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Cell-wall antigens in mesophyll cells and mesophyll-derived protoplasts of sugar beet: possible implication in protoplast recalcitrance?

Received: 18 October 2002 / Revised: 10 February 2003 / Accepted: 11 February 2003 / Published online: 3 April 2003
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Abstract We have investigated the possible relation between plant cell-wall constituents and the recalcitrance of the cell to regenerate organs and whole plants in vitro. A temporal and spatial expression of several carbohydrate epitopes was observed both within leaf tissue used for protoplast isolation and within new walls reformed by recalcitrant mesophyll protoplasts of sugar beet (*Beta vulgaris* L.); these include four pectic epitopes, one xyloglucan (rhamnogalacturonan I) epitope, two carbohydrate motifs of arabinogalactan proteins (AGPs) and callose. The walls of mesophyll cells and newly formed walls of protoplasts were similar with respect to the presence of large amounts of pectins recognized by JIM7 antibodies, the scarcity of JIM5-pectins and the complete absence of LM5-responding pectin molecules. Their main differences were the significantly higher accumulation of LM6-recognizing pectins and the very conspicuous greater accumulation of AGPs and callose in walls deposited by protoplasts than in those synthesized by donor cells.

Keywords *Beta vulgaris* L. · Cell wall · Polysaccharides · Recalcitrance in vitro

Abbreviations AGPs: Arabinogalactan proteins · BSA: Bovine serum albumin

Introduction

The plant cell wall is a dynamic structure that undergoes continuous changes in its chemical composition and molecular organization. These processes involve a coordinated series of biochemical reactions that result in the biosynthesis and degradation as well as the turnover of cell-wall components (Carpita and Gibeaut 1993). Changes in cell-wall composition and architecture are considered to be an integral part of the differentiation process and may therefore determine morphogenetic events during plant development (Fleming et al. 1997; Carpita et al. 2001; Roberts 2001).

Plant protoplasts provide an excellent model to study the correlation between cell-wall composition and cell behaviour in vitro, since successive stages of cell-wall synthesis and deposition can be traced with precision. In the investigation reported here we used material that consisted of mesophyll cells and developing mesophyll-derived protoplasts of sugar beet (*Beta vulgaris* L.). Cultured protoplasts of this species display a very low plating efficiency, ranging from 0.04% to 1.2%, and show an extremely limited capability to enter morphogenetic processes (Krens et al. 1990; Lenzner et al. 1995; Jądzewska et al. 2000). They are thus considered to be recalcitrant protoplasts.

Wall regeneration by protoplasts has been investigated in several species, mainly by means of biochemical methods including chromatographic analysis of polysaccharide components and the uptake of radiolabelled sugars into polysaccharide fractions (Takeuchi and Komamine 1978; Shea et al. 1989; Mock et al. 1990; Katsirdakis and Roubelakis-Angelakis 1992; David et al. 1994). However, because of the structural complexity of cell-wall polysaccharides and proteins, these methods do not always make it possible to identify particular sugars as components of specific carbohydrate polymers. The use of monoclonal antibodies against oligosaccharidic epitopes and the immunocytochemical detection of wall antigens in muro, seem—at the present time—to be the most informative tools to identify specific structural

Communicated by H. Lörz

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motifs that may have regulatory functions in cell development (Pennell and Roberts 1995; Knox 1996).

In our attempt to determine whether the lack of morphogenetic potential of protoplasts might be predicted on the basis of molecular characteristics of the cell wall, we used immunocytochemical methods to trace and characterize the cell-wall constituents. This article reports the pattern of temporal and spatial expression of several polysaccharide structural motifs in both leaf tissues used for protoplast isolation and in new walls being formed during the development of mesophyll protoplasts of sugar beet. Immunofluorescence and immunogold techniques were used to trace the presence and subcellular distribution of pectic epitopes recognized by the JIM7, JIM5, LM5 and LM6 antibodies, the xyloglucan (rhamnogalacturonan I) epitope recognized by the CCRC-M1 antibody, two carbohydrate epitopes characteristic of AGP that bind the JIM13 and LM2 antibodies and the presence and distribution of callose polymers. The results are discussed in terms of the biological and molecular events known to typify protoplast development *in vitro*.

Materials and methods

Plant material

Axenic shoot cultures of male-sterile, diploid genotype 491D of sugar beet (*Beta vulgaris* L.) were initiated and maintained as described by Jądzewska et al. (2000). The experimental material consisted of leaves of 2-week-old shoots, which were either immediately fixed or used as a source tissue to isolate mesophyll protoplasts. In addition, 4-week-old leaves were used to check the expression level of LM6 molecules.

Protoplast isolation and culture

Leaves were cut into approximately 1-mm strips and immediately placed in an enzyme solution containing of 0.3% cellulase R-10 and 0.4% macerozyme R-10 (both from Yakult Honsha, Tokyo), 30 mM 2-(*N*-morpholino)ethanesulfonic acid and 0.45 M sorbitol. All components were dissolved in a salt solution (CPW) according to Frearson et al. (1973).

Leaf tissues were digested for 6–8 h at 27°C in the dark, with gentle shaking. The protoplasts were then collected by filtering the macerated tissue through a 20- μ m nylon cloth, then centrifuged at 800 rpm for 10 min. The protoplast pellet was resuspended in CPW solution and centrifuged again; this step was repeated twice. Following the last centrifugation, the protoplasts were washed carefully and suspended at a density of 1.5×10^5 ml⁻¹ in liquid Murashige and Skoog (1962) medium supplemented with 5 μ M α -naphthaleneacetic acid, 2 μ M 6-benzylaminopurine, 100 μ M *n*-propyl gallate and 0.45 M sucrose. Cultures were kept at 25°C in the dark.

In vivo labelling with anti-AGP antibodies

Freshly isolated protoplasts and 4-day-old protoplasts were washed several times in CPW solution and then suspended in culture medium at pH 7.2. The material was blocked in 5% BSA in culture medium for 1 h at room temperature, and subsequently incubated for 1.5 h with JIM13 or LM2 antibodies diluted 1:10 in the medium with 0.1% BSA. The protoplasts were washed with medium several times and incubated for 1 h with FITC-anti-rat antibody, diluted

1:50, in the presence of 0.1% BSA. The excess secondary antibody was removed by repeated rinsing with pure medium. In control samples, labelling with the primary antibody was omitted.

Fixation of leaves and protoplasts

The leaves were sliced into pieces of several square millimeters in size and fixed in a mixture of 0.25% glutaraldehyde and 4% paraformaldehyde in 0.05 M Pipes buffer, pH 7.2. Fixation lasted for 24 h: an initial 3-h period at room temperature under slight vacuum was followed by 21 h at 4°C.

Protoplasts were fixed immediately after isolation, which was carried out after 4 days of culture when the protoplasts had started to resynthesize a new cell wall, and after several weeks of culture when callus aggregates had formed. Protoplasts and protoplast-derived cells were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.05 M Pipes buffer for 2 h at room temperature. After fixation, both the leaves and protoplasts were rinsed several times in buffer, dehydrated in an ethanol series, infiltrated and embedded in LR gold resin.

Immunocytochemical detection of cell-wall components

Several monoclonal antibodies were used to detect specific polysaccharide components within the walls of mesophyll cells and regenerating mesophyll-derived protoplasts. These were: (1) JIM5, showing optimal binding to pectins with 31–40% esterification (Willats et al. 2000); (2) JIM7, showing optimal binding to pectins with 15–80% esterification (Willats et al. 2000); (3) LM5, detecting neutral pectins with four residues of (1 \rightarrow 4)- β -D-galactose (Willats et al. 1999); (4) LM6, detecting neutral pectins with five residues of (1 \rightarrow 5)- α -L-arabinose (Willats et al. 1999); (5) CCRC-M1, detecting xyloglucans (rhamnogalacturonans I) with terminal α -(1 \rightarrow 2)-fucosyl residues (Puhlmann et al. 1994); (6) anti- β -(1 \rightarrow 3)-glucan-detecting β -(1 \rightarrow 3)-glucans (callose); (7) LM2, binding to carbohydrate epitopes of AGP that contain β -linked glucuronic acid (Yates et al. 1996); (8) JIM13, binding to AGPs that contain the GlcpA- β (1 \rightarrow 3)-D-GalpA- α (1 \rightarrow 2)-L-Rha motif (Yates et al. 1996).

The JIM5, JIM7, and JIM13 antibodies were kindly provided by Dr. K. Roberts (John Innes Center Institute, UK); LM2 was provided by Dr. P. Knox (University of Leeds, UK); CCRC-M1 was provided by Dr. M. Hahn (University of Georgia, USA); LM5 and LM6 were obtained from Plant Probes (Leeds, UK); anti- β -(1 \rightarrow 3)-glucan was from Australia Biosupplies (Parkville, Australia).

Immunocytochemical reactions were performed on semi-thin (0.5 μ m) and ultra-thin (70–90 nm) sections. The experimental procedures were exactly as those described by Majewska-Sawka et al. (2002).

Results

Walls of mesophyll cells within the leaf

Both palisade and spongy mesophyll cells were strongly labelled with the JIM7 antibody (Fig. 1A). Gold particles were distributed randomly across the entire width and length of the wall (Fig. 1B) and were similar in abundance in various cells and different regions of the wall. Intercellular spaces were completely devoid of label. The JIM5 antibody was recognized in only some walls and some regions of the cell walls, and the intensity of labelling differed in different areas of the wall surrounding the same cell (Fig. 1C). The distribution of gold

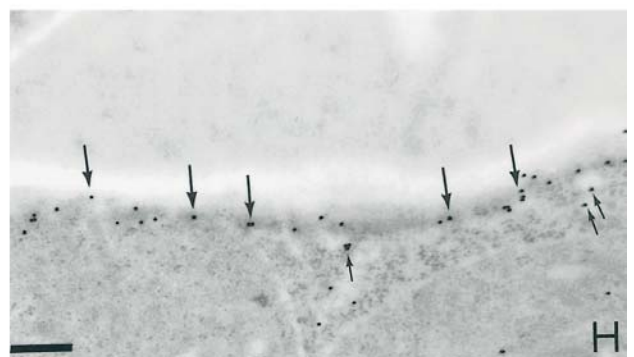
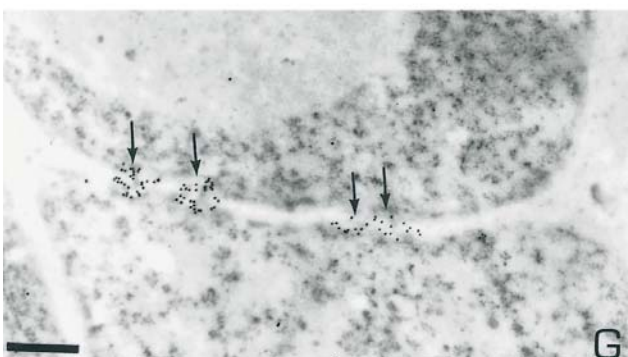
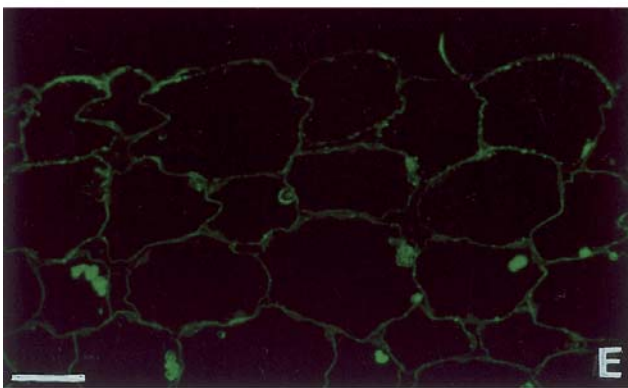
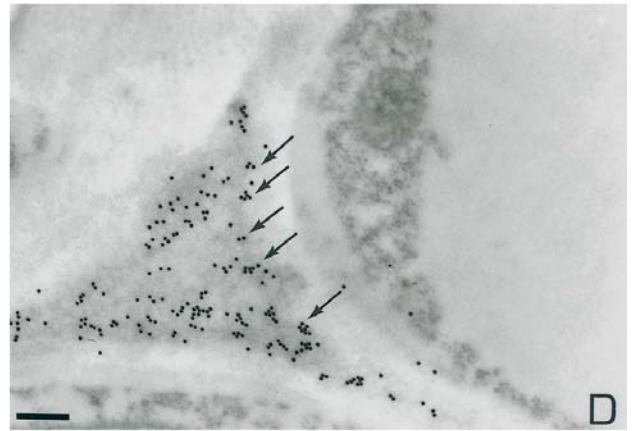
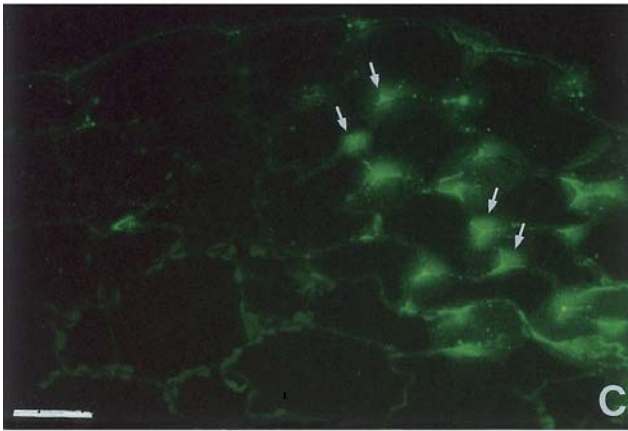
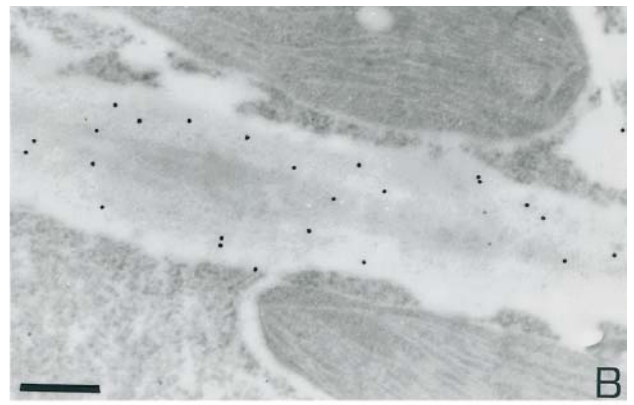
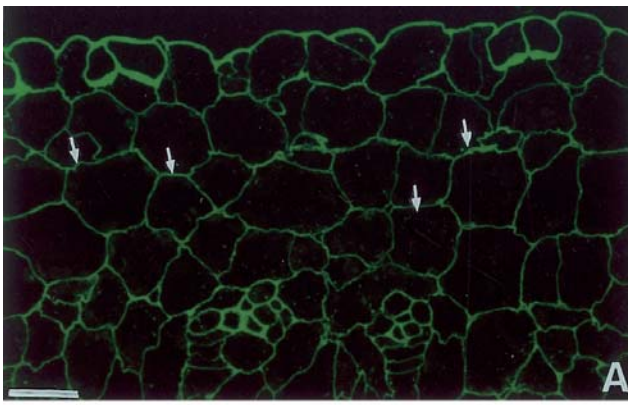


Table 1 Immunolocalization of carbohydrate epitopes within mesophyll cells and mesophyll-derived protoplasts of sugar beet, genotype 491D

Cell/tissue type	Antigen detection ^a as determined by the intensity of antibody binding							
	JIM13	LM2	JIM5	JIM7	LM5	LM6	CCRC-M1	Anti- β -(1→3)-glucan
Leaf mesophyll	+	+	+	++	–	+	+	+
Mesophyll protoplasts:								
Freshly isolated	+	+	–	–	–	–	–	–
Four-days-old	++	++	–	–	–	–	+	++
Protoplast-derived callus	++	++	–	++	–	++	+	+

^a+, Present; ++, present and very abundant; –, not present or present in trace amounts

particles was clearly limited to the middle lamella of primary walls and to the intercellular spaces (Fig. 1D). Reactions with the LM6 antibody resulted in no labelling or very weak labelling of only some cells in 2-week-old leaves, but the synthesis of neutral pectins with five residues of (1→5)- α -L-arabinose clearly increased as the leaf aged (Fig. 1E). No signal could be found in the walls of mesophyll cells after incubation with the LM5 antibody.

Xyloglucans with terminal fucosyl residues were scarce in mesophyll cell walls, and only a few gold particles were observed in some cells. The abundance of gold particles was variable in different cells and different regions of the same wall (Fig. 1F).

Labelling with the anti- β -(1→3)-glucan antibody produced only localized signals within mesophyll cell walls, and sporadic reactions within young walls formed between two daughter cells immediately after cytokinesis. As revealed by the electron microscope, this circumscribed labelling corresponded to plasmodesmata connections (Fig. 1G).

The presence of AGPs was traced with the JIM13 and LM2 antibodies. Both reagents produced labelling in all leaf cells, and the intensity of the signal was similar throughout the mesophyll tissue. These proteoglycan epitopes were observed mainly in the plasma membranes and, to a much lesser extent, in the cytoplasmic vesicles (Fig. 1H). The results of immunocytochemical analyses of particular epitopes within the leaf tissue are presented in Table 1.

Fig. 1A–H Immunolabelling of leaf sections with a panel of monoclonal antibodies reveals the presence and distribution of carbohydrate epitopes within the walls. **A** The signal (*arrows*) resulting from JIM7 binding is present in all mesophyll cells, **B** gold particles are distributed randomly across and in the walls, **C** the JIM5 antibody binds only to the walls of some cells, **C**, **D** the marker frequently accumulates in the intercellular spaces (*arrows*), **E** LM6-recognizing molecules are abundant within mesophyll cell walls, but only in relatively old leaves, **F** the CCRC-M1 antibody is only weakly bound to the walls of mesophyll tissue, and the gold marker is unevenly distributed in different zones of the wall (*arrows*), **G** callose is distributed in localized areas within the walls corresponding to plasmodesmata connections (*arrows*), **H** the anti-AGP JIM13 antibody binds strongly to mesophyll tissue, mainly to the plasma membrane (*arrows*) and, to a much lesser extent, to the cytoplasmic vesicles (*small arrows*). Bars: 20 μ m (**A**, **C**, **E**); 0.25 μ m (**B**, **D**, **F–H**)

Walls regenerated by mesophyll protoplasts

Freshly isolated mesophyll protoplasts were spherical, ranging in diameter from 15 μ m to 20 μ m (Fig. 2A). They were filled with numerous chloroplasts, some of which contained tiny starch granules. Electron microscopic observations showed that the protoplasts were delineated by the plasma membrane and contained no remnants of the cell wall (Fig. 2B). Immediately after isolation, the only components that could be detected—among those studied by us—were two carbohydrate epitopes of AGP recognized by the LM2 and JIM13 antibodies. Both were found at the plasma membrane and in cytoplasmic vesicles (Fig. 2B).

After 4 days of culture, the most visible change in the protoplasts was the appearance of one or several large vacuoles, which squeezed the cytoplasm against the plasma membrane. Other events were the conspicuous accumulation of starch within the chloroplasts and deposition of the incipient components of a new wall at the protoplast surface (Fig. 2C). Two AGP epitopes were relatively more abundant than in freshly isolated protoplasts and were also detected in numerous protoplasts by *in vivo* immunolabelling (Fig. 2D, E). β -(1→3)-glucan was a particularly abundant polysaccharide synthesized by the protoplasts in this early phase of development. It was deposited at the surface of the plasma membrane either as discrete patches or in the form of a continuous layer around the protoplasts (Fig. 2F, G). The fucosylated epitope of xyloglucans was not detected with the immu-

Fig. 2A–I Freshly isolated protoplasts (**A**, **B**) and 4-day-old protoplasts (**C–I**) illustrate the temporal sequence of carbohydrate epitope deposition at the protoplast surface. **A**, **B** Freshly isolated protoplasts (**A**) are delineated by the plasma membrane and labelled with the anti-AGP JIM13 antibody (**B**, *arrows*). **C–I** Four-day-old protoplasts show conspicuous accumulations of starch (*s*) within plastids (*p*), mitochondria (*m*) with well-developed cristae, and incipient new wall material at the surface (**C**, *arrows*); labelling with LM2 *in vivo* (**D**, *arrows*) documents the presence of AGP, whereas control reactions result only in red autofluorescence of chloroplasts (**E**); callose is abundant, as revealed by both the immunofluorescence (**F**, *arrows*) and immunogold techniques (**G**, *arrows*); CCRC-M1 labels the contents of cytoplasmic vesicles (**H**, *arrows*); but the epitopes it recognizes are not observed on the protoplast surface; control reactions with the anti-mouse secondary antibody coupled to colloidal gold result in no signal (**I**). Bars: 20 μ m (**A**, **D–F**); 0.5 μ m (**C**, **G**), 0.25 μ m (**B**, **H**, **I**)

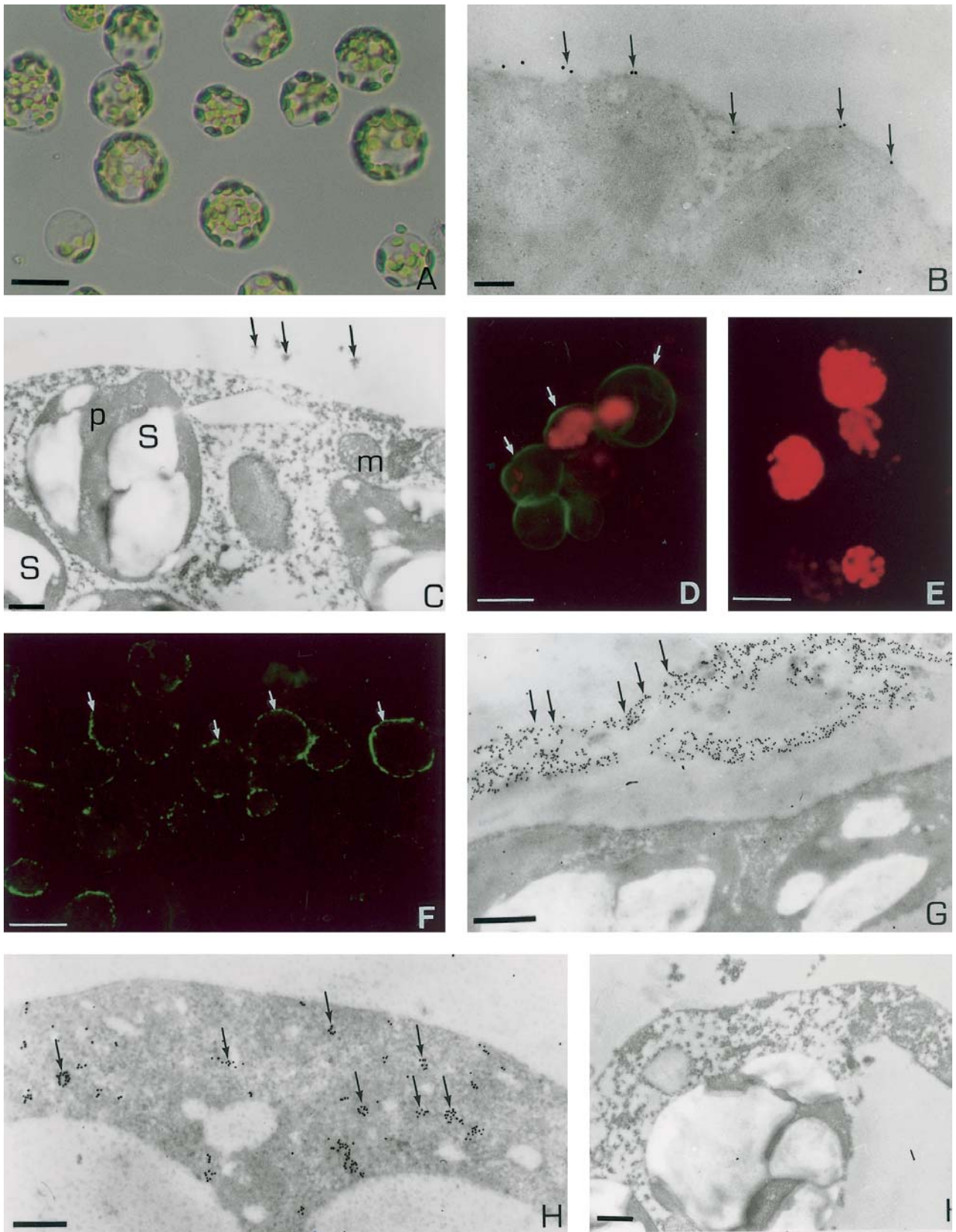


Fig. 2A-I Legend see page 949

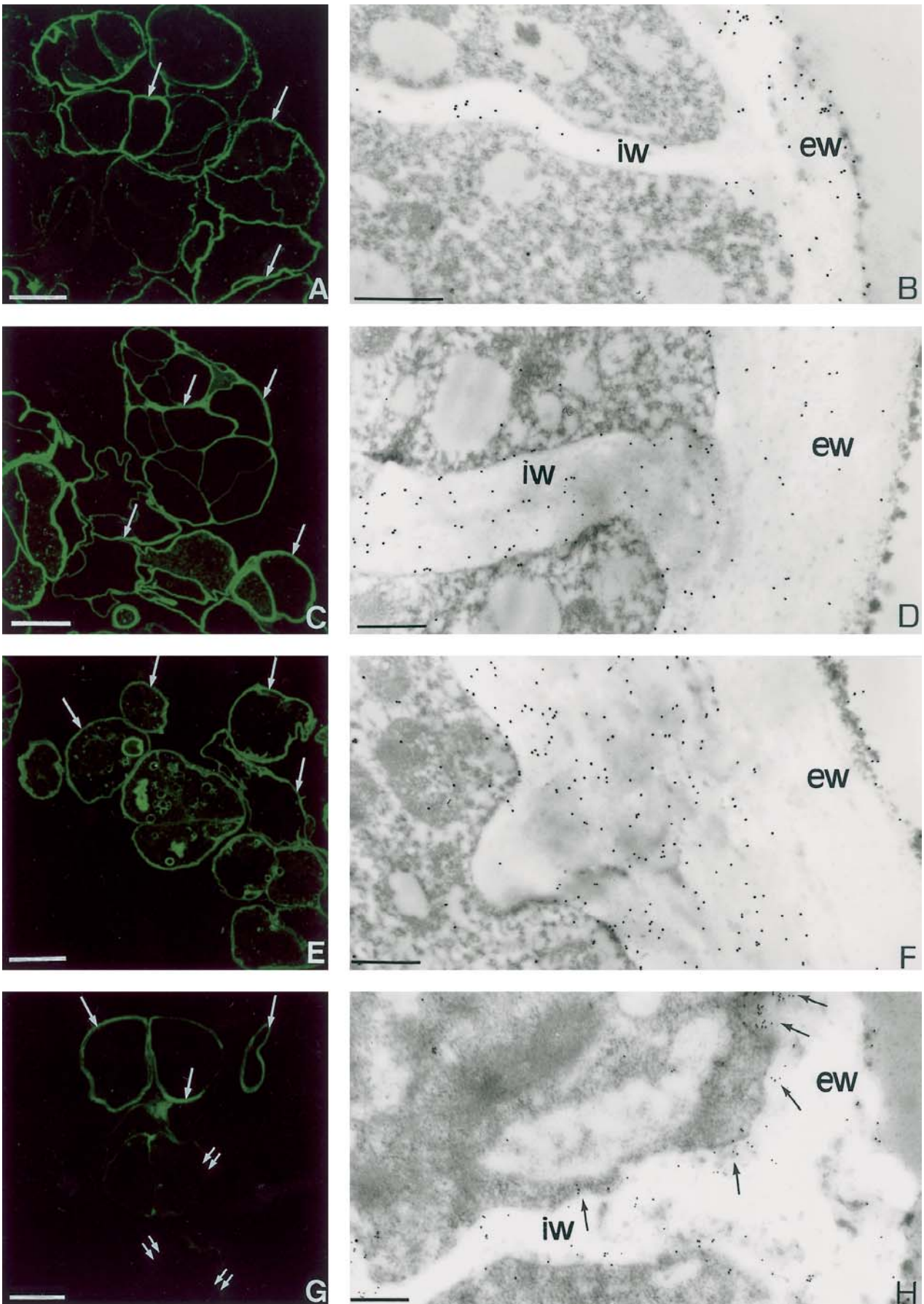


Fig. 3A-H Legend see page 952

nofluorescence technique, but careful examination of sections with an electron microscope revealed the presence of numerous gold particles within cytoplasmic vesicles, mainly in the vicinity of the plasma membrane (Fig. 2H). Control reactions performed by omitting the primary antibody and using a gold-conjugated secondary antibody resulted in no labelling (Fig. 2I). Reactions with the JIM7 antibody resulted in the sporadic appearance of a few gold particles outside the protoplast membrane (not shown), whereas JIM5, LM5 and LM6 did not produce any labelling.

The protoplasts divided after about 10 days of culture, and after a further 2–3 weeks small callus colonies had formed. The presence and precise distribution of carbohydrate antigens in completely regenerated cell walls is documented in Fig. 3 and summarized in Table 1. Pectins detected by the JIM7 antibody were abundantly deposited in the walls surrounding callus cells. They were found both in young, thin walls formed just after cell division and in older, much thicker walls that surrounded aggregates of several cells and remained in direct contact with the environment (Fig. 3A, B). The label was distributed more or less homogeneously in the walls. Intense signals were found in most cells after the immune reaction with the LM6 antibody, which binds to five arabinose residues within neutral side chains of pectins (Fig. 3C). As revealed by electron microscopy (Fig. 3D), the distribution of gold particles was similar to that described for JIM7. Pectic epitopes recognized by the JIM5 and LM5 antibodies were detected only sporadically in a few cells, and the labelling was extremely weak (not shown).

Fully developed walls synthesized by developing protoplasts were strongly labelled with two anti-AGP antibodies (Fig. 3E). Gold particles were more abundantly distributed in the wall area close to the plasma membrane in comparison to the external part of the wall (Fig. 3F). Epitopes of xyloglucan (rhamnogalacturonan I) recognized by CCRC-M1 were abundant in some cells but totally absent in other callus cells (Fig. 3G). Within one aggregate composed of several cells, these molecules were found both in young walls and in thick external walls (Fig. 3H). When present, they showed a characteristic distribution with relatively large amounts appearing in the wall zone adjacent to the plasmalemma and much smaller amounts in the thickness of the wall (Fig. 3H).

◀ **Fig. 3A–H** Immunolabelling of protoplast-derived callus with a panel of monoclonal antibodies. The signal resulting from JIM7 (**A, B**) and LM6 binding (**C, D**) is present in almost all callus cell walls (**A, C**, arrows). Gold particles mark both the thick, external walls in direct contact with the environment (*ew*) and the thinner, internal walls (*iw*) separating two daughter cells. The signal resulting from JIM13 binding is present in almost all callus cell walls (**E**, arrows). Gold particles are not homogeneously distributed across the wall (*ew*) but accumulate in the zone close to the plasma membrane; weak labelling of the cytoplasm can also be seen (**F**). Reactions with CCRC-M1 produce signals in only a few cells (**G**, arrows), whereas the others remain unlabelled (*small double arrows*). Gold particles frequently accumulate in the wall zones (*ew, iw*) adjacent to the plasma membrane (**H**, arrows). Bars: 20 μm (**A, C, E, G**); 0.5 μm (**B, F, H**), 0.25 μm (**D**)

Discussion

This paper represents part of ongoing studies designed to elucidate the relation between cell-wall composition and the development of cells in vitro, in terms of their recalcitrance to differentiate into new tissues and organs. We used immunocytochemical methods to characterize and compare the carbohydrate structure of cell walls in two culture systems: (1) leaf mesophyll tissue and (2) mesophyll-derived protoplasts. The latter are rarely able to re-form walls, divide and produce non-regenerable calli. The most noteworthy properties of this new protoplast wall—compared to the intact leaf—were the significantly higher accumulation of LM6-recognizing pectins, the larger contribution of callose and the much more conspicuous accumulation of AGPs.

Pectins constitute the main component of the primary cell walls and are responsible for their mechanical properties and involved in the regulation of morphogenetic processes (Stolle-Smits et al. 1999; Ermel et al. 2000). Protoplasts and cells cultured in vitro mostly secrete esterified pectins, and considerable amounts of these substances are released into the medium (Shea et al. 1989; David et al. 1995; Stacey et al. 1995). We have demonstrated that mesophyll-derived protoplasts of sugar beet synthesize large amounts of pectins that bind the JIM7 and LM6 antibodies and only traces of pectins that react with JIM5.

In carrot suspension cells, the content and structure of neutral side chains within pectins, i.e. α -(1 \rightarrow 5)-L-Ara or β -(1 \rightarrow 4)-D-Gal, have been correlated with the strength of intercellular contacts and, consequently, with the differentiation process. Non-embryogenic, actively proliferating cells of cv. Early Nantes were shown to be rich in arabinose, whereas galactose constituted the predominant sugar in differentiated, elongated cells that developed after 2,4-dichlorophenoxyacetic acid was removed from the medium (Willats et al. 1999). In recalcitrant, well-proliferating protoplast-derived calli of sugar beet, the deposition of α -(1 \rightarrow 5)-L-Ara motifs is also very intense, a finding consistent with the results of Willats et al. (1999). However, suspensions of another carrot cultivar, US-Harumakigosun, were shown to contain large amounts of arabinose only in highly embryogenic, compact clusters or in somatic embryos, whereas non-embryogenic, loosely attached cells contained large amounts of galactose (Kikuchi et al. 1995; Iwai et al. 1999). This discrepancy may reflect genotype-related differences or may result from the different culture conditions used by the two research groups.

Regulated growth of the cell wall also depends on xyloglucan metabolism, a process that is tissue-specific (Pauly 2001). Molecules with terminal fucosyl residues are present in sugar beet mesophyll cells and in some mesophyll-derived protoplasts, but in relatively low amounts. As shown in our electron micrographs, xyloglucan molecules are initially localized within Golgi-derived vesicles and later found in the re-formed wall. Similar cytoplasmic locations of these epitopes were reported for

cells of *Trifolium pratense* (Moore and Staehelin 1988) and *Arabidopsis thaliana* (Freshour et al. 1996) and for protoplasts of tobacco line BY-2 (Sonobe et al. 2000). Recently, the same secretion pathway was described for endoxyloglucan transferase, a key enzyme involved in the formation of xyloglucan-cellulose network within primary walls (Yokoyama and Nishitani 2001).

Arabinogalactan proteins are abundantly synthesized by plant cells and protoplasts cultured in vitro and are located within the plasma membrane, inside the newly regenerating cell wall and, to much lower extent, in the cytoplasm, the latter site probably reflecting their transport from Golgi structures or from the endoplasmic reticulum towards the plasma membrane (Šamaj et al. 1998, 2000). Our data complete earlier reports that type II arabinogalactans—the main components of AGPs—are actively secreted by protoplasts of *Vinca rosea* and *Daucus carota* (Takeuchi and Komamine 1978; Shea et al. 1989; Mock et al. 1990). In previous studies of sugar beet suspension-derived protoplasts and mesophyll-derived protoplasts of lines different from the one used in the present study, we also demonstrated the involvement of two carbohydrate epitopes of AGPs in the formation of new cell walls of non-embryogenic cells, and their essential role in the proper assembly of cell wall polysaccharides (Butowt et al. 1999).

We document here the sequence of synthesis and deposition of several polysaccharide epitopes within primary walls formed at the surface of recalcitrant sugar beet protoplasts growing under well-defined experimental conditions. Arabinogalactan proteins containing JIM13 and LM2 epitopes as well as pectic polysaccharides that possess arabinose-rich side chains are the most abundant components of mesophyll protoplast-derived cells. These carbohydrate motifs are probably important for the formation and proper assembly of the incipient wall and provide a network for the attachment of other wall components. Our observations constitute, to our knowledge, a novel contribution to our still limited understanding of events leading to the regeneration of new cell walls. The specific polysaccharide compounds synthesized by sugar beet protoplasts may have an influence on tissue properties in vitro, particularly on the strength of intercellular attachments—hence the recalcitrance in morphogenic responses. Studies now in progress will determine the composition of the cell wall in highly morphogenic cells of sugar beet and, subsequently, whether specific components have direct implications for further cell growth or the lack of morphogenic response.

Acknowledgements This study was supported by The Polish Committee for Scientific Research (project no. 5-P06A 040-17). The authors thank Dr. K. Roberts (John Innes Centre, UK) for providing the JIM5, JIM7 and JIM13 antibodies, Dr. P. Knox (University of Leeds, UK) for the LM2 antibody, and Dr. M. Hahn (University of Georgia, USA) for the CCRC-M1 antibody. We acknowledge M.I. Rodriguez-Garcia (Estación Experimental del Zaidin, CSIC, Spain) and A. Tretyn (University of Nicolas Copernicus, Poland) for the use of electron microscopes. We also

thank K. Shashok and Sz. Andrzejewski for correcting the English version of the manuscript.

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