The Plant Journal (1995) 8(6), 891–906

Dynamic changes in cell surface molecules are very early events in the differentiation of mesophyll cells from *Zinnia elegans* into tracheary elements

Nicola J. Stacey[1], Keith Roberts[1], Nicholas C. Carpita[2], Brian Wells[1] and Maureen C. McCann[1,*]
[1]*Department of Cell Biology, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK, and*
[2]*Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907, USA*

Summary

The *Zinnia* mesophyll cell system consists of isolated leaf mesophyll cells in culture that can be induced, by auxin and cytokinin, to transdifferentiate semi-synchronously into tracheary elements (TEs). This system has been used to establish the precise time point at which the TE cell fate becomes determined, and then changes have been looked for in cell-wall composition and architecture that are associated with the establishment of competence, determination, and differentiation with the transition from primary to secondary cell wall formation. At very early stages in this time course, changes in the repertoire of proteins and polysaccharides both in the cell wall and secreted into the culture medium were found.

Changes in the secretion of pectic polysaccharides, xyloglucans and arabinogalactan proteins (AGPs) have been detected using the monoclonal antibodies JIM 7, CCRC-M1 and JIM 13, that recognize these three classes of cell-wall molecule, respectively. Twenty-four hours before secondary thickenings are visible, an AGP is present in the primary walls of a subpopulation of cells, and is secreted into the culture medium. This molecule is present in the secondary thickenings of mature TEs but not in their surrounding primary walls. Methyl-esterified pectic polysaccharides are present in all cell walls and are secreted into the culture medium throughout the time course of differentiation, though at an increased rate in inductive medium. However, sugar and linkage analysis of culture media shows that a relatively unbranched rhamnogalacturonan is enriched in inductive medium around the time of determination and increases rapidly in concentration. The amount of fucosylated xyloglucan in cell walls increases during the time course, but appears in inductive medium 24 h earlier than in control medium and may have a subtly different structure. The fucose-containing epitope on the xyloglucan disappears abruptly and entirely from inductive medium 6 h before any secondary thickenings are visible in the cells. The disappearance of the epitope is correlated with secretion of several hydrolytic enzyme activities.

In *Zinnia* leaves, the mesophyll cell walls contain neither the fucosylated xyloglucan nor the AGP, although methyl-esterified pectin is present. All three epitopes are expressed in the vascular bundles, and the AGP is specifically localized in the xylem cells. Fucosylated xyloglucan is also present in the epidermal tissue, and the AGP is present in guard cells.

The dynamic behaviour of these specific cell-wall molecules is tightly correlated with differentiation events *in vitro*, and can be clearly distinguished from the production of new wall material found in expanding and elongating cells. The precise timing of the appearance and disappearance of these proteins and polysaccharides compared with the point of cell-fate determination provides us with a series of cell-surface markers for cell states at very early times in the transdifferentiation pathway.

Received 24 March 1995; revised 17 August 1995; accepted 27 September 1995.
*For correspondence (fax +44 1603 451704; e-mail mccannm@bbsrc.ac.uk).

Introduction

In plants, different cell types are distinguished by their specialized cell walls. However, very little is known about the basic architecture of cell walls, the changes in that architecture that occur during growth and differentiation, and the influence of changes within the wall on processes within the cell. In recent years, it has become clear that the extracellular matrix and its components can have a profound influence on plant development (Roberts, 1994). In the two-cell *Fucus* embryo, the fate of the thallus cell can be redirected to a rhizoid cell fate by placing the protoplast in contact with the cell wall from the rhizoid cell (Berger *et al.*, 1994). A cell-wall proteoglycan is associated with the de-suppression of cell proliferation in leafy liverworts (Basile and Basile, 1993). In flowering plants, Yariv phenylglycosides specifically bind to cell-surface proteoglycans, and in doing so, suppress both division and growth of cells in culture (Serpe and Nothnagel, 1994). Given these examples, cell-surface molecules may play a role in directing cell determination and differentiation. Mesophyll cells from the leaves of *Zinnia elegans* differentiate into tracheary elements (TEs) when cultured in a medium containing a specific ratio of auxin to cytokinin (Fukuda, 1992; Fukuda and Komamine, 1980). This model

system offers controllable semi-synchronous cell differentiation in culture and is suitable both for charting the changes in cell-wall architecture during the transition from primary to secondary cell-wall deposition, and for studying the molecular basis of cell determination (Chasan, 1994).

In intact plants, TEs normally develop from proliferating cells of the procambium and the cambium. The differentiation process is irreversible and is characterized by a series of cytological changes. First, microtubules rearrange in a cortical banding pattern that reflects the position of future secondary thickenings, then cellulose is deposited in the incipient thickenings, then lignification occurs, and finally the cell undergoes programmed cell death (Mittler *et al.*, 1995). Epidermal and mesophyll cell walls are of different chemical composition, thickness and architecture, and are already adapted for their functions within the plant and yet both cell types can be induced to form tracheids *in vitro* (Church and Galston, 1989). It seems probable that some of the cellular events in the time course of *in vitro* xylogenesis must be associated with acquiring competence, then re-determination to a new cell fate, and finally with differentiation-specific events.

The most visible changes in TE differentiation occur at the cell surface, but changes in wall architecture and the influence of these changes on subsequent processes of differentiation have not been analysed, with the exception of the late event of lignification. Deposition of lignin in ordered patterns depends on the prior deposition of cellulose (Roberts *et al.*, 1985; Suzuki *et al.*, 1992), and inhibition of cellulose synthesis by the herbicide 2,6-dichlorobenzonitrile disrupts lignin patterning and causes the loss of xylans from the cellulose-depleted thickenings (Taylor *et al.*, 1992). In addition to increased cellulose and lignin synthesis necessary for secondary wall formation, an increase in total carbohydrate and xylose in alkali-extractable fractions, and a change in the relative proportion of pectic polysaccharides that are EDTA-extractable, are also correlated with the time course of TE formation (Ingold *et al.*, 1988).

Different time courses for differentiation in the *Zinnia* mesophyll cell system have been reported (Fukuda, 1992) and so it is important to define cellular events with respect to definable markers related to developmental time rather than simply in hours. Whilst differentiation is overt cell specialization, a specialization of cell character that is grossly apparent, a cell is said to be determined if it has undergone a self-perpetuating change, of internal character that distinguishes it and its progeny from other cells and commits them to a specialized course of development. The precise time at which cells become irreversibly determined can be established by transferring cells from inductive to non-inductive medium: cells transferred into non-inductive medium after at least 48 h in inductive medium still differentiate (Church and Galston, 1988). Thus, competence and cell determination are established at an earlier stage. The reorganization of microtubules (Roberts *et al.*, 1985; Seagull and Falconer, 1991) and the appearance of differentiation-specific proteins or transcripts (Demura and Fukuda, 1993, 1994; Thelen and Northcote, 1989; Ye and Varner, 1993) have been catalogued as early events, preceding the earliest deposition of cellulose thickenings detected by staining with Calcofluor white.

Using monoclonal antibodies as probes for specific cell wall epitopes, we have identified three classes of cell-wall molecule, present in xylem cells in *Zinnia* leaves and in *in vitro* TEs, that are synthesized and secreted into the culture medium at early times in the course of differentiation. An AGP is secreted 24 h before there are any visible cell-wall thickenings and is also a component of the secondary thickenings later on. Although the other two epitopes, on fucosylated xyloglucan and methyl-esterified pectin, respectively, are also present in cell walls that are expanding, biochemical analysis has shown differences in the macromolecules containing the epitopes; some differentiation-specific polymers are produced. We have also detected the secretion of a large number of proteins into the culture medium at early time-points, and several hydrolytic enzyme activities 6 h before thickenings are visible. We have mapped these events with respect to the developmental course of competence, determination and transdifferentiation.

Results

Time courses of TE formation under different culture conditions

First, we established the time of determination by transferring cells that had been cultured in inductive medium into non-inductive medium. Cells that had been in inductive medium for longer than 48 h would continue to differentiate when transferred, while cells cultured for less than 48 h did not differentiate. These results are in broad agreement with those of Church and Galston (1988). Transferring cells at different times showed that, under our particular culture conditions, 48 h was a reproducibly critical time for cell fate determination. Cell state markers such as Calcofluor white and phloroglucinol staining, as well as the dynamic cell surface changes we describe here, can then be mapped with respect to this time-point as well as to the onset of visible thickenings (Figure 1).

We have used two further controls to determine whether the synthesis and secretion of particular cell-wall molecules correlates with transdifferentiation events or with general growth events associated with cell culture. Cells are stimulated into producing wall polysaccharides upon subculture, so we first used a maintenance medium with reduced levels of cytokinin and auxin for comparison with inductive

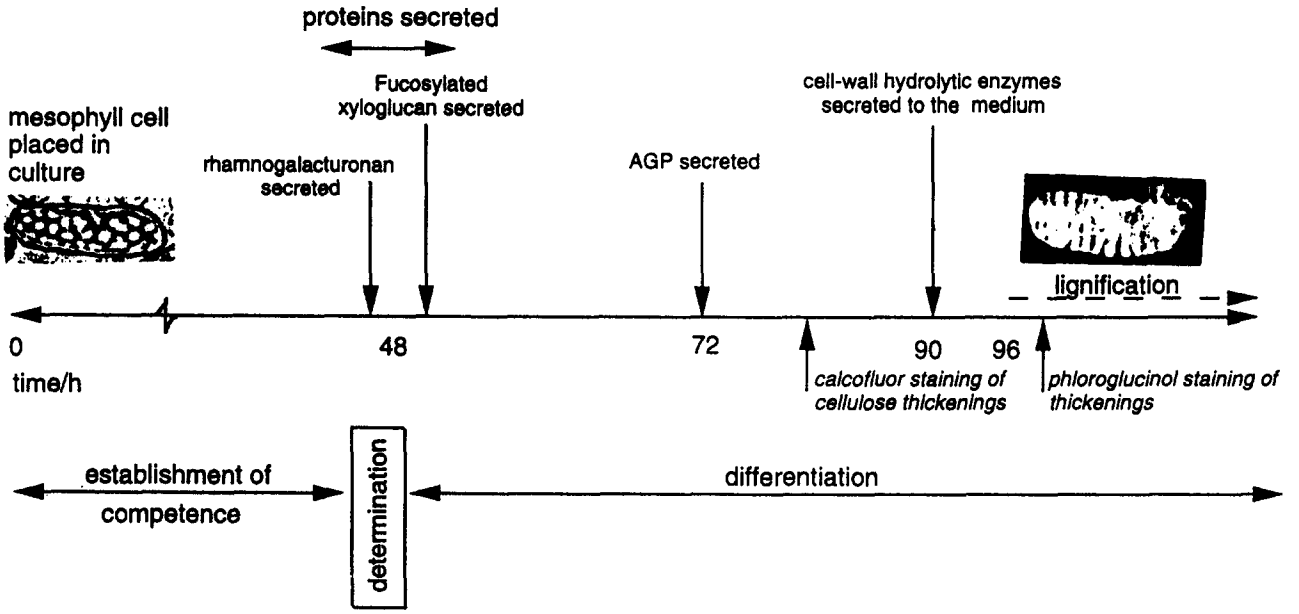

Figure 1. A development time course for the *in vitro* differentiation to TEs of *Zinnia* mesophyll cells.
The observations reported in this paper are shown mapped with respect to classical markers of cell state (in italics) and to the measured point of determination.

medium at all times. In this non-inductive medium, the cells expand and elongate but do not differentiate into TEs. A second control is to compare the range of cell-wall molecules produced by cells that elongate in inductive medium before differentiating. Coating the base of the wells of the culture dish with 1% agar delayed the time course of differentiation by 2 days, during which time cell elongation but not cell division occurred, resulting in many more elongated cells in the population and a lower frequency of differentiation (Figure 2). Embedding *Zinnia* cells in 1% agar gel further slowed this time course with cells favouring elongation over differentiation and taking up to 3 weeks to show secondary thickenings. This seems to be either an inhibitory or mildly toxic effect of the agar (Romberger and Tabor, 1971) since medium that has been washed over agar also slows down cell differentiation in the absence of the agar itself.

Secretion of cell-wall macromolecules

Cells in liquid culture are known to secrete extracellular polysaccharides and proteins, and so analysis of the culture medium provides a convenient 'snapshot' of which soluble polymers are newly synthesized. SDS–PAGE shows that a large number of new proteins appear in the medium at 48 h, at least four of which differ from proteins secreted into non-inductive media (Figure 3). Gels of media from cultures containing high auxin and no cytokinin, and high cytokinin but no auxin, as a function of time are shown in Figure 3 to distinguish between proteins that are simply induced by high levels of one or other hormone and proteins that are induced by their synergistic action. In particular, a strong band at 87 kDa and other bands at around 60, 45 and 40 kDa are only present in inductive medium. However, it should be noted that the samples of all non-inductive media have been concentrated 200-fold before loading on to the gel, to give silver-stained bands of roughly equal intensity to those of inductive medium samples.

Table 1 shows the sugar analysis of polysaccharides secreted into the culture medium at specific times expressed both as nmol amounts per 100 ml of culture medium and as relative mole percentages. The linkage analysis is given in Table 2. The medium was harvested by gently spinning down the cells to avoid the use of filters and autoclaved before dialysis to prevent degradation by hydrolytic enzyme activities. Samples that are not autoclaved show large losses of polysaccharide, particularly at later time-points. Thus, each snapshot represents a dynamic equilibrium between rates of polysaccharide secretion and degradation. More than three times the total amount of polysaccharide is present in inductive medium than in non-inductive medium up to 96 h (Table 1a). However, at a very late time-point (168 h), there are roughly equal amounts of polysaccharide present; probably a consequence of the secretion of a number of hydrolytic enzymes into inductive medium (see later). The relative mole percentages of sugars show major changes in the spectrum of polysaccharides secreted (Tables 1b and 2). In non-inductive medium, a 5-linked arabinan, 4-linked xylan (4-Xyl, 2,4-Xyl), Type II arabinogalactan associated with AGPs (t-, 6-, and 3,6-Gal), and a glucomannan (4-Glc,

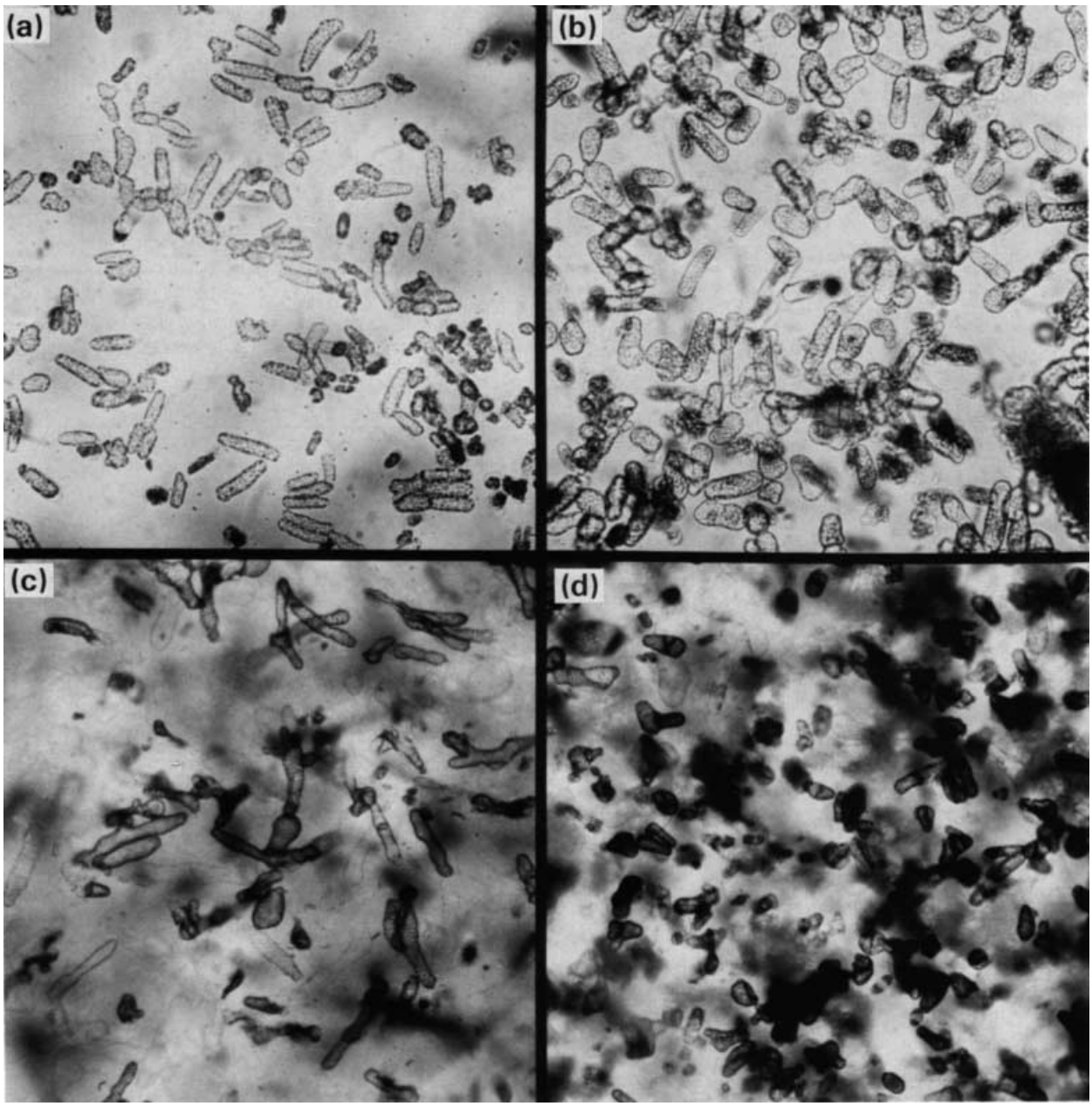

Figure 2. *Zinnia* cells in different culture conditions.
(a) Mesophyll and palisade cells freshly isolated from *Zinnia* leaves.
(b) *Zinnia* cells expand when cultured in non-inductive medium for 5 days but do not differentiate.
(c) Cells cultured in inductive medium in agar-coated wells elongate before differentiating to TEs. Fewer cells differentiate and the time course of differentiation is delayed by 48 h.
(d) Cells cultured in inductive medium differentiate to TEs without significant expansion if the bases of the wells of the culture dish are uncoated.
Magnification ×100.

4-Man), are all secreted and increase in amount in the medium. A small proportion of xyloglucan (t-Xyl, 2-Xyl, t-Fuc, 4,6-Glc) and a large proportion of an unbranched homogalacturonan (4-GalA) increase rapidly after 120 h. The changes in inductive medium are more complex. A branched rhamnogalacturonan (4-GalA, 2-Rhm, 2,4-Rhm) with short side-chains (no Type I arabinogalactan detected), and an arabinan (5-Ara) increase in amount during the time course, with the rhamnogalacturonan being over 10-fold more abundant than in the non-inductive medium. Most of the terminal fucose in the inductive medium must be associated with the rhamnogalacturonan, as there is

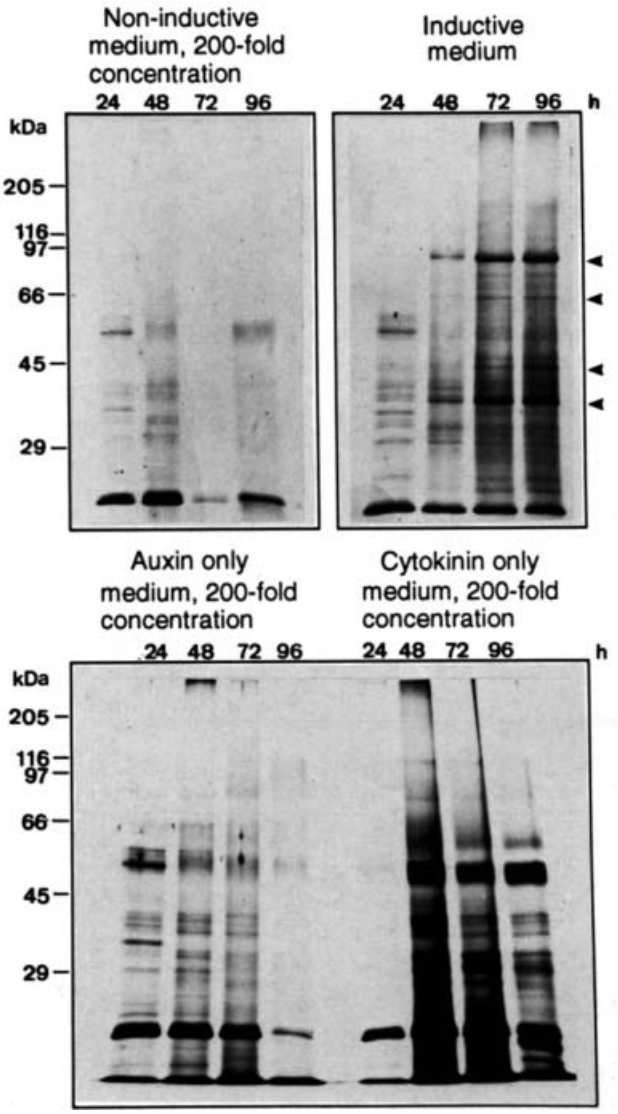

Figure 3. Silver-stained SDS–polyacrylamide gels show that a large number of differentiation-specific proteins are secreted into the culture medium at very early times in the course of differentiation.
The time courses of protein profiles in aliquots of culture medium collected at 24, 48, 72 and 96 h of subculture are shown for inductive medium, non-inductive medium, medium containing 1.0 mg l^{-1} auxin but no cytokinin and medium containing 1.0 mg l^{-1} cytokinin but no auxin. Arrows indicate major bands at 87 kDa and around 60, 45 and 40 kDa that are unique to inductive conditions.

still 0.5 mol% present at 96 h despite the loss of the CCRC-M1 fucosylated xyloglucan epitope from the medium (see later). There is also very rapid synthesis of a Type II arabinogalactan, xylan and xyloglucan, and so the amounts of these at equivalent times are very much greater in inductive than in non-inductive medium. The amount of xylan and xyloglucan halves by 96 h, as a consequence of hydrolytic activities in the medium and as newly synthesized polymers become entrapped in the incipient cellulosic thickenings.

Three specific cell-wall epitopes are present in in vitro *TEs and leaf tracheids*

Even under optimal conditions, only a proportion of the mesophyll cells differentiate with a maximum of about 60%. It is difficult, therefore, with analysis of bulk samples, to know if the observed changes are due to subculture and growth effects in the 40% of undifferentiated cells rather than to differentiation events. Populations of *Zinnia* cells at different time-points during differentiation were low-temperature-embedded (Wells, 1985) for immunogold labelling with three monoclonal antibodies to specific cell-wall epitopes. Using JIM 13, an antibody that recognizes a carbohydrate epitope on an arabinogalactan protein (Knox *et al.*, 1991), the walls of a subpopulation of cells in inductive medium are weakly labelled about 24 h before visible thickenings are present. At later time-points, the walls of a subpopulation of cells are strongly labelled, and in cells which are beginning to lay down secondary thickenings, the AGP is present both in the surrounding primary wall and the thickenings. Figure 4(a) shows that only the subpopulation of cells that have differentiated by 120 h is labelled with the JIM 13 antibody. In fully mature TEs, the AGP is restricted to the secondary thickenings and is absent from the surrounding primary wall (Figure 5a). In non-inductive medium, the AGP is secreted and is present in the cell walls at a very late time point, after 168 h.

After 24 h of culture in inductive medium, walls of some cells are very weakly labelled with the CCRC-M1 antibody, that recognizes fucosylated xyloglucan, but the density of labelling increases markedly during the time course. At 120 h, the walls of all living cells and tracheids in the population are labelled but the walls of dead cells are unlabelled (Figure 4b). Fully mature TEs are weakly labelled in the surrounding primary wall with the antibody but the epitope is excluded from the thickenings themselves (Figure 5c). In some walls, the region of the wall closest to the plasma membrane is most densely labelled.

The JIM 7-reactive epitope, present on methyl-esterified pectin, is present in walls of cells in both inductive and non-inductive medium at all times, although the amount from cell to cell is variable. The primary wall of mature TEs, but not the thickenings, is strongly labelled with JIM 7 (Figure 5b).

We have also immunolabelled sections of *Zinnia* leaf with these three antibodies, using gold/silver enhancement at the light-microscope level, and gold-labelling on grids at the electron-microscope level. Methyl-esterified pectic polysaccharides are present in all cell walls (Figure 6a) but their localization in mesophyll and palisade cells is to the outer layer of the wall (Figure 7d) whereas epidermal and vascular tissues (Figure 7c) show strong labelling throughout the wall. Fucosylated xyloglucan is present in the epidermis and vasculature (Figures 6b and 7a) but the

Table 1. Sugar analysis of secreted extracellular polysaccharides from *Zinnia* cells in inductive and non-inductive culture media

	Non-inductive				Inductive			
Sugar	72 h	96 h	120 h	168 h	48 h	72 h	96 h	168 h
(a) Relative amounts of sugars expressed as nmol per 100 ml culture medium								
Rhm	6	11	14	57	tr	117	477	597
Fuc	2	tr	nd	21	nd	33	41	63
Ara	285	242	546	1678	375	884	1475	2127
Xyl	203	262	586	1642	228	2108	1243	1470
Man	52	144	255	571	191	301	230	303
Gal	67	167	405	2156	197	1049	2350	3787
Glc	291	471	641	2056	384	552	668	993
GalA	105	304	473	3506	59	696	2082	1500
Total	1011	1602	2918	11688	1434	5741	8566	10840
(b) Relative mole percentages of sugars								
Rhm	0.6	0.6	0.5	0.5	tr	2.0	5.6	5.5
Fuc	0.1	tr	nd	0.2	nd	0.6	0.5	0.6
Ara	28.2	15.1	18.7	14.4	26.1	15.4	17.2	19.6
Xyl	19.9	16.4	20.1	14.1	15.9	36.7	14.5	13.6
Man	5.2	9.0	8.7	4.9	13.3	5.2	2.7	2.8
Gal	6.7	10.4	13.9	18.4	13.7	18.3	27.4	34.9
Glc	28.9	29.4	22.0	17.6	26.8	9.6	7.8	9.2
GalA	10.4	19.0	16.2	30.0	4.1	12.1	24.3	13.8

Table 2. Methylation analysis of secreted extracellular polysaccharides from *Zinnia* cells in inductive and non-inductive culture media

		Non-inductive (mol%)				Inductive (mol%)			
Sugar	Linkage	72 h	96 h	120 h	168 h	48 h	72 h	96 h	168 h
Rhm	2-	0.3	0.3	0.2	0.3	nd	1.4	2.1	2.1
	2,4-	0.3	0.3	0.3	0.2	nd	0.6	3.5	3.4
Fuc	t-	0.1	tr	nd	0.2	nd	0.6	0.5	0.6
Ara	t-	8.3	3.1	tr	1.3	13.3	4.3	0.9	1.8
	3-	6.5	0.1	nd	1.6	tr	0.6	0.2	1.8
	5-	8.8	11.2	16.5	9.4	7.7	8.4	14.0	14.5
	3,5-	4.6	0.7	2.2	2.1	5.1	2.1	2.0	2.8
Xyl	t-	tr	tr	tr	tr	tr	1.3	tr	tr
	2-	1.1	0.5	0.7	2.6	nd	2.9	1.5	1.2
	4-	17.3	15.0	18.1	11.5	14.7	29.0	10.8	10.9
	2,4-	1.4	0.9	1.3	1.0	1.2	3.9	1.4	1.5
Man	4-	4.8	8.1	7.7	4.8	3.6	4.6	2.1	2.3
	4,6-	0.4	0.9	1.0	0.1	9.7	0.7	0.6	0.5
Gal	t-	2.2	4.2	2.5	0.5	2.7	0.7	5.4	6.9
	6-	1.1	0.9	0.9	0.2	1.6	4.8	8.4	11.0
	3,6-	3.4	5.3	10.4	17.7	9.4	12.9	13.4	16.9
Glc	t-	0.8	1.0	0.2	0.3	tr	0.6	0.1	0.2
	4-	25.9	22.8	18.1	14.0	24.5	7.0	6.7	8.0
	4,6-	2.2	5.7	3.7	3.3	2.3	1.8	0.9	1.0
GalA	4-	10.4	19.0	16.2	30.0	4.1	12.1	24.3	13.8

epitope is absent from the mesophyll and palisade cells that will differentiate to the TEs *in vitro*. The AGP is present in xylem cells in the vascular bundles and in stomatal guard cells (Figure 6c). Figure 7(b) shows the epitope localized to specific cell walls within the vascular bundle.

Thus, three classes of cell-wall molecule, arabinogalactan

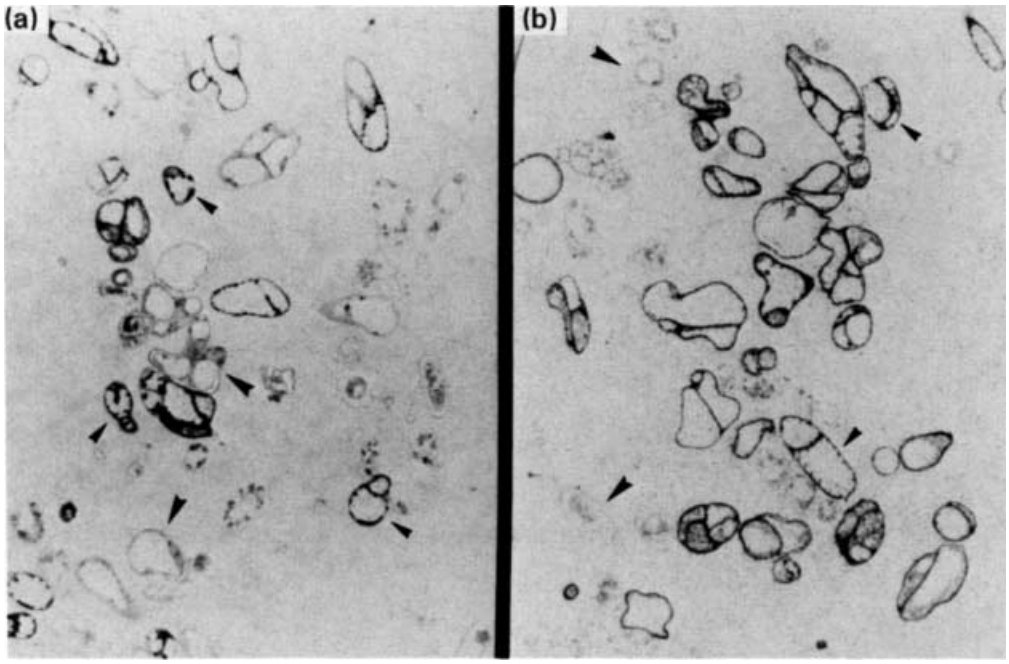

Figure 4. Immunogold/silver-enhanced 1 μm sections of *Zinnia* cells at 120 h culture in inductive medium show the subpopulation of cells that have differentiated label with (a) JIM 13 and (b) CCRC-M1 antibodies.
Labelled material appears black using bright-field microscopy and the sections have been counter-stained with Basic Fuchsin.
(a) Only the subpopulation of cells that have differentiated label with JIM 13 (small arrow). Unlabelled cells indicated by large arrow.
(b) All of the living cells and the TEs in the population label with the CCRC-M1 antibody (small arrow). Some dead cells are present and do not label (large arrow).
Magnification ×160.

proteins, fucosylated xyloglucan and methyl-esterified pectic polysaccharides are components of TEs both *in vitro* and *in vivo*.

Dynamic changes in three secreted cell-wall macromolecules

Aliquots of culture medium sampled during the time course of differentiation were analysed by immunodot-blots on nitrocellulose (Figure 8). Considerable differences can be seen in the secretion pattern of all three epitopes as a function of time, under inductive and non-inductive conditions. Immunodot-blots from cultures containing high auxin and no cytokinin, and high cytokinin but no auxin, had the same secretion pattern as N (non-inductive, maintenance) medium (data not shown). In high-auxin culture conditions, the cells divide rapidly and then elongate after 6 days, whilst in high-cytokinin conditions, the cells simply expand.

The AGP is present in inductive medium 24 h before secondary thickenings are visible and increases in concentration, but is present only weakly in non-inductive medium even at a very late time-point (after 7 days). Fucosylated xyloglucan, recognized by the CCRC-M1 antibody, appears 24 h earlier in inductive medium than in non-inductive medium but dot-blots of hourly time-points sampled between 80 h and 100 h show that the epitope abruptly and entirely disappears at 90 h, 6 h before secondary thickenings become detectable. Linkage analysis (Table 2) shows corresponding mol% decreases in t-Xyl, 2-Xyl, 4,6-Glc and t-Fuc between 72 and 96 h. In contrast, the epitope increases steadily in concentration in non-inductive medium. At later times, the epitope reappears in inductive medium, perhaps secreted by the 40% of cells that have not been determined to differentiate (Figure 8a). Immunodot-blots of culture medium sampled from cultures in wells coated with agar have the same pattern of expression as those from medium of cells in non-agar-coated wells (Figure 8b) but the time-points are delayed by the time taken for the cells to elongate. Also, the quantitative expression of these epitopes is precisely correlated with the ability of cells to differentiate when transferred from inductive to non-inductive medium. The JIM 13-reactive epitope is present at low concentration in the non-inductive medium 7 days after transfer unless the cells have been long enough in inductive medium for them to have become determined to differentiate. Also, the CCRC-M1-reactive epitope only disappears if the cells have become determined to differentiate. The CCRC-M1 antibody has been shown to weakly cross-react with a pectic polysaccharide in sycamore suspension culture medium (Puhlmann *et al.*, 1994), but anion-exchange chromatography of *Zinnia* culture medium

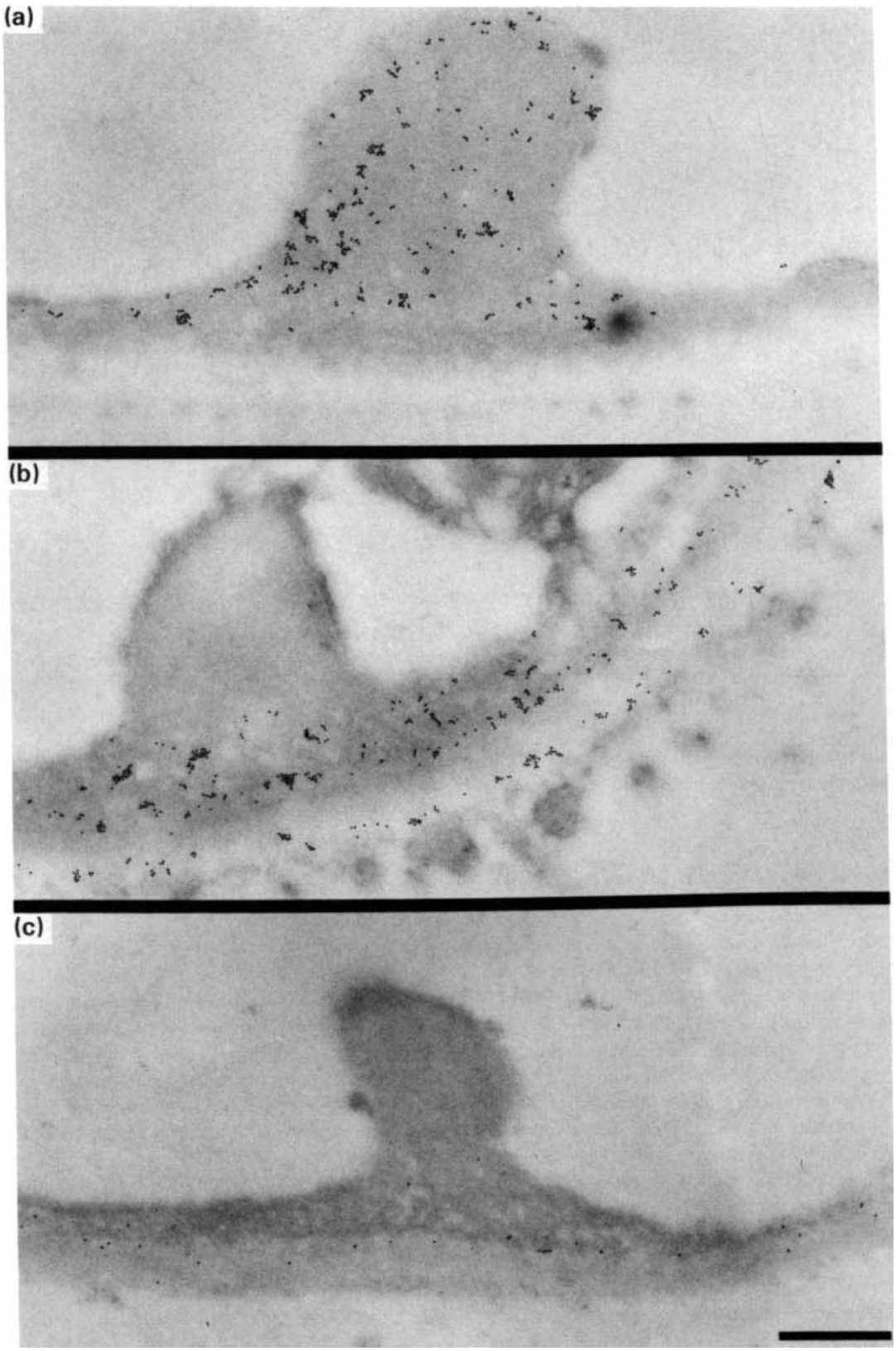

Figure 5. Electron micrographs of thin sections of secondary thickenings in fully developed *in vitro* TEs immunogold-labelled using (a) JIM 13, (b) JIM 7 and (c) CCRC-M1 antibodies.
The JIM 13-reactive epitope is present in the secondary thickening but not the surrounding primary wall, whereas JIM 7 and CCRC-M1 label the primary wall around the tracheid. Scale bar represents 200 nm.

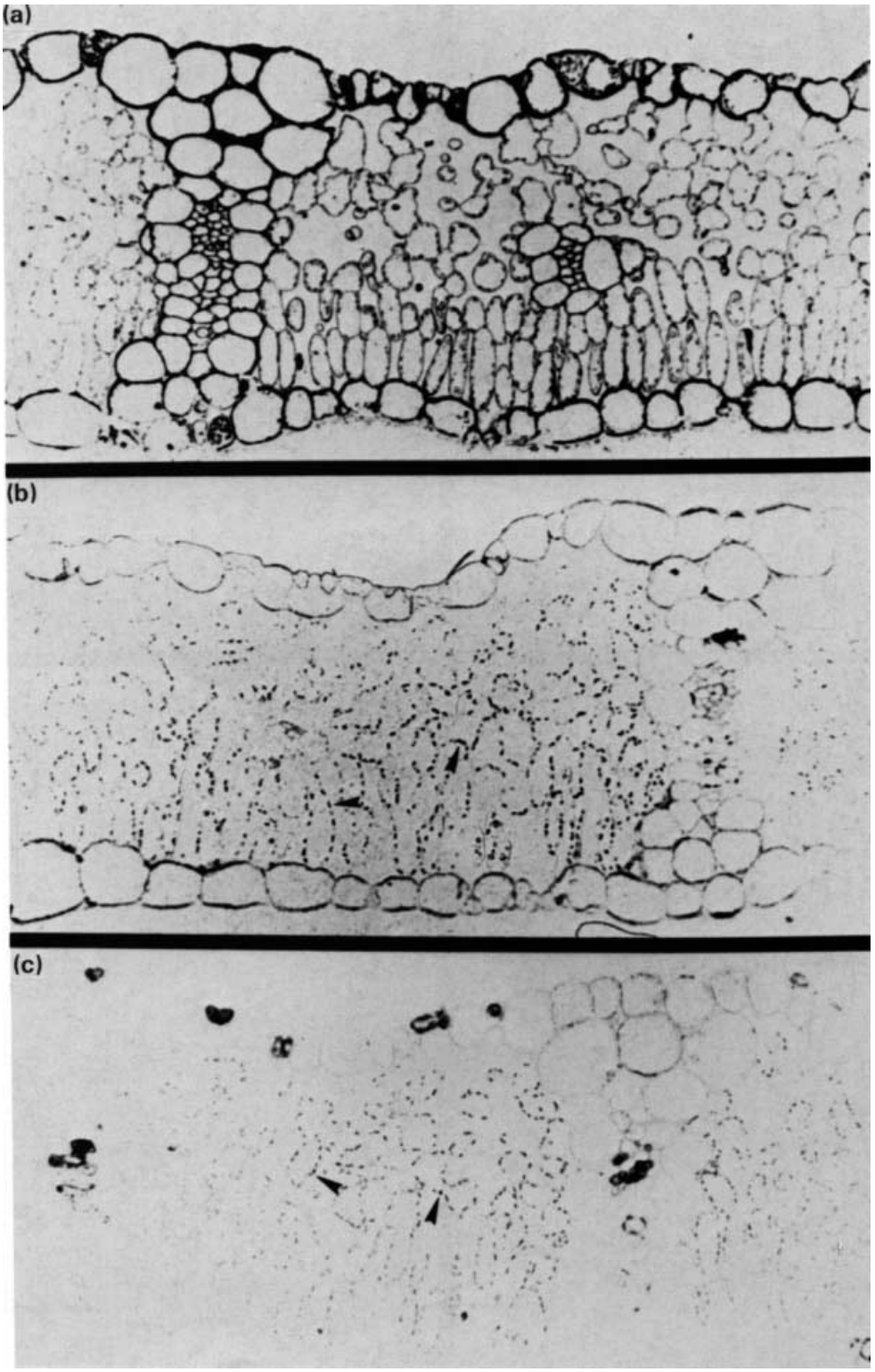

Figure 6. Light micrographs of 1 μm sections of *Zinnia* leaves which have been immunogold-labelled and silver-enhanced.
Immunolabelled material appears black using bright-field microscopy, but the spotty staining inside the cells is an artefact as a result of precipitation of silver grains on the chloroplasts (indicated by arrows on b and c).
(a) JIM 7 labels all cell walls in the leaf.
(b) CCRC-M1 antibody labels epidermis and vasculature but not the mesophyll and palisade cell walls.
(c) JIM 13 labels guard cells in the epidermis and xylem elements in the vasculature.
Magnification ×160.

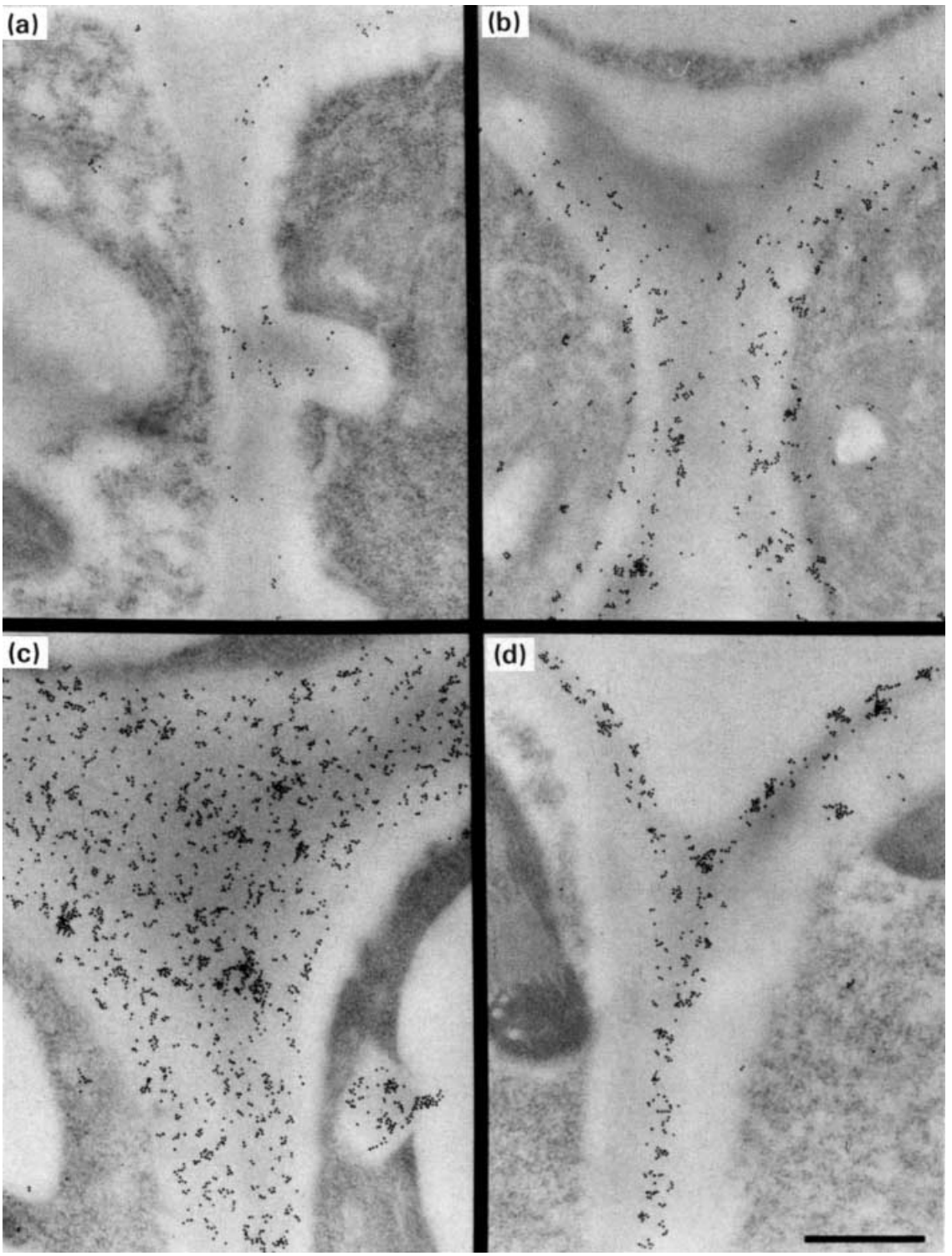

Figure 7. Electron micrographs of immunogold-labelled *Zinnia* leaf cells.
(a) CCRC-M1 antibody labels cell walls in the vasculature.
(b) JIM 13 labels some cell walls in the vasculature but not all.
(c) JIM 7 densely labels all the cell walls.
(d) In palisade cells, the JIM 7 epitope is restricted to an outer layer of the wall. Scale bar represents 200 nm.

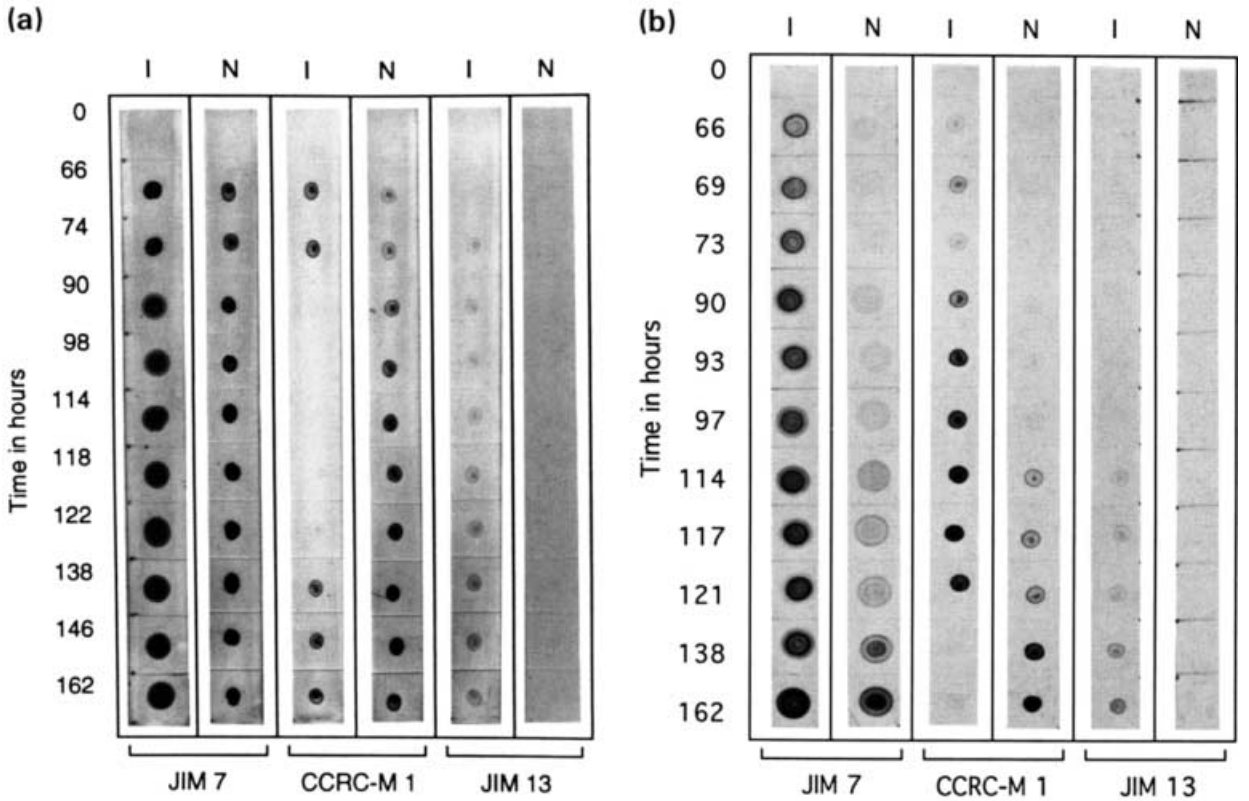

Figure 8. Immunodot-blots of culture medium aliquots sampled during the time course of differentiation and dotted on to nitrocellulose.
(a) The JIM 7 epitope dries in a series of concentric rings on the nitrocellulose, indicating a mixed population of pectins. The CCRC-M1 epitope is secreted 24 h earlier into inductive medium but is removed from the medium at 90 h, precisely 6 h before secondary thickenings are apparent. In non-inductive medium, the epitope increases steadily in concentration. The JIM 13 epitope is secreted into inductive medium at 74 h, about 24 h before secondary thickenings are visible.
(b) Immunodot-blots of culture medium aliquots from cells cultured in wells coated in agar have the same expression patterns but at later times. Cell elongation occurs in inductive medium before 48 h. The JIM 7 epitope in inductive and non-inductive media dries in two distinctive ring patterns, indicating mixed, and different, pectic populations. The CCRC-M1 epitope disappears from inductive medium at 138 h, 6 h before secondary thickenings are apparent, and the JIM 13 epitope is secreted at 114 h.

showed that the CCRC-M1 epitope was contained in a neutral fraction which did not cross-react with anti-pectin antibodies or contain any uronic acid (data not shown).

The methyl-esterified pectic epitope, recognized by the JIM 7 antibody, is present in both inductive and non-inductive media, but the pectins are synthesized and secreted at greater concentration in inductive medium than in non-inductive medium. However, pectins are a heterogeneous group of cell-wall polymers, and methyl esterification is a common modification to the newly synthesized polysaccharides. In contrast to the other two epitopes, the JIM 7-reactive epitope dries on dot-blots (see Figure 8) in a reproducible series of concentric rings with consistent marked differences between the rings present in the inductive and non-inductive medium, and between medium from cultures grown in agar-coated or uncoated wells, suggesting that during the drying of the dots some chromatographic separation of a mixture of pectins occurs on the nitrocellulose. The sugar and linkage analysis shows that a rhamnogalacturonan is secreted in large amounts under inductive conditions (Tables 1 and 2).

Release of hydrolytic enzymes

The fucosylated epitope on xyloglucan increases in concentration in inductive medium until 6 h before any visible sign of cell-wall thickening appears (Figure 8). This abrupt disappearance occurs within 1 h. We considered three possibilities: that the epitope was screened by non-covalent interactions with another secreted polymer, that the epitope has been removed by an α-fucosidase, and that the entire polymer was degraded. We mixed 90-h-old medium with a variety of agents that disrupt non-covalent binding, such as urea, high salts and SDS, but none of these treatments exposed the CCRC-M1 epitope in an immunoassay.

We made the assumption that if the epitope was removed, then enzymes must be present in the medium at this time, and that if aliquots of this medium were mixed with medium samples from earlier time-points, then the secreted enzymes would be able to digest the provided substrate. After mixing a sample of inductive medium taken at 90 h with that from 75 h, the epitope present in the latter disappeared after a 2 h incubation, that is,

the CCRC-M1 antibody no longer cross-reacted with the mixture. This activity in 90-h-old medium was found to be heat-sensitive, protease-sensitive, and was slower at 4°C than at 20°C. Another antibody against a xyloglucan epitope that does not contain fucose (Moore *et al.*, 1988), gave the same labelling pattern as the CCRC-M1 antibody with the epitope disappearing 6 h before visible thickenings and so we conclude that it is likely that the entire molecule is being degraded. In the leaf, all cell walls and the secondary thickenings are strongly labelled with this anti-xyloglucan antibody.

We took aliquots of culture medium at different times, incubated them at room temperature, and then took hourly samples to assay for loss of epitopes. The CCRC-M1-reactive epitope remained present in aliquots of medium up to 90-h-old despite long incubation times but is removed by a 2 h incubation if the medium is older than this, and thus contains secreted enzymes. It is not removed from room temperature incubations of non-inductive medium of any time-point. If enzyme-containing inductive medium is mixed with epitope-containing non-inductive medium then the epitope is also not removed, the CCRC-M1 antibody continues to label dot-blots of the mixture. The conclusion must be that the CCRC-M1 epitope in the non-inductive medium is on a different molecule than the xyloglucan secreted in inductive medium or that the xyloglucan is modified by some substituent that interferes with enzyme binding.

Although the JIM 7-reactive epitope increases in concentration in the medium, there is also turnover of pectic polysaccharides. The JIM 7-reactive epitope is also removed from free-standing isolated medium that has been sampled at time-points later than 90 h, and so the apparent increase in concentration is due to continued synthesis and secretion of excess polysaccharide. Again, we considered the possibility that either the epitope was removed, or that entire molecules were degraded. Incubation of a dot-blot of medium with 50 mM Na_2CO_3 for 1 h at room temperature removes methyl ester groups recognized by the JIM 7 antibody, and exposes JIM 5-reactive sites. As the JIM 5-reactive epitope, a relatively unesterified pectic epitope (Knox *et al.*, 1990), is also removed from free-standing, 90-h-old medium, we conclude that pectin is being degraded rather than just de-esterified.

Discussion

We have established the precise time-point at which the cell fate of *Zinnia* mesophyll cells in culture becomes determined. Events before this can be associated with the acquisition of competence to differentiate, but only at the point of determination does the cell become committed to its new fate. By transferring cells from inductive to non-inductive medium, we showed that 48 h after culture was a reproducibly critical time for cell determination and we have mapped the dynamic cell surface changes described in this paper with respect to this time-point as well as to the onset of visible thickenings (see Figure 1). We have found changes in the repertoire of both protein and polysaccharide cell-wall components, both in the cell wall and secreted into the culture medium, at very early stages of the differentiation of *Zinnia* mesophyll cells to TEs, around the time at which the cells become determined.

Changes in wall architecture occur during isodiametric cell expansion, cell elongation, and the thickening of a growing wall to its mature thickness (McCann *et al.*, 1993, 1994), and so it is important to define whether the synthesis and secretion of particular cell-wall molecules correlate with differentiation events or with general cell expansion. Cells are stimulated into producing wall polysaccharides upon subculture, so we used a non-inductive medium, in which the cells only expand, for comparison with inductive medium at all times. A second control is to compare the range of cell-wall molecules produced by cells that elongate in inductive medium before differentiating. Coating the base of the wells of the culture dish with 1% agar delayed the time course of differentiation by 2 days, enough time for considerable cell elongation to occur. The observed changes in the range of cell-wall proteins and polysaccharides and the dynamic changes in the synthesis and secretion of three particular cell-wall epitopes into the culture medium were strictly correlated with the cells having become determined to TE fate, and were not associated with either cell expansion or with the effects of subculture.

Analysis of the culture medium provides a convenient snapshot of the range of newly secreted proteins and polysaccharides at any point in time. Using SDS–PAGE, we have shown that a large number of new proteins are secreted into the medium at very early times (around the point of determination), at least four of which differ from the proteins secreted into non-inductive media, in particular, a strong band at 87 kDa. Previous studies have found only two polypeptides that are upregulated and two that are downregulated in the course of differentiation, by 2-D gel analysis of proteins isolated from cell walls (Fukuda and Komamine, 1983). However, many bands are apparent on silver-stained 1-D gels of samples from the culture medium that differ from the bands on 1-D gels of samples from control media. The amount of protein secreted into non-inductive media is very small compared with inductive medium. We have also shown that the pectins and xyloglucans are degraded by a burst of hydrolytic enzymes secreted into the medium precisely 6 h before visible thickenings have developed. In *Zinnia* leaves, xylem development may include some degradation of wall material to increase porosity and it is possible that radical

wall remodelling occurs at this time. Ye and Varner (1993) have isolated cDNAs that are upregulated in differentiating *Zinnia* cells by subtractive hybridization of cDNA libraries, but many have no homology to known proteins.

Sugar and linkage analysis of the time course shows that a xylan, an arabinan and a relatively unbranched rhamnogalacturonan are secreted very rapidly by cells in inductive medium, whilst an unbranched homogalacturonan and a glucomannan are secreted by cells in non-inductive medium. The xylan and arabinan are also secreted in non-inductive conditions but at a much slower rate. The amount of xylan and xyloglucan in inductive medium decreases to half between 72 and 96 h, probably as a result of hydrolysis of polysaccharides in the medium and incorporation of newly synthesized polymers into incipient cellulosic thickenings. This is consistent with a previous sugar analysis showing that the major changes occurring in the wall during the time course of differentiation are a large increase in xylan and a change in the proportion of chelator-soluble pectin (Ingold *et al.*, 1988). In flax, increases in the amount of 5-arabinan are also correlated with increases in 4-xylan during fibre development (Carpita, personal communication).

Using monoclonal antibodies as probes for specific cell wall epitopes, we identified three epitopes, present in xylem cell walls in *Zinnia* leaves and in *in vitro* TEs, that are synthesized and secreted into the culture medium in a developmentally regulated manner.

The JIM 13-reactive epitope, a carbohydrate epitope on an arabinogalactan protein, is present in the primary wall of a subpopulation of cells, and is also secreted into the medium 24 h before secondary thickenings are visible. As it is not present in the primary walls of fully mature TEs, it must become entrapped in the secondary thickenings, which label strongly with JIM 13, but be intrinsically soluble from the primary wall. JIM 13 labels cells in the *Arabidopsis* root (Linstead and Roberts, personal communication) and maize coleoptiles (Schindler *et al.*, 1995) that are fated to later become tracheids. In *Zinnia* leaves, the AGP is localized specifically in the xylem and guard cells; two cell types with thickened cell walls and highly specialized functions. The JIM 13-reactive epitope is also secreted in large amounts by some suspension cells, for example, carrot (McCann *et al.*, 1993) and is secreted as a very late event (7 days) in the non-inductive medium. Whether the epitope in these cases is on the same molecular species is not known.

Methyl-esterified pectic polysaccharides are present in all tissues in the *Zinnia* leaf, although they are localized to the outer region of the wall in mesophyll and palisade cells. They are also present in the walls of differentiating and non-differentiating cells *in vitro* at all times, but with varying labelling density. However, methyl esterification is a common modification of pectins, which are a highly heterogeneous class of polymers. Sugar and linkage analysis shows that a relatively unbranched rhamnogalacturonan is secreted into inductive medium, and this may account for the change in the proportion of chelator-soluble pectic polysaccharides in the cell walls reported by Ingold *et al.* (1988).

Computer modelling suggests that the fuc-gal-xyl side-chain of xyloglucan alters the conformation of the glucan backbone to facilitate binding to cellulosic microfibrils (Levy *et al.*, 1991). Modifications to this side-chain are therefore a potential control on the properties of the cellulose/xyloglucan network. Fucose is a minor component of plant cell walls and yet an *Arabidopsis* mutant that lacks fucose in the aerial parts of the plant has drastically reduced tensile strength, one-third that of the wild-type in the leaves (Reiter *et al.*, 1993). It is notable that in *Zinnia* leaves, it is the tissues with a requirement for high tensile strength, the epidermis and the vascular bundles, that contain fucosylated xyloglucan, and this labelling pattern is also seen in wild-type *Arabidopsis* leaves (Wells and McCann, personal communication). The epidermal and vascular walls are much thicker than the mesophyll cell walls, but tissue strength must be a function of cell–cell adhesion as well as of wall thickness and composition. If the presence of fucosyl residues facilitates cross-linking of microfibrils, then cellulose microfibrils from walls of neighbouring cells may become cross-linked together by the xyloglucan molecules, which are extremely long (McCann and Roberts, 1991). In mesophyll and palisade cells, cell–cell adhesion must be restricted so that air spaces can develop. Mesophyll cell walls also have an outer layer of methyl-esterified pectin which may also have a role in restricting cell–cell adhesion, whereas epidermal cell walls appear homogeneous.

The CCRC-M1 antibody only weakly labels the primary walls around mature TEs but not the thickenings. One of the very earliest responses of cells in inductive medium is the synthesis of fucosylated xyloglucan; this is present in the primary wall throughout the time course of differentiation. The fucosylated epitope recognized by the antibody disappears, however, from the culture medium precisely 6 h before any secondary thickenings are visible probably as a result of hydrolytic degradation of the xyloglucan. However, the CCRC-M1-reactive epitopes are complex since the inability of 90-h-old inductive medium to digest the epitope in non-inductive medium, whilst it can digest the epitope from inductive medium, implies that the epitope is on at least two different structures of molecule. The CCRC-M1-reactive epitope is secreted 24 h earlier in inductive medium than in non-inductive medium and in the walls of cells at a time (around 48 h) that is critical for cell determination.

We have found three carbohydrate epitopes and a range of proteins that appear to be developmentally regulated in

the *Zinnia* mesophyll cell system. The expression of these molecules precedes the time when incipient thickenings can be stained with Calcofluor white and therefore represents the earliest indication reported of architectural changes within the wall. The precise timing of the appearance and disappearance of these epitopes provide us with a series of cell-surface markers for cell state, particularly correlated with the time of determination. Their function in cell-wall architecture and possible influence on cell fate determination remains to be elucidated.

In animals, the time of cell determination is classically discovered by transplantation experiments; moving cells to a new test environment. In plants, the cell wall makes this class of experiments effectively impossible. However, in the *Zinnia* system, the ability to move a cell from an inductive culture medium to a non-inductive one, provides a unique comparable opportunity in plants. Cells that have become determined will proceed to their appropriate fate regardless of which medium they are subsequently placed in. What is the nature of the molecules that define the cell's state of determination, and the nature of the memory mechanism that maintains that state? In animals, the latter often depends on the secretion of products into the extracellular matrix that act back on the cell, in a feedback loop, to maintain it in this state. This 'autocrine' memory mechanism underlies the cooperativity of some determination events and it is possible that such a mechanism may be operating in the *Zinnia* system.

Experimental procedures

Preparation of cell cultures

Seeds of *Zinnia elegans* c. Envy (Suttons Seeds Ltd, UK) were germinated in a peat/sand potting compost in a controlled environment room at 20°C with a 16 h day. The first true leaves of 14-day-old plants were removed, surface-sterilized in 5% commercial NaOCl, and rinsed three times in sterile distilled water. Single cells were isolated by gently grinding the leaves in 0.2 M mannitol in a mortar and pestle. The suspension was filtered through two layers of muslin and washed three times in 0.2 M mannitol by spinning at 800 r.p.m. for 2 min. Cells were resuspended in culture medium (Fukuda and Komamine, 1980) with 1.0 mg l^{-1} BAP and 1.0 mg l^{-1} NAA (inductive medium or 1 μg l^{-1} BAP and 1 μg l^{-1} NAA (non-inductive medium) or 1.0 mg l^{-1} BAP or 1.0 mg l^{-1} NAA. Cultures were maintained at 27°C in the dark, shaking at 80 r.p.m., with 3 ml of cell suspension per well in 6-well plates (Sterilin, UK) at a density of 10^6 cells ml^{-1}. Solid medium was supplemented with 1% agar (LabM agar 1; Amersham, UK). To define the point of determination, cells were transferred at different times from inductive to non-inductive medium after washing twice in 0.2 M mannitol.

SDS–PAGE

Aliquots of inductive culture medium or 200-fold concentrated non-inductive media were boiled for 5 min in an equal volume of 2× sample buffer. SDS–PAGE gels (10% acrylamide) were prepared and run according to Laemmli (1970). Gels were stained by the ammoniacal silver method of Oakley *et al.* (1980).

Light microscopy

Light micrographs were taken of cell suspensions *in situ* viewed in bright field with a Zeiss inverted microscope.

Immunogold labelling

The JIM 7 and JIM 13 antibodies have been described previously (Knox *et al.*, 1990, 1991). The CCRC-M1 antibody (Puhlmann *et al.*, 1994) and anti-xyloglucan antibody (Moore *et al.*, 1988) were the kind gift of Dr Michael Hahn, Complex Carbohydrate Research Center, Georgia, and Professor Andrew Staehelin, University of Colorado at Boulder, respectively. Suspension cells were fixed for 1 h in 2% glutaraldehyde in 0.05 M cacodylate buffer pH 7.0, pelleted, and re-suspended in a drop of low-gelling-point agarose before re-fixing for low-temperature embedding (Wells, 1985). Immunogold labelling of thin sections on plastic-filmed gold grids was carried out as described previously (McCann *et al.*, 1992) except that the blocking conditions used for incubations with the CCRC-M1 antibody were 10% sheep serum, and with the other antibodies were 3% BSA, 2% low-fat milk (Boots, UK) and 1% Tween. Pieces of leaf tissue similarly prepared for low-temperature embedding were thick-sectioned for immunogold labelling (McCann *et al.*, 1992) and then silver enhancement (manufacturer's instructions, Bio-Rad silver enhancement kit, UK). Bright-field light micrographs of 1 μm thick resin sections were taken on a Zeiss photomicroscope. Sections of isolated cells were counter-stained with Basic Fuchsin after immunogold labelling and silver enhancement.

Immunodot-blots

Drops of culture medium (3 μl) sampled from wells containing *Zinnia* cells in either inductive or non-inductive medium were dried on to nitrocellulose and assayed for the presence of immunoreactive epitope as described previously (Knox *et al.*, 1990). The blocking agent was 10% sheep serum for all the antibodies.

Sugar and linkage analysis

Culture medium (100 ml) from each time-point was harvested by spinning down the cells at 800 r.p.m., then at 1500 r.p.m., and then autoclaved. The medium was then dialysed against double-distilled water and freeze-dried.

Samples were hydrolysed with 2 M TFA containing 1 μmol myo-inositol (internal standard) for 90 min at 120°C in a heating block. The TFA was evaporated under a stream of N_2, and sugars were converted to their corresponding alditol acetates (Gibeaut and Carpita, 1991a). Other samples were per-*O*-methylated by a modification of the method of Kvernheim (1987) as described previously (Gibeaut and Carpita, 1991b). The alditol acetates were separated by gas-liquid chromatography on a 0.25 mm diameter × 30 m long vitreous silica capillary column of SP-2330 (Supelco, Bellefonte, PA) temperature-programmed from 170 to 240°C at 5°C min^{-1} with a 6 min hold at the upper temperature. The partially methylated alditol acetates were separated on the same column but temperature-programmed from 160°C to 210°C at 2°C min^{-1} and then to 240°C at 5°C min^{-1} with a 4 min hold at

the upper temperature. Injector and transfer lines were at 250°C with He as carrier at 1 ml min^{-1}. Separations were carried out with a Hewlett-Packard Model 5890 gas chromatograph coupled to a Model 5971 mass-selective detector. Electron-impact mass spectra were obtained at 70 eV, and derivative structures were deduced as described in Carpita and Shea (1989).

Acknowledgements

Many thanks to Jan Peart and Anna Cullingford for supplying JIM 7 and JIM 13 antibodies, Dr Michael Hahn for the kind gift of CCRC-M1 antibody, Professor Andrew Staehelin for the anti-xyloglucan antibody, Sue Bunnewell for photography assistance, and Professor J.S. Grant Reid for useful discussions. M.C.M. is funded by a BBSRC/Unilever LINK grant. N.C.C. acknowledges USDA/NRICGP grant 90-37280-5647.

References

Basile, D.V. and Basile, M.R. (1993) The role and control of the place-dependent suppression of cell division in plant morphogenesis and phylogeny. *Mem. Torrey Bot. Club,* **25,** 63–68.

Berger, F., Taylor, A. and Brownlee, C. (1994) Cell-fate determination by the cell-wall in early *Fucus* development. *Science,* **263,** 1421–1423.

Carpita, N.C. and Shea, E.M. (1989) Linkage structure of carbohydrates by gas chromatograph-mass spectrometry (GC-MS) of partially methylated alditol acetates. In *Analysis of Carbohydrates by GLC and MS* (Bierman, C.J. and McGinnis, G.D., eds), Boca Raton, FL: CRC Press, pp. 157–216.

Chasan, R. (1994) Tracing tracheary element development. *Plant Cell,* **6,** 917–919.

Church, D.L. and Galston, A.W. (1988) Kinetics of determination in the differentiation of isolated mesophyll cells of *Zinnia elegans* to tracheary elements. *Plant Physiol.* **88,** 92–96.

Church, D.L. and Galston, A.W. (1989) Hormonal induction of vascular differentiation in cultured *Zinnia* leaf disks. *Plant Cell Physiol.* **30,** 73–78.

Demura, T. and Fukuda, H. (1993) Molecular cloning and characterization of cDNAs associated with tracheary element differentiation in cultured *Zinnia* cells. *Plant Physiol.* **103,** 815–821.

Demura, T. and Fukuda, H. (1994) Novel vascular cell-specific genes whose expression is regulated temporally and spatially during vascular system development. *Plant Cell,* **6,** 967–981.

Fukuda, H. (1992) Tracheary element formation as a model system of cell differentiation. *Int. Rev. Cytol.* **136,** 289–332.

Fukuda, H. and Komamine, A. (1980) Establishment of an experimental system for the tracheary element differentiation from single cells isolated from the mesophyll of *Zinnia elegans. Plant Physiol.* **52,** 57–60.

Fukuda, H. and Komamine, A. (1983) Changes in the synthesis of RNA and protein during tracheary element differentiation in single cells isolated from the mesophyll of *Zinnia elegans. Plant Cell Physiol.* **24,** 603–614.

Gibeaut, D.M. and Carpita, N.C. (1991a) Tracing cell wall biogenesis in intact cells and plants. *Plant Physiol.* **97,** 551–561.

Gibeaut, D.M. and Carpita, N.C. (1991b) Clean-up procedure for partially methylated alditol acetate derivatives of polysaccharides. *J. Chromatography,* **587,** 284–287.

Ingold, E., Sugiyama, M. and Komamine, A. (1988) Secondary cell wall formation. Changes in cell wall constituents during the differentiation of isolated mesophyll cells of *Zinnia elegans* to tracheary elements. *Plant Cell Physiol.* **29,** 295–303.

Knox, J.P., Linstead, P.J., King, J., Cooper, C. and Roberts, K. (1990) Pectin esterification is spatially regulated both within cell walls and between developing tissues of root apices. *Planta,* **181,** 512–521.

Knox, J.P., Linstead, P.J., King, 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.

Kvernheim, A.L. (1987) Methylation analysis of polysaccharides with butyllithium in dimethylsulfoxide. *Acta Chem. Scand. (SerB),* **41,** 150–154.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **227,** 680–685.

Levy, S., York, W.S., Stuikeprill, R., Meyer, B. and Staehelin, L.A. (1991) Simulations of the static and dynamic molecular-conformations of xyloglucan—the role of the fucosylated side-chain in surface-specific side-chain folding. *Plant J.* **1,** 195–215.

McCann, M.C. and Roberts, K. (1991) Architecture of the primary cell wall. In *The Cytoskeletal Basis of Plant Growth and Form* (Lloyd, C.W., ed.). London: Academic Press, pp. 109–129.

McCann, M.C., Wells, B. and Roberts, K. (1992) Complexity in the spatial localization and length distribution of plant cell-wall matrix polysaccharides. *J. Microsc.* **166,** 123–136.

McCann, M.C., Stacey, N.J., Wilson, R. and Roberts, K. (1993) Orientation of macromolecules in the walls of elongating carrot cells. *J. Cell Sci.* **106,** 1347–1356.

McCann, M.C., Shi, J., Roberts, K. and Carpita, N.C. (1994) Changes in pectin structure and localisation during the growth of unadapted and NaCl-adapted tobacco cells. *Plant J.* **5,** 773–785.

Mittler, A., Shulaev, V. and Lam, E. (1995) Coordinated activation of programmed cell-death and defense-mechanisms in transgenic tobacco plants expressing a bacterial proton pump. *Plant Cell,* **7,** 29–42.

Moore, P.J., Darvill, A.G., Albersheim, P. and Staehelin, L.A. (1988) Immunogold localization of xyloglucan and rhamnogalacturonan I in the cell walls of suspension-cultured sycamore cells. *Plant Physiol.* **82,** 787–794.

Oakley, B.R., Kirsch, D.R. and Morris, N.R. (1980) A simplified ultra-sensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* **105,** 361–363.

Puhlmann, J., Bucheli, E., Swain, M.J., Dunning, N., Albersheim, P., Darvill, A.G. and Hahn, M.G. (1994) Generation of monoclonal antibodies against plant cell-wall polysaccharides. I. Characterization of a monoclonal antibody to a terminal α-(1-2)-linked fucosyl-containing epitope. *Plant Physiol.* **104,** 699–710.

Reiter, W.-D., Chapple, C.C.S. and Somerville, C.R. (1993) Altered growth and cell walls in a fucose-deficient mutant of *Arabidopsis. Science,* **261,** 1032–1035.

Roberts, K. (1994) The plant extracellular matrix: in a new expansive mood. *Curr. Opinions Cell Biol.* **6,** 688–694.

Roberts, K., Burgess, J., Roberts, I. and Linstead, P. (1985) Microtubule rearrangements during plant cell growth and development: an immunofluorescent study. In *Botanical Microscopy 1985* (Robards, A.W., ed.). Oxford: Oxford University Press, pp. 263–283.

Romberger, J.A. and Tabor, C.A. (1971) The *Picea Abies* shoot apical meristem in culture. I. Agar and autoclaving effects. *Am. J. Bot.* **58,** 131–140.

Schindler, T., Bergfield, R. and Schopfer, P. (1995) Arabinogalactan proteins in maize coleoptiles: developmental relationship to cell death during xylem differentiation but not to extension growth. *Plant J.* **7,** 25–36.

Seagull, R.W. and Falconer, M.M. (1991) *In vitro* xylogenesis. In *The Cytoskeletal Basis of Plant Growth and Form* (Lloyd, C.W., ed.). London: Academic Press, pp. 183–194.

Serpe, M.D. and Nothnagel, E.A. (1994) Effects of Yariv phenylglycosides on *Rosa* cell suspensions: evidence for the involvement of arabinogalactan-proteins in cell proliferation. *Planta*, **193**, 542–550.

Suzuki, K., Ingold, E., Sugiyama, M., Fukuda, H. and Komamine, A. (1992) Effects of 2,6-dichlorobenzonitrile on differentiation to tracheary elements of isolated mesophyll cells of *Zinnia elegans* and formation of secondary cell walls. *Physiol. Plant.* **86**, 43–48.

Taylor, J.G., Owen, T.P., Koonce, L. and Haigler, C.H. (1992) Dispersed lignin in tracheary elements treated with cellulose synthesis inhibitors provides evidence that molecules of the secondary cell wall mediate wall patterning. *Plant J.* **2**, 959–970.

Thelen, M.P. and Northcote, D.H. (1989) Identification and purification of a nuclease from *Zinnia elegans*: a potential molecular marker for xylogenesis. *Planta*, **179**, 181–195.

Wells, B. (1985) Low temperature box and tissue handling device for embedding biological tissue for immunostaining. *Micron Microscopica Acta*, **16**, 49–53.

Ye, Z.-H. and Varner, J.E. (1993) Gene expression patterns associated with *in vitro* tracheary element formation in isolated single mesophyll cells of *Zinnia elegans*. *Plant Physiol.* **103**, 805–813.