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The ins and outs in membrane dynamics: tubulation and vesiculation

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Living cells constantly adjust the composition and size of their membrane systems to accommodate the demands for the housekeeping activities, to expand and reduce cell size, and to commit the cell for division. Although it is well known that vesicles are the vehicles to deliver and retrieve lipids and proteins to and from the membranes, the mechanisms allowing vesicles to pinch off from membranes or fuse into a flat lipid bilayer have been poorly understood, particularly in plants. Recent studies on dynamins and dynamin-related proteins in animals and plants now allow new concepts in membrane dynamics to be considered.

Dynamics of membrane biogenesis

The dynamics of membrane biogenesis and maintenance in a eukaryotic cell requires a constant turnover of membrane constituents mediated by the processes of exocytosis and endocytosis. In each of these processes, vesicular packages composed of lipid bilayers and assorted membrane proteins are pinched off of the membranes of one part of the cell and delivered like cargo to other subcellular or extracellular compartments that are undergoing expansion or reconstruction. Throughout most of the cell cycle, when the volume of the cell is increasing, much of this trafficking is directed outwards towards the plasma membrane. However, at a precisely defined period of time during cytokinesis in plants, the exocytic machinery is redirected from the plasma membrane to the nascent cell plate. This compartment is formed *de novo* at the midplane of the cell by homotypic fusion of Golgi-derived vesicles. To prevent the fusing vesicles from ballooning outward and interfering with cell plate construction, the membranes assembling here are shaped into a latticework of tubules and sheets by dynamins and dynamin-related proteins [1].

Dynamin-related proteins (DRPs) comprise a large family of high molecular weight GTPases that play a central role not only when the plasma membrane is formed at the cell plate but also during the division of organelles such as chloroplasts, mitochondria and peroxisomes [1]. The basic feature of this group of proteins is that they form helical structures able to wrap around the membranes and either tubulate them or pinch them off of larger membrane sheets. Although different DRPs appear to be dedicated to each function, the enzymological mechanisms producing either vesicles or tubules might be more similar than they appear at first glance. Recent studies have shown that pinchase can be converted to tubulase simply by reducing GTPase activities by introducing a mutation in the GTPase domain, or by carrying out the reaction in the presence of GTP-γ-S.

This brief review is not intended to offer a comprehensive summary of membrane trafficking and cell plate formation (reviewed in Refs [2–7]) but rather to present an opinion concerning new concepts in membrane dynamics in light of recent studies on dynamins and DRPs in animals and plants.

Tubulases and pinchases of the membranes

Vesicle tubulation refers to the process by which lipid bilayers connecting free vesicles or vesicular buds to other membranes are drawn out into long tubes (Figure 1c). Tubulized membrane structures have been observed *in vivo* at the nascent cell plate in plants (Figure 1e–i) [6,8,9], in the plasma membrane of animal nerve terminals in the presence of GTP-γ-S [10], and in fruit flies expressing a clathrin antisense RNA (Figure 1n) [11]. Similar structures can also be created *in vitro* by purified animal dynamins acting on endocytic vesicles (Figure 1k) [12] or liposomes [13–15]. However, both plants and animals have proteins called phragmoplastin and dynamin-like protein DLP1, respectively, which are specifically dedicated to the formation of membrane tubes (Figure 1a,c). Phragmoplastins are believed to be responsible for the formation of the hourglass and dumbbell-shaped vesicle–tubulo–vesicle (VTV) structures observed at the forming cell plate [2,5,8,9,16], whereas DLP1 is able to tubulate membrane both *in vivo* and *in vitro* [17].

All DRPs including phragmoplastins are able to form helical structures [16] that wrap around the vesicles to create either tubes or vesicles [14]. The kind of products produced is determined in part by GTP binding and hydrolysis activity. Both phragmoplastins and dynamins are known to possess relatively high GTPase activities when they are in a dissociated state [18,19]. However, when this activity is reduced by mutations in the GTP-binding domain, incubation with GTP-γ-S, or depletion of clathrin, dynamins can function as a tubulase generating long tubules on the plasma membrane [10,11]. Conversely, the expression of a dominant-negative mutant of phragmoplastin causes the accumulation of tubular membrane structures throughout the cell plate (Figure 1 o–q) [20].

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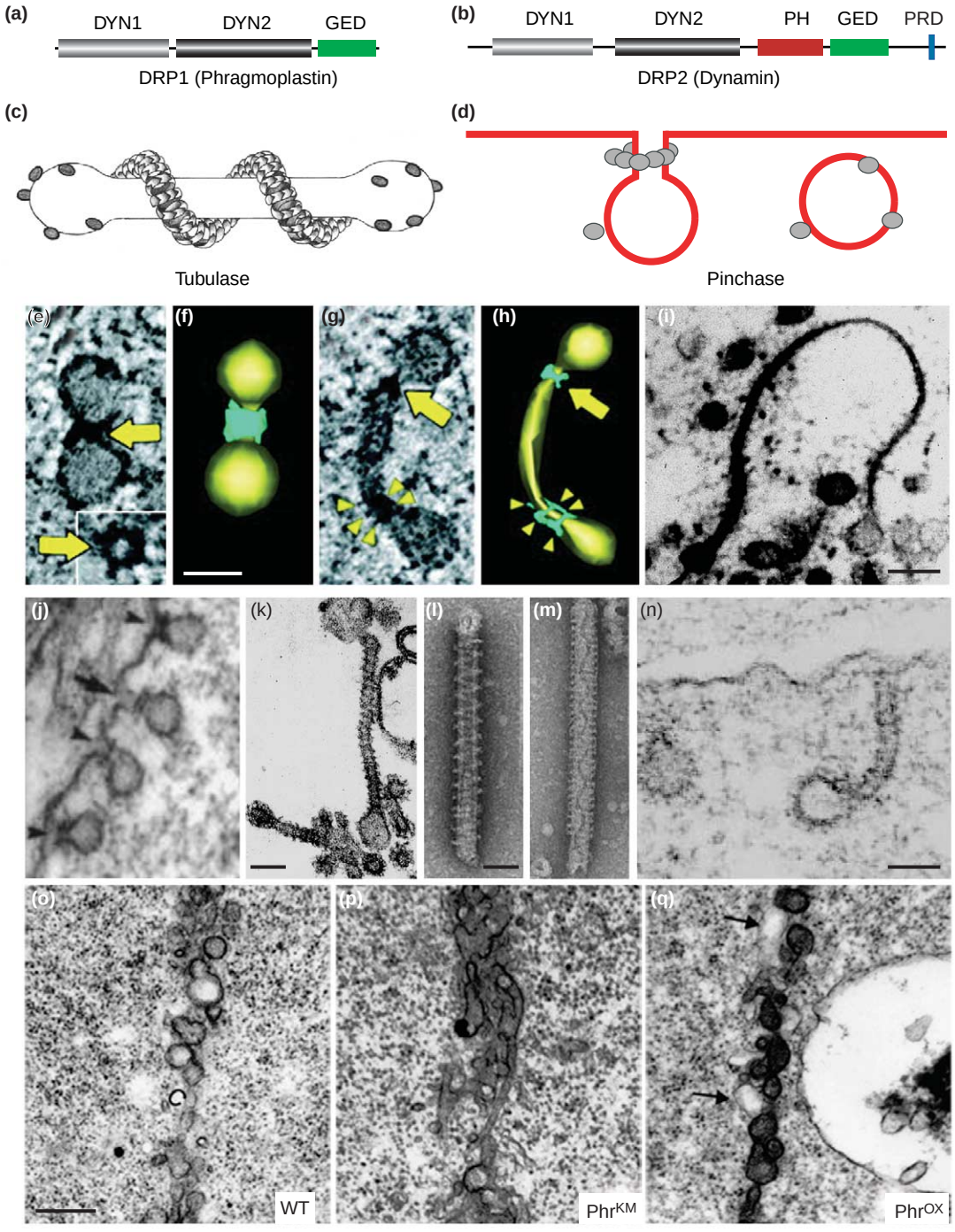

Figure 1. Vesicle tubulation and membrane vesiculation are mediated by structurally related phragmoplastins and dynamins. **(a,b)** *Arabidopsis* DRP1 is closest to the soybean phragmoplastin [7,18]. DRP2, the *bona fide* plant dynamin, contains all the functional domains found in the animal dynamins [32,61]. Abbreviations: DYN1, dynamin homology domain 1; DYN2, dynamin homology domain 2; GED, GTPase-effector domain; PH, pleckstrin homology; PRD, proline-rich domain. **(c,d)** *Arabidopsis* DRP1 acts as a tubulase and forms highly ordered spiral polymers on the surface of vesicles leading to the formation of tubular structures. DRP2, a pinchase, forms a collar ring surrounding the junction region between a budding endocytic vesicle and the plasma membrane. **(e–i)** Phragmoplastin tubulase observed at the forming cell plate of *Arabidopsis* meristem cells (e–h) [6] and tobacco BY-2 cells (i) [8]. Tomographic slices (e,g) and models (f,h) of the hourglass-shaped vesicle wrapped with the phragmoplastin spring (e,f) and the long dumbbell-shaped vesicular tubule wrapped with two collars (g,h), one adjacent to each bulbous end [6]. Tomographic slices are digital electron micrographs of thin-sections, whereas image models are generated by computer software using image series taken while the specimen section is tilted from $+60^{\circ}$ to -60° at 1° intervals [6]. Long dumbbell-shaped tubules observed at the nascent cell plate of tobacco BY-2 cells (i) [8]. **(j)**, Dynamin pinchase in fruit flies. A conditional mutant of *Drosophila* dynamin (*shibire*^{ts}) blocks the endocytosis of synaptic vesicles, allowing better observation of the vesiculation event mediated by the dynamin collar ring [62]. **(k–n)** Conversion of a pinchase to a tubulase in the presence of a GTP analog or in the absence of clathrin in animal cells. Tubular structures can be formed by dynamins *in vitro* using endocytic vesicles from rat brains in the presence of GTP-γ-S (k) [12] or using lipid nanotubes in the presence of GDP-dynamin (l) and GTP-γ-S-dynamin (m). Note the more tightly packed dynamin springs in (m) than in (l) [15]. Tubules can also be formed in animal cells expressing clathrin antisense RNA (n) [11]. **(o–q)** Disruption of cell plate formation in tobacco cells overexpressing soybean phragmoplastin tubulase (Phr^{OX}) or its dominant-negative allele (Phr^{KM}). Note the accumulation of thin and intertwined tubule-like structures in Phr^{KM} [20], and the more electron-dense cell plate as well as accumulated multivesicular bodies (arrows) in Phr^{OX} cells as compared with the cell plate of wild-type (WT) cells [36]. Scale bars: (e–h) =50 nm; (i) =100 nm; (k) =100 nm; (l–m) =50 nm; (n) =100 nm; (o–q) =450 nm. Panels (e–q) are reproduced, with permission, from Refs [6,8,11,12,15,36,62].

Under natural physiological conditions, the decision to act as a tubulase or a pinchase might rely either on auxiliary proteins that can regulate GTPase activities or on the presence of Ca^{2+} that is known to affect dynamin GTPase activity [21]. Both phragmoplastins and dynamins contain a GTPase-effector domain (GED) [1] through which the GTPase activity is regulated. Dynamins contain a pleckstrin homology (PH) domain for binding to phosphoinositides, and a proline-rich (PR) motif (RXPXXP) for interacting with the SH3 domains of proteins such as amphiphysins and endophilins in animal cells [22]. By contrast, animal DLP1, soybean phragmoplastins and *Arabidopsis* DRP1 do not contain either PH or PR domains [1,18,23]. Phragmoplastins interact with a different set of proteins including a UDP-glucose transferase [24] (a subunit of the cell plate-specific callose synthase [25,26]), a novel RNA-binding protein and a sumoylation enzyme (D.P.S. Verma and Z. Hong, unpublished). Phosphorylation of the PR motif of dynamins by cyclin-dependent kinase 5 (Cdk5) blocks its interaction with amphiphysin and inhibits clathrin-mediated endocytosis of the synaptic vesicles [27]. Although there is less direct evidence that phragmoplastins and dynamins are regulated by Ca^{2+}, the cell plate has been shown to contain high concentrations of this ion bound to the membrane [28,29]. Calcium might regulate the GTPase activities of phragmoplastin and dynamins via calcium- and calmodulin-dependent protein kinases and phosphatases such as calcineurin [21,30], or directly control pinchase given that it has been shown to inhibit dynamin GTPase activity [31].

The majority of dynamins in animals function as pinchases (Figure 1b,d) that hydrolyze GTP and provide mechanical force to squeeze the vesicles off the plasma membrane [14,32]. A plant DRP that might act as a pinchase is present at the cell plate, presumably associated with the clathrin-coated vesicles participating in endocytosis and membrane recycling [20]. Vesiculation at the Golgi might also require the participation of a *bona fide* plant dynamin molecule, DRP2A (i.e. ADL6) acting as pinchase [33].

Role of homotypic vesicle fusion in initiation and expansion of cell plate

Vesicle tubulation appears to play a pivotal role in the creation and expansion of the cell plate. During cell plate initiation, Golgi-derived vesicles are transported to the division plane. These vesicles do not have a target membrane to fuse with, therefore, homotypic fusion (i.e. fusion between the same type of vesicles) must occur (Figure 2a). In other homotypic fusion systems such as the yeast mitotic vacuole fusion, two small vacuoles fuse with each other, giving rise to the formation of a larger, round vacuole [34]. By contrast, homotypic fusion of vesicles at the cell plate results in the formation of 'hourglass' and 'dumbbell-shaped' tubular structures via phragmoplastin-mediated tubulation of the fused vesicles (Figure 1e–i) [6,8,9,16]. The VTV structures thus generated then expand and form the consolidated tubulo-vesicular network (TVN) [2,35]. Fusion of VTV is initiated in the center of the phragmoplast and extends in a centrifugal manner to the parental cell wall. At the growing edges, vesicles do not directly fuse with the consolidated TVN. Thus, homotypic fusion of vesicles and tubulation of the fused vesicles are two distinct and essential steps in building the cell plate (Figure 2a). Because the completion of the cell plate takes place over a comparatively short time (about an hour) the homotypic fusion and tubulation machineries must be highly efficient.

Exocytosis and endocytosis at the cell plate

In addition to homotypic fusion, building a cell plate also involves an analogous heterotypic process in which Golgi-derived vesicles undergo direct fusion with the proto-type plasma membrane of the forming cell plate. The proto-type plasma membrane refers to the membrane surrounding the tubular structures of the cell plate (Figure 2a) as opposed to the 'mature' and 'flat' plasma membrane on the cell surface. Heterotypic vesicle fusion at the cell plate resembles the process of exocytosis at the plasma membrane [7]. The vesicles involved in heterotypic fusion versus homotypic fusion might differ in their membrane composition as well as the cargo they carry. At the same time, endocytosis is required to retrieve excess membrane and to recycle membrane components (phospholipids and membrane proteins) from the proto-type plasma membrane of the cell plate. The two processes (exocytosis and endocytosis) have to be precisely coordinated to build a functional cell plate compartment. We have observed that overexpression of phragmoplastin in tobacco perturbs membrane recycling, resulting in the accumulation of multivesicular bodies (MVB) in the cell [36]. Accumulation of these MVBs in the path of growing ends of the plate in turn creates an oblique cell plate [37] and affects cell differentiation in the apical meristems [36,38].

Two sets of protein complexes have been implicated in the control of exocytosis at the cell plate: SNARE proteins that mediate membrane fusion events and exocyst proteins that determine the site of exocytosis. An exocyst is a complex of eight distinct subunits in yeast and animals [39,40] that unlike tSNAREs is not distributed uniformly around the inner leaflet of the cytoplasmic membrane but instead is confined to areas where vesicles are destined to fuse [40]. The cytokinesis-specific SNARE complexes in *Arabidopsis* are composed of KNOLLE (a t-SNARE), Keule (a Sec1-like protein), SNAP33 (a SNAP25 homolog) and NPSN11 (a plant-specific SNARE) [3,41–45]. By comparison, little is known about the exocyst complex in plants. A search of the *Arabidopsis* database identified orthologs of all eight subunits of the exocyst complex, which suggests that this mechanism is conserved among all eukaryotic cells. GFP-tagged AtSec6 and AtExo70 are localized as particulate structures on the surface of the plasma membrane [46]. Recent investigations into cell plate formation by electron tomography have revealed the possible involvement of exocyst-like particles tethering vesicles to the newly formed cell plate (Figure 3a–c) [6].

Our knowledge of the proteins that participate in the control of exocytosis and endocytosis or membrane recycling at the cell plate is still limited. *Arabidopsis* has two genes (*DRP2A* and *DRP2B*) for the *bona fide* plant dynamins that have all the functional domains present in

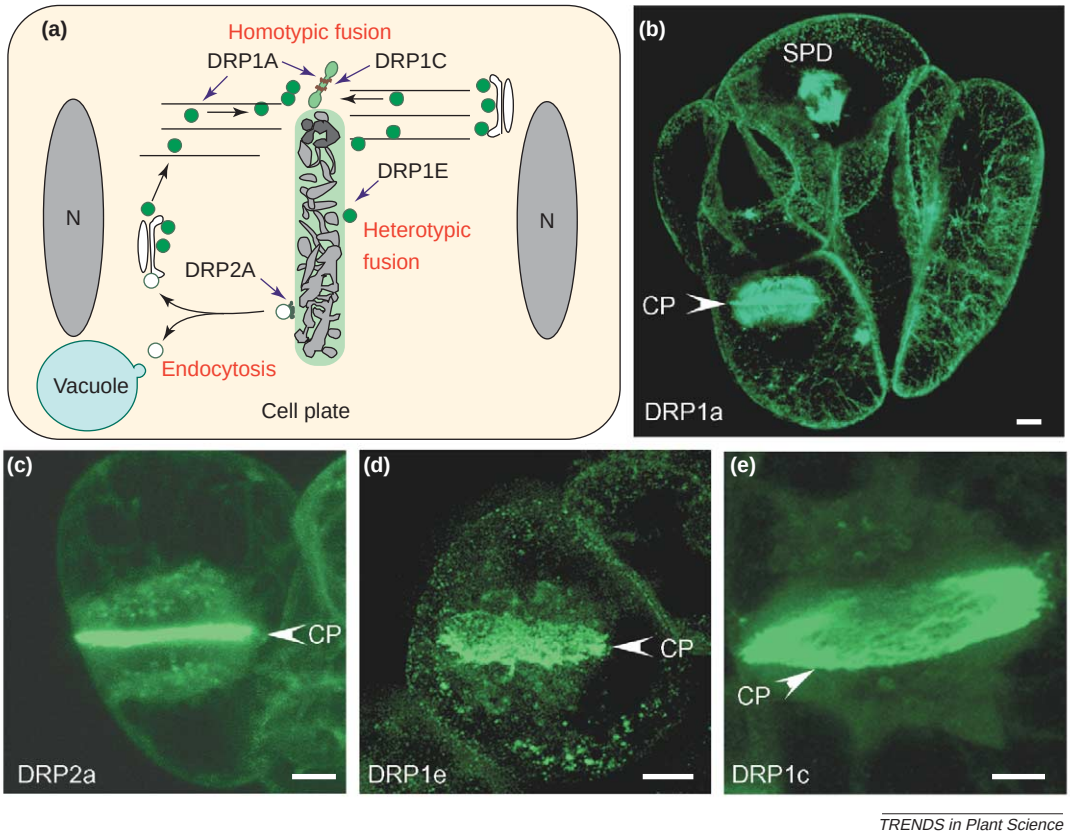

Figure 2. Homotypic fusion, heterotypic fusion and membrane recycling at the cell plate. **(a)** Building a functional cell plate requires the coordination of multiple traffic pathways for vesicle delivery and membrane recycling. Fusion of homotypic vesicles occurs at the initial stage of cell plate formation, as well as at the growing edge of the cell plate. Heterotypic fusion between vesicles and the target membrane (the proto-type plasma membrane of the cell plate) occurs in the inner region of the cell plate. The endocytic machinery operates actively to recover excess membranes and to retrieve unwanted membrane components. **(b)** Confocal image showing the association of GFP-tagged DRP1A with the microtubule and cell plate [20]. DRP1A is found on the spindle (SPD) in metaphase, and is associated with the phragmoplast microtubule arrays and the cell plate (CP) from late metaphase to early anaphase. This protein might provide an anchor for Golgi-derived vesicles to attach to the microtubule. At the growing edges of the cell plate, DRP1A controls the formation of the dumbbell-shaped VTV (vesicle–tubular–vesicle) structures that are formed by the homotypic fusion of cell plate vesicles. **(c)** Confocal image showing the association of DRP2A, the plant dynamin, with the cell plate in transgenic tobacco BY-2 cells. DRP2A might function like the dynamin pinchase in animal cells and provides a mechanical force to sever the endocytic vesicles from the cell plate. **(d,e)** Different localization patterns of phragmoplastin-like tubulases at the forming cell plate [20]. DRP1C is largely localized in the growing edges of the cell plate, forming a ring-like structure (e), whereas DRP1E is found in the inner region of the cell plate and is associated with the tubular-network (d). Scale bars (b–e) =10 μm. Panels (b–e) are reproduced, with permission, from Ref. [20].

animal dynamins, including a PH domain in the middle of the molecule and a PR motif at the C-terminus (Figure 1b). By analogy to animal dynamins, these two genes are believed to participate in clathrin-coated vesicle-mediated endocytosis in plants. At cytokinesis, DRP1A is concentrated to the nascent cell plate [20]. Overexpression of GFP–DRP2A results in the formation of giant cells (Figure 3d). These cells are multinucleate, contain large vacuoles and accumulate multivesicular endosomes (MVE) (Figure 3f). These giant cells might be formed as a result of excessive endocytosis, leading to the expansion of endosome-like vacuoles. Because the budding of vesicles from Golgi also requires the involvement of DRPs [19,33,47] and because a dominant negative mutant of phragmoplastin prevents budding of such vesicles from Golgi [20], the role of this group of proteins in vacuolar membrane recycling is also likely [48].

Redirection of the exocytic traffic to the forming cell plate

Cytokinesis in plants involves reorganization of the cytoskeleton, the assembly of the phragmoplast and the re-positioning of the exocyst complex. There are two possible ways that the exocyst complex can be moved to its new location: (i) the whole complex can be transferred from the plasma membrane to the forming cell plate during cytokinesis; or (ii) the complex dissociates so that its components can be transported to the cell plate and reassembled into functional complexes at the new location. In addition to the eight core components of the exocyst (Figure 3a–c), other regulatory proteins might also bind 'transiently' to this complex at the cell plate.

In animal cells, the exocyst complex is found only in the exocytosis-active sites of the plasma membrane, whereas the SNARE complex is evenly distributed on the plasma membrane [40]. This led to the conclusion that the exocyst complex determines the site for the exocytic vesicle to dock while the SNARE complex carries out the fusion of the membranes. This model implies that relocation of the exocyst complex alone might be sufficient to direct the exocytic vesicle traffic to the forming cell plate and that the SNARE complex might merely be an integral part of the cell plate-specific vesicles. This has been suggested by recent observations on the expression of

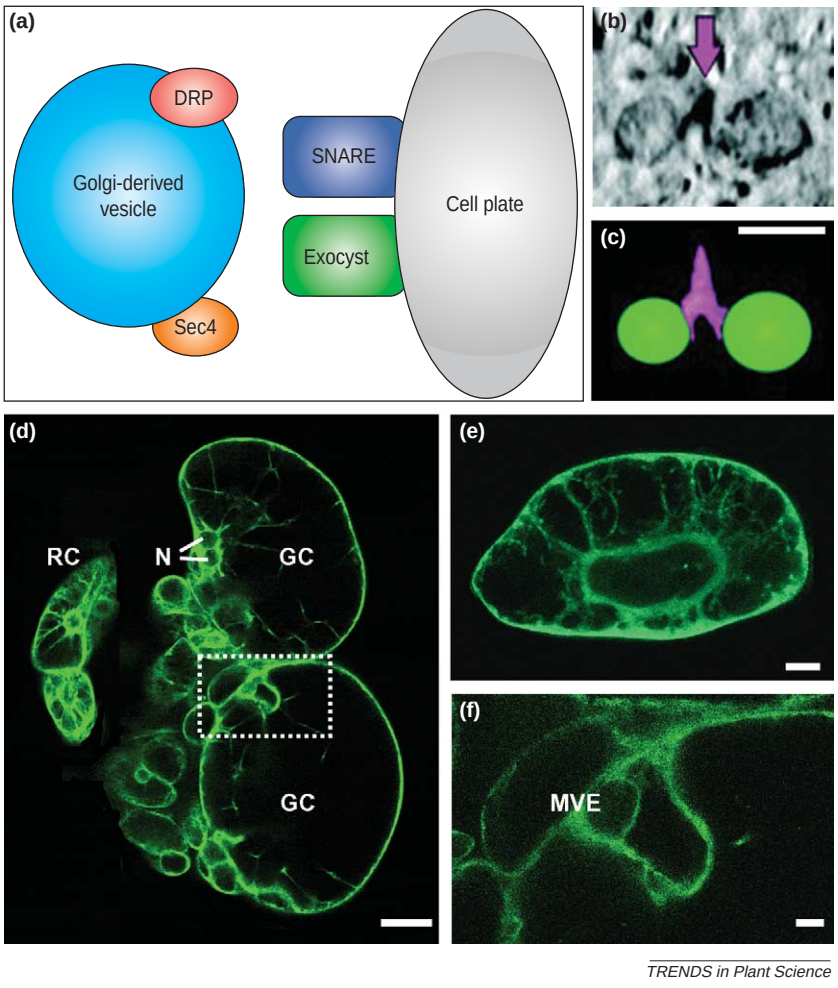

Figure 3. Molecular machinery of exocytosis and endocytosis. **(a)** Two protein complexes are involved in the heterotypic fusion between vesicles and the proto-type plasma membrane of the cell plate. The cytokinesis-specific SNARE complex in *Arabidopsis* is composed of four protein helices: KNOLLE (a syntaxin), KEULE (a Sec1-like protein), SNAP33 (a SNAP25 homolog) and NPSN11 (a plant-specific SNARE). *Arabidopsis* DRPs and Sec4 homologs that are present in cell plate vesicles might play a role in exocytosis. The location of the exocyst determines the site of exocytosis whereas the SNARE mediates the fusion event of the membranes. **(b,c)** Tomographic slice and colored model of cell plate vesicles connected by a 'Y'-shaped tethering complex (purple arrow) that could be the exocyst complex [6]. These images were taken at the cell plate in *Arabidopsis* using electron microscope and 3-D-image-processing software. **(d–f)** Formation of giant cells caused by overexpression of DRP2A, the plant dynamin. When expressed in tobacco cells, DRP2A is found in the plasma membrane and in the cytoplasm in a punctuate pattern (e). At cytokinesis, the protein is targeted to the cell plate (Figure 2c). About 5–10% of the cells expressing GFP–DRP2A are giant cells (GC); these giant cells appear to form as a result of excessive endocytosis at the plasma membrane. As a consequence, vacuoles become enlarged and the cell plate formation is disrupted. These cells undergo 1–2 rounds of mitosis without forming a cell and become multinucleate (d). Multivesicular endosomes (MVE) accumulate and eventually fuse to the vacuoles [20]. Boxed region in (d) is shown in (f). Abbreviations: N, nucleus; RC, regular size cells. Scale bars: (b,c) =50 nm; (d) =50 μm; (e,f) =10 μm. Panels (b,c,e) are reproduced, with permission, from Refs [6,20].

cytokinesis-specific SNARE components in *Arabidopsis*. KNOLLE protein, a t-SNARE syntaxin, is found in a punctuate pattern at mid phase of the cell cycle and at the nascent cell plate during telophase [44]. The transcription of this gene is regulated in a cell cycle-specific manner. Its mRNA accumulates during M-phase to produce proteins to be used in cytokinesis and is then degraded rapidly to prevent the accumulation of the protein following cytokinesis [49]. Constitutive expression of the *KNOLLE* gene does not rescue the *knolle* mutant phenotype and mislocalizes the KNOLLE protein to the plasma membrane [49]. KEULE, a Sec1 homolog, interacts with KNOLLE syntaxin and is highly expressed in meristems [41,50]. Two other components of the cytokinesis-specific SNARE, SNAP33 and NPSN11, are also expressed in actively dividing cells [42,43]. Thus, it seems that during cytokinesis, a new set of cell plate-specific SNARE components are synthesized and targeted to the plane of division along with the Golgi-derived vesicles. Human septins have been shown to interact with the exocyst and direct the complex to the site of polarized growth [51]. It is important to know if plant septin homologs and other proteins interact with the exocyst and regulate the localization and activity of this complex. Septin homologs exist in *Arabidopsis* but their function remains to be determined.

Future perspectives

There has been significant progress in research on DRP GTPases over the past ten years since phragmoplastin and DRP1A (i.e. ADL1a) were first characterized in plants [18,52]. However, many important questions remain to be answered. First, DRPs do not act alone. They form homo-oligomers *in vivo* [37] and *in vitro* [16], as well as hetero-oligomers at least *in vitro* [20]. This hetero-oligomerization occurs not only between members of the same subfamily but also between subfamilies such DRP1A

(a tubulase) and DRP2A (a pinchase) [20]. It is not known if such cross-subfamily hetero-oligomerization occurs *in planta* and whether these interactions play a significant role in membrane biogenesis. Second, DRPs interact with other proteins and possibly each member of the DRP family interacts with a different set of proteins. Identification of all these partners will help us to understand how DRPs are regulated. Third, DRP5A (i.e. ARC5) and DRP3A (i.e. ADL2a) [1] are known to function as 'constrictases' in the division of chloroplasts, mitochondria and peroxisomes [53–58] but little is known about how these activities are regulated. Fourth, research on the DRP4 subfamily in plants [1], which resembles the antiviral protein [59] Mx, has yet to clarify whether they function as a pinchase, tubulase, constrictase or something else, and whether this activity is able to affect viral infection and proliferation in plants. Fifth, because DRPs have so far not been linked to the vesiculation required for coatomer-protein complex (COP)-coated vesicle transport between the translational ER and the *cis*-Golgi, it is possible that vesiculation in these subcellular locations employs as yet unidentified DRPs. Finally, we must keep in mind that the budding yeast contains several dynamin-like proteins but lacks a dynamin ortholog. Endocytosis in this organism is instead controlled by epsin and amphiphysin homologs in addition to a functional actin cytoskeleton [60]. How the pinchase role of dynamin is replaced by these unrelated proteins in yeast is unclear. However, their use in this way indicates that nature might have ways of shaping membrane structure using proteins completely unrelated to those described here.

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