 4 *b*) and inner face of the tonoplast (Fig. 4 *a*). Gold particle distribution upon the cell walls did not conform to an obvious pattern in most of the reactive cells, but in some, which were dead and which contained thickened cell walls, it was more dense towards the cell wall periphery (data not shown).

To partially characterize the JIM8-reactive cell wall antigen, we extracted embryogenic suspension cultures with a series of solvents, and tested each of the extracts with mAbs. The dot blots showing the results of these tests are in Fig. 5. The dots that have been treated with periodic acid and stained with Schiffs reagent (PAS reaction) show that some cell wall carbohydrates were extracted with each of the eight solvents (Fig. 5 *a*), those labeled with JIM5 showed that homogalacturonan (the JIM5-reactive antigen) was preferentially solubilized with EDTA, as is characteristic of pectin (Fig. 5 *b*), and the JIM8 dot immunoblots demonstrated that the JIM8-reactive cell wall antigen was partially soluble in water but completely extracted by EDTA and sodium carbonate (Fig. 5 *c*), the latter of which deesterifies and solubilizes additional pectins from the cell wall (Redgwell and Selvendran, 1986). Neither the JIM5 nor JIM8 cell wall antigens entered 10% protein gels (data not shown), demonstrating that both antigens are probably high molecular weight polysaccharides.

Acquisition of the JIM8 Cell Wall Epitope

When explants of carrot hypocotyls were transferred to liquid growth medium, the JIM8-reactive cell wall epitope appeared on the callus cells which formed at the cut surfaces or the flanks of the explants after ~10 d (data not shown). The callus contributed single cells and groups of cells to the suspension cultures so that, in the following 28 d, all the cell types characteristic of mature suspension cultures became present and the proportion of JIM8-reactive single cells reached ~16%. After another 12 wk, the differences between the cell classes became less evident and the proportion of JIM8-reactive cells diminished, eventually to nil. Embryogenic potential changed in the same way but lagged behind the variations in the JIM8-reactive cells by ~20 d (Fig. 6). The initial proportion of JIM8-reactive cells was greater when callus from solid-phase culture was used to start the suspension cultures (data not shown).

Developmental Regulation

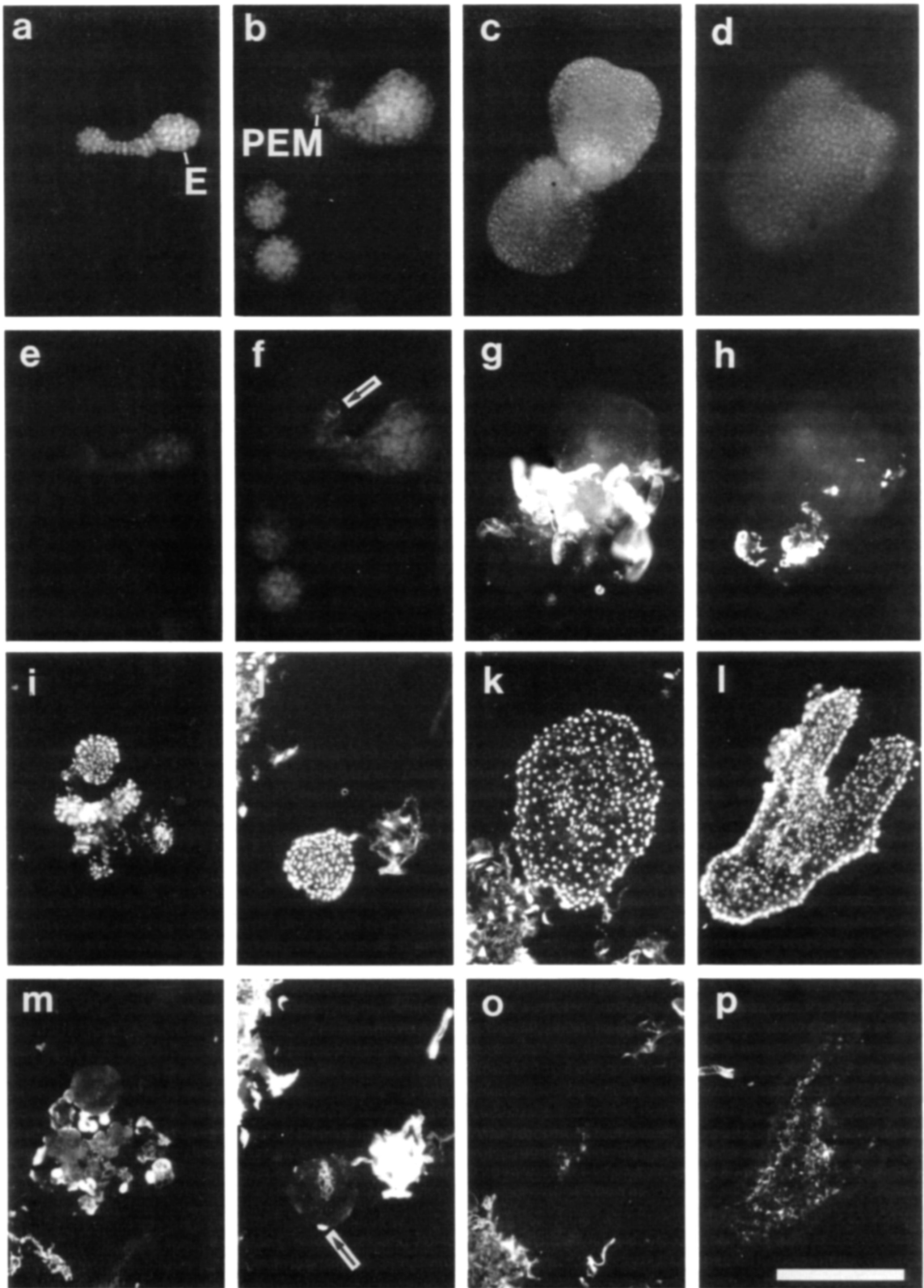
To try to establish whether a sub-population of the JIM8-reactive cells had acquired embryogenic competence, we transferred samples of sieved suspension cultures, consisting of small single cells (of which >90% contained the JIM8 cell wall epitope) and occasional divided cells and cell quartets (which did not react with JIM8) into the preculture conditions appropriate for the formation of somatic embryos from single cells (Nomura and Komamine, 1985). As shown in Fig. 7, JIM8-reactive cell clusters then formed in 2–3 d (Fig. 7, *a* and *b*), and JIM8-unreactive proembryogenic masses appeared to develop from them within another 4 d (Fig. 7, *c* and *d*). Somatic embryos grown from these proembryogenic masses did not contain the JIM8-reactive cell wall epitope either at the embryo surface or, when sections were examined, in any location other than at some plasma membranes in the ground tissue (data not shown).

Discussion

Heterogeneous Plant Cell Antigens React with JIM8

The microscopical and extraction analyses that we have performed on embryogenic carrot suspension cultures demonstrate that the cell wall epitope recognized by JIM8 is a component of a cell wall antigen or antigens. The epitope is thought to be a galactose-rich carbohydrate known to be present in three different plasma membrane AGPs in sugar beet (Pennell et al., 1991), and in the soluble AGP 1 secreted by the non-embryogenic carrot suspension culture termed L1 (Knox et al., 1991). The reactive site in the cell wall therefore identifies a new class, the third so far described, of plant cell antigen found to contain the JIM8 epitope.

The identity of the JIM8 cell wall antigen has not been clearly resolved in this paper, but its EDTA extractability and its inability to enter protein gels suggests either that it belongs to the heterogeneous and complex class of plant cell wall polysaccharides, the pectins, or that it is a glycoprotein which is tightly bound to pectin. As such, it forms a new class of developmentally regulated cell wall antigens to go alongside those so far described, the glycine-rich proteins (GRPs), the proline-rich proteins (PRPs) and the hydroxyproline-rich glycoproteins (HRGPs) (Cassab and Varner, 1987; Keller et al., 1989; Hong et al., 1989; Ye and Varner, 1991; Ye et al., 1991). Of the pectins known to be present in the walls of suspension-cultured cells and extractable by EDTA, the main one is homogalacturonan (the JIM5-reactive antigen we have localized in this study for control purposes), but this is not the JIM8-reactive cell wall antigen because it does not inhibit JIM8 binding and it is not cell specific. Other candidate pectin antigens include the rhamnogalacturonans (RGs). These are moderately effective inhibitors of JIM8 binding (Pennell et al., 1991), but they have not yet been localized in carrot suspension cultures to see if they correspond in position to the antigens which bind JIM8. Also, since polysaccharide gums such as gum karaya and gum arabic are the most effective JIM8 inhibitors, it is pos-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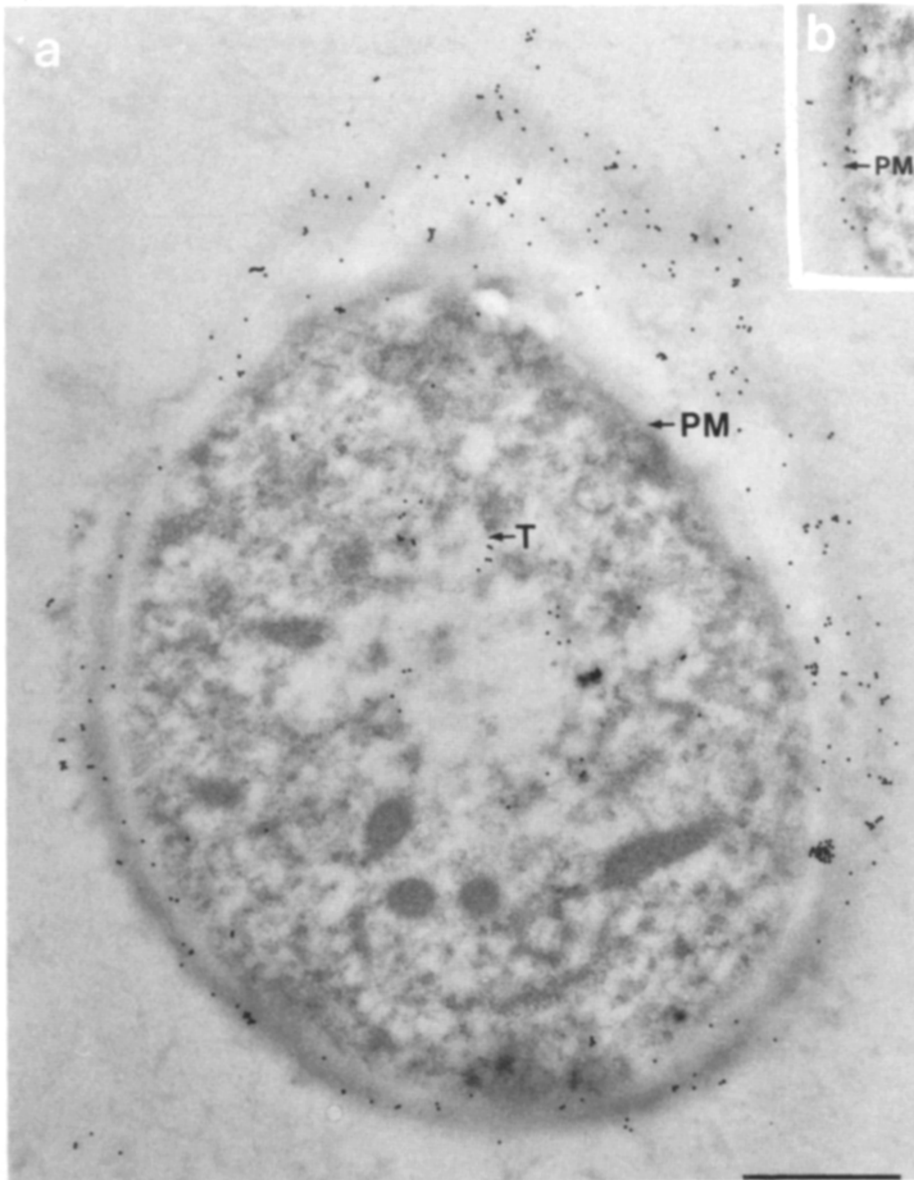

Figure 4. Transmission electron micrographs of resin sections of single cells labeled with JIM8 and colloidal gold particles, showing intracellular localization of the JIM8 epitope. (*a*) Whole-cell profile, demonstrating that the JIM8 epitope is present only throughout the cell wall and at the tonoplast (*T*). (*b*) Detail of cell surface, characteristic of most cells, showing that the JIM8 epitope is also at the outer face of the plasma membrane (*PM*). Bar, 1 μm.

sible that the cell wall JIM8-reactive antigen is an AGP-related molecule that is tightly bound to pectin.

Expression of the JIM8-reactive Cell Wall Epitope Correlates with Suspension Culture Embryogenic Potential

By performing immunofluorescence on different carrot suspension and embryo cultures, we located the JIM8-reactive cell wall epitope in well-defined kinds of single cells that correspond to specific cell types known to be present in embryogenic carrot suspension cultures (Nomura and Komamine, 1985). They are clearly distinct from those that contain the 52–54-kD EP1 cell wall glycoprotein, the elongated and vacuolated cells (van Engelen et al., 1991), and from those that express the EP2 gene, specific cells in proembryogenic masses and somatic embryos (Sterk et al., 1991).

Figure 3. Somatic embryos grown from proembryogenic masses. Rows 1 and 2 show intact embryos (*E*) stained by immersion with DAPI (row 1) and JIM8 (row 2), and rows 3 and 4 show sections of embryos, at approximately equivalent developmental stages to those in the first 2 rows, stained with DAPI (row 3) and JIM8 (row 4). (*a*, *e*, *i*, and *m*) Pre-globular stage; (*b*, *f*, *j*, and *n*) globular stage; (*c*, *g*, *k*, and *o*) heart stage; and (*d*, *h*, *l*, and *p*) torpedo stage. When intact embryos are labeled with JIM8, only heart and torpedo stages were found to contain strongly reactive cells (*g* and *h*), some of which are callus cells (*h*). Some of the proembryogenic masses (*PEM*) associated with the pre-globular and globular stage embryos, but not the embryos themselves, contain weakly reactive cells (*f*, *arrow*). When the embryos were sectioned and labeled with JIM8, exposing the developing protoderm below the cuticle to the antibody solutions, the JIM8-reactive cell wall epitope was not detected in any cells apart from those proliferating into embryogenic callus at the embryo surfaces (*n*, *arrow*). The JIM8-reactive epitope was present in parts of the undifferentiated ground tissue inside the somatic embryos, but not in the protoderm, of globular (*n*), heart (*o*), and torpedo (*p*) embryos; in these cells it is a plasma membrane AGP component. Bar, 250 μm.

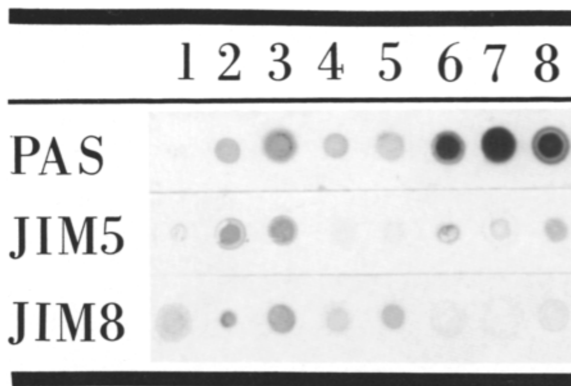

Figure 5. Solubility properties of the JIM8-reactive cell wall antigen, determined by dot-immunoblotting. (*1–8*) Cell wall extracts, solubilized with H_2O (2 h), 50 mM EDTA (2 h), 50 mM EDTA (18 h), 50 mM Na_2CO_3 (2 h), 50 mM Na_2CO_3 (18 h), 1 M KOH (2 h), 1 M KOH (18 h), and 4 M KOH (18 h) from an embryogenic suspension culture. Dots represent the proportions of polysaccharide extracted at each step. The PAS stain demonstrates the efficiency of the extractions. The JIM5 immunoblot shows the preferential extraction of cell wall homogalacturonan with EDTA. The JIM8 immunoblot shows that the JIM8 cell wall antigen is also preferentially solubilized with EDTA, but that residual JIM8 cell wall antigen is extracted along with the other pectin fractions.

Cells containing the JIM8-reactive cell wall epitope are only present in embryogenic suspension cultures, and appear to be present in all embryogenic cultures not only of carrot but of some other species as well (McCabe, P. F., and R. I. Pennell, unpublished observations). Non-embryogenic cultures obtained by either prolonged periods of sub-culturing (when embryogenic potential inevitably declines), or, as for ts11, by mutagenesis, did not contain the JIM8 cell wall epitope, indicating that the epitope is correlated specifically with embryogenic potential rather than with time in culture. The presence of the JIM8-reactive cell wall epitope in approximately only 2% of the cells in the moderately embryogenic revertant of the ts11 culture, ts11R (LoSchiavo et al., 1990), supports this correlation, but it also shows that reacquisition of the epitope need not require that the tissue culture cells be recycled through a regenerated multicellular plant. The carrot EP2 gene is regulated in a similar way (Sterk et al., 1991). The JIM8-reactive cell wall epitope was regularly localized in cells that were occasionally present at the surface of somatic embryos. We consider some of these cells to be callus cells, presumably reflecting the high embryogenic potential of the embryo cells themselves (Pretova and Dedicova, 1992), but many others may be divided JIM8-reactive cells that persist from the stage when the proembryogenic mass initials were determined. The significance of these observations is clear; they demonstrate a direct correlation between expression of the JIM8-reactive cell wall epitope and embryogenic potential, but they rule out a correlation with cell proliferation, since this process takes place in non-embryogenic suspension cultures as well.

In Embryogenic Suspension Cultures, a Complex Set of Cells Contain the JIM8-reactive Cell Wall Epitope

The JIM8-reactive cells we describe in this paper belong to four well-described cell types (three single cell types and one cell type in cell clusters) that occur in embryogenic carrot suspension cultures. The small spherical JIM8-reactive single cells resemble type 1 cells, known to have highest embryogenic potential, and most of those that are oval or elongated resemble type 2 and type 3 cells, which have lower embryogenic potential (Nomura and Komamine, 1985; Komamine et al., 1990). It is known that some cell clusters shed surface cells into the growth medium (Halperin and Wetherell, 1965), and it may be that the JIM8-reactive cells at the cluster surfaces are those that are liberated. Once released, they could be identified as type 1 cells. Our data therefore imply that the spherical JIM8-reactive single cells are type 1 cells, apparently able to divide and cut off proembryogenic mass initials or, failing that, able only to elongate and die. Two other observations support this view. The first is the way in which changes in the abundance of JIM8-reactive single cells anticipate equivalent changes in embryogenic potential defined as the number of somatic embryos that develop from a fixed number of proembryogenic masses (de Vries et al., 1988*b*), and the second is the ability of the cells in our single cell cultures to give rise to proembryogenic masses in the pre-culture conditions necessary for the direct regeneration of embryos from single cells (Nomura

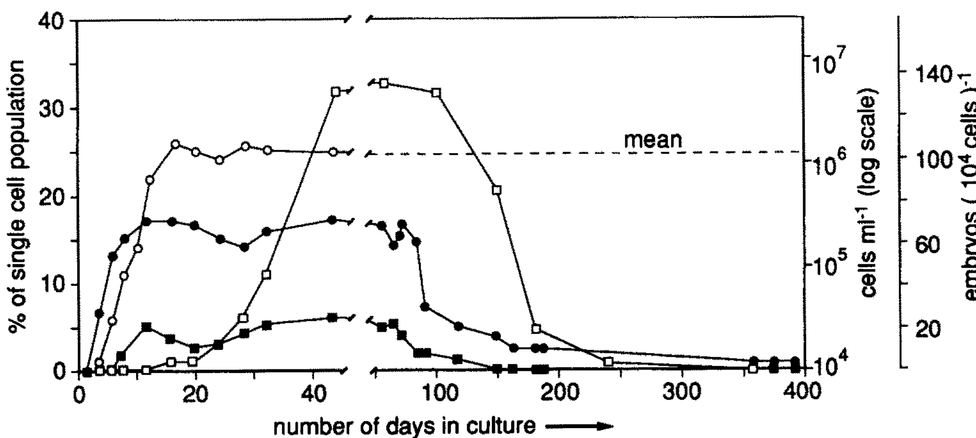

Figure 6. Temporal expression patterns of the JIM8 cell wall epitope from culture initiation to a time >1 yr (25 sub-cultures) later. The changes in cell density are shown by the open circles, depicting cells ml^{-1}. This line is broken to represent mean cell density at sub-culture. The changes in the proportion (of total cells) of JIM8-reactive single cells are shown by the closed circles. The changes in the embryogenic potential of the suspension culture, depicting the number of cells regenerating from the proembryogenic masses present among 10^4 single cells, are shown by the open squares. The closed squares show changes in the proportion of the thick-walled cells, their numbers being related to the embryogenic potential rather than to cell density.

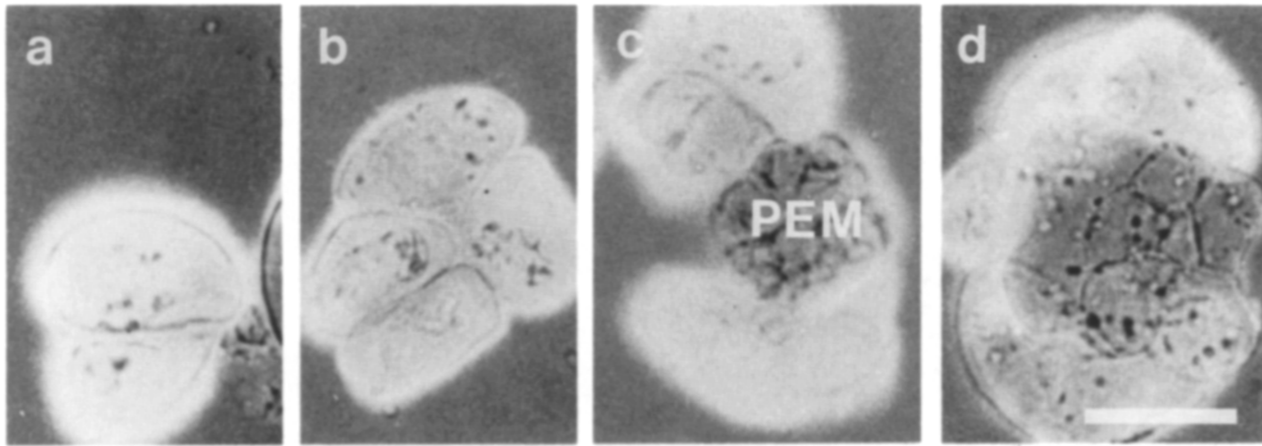

Figure 7. Developmental regulation of the cell wall JIM8 epitope in the growth medium appropriate for somatic embryo formation from single cells. Different cell clusters are shown in *a–d* as they develop during a period of 1 wk. (*a* and *b*) Growing cell clusters in which all the cells contain the JIM8 epitope; (*c* and *d*) larger cell clusters from which a proembryogenic mass (*PEM*) has arisen. Bar, 10 μm.

and Komamine, 1985). However, we have not directly proven this to be true, and it is still possible, though unlikely, that JIM8-reactive single cells are formed together with the truly totipotential cells from an as yet unidentified precursor cell type that is present in very low abundance in embryogenic suspension cultures. Sieving experiments suggest that up to 5% of type 1 cells do not contain the JIM8 cell wall epitope (Phillips, R., and A. Komamine, personal communication), and it is plausible that these are the antecedents of the JIM8-reactive cells. However, they could also be proembryogenic mass initial cells. To discriminate between these options, serial observations on stationary cultures of JIM8-labeled cells are required.

Many of the cells we score as type 2, including all of those with thick cell walls, are dead, apparently having undergone a programmed form of cell death (Raff, 1992). The proportion of JIM8-reactive type 2 cells is higher than that of the JIM8-reactive type 3 cells which we believe develop from them, the difference presumably being due to the gradual dissipation of the JIM8 cell wall antigen during the elongation process or to the multiple developmental origins of the cells we score as type 2. There is some evidence in the relationship of the thick-walled cells (which we score as type 2) to embryogenic potential that these cells arise from key cell divisions that also cut off proembryogenic mass initials. These divisions may be asymmetric (Nomura and Komamine, 1986). Such divisions also give rise to the suspensor and embryo proper (Steeves and Sussex, 1989) and to the megaspores (Bell, 1992) in seed-bearing plants, and are well known to represent cell fate divergence points in both plant and animal development (Horwitz and Herskowitz, 1992).

JIM8 Identifies an Obligatory Cell State for Cells in the Developmental Pathway to Somatic Embryogenesis

The data we present in this paper demonstrate that the JIM8-reactive cell wall epitope is a marker for embryogenic potential in carrot suspension cultures. We suggest that the epitope identifies a particular cell state that is transitional between explanted tissue cells and somatic embryos, as well as many of the undivided descendants of this cell state, as shown in Fig. 8. On entering this state, cells seem to have a binary choice. They may either elongate (sometimes with occasional cell divisions) and eventually die (pathway 2), or they may divide to form a daughter cell, in state D, with a thickened cell wall and proembryogenic mass initial cell, in state C (pathway 3). All of these cells except the state C cell contain the JIM8-reactive cell wall epitope. The frequency with which a state B cell will follow either of the two pathways 2 and 3 appears to be stochastic, and in our cultures a state B cell is approximately fifteen times more likely to follow pathway 2 than pathway 3.

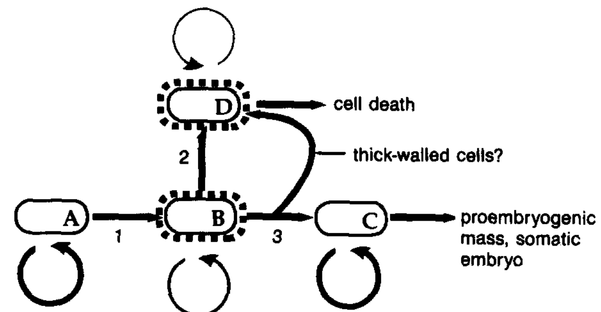

Figure 8. Schematic model of four cell states in embryogenic carrot suspension cultures, and their interrelationships. Cells that contain the JIM8-reactive cell wall epitope are indicated by crenellations. The proportion of each of the cell states present in a suspension culture depends on the cell density and the availability of exogenous growth regulators. Cell state A is a hypothetical and as yet unidentified cell state that represents the immediate precursors of the state B cells. Cell state A is JIM8 unreactive and would be found in explant callus or in an immature embryo. In an auxin-containing suspension culture, cells in state A can be induced to switch (pathway 1) to state B. Cell state B contains type 1 cells (Nomura and Komamine, 1985), and is JIM8-reactive. Most state B cells then switch (pathway 2) to cell state D, in which they elongate, perhaps with occasional divisions, and then die. Type 2 and type 3 cells (Nomura and Komamine, 1985) are in state D. But a small proportion of state B cells (6–7% of them in our cultures) divide, perhaps asymmetrically (pathway 3), to produce another state D cell and a newly JIM8-unreactive state C cell. The state C cell is the proembryogenic mass initial cell, which may go on to develop while still attached to its sister, JIM8-reactive, state D cell. We think that all state A-D cells are capable of varying amounts of cell division (thickness of circular arrows).

Our hypothesis predicts that the only plant cells that might contain the JIM8-reactive cell wall epitope in addition to those in embryogenic suspension cultures are various parthogenetic initials, since they too are transitional cell types in the re-acquisition of the totipotential condition (Goldberg, 1988; Steeves and Sussex, 1989). Also our hypothesis does not yet suggest a function for the JIM8-reactive cell wall antigen. However, there are many examples in animal systems of extracellular matrix molecules that regulate cell function, and there is some evidence that cell wall modifications exert important controls over somatic embryogenesis (van Engelen and de Vries, 1992). Therefore, at present, the JIM8-reactive cell wall epitope is a useful marker for an early determinative event in carrot somatic embryogenesis.

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