Synthetic Lipid (DOPG) Vesicles Accumulate in the Cell Plate Region But Do Not Fuse\[W][OA]

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The cell plate is the new cell wall, with bordering plasma membrane, that is formed between two daughter cells in plants, and it is formed by fusion of vesicles (approximately 60 nm). To start to determine physical properties of cell plate forming vesicles for their transport through the phragmoplast, and fusion with each other, we microinjected fluorescent synthetic lipid vesicles that were made of 1,2-dioleoyl-sn-glycerol-3-phospho-ethanolamine (DOPG) into Tradescantia virginiana stamen hair cells. During interphase, the 60-nm wide DOPG vesicles moved inside the cytoplasm comparably to organelles. During cytokinesis, they were transported through the phragmoplast and accumulated in the cell plate region together with the endogenous vesicles, even inside the central cell plate region. Because at this stage microtubules are virtually absent from that region, while actin filaments are present, actin filaments may have a role in the transport of vesicles toward the cell plate. Unlike the endogenous vesicles, the synthetic DOPG vesicles did not fuse with the developing cell plate. Instead, they redistributed into the cytoplasm of the daughter cells upon completion of cytokinesis. Because the redistribution of the vesicles occurs when actin filaments disappear from the phragmoplast, actin filaments may be involved in keeping the vesicles inside the developing cell plate region.

In plant cells, the cell plate constitutes the new cell wall with plasma membranes that separates the cytoplasm of the two daughter cells during cytokinesis. It is formed by the fusion of membrane vesicles of approximately 60 nm in diameter that contain a variety of hemicelluloses and pectins, and have callose and cellulose synthesizing enzyme complexes in their membrane (Zuo et al., 2000; Verma, 2001; Yokoyama and Nishitani, 2001).

The cell plate is built up in the middle of a structure called the phragmoplast, often at an equatorial plane in the cell. The phragmoplast is a cytoplasmic dense area containing microtubules, actin filaments, endoplasmic reticulum (ER), and cell plate forming vesicles (Schopfer and Hepler, 1991; Samuels et al., 1995; Staehelin and Hepler, 1996; Segui-Simarro et al., 2004; Jürgens, 2005). Other organelles, such as the Golgi bodies, mitochondria, and the vacuole stay outside the phragmoplast. The phragmoplast is initiated at late anaphase from the antiparallel overlapping microtubule and actin filament arrays at the spindle midzone, the plus-ends of cytoskeleton polymers facing the accumulating vesicles that form the cell plate (Kakimoto and Shibaoka, 1988; Baskin and Cande, 1990; Zhang et al., 1990, 1993; Wick, 1991; Cleary et al., 1992; Hepler et al., 1993; Sano et al., 2005). During expansion of the cell plate, microtubules disappear from the center of the phragmoplast as soon as a cell plate has formed (Zhang et al., 1990, 1993; Cleary et al., 1992; Hepler et al., 1993; Granger and Cyr, 2000; Ueda et al., 2003), whereas actin filaments become shorter but remain present in the whole area throughout cell plate formation (Hepler et al., 1993; Zhang et al., 1993).

The process of cell plate formation with its intermediate stages is well studied (Staehelin and Hepler, 1996; Verma, 2001; Jürgens, 2005). The vesicle fusion in the middle of the phragmoplast is mediated by tethering SNARE complexes (Waizenegger et al., 2000) in a region that has been termed the cell plate assembly matrix (CPAM) in electron tomography studies (Otegui et al., 2001; Segui-Simarro et al., 2004).

With the help of dynamin-like molecules, the fusion of vesicles leads to the formation of membrane fusion tubules with a diameter of 20 nm (Samuels et al., 1995; Gu and Verma, 1996). The fusion tubes fuse with other vesicles and form the tubulo-vesicular network that is

\textsuperscript{1} This work was supported by the FOM Institute for Atomic and Molecular Physics, Amsterdam (to A.M.C.E.).

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\textsuperscript{[W]} The online version of this article contains Web-only data.

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www.plantphysiol.org/cgi/doi/10.1104/pp.108.119842
transformed into a tubular network and later into a fenestrated membrane sheet. This transformation involves the removal of excess membrane via clathrin-coated vesicles and the deposition of callose (Samuels et al., 1995; Otegui et al., 2001; Seguí-Simarro et al., 2004). The cell plate grows centrifugally toward the cell borders due to the fusion processes of later arriving vesicles, until it attaches to the plasma membrane and cell wall of the mother cell (Staehelin and Hepler, 1996).

For years, it was believed that cell plate forming vesicles are only Golgi derived (Whaley and Mollenhauer, 1963), but recently it was proposed that endocytosis was involved in this process (Dhonukshe et al., 2006). However, experiments in which specific trafficking blocking drugs were used in combination with visualization of cytokinesis-specific proteins have clearly confirmed the Golgi nature of the cell plate forming vesicles and shown that endocytosis is not involved in cell plate formation (Reichardt et al., 2007). For the mechanism of vesicle transport through the phragmoplast it has been suggested that phragmoplast microtubules are directly responsible and that actin-myosin plays a role in the proper attachment of the cell plate to the parental cell wall (Otegui et al., 2001; Molchan et al., 2002). However, injection of the actin-binding protein profilin strongly suggested that actin filaments in the phragmoplast are also crucial for transport of cell plate forming vesicles to the cell plate (Valster et al., 1997). Apart from being transport vehicles, the tight array of microtubules and actin filaments in the phragmoplast might also act as a sieve, allowing only the right-sized vesicles to move into the region where fusion occurs, and act as a scaffold for the forming cell plate.

Here, we describe results obtained with the injection of synthetic phospholipid vesicles into plant cells to start to determine possible necessary and sufficient physical properties of cell plate forming vesicles for their transport through the phragmoplast and for their fusion with each other and with the existing cell plate. We used phospholipid vesicles made of 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DOPG) that partly mimic the behavior of endogenous vesicles during cell plate formation. We show that upon injection, DOPG vesicles are transported to and through the phragmoplast and accumulate in the region of the existing growing cell plate. However, unlike endogenous vesicles, synthetic vesicles redistribute into the cytoplasm of the daughter cells upon completion of cytokinesis. This suggests that the phragmoplast is not selective for lipid membrane composition or for integral membrane proteins. Interestingly, the synthetic vesicles are kept in the cell plate region during its formation. In this process the CPAM could be involved. Because vesicles keep moving to the center of the cell plate during late telophase when the microtubules have left that area but actin filaments are still present, we envision a role for the actin cytoskeleton in this process.

RESULTS

Synthetic Lipid Vesicles (DOPG) Distribute throughout the Cytoplasm of Interphase Tradescantia virginiana Stamen Hair Cells

We made synthetic vesicles from negatively charged (anionic) DOPG, labeled with 2% fluorescent phosphocholine Bodipy FC12-HPC, with a size of 60 nm (±7 nm; Fig. 1) to study physical properties of the vesicles that are required for transport through the phragmoplast and the formation of the cell plate. The vesicles did not fuse or aggregate with each other, even after 7 d in the buffer used for microinjection (data not shown). Synthetic lipid (DOPG) vesicles injected into young elongating interphase stamen hair cells of T. virginiana distributed evenly in the cytoplasm within 5 to 10 min after microinjection and were visible as individual fluorescent speckles (Fig. 2, A and B). The injection of these vesicles was not lethal to the cells, nor did it affect cytoplasmic streaming or the cytoarchitecture (Fig. 2A; differential interference contrast [DIC] images). Inside the cells, the vesicles also seemed to be stable because their fluorescence was not incorporated into the plasma membrane, tonoplast, ER, or other organelles (mitochondria and plastids) that can be seen with DIC microscopy. In addition, the vesicles did not fuse together in the cytoplasm, as no obvious conglomerates were formed and the number of speckles did not markedly drop over time. Microinjected fluorescent vesicles moved through the cytoplasm for at least 1.5 h after injection.

The average velocity of vesicles in the cytoplasm was 0.74 ± 0.04 (SE) μm/s, which is comparable to, but consistently slower than, that of visible organelles with a size of approximately 1 to 2 μm in these cells (1.12 ± 0.06 [SE] μm/s) and in cells that were not injected (1.24 ± 0.12 [SE] μm/s; Fig. 2C). The movement of DOPG vesicles and organelles in injected cells was
similar (Supplemental Fig. S1). This suggests that the injected vesicles may be coated with motor proteins and move on cytoskeletal tracks like organelles do, or else, move by hydrodynamic flow produced by the moving organelles.

**Synthetic Lipid Vesicles Accumulate in the Cell Plate Region**

Upon microinjection into cells in early-to-late anaphase, synthetic lipid vesicles moved from the microinjection site and distributed evenly in the cytoplasm. Within 5 to 10 min after injection into anaphase cells, synthetic lipid vesicles spread throughout the cytoplasm and between the chromosomes in the spindle (Fig. 3). When injected cells entered telophase, the vesicles accumulated at the spindle midzone and were seen as a broad band of fluorescence. In DIC microscopy, a cell plate could not yet be observed at this stage (Fig. 3A; 22 min). This band of accumulated synthetic vesicles subsequently narrowed at the same time when the young cell plate became visible with DIC microscopy. This was seen in all injected cells (n = 24 cells). Occasionally, an additional accumulation of lipid vesicles was seen around the edges of the growing cell plate (data not shown).

The accumulation of injected synthetic lipid vesicles in the cell plate region was specific; vesicles did not
just fill the accessible volume like coinjected fluorescent dextran did (Fig. 3C).

We observed a uniform distribution of fluorescence throughout the cell plate region, i.e. there was no black line in the middle, which suggests that injected vesicles accumulated not only on the surface of the developing cell plate, but also within the fenestrated cell plate. We compared the width of the vesicle accumulation zone with that of the cell plate itself, as measured with FM4-64 labeling. FM4-64 is a fluorescent lipophilic styryl dye that labels the developing cell plate when applied from the outside of the cell (Bolte et al., 2004). FM4-64 did not label DOPG vesicles in vitro and in vivo (Fig. 4, A and B). We followed the behavior of the injected synthetic lipid vesicles during cell plate formation and compared this with the cell plate labeling of externally applied FM4-64 (Fig. 4C).

The accumulation of injected lipid vesicles in the cell plate region coincided with FM4-64 labeling of the cell plate. However, the region of synthetic vesicle accumulation was slightly, but consistently thicker than the FM4-64-labeled cell plate (1.5 \( \mu \)m versus 0.8 \( \mu \)m; Fig. 4D).

The injection of synthetic lipid vesicles did not hinder or delay cell plate formation. In all vesicle injected cells, cell plate formation took on average 27 min \( \pm \) 3 s, which did not differ from the experimental controls (Fig. 5). Furthermore, the initial appearance, the growth, and the attachment of the cell plate to the parental cell wall were all normal. Interestingly, the moment of injection did not have any influence on the accumulation of vesicles in the cell plate region. Injected vesicles accumulated in the whole cell plate region when injected at different stages of cell division (Table I), even in cells in which a cell plate was already visible at the time of injection (Fig. 6A). Further, synthetic vesicles were not redistributed back into the cell as long as the phragmoplast existed. Because microtubules are mostly present at the periphery of the torus-shaped phragmoplast, they are not likely to be instrumental in bringing the vesicles to the center or keeping them there at this later stage of cell plate formation (Fig. 6B).

To exclude that fluorescently labeled Bodipy FC12-HPC phosphocholine lipid or free Bodipy dye could label the cell plate without being part of the synthetic vesicles, we injected the vesicles into the interphase cell, neighboring a cell in anaphase (Fig. 7). Upon injection, some fluorescence was transferred from the injected cell into the neighboring cells. This could be free Bodipy FC12-HPC, which has a molecular mass of 0.895 kD and is close to the exclusion limit (0.8 kD) of plasmodesmata (Pheasant and Hepler, 1987). In the dividing cell, no labeling of the cell plate occurred, which further underpins that the observed fluorescence in the cell plate region comes from the accumulation of injected fluorescent vesicles, and not from free dye.

**Synthetic Lipid Vesicles Redistribute inside the Daughter Cells after Cell Plate Attachment**

At the end of cytokinesis, when the cell plate attached to the parental plasma membrane and cell wall, the lipid vesicles redistributed completely into the cytoplasm, while the FM4-64 labeling remained in the plasma membranes lining the young cell wall (Fig. 8). The fluorescent band of lipid vesicles first broadened (Fig. 8A; 2 min), then two clouds of fluorescence were formed one on each side of the new cell wall (Fig. 8A; 4–8 min), and finally the fluorescence completely
redistributed in the cytoplasm of the cells. This shows that although synthetic lipid vesicles accumulate in the fenestrated cell plate, they do not fuse together with endogenous vesicles to form the cell plate.

We analyzed the fluorescence intensities of a region of 1.5 × 5 μm of the phragmoplast over time, including the cell plate, in cells injected with fluorescent synthetic vesicles and labeled with FM4-64 (Fig. 9). During the initial phase of cell plate growth and expansion, injected DOPG vesicles and FM4-64 labeled membranes showed the same distribution (Fig. 4B). The thickness of the cell plate of synthetic vesicles and FM4-64 labeling, which was measured as the width at half-height of a Gauss curve fitted to the fluorescence intensity profile (see also Fig. 4C), stabilized after 10 to 15 min at 0.5 to 0.7 μm (Fig. 9A). About 30 to 35 min after initiation, the cell plate attached to the parental plasma membrane. At this moment the DOPG vesicles lost their confinement to the cell plate region and concomitantly the width of the fluorescence peak broadened. On the other hand, the thickness of cell plate as measured with FM4-64 did not broaden and stayed approximately 0.5 μm. The disappearance of the synthetic vesicles from the new cell wall between the two daughter cells is also evident from the fluorescence intensity measurements over time (Fig. 9B). Starting at 25 to 30 min, the maximum fluorescence intensity at the cell plate decreased steadily to the

Figure 4. FM4-64 does not label the synthetic lipid vesicles in vitro and in vivo, but the accumulation of injected lipid vesicles in the cell plate region coincides with FM4-64 labeling of the cell plate. A and A1, Bodipy FC12-HPC-labeled DOPG vesicles (A) incubated for 1.5 h with 2 μM FM4-64 in microinjection buffer are not labeled with FM4-64 (A1). Bar = 10 μm. Injected fluorescently labeled DOPG vesicles (B) are not labeled with FM4-64 (B1) in a cell with a phragmoplast. The vesicles marked with white circles do not colocalize with FM4-64 labeling. The cell plate and plasma membrane, however, are clearly labeled with FM4-64. Bar = 5 μm. C, The cell plate is labeled by injected lipid vesicles and with FM4-64. The synthetic lipid vesicles accumulate in the region of the cell plate at the same time as the cell plate is labeled with FM4-64; both labels correspond to the cell plate visible in DIC microscopy. Time is in minutes; bar = 10 μm; n = 3 cells. FM4-64 concentration is 2 μM (in A1, B1, and C), added to the surrounding medium 15 min before injection during anaphase. D, Fluorescence intensity profiles of vesicles, FM4-64, and Alexa-568 dextran (10 kD) through the phragmoplast. The line profiles were taken from cells in late telophase, before the cell plate was attached to the parental cell wall. Three regions of 1 × 4 μm perpendicular to the phragmoplast were selected from the images. The average fluorescence intensity of each horizontal row of pixels was plotted versus its vertical position. The accumulation of vesicles in the cell plate region coincides with the FM4-64 labeling. However, the synthetic vesicles in the cell plate show a broader peak of fluorescence than the FM4-64 labeling. The dextran clearly does not enter the cell plate region.
initial levels, whereas the FM4-64 labeling remained constant during the whole period. The decrease in fluorescence of the synthetic vesicles in the cell plate region was not caused by the bleaching of Bodipy; the fluorescence intensity of synthetic vesicles in the cytoplasm of the same cells did not change over time. This shows that injected vesicles only accumulate in the cell plate region, but do not fuse with each other, with the endogenous cell plate forming vesicles, or with the developing cell plate. They are again distributed throughout the cytoplasm when the cell plate attaches to the parental plasma membrane and cell wall, and the maturation of the young cell wall begins, at the moment that the typical phragmoplast actin cytoskeleton also disappears.

**DISCUSSION**

**Injected Synthetic Vesicles Move through the Cytoplasm with Velocities Comparable But Slightly Less Than Organelles**

We used synthetic DOPG vesicles to study basic physical properties that vesicles require to enter, move through, and accumulate in the cell plate region. We prepared 60-nm DOPG vesicles with the same size as endogenous Golgi vesicles that form the cell plate (Reichardt et al., 2007). We showed that these synthetic vesicles were stable in vitro and could be successfully microinjected into plant cells. The vesicles were not toxic to the cells and they did not disturb cellular processes.

The injected lipid vesicles did not fuse together or with endogenous membranes, but moved through the cytoplasm of interphase *T. virginiana* stamen hair cells at slightly lower velocities than large endogenous organelles. The presence of the synthetic DOPG membrane did not affect the movement of these endogenous organelles in the cytoplasm (Figs. 2 and 3).

Although we injected uncoated lipid vesicles, these vesicles may be coated in the cells with cytoplasmic proteins. The fast distribution of the vesicles and their movement inside the cytoplasm could be caused by motor protein binding and subsequent transport activity. It is known that myosin-I prefers to bind anionic lipids (Adams and Pollard, 1989), like the DOPG that we used for our vesicles. Pull-down assays with DOPG-coated magnetic beads in cytoplasmic extracts confirm the binding of several proteins to the membrane (Supplemental Fig. S2). On the other hand, it is unlikely that endo- or trans-membrane proteins, which may be needed for binding of these motor proteins, are inserted into the membranes of injected synthetic vesicles, but it has been shown that the Chara myosin globular tail domain binds to vesicles made from acidic phospholipids (Nunokawa et al., 2007). Furthermore, we cannot exclude the contribution of hydrodynamic flow on vesicle movement. Cytoplasmic movement by hydrodynamic flow is an additional way of transport and may well contribute to transport of large structures like vesicles and even organelles (Houtman et al., 2007; Esseling-Ozdoba et al., 2008).

**Synthetic Vesicles Are Transported through the Phragmoplast and Accumulate in the Cell Plate Region**

Although their membrane composition and content are different from endogenous vesicles, microinjected synthetic DOPG vesicles passed through the phragmoplast structure of microtubules, actin filaments, and ER membranes, and accumulated at the developing cell plate. They did not disturb the transport of endogenous vesicles toward the cell plate because the initiation and the subsequent development of the cell plate was not disturbed in any of the injected cells (as visualized with DIC and FM4-64 and with regard to time; Figs. 4–6; Table I).

The specific accumulation of synthetic vesicles in the cell plate region of the phragmoplast, instead of filling the accessible volume (Supplemental Fig. S3), points to two processes, namely, the directional transport of these vesicles inside the phragmoplast toward the cell plate and the inhibition of transport away from the cell.

**Table I. Lipid vesicles accumulate in the cell plate region upon injection at different stages of cell division**

<table>
<thead>
<tr>
<th>Stage of Cell Division When Injected with Vesicles</th>
<th>Percentage of Cells with Vesicle Accumulation at the Cell Plate</th>
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<tbody>
<tr>
<td>Prophase</td>
<td>100% (<em>n</em> = 3)</td>
</tr>
<tr>
<td>Metaphase</td>
<td>100% (<em>n</em> = 9)</td>
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<tr>
<td>Anaphase</td>
<td>100% (<em>n</em> = 24)</td>
</tr>
<tr>
<td>Telophase</td>
<td>100% (<em>n</em> = 7)</td>
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plate. Because the vesicles were transported through the cytoplasm after they were injected, it could well be that these vesicles were, as we discussed above, coated with cytoplasmic motor proteins that caused their movement along the phragmoplast microtubule and/or actin cytoskeleton. However, the mode of transport of endogenous vesicles through the phragmoplast is still unclear. With electron microscopy (EM), it has been observed that some vesicles are associated with phragmoplast microtubules, suggesting the microtubules as the cytoskeletal element along which the vesicles are transported, but some other vesicles apparently were not associated with microtubules (Otegui et al., 2001), but both vesicles and microtubules are present in high numbers. Actin filaments were not considered in those studies because it was not possible to preserve them for EM observations (Seguí-Simarro et al., 2004). Based on these EM studies, it is not possible to assess if actin filaments, which are located in arrays parallel with the microtubules in the phragmoplast, are involved in vesicle transport. Synthetic vesicles injected at different stages of cell division, even at late telophase when the cell plate was already partially formed, accumulated at the same time in the whole cell plate region. This suggests that the DOPG vesicles at late telophase are transported toward the cell plate along the remaining actin filaments, at least in that part of the phragmoplast from which microtubules have already disappeared (T. virginiana, Zhang et al. [1990]; tobacco [Nicotiana tabacum] BY2 cells, Granger and Cyr [2000]). In that middle part, the actin cytoskeleton is still present as shown with microinjections of fluorescent phalloidin (Zhang et al., 1993), which we confirmed in living cells of tobacco BY-2 suspension cells using a GFP::FABD construct for visualization of the actin cytoskeleton, and a spinning disc microscope (Fig. 10). One might also think that in this situation injected vesicles are transported to the cell plate through the margins of the phragmoplast in which there are still microtubules present and subsequently move laterally along the surface of the developing cell plate. However, in those cells that were injected at late telophase, we never observed a flow of fluorescence from the phragmoplast margins toward the microtubule-free middle of the cell plate region, excluding the possibility that synthetic vesicles only “incorporated” at the margins and moved laterally along the cell plate. This demonstrates that it could well be that vesicles use actin filaments to be transported through the phragmoplast toward the cell plate. This transport also could be in the form of hydrodynamic flow produced by endogenous vesicles moving along the actin phragmoplast cytoskeleton toward the cell plate, as our results indicate for vesicle movement in interphase cells and prove for molecules the size of GFP (Esseling-Ozdoba et al., 2008).
Although all our data suggest a role for the actin cytoskeleton in keeping vesicles in the cell plate region, we cannot exclude the possibility that the negative charge of DOPG vesicles contribute to its accumulation in the cell plate region.

**Synthetic Vesicles Do Not Fuse with the Cell Plate**

Although the injected synthetic vesicles were transported through the phragmoplast and accumulate in the cell plate region, they did not fuse with the developing cell plate, but redistributed again throughout the cytoplasm upon attachment of the cell plate to the parental cell wall (Fig. 8). This redistribution strongly suggests that these vesicles cannot fuse with endogenous vesicles/cell plate or with each other. The latter was already indicated by the fact that the vesicles did not fuse in vitro, even after 7 d. In injected cells, the DOPG vesicles probably reside in the tubulo-vesicular network, the tubular network, and finally in the fenestrae (Samuels et al., 1995; Staehelin and Hepler, 1996; Seguí-Simarro et al., 2004) of the developing cell plate. Whether the region of DOPG vesicle accumulation coincides with the CPAM, which is suggested to be responsible for regulation of the fusion events in the cell plate region (Seguí-Simarro et al., 2004), is difficult to determine. The CPAM, including the cell plate proper, has a total width of 155 nm (Seguí-Simarro et al., 2004), while the width of a cell plate determined from fluorescence of DOPG vesicles is 0.5 to 0.7 μm and a cell plate labeled with FM4-64 is 0.5 μm.

Our observation that injected vesicles cannot fuse with endogenous vesicles in the developing cell plate was to be expected. Firstly, the injected vesicles lack trans-membrane SNARE proteins and are probably

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**Figure 7.** Bodipy FC12-HPC alone does not label the cell plate. A cell below a dividing one was injected with fluorescent synthetic vesicles. No labeling of the cell plate is visible. The probe only accumulates in the cell plate when it is incorporated into vesicles. Insert, Detail of the phragmoplast of the cell that neighbors the injected cell imaged at 32 min after injection, showing no incorporation of Bodipy FC12-HPC into the cell plate. Time is in minutes; bar = 20 μm.

**Figure 8.** Redistribution of DOPG lipid vesicles after the cell plate is attached to the parental cell wall. The fluorescence of microinjected synthetic vesicles (A) is redistributed to the cytoplasm of the daughter cells while the labeling with FM4-64 (B) stays in the cell plate region. C, A merged image of A and B. The cell plate is visible with DIC microscopy (D). The FM4-64 concentration is 2 μM and was added to the surrounding medium 5 min before vesicle injection during telophase. Time is in minutes; time 0 is arbitrarily chosen; bar = 10 μm.
not able to acquire them after injection. SNAREs are necessary for the fusion of cell plate forming vesicles because the Arabidopsis SNARE mutants knolle and keule have unfused cytokinetic vesicles in the cell plate region (Assaad et al., 1996; Lukowicz et al., 1996; Lauber et al., 1997; Waizenegger et al., 2000). Secondly, only homotypic fusions may occur in the cell plate region, which means that only the similar vesicles are able to fuse as has been observed with electron tomography (Seguí-Simarro et al., 2004) and reviewed by Albertson et al. (2005). Our synthetic vesicles are physically identical to each other and do not fuse with each other or other membranes in medium and cells in interphase. We wondered whether their close proximity and the environment of the phragmoplast would make them fuse to each other in the cell plate region of dividing cells. Based on these observations we conclude that close proximity alone and in combination with the specific environment in the cell plate region is not sufficient for fusion of DOPG vesicles.

F-Actin May Keep Unfused Vesicles in the Cell Plate Region

During the centrifugal growth of the cell plate, the centrally located microtubules depolymerize and reassemble at the periphery of the phragmoplast, which then becomes torus shaped (second phase; Valster and Hepler, 1997; Kost and Chua, 2002). The microtubules would therefore not be able to keep unbound vesicles from diffusing away from the central region of the cell plate. This suggests that another mechanism is responsible for keeping the synthetic vesicles close to the cell plate region. We hypothesize that actin filaments could play this role of keeping the synthetic vesicles in the cell plate region, just as they also could act as a scaffold for the fragile developing cell plate (Samuels and Staehelin, 1996).

The breakdown of actin filaments in the phragmoplast occurs uniformly along the whole width at the moment when the cell wall is completed, rather than in a centrifugal pattern (Cleary et al., 1992; Hepler et al., 1993; Zhang et al., 1993). A similar mechanism has been proposed for keeping exocytotic vesicles close to the growing tip of tip-growing cells, where actin filaments deliver vesicles to the vesicle-rich region in the pollen tube (Geitmann et al., 2000) and root hair tips (Miller et al., 1999) and keeps them in the tip (Ketelaar et al., 2003). Actin filaments in the phragmoplast could be involved in delivering vesicles to the cell plate and keeping them there, while at the same time keeping larger organelles away from the developing cell plate, thus acting as a physical sieve for these vesicles and a barrier for the larger organelles that otherwise would obstruct the accumulation processes. These roles of actin filaments in the phragmoplast are supported by experiments in which cells during cytokinesis were injected with excess amounts of the G-actin binding protein profilin (Valster et al., 1997). Upon injection of profilin, the actin filaments in the phragmoplast depolymerized, resulting in a delay in the cell plate formation or a complete absence of the cell plate.

We have shown that DOPG vesicles are a useful tool to study the processes involved in cell plate formation. Using these synthetic vesicles, we uncovered what to our knowledge is a possible new role for the phragmoplast actin cytoskeleton in cell plate formation. The next challenge is to elucidate the exact origin(s) and the
nature of the endogenous vesicles that form the cell plate. As one of the direct experimental approaches, we propose to inject vesicles with experimentally defined surfaces (e.g., specific phospho- and/or glycolipid compositions and embedded and/or [covalently] attached proteins) to analyze the vesicle transport mechanism and the requirements for vesicles to fuse and form the cell plate.

MATERIALS AND METHODS

Plant Material

Tradescantia virginiana plants were grown in a growth chamber with a 16-h photoperiod at 25°C and 8-h dark period at 18°C and 75% to 80% relative humidity. Stamens hairs with dividing cells in the apical region were dissected from immature flower buds with a length of approximately 5 mm. For microinjection experiments, we immobilized these stamen hairs in a thin layer of 1% low-temperature gelling agarose (BDH Laboratory Supplies) in culture medium (5 mM HEPES, 1 mM MgCl₂, and 0.1 mM CaCl₂, pH 7.0) and 0.025% Triton X-100 (BDH Laboratory Supplies), following the procedure described by Vos et al. (1999). The layer of agarose with stamen hairs was solidified by cooling at 4°C for 10 to 15 s and then flooded with culture medium.

Preparation of Synthetic Vesicles, Dextran, and FM4-64

Synthetic vesicles consisted for 98% of the anionic nonfluorescent phospholipid DOPG (Avanti Polar Lipids) and for 2% of the fluorescent phospho- choline Bodipy FC12-HPC (excitation maximum at 503 nm, emission maximum at 512 nm; Molecular Probes). Bodipy FC12-HPC was especially chosen because it labels the acyl chain rather than the hydrophilic head of the phospholipid. It therefore prevents the movement of the fluorescent probe between lipid layers (jumping). DOPG was purchased as a chloroform solution of a sodium salt. Bodipy FC12-HPC was purchased as a powder and was then dissolved in ethanol. Phospholipids were mixed together and dried onto a glass paper. The grids were plunge-frozen in liquid propane and after short storage in microinjection buffer. The excess fluid from the film was removed with filter grids covered with holey carbon film were submerged in the vesicle solution and mounted onto a pressure injector consisting of a microneedle holder (Eppendorf) and a 2-ml micrometer syringe (Gilmont Instruments) connected via a water-filled fine polyethylene tubing. The position of the pressure injector was controlled by a hydraulic micromanipulator (model N0-305; Narashige Scientific Instruments).

Microinjection

The microinjection experiments were conducted according to Vos et al. (1999). In short, needles were pulled from borosilicate capillaries with filament (World Precision Instruments) by using a vertical pipette puller (model 700C; David Kopf Instruments). The opening of these needles was 200 to 240 nm (measured from scanning EM images; data not shown). The needles were back-filled with the experimental solution and water, and mounted onto a pressure injector composed of a microneedle holder (Eppendorf) and a 2-ml micrometer syringe (Gilmont Instruments) connected via a water-filled fine polyethylene tubing. The position of the pressure injector was controlled by a hydraulic micromanipulator (model N0-305; Narashige Scientific Instruments).

Microscopy, Imaging, and Data Analysis

Microinjections were performed on inverted microscopes. Images were collected with a Cell-MAP IC (Bio-Rad) confocal laser-scanning microscope, coupled to an Eclipse TE2000-S (Nikon) or with an LSM 5 Pascal confocal laser-scanning microscope coupled to an Axiovert 200 microscope (Zeiss). For Bodipy/ Alexa-568 dextran or FM4-64 dual scanning we used the excitation/ emission combination of 488/520 to 540 nm BP and 532/560 nm LP (Cell-MAP IC) or the combination of 488/505 to 550 nm BP and 543/560 nm LP (HFT 488/NFT 545/HFT 545; LSM 5 Pascal). Images were obtained with a 1.4 NA 60× or 1.4 NA 63× oil immersion objective, collected by Kalman averaging of two to three full scans (Cell-MAP IC) or with scan speed 7 (LSM 5 Pascal). Images were taken at 2- or 3-min intervals, which allowed observation of developing cell plates for long periods of time without disturbing the cell plate formation process. Images were processed and analyzed with the software programs Confocal Assistant 4.02 (written by Todd Clark Brede), Adobe Photoshop 5.0 and 8.0 (Adobe Systems), and Image J (version 1.32); National Institutes of Health). For fluorescence intensity plots, rectangles (10 × 50 pixels) of images at different time points were cut out and reduced to 1 × 50 pixels to average the fluorescence. The resulting pixel intensities were saved as text files with Image J, and plotted and curve-fitted in Origin (version 7.5; SIS; OriginLab) using standard Gauss equations.

Images of actin filaments in phragmoplast of BY-2 cells transformed with GFP::FABD were made with a spinning disc confocal microscope (PerkinElmer) coupled to an Eclipse TE2000-S (Nikon).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Similarity of movement of DOPG vesicles and organelles in injected cells.

Supplemental Figure S2. Pull-down assay with DOPG-coated magnetic beads in cytoplasmic extracts confirming the binding of several proteins to the membrane.

Supplemental Figure S3. Accumulation of DOPG vesicles in the cell plate region.

ACKNOWLEDGMENTS

We gratefully thank Richard Kik, Mieke Kleijn, and Frans Leerakers of the Laboratory of Physical Chemistry and Colloid Science, Wageningen University, for help with designing the vesicles and for useful discussions. We thank Magdalena Szechynska-Hebda for initial help with the vesicle microinjections. We also thank Adriaan van Aelst for help with cryo-TEM and John Esseling for critical reading of the manuscript. Received March 26, 2008; accepted June 19, 2008; published June 26, 2008.

LITERATURE CITED


