

# Plasma Membrane Protein Trafficking

Wendy Ann Peer

**Abstract** The plasma membrane is the interface between the cytosol and the external environment. The proteins that reside and function on the plasma membrane regulate the cellular entrance and exit of bioactive molecules, actuate signaling cascades in response to external stimuli, and potentiate interactions between cells. The presence and abundance of proteins on the plasma membrane is regulated by anterograde and retrograde intracellular vesicular trafficking, exocytosis, and endocytosis. The cytoskeleton is an integral component of cellular trafficking mechanisms, as the vesicles and endosomes move on actin filaments or microtubules. Selection and movement of the protein cargo to be trafficked to and from the plasma membrane depends to a great extent on signature organellar targeting motifs within the proteins themselves as well as interactions with various adaptor proteins. Endocytosis is essential not only to the recycling/turnover of plasma membrane proteins, but it also functions in dynamic processes that recycle proteins back to the plasma membrane. Some evidence suggests that transcytotic trafficking mechanisms function in plants, although these are distinct from basolateral – apical redirection mechanisms characterized in animal cells.

## 1 Types of Trafficking at the Plasma Membrane

Protein trafficking at the plasma membrane (PM) involves (1) secretion of proteins to the PM or to the apoplast (exocytosis, secretion, or anterograde trafficking), (2) uptake of proteins at the PM for recycling or regulation of their activity (endocytosis or retrograde trafficking), and (3) moving proteins from one location on the PM to another (transcytosis). The integration of these processes is required for dynamic

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W.A. Peer

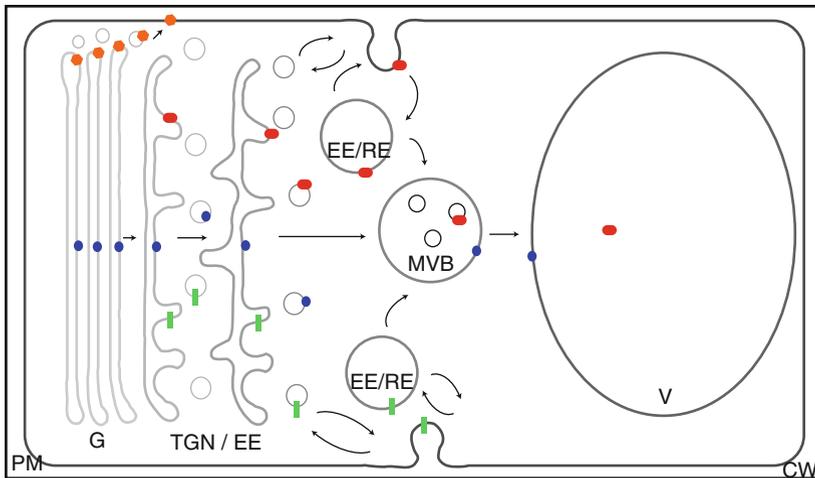
Department of Horticulture and Landscape Architecture, Purdue University, 625 Agricultural Mall Drive, West Lafayette, IN 47907-2010, USA

e-mail: peerw@purdue.edu

maintenance of cellular homeostasis following biotic or abiotic stimuli, such as herbivory, fungal infection, drought stress, or gravitropic or phototropic stimuli.

Secretion is the process by which proteins, lipids, and other molecules are trafficked, usually through the endoplasmic reticulum and Golgi-apparatus, and targeted to the plasma membrane (PM), extracellular space, or other organelles in the cytosol (Fig. 1). Endocytosis is the process by which nutrients, sterols, lipoproteins, peptide hormones, growth factors, and receptor-binding toxins are taken into cells. Endocytosis regulates the abundance and distribution of PM transport and receptor proteins, and it is an important mechanistic component of degradative and recycling mechanisms resulting in reuse of expensive transmembrane proteins and organelle homeostasis (Samaj et al. 2004). Transcytosis is well documented in animal systems where proteins undergo transcytotic mediation of apical to basolateral redirection. Animals have gap junctions, which define the polarity of cells. Although the structural/mechanical basis of cellular polarity has not been elucidated in plants, transcytosis has been documented in plants cells: during embryogenesis, the PIN1 auxin efflux carrier is translocated from opposite sides of the cell via a guanine-nucleotide exchange factor for ADP-ribosylation factor GTPase-dependent transcytosis-like mechanism (Kleine-Vehn et al. 2008a).

Proteins that are targeted to or function at the PM are integral or peripheral membrane proteins. Integral membrane proteins have a membrane spanning helix



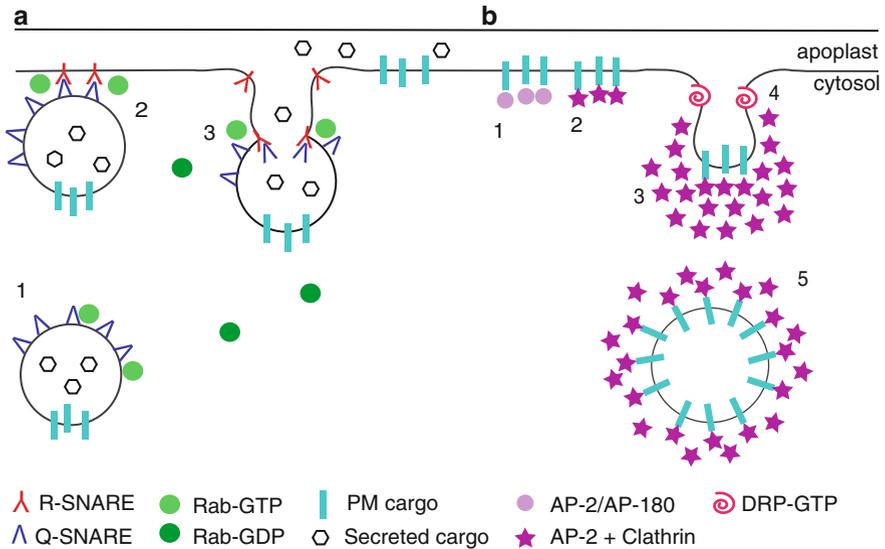
**Fig. 1** Overview of trafficking pathway: ER to Golgi to TGN to PM; ER to PM. (a) simplified view of trafficking of proteins through the endomembrane system to and from the plasma membrane. *Orange polygons* show Golgi to PM traffic. *Blue circles* show Golgi to tonoplast (vacuolar membrane) traffic through the endomembrane system. *Red ovals* show a PM protein that is undergoing turnover in the vacuole. *Green rectangles* show a protein that is constitutively recycled. *G* Golgi, *TGN* trans-Golgi network, *PM* plasma membrane, *EE* early endosome, *RE* recycling endosome, *MVB* multivesicular body (prevacuolar compartment), *V* vacuole, *CW* cell wall. The nucleus, mitochondria, plastids, actin filaments, microtubules and endoplasmic reticulum have been omitted for clarity. *Arrows* indicate the direction of movement

or helices that anchor them to the PM, while peripheral membrane proteins are associated with the membrane through hydrophobic regions of the protein and may or may not be associated with integral membrane proteins. Initially, a protein that is targeted to the PM is synthesized on the rough endoplasmic reticulum (ER) (Fig. 1). From there, the protein may be trafficked to the *cis*-Golgi via COPII-coated vesicles (type I protein) or may be directly targeted to the PM (type II protein). From the *cis*-Golgi, the protein may be trafficked back to the rough ER via COPI-coated vesicles, or may continue through to the *medial*- and *trans*-Golgi compartments. If the protein requires modification for proper function, such as glycosylation by the glycosyl transferases resident in the Golgi stacks, then the protein may undergo multiple rounds of trafficking among the Golgi stacks via COPI-coated vesicles. From the *trans*-Golgi, the protein then traffics through the *trans*-Golgi network (TGN) via clathrin-coated vesicles (CCVs). From there, the TGN/early endosome compartment, the protein may traffic to another compartment or organelle or to the PM. In plants, the TGN and early endosomes (EE) appear to be one compartment in contrast to animals where discrete compartments (identified via markers) have been visualized (Lam et al. 2007, 2009). The TGN/EE compartment is the intersection of the secretory and endocytosis pathways, with proteins from the *trans*-Golgi, PM, prevacuolar compartment, and multivesicular bodies (Lam et al. 2007, 2009). Once at the PM, the protein may remain there or be trafficked back to the TGN/EE compartment through endocytosis via CCVs or receptor-mediated endocytosis. Once there, the protein may be sorted to return to the PM or be targeted to another compartment or organelle. The vesicles move on actin filaments or microtubules (composed of tubulin), which are protein scaffolds of cellular structure.

## 2 Exocytosis/Secretion/Anterograde Trafficking

Exocytosis has several synonyms including secretion and anterograde trafficking. Proteins that are targeted to the PM include peripheral membrane proteins, such as ADP-ribosylation factor Ras GTPases (ARFs), and integral membrane proteins such as transporters, symporters, and channels. The residence time of the proteins on the PM in living cells may be transitory (e.g., ARFs) to semipermanent for the functional lifetime of the protein (e.g., PM H<sup>+</sup> ATPase). Exocytosis is required for the formation of the cell plates in newly divided cells and also for the polar growth of the tips of root hairs and pollen tubes. Secreted proteins and peptides are destined for the apoplast, such as cell wall remodeling enzymes or rhizosphere (reviewed in Mathesius 2009).

In order for the protein to traffic to the PM, the protein must be associated with a vesicle. There are several types of vesicles, including CCVs and exocysts, which have unique protein coats. Vesicle formation usually requires the adaptor protein (AP) complex to form CCVs, while vesicle fusion requires the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. Both vesicle



**Fig. 2** Vesicle fusion and formation. **(a)** An example of vesicle fusion with the PM. Vesicle fusion requires the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. In this example, the Q-SNARE on the vesicle is recognized by the R-SNARE on the PM (1, 2). Then GTP is hydrolyzed by Rab GTPase, and the vesicle fuses with the plasma membrane (3). The GDP-bound Rab is then released from the membrane to be regenerated in the Rab GTP-bound form. After vesicle fusion with the PM, proteins are localized at the PM or cargo within the vesicle is secreted to the apoplast. **(b)** An example of vesicle formation from the PM. Vesicle formation usually requires the adaptor protein (AP) complex to form clathrin-coated vesicles. In the example, AP complex binds cargo on the PM (1). Then clathrin is recruited to the AP-cargo complex (2). More and more clathrin is recruited until a clathrin-coated pit is formed (3). DRP interacts with the clathrin light chain and DRP-clathrin-mediated endocytosis occurs in plants, although it is not known if the DRP-GTPase functions in the same way in plants. DRP hydrolyzes GTP, and that hydrolysis may provide the energy for vesicle scission (4). Then the clathrin-coated vesicle is released (5)

formation and fusion require energy. Figure 2 illustrates vesicle fusion and formation (budding/scission).

## 2.1 Clathrin-Coated Vesicles

Plant CCVs (50–90 nm) are smaller than their mammalian counterparts (120 nm), most likely because of the rigid cell wall and turgor pressure of plant cells (1–4.5 MPa). Three heavy (180–190 kDa) and three light (30–50 kDa) clathrin chains make up the basic unit of the clathrin coat in both plants and animals, but the chains are 10–15 kDa larger in plants (Holstein et al. 1994). Clathrin coats can spontaneously assemble in low ionic strength and low pH buffer, but clathrin

assembly requires adaptor proteins (AP) under physiological conditions to recruit cargo proteins. CCVs also function in secretion.

The mechanism of CCV assembly and uncoating has been studied in yeast and animal cells, but it has largely been inferred in plants. ADP-ribosylation factor Ras GTPases (ARFs) can stimulate phosphatidylinositol production in the membranes, particularly phosphatidylinositol 4-phosphate (PtdIns4P) in *trans*-Golgi and phosphatidylinositol 4,5 biphosphate (PIP2) in the PM, where they function as coat protein-docking sites. In mammals, activated GTP-bound ARF1 recruits AP-1, GGA, and clathrin at the *trans*-Golgi, and ARF6 recruits AP-2, clathrin, and other components at the PM. In mammals, direct interactions between ARF6 and H<sup>+</sup> V-ATPase subunits are dependent on luminal pH, establishing a link between endosomal acidification, recruitment of coat proteins, and trafficking (Hurtado-Lorenzo et al. 2006; Recchi and Chavrier 2006; Marshansky 2007). Uncoating of CCVs occurs via the activity of Hsc70 and its cofactor auxilin before fusion with specific endosomes/organelles (Ungewickell et al. 1995). In mammalian cells, dissociation of clathrin coats and APs from early endosomes (pH 5.9–6) may be dependent on acidification of CCVs derived from the PM (pH 7.0) (Lemmon 2001; Schlossman et al. 1984). In plant cells, CCVs derived from the PM are thought to retain the extracellular pH of 5.0–5.5, which is already lower than acidified mammalian early endosomes, suggesting differences between AP/clathrin-mediated vesicle trafficking and sorting in plants and animals.

## 2.2 *Adaptins and Adaptor Protein Complexes*

AP complex function has been extensively studied in animals and yeast (Murphy et al. 2005). Adaptor proteins can be classified into two groups: monomeric and heterotetrameric. Animals have several monomeric adaptors like AP180,  $\beta$ -arrestin, GGA, and stonins, but plants have only one known monomeric adaptor, an ortholog of AP180 (Barth and Holstein 2004).

Four heterotetrameric adaptor complexes have been identified: AP-1, AP-2, AP-3, and AP-4. Each of the AP complexes is made up of two large adaptin subunits (one of  $\gamma/\alpha/\delta/\epsilon$  and one  $\beta$ 1–4, respectively, 90–130 kDa), one medium adaptin ( $\mu$ 1–4, 50 kDa), and one small adaptin ( $\sigma$ 1–4, 20 kDa). AP-1, AP-2, and AP-3 are found in all eukaryotes, but AP-4 is found in mammals, plants, birds, and slime mold but not in insects or yeast (Robinson and Bonifacino 2001). All four AP complexes have been shown to associate with clathrin, although a clear clathrin-binding motif has not been identified in AP-4 (Barois and Bakke 2005). AP-1, AP-3, and AP-4 associate with the TGN and other vesicular compartments, and AP-2 associates with the PM and is responsible for rapid endocytosis from the membrane (Robinson and Bonifacino 2001). Essentially, AP-1 mediates secretion from TGN to endosomes. AP-2 mediates endocytosis from the PM in mammals and yeast. AP-3 mediates trafficking from *trans*-Golgi to the vacuole in yeast. AP-4 function is not well characterized.

Knockout mutations in the adaptins of the AP-1 and AP-2 complex are lethal in the embryos of mice and insects (reviewed in Ohno et al. 2006). Deletion of AP-3 adaptins results in pigmentation defects in humans, mice, and fruit flies, but it is lethal in the embryo of nematodes, and results in neurological defects in mice (Ohno et al. 2006). Deletions in AP-4 adaptins have not been recovered in animal model systems, but AP-4 appears to have some role in Golgi to lysosome trafficking (Barois and Bakke 2005) and basolateral redirection (Simmen et al. 2002), although its function has not been demonstrated.

The Arabidopsis genome encodes orthologs of animal clathrin-mediated vesicular trafficking proteins (Boehm and Bonifacino 2001). Adaptin orthologs in Arabidopsis identified by propeptide sequence alignments (Sanderfoot and Raikhel 2002; Boehm and Bonifacino 2001) indicate that there are four AP complexes and, therefore, four  $\beta$ ,  $\sigma$ , and  $\mu$ -adaptin isoforms. There are also three  $\gamma$ , two  $\alpha$ , and one each  $\delta$  and  $\epsilon$  adaptin. Tentative annotations of the adaptins were based on similarity with animal adaptins. However, these assignments are based on differences in a small number of residues, and experimental evidence is needed for either validation or reassignment. Of the four  $\beta$ -adaptins,  $\beta 1$  and  $\beta 2$  have 92% sequence identity (MatGAT, Campanella et al. 2003). High sequence identity of  $\beta 1$  and  $\beta 2$  suggests that they may be in AP-1 and/or AP-2 (Boehm and Bonifacino 2001). Recently, Dacks et al. (2008) concluded that  $\beta 1$  and  $\beta 2$  arose from a gene duplication event. Therefore, assignment of the  $\beta$ -adaptin isoforms to AP complexes based on experimental evidence is an outstanding question.

The AP180,  $\mu$ , and  $\sigma$ -adaptins from Arabidopsis have been characterized (Happel et al. 2004; Barth and Holstein 2004; Holstein and Oliviusson 2005), and structural subunits  $\alpha$ ,  $\delta$ ,  $\gamma$ -adaptins have been partially characterized by inference (Song et al. 2009; Lee et al. 2007).  $\beta$ -adaptins in plants were first identified by Holstein et al. (1994) but have not been investigated further. These reports suggest that AP-1 mediates TGN to vacuole trafficking, while AP-3 mediates TGN to vacuole trafficking via a different vesicular population than AP-1. AP-2 functions in endocytosis. AP-4's function is unknown.

### 2.3 Vesicle Fusion with the Plasma Membrane

Once the vesicle traffics to the PM, the vesicle docks at the PM prior to fusion. The soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes are composed of Q-SNAREs (Qa, Qb, Qc) and R-SNAREs, categorization based on sequence motifs (Fasshauer et al. 1998, Bock et al. 2001). Q-SNAREs are syntaxins of plants (SYPs) associated with synaptosome-associated proteins (SNAPs) at the PM, while R-SNAREs are vesicle-associated membrane proteins (VAMPs), which contain a longin domain required for subcellular sorting and vesicle targeting (Uemura et al. 2005). Q-SNAREs and R-SNAREs form complementary pairings that regulate specificity of docking for vesicle fusion and form a ternary complex with SNAPs to facilitate vesicle fusion (Kwon et al. 2008).

The Arabidopsis genome has 54 SNARE genes (18 Qa-SNAREs/Syntaxins, 11 Qb-SNAREs, 8 Qc-SNAREs, 14 R-SNAREs/VAMPs and 3 SNAP-25s) (Uemura et al. 2004), many of which are plant-specific and those associated with the PM have roles in cytokinesis, hormone responses, and pathogen resistance (Collins et al. 2003). Nine Qa-SNAREs are on the PM: SYP111/KNOLLE, SYP112, SYP121, SYP122, SYP123, SYP124, SYP125, SYP131, and SYP132 (Uemura et al. 2004). Some of these Qa-SNAREs show ubiquitous expression and uniform localization on the PM (SYP132), while others show polarized localization in the growing tips of root hairs (SYP123) or pollen tubes (SYP131) (Enami et al. 2009), suggesting diverse function and selective recognition of vesicles prior to fusion. Four Qb-SNAREs (VTI11, NPSN11, NPSN12 and NPSN13) are on the PM, while VTI12 regulates transport between TGN/EE compartment and the PM (Uemura et al. 2004). Thus far, one Qc-SNARE (SYP71) has been characterized as on the PM as well as the ER (Suwastika et al. 2008), suggesting that it regulates trafficking between the ER and PM that bypasses the Golgi/TGN pathway. SNAP-25s (SNAP29, SNAP30, SNAP33) are targeted to the PM by posttranslational modification (Gonzalo et al. 1999).

R-SNAREs have a longin domain, as mentioned above, and five are associated with the PM (VAMP721, VAPM722, VAPM724, VAPM725, VAMP726) (Uemura et al. 2004). However, a subclass of the VAMP72 group does not have a SNARE motif in the central region (Vedovato et al. 2009). These non-SNARE longin proteins are plant-specific (phytolongins), suggesting that additional components are involved in vesicle sorting, targeting, and subsequent fusion. Q-SNAREs and SNAP-25s can have polar localization and associations with lipid rafts, presumably for targeted R-SNARE delivery of cargo to these lipid domains.

Vesicle fusion requires energy, typically through the hydrolysis of GTP via the Rab family of GTPases, a subset of the Ras GTPase superfamily (Rutherford and Moore 2002). Rabs are small GTP-binding proteins that cycle between the active GTP-bound state and inactive in the GDP-bound state. GTP-bound Rab associates with the PM and also recruits other factors to the PM, while GDP-bound Rab dissociates from the PM (Rutherford and Moore 2002). The Arabidopsis genome encodes 57 Rab proteins. The Rab-A family functions in TGN/EE to PM trafficking, with the Rab-A2 and Rab-A3 GTPases also playing a role in cell plate formation (Chow et al. 2008), while the Rab-A4d GTPase is instrumental in regulating polarized growth, such as root hair tips and pollen tubes (Preuss et al. 2004; Szumlanski and Nielsen 2009). Rab-E is also involved in post-Golgi secretion to the PM and appears to have a role in plant defence (Speth et al. 2009). GTP-bound Rab-E interacts with phosphatidylinositol-4-phosphate (PtdIns4P) 5-kinase 2 (PIP5K2) on the PM and stimulates PIP5K2 kinase activity (Camacho et al. 2009). The interaction between active Rab-E GTPase and PIP5K2 may increase localized PtdIns(4,5)P<sub>2</sub> production on the PM (Camacho et al. 2009). This may enhance endocytosis and thereby balance the rates of exocytosis and endocytosis (Zoncu et al. 2007; Camacho et al. 2009). After vesicle fusion, GTP-bound Rab is regenerated and can return to the TGN/EE, where it can participate in another round of vesicle fusion.

Acidification mechanisms regulate vesicle trafficking and fusion in yeast, animals, and plants. The vacuolar V-ATPase is required for secretion, endocytosis, Golgi organization, and vacuole function in embryogenesis (Dettmer et al. 2005, 2006; Strompen et al. 2005). V-PPase AVP1 has a role in secretion of the PM H<sup>+</sup>ATPase (Li et al. 2005). In addition, the V-ATPase and V-PPase may physically interact (Fischer-Schliebs et al. 1997), which has further implications for regulatory mechanism of pH on trafficking.

## 2.4 *Exocyst*

The exocyst, sometimes referred to as the Sec6/8 complex, is a specialized complex that is involved in tethering vesicles to PM prior to SNARE docking and subsequent membrane fusion. It is a heteromeric complex composed of eight proteins in yeast and mammals with homologs in plants: Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p (Hála et al. 2009; Samuel et al. 2009; Chong et al. 2009). Seven of these have been experimentally identified in the complex thus far, with Exo84p as the outlier (Hála et al. 2009).

SEC6 and SEC8 localize in the growing tips of tobacco pollen tubes (Hála et al. 2009). In yeast two-hybrid assays, EXO70A1/SEC3a, SEC15b/SEC10, and SEC6/SEC8 pairs showed strong interactions (Hála et al. 2009). The mutational data suggests that exocysts are involved in many cellular functions ranging from cell wall formation to polar auxin transport to self-incompatibility (reviewed in Hála et al. 2009; Samuel et al. 2009). Chong et al. (2009) demonstrated that exocyst subunits Sec15 and Exo70 colocalized with SNAREs in transient expression in BY-2 cells, and Exo70 can recruit Sec5, Sec8, Sec15, and Exo84 components to form the exocyst (Chong et al. 2009). Analysis of loss-of-function mutants, including *exo84p*, shows pleiotropic defects including pollen germination and pollen tube growth (Hála et al. 2009).

## 2.5 *Secretory Vesicle Cluster*

The secretory vesicle cluster (SVC) is a linked set of secretory vesicles distinct from the Golgi and TGN/EE, although it appears to originate from the TGN/EE in tobacco BY-2 cell cultures (Toyooka et al. 2009). The SVC is characterized by several markers: the secretory carrier membrane protein 2 (SCAMP2), the SNARE SYP41, and the small GTPase Rab11-D; SVCs are not associated with CCVs (Toyooka et al. 2009). Among the markers that characterize SVCs are JIM7, a monoclonal antibody against homogalacturonan of pectic polysaccharides (Clausen et al. 2003), a cell wall component. SCVs appear to be involved in mass secretion to the PM in nondividing cells and are targeted to the cell plate in dividing cells (Toyooka et al. 2009) consistent with a role in secreting pectins.

## 2.6 *The Microtubule-Associated Cellulose Synthase Compartment*

The plasma membrane is the interface between the cytosol/symplast and cell wall/apoplast. The proteins on the PM are among the regulators of the components that enter and exit the cell. The cell wall is comprised of carbohydrates (e.g., cellulose, hemicelluloses, pectins), polyphenols (e.g., lignins), minerals (e.g., boron, silica), and sometimes waxes (e.g., suberin, cutin) that are secreted into the extracellular space. Once in the apoplast, these cell wall components often undergo modification due to developmental programs (e.g., cell enlargement, cell loosening in maternal tissues for pollen tube extension) or in response to biotic or abiotic stressors. These changes can be achieved by secretion of cell wall remodeling proteins, which may function enzymatically (cellulases, pectinase) or nonenzymatically (expansions) (reviewed in Lebeda et al. 2001; Sampedro and Cosgrove 2005).

Cellulose is synthesized by rosette-shaped cellulose synthase complexes (CSCs), ~25–30 nm, composed of cellulose synthase proteins (CESAs) on the PM and by a cytosolic component, ~45–50 nm (Mueller and Brown 1980; Bowling and Brown 2008). CESA3 is found in four different compartments using functional fluorescent protein fusions and immunogold labeling techniques: Golgi bodies (Paredez et al. 2006), TGN/EE (Crowell et al. 2009), a unique microtubule associated cellulose synthase compartment (MASC) that originates from the medial- or *trans*-Golgi (Crowell et al. 2009), and a population of small CESA compartment (SmaCCs) (Paredez et al. 2006). Tethered SmaCCs colocalize with microtubules (Paredez et al. 2006), and this tethered population may be synonymous with MASCs. Microtubules have been shown to define the trajectory of the CSCs (Paredez et al. 2006; Gutierrez et al. 2009), and intact, functional microtubules are required for MASC trafficking (Crowell et al. 2009). As the Golgi apparatus moves along the cortical microtubules, the Golgi bodies and TGN/EE pause on discrete sites, which are in proximity to MASCs (Crowell et al. 2009). The pause in trafficking occurs when the Golgi body is beneath the PM and leads to localized increases of CSC density. Crowell et al. (2009) conducted fluorescence recovery after photobleaching (FRAP) experiments and showed that CSC was not inserted randomly into the PM but followed the linear tracks of the microtubules and CSCs were inserted in rows. They went on to hypothesize that CSCs were associated with the TGN/EE only during endocytosis, as has been shown for the auxin transporter PIN2 (Robert et al. 2008), and that MASCs are the result of CSC internalization as MASCs correspond with the decrease in CSCs at the PM.

## 3 Endocytosis/Retrograde Trafficking

Endocytosis results in the internalization of nutrients, sterols, lipoproteins, hormones, receptor-binding toxins, and transport and receptor proteins from the PM. The proteins are trafficked back to the TGN/EE where they are sorted and either

recycle back to the PM or are targeted to another organelle where the protein may be modified and then redirected back to the PM or to another organelle, such as the lytic vacuole or peroxisome. Therefore, endocytosis and exocytosis pathways partially overlap and converge in the TGN/EE (Fig. 1). Coordination of endocytosis and exocytosis is required for homeostasis of the PM, since the PM and its components are taken up into the cell and the PM must be replenished for the mature cell to maintain size and integrity, and therefore function.

Endocytosis can be classified by the types of cargo and molecular machinery driving its internalization: clathrin-mediated endocytosis, caveolae/lipid raft-mediated endocytosis, and fluid phase endocytosis. Fluid phase vesicles are 0.5–2  $\mu\text{m}$  in diameter and larger than CCVs (~30–100 nm) and caveolin vesicles (~50 nm) (Johannes et al. 2002; Dhonukshe et al. 2007). In animals, fluid phase endocytosis is dependent on the concentration of endocytosed soluble molecules, but receptor-mediated endocytosis, including that involving CCVs, is saturable, and thus is consistent with membrane localized receptors. Caveolae are composed of cholesterol, sphingolipids, and GPI (glycosylphosphatidylinositol)-anchored protein-rich microdomains on PM (Brown and London 2000). Although there are no published data about caveolin in plants, structural sterols such as stigmaterol, sitosterol, and sphingolipid are thought to organize lipid rafts in plants instead of cholesterol (Mongrand et al. 2004).

Most endocytotic vesicles originate as CCVs (Brett and Traub 2006), and clathrin-mediated endocytosis is better characterized in mammals than plants. Despite the long-standing evidence of CCVs in plants (Holstein et al. 1994), clathrin-mediated endocytosis has only recently been demonstrated (Dhonukshe et al. 2007; Leborgne-Castel et al. 2008). There are at least two endocytosis pathways in plants: one is characterized by the styryl dye FM4-64, SCAMP1, and Rab-F2 (ARA7/RHA1), and the other is characterized by SCAMP2 (Toyooka et al. 2009).

### 3.1 *Clathrin-Mediated Endocytosis*

The adaptor proteins AP-2 and AP-180 mediate clathrin cage assembly. AP-2 is a heterotetramer like AP-1, AP-3, and AP-4, as described earlier, but AP-180 functions as a monomer and also appears to interact with  $\alpha\text{C}$ -adaptin (AP-2 subunit) (Barth and Holstein 2004). The AP-2 subunit  $\mu\text{2}$  is responsible for cargo selection, and until recently was thought to be the only subunit involved in cargo selection. The  $\beta\text{2}$  subunit has also been shown to participate in cargo selection independent from  $\mu\text{2}$  in mammals, and a point mutation in  $\beta\text{2}$  cannot recruit specific proteins (e.g.,  $\beta$ -arrestin or autosomal recessive hypercholesterolemia protein) to clathrin structures (Keyel et al. 2008).

Cargo selection and sorting signals for recruitment into the clathrin-mediated endocytotic pathway are the tertiary structures of SNAPs, ubiquitin-tagged proteins, and Yxx $\emptyset$  (where  $\emptyset$  is a bulky hydrophobic residue) on the C terminus of

proteins recognized by the AP complexes and the acidic dileucine [DE]<sub>xxx</sub>L [LIM] motifs recognized by  $\alpha$ ,  $\sigma$ 2, and GGAs (Golgi-localizing,  $\gamma$ -adaptin ear homology domain, ARF-interacting proteins) (Keyel et al. 2008; Kelly et al. 2008). The AP-2 complexes appear to have differential binding affinities for the various acidic dileucine motifs and therefore afford specificity for internalization of proteins from the PM. It appears that the affinities of  $\beta$ 2 or  $\mu$ 2 for [DE]<sub>xxx</sub>L [LIM] are based on competitive binding, binding pockets, and the structure of the cargo protein (Kelly et al. 2008).

While vesicle fusion requires energy to overcome the hydrophobic and electrostatic forces required for vesicle fusion, vesicle formation (or scission) can occur spontaneously without ATP or GTP hydrolysis, via chemical changes in the clathrin lattice (Mashl and Bruinsma 1998); the frequency of this occurrence is unknown. More commonly, dynamin and dynamin-related protein (DRP) GTPases are active participants in endocytosis and complete vesicle fission via GTP hydrolyses (Fig. 2).

Dynamin and DRPs are large GTPases with multiple roles in protein trafficking, and cell and organelle division (Konopka et al. 2006), and Konopka et al. (2008) showed that plant dynamins play a role in clathrin-mediated endocytosis. In Arabidopsis, 16 genes, divided into six families, are predicted to encode dynamins (Hong et al. 2003; Gao et al. 2006). DRP2 interacts with the  $\gamma$  subunit of AP-1 (Jin et al. 2001), which suggests a role for DRP2 in clathrin-mediated trafficking to the PM. The mammalian dynamin 1 is most similar to plant DRP2. In contrast, the plant-specific DRP1 subfamily is necessary for cell expansion and division (Kang et al. 2001; 2003) and plays an active role in endocytosis (Konopka et al. 2008). The DRP1 subfamily has five isoforms (A–E), and so far, only partial functional redundancy has been observed (Konopka and Bednarek 2008). Using a combination of approaches and fluorescently tagged DRP1C and the clathrin light chain, Konopka et al. (2008) showed that DRP1C and clathrin light chain are simultaneously recruited to sites on the PM that are active in protein trafficking about 70% of the time, in contrast to the stepwise recruitment observed in mammals. Fluorescence of the fused proteins was not observed following endocytosis, presumably due to dissociation of the proteins from the complexes. The DRP1-clathrin-mediated endocytosis is not directly linked to actin polymerization but is mediated by microtubules.

### ***3.2 Receptor-Mediated Endocytosis***

Receptor-mediated endocytosis, sometimes called ligand-mediated endocytosis, results in the internalization of diverse molecules, such as hormones or peptides. Ligand binding increases the rate of endocytosis of the receptor, which either results in attenuation of the signal transduction cascade initiated at the PM [e.g., G protein-coupled receptors (GPCRs)] or promotion of the signal transduction

cascade if it occurs in the endosome (e.g., FLS2 and BRI1), and therefore effecting changes in transcription, metabolism, or trafficking.

Comparison of the animal and plant models is complicated by a proliferation of mechanisms in mammals, especially those involving GPCRs (Wolfe and Trejo 2007). GPCRs are heterotrimeric proteins that regulate signal transduction, and they are composed of  $G\alpha$ ,  $G\beta$ , and  $G\gamma$  subunits. Recent evidence indicates that a GPCR regulates the signaling response of the plant hormone abscisic acid (Pandey et al. 2009). Interestingly, it appears that the GDP-bound GTGs (GPCR-type G proteins 1 and 2) are the active signaling forms and not the GTP-bound forms. Recently, a nonprototypical G-protein complex has been shown to play a role in disease resistance (Zhu et al. 2009).

The receptor-like kinases (RLK) family has 610 members in Arabidopsis and contains receptor kinases and nonreceptor kinases (receptor-like cytoplasmic kinases), and RLKs in plants have been divided into 15 families (Shiu and Bleecker 2003). RLKs are transmembrane proteins with a cytoplasmic kinase domain, while the extracellular domain is variable. Self-incompatibility in Brassicaceae is regulated by the S-locus cysteine-rich protein (SCR) in the pollen which binds to the S-locus receptor kinase (SRK) in the stigma resulting in inhibition of pollen tube growth (reviewed in Peer and Murphy 2005). Serendipitously, most of the research has been on what turned out to be leucine-rich repeat (LRR) receptors, which have great diversity and function in nearly every aspect of plant growth and development and defense against pathogens. In one instance, there appears to be an overlap in the endocytotic pathways of BRI and FLS2 via BAK1/SERK1 (BRI1-associated kinase 1/serine and proline rich receptor kinase 1). BRI1 is the brassinosteroid receptor, and after brassinolide binding to BRI1, BRI1 forms a dimer and phosphorylates BRI1 kinase inhibitor 1, which then dissociates from BRI1. Then BRI1 and BAK1 form a heterodimer complex that undergoes endocytosis (reviewed in Chinchilla et al. 2009). Brassinolide signaling then occurs from the endosomal compartment.

Similarly, flagellin, a bacterial protein that comprises flagella, binds to the FLS2 receptor (flagellin sensitive 2) on the plant PM. Then FLS2 and BAK1 form a heterodimer, which is internalized, and the defense signal in response to the pathogen then occurs from the endosomal compartment (Robatzek 2007; Robatzek et al. 2006; Kwon et al. 2008; reviewed in Chinchilla et al. 2009). It is also possible that FLS2 and BAK1 are associated with each other before ligand binding, and the conformational changes that follow ligand binding produce the stable heterodimer. Systemin, an 18 amino acid peptide derived from prosystemin, is another example of an endogenous peptide ligand to an LRR that is involved in defense responses after mechanical wounding (e.g., herbivory) (Ryan et al. 2002). While BRI1 is able to bind systemin, it appears that the defense response is not elicited via BRI1, and that the yet unidentified systemin receptor is specifically in the vascular tissue, as is prosystemin (Malinowski et al. 2009). Cryptogein is a secreted fungal protein that binds to an unidentified PM receptor and stimulates a signal transduction response of which one of the results is rapid clathrin-mediated endocytosis. Interestingly,

cryptogein induction of CCV endocytosis occurs via reactive oxygen species (Leborgne-Castel et al. 2008).

Phytosulfokine (PSK) is an endogenous five amino acid sulfonated peptide [H-Tyr(SO<sub>3</sub>H)-Ile-Tyr(SO<sub>3</sub>H)-Thr-Gln-OH] that is involved in cell proliferation and elongation (Matsubayashi et al. 2002). High affinity and low affinity PSK receptors were identified (Matsubayashi et al. 1997), and subsequently, an LRR PSK receptor was purified (Matsubayashi et al. 2006). CLV3 (CLAVATA 3) is a 79 amino acid signaling polypeptide along with the LRR's CLV1 and CLV2, which are important for meristem cell maintenance (reviewed in Wang and Fiers 2009). ACR4 (ARABIDOPSIS CRINKLY4) is an LRR required for cell layer organization, and its ligand is CLE40 (CLAVATA/ENDOSPERM SURROUNDING REGION40).

### 3.3 *Sorting and the Return Trip to the PM*

Since the TGN and early endosome (EE) compartments overlap (Lam et al. 2007), both outward- and inward-bound vesicles are present in this compartment. Therefore, there must be a mechanism to sort which cargo continues on to other compartments or returns to the PM. The recycling mechanisms result in the reuse of expensive transmembrane proteins and degradation of the damaged or unneeded proteins. Therefore, there are several trafficking pathways that converge in endosomes: endosome to PM trafficking, endosome to TGN retrieval, and endosome to vacuole targeting.

In addition to the sorting signals involved in cargo selection described above, the ADP-ribosylation factor (ARF)-GTPase and Rho-GTPase of plants (ROP-GTPase) proteins and the proteins regulating their function are essential components regulating traffic to and from the PM. ARF-GEFs (ARF-guanine nucleotide exchange factors) catalyze the GTP-bound form of ARF, while ARF-GAPs (ARF-GTPase-activating proteins) catalyze the GDP-bound form of ARF GTPases, and therefore regulate the activity of the ARFs. Similarly, the ROP-GEFs and ROP-GAPs recycle the active and inactive forms of the ROP-GTPases (reviewed in Yalovsky et al. 2008; Payne and Grierson 2009).

ADP-ribosylation factor-guanine-nucleotide exchange factors (ARF-GEF) such as GNOM and GNOM-LIKE 1 are instrumental in recruiting the protein coats for vesicle formation and also for cargo selection (Donaldson and Jackson 2000; Richter et al. 2007; Teh and Moore 2007). GNOM is involved in recycling of proteins, like the PIN auxin transporters, from endosomes to the PM (Geldner et al. 2003), while GNOM-LIKE 1 is important for trafficking of the ABCB auxin transporters (Titapiwatanakun et al. 2009). The ADP-ribosylation factor-GTPase-activating protein (ARF-GAP) VAN3/SCARFACE (Koizumi et al. 2005; Sieburth et al. 2006) is involved in leaf vein patterning and is required for correct trafficking of auxin efflux carriers and auxin signaling. The ligand for VAN3 appears to be specific phosphoinositides generated by the polyphosphate 5'-phosphatases

COTYLEDON VASCULAR PATTERN2 (CVP2) and CVP2 LIKE1 (CVL1) (Carland and Nelson 2009).

Rho-GTPases of plants (ROPS) are also important in developmental patterning. For example, SCN1, a RhoGTPase GDP dissociation inhibitor (RhoGDI), restricts ROP activity to one focus on the PM to produce a single root hair (Carol et al. 2005), while overexpression of constitutively active ROP2 produces two root hairs in one cell (Jones et al. 2002), indicating that two foci develop on the PM instead of one. ROP2 is also light-regulated, and expression of constitutively active ROP2 is observed on the PM and inhibits stomatal opening, while constitutively inactive ROP2 is cytosolic (Jeon et al. 2008). In animals, GTPases with both ARF-GAP and Rho-GAP domains have been identified, suggesting cross-talk between the pathways (Miura et al. 2002). Although these have not yet been described in plants, mutational analyses of ARF1 indicates genetic interactions with ROP2 and trafficking of PIN2, which is mediated by the ARF-GEF GNOM (Xu and Scheres 2005; Kleine-Vehn et al. 2008b).

There are also sorting signals like the retromer protein complex, characterized by VPS cargo recognition heterotrimer [VPS35 (a-c), VPS26 (a,b), VPS29], and SNX1 (sorting nexin 1) (reviewed in Otegui and Spitzer 2008), which return receptors and other proteins from the MVB to the TGN/early endosome. SNX1 signals are also observed in GNOM-containing endosomes, consistent with SNX1 involvement in recycling of PM proteins. SNX1 signals overlap with the prevacuolar compartment/multivesicular body, and markers for the secretory (BP80) and endocytotic (BR1, PIP2a) pathways show that both overlap in SNX1-containing endosomes (Jaillais et al. 2008), and these data suggest a role in recycling proteins from the MVB back to the PM. However, trafficking inhibitors, such as the fungal inhibitor wortmannin, resulted in mistargeting of the PM proteins to the lytic vacuole (Jaillais et al. 2008).

### **3.4 Endosomes and Multivesicular Bodies**

Following endocytosis, the vesicles fuse with endosomes. The endosomal compartment is comprised of a gradient of endosomal populations, which can be loosely defined as early, late, and recycling endosomes. As discussed above, in contrast to animal cells, and the early endosome and TGN compartments overlap in plants (Lam et al. 2007); therefore, discrete assignment of the membrane populations is not possible. Spatially or functionally discrete endosomes are identified by markers (often ARF-GEFs, RABs or SYPs), which also have overlapping or partial localizations in the endosomal populations. Endosomes are also unique, multifunctional organelles as both the BR1 and FLS2 receptors appear to signal from the endosomes and not from the PM. Recycling endosomes, as discussed above, have been shown to have at least two pathways, GNOM-dependent and GNOM-independent (reviewed in Otegui and Spitzer 2008).

The late endosomes are often synonymous with multivesicular bodies (MVBs), also known as the prevacuolar compartment. MVBs may be the most complex endosome, containing many intraluminal vesicles of proteins and macromolecules resulting from endocytosis. The intraluminal vesicles form when an endosome invaginates and buds into its own lumen. Although the signal(s) for this are complex and not well understood, many of the proteins in the intraluminal vesicles are ubiquitinated. The intraluminal proteins are usually targeted to the lytic vacuole for degradation, although this is yet to be shown for plant PM proteins. Sorting of cargo in MVBs involves the ESCRT (endosomal sorting complexes required for transport) and ESCRT-related CHMP1A and B (charged MVB protein/ chromatin modifying protein 1A and B) proteins (Spitzer et al. 2009). Recently, through molecular genetic and cell biology techniques, Spitzer et al. showed that the auxin carriers PIN1 and 2 and AUX1 are MVB cargo sorted by the ESCRT machinery, and this function is lost in *chmp1a chmp1b* double mutants.

## 4 Role of the Cytoskeleton in Plasma Membrane Protein Trafficking

The cytoskeleton provides the scaffolding or framework for the shape for the cell, and protein trafficking to the PM requires motorized movement of the vesicles to and from the PM. This is hypothesized to occur on either the actin cytoskeleton or on microtubules. Experimental evidence exists for microtubule involvement in secretion to the PM, and microtubule and actin participation in endocytosis from the PM. Although the role of actin in secretion to other organelles has been demonstrated, an active role for actin in secretion to the PM remains an outstanding question (Staiger et al. 2009). The cytoskeleton also provides the framework for new cell plate formation. The cytoskeleton establishes the preprophase band where the new cell plate will form and coordinates vesicle trafficking from the center of the cell plate towards the periphery resulting in two daughter cells.

### 4.1 Actin

A role of actin in secretion to the PM has been hypothesized, as a treatment with actin inhibitors reduces mucilage production (Hawes et al. 2003). However, ROP1 assembly and disassembly occurs via RICs (ROP-interactive CRIB-containing proteins): actin disassembly via RIC3 is required for exocytosis, while RIC4-mediated actin assembly resulted in polar vesicle accumulation during tip growth (Gu et al. 2005; Lee et al. 2008). A role for actin has also been demonstrated for clathrin-mediated endocytosis. The motive force for endocytosis not only involves the clathrin triskelia, and the energy released from GTP hydrolysis via the large and

small GTPases, but also the force generated by actin polymerization (Conner and Schmid 2003). Actin may organize endocytotic “hotspots,” and accessory proteins have been shown to bind to actin (Qualman et al. 2000; Conner and Schmid 2003; Samaj et al. 2004). Use of chemical inhibitors of actin polymerization, such as the fungal toxin latrunculin B, also points to the importance of actin as part of the endocytotic machinery (Blancaflor et al. 2006).

## 4.2 *Microtubules*

However, protein trafficking in plants utilizes both actin and microtubules for secretion and endocytosis. Secretion and endocytosis of the cellulose synthase complex (CSC) occurs via microtubules (Paredes et al. 2006; Gutierrez et al. 2009; Crowell et al. 2009). Cortical microtubules may also carry the complexes that secrete mucilage during germination (McFarlane et al. 2008). Endocytosis of the cellulose synthase complex (CSC) does not appear to occur via clathrin-coated vesicles (CCVs) (Crowell et al. 2009), since the smallest plant CCVs observed are 30 nm in diameter, while CSCs are 25 nm. It seems likely, however, that other adaptor or accessory proteins may be required for CSC endocytosis. Microtubules are also required for clathrin-mediated endocytosis that utilizes dynamins (Konopka and Bednarek 2008). Therefore, microtubules are involved in both clathrin- and nonclathrin-mediated endocytosis, as well as marking the sites of cellulose and pectin secretion.

## 5 **Models of Trafficking**

Cellular trafficking occurs in all living cells to maintain homeostasis and respond to cellular communication and biotic and abiotic stimuli. Rates of endocytosis and exocytosis in root hairs and pollen tubes were estimated by Ketelaar et al. (2008). Based on the amount of membranes and cell wall material needed to be inserted for growth and the amount of extra membrane that would need to be recycled via endocytosis, they calculated that there was an excess of 86.7% membranes in root hairs and 79% in pollen tubes. They then calculated that if secretion were inhibited, the cells would continue to grow for 33 more seconds. Ketelaar et al. tested this experimentally, and measured that growth continued for 30–40 s, in the range that their model predicted. Therefore, trafficking to and from the PM is a dynamic process, and there may also be some lag time in responding to the stimuli as vesicles are already in motion. Trafficking to and from the PM may be induced, as observed in receptor-mediated endocytosis, or constitutive as appears to be the case for some of the PM-localized PIN auxin transporters. Other types of trafficking are specialized and specific to certain cell types or developmental processes, such as the polar tip growth



of pollen tubes and root hairs and the formation of the cell plate during cell division, and responses to pathogens. Models of each type of trafficking are presented in Fig. 3.

## 5.1 Constitutive

An example of a constitutively cycled protein is the auxin efflux carrier PIN1, which also displays polar localization in the stele of root tissues. Proper PIN1 polar localization on the PM is dependent on a brefeldin A-sensitive ARF-GEF GNOM (Geldner et al. 2003). The hormone auxin itself affects the polar localization of PIN1 (Peer et al. 2004), which was attributed to auxin inhibition of endocytosis (Paciorek et al. 2005). Vesicular cycling of PIN1 is also sensitive to the auxin transport and trafficking inhibitor *N*-1-naphthylphthalamic acid and trafficking inhibitors such as brefeldin A (BFA) (Geldner et al. 2003; Peer et al. 2004). Recently, Dhonukshe et al. (2007) manipulated the Adaptor Proteins by over-expression of the heavy clathrin chain and used a kinase inhibitor (tyrphostin A23) that has been shown to inhibit  $\mu$ -adaptin interaction with cargo (Dhonukshe et al. 2007; Ortiz-Zapater et al. 2006). The result was altered rates of CCV-mediated PIN1 endocytosis, although PIN1 appears to lack a  $\mu$ -adaptin-binding site. Therefore, polar and asymmetric localization of auxin transport proteins is mediated by CCVs, and specific adaptins/APs are essential to the establishment and maintenance of cellular polarity in plants. As AP-4 in mammals is proposed to have a role in basolateral redirection, AP-4 may function in analogous, but mechanistically distinct, polar trafficking of membrane proteins in plants.

## 5.2 Induced

KAT1 ( $K^+$  channel) is an example of a PM protein that undergoes induced endocytosis and secretion in guard cells as elegantly demonstrated with the use of functional fluorescently fused proteins, FRAP, and electrophysiological analyses

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**Fig. 3** (continued) trafficking is mediated by the ARF-GEF GNOM-like1, and ABCB19 appears to be stable on the membrane and does not undergo dynamic cycling. (b) Induced trafficking. KAT1 trafficking in guard cells is induced by the hormone abscisic acid (ABA). In the basal or uninduced state, the majority of KAT1 is on the PM, and a subset of KAT1 is in an endosomal population (*left*). Following ABA stimulus, KAT1 is rapidly internalized from the PM into endosomal populations (*right, heavy arrow*). This rapid endocytosis is specific to KAT1, since the PM ATPase remains at the PM. After several hours, KAT1 is recycled back to the PM via SYP121-depend trafficking (*light arrows*). (c) Specialized trafficking. Cytokinesis is an example of specified trafficking. Cytokinesis requires coordination of KEULE/Sec1, KNOLLE/SYP111, SNAP23, Rab-A2, Rab-A3, Rab-F2, and DRP1A and DRP2B for vesicle fusion and endocytosis for nascent PM formation and secretion of callose, cellulose, and pectin for cell plate formation

(Sutter et al. 2006, 2007). The majority of the KAT1 channels reside on the PM and the remainder are in an endosomal population. Following stimulus by the hormone abscisic acid, KAT1, but not the PM H<sup>+</sup> ATPase, undergoes selective and rapid endocytosis to an endosomal compartment. KAT1 then recycles back to the PM over several hours in a SYP121-dependent pathway. This work also showed that KAT1 is localized on the PM in microdomains of 0.5–0.6  $\mu\text{m}$  in diameter. BRI1, discussed above, is another example of a protein that undergoes induced trafficking.

### 5.3 *Specialized*

Specialized trafficking includes polar growth observed in root hairs and pollen tubes, as well as those that occur during cell plate formation during cytokinesis. While the asymmetric localization of PM proteins such as COBRA, PIN1, and PIN2 in the root tip may be considered specialized, little is known about the mechanisms that drive and maintain that localization. Polar tip growth in pollen tubes and root hairs involves coordination of Rabs, Secs, SYPs, and dynamins and microtubules, and has been discussed throughout this chapter.

Cytokinesis also requires coordination of Rabs (Rab-A2, Rab-A3, Rab-F2, Chow et al. 2008), Secs (KEULE/Sec1, Assaad et al. 2001), SYPs (KNOLLE/SYP111, Boutté et al. 2010; Reichardt et al. 2007), and dynamins/DRPs (DRP1A, Konopka and Bednarek 2008; DRP2B, Fujimoto et al. 2008) and microtubules, but additionally, it requires that a cell plate is formed during nascent PM formation to produce two daughter cells. Cell plate formation is dependent on secretion of nascent proteins to the preprophase band and formation of the phragmoplast (a scaffold of the forming cell plate). The targeting of vesicles to the cell plate is viewed as the default pathway in dividing cells (Jürgens 2005). Vesicle fusion results in PM formation, localized regions of callose, followed by callose removal and depositions of pectin and hemicellulose/cellulose and cell wall modifying enzymes like KORRIGAN (Zuo et al. 2000; Robert et al. 2005), resulting in membrane partitioning as the cell plate is formed.

KEULE/Sec1 binds KNOLLE/SYP111 to effect vesicle fusion during cytokinesis (Assaad et al. 2001). KNOLLE (KN) has been used to follow the secretory pathway during cytokinesis, and to show that nascent proteins are secreted to form the cell plate, and KN subsequently was found in MVBs bound for the lytic vacuole, and that endocytosis is not necessary in that process (Reichardt et al. 2007). More recently, it was shown that endocytosis plays a role in restricting KN to the cell division plane (Boutté et al. 2010). Polar localization of other proteins at the cell plate is also observed. For example, ABCB19 has polar localization at the newly formed cell plate, but ABCB19 does not colocalize with KEULE, indicating that ABCB19 is not involved in early cell plate formation (Blakeslee et al. 2007). Protein turnover or processing may also take place at the cell plate as aminopeptidase M1 (APM1) is observed at the forming cell plate during cytokinesis, and loss-

of-function mutants show aberrant planes of cell division (Peer et al. 2009). Therefore, APM1 is required early in cytokinesis for proper planes of cell division, whereas KNOLLE, KEULE, and KORRIGAN are required for completion of cell division.

## 6 Concluding Remarks

Although the past decade has yielded a wealth of new information regarding plasma membrane trafficking mechanisms, there are still many outstanding questions to be resolved. The mechanisms underlying the polar targeting of proteins to PM are yet to be fully elucidated. More substantive elaboration of transcytotic redirection events and evaluation of the contribution of this phenomenon to distinct polar trafficking pathways are required. More extensive characterization of the motif(s) that are required for cargo selection and targeting is a priority, as earlier models assuming that cargo selection is primarily mediated by  $\mu$ -adaptins must be reevaluated in light of evidence that cargo binding can be demonstrated for all mammalian adaptor protein isoforms. Regulatory combinations of the many potential CLE and LLRs, which act synergistically or antagonistically to maintain the meristem or cell identity must be explored. It must also be determined how many different types of endosomal compartments are really present and whether they are really distinct compartments or a gradient of membrane populations characterized by protein concentrations. Finally, the contribution of membrane subdomains to trafficking mechanisms must be evaluated. Emerging new techniques and technologies to dynamically resolve the identity and spatial distribution of vesicle populations and membrane subdomains as they interact with the cytoskeleton are expected to resolve many of the outstanding questions currently confronting plant cell biologists and provide a framework for the identification and engineering of plants that can meet the needs of a growing human population without sacrificing biodiversity.

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