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Cell wall pectins and xyloglucans are internalized into dividing root cells and accumulate within cell plates during cytokinesis

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Summary. Recently, we have reported that cell wall pectins are internalized into apical meristem root cells. In cells exposed to the fungal metabolite brefeldin A, all secretory pathways were inhibited, while endocytic pathways remained intact, resulting in accumulation of internalized cell wall pectins within brefeldin A-induced compartments. Here we report that, in addition to the already published cell wall epitopes, rhamnogalacturonan I and xyloglucans also undergo large-scale internalization into dividing root cells. Interestingly, multilamellar endosomes were identified as compartments internalizing arabinan cell wall pectins reactive to the 6D7 antibody, while large vacuole-like endosomes internalized homogalacturonans reactive to the 2F4 antibody. As all endosomes belong topographically to the exocellular space, cell wall pectins deposited in these "cell wall islands", enclosed by the plasma-membrane-derived membrane, are ideally suited to act as temporary stores for rapid formation of cell wall and generation of new plasma membrane. In accordance with this notion, we report that all cell wall pectins and xyloglucans that internalize into endosomes are highly enriched within cytokinetic cell plates and accumulate within brefeldin A compartments. On the other hand, only small amounts of the pectins reactive to the JIM7 antibody, which are produced in the Golgi apparatus, localize to cell plates and they do not accumulate within brefeldin A compartments. In conclusion, meristematic root cells have developed pathways for internalization and recycling of cell wall molecules which are relevant for plant-specific cytokinesis.

Keywords: Brefeldin A; Cell plate; Cell wall; Cytokinesis; Endocytosis; Golgi apparatus; Pectin.

Introduction

Plant cells generate their walls through secretory pathways that begin in the endoplasmic reticulum (ER), pass through compartments of the Golgi apparatus (GA), and then terminate with anterograde vesicles which fuse with the plasma membrane (PM), extruding their contents into the walls. For many years, the central tenet of cell wall formation has been that newly synthesized cell wall molecules released on the external face of the plasma membrane merge with the already present cell wall and remain there as long as the cell is integrated within a given plant tissue. According to this classical model, the only way cell wall remodeling can be accomplished in plants is via in muro enzymatic activities within the cell wall itself.

Embedding techniques using Steedman's wax enabled us to monitor diverse antigens both in the cytoplasm and within cell walls of maize root apices (Baluška et al. 1992, 1997; Jahn et al. 1998; Šamaj et al. 1998, 2000; Reichelt et al. 1999; Mews et al. 2000). Combined with the currently available antibodies recognizing diverse epitopes unique to cell wall pectins, this technique allowed us to make the surprising discovery that cell wall pectins can be actively internalized in root apex cells (Baluška et al. 2002, Yu et al. 2002, Šamaj et al. 2004). The unique property of brefeldin A (BFA) is to effectively block anterograde secretory pathways while allowing endocytosis to continue, resulting in the cytoplasmic accumulation of all recycling molecules which then become sequestered within BFA compartments (reviewed by Nebenführ et al. 2002, Geldner 2003, Šamaj et al. 2004). Using this drug, we found that cell wall pectins, cross-linked with calcium and boron, accumulate within BFA compartments in maize and wheat root apical cells (Baluška et al. 2002, Yu et al. 2002, Šamaj et al. 2004).

This new twist in cell wall biology was possible in part because of the recent advances made in studies of endocytosis and vesicle recycling in plant cells, resulting from the identification and characterization of the *gnom/emb30*

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mutant of *Arabidopsis thaliana* (Mayer et al. 1993, Shevell et al. 1994). Cloning of the *gnom/emb30* gene identified a BFA-sensitive exchange factor (GEF) belonging to the GGG class of small GTPases of the ARF family (Jürgens and Geldner 2002, Jürgens 2004). Intriguingly, ARF-GEF GNOM localizes to plant endosomes (Geldner et al. 2003) and is essential for both the structure and the functioning of these endosomes, which rapidly recycle certain plasma membrane proteins and cell wall pectins (Geldner et al. 2003, Šamaj et al. 2004). Importantly, cell wall pectins seem to use the same vesicular pathways as these plasma membrane proteins, as they colocalize in BFA compartments (Šamaj et al. 2004). Therefore, it is not surprising that cells of the *gnom/emb30* mutant assemble aberrant cell walls, since recycling cell wall pectins, in particular, are abnormally localized (Shevell et al. 2000, Baluška et al. 2002). A plausible explanation of these observations is that the recycling of cell wall pectins is essential for the proper assembly of cell walls. Therefore, we can conclude that cell wall pectins are internalized into meristematic root cells and then obviously follow the same recycling pathway taken by plasma membrane proteins.

In order to extend our still rudimentary knowledge of this intriguing phenomenon, we have investigated here further pectic epitopes which are internalized into dividing cells of root apices. The 6D7-reactive epitope has been reported to accumulate within endosome-based BFA compartments (McCurdy 1999). We reveal that the 6D7 antibody recognizes cell wall pectins enriched with arabinan moieties that are internalized in root cells via multilamellar endosomes. Moreover, we document large-scale internalization of cell wall pectins with the 2F4 monoclonal antibody, which recognizes homogalacturonans with a low degree of esterification (Liners et al. 1989, 1992). Last but not least, xyloglucans are also internalized from cell walls. Unfortunately, the physiological significance of endocytosis of cell wall pectins and other cell wall molecules remains largely obscure. As revealed by our study, one possible role of the internalized cell wall pectins and xyloglucans is to serve as a ready source of material for the rapid cell plate formation that occurs during plant cytokinesis.

Material and methods

Plant material and inhibitor treatments

Maize grains (*Zea mays* L. cv. Alarik) obtained from Force Limagrain (Darmstadt, Federal Republic of Germany) were soaked for 6 h and germinated in well moistened rolls of filter paper for 4 days in darkness at 20 °C. Young seedlings with straight primary roots, 50–70 mm long, were selected for inhibitor treatments and subsequent immunolabeling studies. Unless stated otherwise, all chemicals were obtained from Sigma Chemicals (St. Louis, Mo., U.S.A.). For pharmacological experiments, root apices were submerged into appropriate solutions at room temperature. For BFA treatment, we used a 10^{-2} M stock solution (made in dimethyl sulfoxide), further diluted in distilled water to achieve an effective working solution of 10^{-4} M immediately before submergence of root apices for 2 and 6 h. β-Glucosyl Yariv reagent and its unreactive analogue $(\beta\text{-D-Man})_3$ were used at a concentration of 10^{-5} M for 3 h, latrunculin B was used at 10^{-5} M for 3 h, oryzalin at 10^{-5} M for 3 h, and colchicine at 10^{-3} M for 3 h.

Daucus carota L. seeds were surface sterilized by a 10 min treatment with 50% (w/v) sodium hypochlorite in water. After several washes with sterile water, the seeds were germinated in light at 25 °C on petri dishes containing 0.7% (w/v) agar in water.

Mono-, oligo-, and polysaccharides

Monosaccharides, disaccharides, and polymeric carbohydrates, such as citrus pectins DE 34%, DE 65%, and DE 92% (P-9311, P-9436, P-9561), apple pectin (P-2157), and polygalacturonic acid (P-3889) were purchased from Sigma. Sugar beet pectin (RU 320) was from Herbstreith & Fox. Arabinan preparation 1, with the following composition (weight percent): Rha, 3.5; Ara, 48.8; Gal, 9.6; Glc, 2.4; GalA, 8.0; and arabinan preparation 2, with the following composition (weight percent): Rha, 3.2; Ara, 40.4; Gal, 12.2; Glc, 4.6; GalA, 9.7; were from British Sugar, Peterborough, U.K. Two rhamnogalacturonan I (RGI) preparations were kindly provided by Dr. C. Renard (INRA, Le Rheu, France): RGI 1 (moles percent) after trifluoroacetic acid TFA hydrolysis: Rha, 4.0; Ara, 47.9; Gal, 10.5; Glc, 0.3; GalA, 20.9; proteins, 8.2; ash, 7.3; and RGI 2 (moles percent) after trifluoroacetic acid TFA hydrolysis: Rha, 21.1; Ara, 1.7; Gal, 25.2; GalA, 52.0. Oligo-arabinans of DP 2, 3, 4 and 8 were from Megazyme, Ireland.

ELISA assays for determining the identity of the 6D7 epitope

Wells of Maxisorp ELISA (enzyme-linked immunosorbent assay) plates (Nunc, Denmark) were coated overnight at 4 °C with 100 μl of a polymer solution (100 μg/ml) in phosphate-buffered saline (PBS) (5.84 g of NaCl, 4.72 g of Na_2HPO_4, 2.64 g of $NaH_2PO_4 \cdot H_2O$, pH 7.2, per liter). Wells were rinsed three times with PBS, blocked by incubation with 250 μl of 1% (w/v) casein hydrolysate in PBS (C-PBS) for 2 h at room temperature (RT) and rinsed again three times with PBS. 6D7 antibody supernatants, diluted 20 times in C-PBS, were added to wells and allowed to stand for 2 h at RT. Wells were washed three times with PBS containing 0.1% (w/v) Tween 20 and three times with PBS. Bound antibodies were detected by incubating for 1 h at RT with 100 μl of a horseradish peroxidase-conjugated anti-mouse antibody (Amersham-Pharmacia Biotech.) per well diluted 1 : 5000 in C-PBS. After three washes with PBS containing 0.1% Tween 20 and three washes with PBS, the peroxidase activity was detected with 100 μl of K-blue substrate (Neogen Corp., Lansing, Mich., U.S.A.) per well. The reaction was stopped after 20 min in the dark with 100 μl of 1 M HCl per well and absorbances were measured at a wavelength of 450 nm.

For competitive assays, wells of Maxisorp ELISA plates (Nunc) were coated overnight at 4 °C with 100 μl of arabinan 1 (100 μg/ml) in PBS. Wells were rinsed three times with PBS, blocked by incubation with 250 μl of 1% (w/v) C-PBS for 2 h at RT and rinsed again three times with PBS. Competitive mixtures, prepared as described below, were added to the wells and left for 2 h at RT. The rest of the protocol was performed exactly as described above for determination of the pectic domain.

To prepare the competitive mixtures, 6D7 antibody was diluted 10 times in C-PBS and mixed (v/v) with oligo-arabinans (DP 2, DP 4, DP 8) or arabinan 1 polymers prepared in C-PBS. These mixtures were vortexed briefly and allowed to stand for 30 min at RT before being added to the microwells of the ELISA plates. Control mixtures with no oligoarabinans or arabinose polymers were included in the test. Each type of ELISA assay (noncompetitive and competitive) was performed twice.

Cytochemical analysis

For epitope characterization, ultrathin sections collected on gold grids were incubated for 10 min with saturated aqueous sodium-*meta*-periodate (Sigma). The sections were washed in distilled water and then incubated for 10 min at RT in 50 mM $NaBH_4$ in PBS. The sections were washed again in distilled water, then processed for immunogold labeling as described above. Phosphotungstate staining of the plasma membrane was performed using the method described by Roland et al. (1972). Sections collected on gold grids were incubated in 1% (v/v) aqueous periodic acid for 30 min at RT. The sections were washed extensively with distilled water, then incubated for 10 min in 1% (w/v) phosphotungstic acid in 10% (w/v) chromic acid. Sections were again washed extensively with distilled water, allowed to dry, and then viewed.

Immunogold electron microscopy of 6D7

Wheat (*Triticum aestivum* L. cv. Kite) seeds were germinated on moist paper toweling in darkness at RT for 3–4 days. Root tips were fixed in 2% (w/v) paraformaldehyde and 1.5% (w/v) glutaraldehyde in 50 mM phosphate buffer, pH 6.8, for 2–3 h, washed in the same buffer, and postfixed in 1% (w/v) osmium tetroxide for 1 h. Tissue was dehydrated through an ethanol series and then embedded in LR-White resin. For some immunolabeling trials, root tips were processed without postfixation in osmium tetroxide. Sections were processed for immunogold labeling with either hybridoma supernatant from line 6D7 (McCurdy 1999), diluted 1 : 2 or 1 : 5 in PBS–bovine serum albumin (BSA), or affinity-purified 6D7 antibody at 5 μg/ml. As a secondary antibody, anti-mouse immunoglobulin G (IgGs) coupled to 10 nm diameter colloidal gold (Sigma) was used. For preabsorption experiments, 5 μg aliquots of affinity-purified 6D7 antibody were incubated overnight at 4 °C with either 5 μg or 20 μg of apple pectin fraction (Sigma).

Immunogold electron microscopy of 2F4

Root apex segments, 4 mm long, were fixed for 3 h at RT with 2.5% (w/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.05 M cacodylate buffer, pH 6.8. They were washed twice for 10 min each in cacodylate buffer and were postfixed with 1% (w/v) OsO_4 in the same buffer for 1 h at 4 °C. The hypocotyl segments were then washed three times for 10 min with the buffer and three times for 10 min with H_2O before being dehydrated in an ethanol series and embedded in LR-White resin, as described previously (Liners and Van Cutsem 1992). Ultrathin sections (50 to 70 nm) were cut with a LKB Ultratome III and were collected on uncoated gold or nickel grids (300-mesh). Sections were viewed at 60 kV in a Philips EM 301 microscope.

Pectic polysaccharides were sequentially extracted from glutaraldehyde-paraformaldehyde fixed samples. After the two 10 min washes in cacodylate buffer and a 5 min wash in H_2O, four fixed samples were placed in Eppendorf tubes and were incubated, with shaking, in 1 ml of the following extracting solutions: 0.02 M cyclohexanediaminetetraacetic acid (Na salt, pH 4.9) at RT for 2 h; 0.05 M Na_2CO_3 (pH 10.3) at 4 °C for 2 h, then 0.05 M Na_2CO_3 at RT for 2 h. Between each extraction step, the samples were centrifuged for 10 min at 7500 **g** and the supernatants were removed. Some specimens were only incubated in one of the extracting media, to test the individual effect of each of these treatments. Extracted samples were washed twice for 5 min in cacodylate buffer, postfixed with OsO_4, and processed for electron microscopy as above. Control samples were kept in cacodylate buffer during the extraction process. Ultrathin sections collected on nickel grids were labeled with 2F4 ascites, as previously described (Liners and Van Cutsem 1992).

Indirect immunofluorescence microscopy

Apical root segments (7 mm) encompassing the major growth zones were excised into 3.7% formaldehyde prepared in stabilizing buffer (SB) (50 mM piperazine-N,N′-bis(2-ethanesulfonic acid), 5 mM $MgSO_4$, 5 mM EGTA, pH 6.9) and fixed for 1 h at room temperature. Following rinsing in SB, the root apices were dehydrated in a graded ethanol series diluted with PBS. They were then embedded in Steedman's wax and processed for immunofluorescence (for details, see Baluška et al. [1992]). To enable efficient penetration of antibodies, sections were dewaxed in absolute ethanol, passed through a graded ethanol series diluted with PBS, and then placed in SB for 30 min. After a 10 min rinse with absolute methanol at −20 °C, the sections were transferred to SB containing 1% BSA for 30 min at room temperature.

Sections were then incubated with the following primary antibodies: 6D7 monoclonal antibody diluted 1 : 2, JIM5 and JIM7 monoclonal antibodies diluted 1 : 20, LM5 monoclonal antibody diluted 1 : 20, 2F4 monoclonal antibody (Liners et al. 1989), anti-XG (Sonobe et al. 2000), AFR1, and anti-rhamnogalacturonan II (RGII) polyclonal antibodies (Baluška et al. 2002) diluted 1 : 100. With the exception of 2F4 monoclonal antibodies for which a T-Ca-S (20 mM Tris-HCl, pH 8.2, 0.5 mM $CaCl_2$, 150 mM NaCl) buffer was used in all steps of the labeling experiment, all primary antibodies were diluted in PBS. The buffers were supplemented with 1% BSA. Sections were incubated in primary antibody for 1 h at RT. After rinsing in T-Ca-S or PBS, the sections were incubated for 1 h with fluorescein isothiocyanate-conjugated anti-mouse IgGs (6D7, 2F4), anti-rat IgGs (JIM5, JIM7, LM5), or with anti-rabbit IgGs (XG, RGII), each raised in goat and diluted 1 : 100 in appropriate buffer containing 1% BSA. A further rinse in T-Ca-S or PBS (10 min) preceded a 10 min treatment with 0.01% Toluidine Blue to diminish autofluorescence of the root tissues. The sections were then mounted using an anti-fade mounting medium containing *p*-phenylenediamine (Baluška et al. 1992). Sections were examined with an Axiovert 405M inverted microscope (Zeiss, Oberkochen, Federal Republic of Germany) equipped with epifluorescence and standard fluorescein isothiocyanate excitation and barrier filters.

Results

6D7 antibody recognizes pectin epitope internalized into cells via multilamellar endosomes

Electron microscopy (EM) revealed that 6D7-reactive pectins are localized throughout the entire cell wall in young meristematic wheat root cells (Fig. 1A). However, the signal from the cell wall became progressively weaker during cellular development, while the middle lamella retained a strong signal (Fig. 1B). Besides cell walls, the most strongly labeled subcellular structures were distinct multilamellar structures localized near the plasma membrane (Fig. 1C). Typically, adjacent GA was devoid of labeling (Fig. 1D), although other GA was often labeled (data not shown). The distribution of 6D7-positive gold particles implies that these multilamellar structures were derived from the plasma membrane coincident with massive internalization of 6D7 pectins from the cell wall (Fig. 1E).

In order to confirm the putative PM origin of the membranes of these multilamellar structures, we performed cytochemical tests using phosphotungstate stain (Roland et al. 1972). In addition to the PM, membranes of multilamellar structures and adjacent smaller vesicular structures were stained with the precipitate (Fig. 2A). When ultrathin sections were treated with sodium-*meta*-periodate before the

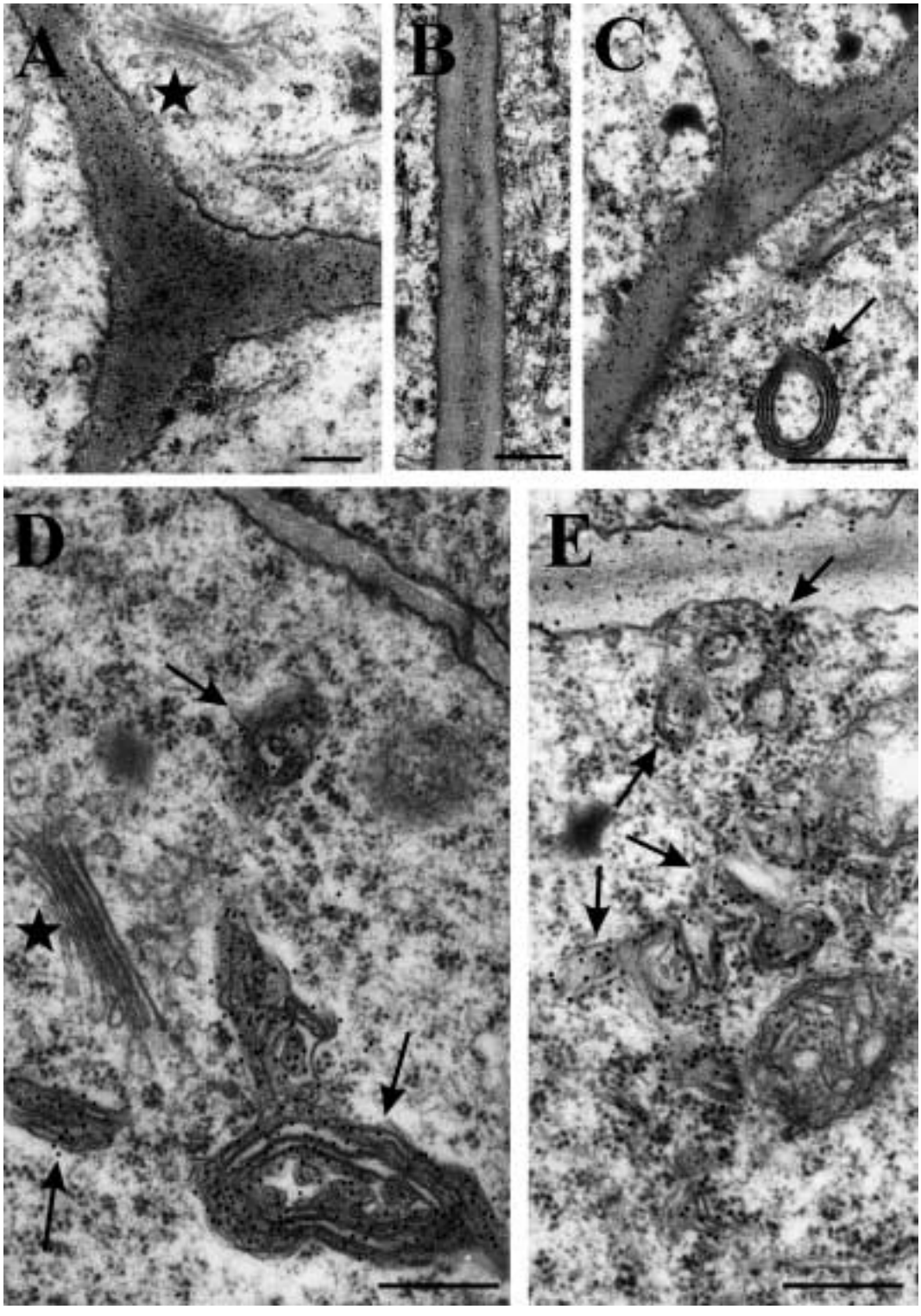

Fig. 1A–E. Immunogold labeling of 6D7 pectins in wheat root apices. **A** and **B** 6D7 antibody labels the entire cell wall in meristematic root cells, while only the middle lamella region is labeled in walls of mature root cap cells. **C–E** 6D7 decorates multilamellar structures which occur individually in the cytoplasm (arrow in **C**) or as extensive networks (arrows in **D** and **E**) ramifying from the PM deep into the cytoplasm. Note that the GA stacks are not labeled with the 6D7 antibody (stars in **A** and **D**). Bars: 0.5 μm

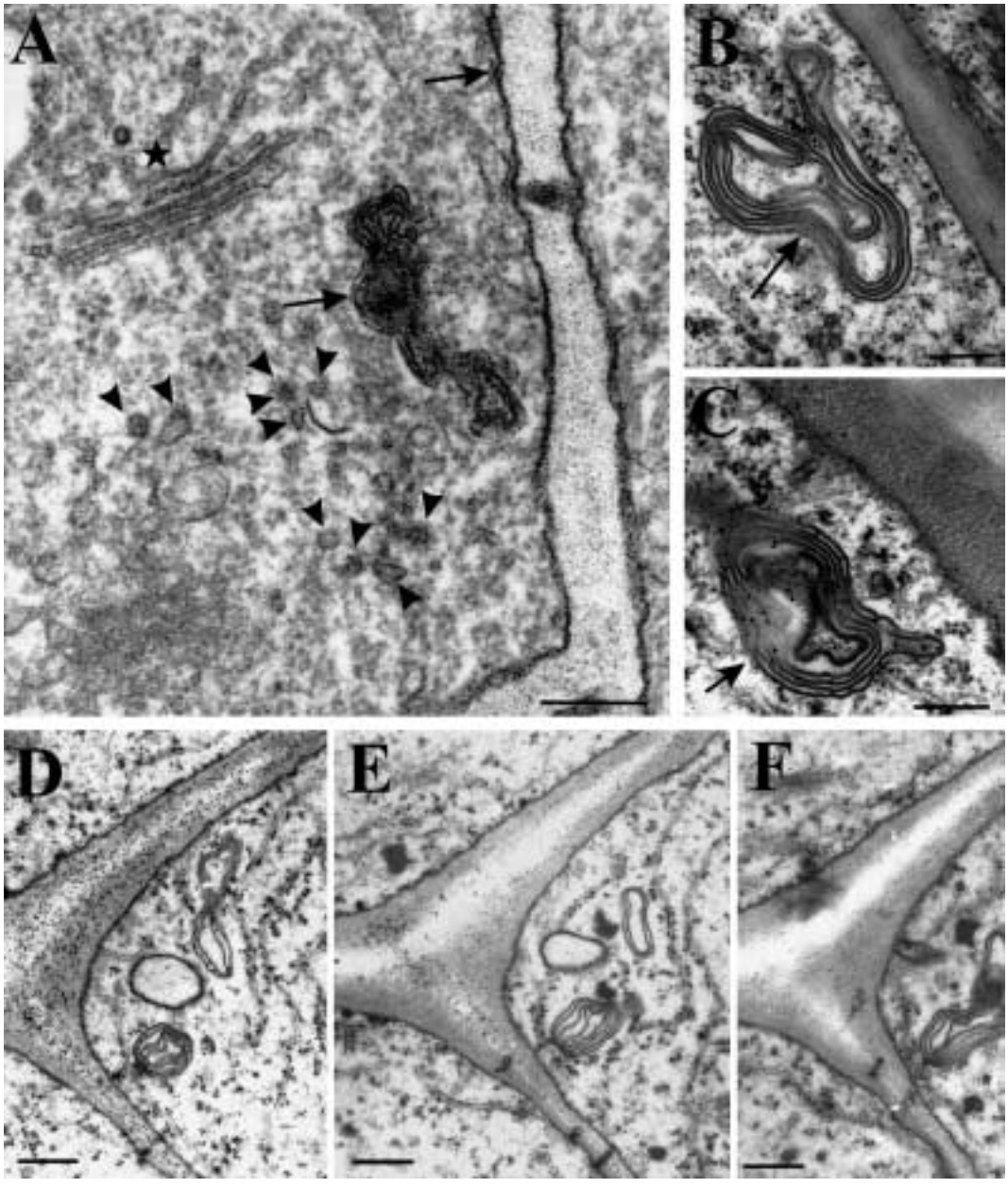

Fig. 2A–F. Pectin-based nature of the 6D7 epitope internalized from cell walls. **A** Section stained with the phosphotungstate stain reveals that multilamellar structures (lower arrow) are derived from the plasma membrane (upper arrow). Note that Golgi membranes are not heavily stained, while several vesicles adjacent to the multilamellar structure are stained (arrowheads). **B** and **C** Reactivity of the 6D7 antibody with multilamellar structures (arrow in **C**) can be abolished by prior treatment of sections with sodium *meta*-periodate (arrow in **B**), indicating its carbohydrate nature. **D–F** Immunolabeling of cell walls and of multilamellar structures can be inhibited by preincubation of the antibody with crude pectin (**D**, 6D7 alone; **E**, 6D7 plus 5 μg of pectin; **F**, 6D7 plus 20 μg of pectin), revealing the pectic nature of the 6D7 epitope. Bars: A and D–F, 0.5 μm; B and C, 0.25 μm

6D7 antibody (Fig. 2B), 6D7-positive gold particles were absent, showing the carbohydrate-like nature of the recognized molecules (compare Fig. 2B with Fig. 2C). Mild periodate oxidation at acidic pH has been shown to cleave carbohydrate vicinal hydroxyl groups without altering the structure of the polypeptide chains (Woodward et al. 1985).

ELISA assays reveal sequence of three arabinosyl residues representing the 6D7 epitope

The 6D7 antibody showed strong recognition of two arabinan fractions and an RGI preparation rich in arabinose (RGI 1), whereas sugar beet pectin and an RGI preparation

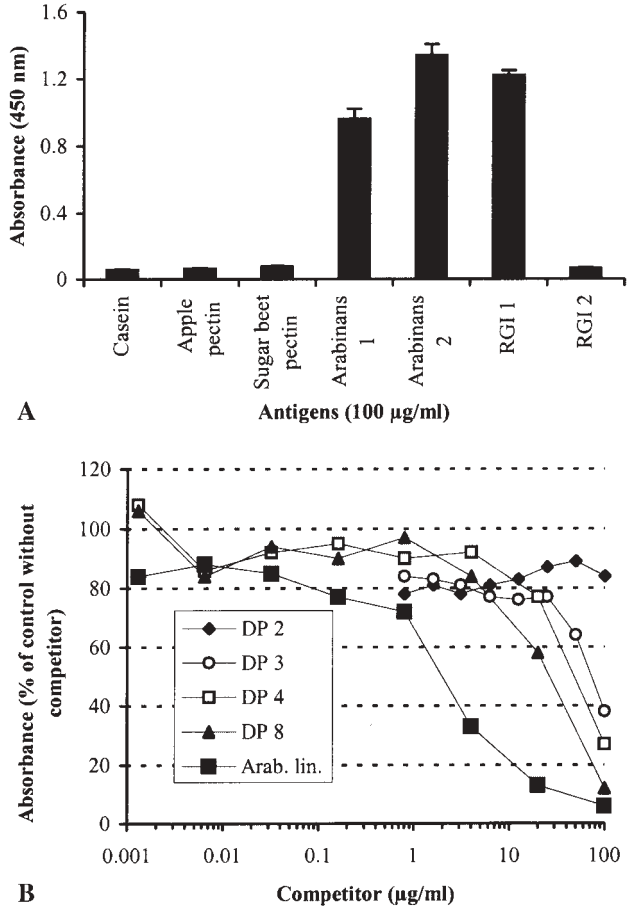

Fig. 3A, B. ELISA assays for determining the identity of the 6D7 epitope. **A** Recognition of different pectic polymers by the 6D7 antibody used at different dilutions. Bars indicate standard deviation (n = 4). **B** Competitive ELISA tests: effect of the concentration of oligoarabinans DP 2 (◆), DP 3 (○), DP 4 (□), and DP 8 (▲) and of arabinan polymers (■) on the recognition of arabinans by 6D7 antibodies. Results (means of duplicates) are expressed as percentages of control (antibodies without competitors)

with a low arabinose content (RGI 2) were virtually unrecognized (Fig. 3A), as were other pectic polymers, such as polygalacturonic acid and citrus pectins with 35%, 75% and 95% methyl-esterification (not shown). Competitive assays, performed to confirm the binding of 6D7 antibodies to a sequence of arabinose residues, showed that arabinose dimers (DP 2) did not inhibit the recognition of arabinans adsorbed to the microwells, whatever the concentration used (up to 100 µg/ml) (Fig. 3B). In contrast, 50% inhibitory concentrations of about 70 and 50 µg/ml were obtained for arabinan DP 3 and 4 oligomers, respectively. Lower amounts of DP 8 oligomers and arabinan polymer (less than 8 µg/ml) were needed for 50% inhibition of 6D7 antibody binding to immobilized arabinans. These results demonstrate that the 6D7 antibody recognizes a sequence of arabinose residues. The 6D7 epitope is probably made up of 3 arabinosyl residues.

Development-, tissue-, and cell-cycle-specific localization of the 6D7 pectin epitope

In immunofluorescence, 6D7-reactive pectins localized to cell walls throughout maize root apices. Nevertheless, the density of the 6D7 signal varied according to the stage of development, with the strongest signal found in developmentally younger cells (Fig. 4A, B). In more advanced root cap cells, labeling became more apparent in the cytoplasm than that seen in younger root cap cells (Fig. 4A). When the PM was included within the section plane, transverse (with respect to the root axis) labeling patterns of 6D7 pectin were apparent (Fig. 4B), closely resembling the known distribution of cortical microtubules in such cells (Baluška et al. 1992). Besides strongly labeled cell walls, cytoplasmic spots of variable size were typically found at cell peripheries (Fig. 4B, C).

BFA treatment (100 µM for 2 h) of maize root apices induced dramatic redistribution of 6D7-reactive pectins (Fig. 4D). Meristematic cells accumulated 6D7-reactive pectins in the form of small dots that progressively coalesced into large compartments (Fig. 4E, F; see also McCurdy [1999: fig. 3B, D]). While cells still showed PM-associated labeling (Fig. 4F), these compartments were irregularly shaped and their surfaces appeared uneven (Fig. 4F). In contrast, root cap cells (Fig. 4D), as well as metaxylem cells and elongating root body cells (data not shown), did not assemble BFA compartments, and 6D7-reactive pectins accumulated in the cytoplasm and within vacuoles. 6D7-reactive BFA compartments were also observed in nongrowing and metabolically inactive cells of the quiescent center (Fig. 4D).

In order to demonstrate that the internalization of 6D7 cell wall pectins represents endocytosis, we used low temperature and latrunculin B as two inhibitors of endocytosis (for plant cells, see Baluška et al. [2002]). Both treatments significantly inhibited internalization of 6D7 pectins, as evidenced by smaller BFA compartments which were typically associated with cellular peripheries (compare Fig. 4G with Fig. 4H, I, J). Moreover, these small BFA compartments associated preferentially with cross walls, suggesting that the high rate of internalization and recycling of cell wall pectins occurs selectively at cross walls (Fig. 4I, J). In contrast, cells devoid of microtubules after oryzalin treatment formed extremely large BFA compartments that occupied a considerable portion of the cytoplasmic space (compare Fig. 5D, E, F, G with Fig. 4G). Thus, microtubules are not essential for endocytosis of 6D7-reactive pectins in dividing root cells.

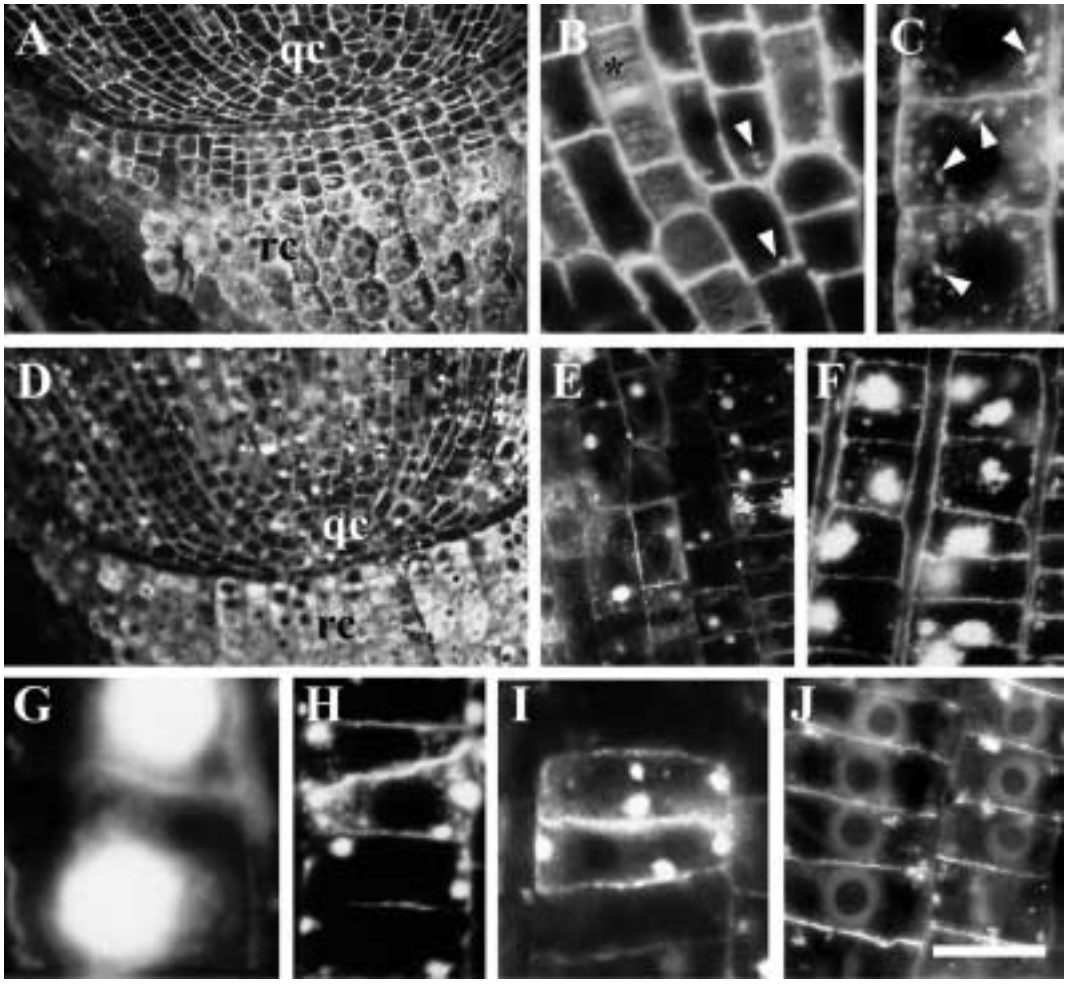

Fig. 4A–J. Subcellular and tissue-specific distributions of 6D7 pectins in cells of maize root apices. **A–C** 6D7 pectins localize preferentially to cell walls of all cells of the root apical meristem, and also of the quiescent center (*qc*). 6D7 pectins are often arranged into transverse arrays with respect to the root axis (asterisk in **B**). **C** Arrowheads highlight large 6D7 endosomes. **A** and **B** In root cap (*rc*) cells, in addition to the cell wall-associated signal, strong cytoplasmic labeling, with the exception of vacuoles, is apparent. **D–F** BFA-treated cells accumulate 6D7 pectins in BFA compartments, while they are depleted from cell walls. **D** 6D7-positive BFA compartments form even in nongrowing and metabolically inactive quiescent center (*qc*) cells. **E** and **F** Cortex cells (**F**) form larger BFA compartments than stele cells (**E**). **G** Root cortex cells devoid of microtubules before BFA treatment assemble extremely large 6D7-positive BFA compartments. **H** and **I** In contrast, cells devoid of F-actin before BFA exposure form smaller 6D7-positive BFA compartments which are restricted to cellular peripheries. **J** Cold treatment also prevents formation of large BFA compartments and the few smaller compartments are typically associated with cross walls. Bar in J: for A and D, 60 μm; for B and F, 20 μm; for C, 13 μm; for E, 8 μm; for G–J, 15 μm

All cell wall pectin epitopes internalizing from cell walls accumulate within cell plates

In dividing cells, cytokinetic cell plates (Fig. 5A) and young cell walls (Fig. 5B) were the most prominent subcellular domains accumulating 6D7 pectins. Again, cytoplasmic spots were also labeled at cellular peripheries, especially near young cell walls (Fig. 5B). Characteristically, four distinct 6D7-reactive aggregates occurred invariably around maturing cytokinetic cell plates (Fig. 5B). Besides 6D7, RGII pectins cross-linked with boron and calcium (Fig. 5C–E), LM5-reactive pectins (Fig. 5F, G), 2F4-reactive pectins (Fig. 5H, I), and JIM5-reactive pectins (Fig. 5J) also accumulated within cell plates. Surprisingly, secretory GA-based pectins did not (Fig. 5K). Importantly, the BFA-target ARF1 was also found within cell plates (Fig. 5L) and endocytic pectin compartments induced at their growing margins after BFA treatment (Fig. 5M, N). Since cell wall pectins cross-linked with boron and calcium are internalized, it might be proposed

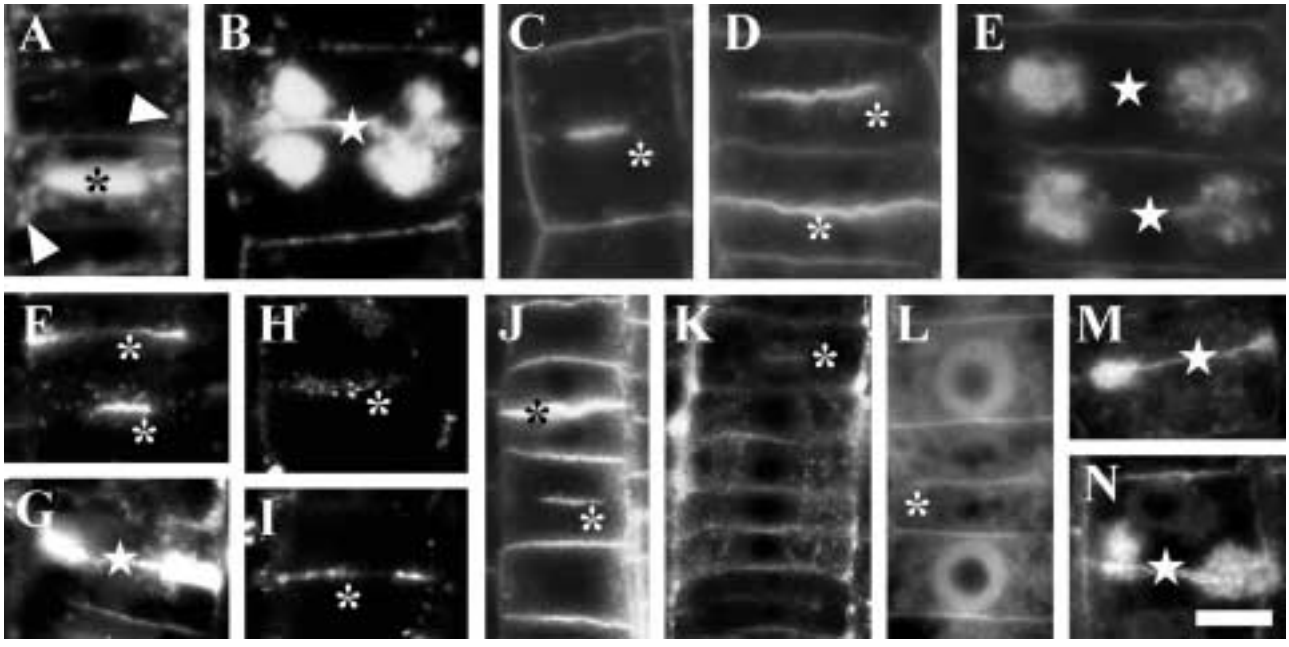

Fig. 5A–N. Cell wall pectins accumulate within cell plates and BFA compartments in cells of maize root apices. **A** During the cell cycle, 6D7 pectins accumulate abundantly within the assembling cell plates. **B** BFA-treated cell plates assemble BFA compartments at the leading edges of growing cell plate. **C–E** RGII pectins cross-linked with boron also accumulate within cell plates and assemble in BFA compartments at their leading edges. **F** and **G** LM5 pectins in cell plates and their BFA compartments. **H** and **I** 2F4 pectins in early (**H**) and late (**I**) cell plates. **J** Cell wall-based JIM5 pectins are abundant in both early (white asterisk) and mature (black asterisk) cell plates. **K** GA-based JIM7 pectins associate only weakly with cell plates, while small spots distributed throughout the cytoplasm correspond to GA stacks. **L–N** The cellular target of BFA is ARF1 GTPase, which also accumulates within both cell plates (**L**) and BFA-induced compartments (**M** and **N**) assembled at the leading edges of the extending cell plate. Asterisks mark cell plates in control cells, while stars highlight cell plates in BFA-treated cytokinetic cells. Bar in N: for A, C, and H–K, 15 μm; for F, 18 μm; for B, E, and G, 10 μm; for D, 12 μm

that one purpose of internalizing cell wall pectins is to provide cytokinetic cells with ready-to-use building blocks for the formation of a cell plate, which is known to happen rapidly, even in turgid cells.

Cell wall homogalacturonans are massively internalized into root cells and accumulate abundantly within peripheral vacuole-like endosomes and cell plates

In order to test if the large-scale internalization of 6D7-reactive cell wall pectins is unique for this epitope, we analyzed in detail the localization of 2F4-reactive pectins, which are able to adopt calcium-induced conformations through their polygalacturonic acid domains (Liners et al. 1989, 1992). In block de-esterification treatments performed on glutaraldehyde-paraformaldehyde-fixed carrot root apices allowed the 2F4 antibodies to heavily label cell walls in tissue sections (Fig. 6A–D). Intriguingly, 2F4-labeled pectins were also found to be internalized via large invaginations into root cells on a large scale, accumulating abundantly within peripheral vacuole-like endosomes (Fig. 6B–F). The adjacent vacuoles and other organelles, as well as the rest of the cytoplasm, were free of any gold particles (Fig. 6B, F). Importantly, 2F4-labeled pectins were enriched within cell plate vesicles and early cell plates (Fig. 6G, H) during cytokinesis.

Xyloglucans are internalized from walls and accumulate in cell plates and BFA compartments

Xyloglucans visualized by labeling cell plates with antibodies (see also Sonobe et al. 2000) were very abundant, especially at the cross walls of xylem elements and of cells in the middle and outer cortex (Fig. 7A). After BFA treatment, almost all xyloglucans were removed from cell walls into BFA compartments (Fig. 7B), revealing a surprisingly high rate of xyloglucan recycling in the root cells. In accordance with previously published data for tobacco BY-2 cells, xyloglucans accumulated abundantly in the early cell plates of cytokinetic maize root cells (Fig. 7C, D).

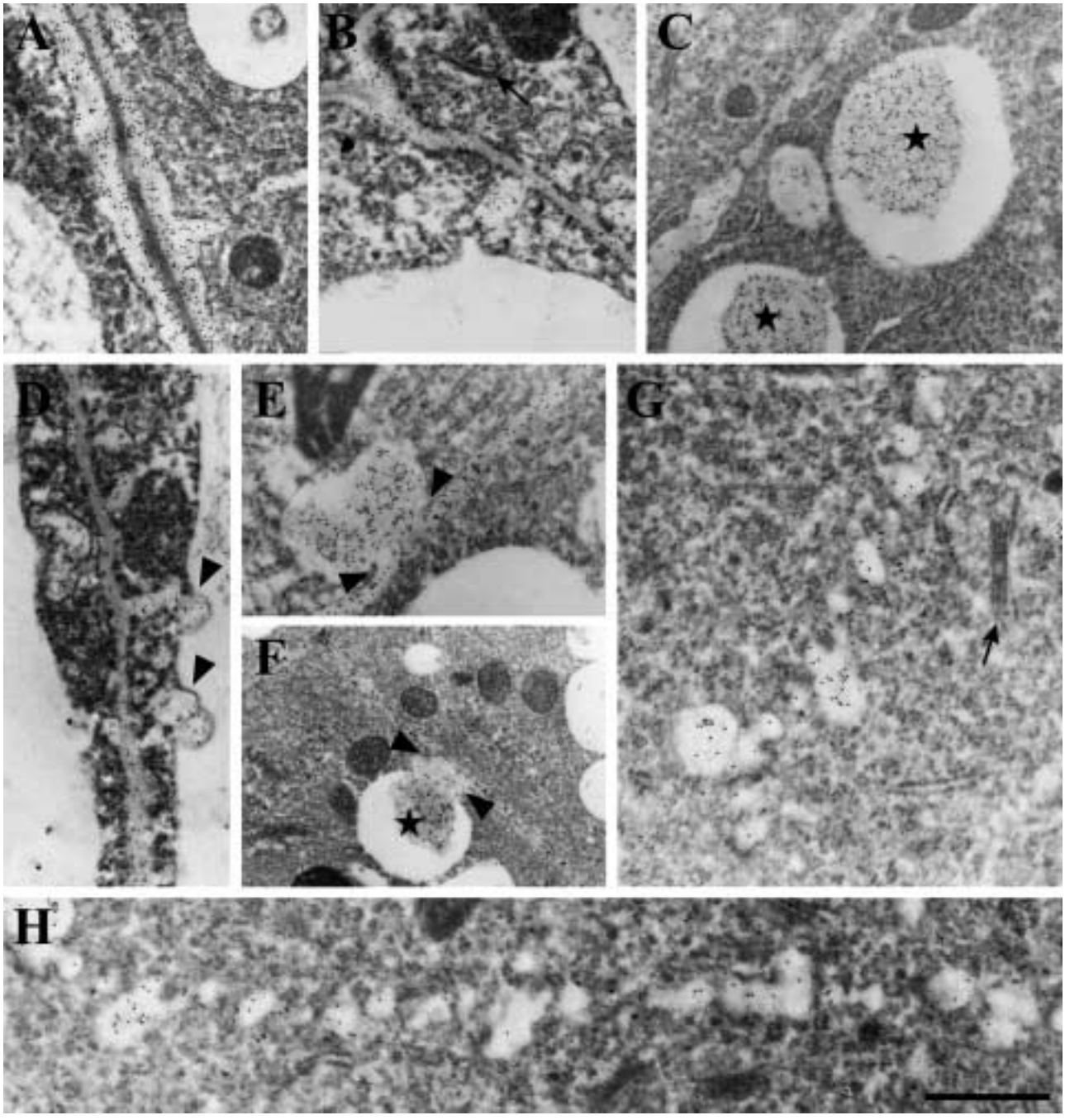

Fig. 6A–G. Immunogold labeling of 2F4-reactive pectins after in-block de-esterification treatment of carrot root apices. **A** and **B** 2F4 labeling localizes almost exclusively to cell walls and large invaginations of the PM. **C** 2F4-labeled pectins are internalized into small, peripheral, vacuole-like endosomes, whereas adjacent vacuoles and other organelles, as well as the rest of the cytoplasm except the GA, are free of any gold particles (see also panels B and F). **D** and **E** High-magnification views of the internalization of 2F4 pectins from cell walls into peripheral vacuole-like endosomes. **F** Low-magnification view of 2F4 pectins undergoing a large-scale internalization from the cross wall into large, peripheral, vacuole-like endosomes. **G** and **H** Accumulation of 2F4-reactive pectins within assembling cell plates. Bar in H: for A, B, D, F, and H, 0.6 μm; for C, E, and G, 0.4 μm

Discussion

Recently we reported that several cell wall pectin epitopes, including JIM5- and LM5-reactive pectins and RGII dimers cross-linked with boron, are actively internalized into dividing root cells (Baluška et al. 2002, Yu et al. 2002). Here we have extended this phenomenon by documenting large-scale internalization of cell wall pectins reactive to the 6D7 (McCurdy

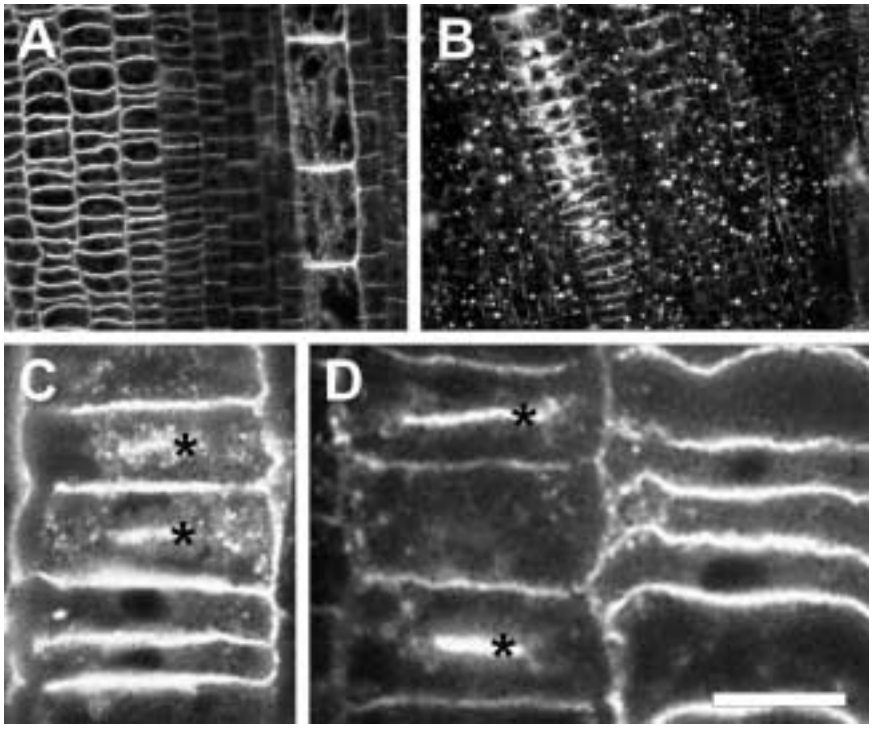

Fig. 7A–C. **A** Xyloglucans are very abundant in cross walls of xylem elements and of cells in the middle and outer cortex. **B** In BFA-treated cells, almost all xyloglucans internalize into abundant BFA compartments. **C** and **D** In cytokinetic cells, xyloglucans are localized within compartments sized between about 500 and 1000 nm, which by their fusion build both the early and late cell plates. Bar: 15 μm

1999) and 2F4 (Liners et al. 1989, 1992) antibodies. The epitope of the 6D7 antibody was identified here by ELISA as an arabinan moiety of probably three arabinosyl residues found in RGI pectins, while the 2F4 epitope is specific to cell wall polygalacturonic acid (PGA) chains (Liners et al. 1989, 1992). Moreover, cell wall xyloglucans are also internalized from cell walls and accumulate within early cell plates (this study, Sonobe et al. 2000). The immediate question emerging from these findings is that about the purpose of firstly secreting macromolecules into the exoplasmic space and then accomplishing tedious internalization against turgor pressure.

We discuss here the two most plausible reasons why dividing plant cells internalize cell wall macromolecules. The first relates to tight control of the mechanical properties of cell walls. In order to maintain a loosened wall structure to enable extensive elongation after ceasing mitotic divisions, it is necessary to actively maintain low levels of pectin cross-linking within cell walls. Endocytosis of cross-linked pectins and acidic pectin-xyloglucan complexes, removal of cross-linkages, and subsequent recycling would fulfill this requirement without loss of molecules and expending energy. The second reason relates to plant-specific cytokinesis of turgid cells and the requirement to rapidly build a new cell wall de novo during plant cytokinesis. In fact, this requirement demands construction of a new extracellular space in the cell's mid-region, which then expands towards, and fuses with, parental cell walls, all of which occurs within an extremely short time frame (Jürgens and Pacher 2003). In this context, endocytic vesicles filled with ready-to-use cell wall macromolecules would be ideally suited to provide building blocks for rapid formation of the cytokinetic cell plate.

This concept of a pool of internalized pectins providing a ready supply of cell wall material is supported by observations made in other systems where pectins are known to be rapidly disposed and recycled, such as the rapid guard cell movements during stomatal opening and closing (Shope et al. 2003), rapid papilla formation during pathogen penetration attempts (Collins et al. 2003), and rapid tip growth of root hairs and pollen tubes with pectic cell walls (Roy et al. 1999, Holdaway-Clarke et al. 2003). However, as discussed briefly in the outlook section of this discussion, there are also less obvious reasons why plant cells should internalize cell wall pectins, including general stress responses, propagation of pectic signals, and transport of auxin.

Large-scale internalization of cell wall pectins into multilamellar endosomes and peripheral vacuole-like endosomes

In our previous study, we observed that internalized cell wall pectins accumulate within BFA compartments together with recycling plasma membrane proteins, such as plasma

membrane H^+-ATPase and auxin transporters from the PIN family (Baluška et al. 2002, Šamaj et al. 2004). Here we document, using immunogold EM analysis, that cell wall pectins recognized by 6D7 and 2F4 antibodies are internalized into dividing root cells on a large scale and accumulate within multilamellar endosomes and small peripheral vacuole-like endosomes. Similarly, JIM5-reactive pectins, which also accumulate in BFA compartments and within cell plates, have been reported to localize to plasma membrane invaginations and multivesicular bodies in *Datura suaveolens* stylar transmitting tissue (Hudak et al. 1993). Those authors also showed that GA-based JIM7-reactive pectins do not show this localization and that plasma membrane invaginations and multivesicular bodies contain carbohydrates and are filled with fibrillar material resembling cell wall material. Similar fibrillar material of cell wall origin, identified as pectin arabinogalactans, has been observed in multilamellar compartments invaginating into vacuoles of bean root cells and accumulating within cell plates (Northcote et al. 1989). Besides cell wall pectins, arabinogalactan proteins (AGPs) have also been reported to be internalized from cell walls via multivesicular bodies (Sedgley and Clarke 1986, Herman and Lamb 1991). Moreover, large-scale internalization of cell wall material into small vacuoles is a feature characteristic for cell wall thinning during bulge formation in trichoblasts initiating root hairs (Ciamporová et al. 2003). Internalization of exoplasmic polysaccharide-based material and fluids via multilamellar and multivesicular carriers has also been described for zucchini and rice root cells, where this process was proposed to be relevant for the uptake of nutrients (Coulomb and Coulomb 1976, Nishizawa and Mori 1977).

As these processes are especially prominent in osmotically stressed cells (Ciamporová and Mistrik 1993) and those under chilling stress (Stefanowska et al. 2002), one is tempted to propose that multilamellar and multivesicular carriers allow stressed plant cells to regain osmotic balance via internalization of osmotically active substances or to cope with cold stress. Moreover, such large-scale internalization of PGA pectins closely resembles the internalization of aluminum (Vazquez 2002) and silicon (Neumann and De Figueiredo 2002). The dynamic actin cytoskeleton is essential for endocytosis in plants (Šamaj et al. 2004), and the unconventional plant-specific myosin VIII may function as an endocytic motor (Baluška et al. 2004). All these observations illustrate the need to develop integrated and complex models to explain how components of the actin cytoskeleton may contribute to cell wall assembly and remodeling processes.

Although the current view of the plant secretory pathway solely considers the ER–GA–trans-Golgi network–PM direction, several new studies suggest signal-induced secretion from either endosomes or vacuoles. Secondly, several data suggest that a direct vesicular secretory pathway may also operate from late endosomes and vacuoles in plants (Echeverría 2000). Plant multivesicular bodies might participate in papilla formation in *Pseudomonas syringae*-infected lettuce mesophyll cells (Bestwick et al. 1995). Moreover, there are older reports of fusion of multivesicular bodies with the incipient plasma membrane during cytokinetic cell plate formation (Lehmann and Schulz 1969). Relevant in this respect are several studies describing large vesicles fusing with papilla deposits which, as revealed by the diaminobenzidine reaction, contain both peroxidases and reactive oxygen species (Thordal-Christensen et al. 1997, Hückelhoven et al. 1999, Collins et al. 2003). Interestingly, peroxidase is present not only in cell walls but also within vacuoles, provacuolar membranes, and large vesicles underlying the plasma membrane (Andrews et al. 2002). Prevacuolar compartments were recently identified as multivesicular bodies (Tse et al. 2004) which also represent endocytic organelles in plant cells (Tanchak and Fowke 1987). Moreover, peroxidases have high affinity for calcium–cross-linked pectins (Carpin et al. 2001, Dunand et al. 2002). Therefore, we can tentatively propose that endosomes and vacuoles enriched with internalized cell wall pectins might represent storage compartments allowing rapid recruitment of ready-to-use cell wall molecules in situations where rapid secretion is needed, such as during papilla formation. Schulze-Lefert and colleagues (Collins et al. 2003) pointed out that papilla formation resembles the situation in cytokinetic plant cells where KNOLLE mediates homotypic fusion of cell plate vesicles (Jürgens 2004). In fact, homotypic fusion of pectin-enriched endosomes emerges as a relevant mechanism for both cell plate formation (this report) and stomata opening (Shope et al. 2003).

Cell wall pectins and xyloglucans accumulate within cell plates during plant cytokinesis

Topographically, the endosomal interior belongs to the exocellular space and endosomal limiting membranes are derived from the plasma membrane. Therefore, it is not surprising that cell wall macromolecules are enriched within plant endosomes. These should be considered as "islands" of exocellular space temporarily placed within the cytoplasm and being ready to be used in situations requiring extremely rapid buildup of cell walls. In fact, plant cells face this situation regularly during cytokinesis when they build a new cell wall in their interior within minutes (Jürgens and Pacher 2003). Another similar situation is the opening and closing of stomata via dramatic and rapid increases and decreases in the

size of guard cells (Shope et al. 2003). As we discuss below, endosomes and internalized pectins emerge as important components of these processes in both situations.

Early cell plates of cytokinetic plant cells are predominantly pectic structures which are formed via homotypic fusions of cell plate vesicles (Matar and Catesson 1988). The early dogma stated that these cell plate vesicles are derived solely from the GA (Whaley and Mollenhauer 1963). Surprisingly, critical scrutiny of published data reveals that this is based entirely on circumstantial evidence. The only justification for this tenet are EM observations showing that cell plate vesicles structurally resemble GA-derived vesicles. However, there are serious experimental discrepancies with this GA–cell plate dogma. BFA blocks GA-based secretion almost immediately but still allows the completion of cell plate formation (Yasuhara et al. 1995). Importantly, cell plate vesicles fuse via tubular protrusions formed via dynamin-like fragmoplastin (Verma 2001). GA-derived vesicles are not known to associate with dynamins, to form tubular protrusions, or to accomplish homotypic fusions. On the other hand, all these features are characteristic for endosomes. Older in vivo data showed that cell plate vesicles are much larger (about 1 μm) than those identified in fixed EM samples (about 0.1 μm), suggesting that fixation of biological samples results in fragmentation of cell plate vesicles (Bajer 1965, Bajer and Allen 1966).

In addition, the endocytic marker FM4-64 (Fischer-Parton et al. 2000, Geldner et al. 2003, Šamaj et al. 2004, Tse et al. 2004) accumulates within the early cell plates (Belanger and Quatrano 2000), and older studies documented coated vesicles (Franke and Herth 1974) or multivesicular bodies (Lehmann and Schulz 1969) fusing as cell plate vesicles to initiate the early cell plate. Last but not least, early cell plates accumulate large amounts of pectin epitopes generated in, and internalized from, cell walls (this study), but show weak labeling for GA-localized pectins (this study, Moore and Staehelin 1988, Vaughn et al. 1996). Importantly, the cytokinetic *cyt1* mutant (Nickle and Meinke 1998) and the *emb30/gnom* (Shevell et al. 1994) mutants impaired in endocytic vesicle recycling (Geldner et al. 2003) both show aberrant distribution of cell wall–endosome pectins (Baluška et al. 2002) reactive to the JIM5 antibody, but not of GA–cell wall pectins reactive to the JIM7 antibody (Nickle and Meinke 1998, Shevell et al. 2000).

The scenario proposed here, that endosomes enriched with pectins drive cell plate initiation, would also explain the presence of endocytic motifs in KNOLLE and KORRIGAN, two proteins implicated in cell plate formation (Zuo et al. 2000, Batoko and Moore 2001, Zheng et al. 2002, Jürgens 2004). Moreover, KORRIGAN has also been reported to localize to endosomes (Mølhøj et al. 2002). Although KNOLLE was reported to localize specifically to the GA (Lauber et al. 1997, Völker et al. 2001), inspection of published images of KNOLLE localization reveals that the KNOLLE-positive structures (Lauber et al. 1997: fig. 3, Völker et al. 2001: fig. 5A, B, Heese et al. 2001: fig. 6F, Zheng et al. 2002: fig. 5N, Rancour et al. 2002: fig. 4A) are too large to represent GA and resemble rather 6D7-positive endosomes (compare Figs. 4C and 5I, J in the present study). Moreover, biochemical fractionation failed to associate KNOLLE with GA-derived membranes (Zheng et al. 2002). An endosomal localization of KNOLLE is strongly supported not only by the presence of the endocytic motifs but also by the fact that this cytokinesis-specific syntaxin accumulates within endocytic BFA compartments (Geldner et al. 2001), which are also enriched with endocytic pectins (this study) and the molecular target of BFA action, namely, ARF1 GTPases (Jürgens and Geldner 2002, Jürgens 2004). Moreover, KNOLLE interacts with AtSNAP33, the *Arabidopsis thaliana* homolog of the tSNARE SNAP25, which is essential for plant cytokinesis and colocalizes with PIN1 in endocytic BFA compartments (Heese et al. 2001). The endocytic localization of KNOLLE has been confirmed by the systematic analysis of subcellular localization of all SNARE molecules expressed in *Arabidopsis thaliana* (Uemura et al. 2004).

In addition to their role in cytokinetic cells, xyloglucan- and pectin-enriched endosomes enclosed with PM-derived membrane represent excellent ready-to-use cell wall islands for the rapid opening and closing of guard cells. Recently, it was shown that recycling of endosomes between the PM and some internal compartments drives opening and closing of stomata (Shope et al. 2003). Interestingly, guard cell walls are very abundant in pectins (Majewska-Sawka et al. 2002) and arabinan pectins are essential for guard cell dynamicity (Jones et al. 2003). Last but not least, the tip growth of root hairs and pollen tubes is based on dynamic and flexible pectin-based cell walls depleted in other wall components. Importantly, esterified pectins accumulate at the tips of growing pollen tubes and there is a gradient of de-esterification and calcium cross-linking towards their bases (Roy et al. 1999, Holdaway-Clarke et al. 2003). The acidic environment of endosomes internalizing cross-linked pectins, which are covalently linked to xyloglucans (Femenia et al. 1999, Thompson and Fry 2000), is predicted to allow processing of these complexes followed by recycling of individual cell wall macromolecules back to the growing cell wall. Therefore, as during plant cytokinesis, these endosomes represent ideal building blocks supporting the extremely rapid growth of these tip-growing cells.

Outlook

Oligogalacturonides are well known to act as signal molecules inducing electric responses in plant cells, affecting cytoplasmic calcium and pH levels, releasing reactive oxygen species, and regulating gene expression (Messiaen and Van Cutsem 1993, 1994, 1999; Messiaen et al. 1993; Van Cutsem and Messiaen 1994; Thain et al. 1995; Brüdern and Thiel 1999; Lee et al. 1999). In many respects, this pectic signaling is very similar to auxin signaling. Similarly, xyloglucan-derived oligosaccharides serve as effective signaling molecules in plants.

Importantly, the pectic signaling cascade is initiated at the PM via receptor-mediated endocytosis (Horn et al. 1989, Low et al. 1993, Van Cutsem and Messiaen 1994). This fits with the currently emerging concept that endocytosis is tightly linked with signaling (for recent reviews for plants, see Geldner [2003] and Šamaj et al. [2004]). Intriguingly, internalized cell wall pectins accumulate within the same BFA compartments as the putative auxin efflux carriers PIN1 and PIN2 (Baluška et al. 2002, Boonsirichai et al. 2003, Šamaj et al. 2004).

Since endosomes and endocytic vesicles represent de facto cell wall islands enclosed by their PM-derived membrane, it can be proposed that endosomes filled with cell wall pectins represent a ready-to-use reservoir of building blocks for rapid cell wall formation during plant cytokinesis. Finally, internalization of cell wall pectins is potentially also related to other diverse processes requiring rapid assembly of cell walls. Well-designed future studies are essential in order to further unravel this fascinating, but until now largely neglected, topic of plant biology.

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