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Budding and braking news about clathrin-mediated endocytosis

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Clathrin-mediated endocytosis (CME) is the predominate mechanism of endocytosis in eukaryotes, but an understanding of this mechanism in plants has lagged behind yeast and mammalian systems. The generation of Arabidopsis mutant libraries, and the development of the molecular tools and equipment necessary to characterize these plant lines has led to an astonishing number of new insights into the mechanisms of membrane trafficking in plants. Over the past few years progress has been made on identifying, and in some instances confirming, the core components of CME in plants. This review focuses on the recent progress made in the understanding of the mechanism and regulation of CME in plants.

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Introduction

Plant morphogenesis is highly dependent upon clathrin-mediated endocytosis (CME) for regulated and constitutive uptake and recycling of plasma membrane (PM) proteins critical for polarized auxin transport, nutrient uptake, hormone and pathogen signaling and other cellular processes such as the construction of the cell plate during cytokinesis [1,2]. Although evidence is also emerging for the existence of clathrin-independent endocytic pathways in plants [3–5] this review will focus specifically on recent advances in our understanding of plant CME. More than a decade of research has led to our current understanding of the complex network of proteins and lipids required for endocytosis in yeast and mammalian cells. By comparison our knowledge of the mechanism of CME in plants is rudimentary. However, significant progress has been made in recent years towards understanding the CME machinery (Table 1) and its regulation in plants and this progress is highlighted here.

Work in a variety of systems suggests that the mechanism of CME can be divided into steps which include binding of heterotetrameric adaptor protein 2 (AP-2) complex/clathrin to the PM, cargo recognition/recruitment, vesicle maturation, scission, and CME machinery dissociation (Figure 1). In mammalian cells, clathrin-coated pit (CCP) initiation is mediated by binding of a preassembled complex comprised of a few AP-2 complexes and 1-2 clathrin triskelia to phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] on the PM [6]. This binding is AP-2-dependent. Recruitment of additional AP-2-clathrin complexes subsequently recruits cargo molecules, and clathrin undergoes polymerization thereby inducing membrane curvature [7].

Following initial AP-2 and clathrin recruitment, CME accessory proteins, including AP180, FCHO, intersectin, epsin and EGFR pathway substrate 15 (Eps15) proteins associate to the rim of the assembling coated pit [8], stabilizing curvature of the budding vesicle bilayer and clathrin coat [6]. Actin, may under certain conditions aid in maturation and budding [9•]. Once the budding vesicle neck has narrowed, the GTPase dynamin mediates vesicle scission and the clathrin cage, along with the CME machinery, dissociate from the vesicle through the action of auxilin and Hsc70 [10].

Adaptor protein 2 (AP-2) complex

Arabidopsis possesses four adaptor protein (AP) complexes, designated AP-1–4. A fifth putative AP complex has recently been predicted by genome analysis in Arabidopsis [11•]. The heterotetrameric AP complexes consist of two large, one medium, and one small subunit. The characterization of the AP complexes in mammals, yeast and plants has shown these complexes are involved in clathrin-dependent and clathrin-independent post-Golgi and endosomal vesicular trafficking. On the basis of their evolutionary conservation, the plant AP complexes have long been postulated to localize and function analogously to their mammalian counterparts. However, only very recently has the localization and functional analysis of AP-1, AP-2 and AP-3 in plants been reported. Below we discuss the recent data showing that AP-2 indeed functions in plant endocytosis. Research on the other plant AP complexes, specifically AP-1 [12,13,14,15•] and AP-3 [16–18], suggest these complexes do not participate in CME and as a result are beyond the scope of this review. Research performed in mammalian systems suggests AP-4 and AP-5 do not participate in CME but their role in plant membrane trafficking remains to be determined [11•].

As noted above, of the five plant AP complexes, only AP-2 has been shown to function directly in CME. *In planta*

Table 1

Arabidopsis genes involved in assembly and regulation of CME.

Protein	Arabidopsis genes	AGI number	Function in CME	References
Core components				
Clathrin	*CHC1*	At3g11130	Form a triskelion structure, which assembles into a cage during CME	[15•,24•,68,69]
	CHC2	At3g08530		
	CLC1	At2g20760		
	CLC2	At2g40060		
	CLC3	At3g51890		
AP-2	*AP-2A1*	At5g22770	Adaptor complex that interacts with the membrane, clathrin, and CME accessory proteins and cargo	[20•,23•,69]
	AP-2A2	At5g22780		
	AP1/2B1	At4g11380		
	AP1/2B2	At4g23460		
	AP-2M	At5g46630		
	AP-2S	At1g47830		
Dynamins	*DRP1A*	At5g42080	Putative role in CCV scission	[70]
	DRP2B	At1g59610		
	DRP1C	At1g14830		
	DRP1E	At3g60190		
TPLATE	*TPLATE*	At3g01780	Adaptin-like protein, interacts with clathrin	[71,72]
EH domain containing protein		At1g21630		[50]
AtEHD1*		At3g20290		[73,74]
AtEHD2*		At4g05520		[62,63]
CME regulation				
ABP1	*ABP1*	AT4g02980	Auxin regulator of CME	[54,57••,58,59••]
SPIKE1	*SPIKE1*	AT4g16340	GEF for ROP6	[60••]
ROP2	*ROP2*	AT1g20090	GTPase	[48,57••,75]
ROP4	*ROP4*	AT1g75840	GTPase	[48,75]
ROP6	*ROP6*	AT4g35020	GTPase	[57••,58,60••,76]
RIC1	*RIC1*	AT2g33460	Interacts with active ROP6 to inhibit CME in root cells	[57••,76]
RIC4	*RIC4*	AT5g16490	Interacts with active ROP2/4 to inhibit CME in pavement cells	[48,57••,75]
PIP5K	*PIP5K6*	At3g07960	Phosphatidylinositol-4-phosphate 5 kinase 6, CME regulation	[77]
PI4K	PI4K1	At5g64070	Phosphatidylinositol-4-kinase, CME regulation	[77]

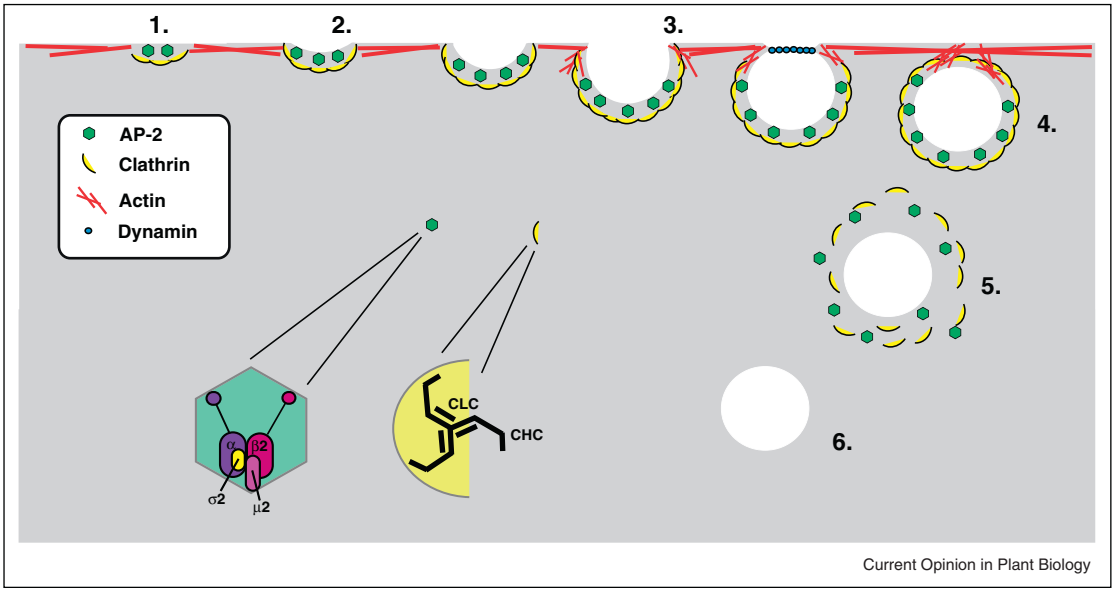
* Additional ENTH and ANTH domain-containing homologs exist in the Arabidopsis genome but are not listed in this table.

co-localization and co-immunoprecipitation studies utilizing fluorescent protein (FP) tagged-CLC2, tagged-large AP-2A, tagged-medium AP-2M, and/or tagged-small AP-2S-AP subunits has demonstrated that the AP-2 complex associates with clathrin at the PM and is involved in CME [19,20•,21,22,23•]. Bashline *et al.*, 2013, also observed that FP-tagged AP-2M colocalized with the cellulose synthase subunit (CESA6) in an undefined intracellular compartment. However, as the intracellular localization of AP-2M was only detected in lines expressing FP-tagged CESA6 and was not observed in other studies ([20•,21,22,23•]), the functional significance of this observation and the nature of this compartment warrants further investigation. Analysis of mutants defective for the large, medium and small AP-2 subunits confirmed that AP-2 is involved in the endocytosis of the auxin transporter PIN2 [21], the brassinosteroid receptor BRI1 [20•], and CESA6 [19], providing further evidence that CME in plants is required for auxin transport, brassinosteroid signaling, and cellulose metabolism.

Clathrin

Clathrin consists of three heavy chain subunits, which are the main structural component of the clathrin vesicle coat, and three light chain molecules that interact with the heavy chains to self-assemble into a three-legged triskelion structure. *Arabidopsis* possesses two functionally redundant clathrin heavy chain (*CHC1* and *CHC2*) and three clathrin light chain (*CLC1–CLC3*) genes. Although *CHC1* and *CHC2* are functionally redundant, *chc2* seedlings display defects in cotyledon organogenesis and embryogenesis, whereas *chc1* seedlings display a similar morphology to wild-type [24•]. In mammalian and yeast systems, CLCs have been proposed to regulate clathrin-coat formation as well as stabilize clathrin trimer formation [25–28]. Plant mutants defective for *clc1* display a pollen lethal phenotype, whereas *clc2* and *clc3* lines generate viable pollen but display pleiotropic defects in plant development. Homozygous *clc2clc3* mutant lines are also viable and display similar but slightly more severe defects in plant development, relative to the single mutant lines, suggesting functional redundancy [15•]. These developmental phenotypes displayed in *chc* and *clc* mutant plants are due partly to aberrant localization of the auxin efflux carrier PIN proteins and the resulting alteration in the vectoral transport of auxin *in planta* [15•,24•].

Figure 1



Model for clathrin-mediated endocytosis. AP-2 binds the membrane through interactions with PI(4,5)P2 marking sites for CCV formation, while recruiting clathrin. The clathrin lattice continues to assemble as the budding vesicle begins to form. Actin polymerization may aid in overcoming high turgor pressure. Scission of the CCV from the membrane is performed by dynamin-related proteins. After the CCV is pinched off from the membrane it loses its clathrin coat. Numbers depict order of CCV assembly. Subunits of the clathrin lattice and the AP-2 complex are depicted in detail.

Cargo recognition motifs: ubiquitin signals internalization and degradation

Although multiple cargo recognition sequences are present on an assortment of cargos, such as short peptide sequences recognized by subunits of the AP-2 complex, in this review we focus primarily on the role of ubiquitin in endocytosis, for an in-depth review of other endocytic sorting signals we direct the reader to [29].

Ubiquitination is a post-translational modification, which results in the covalent linkage of an ubiquitin molecule to a lysine residue on a target protein. This reaction is catalyzed by a series of enzymes, ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and finally a ubiquitin ligase (E3) resulting in the formation of an isopeptide bond between the C-terminus of ubiquitin and the free amine of a lysine residue on the target protein [30].

At the PM, cargo molecules can be ubiquitinated, which functions as a sorting signal for internalization. In metazoans, ubiquitin can be recognized through ubiquitin binding domains contained in eps15 and epsin [31]. In addition to binding cargo, Eps15 and epsin also bind AP-2, linking cargo to CME assembly [32]. After internalization, cargos are trafficked to the endosome where they can be recycled back to the PM or directed to numerous cellular locations. Recent data demonstrates that plants utilize similar sorting mechanisms as metazoans. For example, it was shown that the ubiquitination of the Iron regulated transporter 1 (IRT1) was critical for its constitutive internalization and cycling between the PM and the TGN/EE and its vacuolar degradation [33]. IRT1 ubiquitination and degradation are dependent on IRT1 Degradation Factor, IDF1, a RING E3 Ubiquitin Ligase, which interacts with IRT1 at the PM [34].

At the endosome, ubiquitin-modified cargos are sorted into the lysosomal/vacuolar degradation pathway via four endosomal sorting complexes required for transport (ESCRT-0-III) [35]. Although direct interactions between ubiquitin and ESCRT in plants has not been shown, recently it has been demonstrated that a C-terminal ubiquitin fusion can also serve to signal the internalization and degradation, through vacuolar sorting, of the resident PM protein TMD23 [36].

Additionally, outside its role at the endosome, recent data in metazoans demonstrates that a subset of ESCRT-0 functions to recognize cargos at sites of CME that are destined for degradation [37]. Interestingly, plants lack clear ESCRT-0 homologs, however, the plant genome encodes multiple Tom1 like genes, which may be a functional homolog of ESCRT-0 in plants [38•]. Consistent with this, interaction and co-localization of Tom1 and ESCRT-I subunits have been demonstrated in a recent study in which yeast two-hybrid analysis together with fluorescence microscopy were used to determine an ESCRT interaction network. These observations provide compelling support for the idea that Tom1 may serve an

analogous role in plants as ESCRT-0 does in other systems [39]. Other than an absence of ESCRT-0, plants contain homologs to all other ESCRT complexes. For a more in depth review of the ESCRT machinery in plants we refer readers to [38•].

Roles of the actin cytoskeleton in CME

The requirement for actin in CME varies substantially among organisms. In budding yeast, there is a strict actin requirement for CME to overcome the high turgor pressure (0.6 MPa [40]) of the cell [41]. In metazoans, CME dependence on actin varies among cell types. Recently, it has been demonstrated that an increase in osmotic pressure in metazoans can influence the actin requirement for CME; as the turgor pressure inside the cell is artificially increased, the requirement for actin becomes more pronounced [9•]. Surprisingly, in plant cells, which exhibit similarly high turgor pressures as those observed in budding yeast (0.63 MPA for epidermal root cells [42]), the requirement for actin in CME is unclear.

Pharmacological studies using actin depolymerizing agents showed that the endocytosis of Lucifer Yellow was inhibited in maize [43] suggesting that actin may function similarly in CME as it does in budding yeast. Furthermore, inhibition of actin polymerization with low concentrations of latrunculin B (latB) (1 mM) resulted in an increase of PM-associated CCP lifetimes, whereas higher concentrations (50 μM), which are enough to prevent cytoplasmic streaming, resulted in drastically increased lifetimes of CCPs [44,45]. It is worth noting that treatment with the general myosin inhibitor, 2,3-butanedione monoxime, which also blocks cytoplasmic streaming, likewise has a major effect on CCP lifetimes [44] as well as inhibition of flagellin receptor, FLS2 internalization [46•]. Consistent with this, in cells treated with latB, the levels of PM-associated CesA were unaffected despite the fact that CesA trafficking to the plasma membrane was significantly reduced, suggesting that the internalization of CesA is also dependent on actin polymerization [45]. Conversely, the actin-stabilizing agent, TIBA, was found to reduce the internalization rate of PM marker dyes, including FM4-64 in BY-2 cells [47]. Additionally (as discussed below), promotion of cortical actin filament accumulation inhibits the internalization rate of PIN1 from the PM suggesting that actin fibers can locally act as 'brakes' to inhibit CME [48]. Paradoxically, in this same study [48], CME was unaffected in wild-type lines treated with the actin depolymerizing agents, latB or jasplakinolide. The inhibition of CME by cortical actin network formation is consistent with the observation in renal epithelial BSC1 cells, whereby relaxation of the cortical actin network resulted in decreased CCP lifetimes [49]. Taken together, these seemingly contradictory sets of data indicate that perturbation of actin cytoskeleton dynamics, whether by stabilizing or de-stabilizing it, has inhibitory effects on endocytosis. Thus a highly dynamic actin cytoskeleton may be necessary for functional CME in plants.

Although some players involved in actin-assisted CME in yeast and mammalian cells have homologs in the plant genome, such as: Arp2/3 (actin nucleator) and dynamins, many others including intersectin, Las17 (WASP), and the CLC binding protein, Hip1r, are lacking [43,50]. Further complicating the issue is that Arp2/3 may not participate in actin-assisted CME, based on the relatively mild phenotypes of *arp2/3* mutants and its nucleators relative to mutants in other components of the CME machinery. For a more in-depth analysis on this topic see [51]. Further characterization of the role of actin dynamics in CME awaits the identification of proteins that link these two processes.

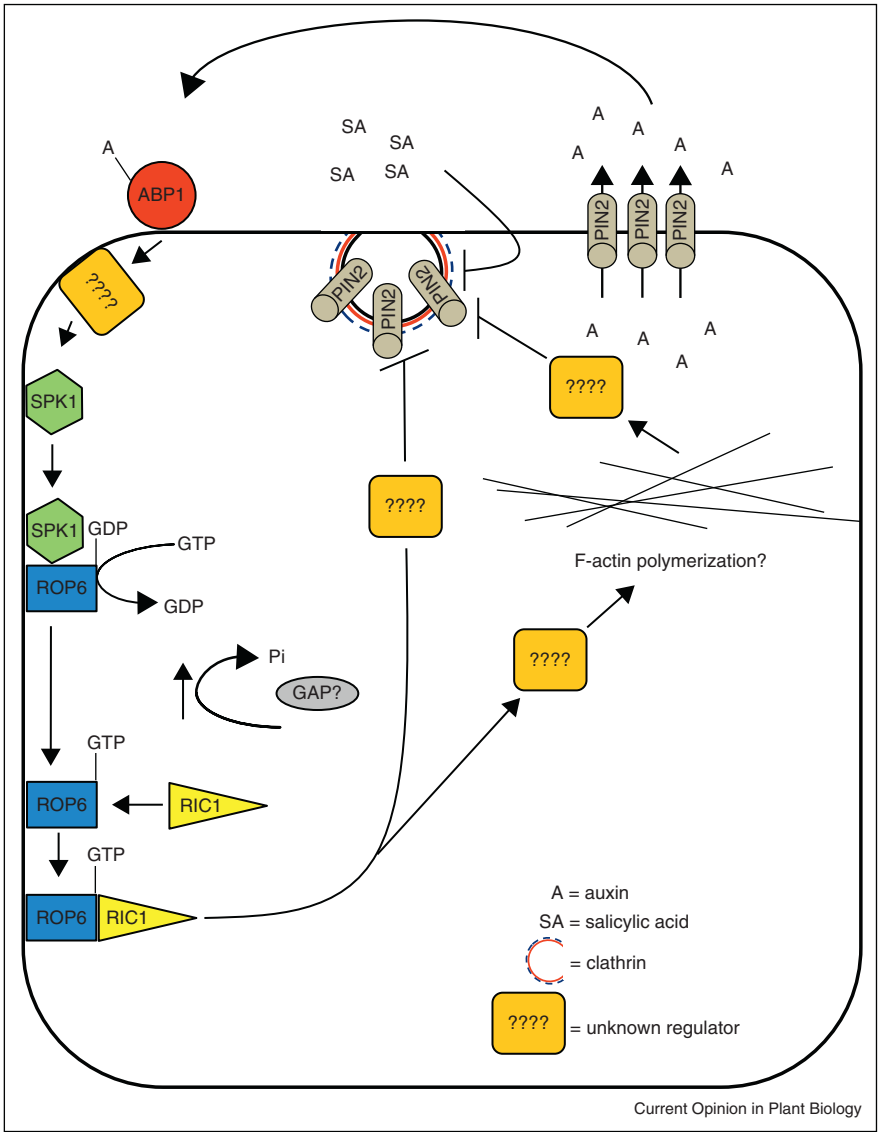
Regulation of CME

The phytohormones, salicylic acid (SA) [52], strigolactone [53], and auxin [54], regulate CME in plants. SA, which is important in pathogen-induced plant immunity [52], has recently been shown to negatively regulate CME. This process is independent of transcription and de novo protein synthesis, does not required signaling components ABP1 or NPR1-3, and is distinct from the BFA-insensitive endocytosis mechanism of the activated FLS2 receptor protein [55]. The identification of the molecular components involved in SA-mediated inhibition of CME remains to be elucidated. Interestingly, the mRNA expression levels of one (*ZmCHC1*) of two (*ZmCHC1 and ZmCHC2*) maize *CHC* homologs increase significantly in the presence of SA or abscisic acid [56•]. Putative SA and abscisic acid cis-acting elements were identified upstream of *ZmCHC1* but not *ZmCHC2*. How SA or abscisic acid regulation of *CHC* affects CME in maize or *Arabidopsis* remains to be elucidated.

The regulation of CME by auxin contributes to the polar distribution of PIN auxin transporters in root and pavement cells (PCs) [24•,57••]. In root cells, auxin negatively regulates the CME of PIN1 and PIN2 through a transcription independent, AUXIN BINDING PROTEIN 1 (ABP1)-mediated regulatory pathway, [15•,58,59••] (Figure 2). Although ABP1 has a KDEL-endoplasmic reticulum (ER) retention motif, a subpopulation of ABP1 that has escaped from the ER, associates with the outer surface of the PM and stimulates PIN endocytosis in the absence of auxin [54,59••]. Conversely, in the presence of auxin, the association of CLCs and CHCs is disrupted at the PM [15•,59••] as well as at the TGN/EE [15•] thereby inhibiting clathrin-mediated trafficking.

Insight into the mechanism of how auxin binding to ABP1 may regulate CME has also come from recent data showing that auxin/ABP1 activates the guanine nucleotide exchange factor (GEF), SPIKE1, which in turn activates the interaction of the small GTPase, ROP6,

Figure 2



A model for the regulation of the polar localization of PIN2 in root epidermal cells. The ABP1-SPIKE1-ROP6-RIC1 pathway is activated by auxin, which results in the negatively regulation of CME and stimulation of actin polymerization. A hypothetical GTPase-activating protein (GAP), responsible for stimulating the GTPase activity of ROP6 is shown. Additional 'unknown regulators' are depicted at steps in the pathway where protein-protein interaction data is lacking and an addition protein could participate. Although the mechanism of regulation by salicylic acid (SA) is unknown, the negative regulation by SA is depicted.

with its effector protein RIC1 [58,59••,60••]. Loss of function mutations in for SPIKE1, ROP6 and RIC1 induced PIN2 internalization in root cells, suggesting this pathway regulates CME since PIN2 internalization is CME-dependent [24•,60••]. This internalization was not suppressed by auxin application [60••]. Auxin/ABP1 thus not only disrupts clathrin coat assembly but also inhibits CME via activation of the ABP1-SPKE1-ROP6-RIC1 pathway, which interestingly, also promotes localized actin polymerization in root cells [60••]. Whether the ROP6-RIC1 protein complexes or actin polymerization directly regulate clathrin triskelia membrane association and/or CCV formation remains to be determined.

Intriguingly, the ADP-ribosylation factor (ARF) ARF1 and its effectors may play a role in the coordinate regulation of CME and actin dynamics. ARF's are small GTPases that function as regulators of vesicle transport, phospholipid metabolism and actin dynamics [61]. In plants, disruption of ARF1 function, by overexpression of GDP-locked dominant-negative *ARF1T31N* inhibits the internalization of the marker dye FM4-64 [62] and

mislocalization of the cell plate syntaxin, KNOLLE, during late cytokinesis due to defects in endocytosis [63]. Furthermore, regulators of ARF, including the ARF-GEF, GNOM and ARF-GAP, VAN3, which interacts with the dynamin-related protein, DRP1 [64], localize to CCP and are required for endocytosis of PM proteins including the auxin efflux PIN proteins [65]. ARF1 has also been demonstrated to be required for the localization and function of ROP2 during root hair expansion [62] suggesting that it may coordinate the formation of CCVs and actin dynamics during CME.

Conclusions

More than a decade of research has led to the current understanding of the complex network of proteins and lipids required for endocytosis in yeast and mammalian cells. By comparison our knowledge of the mechanism of CME in plants was up to very recently, rudimentary. Perhaps the lag in our understanding of the mechanisms of plant CME in plants was delayed because of the idea that endocytosis was improbable due to the high turgor pressure in plant cells, despite the morphological and biochemical data showing the existence of clathrin and clathrin-coated vesicles associated with the PM [66,67]. Alternatively, progress in understanding CME in plants may have awaited the molecular, genetic and imaging tools necessary to dissect this complex process, including the identification of bona fide endocytosed cargo that took many years to develop. With these tools has come the rapid identification and characterization of the core CME machinery and its regulation that have evolved to meet the unique requirements necessary for plant morphogenesis and growth. We look forward to the continued advances in our understanding of plant CME, which will provide new insight into plant-specific as well as fundamental aspects of CME in all eukaryotes.

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- •• of outstanding interest

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