Review

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Endocytosis and its regulation in plants

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Endocytosis provides a major route of entry for membrane proteins, lipids, and extracellular molecules into the cell. Recent evidence indicates that multiple cellular processes require endocytosis, including nutrient uptake, signaling transduction, and plant–microbe interactions. Also, advanced microscopy, combined with biochemical and genetic approaches, has provided more insights into the molecular machinery and functions of endocytosis in plants. Here we review mechanisms of the clathrin-dependent and membrane microdomain-associated endocytic routes in plant cells. In addition, degradation of endocytosed proteins and endosomal sorting complex required for transport (ESCRT)-mediated vesicle formation at the endosome are discussed. Finally, we summarize the essential roles of various regulators during plant endocytosis.

Multiple, complex endocytic pathways in plants

Endocytosis involves the internalization or uptake of plasma membrane (PM) proteins or extracellular materials into the cell via a series of vesicle compartments and thus plays an essential role in cell-to-cell communication and cellular responses to environmental stimuli [1].

In the past decade, substantial progress has been made in the characterization of the plant components involved in clathrin-mediated and membrane microdomain-associated endocytic pathways [2,3] (Figure 1). Several PM-resident receptors and transporters have been identified as endocytic cargoes, including leucine-rich repeat receptor-like kinases (RLKs) such as brassinosteroid (BR) insensitive 1 (BRI1) and flagellin sensing 2 (FLS2) in *Arabidopsis thaliana* and ethylene inducing xylanase receptor (LeEIX2) in tomato (*Solanum lycopersicum*) [4–6]. Other endocytic cargoes in *Arabidopsis* include a boron transporter (BOR1), an iron-regulated transporter 1 (IRT1), a plant aquaporin (PIP2;1), an ammonium transporter (AMT1;3), and respiratory burst oxidase homolog D (RbohD) [7–11]. Compared with the well-defined endocytic networks in animals, our understanding of endocytic mechanisms and their physiological roles in plants has lagged far behind. This review summarizes the recently identified core components of endocytic machineries and links these endocytic routes to crucial cellular processes involved in plant development and responses to environmental cues.

The clathrin-mediated endocytic pathway: main point of entry into the plant cell

Similar to animal cells, clathrin-mediated endocytosis (CME) (see Glossary) is the major route in plants and starts with the initiation of invagination of clathrin-coated membrane, also termed clathrin-coated pits (CCPs) [2] (Figure 2). Several models of CCP formation remain the subject of ongoing debate. One model proposes that the highly conserved adaptor protein complex 2 (AP2), which

Glossary

Clathrin-coated vesicles (CCVs): vesicles that form at the PM, *trans*-Golgi network, and endosome. The outer surface of these vesicles is coated with a clathrin cage. CCVs mediate the vesicular transport of various cargoes between the *trans*-Golgi network, endosomes, and PM.
Clathrin-mediated endocytosis (CME): the primary endocytic route into cells through which extracellular materials are packaged into CCVs and taken up into cells. CME occurs in all known eukaryotic cells and is essential to eukaryotic life.
Detergent-resistant membranes (DRMs): also known as detergent-insoluble membranes (DIMs); a term used to refer to the insoluble fraction of membranes when extracting membrane by mild detergent. Proteins identified in the DRM fraction are usually proposed to be associated with membrane microdomains.
Endocytosis: a process by which cells take up extracellular materials and cell surface proteins via vesicles. Endocytosis is essential for the regulation of signal transduction, the maintenance of PM homeostasis, and the transport of important molecules from outside the cell to the appropriate compartment in the cytoplasm.
Fluorescence correlated spectroscopy (FCS): a powerful technique that analyzes the inherent correlations of fluctuating fluorescence signals when fluorophores transition into and out of a tiny excitation volume or area; it can be used to examine the dynamic behavior of fluorescently labeled molecules in living cells, such as molecular concentrations, diffusion, and interactions between fluorescent molecules.
Membrane microdomain: a specified compartment within the PM that displays characteristics distinct from those of the surrounding membrane, such as protein and lipid composition and organization. It is proposed to be the signal transduction platform that plays a key role in many biological processes.
Total internal reflection fluorescence microscopy (TIRFM): an elegant optical technique that utilizes evanescent waves to illuminate the sample. The energy of an evanescent wave decays exponentially with distance from the interface between the coverslip and the sample, which excites only those fluorophores in proximity to the coverslip. This substantially decreases background fluorescence and significantly increases the signal-to-noise ratio. The technique is well suited to monitoring the dynamic biological processes near the PM.

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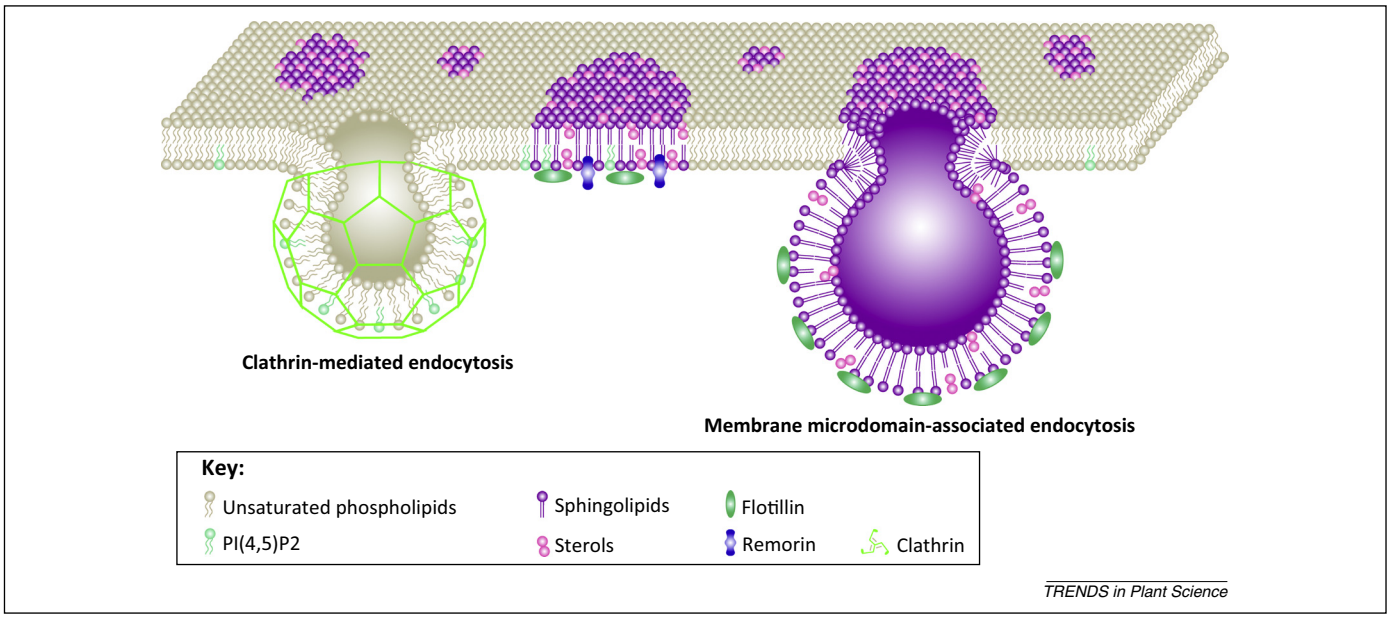

Figure 1. Endocytic pathways identified in plants. In plants, two endocytic pathways have been identified, clathrin-mediated endocytosis (CME) and membrane microdomain-associated endocytosis. Similar to animal cells, CME is the main mechanism for the entry of extracellular material into plant cells. Membrane microdomains are nanodomains at the plasma membrane (PM) that are enriched in sterol and sphingolipids and have been proposed to exist in plant cells. Two membrane microdomain marker proteins, flotillin and remorin, have been identified and flotillin has been shown to participate in a clathrin-independent endocytic pathway in plant cells.

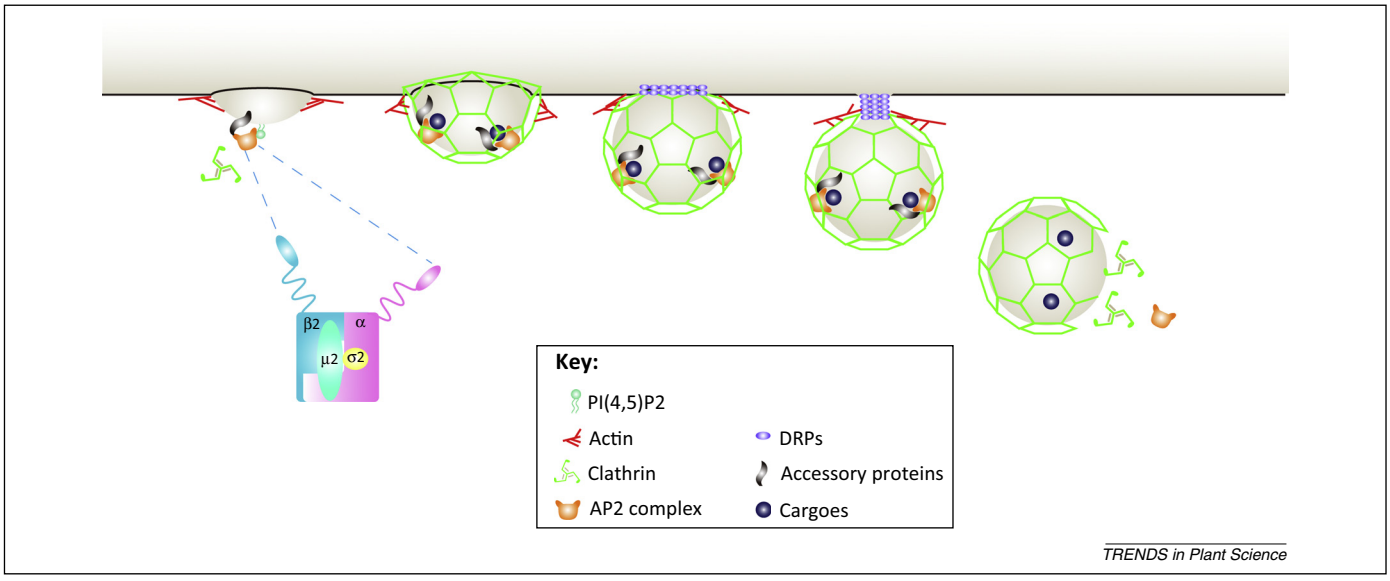

Figure 2. A proposed model of clathrin-mediated endocytosis (CME) in plants. CME can be divided into five steps. (1) Clathrin-coated endocytic vesicle formation starts with the association of adaptor protein complex 2 (AP2) with the plasma membrane (PM) via binding to phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2]. (2) The membrane-associated AP2 recruits clathrin and the unidentified accessory proteins. After initiation, AP2 binds to various cargo proteins through its σ2 and μ2 subunits. With the aid of accessory proteins, AP2 continues to recruit clathrin, which polymerizes and forms a clathrin coat around the newly formed membrane invagination. (3) When the clathrin-coated vesicles (CCVs) mature, the GTPase dynamin-related protein (DRP) is recruited at the neck of the vesicle and (4) is responsible for the detachment of the vesicle from the PM. (5) Once the vesicles have been pinched off, the coated components are disassembled and release the cargo-containing endocytic vesicles into the cytoplasm. In plants, the cortical actin cytoskeleton has been implicated in the regulation of clathrin-coated pit (CCP) dynamics at the PM.

comprises α, β2, σ2, and μ2 subunits, serves as a central player in the initiation of CCP nucleation [12–14] (Table 1). Another model proposes that a well-defined CCP initiation site pre-exists on the PM before the recruitment of AP2. Evidence supporting this model came from the identification of the CCP nucleator Fer/Cip4 homology domain-only protein (FCHo) in mammalian and yeast cells [15]. FCHo has not yet been identified in plants and thus whether the FCHo-centric model exists in plant cells remains to be investigated.

After the establishment of CCP nucleation modules, CME proceeds through cargo selection. The endocytic site has precise cargo-sorting machinery to ensure that CME accurately and selectively internalizes its diverse cargoes. A cargo-selective clathrin adaptor specifically binds to the sorting motifs in the cargo proteins and gathers the transmembrane cargoes into clathrin-coated vesicles (CCVs) [16]. Plant CCVs are not only formed on the PM but also assembled in the *trans*-Golgi network (TGN), where they are involved in various post-Golgi trafficking processes

Table 1. Components of endocytosis in plants

Function	Protein	*Arabidopsis* gene (accession no)	Refs
Adaptors	AP2 complex	*AP2 α1* (At5G22770)	[4,13,94]
		AP2 α2 (At5G22780)	
		AP2 β (At4G23460)	
		AP2 μ (At5G46630)	
		AP2 σ (At1G47830)	
	AtEHD	*AtEHD1* (At3G20290)	[6]
		AtEHD2 (At4G05520)	
	TPLATE	*TPLATE* (At3G01780)	[90]
	Epsin homology (EH) domain-containing protein	*AtEH1* (At1G20760)	[91]
		AtEH2 (At1G21630)	
	TPLATE complex muniscin-like protein	*TML* (At5G57460)	[91]
	Longin-like protein interacting with TPLATE adaptor	*LOLITA* (At1G15370)	[91]
	WD40/YVTN repeat domain-containing protein	*TWD40-1* (At3G50590)	[91]
		TWD40-2 (At5G24710)	[91]
Accessory proteins	AP180	*AP180* (AT1G05020)	[19]
	Epsin-like proteins	*AtECA1* (At2G01600)	[20]
		AtECA2 (At1G03050)	
		AtECA4 (At2G25430)	
	TPLATE-associated SH3 domain-containing protein	*TASH3* (At2G07360)	[91]
	Putative clathrin assembly protein	*CAP1* (At4G32285)	[91]
Clathrin coat	Clathrin	*CHC1* (At3G11130)	[95]
		CHC2 (At3G08530)	
		CLC1 (At2G20760)	
		CLC2 (At2G40060)	
		CLC3 (At3G51890)	
Scission	Dynamins	*DRP1A* (At5G42080)	[21,57]
		DRP1C (At1G14830)	
		DRP1E (At3G60190)	
		DRP2A (AT1G10290)	
		DRP2B (At1G59610)	

[17]. Recent work demonstrated that the endocytic sorting motifs YXXØ (Y, tyrosine; X, any amino acid; Ø, an amino acid with a bulky hydrophobic side chain) occurs in LeEIX2 and AtBOR1 and is required for their internalization [7]. However, cargo-specific adaptors have not yet been identified in plants.

Following CCP initiation and cargo selection, maturation of CCPs involves further clathrin coat assembly and the recruitment of additional accessory proteins. AP2 and the accessory proteins recruit clathrin directly from the cytosol to the CCP nucleation sites [18]. Besides the AP2 complex, plant genomes also encode conserved orthologs of mammalian monomeric adaptors, such as the epsin N-terminal homology domain, and AP180 N-terminal homology domain-containing proteins (Table 1). For example, *Arabidopsis* AP180 can bind clathrin and promote clathrin assembly *in vitro* [19]. A recent study found that *Arabidopsis* epsin-like clathrin adaptor1 (AtECA1) may act as an adaptor protein in clathrin coat assembly during cell plate formation [20]. However, the specific cargo proteins for AtECA1 during CME have not been identified in plants.

After cargo selection and clathrin assembly, the mature CCVs detach from the PM and enter the cytosol. Dynamin and dynamin-related proteins (DRPs), a large family of GTPase proteins, mediate membrane tubulation and scission. Notably, DRP1 and DRP2 were shown to be involved in CME in *Arabidopsis* [21] (Table 1). Unlike dynamin1 in animal cells, all of the identified DRPs (DRP1A, DRP1C, and DRP2B) involved in CME exhibit a gradual increase in fluorescence intensity that occurs concurrently with clathrin recruitment in plants [21]. These observations suggest that plant DRP1 and DRP2 mediate the scission of CCVs from the PM and also play a role at the clathrin assembly stage during CME.

Membrane microdomain-associated endocytosis: an alternative route of entry into plant cells

Emerging evidence shows that, similar to animal cells, plant cells have additional endocytic pathways besides CME. For example, in *Arabidopsis* the membrane microdomain-associated flotillin1 (Flot1) participates in clathrin-independent endocytosis [22]. Flotillins, a family of proteins containing the stomatin/prohibitin/flotillin/HflK/C (SPFH) domain, were detected in the detergent-resistant membrane (DRM) fraction [23]. A previous study demonstrated that plant flotillin is involved in symbiotic bacterial infection in *Medicago truncatula* [24]. Further studies revealed that internalization of *Arabidopsis* PIP2;1 and AMT1;3 was partially associated with the Flot1-associated endocytic pathway [9,10], indicating involvement of membrane microdomains in the regulation of signal transduction via endocytosis. In tomato, sucrose transporter SlSUT2-interacting proteins are shown to be associated with membrane microdomain and intracellular vesicles, indicating that SlSUT2 may be internalized into the cell via the membrane microdomain-associated pathway [25]. Under salt stress, the cross-correlation of *Arabidopsis* RbohD with the membrane microdomain marker Flot1

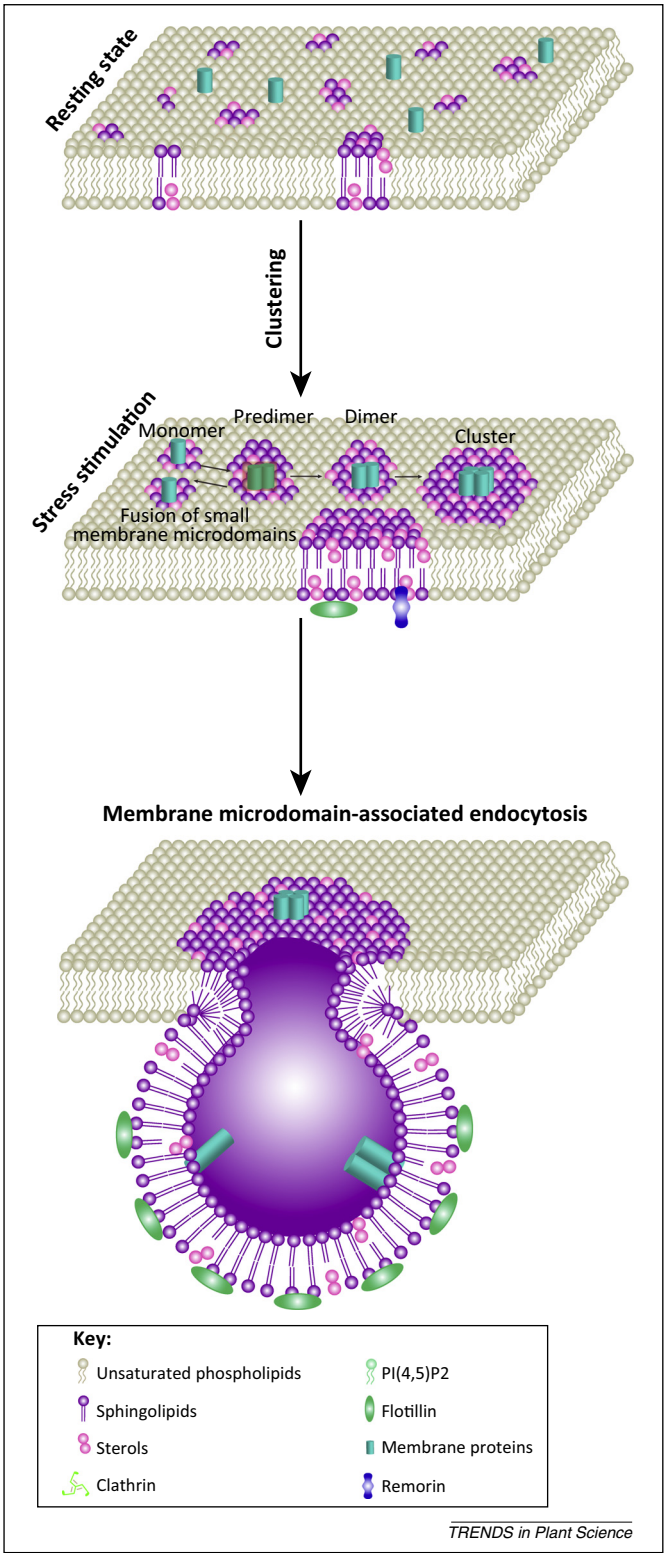

Figure 3. Clustering and endocytosis corporately regulate the activity of plasma membrane (PM)-localized receptors and transporters. In the resting state, many PM proteins reside on the PM as monomers and undergo constitutive endocytosis via clathrin-mediated endocytosis (CME). When stimulated, some of these proteins form dimers or predimers on the PM that will further assemble into clusters. These clusters are segregated into membrane microdomains, which will reduce the dynamics of these proteins. Subsequently, these clustered proteins are internalized via membrane microdomain-associated endocytic pathways. Endocytosis and clustering provide an efficient mechanism of controlling the activity of membrane proteins in response to environmental changes.

increased, suggesting that salt stress stimulates the internalization of RbohD through the microdomain-associated pathway [11]. Furthermore, treatment with the CME inhibitor Tyrphostin A23 and overexpression of HUB, a dominant-negative version of the clathrin heavy chain (CHC) do not affect endocytic uptake of glucose in BY-2 cells, suggesting that the uptake of glucose as a nutrient in plant cells occurs via clathrin-independent endocytosis [26].

Protein clustering occurs in many membrane microdomains [27]. When binding to their ligands, these receptors form active oligomers that ultimately initiate cellular responses to the extracellular stimuli [28]. However, recent studies indicate that some membrane proteins reside on the PM as preformed dimers (an inactive form) that are primed for activation on ligand binding [29]. Interestingly, following stimulation the receptor dimers or trimers can further aggregate into clusters, and receptor clustering functions as an important regulator of signal transduction in bacterial and animal cells [27].

Similarly, other plant PM proteins, such as transporters, can also form clusters in membrane microdomains (Figure 3). It has been shown that the *Arabidopsis* KAT1 K^+ channel has a non-uniform distribution on the PM and forms punctate structures of around 0.5–0.6 μm [30]. Moreover, KAT1 clusters are probably linked to its function on the PM [31]. Also, the sucrose transporter SUT1, which is required for phloem loading, was found to concentrate in the membrane microdomain region in clusters of diameter 200–300 nm [32]. Single-molecule subunit counting revealed that PIP2;1 occurs as a mixture of multimeric forms on the PM [9]. The remorin proteins do not have transmembrane domains but all contain a conserved C-terminal domain [33]. More recently, immunogold labeling demonstrated that remorin forms clusters in the cytosolic leaflet of the PM and that these clusters are sensitive to treatment with the sterol-depleting agent methyl-β-cyclodextrin (mβCD) [34]. Furthermore, remorin also forms clusters on the PM to regulate the perception of Nod factors (NFs) during *Sinorhizobium meliloti* infection of *M. truncatula* [35].

Clustering in microdomains may also regulate protein activity. For example, by measuring the maximum bleaching steps of AMT1;3-EGFP, Wang *et al.* found that AMT1;3 exists as trimers on the PM [10]. Moreover, clathrin-dependent and membrane microdomain-associated endocytic pathways internalize these AMT clusters, leading to shut off of the active AMT on the PM and thus protecting plants against toxic levels of ammonium [10]. Recently, single-particle analysis demonstrated that GFP-RbohD assembled into clusters and then internalized into the cytoplasm via membrane microdomains under salt stress, providing insight into the regulation of RbohD activity [11]. These data indicate that clustering and endocytosis may act as a common mechanism underlying the regulation of PM-localized receptors and transporters (Figure 3).

Extracellular signals affect the endocytic routes of membrane proteins

Numerous endocytic pathways can internalize exogenous materials or regulate signaling at the cell surface in eu-

karyotic cells [36]. In addition to the well-studied clathrin-dependent endocytosis pathway, emerging research has revealed several clathrin-independent pathways, including membrane microdomain-associated endocytosis, fluid-phase endocytosis, and phagocytosis-like uptake of rhizobia in plants [2,3,37,38]. Increasing evidence suggests that PM proteins select the endocytic route for their internalization depending on environmental stimuli. The first evidence for this came from single-particle analysis of PIP2;1 [9]. These data indicate that CME and membrane microdomain-associated endocytosis tightly control the abundance of PIP2;1 on the PM and the contribution of these two pathways to its internalization depends on extracellular signals perceived by the cells [9]. Similarly, AMTs also have multiple routes for internalization [10]. More recently, it has been demonstrated that clathrin- and membrane microdomain-associated endocytic pathways cooperatively regulate RbohD dynamics [11].

Intracellular destinations of endocytosed proteins

The endocytic recycling machinery in plants

PM-localized receptors play an essential role in signal transduction and development in plants [39]. For example, the auxin transporter PIN-FORMED (PIN) is internalized by CME and undergoes constitutive recycling between the PM and the TGN/early endosome (EE) through an ARF-dependent endocytic/endosomal pathway. Furthermore, the ARF-GEF GNOM, which is localized in the recycling endosomes, is involved in the constitutive recycling of PIN proteins [37]. However, the subcellular localization of GNOM differs from both the TGN/EE and the late endosome (LE). Interestingly, the GNOM-dependent recycling pathway is specifically responsible for recycling PINs, as Brefeldin A (BFA) does not affect the intracellular trafficking of auxin influx carrier 1 (AUX1) [40]. A more recent study also showed that GNOM is involved in endoplasmic reticulum (ER)–Golgi trafficking, which is essential for PIN1 basal polarity establishment [41]. By contrast, treatment with endosidin 1 interferes with the endosomal recycling of AUX1 via the SYP61/VHA-a1-labeled EE but does not affect PIN1 recycling [42]. These results suggest that distinctly internalized membrane proteins may use different intracellular trafficking routes in plants.

Degradation of endocytosed proteins: ESCRT-mediated vesicle formation at the endosome

Some endocytosed proteins recycle back to the PM and some proteins are degraded in the vacuole. Ubiquitin functions as a critical signal for the internalization and intracellular sorting of PM-resident proteins [43]. ESCRT complexes mediate the formation of intraluminal vesicles (ILVs) within the endosome and ILVs are responsible for the degradative sorting of monoubiquitinated membrane proteins (Figure 4). Plants have three ESCRT complexes (I, II, and III) but lack ESCRT-0, suggesting a relatively conserved role of the ESCRT sorting machinery across kingdoms. In metazoans and fungi, ILV budding starts with the binding of monoubiquitinated cargoes via ESCRT-0, whereas plants use different mechanisms to initiate ILV formation. The *Arabidopsis* genome contains nine TOM-like proteins with conserved Vps-27, Hrs, and STAM (VHS) and GGA and TOM (GAT) domains, which may provide an alternative mechanism for the recognition of monoubiquitinated cargoes in the absence of ESCRT-0 [44]. However, the roles of TOM-like proteins in the binding of the monoubiquitinated cargoes and the initiation of ILVs remain to be demonstrated experimentally.

The well-characterized plant ESCRT-I protein ELCH (ELC) is the *Arabidopsis* homolog of Vps23 in yeast and Tsg101 in animals. Coimmunoprecipitation and yeast two-hybrid assays showed that ELC interacts with VPS37-1 and VPS28-1, putative components of ESCRT-I in *Arabidopsis*. Moreover, ELC can bind ubiquitin and colocalize with the EE marker Ara6 and the LE marker Ara7, indicating that ELC is a component of ESCRT-I in plants [45]. A recent study showed that VPS37-1 colocalizes with FLS2 at the endosome, indicating a role of ESCRT-I in flg22-activated stomatal defenses in plant immunity [46].

The functions of ESCRT-II constituents in plants remain elusive. VPS22/EAP30 protein is a major component in ESCRT-II in both yeast and mammalian cells and recent work showed that loss of OsVPS22 causes seedling lethality and severe reduction in shoot and root growth, implying that OsVPS22 has an essential role in plant development [47]. Strikingly, the ESCRT-II and Vps20 complex directs Vps32 polymerization specifically to the highly curved membrane as a single string of monomers [48].

The ESCRT-III related protein CHMP1 has been characterized in plants [49]. SAL1, a CHMP1 homolog in maize, plays an essential role in controlling the number of aleurone cell layers in endosperm via regulating the sorting of the internalized PM receptor kinase CR4 for degradation [50]. *Arabidopsis* double homozygous *chmp1a chmp1b* mutants are defective in the establishment of bilateral symmetry and ILV formation. Also, CHMP1A interacts with ESCRT-related proteins *in vitro* and functions in the degradative sorting of auxin transporters including PIN1, PIN2, and AUX1 [49].

Plants also have a homolog of Vps4/SKD1, which provides the energy for dissociation of ESCRT-III from the membrane. For example, *Arabidopsis* SKD1 was found to play a critical role in the dissociation of the ILVs from the endosomal membrane [51]. Recent observations indicate that the associated molecule with the SH3 domain of STAM3 (AMSH3) interacted with ESCRT-III, which functions in ubiquitin-mediated endocytic degradation [52,53]. A more recent study demonstrated a plant-specific ESCRT component, FYVE domain protein required for endosomal sorting 1 (FREE1), that is essential for both multivesicular body (MVB) biogenesis and autophagic degradation [54].

Regulators of plant endocytosis

The emerging role of the cytoskeleton in plant endocytosis

The cytoskeleton plays vital roles in endocytosis and membrane remodeling [55]. The first hint of the involvement of actin filaments in plant endocytosis came from the use of pharmacological agents to disrupt actin turnover [37,56]. Low concentrations of Latrunculin B, an inhibitor of actin polymerization, significantly prolonged the lifetime of CCPs. Conversely, high concentrations of Latrunculin B

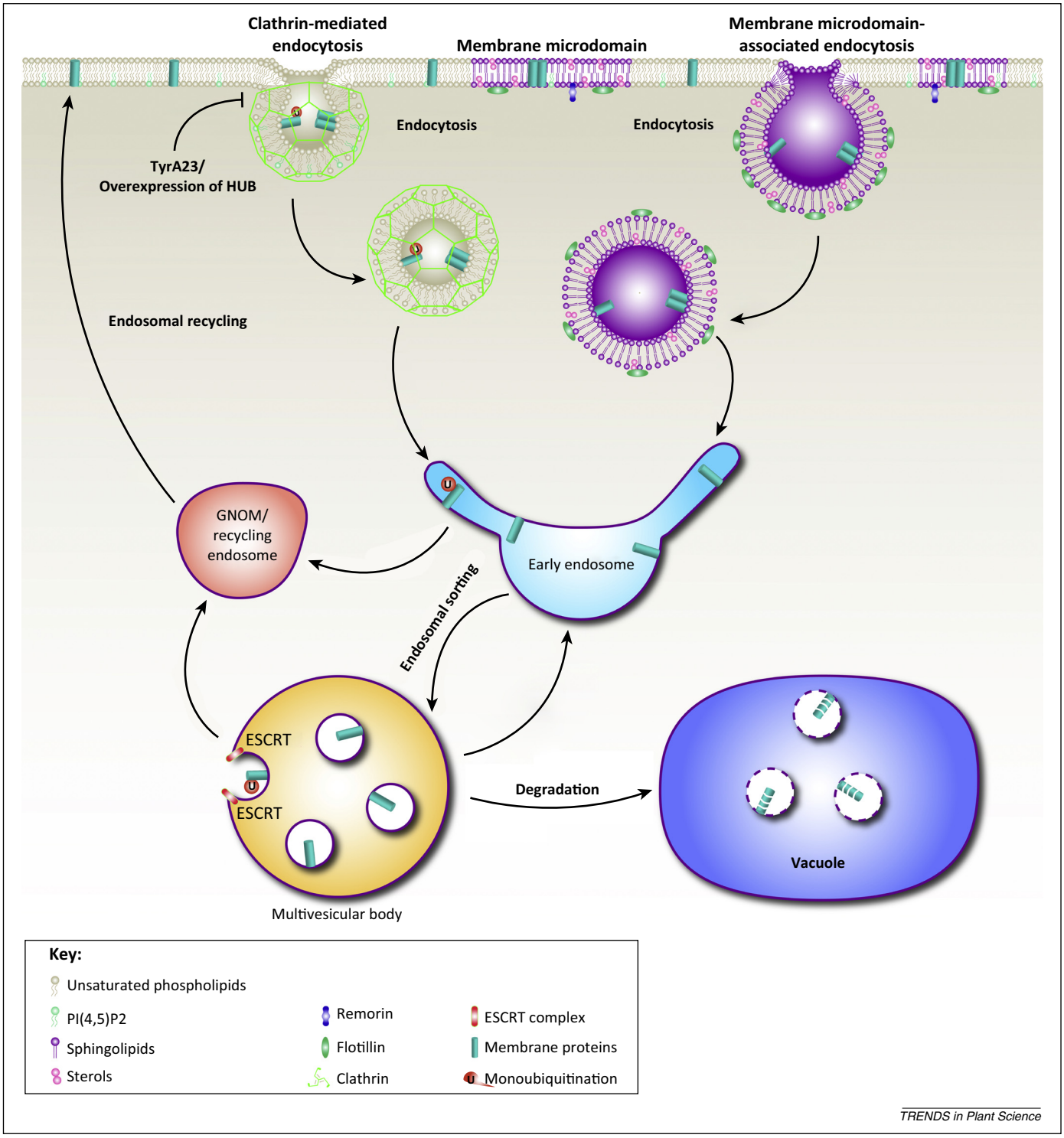

Figure 4. The destinies of internalized cargo proteins. Various kinds of membrane protein are internalized into the cytoplasm via endocytosis and transported to the early endosome, where the fates of these proteins are determined. Depending on extracellular signals, these cargo proteins are translocated to distinct destinations. Some cargo proteins are recycled back to the plasma membrane (PM). Monoubiquitinated cargo proteins are recognized by the endosomal sorting complex required for transport (ESCRT) complex and recruited to the intraluminal vesicles (ILVs) and eventually transported to the vacuole for degradation.

inhibited processes that require cytoplasm streaming [57]. Morphogenetic analysis in leaf pavement cells revealed that the ROP GTPase inhibits CME of PIN1 via accumulation of cortical actin filaments [58–60]. However, the link between CME and actin polymerization was probably not mediated by the Arp2/3 complex in plants as loss-of-function mutants of ARP2/3 subunit genes in *Arabidopsis* displayed only a mild phenotype [61].

In addition, microtubules may also affect plant endocytosis. For example, the microtubule inhibitor oryzalin significantly affects the lifetime and mobility of CCPs on the PM in *Arabidopsis* root cells [57]. A recent observation showed a ubiquitous microtubule-endosome association and microtubule-regulated PIN abundance on the PM in *Arabidopsis* [62]. More strikingly, in addition to the effect on endocytosis, exocytosis is also affected by microtubules

Table 2. Regulators of endocytosis in plants

Regulator	Description	*Arabidopsis* gene (accession no)	Refs
Cytoskeleton	Actin-related protein	*ARP2/3* (AT1G29170)	[61]
Small G protein	Rop	*ROP2* (AT1G20090)	[58–60]
		ROP4 (AT1G75840)	
		ROP6 (AT4G35020)	
	Rop-interactive protein	*RIC1* (AT2G33460)	[96]
		RIC4 (AT5G16490)	
	Rop-GEF	*SPIKE1* (AT4G16340)	[60]
	Rab-Fs	*Rab-F2a/Rha1* (At5G45130)	[97]
		Rab-F2b/Ara7 (At4G19640)	
	Rab-As	*Rab-A1c* (At5G45750)	[70]
		Rab-A2a (At1G09630)	[98]
		Rab-A3 (At1G01200)	
	ARF-GAP vascular network defective 3 (VAN3)	*VAL1* (At5G61980)	[71]
		VAL2 (At1G60860)	
		VAL3 (At1G10870)	
	ARF-GEF GNOM/GNOM-like proteins	*GNOM* (AT1G13980)	[71]
		GNL1 (AT5G39500)	
Environmental cues	Pathogen	*FLS2* (AT5G46330)	[76]
	Gravity	*PIN2*	[78]
		PIN3	[77]
Plant hormone	Auxin	*ABP1* (AT4G02980)	[59,81,96]
	Cytokinin	–	–
	BRs	*BRI1* (AT4G39400)	[86]
	SA	–	–
Membrane factors	Inositol 5-phosphatases	*PIP5K6* (At3G07960)	[99]
		PI4Kβ1 (At5G64070)	

in tobacco pollen tubes [63]. However, the exact mechanisms underlying the regulation of endocytosis or exocytosis by the actin or microtubule cytoskeleton in plants remain to be defined.

Ubiquitination-mediated plant endocytosis

Ubiquitin, a highly conserved 76-amino acid polypeptide, covalently attaches to target proteins and also regulates the internalization of PM proteins in mammalian and plant cells [64]. Evidence for the role of ubiquitination in plant endocytosis comes from studies of IRT1, which can be monoubiquitinated; mutations of its ubiquitination sites (K154 and K179) increase its stability on the PM, suggesting that monoubiquitination regulates the endocytosis of PM-localized IRT1 [8]. Furthermore, artificial fusion of a single ubiquitin to the *Arabidopsis* PM-localized ATPase triggers its internalization [65]. A recent study showed that dynamic ubiquitination acts as a primary signal for the internalization of PIN2 protein, which is crucial for auxin transport and adjustment of root growth to environmental conditions [66]. In plants, other PM proteins, such as BOR1 and FLS2, can be ubiquitinated [67], but less evidence shows any effect of ubiquitination on their endocytosis. However, the plant homologs of the endocytic adaptors that recognize ubiquitinated cargoes in animal cells (epsin) do not contain UBDs [68], indicating that plants use specific endocytic mechanisms to mediate the internalization of ubiquitinated cargoes.

Small G proteins in plant endocytosis

In *Arabidopsis*, the small G protein family includes 93 members, which fall into Arf, Rop, Rab, and Ran classes. However, their endocytic organization and regulation differs from that of animals. For example, Ara7 (Rab-F2b) and Rha1 (Rab-F2a), the mammalian Rab5 GTPase homologs, act in later endocytic pathways [69]. Notably, Rab-A proteins (Rab-A1c/A2a/A3) act in various endocytic pathways, including internalization to EEs, further transport to LEs, or recycling back to the PM [70]. The machinery of the ARF small G protein family associates with CME in *Arabidopsis*. Furthermore, loss of vascular network defective 3 (VAN3) or GNOM leads to decreased internalization of PIN1 and FM4-64, suggesting that the Arf GTPase-based regulatory machinery is involved in plant endocytosis [71] (Table 2). However, plants lack homologs of the animal Arf6 proteins that function in endocytosis [72]; therefore, which Arf proteins cooperate with VAN3 and GNOM to regulate plant endocytosis remains unknown.

Environmental cues regulate plant endocytosis

In plants, pathogens could regulate endocytic trafficking of the pathogen-associated molecular pattern-mediated pattern-recognition receptors [73,74]. For example, FLS2 in *Arabidopsis* recognizes bacterial flagellin [75]. When stimulated with flg22, FLS2-GFP is internalized from the PM into intracellular mobile vesicles, suggesting that endocytosis and subsequent degradation regulate FLS2 activity [5]. Recently, imaging analysis showed that FLS2-GFP fluorescence was still observed in the endosomes 120–200 min after treatment with flg22 [76]. These data indicated that pathogens could trigger innate immune responses via regulating the endocytosis of receptors in plants.

Studies on PIN protein polarity establishment identified gravity as another plant-specific regulator of endocytosis (Table 2). Plant roots reoriented to horizontal will bend in response to gravity by redirecting auxin flux in the

root tip [77]. The gravity-induced relocation of PIN2 required endocytic trafficking [78]. Moreover, by live-cell imaging, together with pharmacological and genetic approaches, it was shown that gravity-induced PIN3 polarization requires rapid, transient internalization of PIN3 rather than protein degradation or *de novo* synthesis [77]. These data suggest that gravity can regulate endocytic trafficking of PIN proteins and mediate plant responses to gravity stimulation.

Plant hormone signaling and endocytosis

Besides the components involved in endocytosis, plant signaling can also induce feedback regulation of the endocytic machinery. Auxin was tested to inhibit the internalization of the endocytic tracer FM4-64 and several PM proteins such as PIN1, PIN2, PM-ATPase, and PIP2 [79]. In addition, the internalization of PIN1 is clearly blocked in root protophloem cells that contain higher concentrations of auxin than the surrounding cells [80]. This suggests that auxin can regulate the endocytic cycling of its own efflux carriers, the PIN proteins, via modulating the endocytic machinery. The auxin receptor auxin-binding protein 1 (ABP1) is required for the recruitment of clathrin to the PM and auxin acts as a plant-specific regulator of CME via binding to ABP1 [81].

Cytokinin is an important regulator of plant endocytosis and interacts with the auxin pathway, primarily at the transcriptional regulation level [82]. Marhavý *et al.* recently showed that cytokinin regulated PIN1 endocytic recycling and redirected PIN1 for degradation. Stimulation of PIN1 degradation provides a specific mechanism to rapidly modulate the distribution of auxin in cytokinin-mediated developmental processes [83]. Interestingly, cytokinin enhances PIN1 depletion at special polar domains, thus rearranging PIN1 polarity and controlling the direction of auxin transport, revealing a novel cytokinin-driven polarization mechanism in plant development [84].

The essential plant steroid hormones BRs have crucial roles in a wide range of developmental and physiological processes [85]. The major BR receptor, BRI1, mainly localizes to the PM and EE compartments in *Arabidopsis* root cells [86]. Furthermore, BFA can induce the accumulation of BRI1-GFP in specific BFA compartments, demonstrating BRI1 trafficking via endosomes [86]. Using a fluorescent BR analog, Alexa Fluor 647-castasterone, a recent study visualized the endocytosis of BRI1-AFCS (Alexa Fluor 647-castasterone, a fluorescent BR analog) complexes in living plant cells and revealed clathrin-dependent endocytic regulation of BR signaling from the PM [87].

Classically, salicylic acid (SA) mediates plant defenses against pathogens [88]. However, a recent study found that SA treatment also interferes with endocytic cycling of PM-localized proteins [89] (Table 2). Further investigation demonstrated that the effect of SA on endocytosis is not due to the traditional SA signaling transduction pathway for transcription regulation but instead involves its effect on the recruitment of clathrin on the PM, a key component in CME. SA treatment significantly reduces the incidence of clathrin light chain (CLC) and CHC on the PM, suggesting that SA affects endocytosis through clathrin [89].

Concluding remarks and future challenges

The field of plant endocytosis has seen enormous progress in the past decade with the identification of many critical PM-resident proteins regulated by endocytosis. Recently, one of the adaptin-like proteins, TPLATE, which is similar to the adaptin/coatomer proteins and is involved in cell plate formation, was identified as a plant-specific adaptor complex for endocytosis [90–92] (Table 1). However, it is unknown whether other plant-specific accessory proteins for endocytosis exist or how they are temporally and spatially organized during endocytosis. Given the diversity of endocytic pathways in animal cells, other endocytic pathways may exist in plant cells. Some of these endocytic pathways may occur in a cell- or tissue-specific manner and mediate the internalization of specific cargoes. The recent application of advanced imaging methods such as total internal reflection fluorescence microscopy (TIRFM), fluorescence correlated spectroscopy (FCS), and super-resolution microscopy imaging [93] in living plant cells will better elucidate the detailed molecular mechanisms of distinct endocytic routes and provide deeper insights into the role of endocytosis in plant development and environmental responses.

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