

F-Actin-Dependent Endocytosis of Cell Wall Pectins in Meristematic Root Cells. Insights from Brefeldin A-Induced Compartments¹

František Baluška*, Andrej Hlavacka, Jozef Šamaj, Klaus Palme, David G. Robinson, Toru Matoh, David W. McCurdy, Diedrik Menzel, and Dieter Volkmann

Plant Cell Biology, Institute of Botany, University of Bonn, Kirschallee 1, D-53115 Bonn, Germany (F.B., A.H., D.M., D.V.); Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Akademická 2, SK-95007 Nitra, Slovakia (J.Š.); Max-Delbrück-Laboratorium in der Max-Planck-Gesellschaft, D-50829 Köln, Germany (K.P.); HIP Zellbiologie, University of Heidelberg, Im Neuenheimer Feld 230, D-69120 Heidelberg, Germany (D.G.R.); Laboratory of Plant Nutrition, Kyoto University, Kyoto 606-01, Japan (T.M.); and School of Environmental and Life Sciences, The University of Newcastle, Newcastle, New South Wales 2308, Australia (D.W.M.)

Brefeldin A (BFA) inhibits exocytosis but allows endocytosis, making it a valuable agent to identify molecules that recycle at cell peripheries. In plants, formation of large intracellular compartments in response to BFA treatment is a unique feature of some, but not all, cells. Here, we have analyzed assembly and distribution of BFA compartments in development- and tissue-specific contexts of growing maize (*Zea mays*) root apices. Surprisingly, these unique compartments formed only in meristematic cells of the root body. On the other hand, BFA compartments were absent from secretory cells of root cap periphery, metaxylem cells, and most elongating cells, all of which are active in exocytosis. We report that cell wall pectin epitopes counting rhamnogalacturonan II dimers cross-linked by borate diol diester, partially esterified (up to 40%) homogalacturonan pectins, and (1→4)-β-D-galactan side chains of rhamnogalacturonan I were internalized into BFA compartments. In contrast, Golgi-derived secretory (esterified up to 80%) homogalacturonan pectins localized to the cytoplasm in control cells and did not accumulate within characteristic BFA compartments. Latrunculin B-mediated depolymerization of F-actin inhibited internalization and accumulation of cell wall pectins within intracellular BFA compartments. Importantly, cold treatment and protoplasting prevented internalization of wall pectins into root cells upon BFA treatment. These observations suggest that cell wall pectins of meristematic maize root cells undergo rapid endocytosis in an F-actin-dependent manner.

Eukaryotic cells perform endomembrane flow accomplished by vesicles shuttling among endoplasmic reticulum (ER), Golgi apparatus (GA), the plasma membrane (PM), and endosomes (for plants see Robinson et al., 1998; Hawes et al., 1999). These compartments and pathways of endomembrane flow are highly conserved in unicellular yeast, higher plants, and animals (for plant cells, see Robinson et al., 1998; Hawes et al., 1999). A major breakthrough in our current understanding of this complex endomembrane flow was provided by rediscovery of the fungal metabolite brefeldin A (BFA; Fujiwara et al., 1988). BFA action prevents vesicle formation in the exocytosis pathway by stabilizing abortive complexes between conserved ADP ribosylation factor 1

(ARF1) and the Sec7 domain of its guanine nucleotide exchange factor during the assembly of coat protein complexes of budding vesicles (for plants see, Pimpl et al., 2000; Robineau et al., 2000). Because of this action, BFA inhibits anterograde vesicular pathways while allowing endocytosis and some retrograde pathways to proceed further (Miller et al., 1992; Gaynor et al., 1998; Belanger and Quatrano, 2000). Moreover, BFA inhibits the endosome to vacuole transport in budding yeast (Gaynor et al., 1998).

The introduction of BFA to investigate the cell biology of endomembrane flow in plant cells occurred some years later (Satiat-Jeunemaitre and Hawes, 1992), but most of the major findings concerning the effects of this drug in animal and yeast systems have been confirmed for plant cells. For example, low concentrations of BFA (<10 μg mL⁻¹) effectively inhibit secretion attributable to blockage of the ER to GA step (Driouich et al., 1993; Boevink et al., 1999), whereas higher BFA levels (>50 μg mL⁻¹) are needed to inhibit the GA to PM transport step (Boevink et al., 1998) and to induce vesiculation of GA stacks (Satiat-Jeunemaitre and Hawes, 1992). This is accompanied by redistribution of GA proteins

¹ This work was supported by the Deutsches Zentrum für Luft- und Raumfahrt (Köln, Germany; to F.B. and D.V.) and by the Slovak Academy of Sciences, Grant Agency Vega (Bratislava, Slovakia; grant nos. 2031 and 2/616/99 to F.B. and J.Š.).

* Corresponding author; e-mail baluska@uni-bonn.de; fax 49-228-739004.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.007526.

into ER (Boevink et al., 1998, 1999; Ritzenthaler et al., 2002; Saint-Jore et al., 2002). Similar to other eukaryotic systems, endocytosis remains intact in BFA-treated plant cells (Satiat-Jeunemaitre and Hawes, 1992; Steinmann et al., 1999; Belanger and Quatrano, 2000). Before the formation of GA-ER hybrid organelle, the trans-most GA cisterna is lost (Ritzenthaler et al., 2002) and apparently contributes to the formation of perinuclear vesicular bodies (Satiat-Jeunemaitre and Hawes, 1992; Wee et al., 1998; Geldner et al., 2001). Such compact areas of densely packed heterologous vesicles were named "BFA compartments" (Satiat-Jeunemaitre and Hawes, 1993), and they represent the most dramatic morphological response of plant cells to high BFA levels. Importantly, BFA compartments form before GA disintegration (Geldner et al., 2001), and ER elements do not participate in the formation of BFA compart-

ments (Henderson et al., 1994). These observations strongly suggest that there must be an additional membranous source that feeds into these compartments of plant cells. Our data suggest that this source is the PM.

The nature of BFA compartments remains controversial also because several other studies failed to report such compartments, even in plant cells that have their GA totally disassembled in response to BFA treatment (Rutten and Knuiman, 1993; Yasuhara et al., 1995; Boevink et al., 1998; Kartusch et al., 2000). In our study, we have addressed these issues using an embedding-sectioning technique based on Steedman's wax (Baluška et al., 1997), which enables reliable development- and tissue-specific localization of diverse intracellular (for tubulin and actin, see Baluška et al., 1992, 1997) and cell wall (Šamaj et al., 1998; this study) antigens in the context of intact root

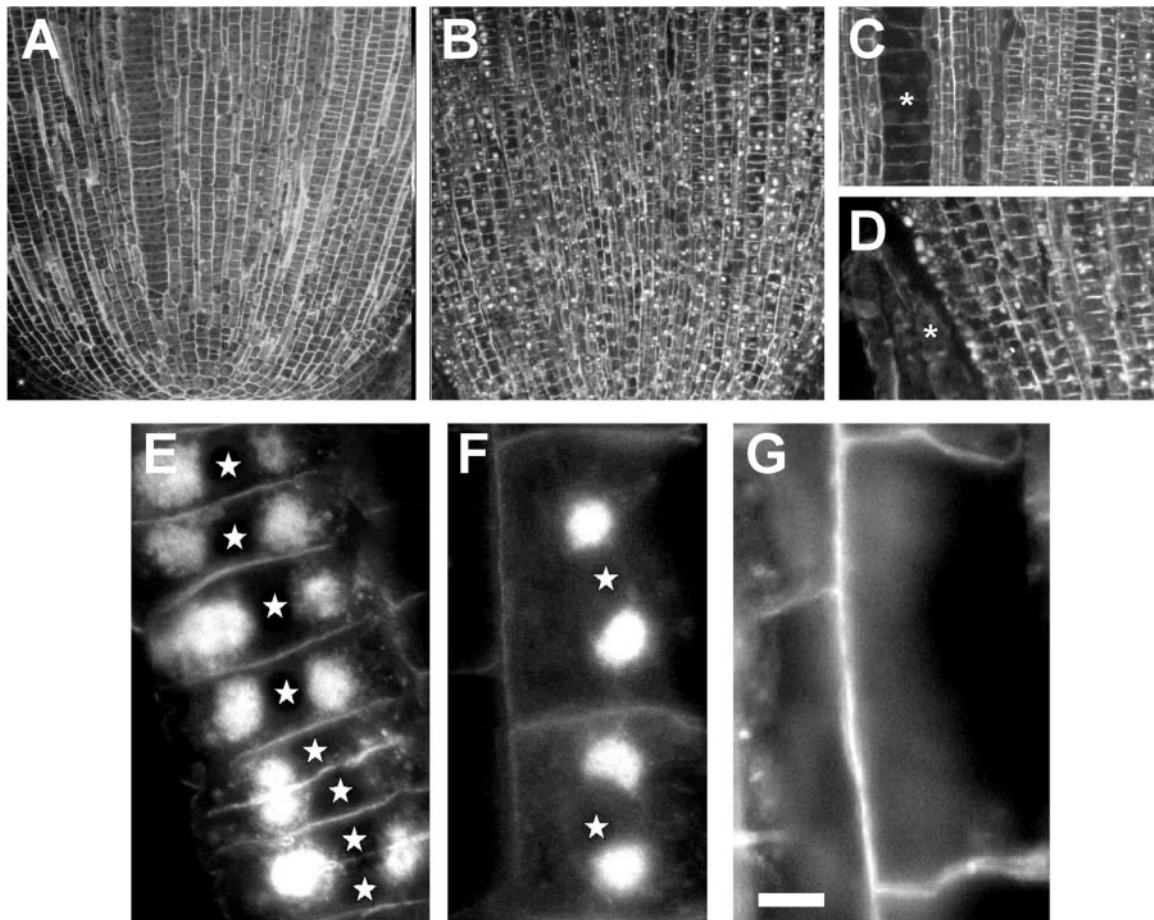


Figure 1. Development- and tissue-specific distributions of RGII-borate pectins in cells of control (A) and BFA-treated (B–F) maize root apices. A, RGII-borate pectins localize preferentially to cell walls of all cells of the root apical meristem. B, In BFA-treated roots, all meristematic cells accumulate RGII-borate pectins within BFA compartments. The only exceptions to this feature are metaxylem elements (asterisk in C) and secretory cells of the root cap periphery (asterisk in D). Prominent BFA compartments are found in epidermis cells in the meristem (E) and in the apical part of the elongation region (F). Note the switch in positioning of BFA compartments in post-mitotic epidermis cells in E and F. In contrast, all other elongating root cells are devoid of BFA compartments; for cortical cells see G. The basal-apical root axis of each cell (in this and all other figures) runs from the top to bottom of the page. Stars indicate nuclei. Bar = 10 μm in A and G; 46 μm in B through D; 12 μm in E; and 15 μm in F.

Table 1. Summary of antibodies used and localization of their antigens

Antibody	Epitope	Predominant Localization	Accumulation in BFA Compartments
RGII	Pectin: rhamnogalacturonan II dimer cross-linked with boron	Cell wall	Yes
JIM5	Pectin: partially esterified (up to 40%) homogalacturonan	Cell wall	Yes
JIM7	Pectin: esterified (up to 80%) homogalacturonan	GA, vesicles	No
LM7	Pectin: non-blockwise de-esterified homogalacturonan	Cell wall	No
LM5	Pectin: (1→4)- β -d-galactan of RG I	Cell wall	Yes
LM2	AGP: α -linked glucuronic acid residue	ER, GA, vesicles, cell wall, PM	No
MAC207	AGP: GlcpA- β (1→3)-d-GalpA- α (1→2)-l-Rha residues	ER, GA, vesicles, cell wall, PM	No
HDEL	ER proteins	ER, vesicles	No
ARF1	ARF1	GA, vesicles, PM	Yes
58K	cis-Golgi marker	GA	No
PM-H ⁺ ATPase	PM-H ⁺ ATPase	PM, vesicles	Yes
PIN1	Auxin efflux carrier	PM, vesicles	Yes

apices. Taking the advantage of BFA and pectin antibodies reactive to cell wall pectin epitopes (Jones et al., 1997; Matoh et al., 1998; Willats et al., 2001), we report that cell wall pectins represent the first complex macromolecules that are shown to be internalized into the cytoplasm of meristematic plant cells.

RESULTS

Development- and Tissue-Specific Distributions of BFA Compartments

Rhamnogalacturonan II (RGII) antibody recognizes cell wall rhamnogalacturonan pectins cross-linked by

a borate diol diester formed within cell walls in muro (Matoh et al., 1998). We have taken advantage of this antibody to probe whether cell wall pectins accumulate within intracellular BFA compartments. We report that the RGII antibody labeled predominantly cell walls in control root apices (Fig. 1A). Importantly, in BFA-treated roots, RGII antibody recognized prominent BFA compartments in all meristematic root cells (Fig. 1, B–D). In contrast, RGII-positive BFA compartments did not form in cells having active exocytosis like post-mitotic metaxylem elements (Fig. 1C) and secretory root cap cells (Fig. 1D). Interestingly, BFA compartments ultimately achieved pe-

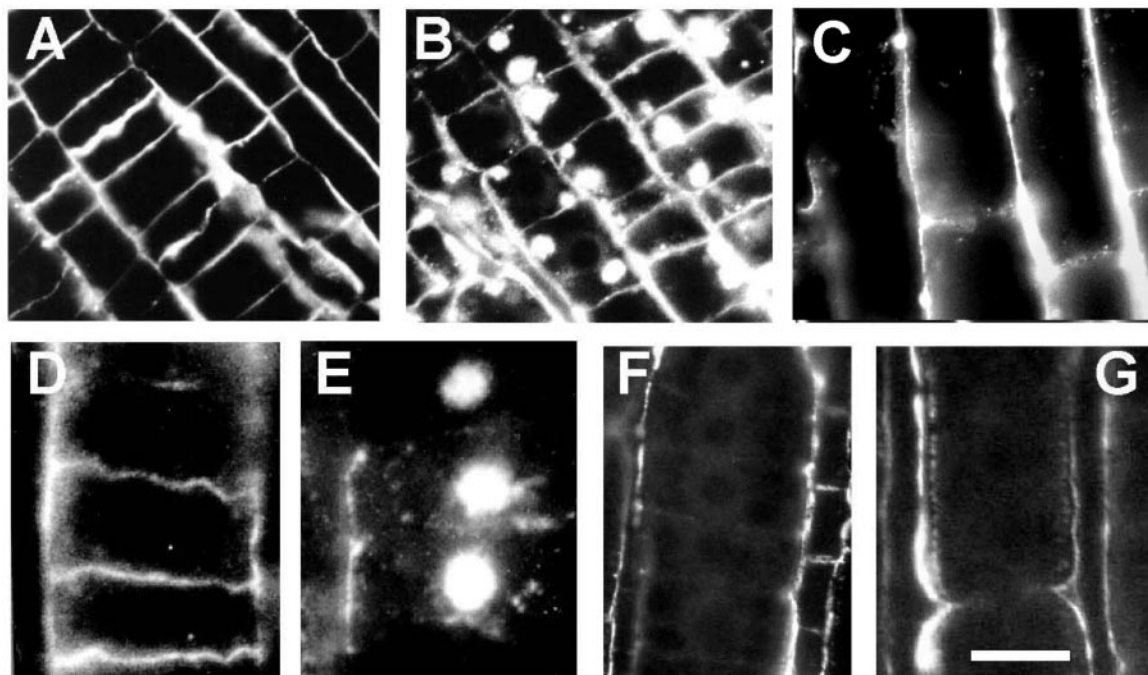


Figure 2. Distributions of JIM5- (A–C), LM5- (D and E), and LM7-reactive (F and G) pectins in control (A, D, and F) and BFA-treated (B, C, E, and G) root apices. B and C, In BFA-treated root apices, JIM5-reactive pectins accumulate within BFA compartments in all meristematic cells (B) but not in elongating cells (C). D and E, LM5-reactive pectins redistribute almost completely from cell walls (D) into BFA-induced compartments (E). F and G, In contrast, LM7-reactive pectins do not accumulate within BFA compartments and remain in cell walls also in BFA-treated cells. Bar = 20 μ m in A and B; 40 μ m in C; and 11 μ m in D through G.

rinuclear positions and maintained the longest distance from each other at the opposite sides of centrally positioned nuclei matching cellular polarity axes (Fig. 1, E and F). In the elongation region, only epidermal cells embarking on root hair formation formed BFA compartments (Fig. 1F), whereas all other rapidly elongating cells did not form BFA compartments (Fig. 1G).

Epitope-Specific Accumulation of Cell Wall Pectins within BFA Compartments

We have tested further cell wall pectin epitopes for their localization in control and BFA-treated root apices. Partially esterified (up to 40%) homogalacturonan pectins are recognized by monoclonal JIM5 antibody (Willats et al., 2001; for an overview of antibodies used, see Table I). In control root apices, JIM5-reactive pectins located exclusively to cell walls of cortex cells, whereas the signal in stele cells was rather faint (Fig. 2A). Root cap and epidermal cells did not react with JIM5 antibody (data not shown). This situation changed after exposure to BFA, where JIM5-reactive pectins accumulated within BFA compartments in all JIM5-positive cells of the apical meristem (Fig. 2B). At variance with meristematic cells, BFA-treated elongating cells retained JIM5-reactive pectins within their cell walls (Fig. 2C). LM5 antibody recognizes (1→4)- β -D-galactan side chains of rhamnogalacturonan I (RGI; Jones et al., 1997; see Table I) and labeled predominantly cell walls in control cells (Fig. 2D). In contrast, labeling by this antibody was almost exclusively confined to large BFA compartments in BFA-treated cells (Fig. 2E). Importantly, cell wall labeling diminished considerably after accumulation of LM5-reactive pectins within intracellular BFA compartments (Fig. 2, D and E). In contrast, non-blockwise de-esterified homogalacturonan pectins reactive to LM7 monoclonal antibody (Willats et al., 2001), which also labeled exclusively cell walls in control root apices (Fig. 2F), did not accumulate within BFA compartments (Fig. 2, F and G).

Actin Filaments Are Essential for Internalization of Cell Wall Pectins

Root cells devoid of F-actin because of their exposure to latrunculin B (Baluška et al., 2001) failed to internalize JIM5-reactive cell wall pectins and to accumulate them within BFA compartments (Fig. 3, A and B). In contrast, oryzalin-treated cells devoid of microtubules formed normal or sometimes even slightly larger JIM5-reactive BFA compartments (Fig. 3D) than those found in control root cells (Fig. 3C).

Distributions of GA-Derived Exocytotic Pectins in Control and BFA-Treated Cells

JIM7-reactive (up to 80% esterified homogalacturonan) pectins localized to intracellular spots (Fig. 4A)

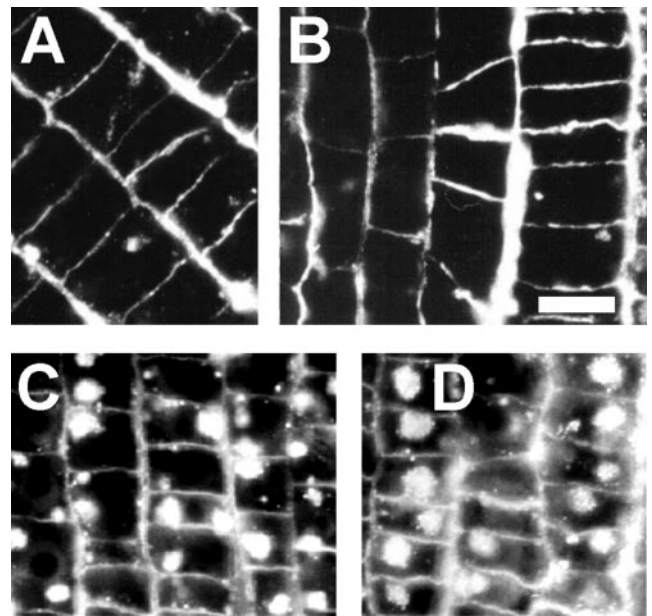


Figure 3. Effects of latrunculin B (A and B) and oryzalin (D) on accumulation of JIM5-reactive pectins within BFA compartments. A and B, Cells devoid of F-actin in latrunculin B pretreated root apices do not internalize JIM5-reactive pectins both in cortex (A) and stele (B). C and D, Cells of oryzalin pretreated roots (D) form even slightly larger JIM5-positive BFA compartments than cells treated only with BFA (C). Bar = 25 μ m.

representing presumably GAs where homogalacturonan pectins are synthesized in their esterified form (Goubet and Mohnen, 1999). In control root cells, pectins reactive to JIM7 antibody did not associate abundantly with cell walls. This characteristic distribution pattern changed only slightly in cells of BFA-treated roots when accumulation of JIM7-reactive spots into a small number of larger aggregates was scored occasionally (Fig. 4B). Importantly, typical compact, roundish, and large BFA compartments were never detected with the JIM7 antibody suggesting that GA is not the major source of membranous structures accumulating within BFA compartments.

GA and ER Are Not Major Contributors to Formation of BFA Compartments

To further explore possible contributions of GA and ER elements to the formation of BFA compartments, we used two arabinogalactan protein (AGP) antibodies (MAC207 and LM2) that label the endomembrane system of maize (*Zea mays*) root cells (Šamaj et al., 2000). Final stages of AGP synthesis occur in GA, after which these complex molecules are secreted into the extracellular space. As a visual marker for ER elements, we used the HDEL antibody (Napier et al., 1992; Table I). Importantly, these GA- and ER-related molecules did not accumulate within BFA compartments of maize root cells (Fig. 4, C–E). These findings confirm that both the GA and ER do

not contribute substantially to the formation of BFA compartments in maize root cells (for similar data, see Henderson et al., 1994).

Recycling PM Proteins Accumulate within BFA Compartments

To look for other molecules accumulating within BFA-induced compartments, we have probed subcellular distributions of PM-associated proteins that are expected to perform recycling. Consistent with our expectations, antibodies raised against both the PM H^+ -ATPase and PIN1 auxin-efflux carrier labeled the PM in control cells (data not shown) and BFA compartments in treated cells (Fig. 4, F–H). The PM H^+ -ATPase (Fig. 4, F and G) and the PIN1 auxin efflux carrier (Fig. 4H) accumulated within BFA compartments after 2 h exposure to BFA (for similar results in Arabidopsis, see Geldner et al., 2001). Depolymerization of F-actin with latrunculin B inhibited accumulation of these recycling PM proteins within BFA compartments (data not shown).

ARF1, But Not cis-Golgi Marker, Accumulates within BFA Compartments

In addition, we have analyzed distributions of two Golgi-associated molecules in BFA-treated maize

roots. ARF1, a small GTPase of the Ras family (Robineau et al., 2000), is the actual target of BFA action in eukaryotic cells localized also to TGN (for maize, see Pimpl et al., 2000). In cells of maize root meristem, ARF1 localized diffusely throughout the cytoplasm but also to the PM. Upon BFA treatment, ARF1 accumulated within BFA compartments and got depleted from the cytoplasm and the PM (Fig. 5, A and B). In contrast, cis-Golgi marker 58-K protein (Saraste et al., 1987; Li and Yen, 2001) did not accumulate in BFA compartments (Fig. 5, C and D).

Protoplasting and Cold Treatment Prevent Accumulation of Cell Wall Pectins within BFA Compartments

To provide further experimental evidence for endocytosis of cell wall pectins, we exposed wall-less protoplasts of meristematic maize root cells to BFA. As predicted, we did not score any intracellular accumulation of RGII-borate pectins and LM5-reactive cell wall pectins in BFA-treated protoplasts (Fig. 6, A–F). In the case of JIM5-reactive pectins, we occasionally found small intracellular aggregates distributed throughout the protoplasts that, however, never coalesced into large BFA compartments (data not shown).

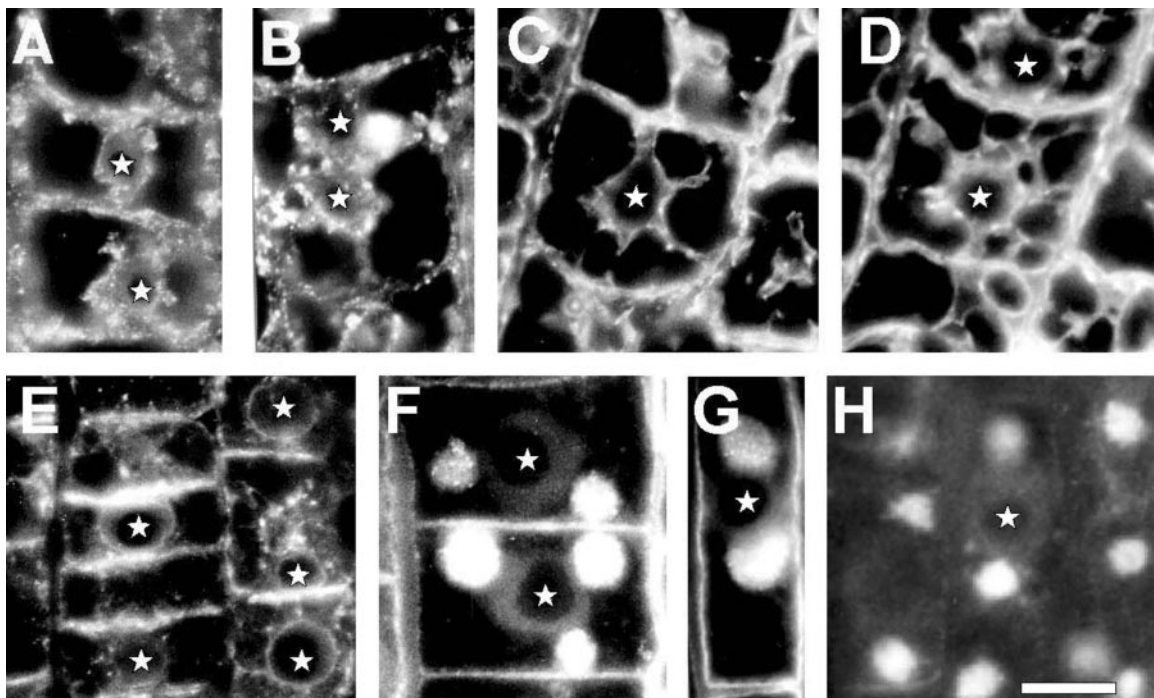


Figure 4. Distribution of JIM7-reactive pectins (A and B), GA-derived AGPs (C and D), ER-based HDEL proteins (E), and PM-associated recycling proteins (F through H) in control (A) and BFA-treated (B through H) cells. In control cells, JIM7 antibody recognizes numerous spots distributed throughout the cytoplasm (A), and this pattern does not change dramatically in BFA-treated cells (B). C and D, Secretory AGPs reactive to MAC207 (C) and LM2 (D) antibodies and ER-based HDEL proteins (E) do not accumulate within BFA compartments of BFA-treated root cells. F through H, In contrast, both PM- H^+ -ATPase (F and G) and PIN1 auxin efflux carrier (H) accumulate abundantly within BFA compartments. Stars indicate nuclei. Bar = 20 μm in A through E; 11 μm in F and H; and 23 μm in G.

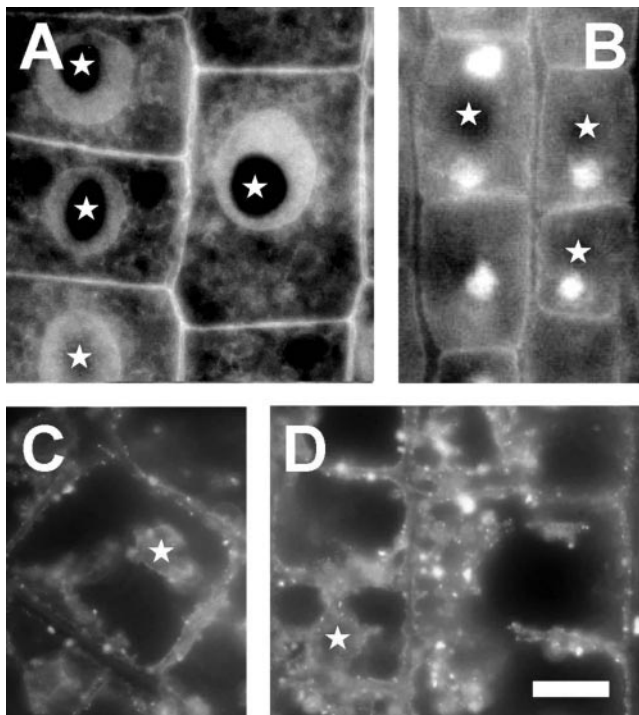


Figure 5. Distribution of ARF1 (A and B) and 58K (C and D) GA proteins in control (A and C) and BFA-treated (B and D) cells. Although ARF1 accumulates prominently within BFA compartments (B), 58K antibody labels spots distributed throughout the cytoplasm that correspond to GA (C), and this pattern does not change dramatically in BFA-treated cells (D). Stars indicate nuclei. Bar = 8 μm in A; 12 μm in B; and 17 μm in C and D.

Additional evidence in favor of the active endocytotic internalization of cell wall pectins was provided using cold treatment of maize roots. A hallmark of clathrin-supported endocytosis is its sensitivity to low temperature (Wileman et al., 1985). BFA treatment at low temperature prevented intracellular internalization of cell wall pectins in all root meristem cells (Fig. 7, A–D). Low temperature similarly blocked accumulation of PM H^+ -ATPase and PIN1 auxin-efflux carrier within BFA compartments (data not shown).

DISCUSSION

We report here that BFA compartments do not form in all root apex cells. Intriguingly, active secretory cells, like elongating root body cells and root cap periphery cells, do not form characteristic BFA compartments. The only exceptions to this rule are epidermal cells embarking on root hair formation. In contrast, all meristematic root cells formed BFA compartments, which ultimately achieved perinuclear positions. Surprisingly, cell wall pectins accumulated in these large compartments. In addition to cell wall pectins, PM-associated proteins undergoing internalization-mediated recycling (Geldner et al., 2001; Friml et al., 2002) also accumulated within BFA

compartments. Therefore, the most plausible explanation for our present data is that membranous structures accumulating in characteristic BFA compartments are predominantly of endocytotic/recycling origin.

Two major implications arise from our present data. First, it is important to be aware that not all intracellular pectins belong to the GA-derived secretory pathway. Some are clearly transported along endocytotic pathways heading for either recycling or degradation. Second, it is apparent that internalization of cell wall pectins must play a key role in the dynamic turnover of pectins in dividing cells of higher plants. Importantly, we document internalization of those pectin molecules which can be cross-linked with boron (RGII pectins) and calcium (RGII-borate pectins and de-esterified homogalacturonan pectins).

BFA Compartments as Endosome-trans-Golgi Network (TGN) Hybrid Organelle

Our data show that BFA compartments accumulate internalized macromolecules via retrograde endocytotic pathway. However, TGN also appears to be involved in the formation of BFA compartments. The most trans-cisterna of plant GA, corresponding to TGN, is rapidly lost via sloughing in response to BFA, and presumably participates in the formation of BFA compartments (see Fig. 12 in Ritzenthaler et al., 2002). In accordance with this notion, JIM84 antibody recognizes a complex carbohydrate epitope generated late in the GA pathway (Fitchette et al., 1999) that does not relocate back into ER during BFA treatment. Instead, the JIM84 antigen ends up within BFA compartments (Satiat-Jeunemaitre and Hawes, 1992) together with the actual BFA target ARF1 (Pimpl et al., 2000; Robineau et al., 2000). In contrast to TGN,

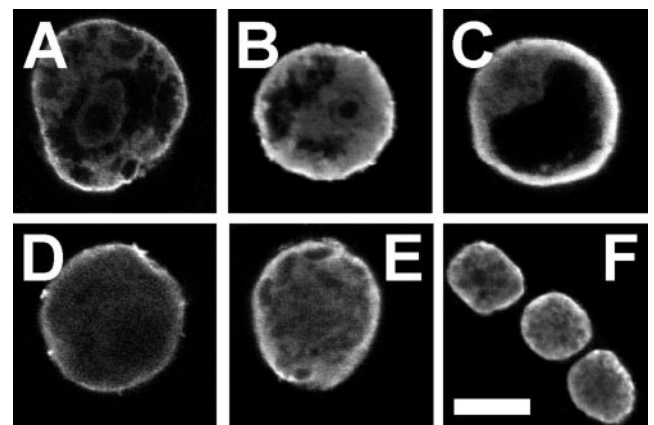


Figure 6. Protoplasts of maize root apex cells do not accumulate cell wall pectins in BFA compartments. JIM5-reactive pectins (A and B), LM5-reactive pectins (C and D), and RGII-borate pectins (E and F) show the same distribution pattern both in control (A, C, and E) and BFA-treated (B, D, and F) protoplasts. Bar = 20 μm in A through E; and 34 μm in F.

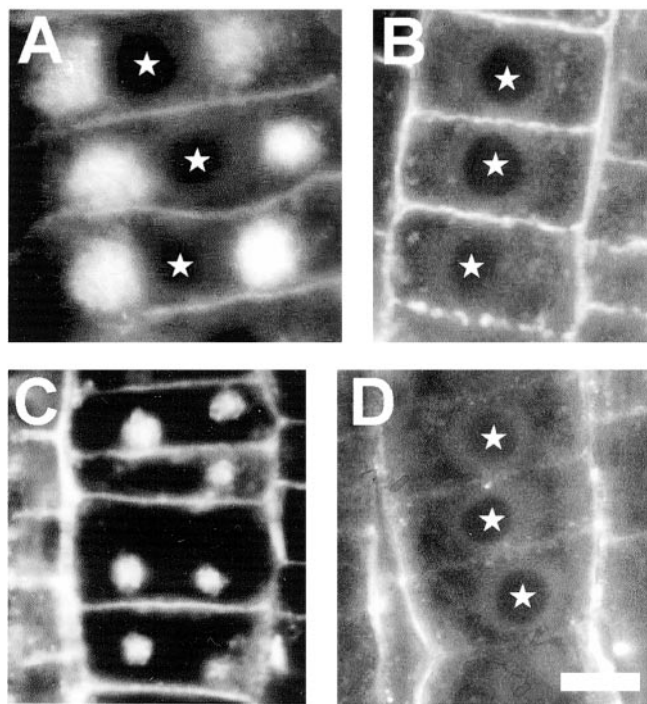


Figure 7. Cold treatment (B and D) prevents internalization of cell wall pectins into BFA compartments (for BFA treatment at room temperature, see A and C). A and B, RGII-borate pectins; C and D, JIM5 pectins. Stars indicate nuclei. Bar = 8 μm in A; 10 μm in B; 12 μm in C and D.

cis- and median-Golgi cisternae merge with ER as reported for BFA-treated tobacco (*Nicotiana tabacum*) cv Bright Yellow-2 cells (Ritzenthaler et al., 2002).

Strong support for this concept, namely that the trans-most (TGN) cisterna of plant GA aggregates together with putative endosomes to form large BFA compartments, is obtained from studies using targeting of mammalian α -2,6-sialyltransferase into plant GA (Wee et al., 1998). This protein localizes exclusively to the trans-most (TGN) cisterna of untreated transgenic Arabidopsis root cells but accumulates within BFA compartments of BFA-treated Arabidopsis meristematic root tip cells (Wee et al., 1998). Accumulation of α -2,6-sialyltransferase within BFA compartments of root cells is apparently dependent on their meristematic nature because post-mitotic leaf cells redistribute the same trans-Golgi marker rather into ER (Saint-Jore et al., 2002). This is in a full agreement with our present finding that characteristic BFA compartments form only in meristematic cells. Furthermore, the dynamin-like protein ADL6 localizes to trans-Golgi and to BFA compartments in Arabidopsis root cells (Jin et al., 2001). In contrast, antibody raised against a cis-Golgi marker 58K protein (Saraste et al., 1987), which recognizes plant GA (Li and Yen, 2001), does not label BFA compartments of maize root cells but is presumably associated with the Golgi-ER hybrid organelle described by Ritzenthaler et al. (2002).

Cell Wall Pectins, But Not GA and ER Molecules, Accumulate within BFA Compartments

We have compared distributions of JIM7- and JIM5-reactive homogalacturonan pectins in cells of control and BFA-treated root apices. In accordance with data from tobacco pollen tubes (Geitmann et al., 1996), BFA compartments accumulate large amounts of low-esterified (up to 40%, JIM5) but not high-esterified (up to 80%, JIM7) homogalacturonan pectins. Importantly, besides JIM5-reactive pectins, RGII dimers cross-linked by a borate diol diester (Matoh et al., 1998) and (1 \rightarrow 4)- β -D-galactan side chains of RGI (Jones et al., 1997) are further pectin epitopes which accumulate within BFA compartments. As boron-cross-linked RGII pectins of cell walls are critical not only for cell wall integrity but, in due course, also for cell growth and overall plant form (O'Neill et al., 2001; Höfte, 2001), their internalization might be expected to have profound impacts on growth and development of plants. Intriguingly in this respect, JIM5-reactive pectins (Knox et al., 1990), LM5-reactive pectins (Bush and McCann, 1999), and RGII pectins cross-linked by a borate diol diester (Matoh et al., 1998) all localize preferentially at the innermost part of cell walls adjacent to the PM and undergo internalization. In contrast, non-blockwise de-esterified homogalacturonan pectins of cell walls, reactive to LM7 antibody, do not localize close to the PM (Willats et al., 2001) and are not internalized into the cytoplasm of dividing cells (this study). Importantly, those cell wall pectin epitopes that are internalized become depleted from walls of BFA-treated root apices. To demonstrate that endocytosis is involved in internalization of these cell wall pectins, we showed that disintegration of F-actin inhibits internalization of cell wall pectins. Further evidence that cell wall pectins are internalized via endocytosis was provided by performing the BFA treatment at 4°C when active processes like endocytosis are blocked (Wileman et al., 1985; Low et al., 1993; Emans et al., 2002). At this temperature, no accumulation of wall pectins within BFA compartments was observed. Finally, protoplasting of meristematic root cells prevented formation of pectin-enriched BFA compartments.

In contrast to extracellular wall pectins, GA-derived JIM7-reactive pectins did not accumulate within typical compact BFA compartments but instead localized into smaller irregular aggregates corresponding presumably to the pleiomorphic GA-ER hybrid organelle pervading the whole cytoplasm (Ritzenthaler et al., 2002). Importantly in this respect, ER-based proteins did not accumulate in BFA compartments (see also Henderson et al., 1994). Antibodies (MAC207 and LM2) raised against cell wall- and PM-associated epitopes of AGPs, secreted via the exocytotic pathway (Samaj et al., 2000), similarly did not label BFA compartments of root cells (at least not after 2 h of BFA treatment). These observations argue

against the concept that GA-derived vesicles represent the major constituents of BFA compartments.

F-Actin, But Not Microtubules, Is Essential for Cell Wall Pectin Internalization

An intact F-actin cytoskeleton is required for endocytosis (Qualmann et al., 2000; for plant cells, see Geldner et al., 2001; Friml et al., 2002). Depolymerization of F-actin with latrunculin B interfered with endocytotic internalization of PM/cell wall-associated molecules. JIM5-reactive cell wall pectins and PM-associated proteins, such as PM-H⁺ ATPase and auxin efflux carrier PIN1, failed to be internalized in the absence of F-actin. These results support the notion that accumulation of internalized pectins within BFA compartments results from the unbalanced recycling of vesicles in BFA-treated cells.

In contrast to F-actin, depolymerization of microtubules did not inhibit endocytosis of cell wall pectins. Indeed, the opposite appeared to be the case in that BFA compartments seemed larger in the absence of cortical MTs. This observation might be explained by the dense arrays of cortical microtubules in plant cells (for maize root cells see Baluška et al., 1992) sterically interfering with the assembly of the endocytic protein complexes at the PM. For instance, the distance between neighboring cortical microtubules is much smaller than the size of coated vesicles of plant cells (Doohan and Palevitz, 1980; Vesik et al., 1996). Thus, a PM devoid of a dense cortical microtubule array might be expected to perform more internalization events.

Recycling PM Proteins Accumulate within BFA Compartments

To further substantiate the idea that endocytosis-driven internalization/recycling contributes significantly to the formation of BFA compartments, we have taken advantage of two well-defined antibodies against PM proteins known, or expected, to perform recycling at the PM. Importantly, PIN1 auxin efflux carrier accumulates rapidly within BFA compartments in cells of *Arabidopsis* embryos (Steinmann et al., 1999). More recent studies on PIN1 and PIN3 (Geldner et al., 2001; Friml et al., 2002) demonstrated that BFA-induced accumulation of PIN1/PIN3 within BFA compartments results from unbalanced endocytosis of a steady-state pool of these molecules that rapidly recycle between the PM and endosomal compartment (Geldner et al., 2001). The identity of this endosome compartment awaits further experimental studies but might be expected to be an early and/or recycling endosome.

Here, we have shown that both PM H⁺-ATPase and PIN1 auxin efflux carriers accumulate within BFA compartments of maize root cells. As recycling PM proteins accumulate within BFA compartments,

it might be expected that the internalized cell wall pectins also accomplish recycling. One plausible scenario would be that boron- and calcium-cross-linked cell wall pectins lose their cross-linkages within endosomes and then return back to cell walls.

CONCLUSIONS

In the present study, we have revealed an unexpected new aspect of pectin behavior in plant cells: internalization of cell wall pectins. Clearly, not all intracellular pectins are on their exocytic pathway, but a portion of these molecules are located within compartments of the endocytic pathway. Our data show that boron-cross-linked RGII pectins are internalized. Calcium-cross-linked homogalacturonan pectins (reactive to 2F4 antibody) also undergo an internalization process in meristematic cells of carrot (*Daucus carota*) root apices (Françoise Liners, personal communication). Because calcium- and boron-cross-linked cell wall pectins are critical for both mechanical strength and porosity of plant cell walls (Kobayashi et al., 1999; Höfte, 2001; O'Neill et al., 2001; Willats et al., 2001), their internalization might prove to be of critical importance for growth, polarity, and morphogenesis.

In plants, dynamic redistribution of pectins between cell walls and the cytoplasm might also have an impact on pectin-based signaling (Messiaen et al., 1993; Van Cutsem and Messiaen, 1994). Interestingly in this context, a pectin-derived elicitor was reported to bind to the PM (Diekmann et al., 1994) and to be taken up into plant cells via receptor-mediated endocytosis (Low et al., 1993). Moreover, besides growth, polarity, and morphogenesis, also cell-to-cell transport via plasmodesmata (Chen et al., 2000), pollen tube guidance (Mollet et al., 2000), and sensitivity of cells toward aluminum toxicity (Schmohl and Horst, 2000) are critical pectin-dependent processes. In light of our findings, it will be important to characterize those mechanisms that drive internalization of cell wall pectins and to unravel how this process impinges on mechanical properties of cell walls and, ultimately, on signaling, cell growth, polarity, and morphogenesis in plants.

MATERIALS AND METHODS

Plant Material and Inhibitor Treatments

Maize (*Zea mays* L. cv Careca S230) grains were soaked for 6 h and germinated in well-moistened rolls of filter paper for 4 d in darkness at 20°C. For cold treatment, seedlings were kept at 4°C for 6 h. Young seedlings with straight primary roots, 50 to 70 mm long, were selected for inhibitor treatments and subsequent immunolabeling studies. Unless stated otherwise, all chemicals were obtained from Sigma Chemicals (St. Louis). 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 (see also Satiat-Jeunemaitre and Hawes, 1992, 1993) before submergence of root apices for 2 and 6 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.

Indirect Immunofluorescence Microscopy

Excised apical root segments (7 mm in length), encompassing the major growth zones, were fixed in 3.7% (w/v) formaldehyde prepared in stabilizing buffer (SB; 50 mM PIPES, 5 mM MgSO_4 , and 5 mM EGTA, pH 6.9) for 1 h at room temperature. After rinsing in SB, the root apices were dehydrated in a graded ethanol series diluted with phosphate-buffered saline (PBS). They were embedded in low-melting-point Steedman's wax and processed for immunofluorescence (for details, see Baluška et al., 1992). After a 10-min rinse with absolute methanol at -20°C , the sections were transferred to SB containing 1% (w/v) BSA for 30 min at room temperature. They were then incubated with the following primary antibodies: anti-Golgi 58K monoclonal antibody (Sigma G2404) diluted 1:50 (w/v), JIM5 and JIM7 monoclonal antibodies (Knox et al., 1990) diluted 1:20 (w/v), LM5 monoclonal antibody (Jones et al., 1997) diluted 1:20 (w/v), LM7 monoclonal antibody (Willats et al., 2001) diluted 1:10 (w/v), RGII polyclonal antibody (Matoh et al., 1998) diluted 1:100 (w/v), LM2 monoclonal antibody (Šamaj et al., 2000) diluted 1:20 (w/v), MAC207 monoclonal antibody (Šamaj et al., 2000) diluted 1:20 (w/v), PM H^+ -ATPase monoclonal antibody (Jahn et al., 1998) diluted 1:100 (w/v), ARF1 polyclonal antibody (Pimpl et al., 2000) diluted 1:100 (w/v), and PIN1 polyclonal antibody raised against auxin efflux carrier of maize diluted 1:100 (w/v). All primary antibodies were diluted in PBS supplemented with 1% (w/v) BSA, and sections were incubated in primary antibody for 1 h at room temperature. After rinsing in SB, the sections were incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgGs (58K and PM H^+ -ATPase), with anti-rat IgGs (JIM5, JIM7, LM2, LM5, LM7, and MAC207), or with anti-rabbit IgGs (RGII, ARF1, and PIN1), diluted 1:100 (w/v; mouse and rabbit antibodies) or 1:20 (w/v; rat antibodies) in PBS containing 1% (w/v) BSA. A further rinse in PBS (10 min) preceded a 10-min treatment with 0.01% (w/v) toluidine blue (made in PBS), which diminished autofluorescence of root tissues. The sections were mounted using an anti-fade medium containing *p*-phenylenediamine (Baluška et al., 1992). Sections were examined with an Axiovert 405M inverted microscope (Zeiss, Oberkochen, Germany) equipped with epifluorescence and standard FITC excitation and barrier filters (BP 450–490, LP 520). Photographs were taken on T-Max film (Eastman Kodak, Rochester, NY) rated at 400 ASA.

Protoplast Preparation and Immunofluorescence

The 50- to 70-mm-long root apices were selected for protoplasts preparation. Root caps were removed, and 2-mm-long segments were used for further protoplast preparation using the method described by Kollmeier et al. (2001). The segments were transferred to the solution containing 1 mM CaCl_2 , 0.5% (w/v) polyvinylpyrrolidone, 0.5% (w/v) BSA, 0.8% (w/v) cellulase, 0.1% (w/v) pectolyase, 8 mM MES-KOH to pH 5.5, and 0.6 M sorbitol. They were incubated at 65 rpm for 60 min at 30°C . The same solution, but without pectolyase, was subsequently added, and another incubation followed for 90 min. The suspension was then filtered through a nylon mesh, and protoplasts were washed three times with washing solution (1 mM CaCl_2 , 5 mM MES/Tris, pH 5.5, and 0.6 M sorbitol). Washing solution was replaced with modified growth medium containing 10^{-4} M BFA, and protoplasts were treated for 2 h. For control, washing solution was replaced with growth medium without BFA.

Immunolabelling of protoplasts was done according to Swanson et al. (1998). Protoplasts were fixed with 4.5% (w/v) paraformaldehyde and 0.5% (v/v) glutaraldehyde for 60 min in PBS containing 0.6 M sorbitol. After washing, they were permeabilized with 0.2% (v/v) Triton X-100 for 30 min. The samples were then incubated with the following primary antibodies diluted in PBS and supplemented with 0.2% (w/v) BSA, JIM5, LM5, and RGII, each at 1:100 (w/v) dilution. After washing, the protoplasts were incubated for 1 h at room temperature with FITC-conjugated anti-rat (JIM5, LM5) and anti-rabbit IgGs (RGII), diluted 1:100 (w/v) in PBS containing 0.2% (w/v) BSA. The protoplasts were mounted using an anti-fade mounting medium containing *p*-phenylenediamine (Baluška et al., 1992). The images were taken using a confocal microscope (TCS 4D, Leica, Heidelberg).

ACKNOWLEDGMENTS

We thank the following colleagues for providing us with antibodies: Keith Roberts (JIM5 and JIM7), Paul J. Knox (JIM5, JIM7, MAC207, LM2, LM5, and LM7), Wolfgang Michalke (PM H^+ -ATPase), and Richard Napier (HDEL).

Received March 22, 2002; returned for revision April 22, 2002; accepted April 25, 2002.

LITERATURE CITED

- Baluška F, Jasik J, Edelmann HG, Salajová T, Volkmann D (2001) Latrunculin B-induced plant dwarfism: plant cell elongation is F-actin dependent. *Dev Biol* **231**: 113–124
- Baluška F, Parker JS, Barlow PW (1992) Specific patterns of cortical and endoplasmic microtubules associated with cell growth and tissue differentiation in roots of maize (*Zea mays* L.). *J Cell Sci* **103**: 191–200
- Baluška F, Vitha S, Barlow PW, Volkmann D (1997) Rearrangements of F-actin arrays in growing cells of intact maize root apex tissues: a major developmental switch occurs in the postmitotic transition region. *Eur J Cell Biol* **72**: 113–121
- Belanger KD, Quatrano RS (2000) Membrane recycling occurs during asymmetric tip growth and cell plate formation in *Fucus distichus* zygotes. *Protoplasma* **212**: 24–37
- Boevink P, Martin B, Oparka K, Santa Cruz S, Hawes C (1999) Transport of virally expressed green fluorescent protein through the secretory pathway in tobacco leaves is inhibited by cold shock and brefeldin A. *Planta* **208**: 392–400
- Boevink P, Oparka K, Santa Cruz S, Martin B, Betteridge A, Hawes C (1998) Stacks on tracks: The plant Golgi apparatus traffics on an actin/ER network. *Plant J* **15**: 441–447
- Bush MS, McCann MC (1999) Pectic epitopes are differentially distributed in the cell walls of potato (*Solanum tuberosum*) tubers. *Physiol Plant* **107**: 201–213
- Chen M-H, Sheng J, Hind G, Handa AK, Citovsky V (2000) Interaction between the tobacco mosaic virus movement protein and host cell pectin methylsterases is required for vital cell-to-cell movement. *EMBO J* **19**: 913–920
- Diekmann W, Herkt B, Low PS, Nürnberger T, Scheel D, Terschüren C, Robinson DG (1994) Visualization of elicitor-binding loci at the plant cell surface. *Planta* **195**: 126–137
- Doohan ME, Palevitz BA (1980) Microtubules and coated vesicles in guard-cell protoplasts of *Allium cepa* L. *Planta* **149**: 389–401
- Driouch A, Zhang GF, Staehelin LA (1993) Effect of brefeldin A on the structure of the Golgi apparatus and on the synthesis and secretion of proteins and polysaccharides in sycamore maple (*Acer pseudoplatanus*) suspension-cultured cells. *Plant Physiol* **101**: 1363–1373
- Emans N, Zimmermann S, Fischer R (2002) Uptake of a fluorescent marker in plant cells is sensitive to brefeldin A and wortmannin. *Plant Cell* **14**: 71–86
- Fitchette AC, Cabanes-Macheteau M, Marvin L, Martin B, Satiat-Jeuemaitre B, Gomord V, Crooks K, Lerouge P, Faye L, Hawes C (1999) Biosynthesis and immunolocalization of Lewis a-containing N-glycans in the plant cell. *Plant Physiol* **121**: 333–343
- Friml J, Wisniewska J, Benková E, Mendgen K, Palme K (2002) Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis*. *Nature* **415**: 806–809
- Fujiwara T, Oda K, Yokota S, Takatsuki A, Ikehara Y (1988) Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *J Biol Chem* **263**: 18545–18552
- Gaynor EC, Chen C-Y, Emr SD, Graham TR (1998) ARF is required for maintenance of yeast Golgi and endosome structure and function. *Mol Biol Cell* **9**: 653–670
- Geitmann A, Wojciechowicz K, Cresti M (1996) Inhibition of intracellular pectin transport in pollen tubes by monensin, brefeldin A and cytochalasin D. *Bot Acta* **109**: 373–381
- Geldner N, Friml J, Stierhof Y-D, Jürgens G, Palme K (2001) Auxin-transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature* **413**: 425–428
- Goubet F, Mohnen D (1999) Subcellular localization and topology of homogalacturonan methyltransferase in suspension-cultured *Nicotiana tabacum* cells. *Planta* **209**: 112–117

- Hawes CR, Brandizzi F, Andreeva AV (1999) Endomembranes and vesicle trafficking. *Curr Opin Plant Biol* 2: 454–461
- Henderson J, Satiat-Jeunemaitre B, Napier R, Hawes C (1994) Brefeldin A-induced disassembly of the Golgi apparatus is followed by disruption of the endoplasmic reticulum in plant cells. *J Exp Bot* 45: 1347–1351
- Höfte H (2001) A baroque residue in red wine. *Science* 294: 795–797
- Jahn Th, Baluška F, Michalke W, Harper J, Volkmann D (1998) Plasma membrane H⁺-ATPase in the root apex: evidence for strong expression in xylem parenchyma and asymmetric localization within cortical and epidermal cells. *Physiol Plant* 104: 311–316
- Jin JB, Kim YA, Kim SJ, Lee SH, Kim DH, Cheong G-W, Hwang I (2001) A new dynamin-like protein, ADL6, is involved in trafficking from the *trans*-Golgi network to the central vacuole in Arabidopsis. *Plant Cell* 13: 1511–1525
- Jones L, Seymour GB, Knox JP (1997) Localization of pectic galactan in tomato cell walls using a monoclonal antibody specific to (1→4)-β-D-galactan. *Plant Physiol* 113: 1405–1412
- Kartusch R, Lichtscheidl IK, Weidinger M-L (2000) Brefeldin A induces callose formation in onion inner epidermal cells. *Protoplasma* 212: 250–261
- Knox JP, Linstead PJ, King J, Cooper C, Roberts K (1990) Pectin esterification is spatially regulated both within cell walls and between developing tissues of root apices. *Planta* 181: 512–521
- Kobayashi M, Nakagawa H, Asaka T, Matoh T (1999) Borate-rhamnogalacturonan II bonding reinforced by Ca²⁺ retains pectic polysaccharides in higher-plant cell walls. *Plant Cell Physiol* 38: 676–683
- Kollmeier M, Dietrich P, Bauer CS, Horst WJ, Hedrich R (2001) Aluminium activates a citrate-permeable anion channel in the aluminium-sensitive zone of the maize root apex: a comparison between an aluminium-sensitive and an aluminium-resistant cultivar. *Plant Physiol* 126: 397–410
- Li Y, Yen L-F (2001) Plant Golgi-associated vesicles contain a novel α-actinin-like protein. *Eur J Cell Biol* 80: 703–710
- Low PS, Legendre L, Heinsteinst PF, Horn MA (1993) Comparison of elicitor and vitamin receptor-mediated endocytosis in cultured soybean cells. *J Exp Bot* 44: 269–274
- Matoh T, Takasaki M, Takabe K, Kobayashi M (1998) Immunocytochemistry of rhamnogalacturonan II in cell walls of higher plants. *Plant Cell Physiol* 39: 483–491
- Messiaen J, Read ND, Van Cutsem P, Trewavas AJ (1993) Cell wall oligogalacturonides increase cytosolic free calcium in carrot protoplasts. *J Cell Sci* 104: 365–371
- Miller SG, Carnell L, Moore HPH (1992) Post-Golgi membrane traffic: Brefeldin A inhibits export from distal Golgi compartments to the cell surface but not recycling. *J Cell Biol* 118: 267–283
- Mollet J-C, Park S-Y, Nothnagel EA, Lord EM (2000) A lily stylar pectin is necessary for pollen tube adhesion to an *in vitro* stylar matrix. *Plant Cell* 12: 1737–1749
- Napier RM, Fowke LC, Hawes C, Lewis M, Pelham HRB (1992) Immunological evidence that plants use both HDEL and KDEL for targeting proteins to the endoplasmic reticulum. *J Cell Sci* 102: 261–271
- O'Neill MA, Eberhard S, Albersheim P, Darvill AG (2001) Requirement of borate cross-linking of cell wall rhamnogalacturonan II for *Arabidopsis* growth. *Science* 294: 846–849
- Pimpl P, Movafeghi A, Coughlan S, Denecke J, Hillmer S, Robinson DG (2000) *In situ* localization and *in vitro* induction of plant COPI-coated vesicles. *Plant Cell* 12: 2219–2235
- Qualmann B, Kessels MM, Kelly RB (2000) Molecular links between endocytosis and the actin cytoskeleton. *J Cell Biol* 150: F111–F116
- Ritzenthaler C, Nebenführ A, Movafeghi A, Stussi-Garaud C, Behnia L, Pimpl P, Staehelin LA, Robinson DG (2002) Reevaluation of the effects of brefeldin A on plant cells using tobacco Bright Yellow 2 cells expressing Golgi-targeted green fluorescent protein and COPI antisera. *Plant Cell* 14: 237–261
- Robineau S, Chabre M, Antonny B (2000) Binding site of brefeldin A at the interface between small G protein ADP-ribosylation factor 1 (ARF1) and the nucleotide-exchange factor Sec7 domain. *Proc Natl Acad Sci USA* 97: 9913–9918
- Robinson DG, Hinz G, Holstein SEH (1998) The molecular characterization of transport vesicles. *Plant Mol Biol* 38: 49–76
- Rutten TLM, Knuiman B (1993) Brefeldin A effects on tobacco pollen tubes. *Eur J Cell Biol* 61: 247–255
- Saint-Jore CM, Evins J, Batoko H, Brandizzi F, Moore I, Hawes C (2002) Redistribution of membrane proteins between the Golgi apparatus and endoplasmic reticulum in plants is reversible and not dependent on cytoskeletal networks. *Plant J* 29: 661–678
- Šamaj J, Baluška F, Volkmann D (1998) Cell-specific expression of two arabinogalactan-protein epitopes recognized by monoclonal antibodies JIM8 and JIM13 in maize roots. *Protoplasma* 204: 1–12
- Šamaj J, Šamajová O, Peters M, Baluška F, Lichtscheidl I, Knox JP, Volkmann D (2000) Immunolocalization of LM2 arabinogalactan protein epitope associated with endomembranes of plant cells. *Protoplasma* 212: 186–196
- Saraste J, Palade GE, Farquhar MG (1987) Antibodies to rat pancreas Golgi subfractions: identification of 58-kD *cis*-Golgi protein. *J Cell Biol* 105: 2021–2029
- Satiat-Jeunemaitre B, Hawes C (1992) Redistribution of a Golgi glycoprotein in plant cells treated with brefeldin A. *J Cell Sci* 103: 1153–1166
- Satiat-Jeunemaitre B, Hawes C (1993) The distribution of secretory products in plant cells is affected by brefeldin A. *Cell Biol Int* 17: 183–193
- Schmohl N, Horst WJ (2000) Cell wall pectin content modulates aluminium sensitivity of *Zea mays* (L.) cells grown in suspension culture. *Plant Cell Environ* 23: 735–742
- Steinmann T, Geldner N, Grebe M, Mangold S, Jackson CL, Paris S, Gälweiler L, Palme K, Jürgens G (1999) Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. *Science* 286: 316–318
- Swanson SJ, Bethke PC, Jones RL (1998) Barley aleurone cells contain two types of vacuoles: characterization of lytic organelles by use of fluorescent probes. *Plant Cell* 10: 685–698
- Van Cutsem P, Messiaen J (1994) Biological effects of pectin fragments in plant cells. *Acta Bot Neerl* 43: 231–245
- Vesk PA, Vesk M, Gunning BES (1996) Field emission scanning electron microscopy of microtubular arrays in higher plant cells. *Protoplasma* 195: 168–182
- Wee EGT, Sherrier DJ, Prime TA, Dupree P (1998) Targeting of active sialyltransferase to the plant Golgi apparatus. *Plant Cell* 10: 1759–1768
- Wileman T, Harding C, Stahl P (1985) Receptor-mediated endocytosis. *Biochem J* 262: 1–14
- Willats WGT, Orfila C, Limberg G, Buchholt HC, van Alebeek GJWM, Voragen AGJ, Marcus SE, Christensen TMIE, Mikkelsen JD, Murray BS, Knox JP (2001) Modulation of the degree and pattern of methylesterification of pectic homogalacturonan in plant cell walls. Implications for pectin methyl esterase action, matrix properties, and cell adhesion. *J Biol Chem* 276: 19404–19413
- Yasuhara H, Sonobe S, Shibaoka H (1995) Effects of brefeldin A on the formation of the cell plate in tobacco BY-2 cells. *Eur J Cell Biol* 66: 274–281