Visualizing microtubule dynamics and membrane trafficking in live and dividing plant cells
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Citation for published version (APA):
Bulk Flow of Endocytic Material from the Plasma Membrane and Cell Wall towards the Cell Plate during Plant Cytokinesis

Chapter 5

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Manuscript under Preparation
ABSTRACT

Cytokinetic plant cells perform an amazing task of building a new cell wall within the cytoplasm in a matter of minutes that physically separates the daughter cells. During the last four decades, the Golgi Apparatus (GA)-based secretory pathway has been considered to be the sole source for this cell plate (primordial cell wall) formation. Strikingly, our real time live cell analysis together with immunolocalization studies reveal fast delivery of internalized plasma membrane (PM) / cell wall (CW) material for cell plate formation. The PM-inserted endocytic tracer FM4-64, CW/endocytic pectins cross-linked with boron and calcium, as well as two different fluid-phase endocytosis markers, all are delivered to the forming cell plates. In addition, co-localization studies using transformed cell lines also show that fusion of endosomes coincides and colocalizes with cell plate initiation in a GA-free zone. Furthermore, fluorescence recovery after photobleaching (FRAP) analysis indicates that endocytosis continues during lateral expansion of growing cell plates. Pharmacological studies and site-directed mutagenic analyses of the endosomal Rab GTPase Ara7 confirm the requirement of the endocytic pathway for the cell plate formation. These multiple independent lines of evidence suggest that besides delivery of GA-derived material, constitutive delivery of endocytic material in form of prefabricated PM/CW material is required for cell plate-formation during plant cytokinesis.
INTRODUCTION

Cytokinesis in immobile plants differs from other higher eukaryotes as a new plasma membrane (PM) and cell wall separating the daughter cells are formed by de novo synthesis within the cytoplasm rather than by inward growth or constriction of existing PM and cell wall (CW) enclosing all plant cells (Frey-Wyssling et al., 1964; Staehelin and Hepler, 1996; Verma, 2001). This process requires creation of 1/3 of the original cell surface area during 4% of the cell cycle duration, and hence demands extremely fast and targeted vesicular trafficking. It is generally assumed that Golgi Apparatus (GA)-derived vesicles are the sole source of the required material and that a plant-specific polarized microtubular array sandwiching the forming cell plate also containing actin, known as the phragmoplast, assists in their delivery (Ledbetter and Porter, 1963). This view of cell plate biogenesis is supported by electron microscopy (EM) observations showing massive accumulation of vesicles with a diameter of 60-80 nm (resembling transport and secretory vesicles) at the spindle and phragmoplast areas during mitosis (Otegui et al., 2001). Brefeldin A (BFA), an inhibitor of secretion, slows down the cell plate expansion (Lippincott-Schwartz et al., 1989; Yasuhara et al., 1995; Yasuhara and Shibaoaka, 2000).

However, there are several observations that cannot be easily reconciled with the notion that GA-derived exocytic vesicles alone drive the plant cytokinesis. First of all, BFA cannot inhibit cell plate initiation (Yasuhara et al., 1995; Yasuhara and Shibaoaka, 2000). Secondly, it is well known that the cell plate is initiated by (presumably homotypic) fusions between individual vesicles via finger-like projections (Staehelin and Hepler, 1996; Otegui et al., 2001). GA-based vesicles are designed to fuse with the PM for its expansion (Thiel and Battey, 1998) but to our knowledge have never been shown to accomplish homotypic fusion via finger-like projections; the latter rather is a property inherent to endosomes (Gorvel et al., 1991). Thirdly, although the early cell plate is almost exclusively a pectin-based structure (Matar and Catesson, 1988), antibodies recognizing GA-based pectins do not label cell plates (Moore and Staehelin, 1988; Samuels et al., 1995). Fourthly, EM tomography revealed the existence of at least two different kinds of vesicles during cell plate formation which could suggest the involvement of multiple membrane sources and trafficking pathways driving cell plate formation (Segui-Simarro et al., 2004).

In addition to these observations, recently there have been a number of studies that have shown that certain components found in cell plates can be endocytosed in interphase cells and that interfering with endocytic motifs on proteins or blocking of endocytic recycling routes affects the localization of key molecules on cell plates. For example, recently, we reported that cell wall pectins are internalized into endosomes in plant cells (Baluška et al., 2002). Furthermore, recent studies show that BFA also
interferes with endocytic recycling routes (Jürgens and Geldner, 2002; Geldner et al., 2003). Moreover, endosomal sorting compartments have been postulated to be involved in recycling superfluous compounds from growing cell plates (Jürgens and Pacher, 2003). Finally, Arabidopsis KORRIGAN containing an endocytic motif that is functionally conserved in many cell types has been shown to be targeted to the forming cell plates and with mutations in its endocytic motif, it was found mainly to be localized at the PM instead of the cell plates (Zuo et al., 2000). Combined, these new findings motivated us to further analyze both the sources of membranes and cell plate pectins as well as the process of endocytosis and cell plate formation in real time in live and dividing plant cells.

RESULTS

Internalization of endocytic and pinocytic markers into cytokinetic cell plates

In a first approach, the endocytic marker FM4-64 that has shown to be the most suitable marker for endocytosis in plants was employed for studying the dynamics of endocytosis during cytokinesis in real time (Emans et al., 2002; Shope et al., 2003; Geldner et al., 2003; Bolte et al., 2004; Meckel et al., 2004). FM4-64 was applied to a well-established dicot tobacco bright yellow-2 (BY-2) cell line, transformed with a GFP fusion protein labeling microtubules (MTs) in vivo, facilitating visualization of the cell cycle stage (Dhonukshe and Gadella, 2003). FM4-64 internalized in the mitotic BY-2 cells and accumulated within the spindle region within a few minutes after application (Fig. 1A). In telophase, it immediately occupied the newly available space between the separating chromosomes and labeled emerging cell plates extensively (even higher than the parental PM), since the very first signs of their appearance (Fig. 1A and Movie S1). Also during its expansion, fusion events of FM4-64 stained endomembrane compartments at the cell plate edges were observed suggesting continuous delivery of endocytosed material during cell plate expansion (Movie S1). Interestingly, FM4-64 was shown to also decorate the cell plates of Fucus zygotes (Belanger and Quatrano, 2000). The continuous delivery of endocytic FM4-64 during cell plate expansion was confirmed using fluorescence recovery after photobleaching (FRAP) analysis. After complete photobleaching of FM4-64 at already initiated cell plates, a fast reappearance of FM4-64 was observed at the cell plate in the bleached area (Fig. 4M and O and Movie S12). To investigate whether besides membrane material also extracellular fluid can be delivered to the cell plate by endocytosis, we used the membrane impermeant pinocytic fluid-phase markers Alexa 633 (Alexa) and Lucifer Yellow (LY). Strikingly, it was observed that both Alexa (Fig. 1B-C) and LY (Fig. 1D) internalized in dividing cells and markedly accumulated within the volume occupied by
Figure 1. Endocytic markers and cell wall associated pectin antibodies label forming cell plates.
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(A) Endocytic plasma membrane marker FM4-64 labeled cell plate from initiation till completion (Movie S1). Microtubules are in green, FM4-64 in red and time in minutes. Bar 5 μM. Note FM4-64-labelled dense vesicular mass in the spindle region gathers together coinciding and colocalizing with cell plate initiation.

(B) Endocytic fluid phase marker Alexa labeled the cell plate volume. (C) Cross-section of cell from B showing no attachment of cell plate to the parental cell wall. Microtubules are in green and Alexa in red. Bar 5 μM.

Internalized LY labeled (D) callosic (E) cell plates. LY is in green and Aniline blue in cyan. RGIII labeled early (J, L) and expanding (F, G) cell plates. JIM5 labeled early (K, M) and expanding (H) cell plates. JIM7 showing almost no labeling of early (N) and minute labeling of expanding (I) cell plates. Pectin antibodies are in green and DAPI in blue.

(O) JIM5 (red) and PIN1 (green) showing co-localization (yellow) on the cell plate.

Cell plates accumulate internalized cell wall pectins but not Golgi-derived secretory pectins

To investigate possible delivery of endocytosed pectic CW material to the forming cell plate, we performed a pectin-immunolocalization study using BY-2 suspension cells and monocot maize root apices. In contrast to mature plant cell walls, early cell plates are enriched in pectins, later also in callose, whereas they hardly contain cellulose (Matar and Catesson, 1988; Moore and Staehelin, 1988; Samuels et al., 1995). Recently, it has been reported that root cells internalize pectins from parental cell wall (Baluška et al., 2002). For the pectin-immunolocalization, we used different sets of antibodies specifically recognizing pectins found in mature cell walls and found in GA-derived vesicles. The first set of antibodies comprise rhamnogalacturonan II (RGII)-antibodies recognizing de novo formed RGII dimers cross-linked by borate diol diester (O’Neill et al., 2001) and JIM5-antibodies recognizing partially esterified (up to 40%) homogalacturonan pectins (Baluška et al., 2002). The GA-vesicle associated pectins were stained using JIM7-antibodies labeling esterified (up to 80%) homogalacturonan pectins. Remarkably, the forming cell plates in both maize and BY-2 cells were strongly labeled with the RGII- and JIM5- antibodies but only weakly with the JIM7 antibody (Fig. 1F-N). Interestingly, at the cell plate, the JIM5-positive signal co-localized with the PIN1 protein (Fig. 1O), which previously was shown to co-localize with plant cell plate-specific syntaxin KNOlle that is involved in cell plate formation (Geldner et al., 2001). The cross-linking of RGII pectins well known to be de novo process occurring exclusively within mature parental cell wall (Kobayashi et al., 1999; O’Neill et al., 2001), suggest that their localization at the cell plate reflects delivery of mature cell wall components by the endocytic pathway. Strong RGII and JIM5 labeling of
cell plates gets weaker as they mature and transform into young cell walls, suggesting recycling of these cross-linked pectins back to the endosomes/parent cell walls.

**GAs but not endosomes are excluded from spindle and early phragmoplast areas**

To further study the involvement of endocytic and exocytic pathways in cell plate formation, we used BY-2 cells transformed with GFP-Ara7, a Rab5 homolog labeling endosomes (Ueda et al., 2001), and/or ST-YFP labeling GAs (Nebenführ et al., 2000), with or without applying FM4-64. The authenticity of endosomal and GA-labeling of these cells was thoroughly investigated and confirmed in interphase cells using a variety of co-localization and drug studies (Fig. 2 and Movies S2-S5). Most striking evidence of this authenticity came from the bi-directional movement (Fig. 2B and Movie S2) and 'kiss and run'-like behavior (Fig. 2C and Movie S3) of GFP-Ara7 labeled endosomes, their colocalization with FM4-64 (Fig. 2D) and the effect of BFA (Fig. 2 E-G, K, W) and phosphatidylinositol-3-kinase (PI-3-kinase)-inhibitors (Fig. 2I-S) on endosome and GA morphology. Remarkably, transient physical interactions were observed between GAs and endosomes (Fig. 2 T-V, Movie S4 and S5). In dividing BY-2 cells during the spindle assembly, GAs remained around the spindle apparatus and never penetrated into its inside space (Fig. 3A, 3E and Movie S6) as also reported in a previous study (Nebenführ et al., 2000). In contrast to GAs, the FM4-64 and Ara7 labeled endosomes always distributed throughout the spindle area and their fusion coincided and colocalized with the cell plate initiation (Fig. 3C-Fand Movie S7 and S8). Once the cell plates were initiated, the GAs moved inwards towards the assembling cell plate (Movie S6 and S8) likely supplying the exocytic products needed for their expansion. During the cell plate expansion, FM4-64 or Ara7 labeled endosomal- and ST labeled GA-organization around the cell plate appeared more complex and interconnected (Fig. 3G and Movie S9) suggesting coupling of both exocytic and endocytic pathways in cell plate expansion.

**Inhibition of secretion via Brefeldin A does not prevent cell plate formation**

Unfortunately, due to the lack of specific in vivo GA-derived cytokinetic vesicle markers, we were unable to visualize the dynamical localization of such vesicles during mitosis that have been assumed to mediate cell plate formation. In order to gain more insight in the respective contributions of endosomal and GA-linked routes in cell plate initiation and subsequent lateral expansion, we used the potent inhibitor of anterograde secretory pathways BFA which is a reversible inhibitor of a subclass of GDP/GTP-exchange factors for small G-proteins of the ARF class, so called ARF-GEF (Peyroche et al., 1999),
Figure 2. Authenticity of endosomal and GA-labeling by various endomembrane markers.
Endocytosis Drives Cytokinesis in Plants

(A) GFP-Ara7 labeled endosomes.
(B) GFP-Ara7 labeled bidirectional endosomal trafficking (Movie S2).
(G) GFP-Ara7 labeled endosomal interactions exhibiting ‘Kiss and Run’ behavior in controls (Movie S3).
(D) Partial co-localization between FM4-64 (red) and GFP-Ara7 (green) labeled endosomes.
(BFA compartment formation induced by 50 µM BFA treatment for 30 min and visualized by GFP-Ara7 single labeled (E), GFP-Ara7 and FM4-64 dual labeled (F) and Ara7 and Alexa dual labeled endosomes (G).
(H-I) ST-YFP labeled GA (green) and FM4-64 labeled endosomes (red) showing minute overlap without extensive colocalization.
(J) ST-YFP labeled GA physical interactions in controls.
(K) ST-YFP labeled GA (green) and FM4-64 labeled endosomes (red) with 50 µM BFA for 30 min showing GA resorbed into ER and FM4-64 labeled endosomes forming BFA compartments.
(L) 10 µM wortmannin treatment for 30 min induces GFP-Ara7 (green) labeled endosomal fusions and enlargements exhibiting prolonged kisses. (M) 10 µM LY294002 for 30 min exhibiting similar effects. (N-O) close-up of endosomal double or triple fusions. Importantly, in mammalian cells treated with wortmannin (Vieira et al., 2003) and in Arabidopsis GNOM mutant cell lines (Geldner et al., 2003), similar blown-up and aggregated Ara7-labeled endosomal structures have been observed. (P) 10 µM wortmannin for 30 min causes similar FM4-64 labeled (red) endosomal enlargements and fusions. (Q) Wortmannin 10 µM for 30 min followed by BFA 50 µM for 30 min results into aggregation of GFP-Ara7 (green) labeled endosomes to initiate BFA compartments. (R) Close-up of cell in Q. (S) ST-YFP labeled GA with wortmannin 10 µM for 30 min showing no change in GA morphology.
(T) GFP-Ara7 labeled endosome (green) and ST-YFP labeled GA (red) localizations in interphase.
(U) GFP-Ara7 labeled endosomal (green) interactions with ST-YFP labeled GA (red) (Movie S4 and S5).
(V) GFP-Ara7 labeled endosomes (green) and ST-YFP labeled GA (red) follow the same tracks (Movie S5).
(W) Upon 50 µM BFA treatment for 30 min, ST-YFP labeled GA (red) reabsorb into ER and GFP-Ara7 labeled endosomal fusions (green) form BFA compartments. Scale bars, in C 1 µm; in B, J, N, O, R and V 2 µm; and in rest 5 µm.

interfering with the recruitment of vesicle coats necessary for vesicle budding and cargo selection (Donaldson and Jackson, 2000). In both mammals and plants one of the first effects of BFA is the loss of COPI-coats from GAs, leading to a breakdown of the physical separation between ER and Golgi, effectively shutting down the secretory pathway within minutes after application (Nebenführ et al., 2002). We treated BY-2 cells with BFA before, after or together with FM4-64. In the BFA-pre-treated cells, FM4-64 still internalized, although less substantially. Still in these cells, both FM4-64 and CW/endocytic pectins, labeled cell plates that were not yet attached to the parental PM (Fig. 4A-B and G-H) but with reduced intensities as compared to non BFA-treated cells (compare Fig. 1A and 4A and Fig. 1G-M and Fig. 4 G-H). In the BFA-pretreated BY-2 cells, the internalized FM4-64 (Fig. 4A), but also RGII-pectins (Fig 4G, H) rarely appeared within individual BFA compartments, suggesting a direct route of endocytic material to the cell plate. In contrast, when BFA was applied to BY-2 cells pretreated with
Figure 3. Endosomes accumulate within the spindle area and fuse to mark the cell plate initiation

(A) ST-YFP labeled GA (green) and FM4-64 labeled endosomes (red) during spindle. Note that GAs remain around the spindle and FM4-64 labeled endosomes occupy the inside space.

(B) FM4-64 labeled endosomes (red) and ST-YFP labeled GA (green) during cell plate initiation (Movie S6).
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GFP-Ara7 (green) and FM4-64 (red) labeled endosomes during spindle (C) and cell plate initiation (D) (Movie S7). Note: FM4-64 and Ara7 stained endosomal fusions coinciding and colocalizing with cell plate initiation.

GFP-Ara7 labeled endosomal (green), ST-YFP labeled GA (blue) and FM4-64 labeled endosomal (red) localizations during spindle (E) and cell plate initiation (F) (Movie S8).

(G) Close-up view of one side of an expanding cell plate with the above triple markers revealing complex interactions between GAs, endosomes and cell plate during its expansion (Movie S9).

Scale bar in G 3 μm, in rest 5 μm.

FM4-64, the internalized FM4-64 labeled large endomembrane aggregates resembling BFA compartments. Remarkably, these aggregates were observed to both participate in cell plate initiation (Fig. 4C and Movie S10) and in the expansion of already initiated cell plates (Fig. 4D-E). When FM4-64 and BFA were applied together during metaphase, the GA-marker was absorbed into the ER, as shown also by others (Ritzenthaler et al., 2002) and FM4-64 decorated cell plates were still initiated (Fig. 4F and Movie S11). Afterwards, as expected, the cell plate expansion slowed down (Yasuhsara et al., 1995; Yasuhara and Shibooka, 2000). In dividing maize root cells, BFA-treatment of cytokinetic cells induced co-localization of CW/endocytic pectins with PIN1 in form of large blobs at the expanding peripheries of the plates resulting in 'cell plate- edge BFA compartments' (Fig. 4I, J). In BFA-treated interphase maize cells, pectin co-localized with PIN1 but not with the GA-marker (β-COP-antibody) (Fig. 4K, L). We quantified the inhibitory effects of BFA on cell plate expansion by performing FRAP analysis of initiated cell plates labeled by FM4-64 in the presence and absence of BFA. Clearly, the FRAP experiments indicate continuous delivery of internalized material in both situations and a partial inhibition of endocytosis by BFA. (Fig. 4M-O and Movies S12, S13).

**Inhibition of protein synthesis does not prevent cell plate formation**

Besides using BFA to block the exocytic pathway, we used cycloheximide to investigate whether internalized material is sufficient to initiate cell plates in the absence of protein synthesis. In shorter cycloheximide pre-incubations (30 min, 1 h), it was found that cell plates could be initiated but upon longer cycloheximide incubations chromosome separation and initiation of the phragmoplast and cell plate were progressively slowed down (Fig 4Q and Movie S14). In similar treatments in maize root cells, RGII-based CW pectins could still label the forming cell plates (Fig. 4P) suggesting their internalization from the parental CW. In prolonged period of treatments (2h or more), the mitotic cells became arrested in the spindle stage. Hence, cell plate formation cannot be studied under prolonged cycloheximide treatments as it obviously interferes with spindle checkpoints. Even in the simultaneous presence of BFA and cycloheximide for 30 min (before adding

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Figure 4. Effects of BFA and cycloheximide on endocytosis and cell plate formation
BY-2 cells (A-H and M-O and Q) and maize root epidermal cells (I-L and P).
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(A-B) FM4-64 (red) internalization and targeting to cell plate after 30 min 50 μM BFA pre-treatment. (B) Cross-section of cell in A.

(C) FM4-64 application for 30 min and subsequent application of 50 μM BFA for 30 min causes FM4-64-stained and internalized membranes (red) to aggregate and initiate the cell plate (Movie S10). (D-E) Same as in C, showing incorporation of FM4-64-stained membrane-aggregates into the cell plate periphery for its expansion. Arrows show BFA compartments. Note GA (green) absorbed into ER that is present at the cell plate.

(F) FM4-64 and 50 μM BFA combined application for 30 min causing GA absorption into ER and FM4-64-stained aggregates participating in cell plate initiation (Movie S11).

(G) RGII (green) and (H) JIM5 (green) cell plate localization in presence of 50 μM BFA for 30 min.

(I) RGII localization (green) and (J) JIM5 (red)-PIN1 (green) co-localization on cell plates and its expanding peripheries.

(K) JIM5 (red) and PIN1 (green) co-localization in BFA compartments.

(L) JIM5 (red) and β-COP (green) localization in presence of 50 μM BFA for 30 min.

Scale bars 5 μm.

FRAP (Movie S12 and S13) of FM4-64 (red) in control (M) and in 50 μM BFA for 30 min pre-treated (N) cells.

(O) Graph representing averages of percentage fluorescence recovery. (P) RGII (green) internalizes in presence of cycloheximide for 2 hr and labels the forming cell plate.

(Q) FM4-64 (red) internalization and labeling to the initiating cell plates in the continuous presence of cycloheximide (1 hr start to 2 hr end). Note that the cell takes more time to initiate the cell plate (Movie 14).

Scale bars in M 6 μm, in N 8 μm and in rest 5 μm. Time is indicated in M and N in seconds and in Q in min.

FM4-64, endocytosis occurred and cell plates were initiated (data not shown), suggesting that the cell plate initiation does not critically depend on intact secretory machinery, supporting the above BFA experiments.

Dominant negative Ara7 Rab GTPase inhibits cell plate formation

Ara7 has previously been shown to be specifically upregulated in Arabidopsis suspension cells during mitosis (Ueda et al., 2001). To test its effect on endocytosis and cell plate formation, we made dominant negative (GDP-stabilized state) versions of Ara7 by either replacing S24N (Stenmark et al., 1994) or S25N. We introduced these mutant Ara7-con structs into cowpea protoplasts by transfections and into BY-2 cells by transformations. In both systems, the mutant Ara7 appeared more in the cytosol than on endosomes (Fig. 5B, H), FM4-internalization through endocytosis was reduced (Fig. 5E-F and K-L), and formation of BFA compartments was less pronounced (Fig. 5C-D and I-J). Interestingly, in cells with low expression levels of dominant negative Ara7, the speed of cell plate formation was reduced (Fig. 5M) without affecting GA morphology or dynamics (data not shown). Upon overexpression of dominant negative Ara7, the cell plate initiation was blocked in
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Figure 5. Effects of dominant negative Ara7 on endocytosis, BFA compartment and cell plate formation

Cowpea protoplasts (A-F) and BY-2 cells (G-M)

(A, G) wild type GFP-Ara7. (B, H) dominant negative GFP-Ara7. Note that wild type GFP-Ara7 labels endosomes while the dominant negative GFP-Ara7 remains mostly cytosolic. 50 μM BFA for 30 min treated cells, expressing wild type GFP-Ara7 form aggregates (C, I) while in dominant negative GFP-Ara7 (D, J) the formation of BFA-induced aggregates is less pronounced.

FM4-64 (red) internalization in cells expressing wild type (E, K) and dominant negative (F, L) GFP-Ara7 (green). Note that in cells expressing wild type GFP-Ara7, FM4-64 has internalized significantly, labeling most of the endosomes also labeled by GFP-Ara7. In cells expressing dominant negative GFP-Ara7 its internalization is severely slowed down.

(M) Slow cell plate formation in cells expressing dominant negative GFP-Ara7 (green) and applied with FM4-64 (red).

Bars 5 μm and time in minutes

mitotic cells and attempts to isolate Arabidopsis plants transformed with dominant negative Ara7 failed whereas control transformations were successful (data not shown). In addition to this site-directed mutagenic manipulation of the endocytic pathway, wortmannin treatment, affecting endosome morphology (Vieira et al., 2003 and this study) also slowed down the cell plate expansion (see Table 1 for all pharmacological treatments). Furthermore, in the presence of endocytic inhibitory sodium azide (SA) and
cold treatments, no cell plates labeled with FM4-64 or Alexa dyes were observed (data not shown). Together, these experiments suggest participation and a functional role of Ara7 and endocytosis in cell plate formation.

### Table 1. Summary of the effect of drugs on the cell plate and BFA compartment formation

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target</th>
<th>Conc. used</th>
<th>Duration</th>
<th>Chromosome alignment</th>
<th>Chromosome separation</th>
<th>Cell plate initiation</th>
<th>Cell plate expansion</th>
<th>BFA compartment formation</th>
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<tr>
<td>Brefeldin A (BFA)</td>
<td>Secretion</td>
<td>50 µM</td>
<td>30 min</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>Slow down</td>
<td>forms</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 hr</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>Slow down</td>
<td>forms</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 hr</td>
<td>No effect</td>
<td>slight effect</td>
<td>slight effect</td>
<td>Slow down</td>
<td>forms</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30 min</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>Slight slow down</td>
<td>forms</td>
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<tr>
<td>Cycloheximide</td>
<td>Protein synthesis</td>
<td>50 µM</td>
<td>1 hr</td>
<td>Partially disorganized</td>
<td>prolonged</td>
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<td>prolonged</td>
<td>forms</td>
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<tr>
<td></td>
<td></td>
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<td>2 hr</td>
<td>disrupted</td>
<td>blocked</td>
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<td>forms</td>
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<tr>
<td>BFA + Cycloheximide</td>
<td>Protein synthesis + secretion</td>
<td>50 µM + 50 µM</td>
<td>30 min</td>
<td>No effect</td>
<td>No effect</td>
<td>No effect</td>
<td>Slow down</td>
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<td>Wortmannin</td>
<td>PI-3 Kinase</td>
<td>10 µM</td>
<td>30 min</td>
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**DISCUSSION**

Our combined results clearly document a new endocytic route participating in cell plate formation since i) the endocytic membrane marker FM4-64, ii) two different fluid-phase markers, iii) cross-linked CW pectins, all are internalized from the PM/CW and rapidly accumulate in cell plates during plant cytokinesis. Moreover, blocking or inhibition of endocytosis via cold treatment, sodium azide, wortmannin, or genetically by expression of dominant negative Ara7, slows down or even abolishes completely both the cell plate initiation and expansion. The *in vivo* colocalization, BFA-experiments and FRAP analysis support the involvement of endocytic route(s) and point out complex interactions between exocytic and endocytic pathways. The primary action of BFA in plants is the splitting of
the GAs into two parts: cis, medial and part of trans stacks are reabsorbed into the ER (as also visualized here using the trans-stack localized ST-GFP marker), whereas the trans GA network (TGN) and components of the endocytic pathway aggregate into BFA compartments (Baluška et al., 2002; Nebenführ et al., 2002; Geldner et al., 2001, 2003). These BFA-compartments indicate a trafficking connection between the TGN and endosomes in plant cells. This connection is also important for delivery of enzymes from the GAs to post-endosomal compartments such as lysosomes or vacuoles (Surpin and Raikhel, 2004). The TGN-endosomal connection can be very direct as shown by our in vivo observations of transient physical interactions between GAs and endosomes (Movie S4 and S5). Most likely, the trapping of the cytokinesis-specific syntaxin KNOLLE within BFA compartments (Geldner et al., 2001) is a consequence of BFA interfering with this trafficking connection. PM-resident proteins such as AUX1, PIN1, PIN3, PM H+-ATPase, as well as CW pectins (Baluška et al., 2002; Nebenführ et al., 2002; Friml et al., 2002; Grebe et al., 2002; Geldner et al., 2001, 2003), all accumulate within BFA compartments, which feed the cell plate expansion. This suggests that the primary mode of action of BFA is to interfere with the vesicular recycling of endocytic material back to the PM. To make the picture even more complex, there is evidence that during cell plate expansion approximately 75% of the membrane (Otegui et al., 2001; Nebenführ et al., 2002) as well as cell wall pectins (this study) initially delivered to the cell plate are recycled back to endomembranes/parent cell walls possibly by direct involvement of recycling endosomes. Also this recycling process can be a target for BFA. The presence of multiple targets for BFA in plant vesicular trafficking is supported by sequence analysis of Arabidopsis ARF-GEFs of which 5 out of 8 are predicted to be BFA-sensitive (Jürgens and Geldner, 2002). One of these BFA-targets is GNOM which has been shown to be required for PIN1-recycling between endosomes and the PM (Geldner et al., 2003). Remarkably, GNOM/EMB30 mutant lines also show defects in distribution of cell wall pectins (Shevell et al., 2000). Interestingly, GNOM is not involved in KNOLLE trafficking, further supporting the involvement of multiple BFA-targets in the formation of BFA-compartments and cell plates (Jürgens and Geldner, 2002). In addition, our FRAP-experiments indicate the presence of both a BFA-insensitive and a BFA-sensitive component in the endocytic route delivering PM material to growing cell plates. The inhibition of the endocytic PM-to-cell plate route by BFA can be direct by affecting those ARF-GEFs, which are involved in plant endocytosis. Since exocytosis and endocytosis need to be balanced at the PM in order to keep its surface area constant, endocytosis can also be indirectly impaired by inhibition of exocytosis. This can be accomplished either by inhibiting direct secretion from GAs to the PM, or by inhibiting the exocytic pathway from TGN through endosomes to the PM. This last endosome-to-PM route has been shown to be BFA sensitive (Jürgens and Geldner, 2002; see our model in Fig. 6).
Our discovery of a new endocytic route in cell plate formation during plant cytokinesis has several implications. Firstly, the differential and dynamic localization of NSPN11, SNARE which interacts with KNOLLE (Zheng et al., 2002), KORRIGAN which is endo-1,4-beta glucanase (Zuo et al., 2000), the dynamin ADL.1A (Kang et al., 2003); but also of PIN1 and KNOLLE in PMs, endosomes, cell plates and BFA-compartment has to be considered in a new perspective of multiple players taking the direct endocytic PM to cell plate route involving endosomal compartments. This would explain the until now perplexing fact that many of these proteins have endocytic sorting motifs. Importantly, this endocytic sorting motifs targets KORRIGAN to cell plate (Zuo et al., 2000) and it was reported that KORRIGAN is localized in intracellular compartments excluding GA (Molhoj et al., 2002).

Secondly, considering the extraordinarily fast speed of the cell plate formation, the use of endosomes enriched with fully matured cell wall pectins and PM-components providing pre-fabricated building blocks (e.g. cell wall pectins, lipids, PM receptors etc.) is
highly efficient. This recycling of 'old' PM components also implies that strictly spoken the cell plate is not entirely de novo synthesized. Besides speeding up the maturation of the cell plate by delivery of fully matured proteins, we propose that the endocytosed PM-components could also be instrumental in defining the nature of the cell plate membrane as a future PM. Once this future destination is established, it might speed up direct fusion with GA-based vesicles (bypassing the endosomal compartment) as well, resulting in GA-mediated 'intracellular cell plate targeted exocytosis' for its fast lateral growth (see our model in Fig. 6).

Thirdly, many aspects of the cell plate-formation in plants display homology to endosomal recycling pathways in mammalian cells; especially those that have been implicated in calcium-regulated PM-repair of torn cell periphery (McNeil and Steinhardt, 2003), and those involved in generating secretory lysosomes (Blott and Griffiths, 2002). In this view, the cell plate can be regarded as some sort of a specialized endosomal compartment which receives material from GAs directly as well as from the PM through sorting and recycling endosomes both of which also receive material from the TGN (see our model in Fig. 6). In addition, the partially BFA-insensitive PM-to cell plate endocytic route also shows similarity to transcytosis observed in mammalian polarized epithelial cells, particularly if one considers the cytokinetic cell plate as a new 'extracellular' space constructed within the parent cell. Future studies aimed at molecular and mechanistic understanding of plant cytokinesis will require concentrate efforts of several laboratories. It will be critical to dissect BFA-sensitivity of the molecular components responsible for the different arrows in our working model. This will definitely put more light on the quest of our understanding of plant cytokinesis, especially of its uniqueness and similarities with other cell systems.

METHODS

Plant material and growth conditions

Maize roots were obtained as described before (Baluška et al., 2002). Tobacco BY-2 cells were cultured and transformed as reported previously (Dhonukshe and Gadella, 2003). Cowpea plants were grown, protoplasts were isolated and transfected as described before (Dhonukshe and Gadella, 2003).

Construction of reporter genes

Construction of GFP-MAP4 was described before (Dhonukshe and Gadella, 2003). In short, GFP-Ara7 in vector pBSIKS+ was excised with HindIII-Xbal and sub-cloned into
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binary vector pBINPLUS. STtmd-YFP in vector pMON was digested with PstI-Smal and cloned into binary vector pCAMBIA 1390. For construction of double reporter gene GFP-ARA7 were excised from vector pBSIIKS+ with HindIII + NotI and STtmd-YFP was excised from vector pMON with NotI-Smal and triple ligated in binary vector pCAMBIA 1390 by using HindIII and Smal restriction sites. Dominant negative versions of Ara7 were made by site directed mutagenesis to replace either S24N or S25N in vector pBSIIKS+. The primers for S24N were TTGGTGCTGGAAAAATAGTCTTGTTGTTACGG/CCTGAAACACACGACTATTTTTCGCAGCACCAAC and primers for S25N were GGTGCTGGAAAATCAAATCTTGTTACGG/CCTGAAACACACGACTATTTTTCGCAGCACCAAC. The mutagenesis was confirmed by sequencing the positive clones. The mutant Ara7 versions were transferred to vector pBINPLUS using HindIII-XbaI.

Fluorescent dyes and inhibitor treatments
FM4-64 (Molecular probes) dissolved in water was applied at 2 μM final concentration for 5 min to the BY-2 cells, cells were washed with BY-2 medium to remove excess dye and were observed immediately. Alexa 633 (Molecular probes; Catalog No. A30634) dissolved in water was applied at 2 μM final concentration and cells were observed immediately. BFA (Sigma), wortmannin (Sigma), LY29402 (Sigma), cycloheximide (Sigma) were used from DMSO dissolved stock solutions and applied to cells at final concentrations of 50 μM, 10 μM, 10 μM and 50 μM respectively, for indicated periods. LY (Sigma) was used at 1% concentration. Sodium Azide (SA) (Sigma) was diluted in water (100 μM). Aniline blue dissolved in Gly/NaOH buffer (pH 9.5) was used at (0.1% (w/v).

Immunofluorescence microscopy
Roots were fixed and processed as described before (Baluška et al., 2002). BY-2 cells were fixed with a mixture of 0.5% (v/v) glutaraldehyde and formaldehyde 1.6% (v/v) in phosphate-buffered saline (PBS) buffer for 15 min. After two washes with PBS the cells were incubated overnight in 4 ml of 0.5% (w/v) NaBH₄ to reduce the autofluorescence of the fixative. The cells were then washed twice with PBS and cell wall was partially digested with 1% (w/v) cellulase in PBS for 30 min. Following two washes with PBS, the cells were allowed to settle onto the multi-well slides coated with 0.1% (w/v) polyethyleneimine. Membranes were permeabilised with 1% (v/v) Triton X-100 in PBS for 15 min. The cells were then washed with PBS and incubated overnight at room temperature with primary antibodies raised against RGII-B-RGII (two
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rhamnogalacturonan II molecules cross-linked with boron) epitope diluted with PBS (1:100), JIM5 and JIM7 epitopes diluted with PBS (1:200), PIN1 epitope diluted with PBS (1:100), β-COP epitope diluted with PBS (1:100) and supplemented with 0.1% (w/v) BSA. Afterwards, the cells were washed with PBS and incubated for 3 hrs at 37°C with anti-rabbit (RGII, PIN1), anti-rat (JIM5, JIM7), and anti-mouse (β-COP) secondary antibodies diluted with PBS (1:100) supplemented with 0.1% BSA, the cells were washed with PBS and stained with DAPI to visualize nuclei. After the final wash the cells were mounted with 0.1% p-paraphenylenediamine containing mounting medium. The cells were then viewed with Zeiss Axiovert 405M microscope equipped with Zeiss AxioCam HR digital camera using 40x objective. Images were captured with Axiovision 3.1 software and processed with Adobe Photoshop 7.0. All the experiments were repeated at least 3 times.

Live cell analysis

For live cell analysis the Zeiss CLSM510 system implemented on an inverted (Axiover t100) microscope was used. The microscopy system, sample preparation, single wavelength scanning, image processing and movie generation was described before (Dhonukshe and Gadella, 2003). Dual and triple color imaging was performed using dual or triple excitation/emission scanning in multitracking mode, respectively. For GFP/ FM4-64 dual scanning, we used excitation/emission combinations of 488 nm/ BP 505-550 for GFP and 543 nm/ LP585 for FM4-64 in combination with the HFT 488/543 primary and NFT545 secondary dichroic splitters. For YFP/ FM4-64 dual scanning, the same settings were used. For GFP/ Alexa dual scanning, we used excitation/emission combinations of 488 nm/ BP 505-550 for GFP and 633 nm/ LP650 for Alexa in combination with the HFT UV/488/543/633 primary and NFT545 secondary dichroic splitters. For GFP /YFP dual scanning, we used excitation/emission combinations of 458 nm/ BP 475-525 for GFP and 514 nm/ BP 530-600 for YFP in combination with the HFT 458/514 primary and NFT515 secondary dichroic splitters. For GFP /YFP/FM4-64 triple scanning, we used excitation/emission combinations of 458 nm/ BP 475-525 for GFP, 514 nm/ BP 530-600 for YFP and 543 nm/ LP650 for FM4-64 in combination with the 80/20 primary, NFT635 secondary, and NFT 515 tertiary dichroic splitters. All filers were from Zeiss. With the last two settings there is marginal bleed through of YFP fluorescence into the GFP channel and no bleed through *vice-versa.* For time-lapse analysis, images were obtained at 1-10 sec time intervals. All the experiments were repeated 3-5 times.
**Fluorescence recovery after Photobleaching (FRAP) analysis**

FRAP experiments were performed on the initiating cell plates that are not attached from any side to the parental plasma membrane to remove the possibility of direct FM flow from the parental plasma membrane to the cell plate membrane. FRAP experiments were performed using 100% laser power of both 488 and 543 nm laser lines on 16 μm × 8 μm rectangle in 4.5 sec to obtain complete photo bleaching of FM-labeled cell plates. 10 images were taken before photobleaching. After photobleaching, images were acquired at 0.8 s time intervals using 1% of 488 and 30% of 543 laser lines in a multitrack mode. The same microscope settings were used for different cells. The average pixel intensity values from the photo-bleached areas were normalized to the average initial fluorescence values. The graphs were assembled using Microsoft Excel and represent averages of the recovery percentages obtained from multiple experiments.

**ACKNOWLEDGEMENTS**

We thank Toru Matoh (Kyoto Univ., Japan) for RGII antibody, Klaus Palme (MPI Cologne, Germany) for PIN1 antibody, Akihiko Nakano (RIKEN, Saitama, Japan) for GFP-Ara7 construct and J. Carette (Wageningen Univ., the Netherlands) for ST-YFP construct. P.D. and T.W.J.G. were supported by NWO FOM-ALW 805.47.012 and by NWO van der Leeuw 835.25.004; F.B., A.H. and M.S. were supported by the European Commission Human Potential Programme (HPRN-CT-2002-00265).

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Supplementary movies can be found online at http://wwwmc.bio.uva.nl once the manuscript is published.