Planta (1996) 200: 167–173

Planta


# Expression of the JIM8 cell wall epitope in carrot somatic embryogenesis

**Marcel A.J. Toonen[1], Ed D.L. Schmidt[1], Theo Hendriks[1], Harrie A.Verhoeven[2], Ab van Kammen[1], Sacco C. de Vries[1]**

[1] Department of Molecular Biology, Agricultural University Wageningen, Dreijenlaan 3, NL-6703 HA Wageningen, The Netherlands
[2] Centre of Plant Breeding and Reproduction Research (CPRO-DLO), Droevendaalsesteeg 1, P.O. Box 16, NL-6700 AA Wageningen, The Netherlands

Received: 29 January 1996/Accepted: 20 May 1996

**Abstract.** Certain single cells in carrot (*Daucus carota* L.) suspension cultures react with the monoclonal antibody JIM8, and it has been proposed that these cells represent a transitional stage in somatic embryo formation. Shortly after isolation of the single cells by sieving, up to 80% of the cells react with JIM8. Within 4 d, JIM8 labelling becomes restricted to 1% of the single cells. To obtain evidence for the proposed correlation between expression of the JIM8 cell wall epitope and somatic embryo formation the developmental fate of carrot single cells labelled with JIM8 was determined by cell tracking. The results, obtained by recording 43 000 cells, show that only few JIM8-labelled cells give rise to embryos, and most somatic embryos develop from cells devoid of the JIM8 cell wall epitope. We therefore conclude that the presence of the JIM8 cell wall epitope does not coincide with the ability of single suspension cells to form embryos.

**Key words:** Arabinogalactan protein epitope – Cell tracking – *Daucus* – Monoclonal antibody (JIM8) – Somatic embryogenesis

## Introduction

Embryogenic suspension cultures of carrot can be obtained by incubation of hypocotyls in culture medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D). After 5–7 d of treatment, cells from the provascular tissue show a reduced vacuolar volume, and have started to divide in both longitudinal and transverse planes to form small isodiametric cells. Cells on the surface of the resulting provascular mass enlarge and detach to give rise to the embryogenic suspension culture. Three days of treatment with 2,4-D is needed to generate an embryogenic cell culture, whereas a shorter treatment leads to organogenesis (Guzzo et al. 1994, 1995). In an established suspension culture, somatic embryos can develop from embryogenic cell clusters (De Vries et al. 1988) and single cells (Backs-Hüsemann and Reinert 1970; Nomura and Komamine 1985; Toonen et al. 1994). Initiation of somatic embryogenesis from single cells requires 2,4-D and re-activation of the cell cycle (reviewed by Dudits et al. 1995). Cells that develop into embryos in the presence of 2,4-D are defined as competent cells. The first morphologically visible sign of embryo development is division of a single cell that subsequently develops into a cluster of small cytoplasm-rich cells. Via successive globular-, heart- and torpedo-stage embryos these cells develop into a plantlet. The small cell clusters do not require externally applied 2,4-D for embryo development and are defined as embryogenic (De Jong et al. 1993; Toonen et al. 1994).

Only a very limited number of cells in a hypocotyl explant or in a single cell population respond to treatment with 2,4-D by becoming competent to develop into somatic embryos. To study the initial processes in somatic embryogenesis it is essential to identify competent cells as early as possible during development. Using cell tracking it has been shown that morphologically distinct single cells can develop into somatic embryos and that cell morphology cannot therefore be used to identify competent cells (Toonen et al. 1994).

In oilseed rape the monoclonal antibody JIM8 has been shown to react with arabinogalactan protein (AGP) epitopes in sexual organs, the eight-celled embryo and a very limited number of other cell types (Pennell et al. 1991). In carrot suspension cultures the JIM8 epitope has been localised on three different cell-membrane AGPs (Pennell et al. 1991) and on AGPs secreted into the suspension culture (Knox et al. 1991). The cell wall epitope, present in spherical, oblate and oval carrot suspension cells (Pennell et al. 1992) has recently been identified as an AGP (R.I. Pennell, personal communication). Spherical and oblong cells showed a uniform distribution of the cell wall epitope while the epitope was present at one or both

Abbreviations: 2,4-D = 2,4-dichlorophenoxyacetic acid; AGP = arabino galactan protein; B5-0 = Gamborg's B5 medium; B5-0.2 = Gamborg's B5 medium supplemented with 0.2 μM 2,4-D; FITC = fluorescein isothiocyanate; PBS = phosphate-buffered saline

*Correspondence to*: S.C. de Vries; FAX: 31 (317) 483 584; E-mail: sacco.devries@mac.mb.wau.nl

cell poles of more-elongated cells. Large single cells and proembryogenic masses did not react with the JIM8 antibody (Pennell et al. 1992). It is unclear whether the JIM8 cell wall AGP epitope is related to the membrane AGPs or to secreted AGPs.

It has been postulated that the JIM8 cell wall epitope marks a transitional state in the formation of embryogenic cells. Pennell et al. (1992) presented a model in which a somatic cell (state A) can obtain the JIM8 cell wall epitope (state B). This cell can either elongate and form a non-embryogenic state-D cell or divide to form a state-C cell devoid of the JIM8 epitope. This state-C cell will develop into the embryo. According to this model, expression of the JIM8 epitope coincides with the transition from somatic cell into competent cell, which probably represents the first and maybe most important stage of somatic embryogenesis. Therefore, JIM8 might be used to identify competent single cells, facilitating further research into the early stages of somatic embryo development. Here we report on the developmental fate of JIM8-labelled cells, determined by semi-automatic cell tracking. The results show that most somatic embryos develop from single cells that are devoid of the JIM8 epitope, indicating that the JIM8 cell wall epitope is not a marker for competent single cells. A role for the JIM8 cell wall epitope as a marker for competency or embryogenic capacity of a cell culture rather than for a competent cell state is discussed.

## Materials and methods

*Plant material and cell culture.* Embryogenic carrot (*Daucus carota* L. cv. Trophy; S&G seeds, Enkhuizen, The Netherlands) suspension cultures were maintained as described previously (De Vries et al. 1988). Single cells smaller then 22 μm were obtained by sieving through polyester sieves (Monodur-PES; Verseidag Techfab, Walbeck, Germany; Toonen et al. 1994). All cultures were incubated in a 16 h light/8 h darkness regime at 25 ± 0.5°C. Embryogenic competence is expressed according to De Vries et al. (1988) as the number of somatic embryos developed per 10 000 cells present at the initiation of the culture. Cell viability was determined by fluorescein diacetate (F7378; Sigma, St. Louis, Mo., USA) as described by Widholm (1972).

*Monoclonal antibodies.* JIM8 antibodies (Pennell et al. 1991) were isolated from JIM8 hybridoma culture supernatant by binding to Bakerbond 40 μm prepscale ABx (7269–02; J.T. Baker Inc., Phillipsburg, N.J., USA) as described by the manufacturer. A column with JIM8-bound ABx was prepared and antibodies were eluted using 20 mM Na-acetate, 500 mM $(NH_4)_2SO_4$, pH 6.8. The JIM8 antibody fraction was dialysed against 0.1 M sodium bicarbonate buffer (pH 9.0) and conjugated to fluorescein isothiocyanate (FITC) using the QuickTag FITC conjugation kit (1248 618; Boehringer, Mannheim, Germany) according to the manufacturer's protocol.

*Immersion immunofluorescence.* Labelling of the JIM8 cell wall epitopes was performed on one-week-old suspension cultures or <22-μm cell fractions. One millilitre of the suspension cultures was transferred into 10 ml phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM $Na_2PO_4$, pH 7.2), Gamborg's B5 medium (B5-0 medium) or B5-0 medium supplemented with 20 mM $Ca^{2+}$. All solutions contained 2% calf serum (56010–010; GibcoBRL, Breda, The Netherlands). After washing, cells were resuspended in 2 ml of the same solution containing 5% JIM8 hybridoma culture supernatant or 2% FITC-JIM8 conjugate. After 1 h, excess unbound JIM8 antibody was removed by washing twice with the washing solution. The JIM8 antibody was localised by resuspending the cells in 0.1% goat anti-rat IgG FITC conjugate (F6258; Sigma) followed by washing to remove excess FITC conjugate. Control experiments were performed using the goat anti-rat IgG FITC conjugate as antibody. Immunofluorescence was observed with a Nikon Optiphot with epifluorescence-optics using a B1a filter set (excitation 470–490 nm, dichromatic mirror 510 nm, barrier filter 520 nm Nikon, Tokyo, Japan) and a low level of phase contrast. Colour micrographs were taken using Kodak Ektachrome 1600.

*Cell immobilisation and in-situ immersion immunofluorescence.* Single cells were immobilised in 0.1% phytagel (P8196; Sigma) in Gamborgs B5 medium supplemented with 0.2 μM 2,4-D (B5-0.2 medium) without $Ca^{2+}$. This suspension was poured onto a 0.2% phytagel-layer in B5-0.2 medium containing 5 mM $Ca^{2+}$ in Petriperm dishes (Hereaus, Hanau, Germany) as described previously (Toonen et al. 1994). After 16 h non-specific binding was blocked by adding onto the phytagel layer an equal volume of 2.5% calf serum in B5-0.2 medium containing 40 mM $Ca^{2+}$. After 30 min the blocking solution was replaced with 10% JIM8 FITC conjugate in B5-0.2 medium containing 20 mM $Ca^{2+}$. One hour later unbound JIM8 was removed by eight washing steps of 10 min each with B5-0.2 medium supplemented with 20 mM $Ca^{2+}$. After recording (see below), excess $Ca^{2+}$ was removed from the immobilised cell culture by washing ten times with basal B5-0.2 medium. Due to this treatment the JIM8 signal was also removed. Subsequent visible light images were recorded for two weeks on alternate days to follow development of the single cells. At day 6, 2,4-D was removed to allow embryogenesis to proceed beyond the globular stage (Toonen et al. 1994).

*Cell tracking.* Cell tracking was performed using a Nikon Diaphot microscope equipped with a BioRad MRC-600 confocal scanning laser system (BioRad, Cambridge, Mass., USA) and a Märzhäuser EM 32 IM cross-table (Märzhäuser, Wetzlar, Germany). The cross-table was controlled by the program MicroScan 1.0 (Agricultural University, Wageningen) running on a Tulip 486 personal computer (Tulip, 's Hertogenbosch, The Netherlands) via a MultiControl 2000–3 unit (Märzhäuser). This set-up allowed scanning of the dishes in the X and Y directions as well as in the Z direction, and the recording of the fluorescent and visible signals simultaneously. Fluorescein isothiocyanate was detected using the FITC filter set (excitation 488 nm, emission 515 nm). The images obtained by the BioRad system were recorded on a VHS video recorder (Hitachi, Tokyo, Japan). Initial experiments were performed using a similar set-up using a Zeiss Axiovert inverted microscope (Zeiss, Oberkochen, Germany).

Video images were transferred to and digitised by a Macintosh 7100 AV PowerPC computer (Apple, Cupertino, Calif., USA) using the VideoMonitor image-grabbing program (Apple). Contrast and brightness of the images was optimised in Photoshop 2.5.1 (Adobe Systems Inc., Mountain View, Calif., USA).

## Results

### *Optimising conditions for in-vivo JIM8 labelling*

*Labelling with JIM8 requires high $Ca^{2+}$.* To allow normal development of single cells labelled with JIM8, labelling conditions have to mimic standard culture conditions used for somatic embryo development. Therefore, labelling of carrot suspension cells in Gamborg B5-0 medium was compared with the labelling in PBS as described by Pennell et al. (1992). In their original immersion immunofluorescence experiments where JIM8 labelling was visualised with a second goat anti-rat IgG FITC conjugate, Pennell et al. (1992) showed that in PBS single cells, loosely attached groups of cells and cells at the

surface of cell clusters reacted with JIM8. Our experiments gave similar results in PBS (Fig. 1a). In parallel, a control labelling experiment with only the goat anti-rat FITC conjugate did not show any specific bright green FITC immunofluorescence signal (Fig. 1b). However, a green/yellow and a red fluorescent background signal was observed. This signal was also visible in the JIM8 labelling under the different conditions applied. The green/yellow signal, caused by phenolic compounds present in the cell walls, was mainly detected in large cell clusters (Fig. 1b, e). Red autofluorescence caused by chloroplasts was observed in a small number of single cells and cell clusters (Fig. 1d, e). The labelling pattern of JIM8 in B5-0 medium (Fig. 1c, d) resembled the non-specific signals observed in the control FITC conjugate labelling rather than the specific JIM8 labelling obtained in PBS. The JIM8 labelling pattern as observed in PBS could be restored by addition of 100 mM NaCl (data not shown) or 20 mM $CaCl_2$ to the B5-0 medium (Fig. 1e, f).

Previously, we have shown that increased $Ca^{2+}$ concentrations in the culture medium have an inhibitory effect on somatic embryo development from single cells. Culturing single cells for two weeks in the presence of 5 mM $Ca^{2+}$ resulted in a 50% decrease in the number of somatic embryos formed (Toonen et al. 1994). Culturing single cells for 4 h in 20 mM $Ca^{2+}$, to allow JIM8 labelling and recording of single cells, and subsequent removal of excess $Ca^{2+}$ did not affect the frequency or development of somatic embryos from single cells (Fig. 2a). The control experiments presented in Fig. 2a showed that there is no effect on the number of somatic embryos after incubation in the JIM8 conjugate, calf serum or with calf serum, $Ca^{2+}$ and JIM8 conjugate (the entire labelling procedure). Morphologically, the embryos derived from the treated single cells were identical to embryos derived from the non-treated control cells.

*Induction of the JIM8 epitope.* In unfractionated carrot suspension cultures, only 0.5–5% of the single cells smaller than 22 μm reacted with the JIM8 antibody. However, isolation of these cells by sieving the suspension culture induced the presence of the JIM8 cell wall epitope and, to our surprise, 70–80% of the single cells then became labelled with the JIM8 antibody. Approximately 7% of the single cells present at the start of the experiment were dead, as determined by viability staining. Large numbers of cells lost the JIM8 epitope with time as shown by the decrease in the percentage of JIM8-reactive cells (Fig. 2b). Twenty hours after JIM8-labelling the number of JIM8-reactive cells varied between 5 and 50% in different experiments, and after 40–90 h the number of JIM8-reactive cells reached a steady-state level of about 1%. These results show that there is a large variation in the speed with which the JIM8 cell wall epitope becomes undetectable by the antibody. During the first week, embryogenic cell clusters developed from single cells. The number of remaining single cells stayed constant but the number of viable cells decreased from 93% to 85% (Fig. 2b), indicating that an additional 7% of the cells died during the first week of culture.

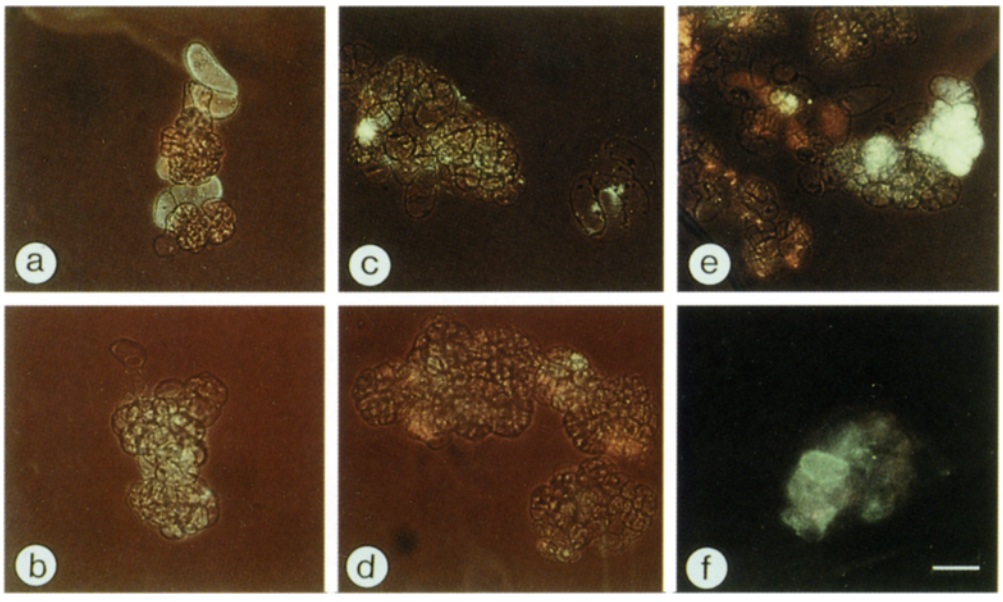


**Fig. 1a–f.** Expression patterns of the JIM8 epitope in carrot suspension cultures. Labelling with JIM8 was performed in PBS medium, B5-0 medium and B5-0 medium supplemented with 20 mM $Ca^{2+}$ to test the effect of high salt concentrations on labelling patterns. One- week-old cell cultures were incubated in calf serum to block a specific binding sites and thereafter either treated with JIM8 hybridoma supernatant followed by the FITC-conjugated second antibody or only with the FITC-conjugated second antibody. **a** Labelling in PBS medium gives a bright green FITC signal, showing expression of the JIM8 epitope. **b** Control labelling in PBS of cells only treated with the goat anti-rat FITC conjugate without JIM8 gives only faint green auto fluorescence signal. **c, d** Labelling with JIM8 in B5-0 medium does not give a specific signal of expression of the JIM8 epitope but some red autofluorescence of chloroplasts is observed. **e, f** Labelling with JIM8 in B5-0 medium supplemented with 20 mM $Ca^{2+}$ restores the specific JIM8 signal as observed for JIM8 labelling in PBS medium. × 500; bar = 20 μm

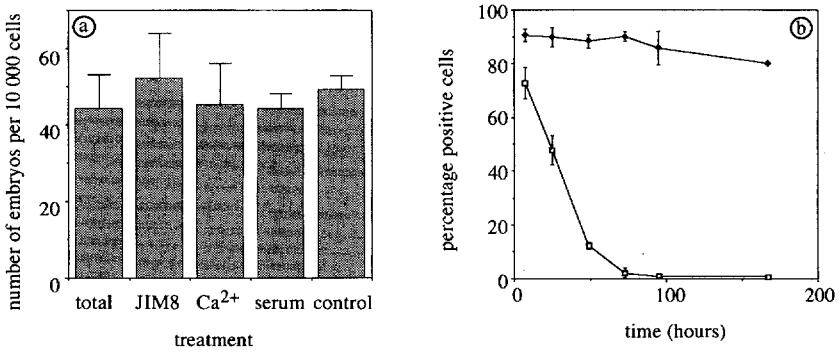


**Fig. 2a,b.** Effects of the different treatments required for in-vivo JIM8 labelling on the number of somatic embryos formed from 10 000 cells. **a** Single cells smaller than 22 μm were incubated in B5-0.2 medium supplemented with 1.25% calf serum and 20 mM $Ca^{2+}$ for 30 min. Subsequently, JIM8-FITC conjugate was added to a final concentration of 5% (total). In addition, single cells were cultured in B5-0.2 medium supplemented with either 5% JIM8-FITC conjugate, 20 mM $Ca^{2+}$, 1.25% calf serum or in basal B5-0 medium (control). After 4 h incubation, all cultures were washed with basal B5-0.2 medium to remove the supplemented compounds and cells were cultured to allow somatic embryo development. The number of embryos scored after two weeks is indicated per 10 000 cells present at day 1 of the experiment. **b** Single cells smaller than 22 μm were isolated by sieving through a 22-μm polyester sieve, concentrated by centrifugation and cultured in 2 ml cultures in B5-0 medium. After the indicated times, samples were labelled with JIM8 and a viability stain was performed. The percentage of JIM8-positive cells (*open squares*) and percentage of viable cells (*closed squares*) at the time after isolation are shown. Data are means ± SD; $n = 3$

*Immobilisation system.* Immediately after isolation of the single-cell fraction by sieving, the cells were embedded in phytagel in B5-0.2 medium (day 0). Embedding in phytagel does not influence somatic embryo development or the frequency of embryo development from single cells (Toonen et al. 1994). Sixteen-20 h after embedding non-specific cell wall epitopes were blocked by calf serum added to the embedded cells in liquid B5-0.2 medium supplemented with $CaCl_2$. Diffusion of these compounds into the phytagel layer gave a final concentration of 1.25% calf serum and 20 mM $Ca^{2+}$ in the single-cell culture. After 30 min the liquid layer was replaced by a 10% JIM8-FITC conjugate in B5-0.2 medium supplemented with 20 mM $CaCl_2$ and 1.25% calf serum. The JIM8-FITC-conjugate was chosen because it precluded a second labelling step with a second FITC-conjugated antibody. Labelling patterns obtained with the JIM8-FITC conjugate were similar to the patterns obtained with JIM8 detected by the goat anti-rat FITC conjugate as described above (data not shown). After 1 h, unbound JIM8 antibodies were removed by extensive washing of the embedded single cells. At this time the percentage of JIM8-reactive cells varied between 5 and 10% in the three different cell-tracking experiments described. Visible light images and fluorescent images of a defined area of the culture dish were recorded using the cell-tracking equipment coupled to the confocal scanning laser microscope (CSLM). After scanning the culture dish, the phytagel layer was washed with basal B5-0.2 medium to remove excess $Ca^{2+}$, calf serum and JIM8-FITC conjugate. After washing, the $Ca^{2+}$ concentration was reduced to the standard 1 mM. Fluorescent signal from the JIM8-FITC conjugate was mainly observed on cell wall remnants. Single cells showed only traces of JIM8-FITC labelling. At day 6, 2,4-D was removed from the culture to allow embryo development beyond the globular stage.

*Cell tracking*

The development of JIM8-labelled single cells into somatic embryos was followed by employing semi-automatic cell tracking. This system allowed the recording of the development of many thousands of individual cells. The cell-tracking system was coupled to a CSLM to detect the JIM8-FITC signal. Damage to the cells due to UV light was prevented by operating the CSLM at low light intensity. After JIM8 labelling the fluorescent and visible light images of several thousand cells present in the scanning area of one culture dish could be recorded in 75 min. In three independent experiments, each experiment consisting of two to four culture dishes, the development of single cells was monitored by recording images every second day for over two weeks.

Of the embedded single cells, 6.9% reacted with the JIM8 monoclonal antibody. This included all morphologically distinct cell types, spherical, oval, elongated and irregular-shaped cells, present in the cell culture. Two types of labelling with the JIM8 monoclonal antibody were observed. (i) Of the reacting cells 3.7% showed JIM8 labelling on patches of the cell wall or at the poles (Fig. 3i, j and k, l). This distribution was observed mainly in oval and elongated cells. (ii) The other 3.2% of the JIM8-reactive single cells consisted of all cell types and showed an equal distribution of the JIM8 epitope over the entire cell wall (Fig. 3e, f and g, h). The cell wall of dead plasmolysed cells (Fig. 3c, d) and cell wall debris from damaged cells (Fig. 4b1) reacted strongly with the JIM8-FITC conjugate. The majority of the embedded single cells (93%) did not react with the JIM8 antibody (Fig. 3a, b).

In total, 45 somatic embryos were seen to develop from a collection of 43 000 single cells. This frequency of somatic embryo development is correlated with the low

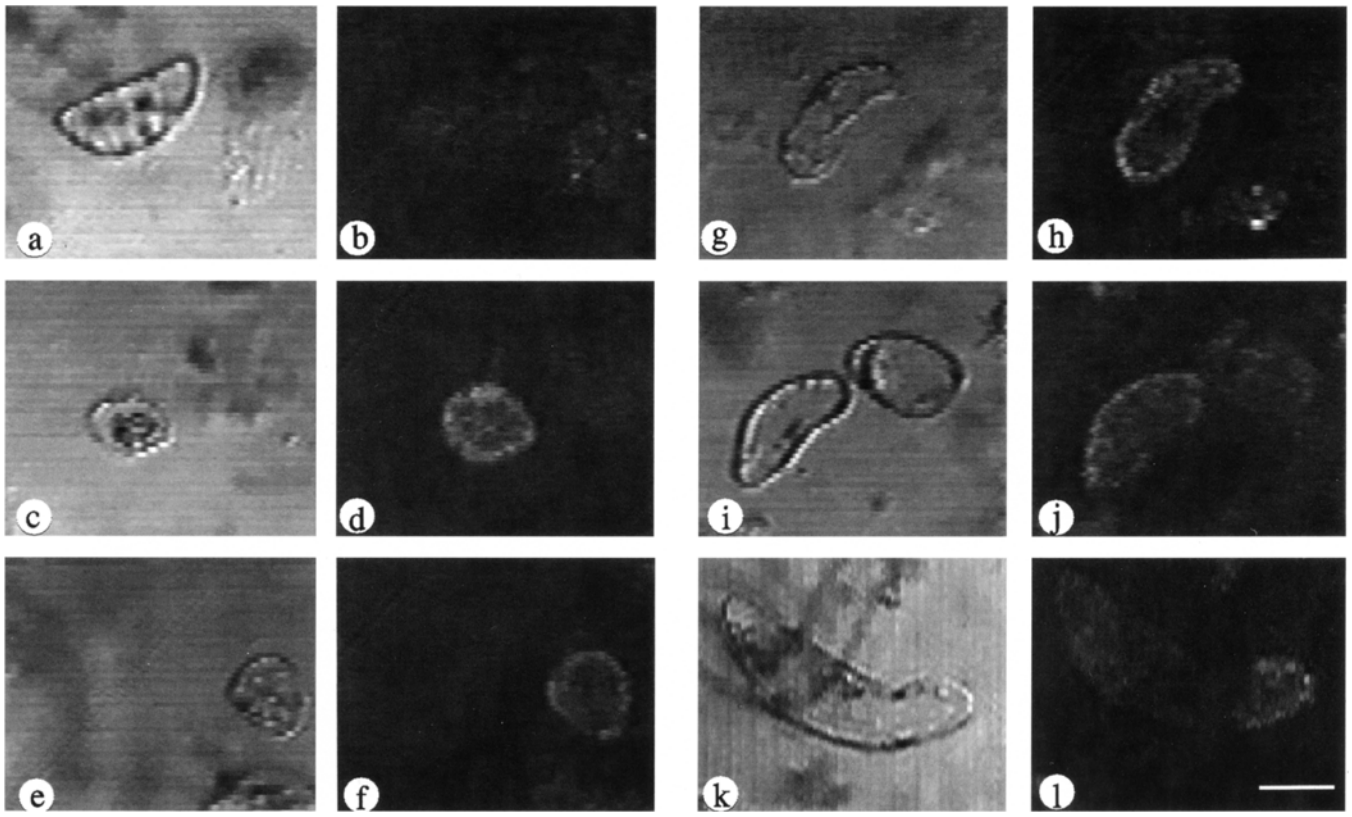


**Fig. 3a–l.** Expression patterns of the JIM8 epitope of embedded single carrot cells labelled with JIM8-FITC conjugate on day 1 of the cell-tracking experiment. First and third columns from the left show visible light images. Second and fourth columns show the JIM8-FITC signal as a white colour. Different types of expression patterns are shown, e.g. an oval cell (**a**); with no expression of the JIM8 epitope (**b**); a dead cell (**c**); showing strong JIM8 expression on the cell wall (**d**); a spherical cell (**e**); expressing the JIM8 epitope at the cell wall all around the cell (**f**); an elongated cell (**g**); expressing the JIM8 epitope at the cell wall all around the cell (**h**); two oval cells (**i**) of which only one shows expression of the JIM8 epitope along one side (**j**); an elongated cell (**k**); showing expression of the JIM8 epitope at one pole of the cell (**l**). × 80; bar = 100 μm

embryogenic capacity of the cell lines used and is not due to JIM8 labelling (Fig. 2a). Of the 45 embryo-forming cells, 42 cells could be identified. The remaining three cells were not in the focal plane at day 1 and therefore could not be classified. No somatic embryos developed from JIM8-reactive single cells, two developed from partially JIM8-positive cells and 38 developed from JIM8-negative single cells. In two cases a somatic embryo developed from a two-celled cluster, morphologically resembling a divided zygote (Fig. 4c). Both cells of this cluster were JIM8 negative.

## Discussion

This paper describes the development of carrot suspension cells labelled with the JIM8 antibody. Directly after isolation of a single cell population by sieving, about 70–80% of the isolated cells were JIM8 reactive. This percentage slowly decreased to about 1% after 40–90 h. In a typical cell-tracking experiment, about 7% of the cells in the total single cell population and 5% of the cells that formed embryos were labelled by the JIM8 antibody. This indicates that there is no direct relation between the presence of the JIM8 epitope in the cell wall and the ability of that cell to develop into a somatic embryo. It can therefore be concluded that the JIM8 monoclonal antibody is not a marker for competent single cells. However, the JIM8 antibody might be used as a marker for embryogenic competence of a total cell population.

A surprising observation was the increase in the number of JIM8-reactive cells after sieving a cell population. In established embryogenic suspension cultures the number of JIM8-reactive single cells smaller than 22 μm was between 0.5 and 5%. In different experiments this percentage increased to 70–80% after isolation of the single cells by sieving and centrifugation. This increase in the number of JIM8-reactive single cells resembles a stress response due to treatment of the cells. The basis of this response is not clear. Expression of the JIM8 epitope could have been caused by de-novo synthesis of new JIM8-containing cell wall AGPs that were subsequently transported to the cell wall. Another possibility is that the JIM8 epitope was present in the cell wall but was masked by other cell wall components, which were removed by mechanical damage during sieving and centrifugation, thus exposing the JIM8 epitope. Cell wall remnants also reacted strongly with the JIM8 antibody, indicating that the JIM8 epitope was already present in the cell walls before the cells burst and that no de-novo synthesis of the epitope is required. Removal of the JIM8 epitope from the cell walls proceeded with variable speed in the different experiments performed. It took 40–90 h to reduce the number of JIM8-

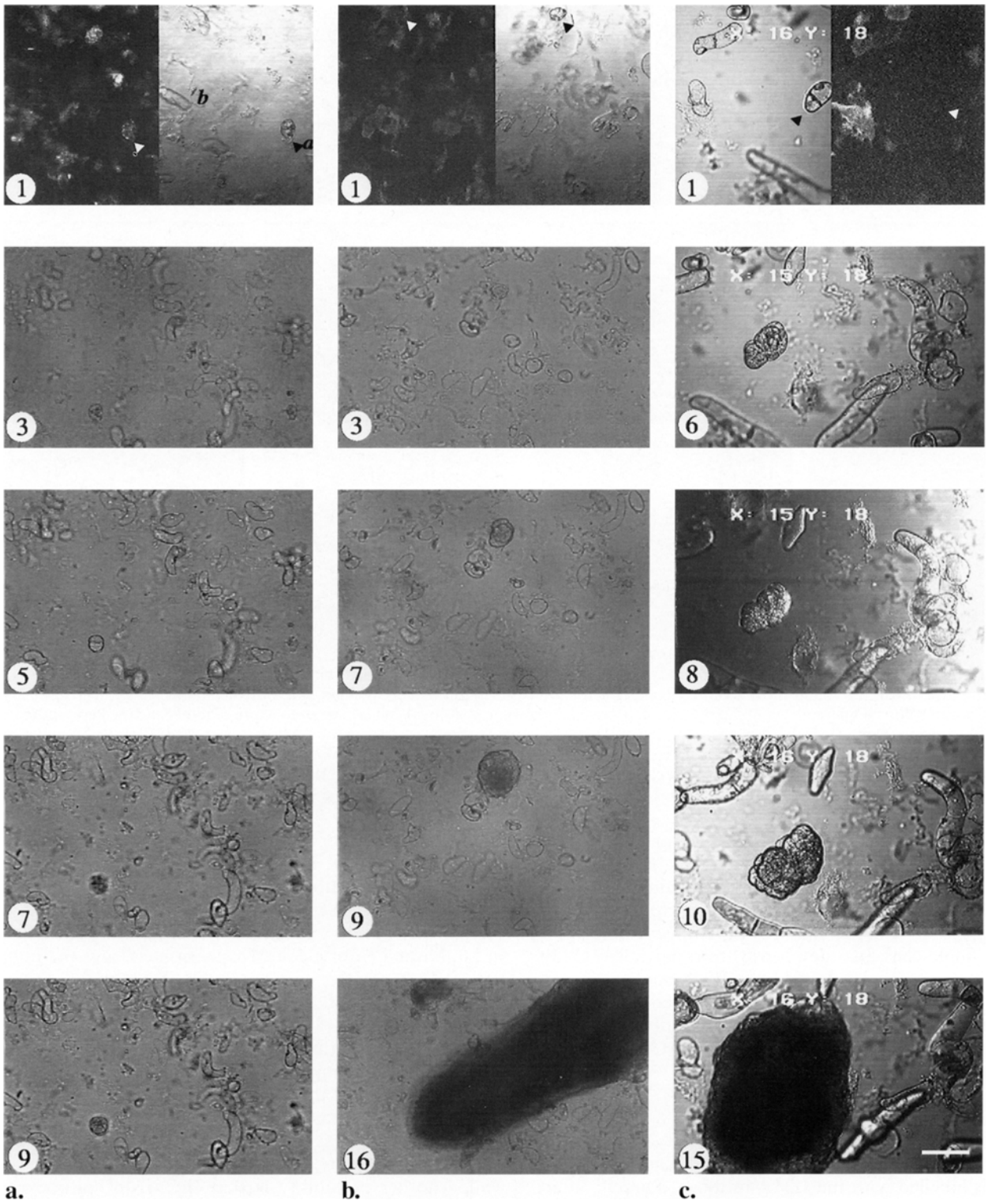


**Fig. 4a–c.** Development of embedded, JIM8-labelled carrot cells into somatic embryos as observed by cell tracking. On day 1, both the visible light image and the JIM8-FITC conjugate fluorescent signal are shown. The fluorescent signal is visualised as white signal. Subsequent development of the embryo, indicated by the day numbers is shown in the lower panels. **a** Development of a partially reactive JIM8-labelled cell into an early globular embryo. The JIM8 signal is visible on the partly reactive cell *a* that develops into the early globular embryo. Negative cells are characterised by the absence of a fluorescent signal (cell *b*). **b** Development of a JIM8-unreactive single cell into a torpedo-stage somatic embryo. **c** Development of an JIM8-unreactive divided cell into a heart-stage somatic embryo. × 540; bar = 20 μm. The numbers on the pictures in *c* are coordinates of the cell tracking system used to identify the image position

labelled cells to the basic level of about 1%. It is possible that the JIM8 epitope, therefore, becomes masked again as a result of either cell wall synthesis or rearrangement of cell wall proteins. Another possibility is that the JIM8 epitope is released into the culture medium, either as total AGP, or as polysaccharides released after the action of degrading enzymes.

Addition of 20 mM $Ca^{2+}$ to the B5-0 medium was required to obtain a JIM8 expression pattern in B5-0 medium that resembled the JIM8 expression pattern in PBS as described by Pennell et al. (1992). A similar effect could be observed in the presence of 100 mM $Na^{+}$. Without additional $Ca^{2+}$, JIM8 labelling in B5-0 medium gave patterns comparable to background signals. High salt concentrations might be required to induce a change of conformation in sugar groups present on JIM8-reactive AGPs. Only after this conformational change might the epitope be recognised by the JIM8 antibody. Another possibility is that high salt masks electrical charges in either the AGP or JIM8 molecule.

While re-activation of the cell cycle (Dudits et al. 1995) and chromosome-reducing mechanisms (Nuti Ronchi et al. 1992; Giorgetti et al. 1995) have been implicated as important mechanisms in competent cell formation, no specific markers are presently available to identify these cells. According to the model presented by Pennell et al. (1992), expression of the JIM8 epitope coincides with the competent cell stage and thus can be used as a molecular marker. However, the experiments described here show that there is no direct correlation between expression of the JIM8 epitope on the cell wall of a cell and the competent cell state. The JIM8 cell wall epitope has been identified as an AGP (R.I. Pennell, personal communication). It has been shown that this type of proteins plays a role during somatic embryo development (Kreuger and Van Holst 1993; Egertsdotter and Von Arnold 1995). When added in nanomolar concentrations, AGPs purified from embryogenic *Daucus* suspension cultures and from dry *Daucus* seed were able to promote the formation of embryogenic cell clusters and somatic embryos in embryogenic and even in previously non-embryogenic carrot suspension cultures (Kreuger and Van Holst 1993, 1995). The AGPs isolated from dry Norway spruce-seeds allowed the transition from a less developed embryo type (group B embryos) into a more mature embryo type (Egertsdotter and Von Arnold 1995). Binding of β-D-glucosyl Yariv reagent to cell wall AGPs of rose suspension cells inhibited growth in a reversible fashion, probably due to suppression of the cell cycle eventually in combination with prevention of cell expansion (Serpe and Nothnagel 1994). These observations hint that AGPs have a function in cell growth and division. Culture-medium AGPs carrying the JIM8 epitope might be developmentally related to the cell wall epitope and produced by specific 'nursing' cells in the culture. Such nursing effects have been described for individual *Brassica* protoplasts, where the frequency of microcallus formation from cytoplasm-rich protoplasts was increased by co-cultivation with a more-vacuolated protoplast type (Spangenberg et al. 1985). In established carrot suspension cultures there is a good correlation between the level of JIM8-reactive cells and the number of somatic embryos formed (Pennell et al. 1992). However, since most somatic embryos develop from JIM8-negative cells this may reflect the embryogenic competence of the entire culture rather than mark individual competent cells. This suggests that acquisition of embryogenic competence involves cells or the product of cells that are themselves not able to develop into somatic embryos.

We thank Marijke Hartog (Dept. of Molecular Biology, Agricultural University Wageningen, The Netherlands) for maintenance of the cell cultures. M.A.J.T. and E.D.L.S. are supported by the Technology Foundation (STW, Utrecht, The Netherlands) and T.H. was supported by the European Commission Biotechnology Program PTP-BIOTECH.

## References

Backs-Hüsemann D, Reinert J (1970) Embryobildung durch isolierte Einzelzellen aus Gewebekulturen von *Daucus carota*. Protoplasma 70: 49–60

De Jong AJ, Schmidt EDL, De Vries SC (1993) Early events in higher-plant embryogenesis. Plant Mol Biol 22: 367–377

De Vries SC, Booij H, Meyerink P, Huisman G, Wilde HD, Thomas TL, Van Kammen A (1988) Acquisition of embryogenic potential in carrot cell-suspension cultures. Planta 176: 196–204

Dudits D, Györgyey J, Börge L, Bakó L (1995) Molecular biology of somatic embryogenesis. In: Thorpe TA (ed.) In vitro embryogenesis in plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 267–308

Egertsdotter U, Von Arnold S (1995) Importance of arabinogalactan proteins for the development of somatic embryos of Norway spruce (*Picea abies*). Physiol Plant 93: 334–345

Giorgetti L, Vergara MR, Evangelista M, LoSchiavo F, Terzi M, Nuti Ronchi V (1995) On the occurrence of somatic meiosis in embryogenic carrot cell cultures. Mol Gen Genet 246: 657–662

Guzzo F, Baldan B, Mariani P, Loschiavo F, Terzi M (1994) Studies on the origin of totipotent cells in explants of *Daucus carota* L. J Exp Bot 45: 1427–1432

Guzzo F, Baldan B, Levi M, Sparvoli E, LoSchiavo F, Terzi M, Mariani P (1995) Early cellular events during induction of carrot explants with 2,4-D. Protoplasma 185: 28–36

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

Kreuger M, Van Holst G-J (1993) Arabinogalactan proteins are essential in somatic embryogenesis of *Daucus carota* L. Planta 189: 243–248

Kreuger M, Van Holst G-J (1995) Arabinogalactan-protein epitopes in somatic embryogenesis of *Daucus carota* L. Planta 197: 135–141

Nomura K, Komamine A (1985) Identification and isolation of single cells that produce somatic embryos at a high frequency in a carrot suspension culture. Plant Physiol 79: 988–991

Nuti Ronchi V, Giorgetti L, Tonelli M, Martini G (1992) Ploidy reduction and genome segregation in cultured carrot cell lines. II. Somatic meiosis. Plant Cell Tissue Organ Cult 30: 115–119

Pennell RI, Janniche L, Kjellbom P, Scofield GN, Peart JM, Roberts K (1991) Developmental regulation of a plasma membrane arabinogalactan protein epitope in oilseed rape flowers. Plant Cell 3: 1317–1326

Pennell RI, Janniche L, Scofield GN, Booij H, De Vries SC, Roberts K (1992) Identification of a transitional cell state in the developmental pathway to carrot somatic embryogenesis. J Cell Biol 119: 1371–1380

Serpe MD, Nothnagel EA (1994) Effects of Yariv phenylglycosides on *Rosa* cell suspensions: Evidence for the involvement of arabinogalactan-proteins in cell proliferation. Planta 193: 542–550

Spangenberg G, Koop H-U, Schweiger H-G (1985) Different types of protoplasts from *Brassica napus* L.: analysis of conditioning effects at the single cell level. Eur J Cell Biol 39: 41–45

Toonen MAJ, Hendriks T, Schmidt EDL, Verhoeven HA, Van Kammen A, De Vries SC (1994) Description of somatic-embryo-forming single cells in carrot suspension cultures employing video cell tracking. Planta 194: 565–572

Widholm JM (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol 47: 189–194