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Never Walk Alone: Clathrin-Coated Vesicle (CCV) Components in Plant Immunity

Gayani Ekanayake,[1,*] Erica D. LaMontagne,[1,2,*] and Antje Heese[1,†]

[1]Division of Biochemistry, Interdisciplinary Plant Group, University of Missouri, Columbia, Missouri 65211, USA; email: gemrb@mail.missouri.edu, heesea@missouri.edu

[2]Current affiliation: Elemental Enzymes, St. Louis, Missouri 63132, USA; email: erica@elementalenzymes.com

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*These authors contributed equally to this article

†Corresponding author

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Abstract

At the host–pathogen interface, the protein composition of the plasma membrane (PM) has important implications for how a plant cell perceives and responds to invading microbial pathogens. A plant's ability to modulate its PM composition is critical for regulating the strength, duration, and integration of immune responses. One mechanism by which plant cells reprogram their cell surface is vesicular trafficking, including secretion and endocytosis. These trafficking processes add or remove cargo proteins (such as pattern-recognition receptors, transporters, and other proteins with immune functions) to or from the PM via small, membrane-bound vesicles. Clathrin-coated vesicles (CCVs) that form at the PM and *trans*-Golgi network/early endosomes have emerged as the prominent vesicle type in the regulation of plant immune responses. In this review, we discuss the roles of the CCV core, adaptors, and accessory components in plant defense signaling and immunity against various microbial pathogens.

INTRODUCTION

Plants are crucial food sources for humans and animals, and they also provide nonfood products such as medicine, biofuel, wood, and textiles. However, plant diseases caused by microbial pathogens can severely reduce crop yield and nutritional quality, resulting in direct economic loss and threatening food security. Notably, pathogen infection is the exception to the rule, largely because plants have evolved multiple layers of defense mechanisms to protect themselves against pathogen infection. If a plant host is compromised in preformed defenses or is unable to induce effective immune responses, a pathogen may invade and colonize the host.

Plants utilize a two-layered immune system comprising pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) to combat potential infections by invading pathogens (21, 30, 51). In PTI, plasma membrane (PM)-localized pattern-recognition receptors (PRRs) detect structurally diverse microbe- or pathogen-associated molecular patterns (PAMPs) that are derived from invading pathogens. PAMP perception initiates a plethora of immune responses that contribute to PTI (24, 44, 130). Concurrently, pathogens inject a battery of effector proteins into the host cytoplasm and extracellular space (apoplast) to interfere with the plant's ability to mount proper PTI responses, in part by targeting key cellular functions of the host (17, 67, 96, 123). In turn, plants have evolved resistance proteins to recognize pathogen effectors directly or indirectly, triggering ETI responses to stop infection when PTI fails (25, 52). Although their mode of activation differs, both PTI and ETI utilize overlapping immune signaling networks; however, ETI responses are more robust and prolonged and have a higher amplitude (53, 84, 110, 111).

During colonization, many plant pathogens propagate in the apoplast within plant leaves or roots (128). Hence, plants have developed multiple defense mechanisms functioning at the host cell surface that are present prior to pathogen infection (preformed defenses) or established during pathogen infection (induced defenses). As a preformed defense, the plant cell wall surrounds host cells and serves as a physical barrier against invading pathogens (48). Similarly, many host antimicrobial compounds, PRRs, and other proteins that are involved in pathogen perception and modulating downstream immune responses are present in the host PM and apoplast prior to pathogen infection (31, 37, 48, 100, 127). During its infection cycle, however, a pathogen can undergo different life stages, which in turn requires the host cell to adjust its defense strategies accordingly. Therefore, a critical aspect of host immunity is a plant's ability to modulate the composition of its cell surface so that it can mount effective immune responses geared toward a particular stage of pathogen infection. Host cell surface remodeling can occur via new protein and/or cell wall carbohydrate synthesis, or it can involve the relocation of existing plant PM and apoplastic proteins (31, 37, 48, 100, 127). These processes are mainly achieved by the addition and removal of proteins or carbohydrates through an elaborate intracellular membrane system. Because this process involves transporting components in small, membrane-bound vesicles between intracellular membrane compartments and the host cell surface, it is referred to as vesicular trafficking. A complex and dynamic vesicle trafficking network ensures that components with immune functions reach their site of function, namely the cell surface, to contribute to effective immunity. For example, if immune cargo enters an erroneous vesicular trafficking route, it will be delivered to an incorrect subcellular compartment, resulting in a nonfunctional component that cannot fulfill its cellular role(s) in defense.

In this review, we first introduce the vesicular trafficking routes that contribute to modulating the cell surface abundance of immune cargo. We then shift our focus to clathrin-coated vesicles (CCVs), which are the best-studied plant vesicle type and form at both the PM and *trans*-Golgi network (TGN) and sort host cargo. The majority of this review highlights the emerging roles of CCV components in plant defense, with an emphasis on immunity against bacteria, fungi, and

Pattern-triggered immunity (PTI): immunity activated in response to pathogen- or host-derived molecular patterns

Effector-triggered immunity (ETI): immunity activated in response to pathogen-derived effectors

Pattern-recognition receptors (PRRs): in plants, plasma membrane–localized immune receptors that recognize diverse pathogen- or plant-derived molecular patterns to initiate immune responses

Pathogen-associated molecular patterns (PAMPs): pathogen-derived conserved molecular motifs that can be polypeptide, lipid, or carbohydrate; perceived as nonself by host PRRs for immune signaling

Vesicular trafficking: movement of cargo from donor to target organelle via small, membrane-bound vesicles

Cargo: soluble or membrane-bound proteins, carbohydrates, lipids, and other components that utilize vesicular trafficking to move from one organelle to another

oomycetes (**Table 1**). We discuss how loss of CCV components alters defense signaling, potentially by changing the subcellular localization of immune cargo (such as PRRs).

OVERVIEW OF VESICULAR TRAFFICKING

During intracellular membrane trafficking, small vesicles bud off a donor membrane and subsequently fuse with a specific target organelle in a highly coordinated fashion. Each vesicle contains soluble components (proteins, carbohydrates, or nutrients) as well as membrane-bound components (proteins and lipids) collectively referred to as cargo in this review. The vesicular trafficking network can be divided into several different intracellular membrane pathways including secretion, vacuolar trafficking, and endocytosis (**Figure 1**) (for reviews, see 39, 59, 112, 114). In secretion and vacuolar trafficking, newly synthesized proteins start their journey in the endoplasmic reticulum followed by transport via the Golgi apparatus to the TGN. Once cargo proteins reach the TGN, vacuolar proteins are sorted away from proteins destined for trafficking to the PM and apoplast.

In endocytosis, cells internalize regions of the PM and apoplast into small endocytic vesicles to remove proteins, lipids, and nutrients from the cell surface. The various types of endocytosis include constitutive endocytosis, during which components are removed from the cell surface in the absence of any stimulus, mostly to counterbalance an increase in the cell surface due to constitutive secretion, as well as ligand-induced endocytosis, during which specific proteins are internalized from the PM in response to a stimulus (or pathogen) as a means to desensitize cells to the stimulus and/or attenuate signaling. Endocytosed components are then trafficked to early endosomes (EEs), where they are sorted for transport to either the PM via endocytic recycling or late endosomes/multivesicular bodies (MVBs) for endocytic degradation in the vacuole. Notably, in plants, the TGN and EE functionally overlap and serve as a point of convergence for secretion and endocytosis. Here, the late steps in endocytic recycling and secretion, namely the transport and fusion of TGN/EE-derived vesicles with the PM, are referred to as exocytosis.

The level of a cell surface component, such as a PM protein, is not static. Rather, its steady-state level is a function of the rates by which (*a*) the newly synthesized protein of interest is delivered to the PM via secretion, (*b*) it undergoes constitutive endocytosis, and (*c*) the endocytosed protein of interest is recycled back from the TGN/EE to the PM. Thus, the combination of these three trafficking pathways will govern a protein's abundance in the PM under naive conditions (i.e., in the absence of a stimulus). During biotic stress, the contributions of these trafficking processes may change, thereby adjusting the protein's abundance in the PM (70, 102, 103, 107). Perturbation in any of these vesicular trafficking pathways will result in altered cell surface levels of a cargo protein with defense functions, which in turn may change the amplitude and/or duration of immune response(s).

The important contributions of vesicular trafficking to plant immunity are illustrated by the increasing number of mutations in plant vesicular trafficking genes that show altered resistance to microbial pathogens (for reviews, see 14, 23, 41, 59, 113, 121, 131). Although our overall knowledge of these trafficking routes has advanced, we still do not fully understand the underlying molecular mechanisms that enable a plant cell to coordinate the transport of a specific immune cargo protein through multiple organelles to reach its site of function. Such coordination is remarkable considering that vesicular trafficking proteins do not function in isolation but rather form larger complexes of a defined set of vesicular trafficking proteins that are responsible for selecting and then transporting specific cargo molecules in a highly coordinated temporal and spatial manner. Moreover, the same cargo will be passed between compartments, trafficked by distinct vesicle types consisting of different sets of vesicular trafficking proteins depending on its donor

Clathrin: CCV core component consisting of three clathrin heavy chains and three clathrin light chains; forms triskelia into a clathrin lattice

Clathrin-coated vesicles (CCVs): vesicle type surrounded by clathrin lattice; form at the plasma membrane or *trans*-Golgi network/early endosomes to mediate endocytosis or post-Golgi transport

Endocytosis: uptake of extracellular and plasma membrane material by invagination into endocytic vesicles that bud off into the cytoplasm

Table 1 CCV components with roles in immune responses

CCV classification	Protein name	Protein abbreviation	Gene number	Species	Subcellular localization	CCV function	Pathosystem, immune cargo	Reference(s) for immune function
Clathrin core components								
Clathrin heavy chain	CLATHRIN HEAVY CHAIN 2	CHC2	*At3g08530*	*Arabidopsis thaliana*	PM, TGN/EE	Clathrin triskelion	*Pto*, *Pto hrc*⁻ FLS2-flg22 PEPR1-AtPep1 *Golovinomyces cichoracearum*	70, 80, 126
Clathrin light chain	CLATHRIN LIGHT CHAIN 2	CLC2	*At2g40060*	*A. thaliana*	PM, TGN/EE	Clathrin triskelion	FLS2-flg22	26
	CLATHRIN LIGHT CHAIN 3	CLC3	*At3g51890*	*A. thaliana*	PM, TGN/EE	Clathrin triskelion	FLS2-flg22	26
Multimeric adaptor proteins								
Adaptor protein-2	ADAPTOR PROTEIN-2 MU-ADAPTIN	AP2μ/AP2M	*At5g46630*	*A. thaliana*	PM	AP-2 subunit at PM; binds YXXΦ motif	VirE2–*Agrobacterium tumefaciens* *Pto avrRpm1* *Pto avrRpt2*	43, 65
Adaptor protein-1	ADAPTOR PROTEIN-1 MU-ADAPTIN1	AP1M1	*At1g10730*	*A. thaliana*	Golgi, TGN/EE	AP-1 subunit at TGN/EE	SPL28-*Xoo*	87
	EPS15-HOMOLOGY DOMAIN 2	AtEHD2	*At4g05520*	*A. thaliana*	PM	CME	LeEix2-EIX	8–10
Monomeric adaptor proteins								
E/ANTH proteins	MODIFIED TRANSPORT TO THE VACUOLE 1	MTV1	*At3g16270*	*A. thaliana*	TGN/EE	CCV assembly, vacuolar targeting	Pathogenesis-related 5 trafficking to vacuole	97
CCV accessory proteins								
Dynamin-related proteins	DYNAMIN-RELATED PROTEIN 1E	DRP1E/EDR3	*At3g60190*	*A. thaliana*	PM, mitochondria	Putative vesicle scission	*Erysiphe cichoracearum*	106
	DYNAMIN-RELATED PROTEIN 2A	DRP2A	*At1g10290*	*A. thaliana*	PM	Putative vesicle scission	*Turnip mosaic virus*	125
	DYNAMIN-RELATED PROTEIN 2B	DRP2B	*At1g59610*	*A. thaliana*	PM	Putative vesicle scission, CME	*Pto*, *Pto hrc*⁻ FLS2-flg22 EFR-elf26 *Turnip mosaic virus*	63, 102, 125
	DYNAMIN-LIKE PROTEIN 5A	GmSDL5A	*Glyma.08g 023300*	*Glycine max*	PM	Putative vesicle scission	*Soybean mosaic virus*	125
	DYNAMIN-LIKE PROTEIN 12A	GmSDL12A	*Glyma.05g 217300*	*G. max*	PM	Putative vesicle scission	*Soybean mosaic virus*	125
	DYNAMIN-RELATED PROTEIN 2	NbDRP2	*Nb05397*	*Nicotiana benthamiana*	PM	Putative vesicle scission, endocytosis	FLS2-flg22	18
Uncoating proteins								
Auxilin-like proteins	AUXILIN-LIKE PROTEIN 2	AX2	*At4g12770*	*A. thaliana*	PM, cytoplasm	Putative uncoating CCV	PEPR1-AtPep1	80
	XA21-BINDING PROTEIN 21	XB21	*Os12g36180*	*Oryza sativa*	ND	Putative uncoating CCV	XB21-*Xoo*	82
Other CCV-associated components								
	STOMATAL CYTOKINESIS-DEFECTIVE 1	SCD1	*At1g49040*	*A. thaliana*	PM	CME, exocytosis	*Pto*, *Pto hrc*⁻ FLS2-flg22 EFR-elf26	57

Abbreviations: CCV, clathrin-coated vesicle; CME, clathrin-mediated endocytosis; EDR3, ENHANCED DISEASE RESISTANCE 3; EFR, EF-TU RECEPTOR; ND, not determined; FLS2, FLAGELLIN SENSING 2; PEPR1, PEP1 RECEPTOR 1; PM, plasma membrane; *Pto*, *Pseudomonas syringae* pv. *tomato* DC3000; SPL28, SPOTTED LEAF 28; TGN/EE, *trans*-Golgi network/early endosome; *Xoo*, *Xanthomonas oryzae* pv. *oryzae*.

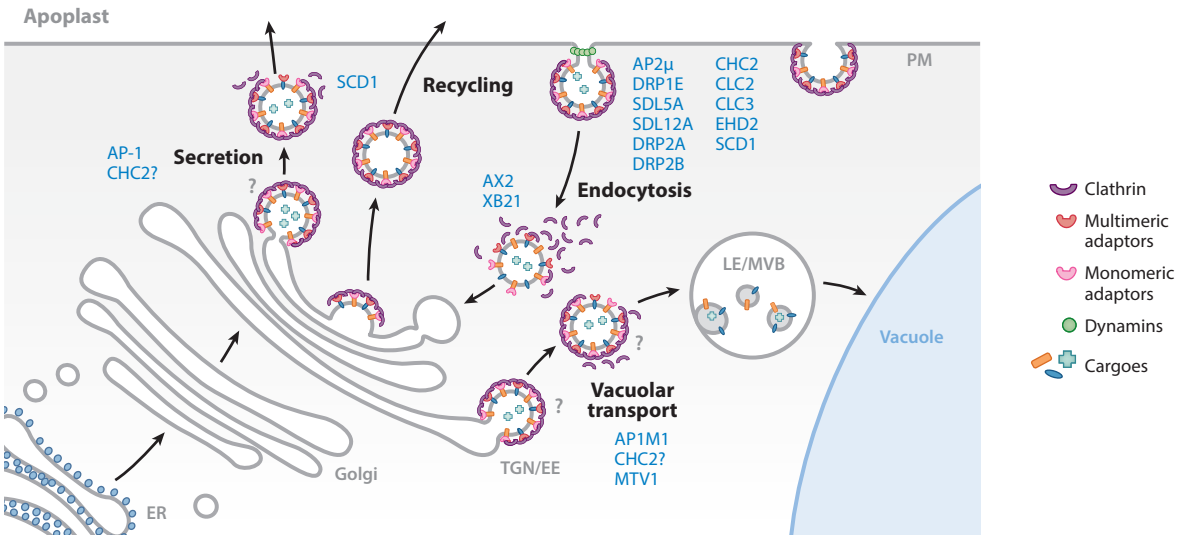

Figure 1

Vesicular trafficking pathways highlighting clathrin-coated vesicle (CCV) components implicated in immune cargo trafficking. CCVs (*purple*) denote subcellular sites at which trafficking may occur via clathrin-mediated processes. Blue lettering indicates subcellular locations of specific CCV components involved in trafficking of immune cargo. Abbreviations: AP-1, ADAPTOR PROTEIN-1; AP1M1, ADAPTOR PROTEIN-1 MU-ADAPTIN 1; AP2μ, ADAPTOR PROTEIN-2 MU-ADAPTIN; AX2, AUXILIN-LIKE PROTEIN 2; CHC2, CLATHRIN HEAVY CHAIN 2; CLC2/3, CLATHRIN LIGHT CHAIN 2/3; SDL5A/12A DYNAMIN-LIKE PROTEIN 5A/12A; DRP1E, DYNAMIN-RELATED PROTEIN 1E; EHD2, EPS15-HOMOLOGY DOMAIN 2; ER, endoplasmic reticulum; LE/MVB, late endosome/multivesicular body; MTV1, MODIFIED TRANSPORT TO THE VACUOLE 1; PM, plasma membrane; SCD1, STOMATAL CYTOKINESIS-DEFECTIVE 1; TGN/EE, *trans*-Golgi network/early endosome; XB21, XA21-BINDING PROTEIN 21.

and target organelle. For example, to secrete a newly synthesized PM-localized PRR, the protein composition of the vesicular trafficking complex that ensures transport from the endoplasmic reticulum to the Golgi (16) differs from that for cargo transport from the TGN/EE to the PM (59, 94). Adding to the complexity, a vesicle trafficking component can function at more than one subcellular localization, as exemplified by CLATHRIN HEAVY CHAIN 2 (CHC2), which contributes to CCV formation and cargo transport at both the TGN/EE and PM (discussed below) (**Table 1**).

FLS2 AS A MODEL IMMUNE CARGO UTILIZING THE VESICULAR TRAFFICKING NETWORK

Arabidopsis FLAGELLIN SENSING 2 (FLS2) has emerged as a model PRR enabling the identification of vesicular trafficking components with novel roles in immune cargo trafficking, PAMP signaling, and immunity against pathogenic bacteria. FLS2 is a receptor kinase that perceives the bacterial PAMP flagellin or its 22–amino acid peptide derivative, flg22, through its extracellular domain with the help of its coreceptor BRI1-ASSOCIATED KINASE 1 (BAK1) (20, 45, 105). Flg22 perception initiates immune signaling that helps restrict bacterial growth. Early flg22 responses include the production of reactive oxygen species (ROS), activation of mitogen-activated protein kinase (MAPK) cascades, and altered accumulation of >1,000 of transcripts. Late responses include callose deposition and transcriptional changes in pathogen-related

FLAGELLIN SENSING 2 (FLS2): plant plasma membrane–localized PRR for bacterial flagellin (or flg22)

flg22: a 22–amino acid PAMP derived from bacterial flagellin; perceived by FLS2 receptor complex as nonself to initiate immune responses

(*PR*) genes (130). Absence (or reduced levels) of functional FLS2 at the PM compromises flg22 signaling, contributing to enhanced susceptibility to flagellated *Pseudomonas syringae* pv. *tomato* (*Pto*) bacteria (15, 102, 136).

Clathrin-mediated endocytosis (CME): process facilitated by clathrin to take up proteins, lipids, and metabolites from the plasma membrane and apoplast to endocytic vesicles

FLS2 requires a functional vesicular trafficking network to fine-tune its abundance in the PM (its site of function) for effective immune responses. In the absence of flg22, FLS2 is constitutively endocytosed and recycled to the PM (12, 14). In response to flg22, however, FLS2 undergoes ligand-induced endocytosis and traffics through endosomal compartments for degradation (12, 22, 26, 102–104). After flg22-induced endocytic degradation of FLS2, newly synthesized FLS2 is delivered to the PM via the secretory pathway (22). Notably, FLS2 uses the TGN/EE as a sorting station during secretion, ligand-independent endocytic recycling, and ligand-induced endocytic degradation (12, 22; E.D. LaMontagne & A. Heese, unpublished results). Of the identified vesicular trafficking components, most are CCV components, thus pointing at a prominent role of clathrin-mediated trafficking for FLS2.

Importantly, some studies have explored how flg22 signaling is linked to FLS2 secretion and endocytosis. Secretion of newly synthesized FLS2 to the PM allows for the establishment of new rounds of flg22 signaling (61, 102, 103, 107). In contrast, ligand-induced endocytosis likely serves to remove activated FLS2 from the PM and help attenuate a subset of flg22 signaling (102, 103). So far, studies indicate that flg22 signaling originates from PM-localized FLS2. No formal evidence exists that FLS2 signaling might also occur from endosomes, which would require FLS2 to traffic from the PM to endosomal compartment(s) to initiate subsets of flg22 responses.

CLATHRIN-COATED VESICLES

Similar to yeast and animals, plants have multiple types of vesicles that differ in their protein coat components. These coat components specify at which donor membrane they form and with which target membrane the vesicles subsequently fuse. The best-studied vesicle type in eukaryotes is the CCV (55, 92), which can form at both the PM and TGN/EE in plants (91). The role of CCVs at the PM in clathrin-mediated endocytosis (CME) is well established (for reviews, see 23, 81, 89). Although their contribution in post-Golgi trafficking is less defined and somewhat controversial (90, 91), CCVs that form at the TGN/EE are implicated in protein trafficking to the vacuole, secretion of newly synthesized cargo, and recycling of endocytosed proteins to the PM (60, 97). In addition, clathrin-mediated trafficking contributes to delivering cell wall, lipid, and protein components to the plant-specific cell plate during cytokinesis (72).

Our general knowledge of the protein composition, structure, and biogenesis of plant CCVs has lagged behind that of animals and yeast (for review of the latter, see 55, 75, 92). However, advances in quantitative live-cell imaging, publicly available automated image analysis platforms, preparation of enriched CCVs from *Arabidopsis* cell culture and seedlings, and the isolation of *Arabidopsis* mutants in genes encoding CCV components have helped expand our limited understanding (6, 19, 28, 50, 62, 79, 88, 109). In addition, the relative ease with which higher-order and single null mutants can be isolated in *Arabidopsis* is enabling the plant field to address functions of CCV components beyond basic cell biology, including analysis of their in planta roles in modulating PM composition in response to plant hormones and nutrients as well as abiotic and biotic stresses (89, 133).

On the basis of these studies, the following model for CCV formation in plants emerged (**Figure 2**). Similar to animals and yeast, the biogenesis of plant CCVs requires the functions of the CCV core, adaptor, and accessory components, some of which are evolutionarily conserved across eukaryotes, whereas others are plant specific (89, 133). The recruitment of each CCV component is carefully coordinated in a spatial and temporal manner. CCV formation can be divided

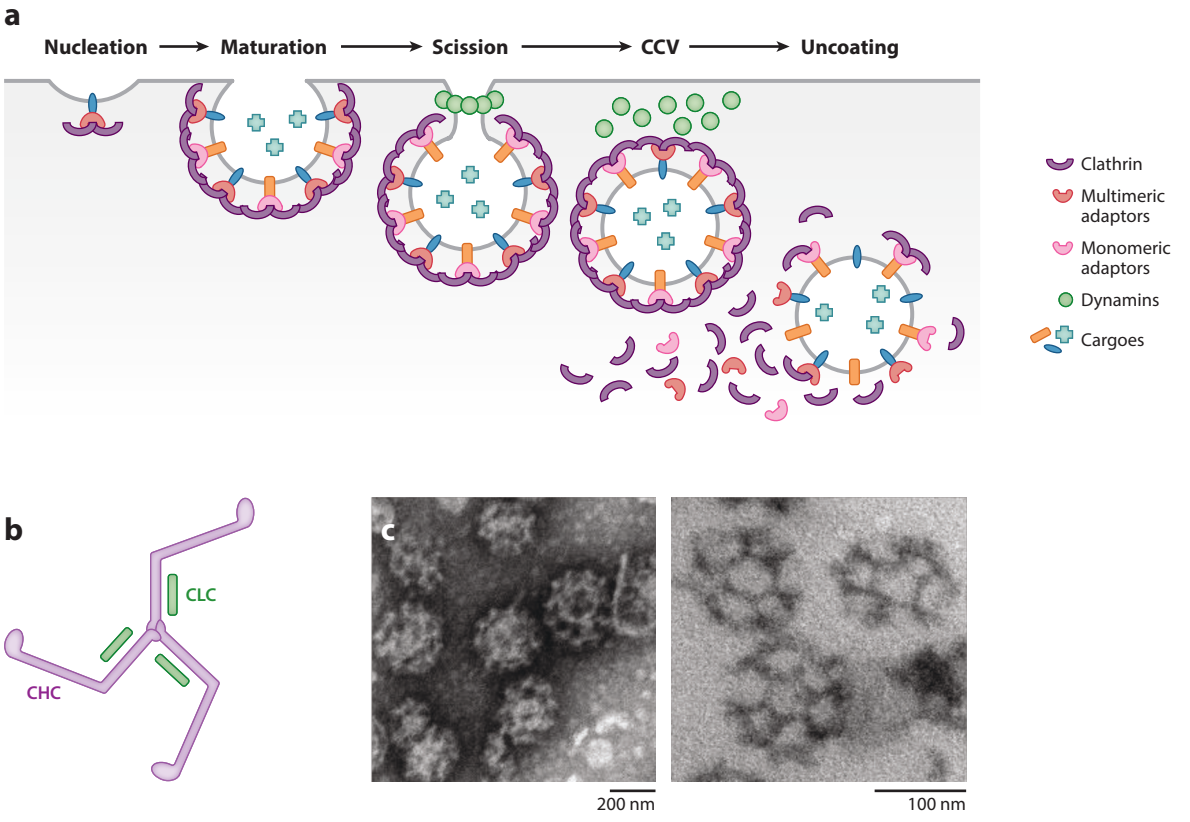

Figure 2

(*a*) Proposed model of CCV assembly and delivery in plants. (*b*) Diagram of a clathrin triskelion composed of three CHC monomers and three CLC monomers. (*c*) Transmission electron microscopy of enriched CCVs from *Arabidopsis*: (*left*) negative-stained CCVs and (*right*) conventional transmission electron microscopy of CCVs. Images provided by Dr. Sebastian Bednarek, University of Wisconsin–Madison. Abbreviations: CCV, clathrin-coated vesicle; CHC, clathrin heavy chain; CLC, clathrin light chain.

into the following steps (**Figure 2*a***): CCV nucleation and cargo selection at the donor membrane, formation of clathrin-coated pits, growth and maturation from the clathrin-coated pit into CCV, release (or scission) of CCV from the donor membrane, and uncoating of CCV to expose vesicle components necessary for subsequent fusion with the target membrane.

CLATHRIN CORE COMPONENTS

The core component, which gives CCV its name, is the evolutionarily conserved clathrin, a hexameric protein complex consisting of three CLATHRIN HEAVY CHAINs (CHCs) and three CLATHRIN LIGHT CHAINs (CLCs) that form a three-legged clathrin triskelion (**Figure 2*b***). Each leg of the clathrin triskelion consists of one CHC that binds near its C-terminal region to one CLC, and three CHC-CLC dimers form the clathrin triskelion through the C termini of CHCs. Multiple clathrin triskelions can then self-assemble into a lattice of pentagons and hexagons to form the characteristic soccer ball (or basket cage)–like structure of CCVs (**Figure 2*c***) (55, 92).

Clathrin-coated pit: invaginated membrane region caused by protein polymerization of clathrin, CCV adaptors, and accessory proteins that encase cargo on the cytosolic side

HUB Overexpression

A useful tool to study overall clathrin function in plants is the overexpression of the CHC HUB domain (HUB-ox), which comprises the C-terminal domain of CHC involved in CLC binding. HUB-ox has a dominant-negative effect, as HUB sequesters cellular CLCs, which in turn disrupts clathrin coat formation at the PM (66) and likely at the TGN/EE. In *Arabidopsis*, HUB-ox impairs constitutive endocytosis of bulk membrane using the fluorescent endocytic dye FM4-64 (56) and internalizing various PM-localized PIN proteins (56). A recent study (65) shows that HUB-ox reduces *Agrobacterium*-mediated transformation, a process by which pathogenic *Agrobacterium tumefaciens* transmits transferred DNA into plant host cells to cause crown gall tumors. HUB-ox impairs endocytic internalization of VirE2 (65), the *A. tumefaciens* effector protein that associates with the host PM and through largely unknown mechanisms reaches the host cytoplasm so that VirE2 can help transport bacterial transferred DNA into the host nucleus. Further insights into HUB-ox function come from studies in *Nicotiana* (tobacco) species in response to two different elicitors, cryptogein and ethylene-inducing xylanase (EIX). As an oomycete elicitor from *Phytophthora cryptogea*, cryptogein binds to an unidentified PM receptor to elicit immune responses (1). When stably transformed in BY-2 tobacco suspension cells, HUB-ox interferes with the formation of cryptogein-induced clathrin-coated pits (10 min post-elicitation) but not with formation of constitutive clathrin-coated pits (1). HUB-ox does not affect early ROS production and extracellular alkalization (a few minutes post-elicitation) but reduces *PR-10* mRNA induction (4 h post-elicitation) (1). Transient HUB-ox also impedes immune responses after exposure to EIX, the 22-kDa fungal elicitor protein from *Trichoderma viride*. EIX-induced cell death (2 h post-elicitation) and electrolyte leakage (24 h post-elicitation) were reduced in *Nicotiana tabacum* L. cv. Xanthi cell culture and leaf epidermal cells, respectively (101). Whether HUB-ox interferes with ligand-induced endocytosis of the EIX receptor LeEix2 and other plant PRRs remains to be determined. When interpreting such results, however, one needs to keep in mind that HUB-ox likely interferes with other clathrin functions in addition to CME.

Clathrin Heavy Chains

Arabidopsis encodes for CHC1 and CHC2, which share >90% amino acid identity and appear to have functional redundancy because *chc1 chc2* double mutants could not be recovered (56). Both CHC1 and CHC2 localize to the PM and TGN/EE (56), corresponding with CCVs forming at these subcellular locations (115). It remains unclear, however, whether CHC1 and CHC2 are present in the same or distinct clathrin triskelions, clathrin lattices, and/or CCVs. On the basis of *chc* single-mutant analyses, CHC2 has emerged as a CCV core component that modulates a variety of hormone, nutrient, abiotic, and biotic responses through cargo trafficking to and from the PM (49, 56, 60, 120) (see below).

CHC2 as a potential target of pathogen effectors. Two large-scale yeast two-hybrid interactome studies have identified CHC2 as a potential target of the pathogen effectors HaRXL62, HaRXLL515, and ATR13 from the oomycete *Hyaloperonospora arabidopsidis* and OEC45 and OEC63 from the obligate biotrophic fungus *Golovinomyces orontii* (77, 124). Future studies need to confirm these effector-CHC2 interactions and determine their biological relevance. However, CHC2 appears to be targeted by multiple effectors from at least two different pathogens, thus supporting the emerging paradigms that (*a*) effectors converge on proteins that represent points of vulnerability in plant immunity and (*b*) pathogens secrete effectors into the host cell to target vesicular trafficking components, thereby promoting host colonization by interfering with trafficking of immune cargo to their site of function (3).

CHC2 in immunity against pathogenic fungi. Notably, the contribution of CHC2 to resistance differs between microbial pathogens, potentially owing to its role in cargo trafficking at the PM and/or TGN/EE. Loss of *CHC2* results in enhanced disease resistance to the fungal pathogen *Golovinomyces cichoracearum*, potentially through its interaction with ENHANCED DISEASE RESISTANCE 4 (EDR4) (126). Both *chc2* and *edr4* single mutants display similar phenotypic defects, indicating that CHC2 and EDR4 are negative regulators of *G. cichoracearum* resistance. Both proteins localize to the PM and TGN/EE, and function in constitutive bulk membrane endocytosis using FM4-64. CHC2 may function with EDR4 in clathrin-mediated trafficking of immune cargo (such as of the MAP3K-like Ser/Thr kinase EDR1), potentially with EDR4 acting as a CCV accessory protein (126).

CCV accessory protein: protein that helps coordinate CCV formation and release from donor membranes; often recruited to CCVs by clathrin or adaptors

CHC2 in immunity against pathogenic bacteria. In contrast to its negative role in *G. cichoracearum* resistance, CHC2 plays a positive role in immunity against *Pto* DC3000 bacterial strains. More specifically, *Arabidopsis chc2* mutant plants display increased susceptibility when spray inoculated with fully virulent, pathogenic *Pto* DC3000 and *Pto* DC3000 *cor*$^{-}$, a strain that lacks coronatine (70). In *Pto* DC3000, coronatine is a bacterial phytotoxin that reopens host stomata, thereby overcoming PAMP-induced stomatal closure and allowing epiphytic pathogenic bacteria to enter the intracellular spaces of plant leaves (40, 74). These immune defects, combined with the fact that flg22-induced stomatal closure is impaired in *chc2* mutants (70), support a role for CHC2 in stomatal defenses in *Arabidopsis*. CHC2 is also implicated in PTI because *chc2* mutants show increased susceptibility to nonpathogenic *Pto* DC3000 *hrcC*$^{-}$ (70) that can neither inject effectors into host cells nor suppress PAMP-triggered responses. An open question is whether CHC2 also contributes to apoplastic immunity, a facet of plant immunity that helps impede multiplication of *Pto* strains in the apoplast of infected tissues.

CHC2 in trafficking of immune cargo and immune signaling against bacterial pathogens. Clearly, CHC2 is required for effective immunity against pathogens, raising the questions: What are the underlying molecular mechanisms for the contribution of CHC2 to immunity, and which immune components require CHC2-dependent trafficking for their function in immunity against *Pto* DC3000? Initial insights come from two recent studies that identified immune cargoes that require CHC2 for their internalization from the cell surface. Loss of *CHC2* impairs internalization of the endogenous damage-associated molecular pattern (DAMP) AtPep1 (80) and the bacterial PAMP flg22 (70) in *Arabidopsis* roots and cotyledons, respectively, showing that CHC2 functions in endocytosis of DAMPs/PAMPs in diverse plant tissues. Both studies utilized biologically active peptide ligands that are fluorescently tagged with 5-TAMRA (5-carboxytetramethylrhodamine) to monitor cellular uptake of these peptide ligands by live-cell imaging. In *Arabidopsis*, the requirement for CHC2 appears to be stronger for endocytosis of flg22 than of AtPep1 (70, 80). Although no direct evidence was provided, these *Arabidopsis* studies indicate that ligand-induced endocytosis of FLS2 and PEPR1 also depends on CHC2, mainly because FLS2/flg22 or PEPR/AtPEP1 are endocytosed simultaneously as receptor-ligand complexes when they colocalize with similar uptake dynamics in wild-type *Arabidopsis* seedlings (70, 80). Further support for the requirement of CHCs in ligand-induced endocytosis of FLS2, PEPR1, and their cognate ligands comes from heterologous expression of these *Arabidopsis* PRRs in *Nicotiana benthamiana* plants, in which gene silencing reduced the expression of six closely related *CHC* genes (*NbCHC1–6*) (70). Whether CHC2 and its related CHCs function in ligand-induced endocytosis of other PM-localized immune receptors such as EF-TU RECEPTOR, LYSIN MOTIF-CONTAINING RECEPTOR-LIKE KINASE 1, CLADOSPORIUM FULVUM-4, or LeEIX2 needs to be determined.

CCV adaptors: proteins that recruit clathrin and CCV accessory proteins to sites of CCV formation to link clathrin with cargo and/or lipids

An important yet open question regards how impaired ligand-induced endocytosis of these PRRs or their cognate ligands correlates with altered DAMP/PAMP signaling and/or susceptibility against *Pto* DC3000 strains in *chc2* mutants. In *chc2* mutants, impaired ligand-induced endocytosis of PEPR1 or FLS2 is expected to result in the increase of at least some early DAMP/PAMP responses owing to the delay in removal of activated PRRs from the PM. Such defects are observed in mutants in DYNAMIN-RELATED PROTEIN 2B (*DRP2B*) for FLS2 (102) (see below) and in endocytic mutants for the animal PRR Toll-like receptor 4 (71). So far, however, the only robust response defects observed in *Arabidopsis chc2* mutants are reduced flg22-induced stomatal closure (intermediate) and callose deposition (late response) as well as impaired AtPep1-induced root inhibition (4 days post-elicitation) (70, 80). Because analysis of immune defects has not been exhausted for these mutants, future studies may uncover new links among CHC2-dependent cargo trafficking, DAMP/PAMP signaling, and immunity against *Pto* DC3000. As noted above, CHC2 likely has roles beyond those affected by CME, including those derived from cargo trafficking from the TGN/EE.

RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD), the PM-localized NADPH oxidase responsible for apoplastic ROS production within minutes after DAMP/PAMP elicitation (54), is also affected in *chc2* mutants. Using variable-angle total internal reflection fluorescence microscopy and single-molecule tracking to quantitatively characterize the localization and dynamics of fluorescently tagged RBOHD, researchers found that loss of *CHC2* leads to a decrease in the lateral mobility as well as an increase in the number and size of fluorescently tagged RBOHD foci in the PM in the absence of any stimulus (42). Interestingly, flg22 elicitation increases the diffusion and clustering of RBOHD within the PM (42), but whether these PM mobility changes are dependent on CHC2 and are necessary to activate RBOHD remains unknown. Because *chc2* mutants display a relatively minor effect on flg22-induced ROS production (70), cellular mechanisms in addition to CHC2-dependent mobility must contribute to the governing of RBOHD activity (54, 86).

CHC1 in plant immunity. Our understanding of CHC1's potential role in plant–microbe interactions is rudimentary, but there are some key findings. *ZmCHC1* mRNA levels are upregulated by the defense hormone salicylic acid (SA) (132), and CHC1 functions in nodule formation in *Lotus japonicus* (117). The conclusion that PAMP-response defects are specific to CHC2, but not CHC1, may need to be revisited in light of a recent study showing that both CHC1 and CHC2 are defective in stomatal function as well as bulk membrane endocytosis and exocytosis (60).

Clathrin Light Chains

Much less is known about the biological role(s) of plant CLCs versus CHCs. *Arabidopsis* encodes *CLC1*, *CLC2*, and *CLC3* (98), the latter two of which are functionally redundant in auxin-related plant development that correlates with *clc2 clc3* double mutants displaying altered subcellular polarity of PIN proteins that serve as auxin efflux carriers (116, 129). A recent study implicates *CLC2* and *CLC3* in ligand-induced endocytosis of FLS2 that appears to be distinct from flg22-induced FLS2 clustering that involves the sterol-associated endocytic pathway of FLS2 (26). It remains to be determined, however, whether single or double *clc2 clc3* mutants alter flg22 signaling and/or plant immunity against microbial pathogens.

CLATHRIN-COATED VESICLE ADAPTORS

Because clathrin cannot bind directly to membrane lipids or cargo proteins, it has to work in concert with other proteins; thus, it never walks alone. Rather, CCV adaptors recruit clathrin

to sites of CCV nucleation to link clathrin with cargo and/or lipids on the donor membrane. In fact, CCV adaptors provide the specificity determining which cargo is selected and the subcellular compartment for subsequent CCV-mediated trafficking. Therefore, many CCV adaptors consist of multiple domains and peptide motifs that provide a platform for interaction with various proteins (either cargo or other vesicle trafficking proteins) and lipids. If these functions are contained within the same protein, then a monomeric CCV adaptor is involved. If multiple proteins (or subunits) need to come together to serve as the link among clathrin, cargo, and lipids, the corresponding protein complex is referred to as a multimeric CCV adaptor (78). Regardless, CCV adaptors do not function alone because several distinct monomeric and multimeric adaptors contribute to the early steps of CCV nucleation and maturation in a precise spatiotemporal sequence (38, 50, 81, 89). *Arabidopsis* CCV adaptors can be either evolutionarily conserved or plant specific.

Critical for cargo sorting, CCV adaptors recognize so-called sorting motifs present in cargo proteins. Sorting motifs can consist of short peptide regions such as the Tyr-based YXXϕ motif (where ϕ represents a bulky hydrophobic amino acid) or the di-Leu [DE]XXXL[LI] motif, both of which are recognized by specific subunits of the adaptor protein (AP) complex (discussed below). In addition, posttranslational modifications such as phosphorylation and ubiquitination can serve as endocytic sorting signals, and APs containing ubiquitin-interaction motifs recognize ubiquitination (55, 78, 92). In plants, mono- and polyubiquitination of cargo proteins have emerged as sorting signals at multiple vesicular trafficking steps, including endocytic internalization, endosomal sorting, and vacuolar targeting of ubiquitinated plant cargo proteins (for reviews, see 33, 81). Cargo ubiquitination is also required for ligand-induced degradation of PRRs such as FLS2 by the E3 ubiquitin ligases PUB12/13 (68), indicating ubiquitin-mediated vesicular trafficking plays some roles in modulating plant immunity (64).

Adaptor protein (AP) complex: evolutionarily conserved heterotetrameric protein complex that links cargo into clathrin-coated pits

TPLATE complex (TPC): plant-specific heterooctameric adaptor complex that drives early steps in clathrin-mediated endocytosis through interactions with clathrin, AP-2, dynamin-related, and other proteins

Multimeric Clathrin-Coated Vesicle Adaptors: Adaptor Protein and TPLATE Complexes

Two classes of multimeric adaptors exist in plants (38, 50, 81, 89). Heterotetrameric AP complexes are evolutionarily conserved among plants, yeast, and animals. By contrast, the more recently discovered heterooctameric TPLATE complex (TPC) may serve as an ancient plant-specific adaptor in endocytosis.

Adaptor protein complexes in the early steps of clathrin-coated vesicle formation. *Arabidopsis* encodes five structurally related but functionally distinct AP complexes (AP-1 to AP-5), each consisting of four AP subunits that are also called adaptins. Of the five AP complexes, AP-1 and AP-2 have roles in CCV-dependent cargo trafficking. AP-1 localizes to the TGN/EE and is implicated in late secretion and vacuolar trafficking (83, 122), with the latter relying on AP-1 γ-adaptin recognizing di-Leu motifs of membrane cargo (83, 122). In contrast, AP-2 functions in CME of diverse cargo proteins by modulating their PM abundance, thereby impacting cellulose biosynthesis, hormone signaling, and immune responses (11, 29, 38). Similar to its mammalian counterparts, *Arabidopsis* AP-2 consists of four subunits: AP2α, AP2β, AP2μ, and AP2σ (35). The AP2σ-clathrin interaction is consistent with the observation that *ap2σ* mutants show developmental seedling defects reminiscent of those described for *chc2* mutants (35).

AP2μ in plant immunity. Because it binds to the endocytic YXXϕ sorting motif of PM proteins, research has focused on characterizing AP2μ, and recent work (65) on VirE2 from pathogenic *A. tumefaciens* has provided direct support for AP2μ function in plant immunity. VirE2 is a secreted bacterial virulence protein that contains two C-terminal YXXϕ motifs, necessary for in

vitro binding to *Arabidopsis* AP2μ. Mutations in either YXXϕ of bacterial VirE2 or host *AP2μ* result in similar phenotypic defects, including impaired endocytosis of VirE2 and reduced tumor formation on *Arabidopsis* root fragments (65). Combined with the HUB-ox data (discussed above) and the fact that VirE2 YXXϕ motifs are conserved across pathogenic *Agrobacterium* species, these results provide evidence that *Agrobacterium* hijacks the plant CME machinery to gain entrance into host cells (65). However, not all plant immune cargo that utilizes CME requires the AP-2 complex for internalization and immune signaling; for example, internalization of TAMRA-PEP1 is not affected in *ap2μ* or *ap2σ* mutants (80). Mutants in these two *AP-2* subunits or in the *AP-1* subunits *AP1M1* or *HAP13* also showed wild-type levels of AtPep1-induced MAPK phosphorylation, indicating that these AP subunits have no apparent role in this immune signaling pathway. However, whether other DAMP/PAMP responses are altered in any *AP* mutants remains unknown.

AP2μ is also required for full ETI mediated by the resistance proteins RPM1 and RPS2 but not RPS4 (43). Specifically, *ap2μ* mutants show impaired ETI responses, including decreased hypersensitive cell death, reduced *PR1* and *PR2* mRNA induction, and increased susceptibility to *Pto* DC3000 carrying the effector genes *avrRpm1* and *avrRpt2* (43). Because RPM1 and RPS2 reside at the cytoplasmic side of the PM but RPS4 functions mostly in the nucleus, AP2μ is implicated in ETI that is mediated by PM-localized resistance proteins (43). Whether these responses involve endocytosis of specific immune receptor components remains to be determined.

Indirect insights into AP2μ function are based on trafficking and response defects for cargo receptors with a mutated YXXϕ sorting motif. For example, the YXXϕ motif is required to internalize LeEIX2 from the PM (101) and to induce hypersensitive cell death (93). However, not all immune receptors utilize their putative YXXϕ motif for immune function. The tomato immune receptor Ve1 that conveys resistance against the soilborne fungal *Verticillium* species does not require its YXXϕ motif in its response to hypersensitive cell death, although the effects on Ve1 endocytosis were not assessed (135). Other resistance proteins or coreceptors of PRR components (such as Cf-4, Cf-9, and BAK1) contain a YXXϕ motif, and future studies need to assess potential role(s) of YXXϕ and AP2μ in endocytosis and/or immune responses dependent on those PRR components.

Tyrphostin A23. In the past years, chemical interference using tyrphostin A23 (TyrA23) has been utilized by many animal and plant labs (12, 26, 42, 65, 101–103, 115) to assess whether a specific PM protein and/or an immune response requires CME and AP2μ in particular. These studies are based on TyrA23 disrupting the interaction between AP2μ and YXXϕ-containing cargo proteins, thereby impeding CME (7). However, interpretation of these TyrA23 experiments needs to be reevaluated in light of a recent study (28) showing that TyrA23 does not specifically target CME. Instead, its mode of action is through mitochondrial uncoupling and protonophoric activity that results in cytoplasmic acidification that appears to be the primary impediment to CME (28; for detailed discussion on potential off-target effects as well as the pros and cons of utilizing TyrA23 and other compounds for chemical interference affecting endocytosis, see 85, 89, 133).

AP-1 in plant immunity. Much less is known about TGN/EE-localized AP-1 subunits and their contribution to plant immunity. However, isolation of *ap1m1* as a lesion-mimic *spotted leaf 28* mutant in rice (87) is consistent with the identification of vesicular trafficking mutants displaying constitutive activation of defense responses that can be associated through as-yet-unknown mechanism(s) with increased SA signaling (57, 134). Whether *spotted leaf 28* mutants have increased SA signaling is unknown, but their rice leaves show significantly enhanced resistance to rice blast *Xanthomonas oryzae* pv. *oryzae*, likely owing to their increased cellular ROS, callose, and phytoalexins in or around the lesions (87).

TPLATE complex in clathrin-mediated endocytosis. In plants, the other multimeric adaptor complex is TPC (11, 38, 95), which is evolutionarily related to the heterohexameric adaptor TSET of *Dictyostelium* slime mold (46). However, TPC is not found in other metazoa, and the eight *Arabidopsis* TPC subunits (TPLATE, TML, TASH3, LOLITA, AtEH1, AtEH2, TWD40-1, and TWD40-2) have no clear homologs in animals or fungi (for a review, see 133). A combination of live-cell imaging and biochemical and genetic interaction studies indicates this multimeric adaptor provides necessary connections to key CME components, including clathrin (CHCs, CLCs), AP2α and AP2σ, two monomeric adaptors of the ANTH (AP180 N-terminal homology) domain family (AtECA4, CAP1), and DRP2 and DRP1 family members (11, 38, 95). Consistent with its colocalization with clathrin at the PM, individual TPC subunits function in CME of cellulose synthases, BRI1, and PINs (11, 38, 95). Notably, the TPC and AP-2 have combinatorial, but also distinct, functions in cargo selection and early steps of CCV biogenesis (11, 38, 95).

TPLATE complex in plant immunity. It remains largely elusive whether TPC subunits participate in CME of immune cargo and/or plant immunity. Although its role in plant defense is unknown, the TPC component AtEH2 contains an ESP15-homology domain similar to that of AtEHD2, previously implicated in plant immune signaling and endocytosis. Transient overexpression of AtEHD2, but not its close paralog EDH1, in *N. benthamiana* impedes endocytosis of FM4-64 and the EIX receptor LeEIX2 (8) as well as ethylene and *PR* gene induction by EIX (but not flg22) (8). This potential dominant-negative effect of EHD2-ox is apparently independent of its EHD (10) but requires SUMOylation of LeEIX2 (9). In further support for EHD2 serving as a negative regulator of EIX responses, plants with virus-induced gene silencing of *NbEHD2* showed increased ethylene and hypersensitive cell death after elicitation with EIX (8).

Monomeric Clathrin-Coated Vesicle Adaptors

Monomeric CCV adaptors combine within a single polypeptide the individual functions present in each subunit of multimeric adaptors. Hence, monomeric adaptors have a modular domain architecture consisting of multiple domains and peptide motifs that are involved in cargo recognition and binding to lipids, clathrin, subunits of multimeric adaptors, and other vesicular trafficking proteins (78). Distinct monomeric adaptors participate in CCV formation at the PM or TGN, often functioning hand in hand with multimeric adaptors (78). However, compared with multimeric adaptors, much less is known about the cellular roles of monomeric adaptors in plants (23, 81, 89, 137). As monomeric adaptors, members of the ENTH/ANTH/VHS superfamily contain an ENTH (EPSIN N-terminal homology), ANTH, or VHS (Vps27, Hrs, and STAM) domain at their N termini, which are involved in lipid binding, induction of membrane curvature, and protein–protein interactions. ENTH/ANTH/VHS proteins also contain various peptide motifs in their unstructured C-terminal domain that allow for AP and clathrin binding (27, 137). Considered an outlier of *Arabidopsis* ENTH proteins (97), *Arabidopsis* MODIFIED TRANSPORT TO THE VACUOLE 1 (MTV1) is implicated in immune cargo trafficking. In agreement with serving in CCV-dependent trafficking events at the TGN/EE, MTV1 copurifies with enriched CCVs and binds to and colocalizes with clathrin (97). Notably, MTV1 participates cooperatively with the ADP-ribosylation factor GTPase-activating protein AGD5 in the transport of several vacuolar cargoes, including pathogenesis-related protein 5 (97). This synergistic activity between MTV1 and ADG5 is notable because *Arabidopsis agd5* mutants display increased penetration rates when exposed to the fungus *Erysiphe pisi* (99). Whether MTV1 or any other ENTH/ANTH/VHS proteins have roles in plant immunity remains to be determined, but our lab has evidence that some but not all ENTH proteins contribute to immunity against *Pto* DC3000 (E.D. LaMontagne & A. Heese, unpublished results).

ACCESSORY COMPONENTS INVOLVED IN CLATHRIN-COATED VESICLE SCISSION, UNCOATING, AND OTHER FUNCTIONS

Dynamin-Related Proteins

Dynamin-related proteins (DRPs): cytosolic large GTPases forming polyhedrical structures around the CCV neck; act as molecular scissors to release CCVs from the donor membrane

After cargo selection, clathrin recruitment, and vesicle maturation, CCVs are released into the cytoplasm through the mechanochemical action of dynamins and dynamin-related proteins (DRPs). These high-molecular-weight GTPases assemble into contractile helical polymers around the neck of the budding CCV and catalyze membrane fission in a GTP-dependent manner (4, 55). Plants encode six DRP families, of which DRP1 and DRP2 family members are implicated in the scission of CCVs (reviewed in 13, 47). The evolutionarily conserved DRP2 family consists of DRP2A and DRP2B, which are considered bona fide (or classical) dynamins because of their five-domain architecture shared with animal dynamins. In contrast, plant-specific DRP1s contain only an N-terminal catalytic GTPase, a middle domain for self-assembly, and a GTPase effector domain, but they lack the pleckstrin homology domain for lipid interaction and a C-terminal proline-rich domain for interaction with SH3 domain proteins present in classical dynamins. Consistent with roles in CME and cytokinesis, both DRP1 and DRP2 family members are found at the PM and cell plate, where they colocalize with clathrin. Further underlining their roles in CME, some DRP1/2s coimmunoprecipitate with the TPLATE-associated SH3 domain–containing protein TASH3 (11, 29, 38). In addition, DRP2s are implicated in post-Golgi trafficking at the TGN/EE. Although DRP2s and DRP1s can interact with each other, it remains unknown whether they heteropolymerize for CCV scission.

DRP2s in plant immunity. DRP2A and DRP2B share 93% amino acid identity and are functionally redundant for *Arabidopsis* gametophytic development (5, 108). However, DRP2A and DRP2B do not have redundant roles in all cellular responses. *AtDRP2B*, but not *AtDRP2A*, is required for flg22-induced ROS production and robust ligand-induced endocytosis of FLS2 (102). Gene silencing of *NbDRP2* family members in *N. benthamiana* also impairs endocytosis of activated FLS2 (18). Consistent with the internalization of activated FLS2 by ligand-induced endocytosis to downregulate signaling (70, 102, 103, 107), delaying the removal of activated FLS2 from the PM correlates with enhanced, early flg22 responses such as increased ROS production and Ca^{2+} flux in *Arabidopsis* (102). Thus, FLS2 endocytosis is linked with early flg22 signaling. Notably, *drp2b* mutants show a noncanonical combination of flg22-immune defects, allowing for the separation of the flg22-signaling network into three branches. Specifically in response to flg22, DRP2B serves as a negative regulator for RBOHD/Ca^{2+}-dependent signaling, has no apparent role in MAPK-dependent responses, and functions as a positive regulator of the late-defense marker gene *PR1* (63, 102). Decreased *PR1* mRNA levels in response to flg22, *Pto* DC3000, and *Pto* DC3000 *hrcC*$^-$ correlate with increased susceptibility to these *Pto* strains in *atdrp2b* mutants (102). However, the extent to which ligand-induced endocytosis of FLS2 contributes to the regulation of these late immune responses remains unknown. The delay in FLS2 trafficking from the PM to endosomes may explain the impaired late gene expression, thus indicating that FLS2 signaling may arise from endosomal compartments. However, one needs to consider the disconnection in timing between the internalization and endocytic degradation of endogenous FLS2 (1 h post-elicitation) versus *PR1* mRNA induction (12–24 h post-elicitation) (102). Thus, for *atdrp2b*, impaired *PR1* gene induction is more likely due to the misregulation/mislocalization of the as-yet-unknown downstream component(s) of the flg22-signaling cascade that may also serve as cargo for AtDRP2B.

The roles of DRP2 in plant immunity differ notably depending on the invading pathogen. During infection by potyviruses, AtDRP2A and AtDRP2B or their orthologs *Glycine max*

(soybean) endocytosis dynamin-like protein 5A (GmSDL5A) and GmSDL12A serve as host factors that enable *Turnip mosaic virus* (TuMV) or *Soybean mosaic virus* to infect their host, *Arabidopsis* or soybean, respectively (125). Specifically, *atdrp2a* and *atdrp2b* (but not *atdrp1*) single mutants show impaired TuMV infection, whereas AtDRP2A/B overexpression results in increased TuMV infection. Providing a partial explanation for these susceptibility phenotypes, AtDRP2s are recruited to the TuMV virus replication complex and interact with viral movement proteins (125). Thus, similar to *A. tumefaciens* bacteria (65), these potyviruses appear to hijack host CCV components for pathogen entry and intercellular movement (125). In addition to TuMV movement proteins, DRP2s can interact (directly or indirectly) with other pathogen-derived proteins because NbDRP2s coimmunoprecipitate with Avr3a, an effector from the oomycete *Phytophthora infestans* that interferes with flg22 signaling and endocytosis of activated FLS2 (18). It will be interesting to determine whether NbDRP2s contribute to resistance against *P. infestans* and serve as an in vivo target(s) of Avr3a, potentially altering the cellular activities of DRP2s.

DRP1s in plant immunity. Among DRP1 family members, only AtDRP1E is implicated in plant immunity given its identification as EDR3 (106). Similar to *edr4* (126), *edr3* displays SA-dependent enhanced cell death responses upon *Erysiphe cichoracearum* (also called *G. cichoracearum*) fungal infection and supports little fungal sporulation (106). For *edr3*, increased fungal resistance is not due to constitutive activation of *PR1* gene induction. Although *edr3* is also more resistant to the necrotrophic fungal pathogen *Botrytis cinerea*, it may not alter immunity against *Pto* strains (106). Notably, phenotypic defects in *edr3* are due to a point mutation in the GTPase domain of DRP1E (106), indicating that its GTPase activity is necessary for immune function. Because a *drp1e* null mutant does not exhibit any resistance defects, *edr3* may be a gain-of-function mutant (106). Whether other DRP1 family members, as well as their GTPase activities, contribute to resistance phenotypes against microbial pathogens remains unknown.

Uncoating Proteins

After scission from the donor membrane, CCVs need to shed their clathrin coat to expose vesicular trafficking proteins that mediate fusion with the target membrane (55, 92). Similar to animals and yeast, *Arabidopsis* AUXILIN-LIKE PROTEIN 1 (AX1) and AX2 are implicated in CCV uncoating (58), likely with the help of HEAT SHOCK COGNATE 70 and SH3 domain–containing proteins such as AtSH3P1 (58). AX1 and AX2 coimmunoprecipitate with CLC1 and partly colocalize with CLC2 at the PM (2). AX1/2-ox impairs CME-dependent endocytosis of multiple cargoes and FM4-64, but their role in CME is derived solely from overexpression studies because *Arabidopsis ax1/2* single and double mutants do not display any apparent defects in development or endocytosis (2).

Auxilin-like proteins in plant immunity. Insights into AUXILIN-LIKE PROTEIN function in plant defense signaling and immunity are also based on AUXILIN-LIKE PROTEIN overexpression. In *Arabidopsis*, AX2-ox impairs uptake of the fluorescently tagged DAMP AtPEP1 and MAPK phosphorylation after AtPEP1 elicitation (80). In rice, the AUXILIN-LIKE PROTEIN Xa21-BINDING PROTEIN 21 (XB21) interacts with Xa21 (82), the immune receptor that confers resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* and may undergo endocytosis (36). XB21-ox results in increased resistance against this pathogen and elevated expression of rice genes likely involved in cell death and vesicle-mediated transport (82). However, silencing of XB21 does not result in altered resistance phenotypes (82).

Other clathrin-coated vesicle–associated components with roles in immunity. Our understanding of CCV accessory proteins in plant immunity is rudimentary. As a vesicle component

that copurifies with CCVs and colocalizes with clathrin at the PM (34, 57, 69, 73), STOMATAL CYTOKINESIS-DEFECTIVE 1 (SCD1) functions with exocyst components and RabE1 GTPases in post-Golgi trafficking to the PM, FM4-64 endocytosis, and cytokinesis (34, 57, 69, 73). SCD1 coimmunoprecipitates with FLS2 (57), indicative of a role in flg22 signaling. However, it remains unknown whether SCD1 contributes to FLS2 transport to/from the PM. Null mutants in *SCD1* are seedling lethal, but conditional *scd1-1* plants containing a point mutation can be utilized for PAMP assays and bacterial infection assays. Interestingly, *scd1-1* mutant plants display noncanonical separation of flg22 responses that mirror mostly those of *drp2b* mutants (57). Consistent with increased flg22-induced *PR* gene induction and constitutive activation of some defense responses, *scd1-1* shows increased resistance against *Pto* DC3000. However, in contrast to typical constitutive expressor of *PR* gene mutants, growth defects in *scd1* mutants are not alleviated by increased temperature or lack of SA (57); this finding provides one of the first examples of a vesicular trafficking protein whose requirement in growth and development could be uncoupled from its role in plant immunity.

DEFENSE HORMONE SALICYLIC ACID IN CLATHRIN-MEDIATED ENDOCYTOSIS

Increasing evidence implicates SA in modulating vesicular trafficking pathways, including CCV formation at the PM and TGN/EE. Prior to SA-dependent upregulation of secreted defense genes, SA coordinately induces gene expression of both endoplasmic reticulum and TGN/EE resident proteins, including vesicular trafficking components, to accommodate the increased demand on the secretory pathway during defense responses (76, 118, 119). This process is also to some extent found in *Zea mays*. Consistent with the presence of SA responsive elements in the *ZmCHC1* promoter, *ZmCHC1* (but not *ZmCHC2*) is upregulated after SA treatment (132). In *Arabidopsis*, SA also upregulates PRR complex components, including FLS2 and its coreceptor BAK1, and is likely to potentiate plant responsiveness to PAMPs (107).

In contrast to its positive effect on the secretory pathway, however, SA inhibits bulk membrane and constitutive endocytosis of several PM proteins, including PIN1 and PIN2 (32, 115). More specifically, increased SA disrupts clathrin-mediated endocytosis by impeding the incidence of CLCs, CHCs, and AP-2, but not TPC subunits, at the PM, indicating SA-sensitive and SA-insensitive CCV trafficking pathways (32, 115). Interestingly, flg22-induced endocytosis of FLS2 is insensitive to SA (32), which is puzzling considering that FLS2 endocytosis in response to flg22 is strongly impaired in a *chc2* mutant (70). SA may interfere with CCV-dependent constitutive (but not ligand-induced) endocytosis of cargo proteins only, but it remains unknown whether SA affects constitutive endocytosis of FLS2 or other immune cargoes.

CONCLUSIONS

Over the past five years, increasing evidence underscores the importance of CCV components in plant immunity against diverse microbial pathogens. However, cargo trafficking via CCVs is complex, as (*a*) clathrin does not function alone but requires the help of organelle-specific CCV adaptors and accessory proteins and (*b*) recruitment of CCV components to form and release a CCV at a target membrane is orchestrated in a precise temporal and spatial fashion. Considering this complexity, we have only scratched the surface in delineating the identity of CCV components and the underlying molecular mechanisms that contribute to a plant's ability to mount effective defenses against invading pathogens. In the future, mutants in CCV components may serve as a refined set of genetic tools that provide deeper insight into the spatial and temporal manner by

which PRRs and other PM-localized proteins with immune functions initiate or attenuate defense responses at the cell surface.

SUMMARY POINTS

1. Increasing evidence shows that clathrin-coated vesicle (CCV) components contribute to effective immunity against diverse plant pathogens, including bacteria, oomycetes, fungi, and viruses.
2. CCVs form at both the plasma membrane and *trans*-Golgi network/early endosome, likely to modulate the abundance of immune cargo at the cell surface for effective initiation and/or attenuation of defense responses.
3. So far, plant immune studies have focused on a few key CCV components: CLATHRIN HEAVY CHAIN 2 (CHC2), ADAPTOR PROTEIN 2μ, and DYNAMIN-RELATED PROTEIN 2 (DRP2).
4. The underlying molecular mechanisms and identity of other CCV adaptors and accessory components that are necessary for CCV function in plant defense remain mostly enigmatic.
5. Underscoring their importance in plant immunity, CCV components such as CHC2 and DRP2 serve as potential targets for pathogen effectors.
6. Viral and bacterial pathogens hijack plant CCV components to gain entry into plant host cells.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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Errata

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