Hitching a Ride on Vesicles: Cauliflower Mosaic Virus Movement Protein Trafficking in the Endomembrane System[1][W][OPEN]

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The transport of a viral genome from cell to cell is enabled by movement proteins (MPs) targeting the cell periphery to mediate the gating of plasmodesmata. Given their essential role in the development of viral infection, understanding the regulation of MPs is of great importance. Here, we show that cauliflower mosaic virus (CaMV) MP contains three tyrosine-based sorting signals that interact with an Arabidopsis (*Arabidopsis thaliana*) μA-adaptin subunit. Fluorophore-tagged MP is incorporated into vesicles labeled with the endocytic tracer *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl)pyridinium dibromide. The presence of at least one of the three endocytosis motifs is essential for internalization of the protein from the plasma membrane to early endosomes, for tubule formation, and for CaMV infection. In addition, we show that MP colocalizes in vesicles with the Rab GTPase AtRAB-F2b, which is resident in prevacuolar late endosomal compartments that deliver proteins to the vacuole for degradation. Altogether, these results demonstrate that CaMV MP traffics in the endocytic pathway and that virus viability depends on functional host endomembranes.

Membrane trafficking is essential in eukaryotic cells. Cellular membranes serve as a delivery system for newly synthesized proteins such as transporters and receptors exiting the endoplasmic reticulum after proper folding. They then transit through the Golgi complex, reaching the plasma membrane (PM) or the tonoplast via intermediate endomembrane compartments. Receptors and transporters returning from the PM are either recycled or targeted to the vacuole for degradation. Delivery and recycling sorting pathways overlap in the trans-Golgi network (TGN)/early endosome (EE), an intermediate compartment for both exocytosis and endocytosis (Reyes et al., 2011). In plant systems, the endoplasmic reticulum and PM provide membrane continuity between cells through the connections made by plasmodesmata (PD), cytoplasmic channels that regulate traffic in the symplasm (Maule et al., 2011).

The selective transport of macromolecules between different compartments of the endomembrane system is mediated by coat proteins promoting the generation of small cargo-trafficking coated vesicles (Spang, 2008). The recognition and recruitment of cargo proteins are mediated by so-called adaptor complexes (AP complexes [AP-1–AP-4]; Robinson, 2004) one of which, AP-1, is localized on the TGN/EE and endosomes, whereas AP-2 is in the PM. The μ-subunit of AP complexes is devoted to cargo protein selection via a specific and well-characterized interaction with a Tyr-sorting signal, YXXΦ, where Φ is a bulky hydrophobic residue and X is any amino acid (Bonifacino and Dell'Angelica, 1999). YXXΦ motifs are present in the cytoplasmic tail of many proteins integral to the PM and TGN/EE and have been found in the movement proteins (MPs) of some viruses (Laporte et al., 2003; Haupt et al., 2005). Plant viruses are obligate parasites that exploit host components to move within the cell and from cell to cell into the vascular system for systemic invasion of the host. Virus movement, which requires the passage of macromolecules through PD connections, is mediated by one or more virus-encoded MPs with the help of the host cytoskeleton and/or endomembranes (Harries et al., 2010). While most MPs act to increase the size exclusion limit of PD to facilitate the passage of the viral nucleoprotein complex, other MPs are assembled in tubules that pass inside highly modified PD and transport encapsidated particles through their lumen.

Here, we focus on this second group of tubule-forming MPs and examine the intracellular trafficking of cauliflower mosaic virus (CaMV) MP. The MP encoded by CaMV forms tubules guiding encapsidated virus particle cell-to-cell transport via an indirect MP-virion interaction (Stavolone et al., 2005; Sánchez-Navarro et al., 2010). However, how CaMV MP (and the other tubule-forming MPs) targets the PM and forms tubules remains to be elucidated. Tubule-forming MPs do not require an intact cytoskeleton for PM targeting

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(Huang et al., 2000; Pouwels et al., 2002) and/or tubule formation (Laporte et al., 2003). However, suppression of tubule formation upon treatment with brefeldin A (BFA), a specific inhibitor of secretion or endocytosis, suggests the involvement of the endomembrane system in correct functioning of some tubule-forming MPs (Huang et al., 2000; Laporte et al., 2003). In this study, we examined the three Tyr-sorting motifs in CaMV MP and show that each of the three domains interacts directly with subunit μ of an Arabidopsis (*Arabidopsis thaliana*) AP complex. Mutations in these domains revert in the viral context to maintain CaMV viability. MP is found in endosomal compartments labeled by AtRAB-F2b (ARA7) and *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl) hexatrienyl)pyridinium dibromide (FM4-64). The presence of at least one functional YXXΦ domain is essential for the localization of MP to endosomes and for tubule assembly but is not required for MP targeting to the PM. We provide several lines of evidence to show CaMV MP trafficking in the endocytic pathway. Our findings are discussed in the light of the recent demonstration that the TGN/EE functions as a major hub controlling secretory and endocytic pathways in plants.

RESULTS

CaMV MP Fusion Protein Labels Motile Spherical and Vesicle Structures

Expression of a GFP-tagged CaMV MP (GFP-MP) in protoplasts produces characteristic foci at the PM, which increase in size with time, suggesting intensive MP targeting and/or cycling activity to the cell periphery. Some of these foci exhibit fluorescent tubules protruding distally from the cell surface that are visible from 10 to 12 h post transfection (hpt), and their number and length increase with time. At 4 hpt, only a small fraction of protoplasts appears fluorescent. In most of these, MP shows a diffuse localization on the PM, although the initial accumulation in foci is visible as small hotspots (Fig. 1A, arrowheads). At this time point, within the same population of fluorescent protoplasts, very few display obvious foci. At 6 to 8 hpt, MP accumulates prevalently in foci (Fig. 1B), and protoplasts with a homogenous distribution over the PM are observed only rarely. Tubule primordia appear at this time point; these elongate and continue forming at the cell periphery, and at 24 hpt, most protoplasts display a typical phenotype with tubules of variable number and length (Fig. 1C). Starting from 10 hpt, a population of motile spherical and vesicle-like structures becomes visible in the cytoplasm of transfected protoplasts (Fig. 1D) and in biolistically bombarded turnip (*Brassica rapa*) plants (Fig. 1E). In turnip leaf tissue with intact cell wall, these structures are preferentially localized at the cell periphery, and some of the vesicles seem to be associated with the PM, from where they might be derived (Fig. 1F). To examine this possibility, we treated *Nicotiana benthamiana*

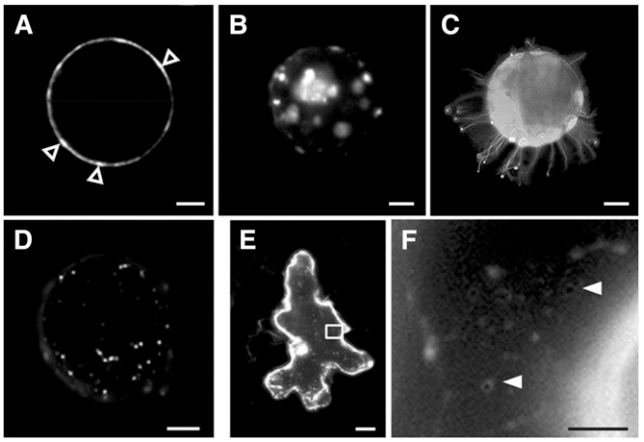

Figure 1. Transient expression of CaMV MP fluorescent fusion protein. A to E, GFP-MP localization in *N. benthamiana* protoplasts at 4 (A), 6 (B), 24 (C), and 12 (D) hpt and in turnip epidermal cells at 24 (E) hpt. Arrowheads in A indicate hotspots of the initial accumulation of GFP-MP in foci. F, Higher magnification image of the inset in E shows vesicles (arrowheads) and spherical bodies. Bars = 20 μm.

protoplasts and leaves expressing GFP-MP with the lipophylic dye FM4-64, a marker for PM internalization and endosomes (Bolte et al., 2004). This dye inserts into the outer leaflet of the surface membrane, from where it becomes internalized within PM-derived vesicles. Therefore, FM4-64-labeled vesicles are, at least in part, a component of the endocytic pathway. FM4-64 colocalized with the large majority but not all of the cytoplasmic structures labeled with GFP-MP in transfected protoplasts (Fig. 2A) and in bombarded epidermal cells (Supplemental Fig. S1). Higher magnification images display colocalization of the MP fusion protein with some dye-labeled vesicles (Fig. 2B). These data indicate that at least a fraction of GFP-MP localizes to PM-derived endosomes and vesicles.

MP Colocalizes with the EE Population

Colocalization with FM4-64 suggests that CaMV MP might be recruited as a cargo protein at the PM and from there be internalized. Interestingly, the amino acid sequence of CaMV MP contains three putative Tyr-based sorting signals (ts1–ts3): YLPL (amino acids 100–104), YGKF (174–177), and YPKF (182–185; Fig. 3A) conforming to the consensus receptor motif YXXΦ that has been shown to interact directly with the μ-subunits of clathrin AP complexes. Sequence comparisons revealed almost perfect conservation of the three motifs among all species of the *Caulimovirus* genus (type member CaMV), a very high percentage of identity or similarity for residues flanking the motifs, and absolute identity of the four residues separating YGKF and YPKF in the species analyzed (Fig. 3B). Based on the results of previous studies that established preferences for the interaction of Tyr-based

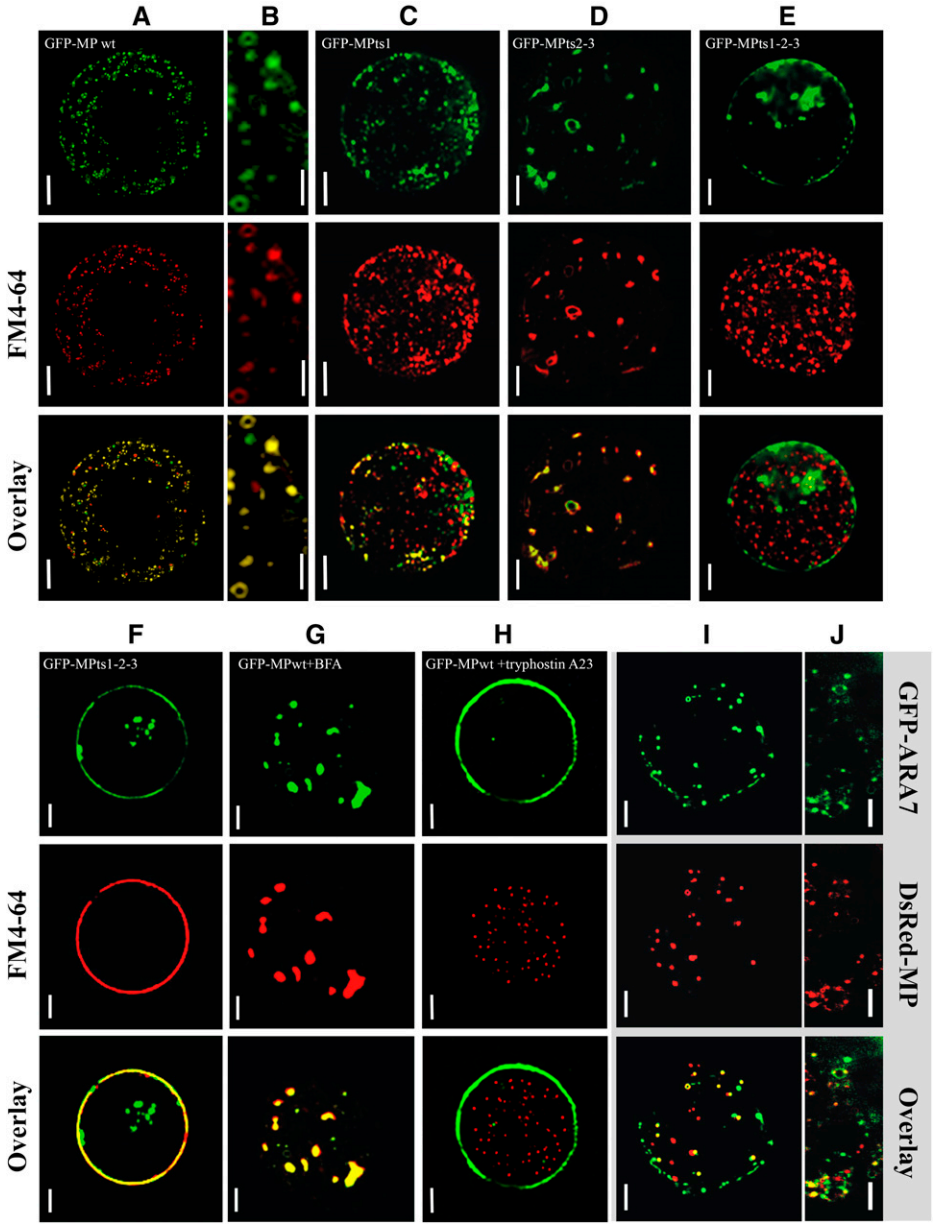

Figure 2. Localization of GFP-MP wild type and Tyr mutants to post-Golgi compartments. A and C to E, Z-series projection of protoplast images showing GFP-MPwt (A), GFP-MPts1 (C), GFP-MPts2-3 (D), and GFP-MPts1-2-3 (E) at 18 to 20 hpt and 30 to 60 min after treatment with FM4-64. B, Magnified views of the images in A showing vesicular and spherical structures in more detail. F, Middle section of protoplast expressing GFP-MPts1-2-3 at 8 hpt and 15 min after treatment with FM4-64. G, Z-series projection of protoplast images showing GFP-MPwt at 20 hpt and 60 min after treatment with FM4-64 and 45 min after BFA administration. H, Middle section of protoplast expressing GFP-MPwt at 20 hpt and 60 min after treatment with FM4-64 and 90 min after tyrphostin A23 administration. I, Z-series projection of protoplast images showing DsRed-MPwt and GFP-ARA7 labeling endosomes and vesicles. J, Magnified views of the images in I showing protein colocalization in vesicles and spherical structures in more detail. Bars = 20 μm except for B and J, where bars = 5 μm.

domains with AP complexes using the yeast two-hybrid system (Ohno et al., 1998), we could not predict for any of the three CaMV MP signals (ts1–ts3) a clear specificity for any of the adaptor medium subunits, although there might be slight preference for the μ-adaptin of the AP-2 complex, which mediates protein internalization from the PM.

To examine the functionality of the three Tyr signals, we prepared six mutants of wild-type MP (MPwt), knocking out the domains one by one, in pairs, or all three together (Fig. 3A). Gly replaced Tyr in all mutants and Phe in ts2 and ts3, whereas the two Leu residues of ts1 were exchanged for Ser and Gly, respectively. To investigate whether the endosome localization of MP was related to Tyr sorting signals, and thus to vesicle transport, we treated protoplasts and leaf tissue transfected with GFP-MP YXXΦ mutants with FM4-64. Independent of which Tyr signal was modified, all three single mutants colocalized with part of the endosome population labeled by FM4-64 (Fig. 2C; Supplemental Fig. S1D), and the same partial colocalization was observed upon transient expression of double mutants (Fig. 2D; Supplemental Fig S1F). This indicates that a single Tyr signal is sufficient to preserve the cargo activity of MP. However, vesicular structures were not observed upon the expression of the triple mutant GFP-MPts1-2-3. The protein remained diffused in the cytoplasm, sometimes accumulating in discrete foci, some of which resembled endosomes, but never colocalizing with the FM4-64-labeled endosome population (Fig. 2E; Supplemental Fig. S1H). These results suggest that MP could recruit cytosolic adaptins via at least one of the

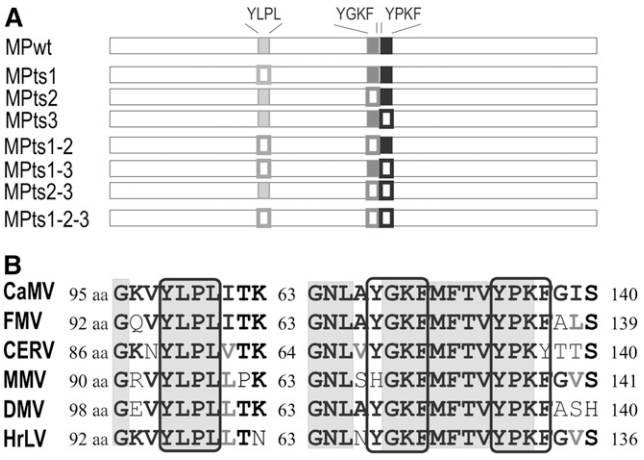

Figure 3. Mapping of YXXΦ signals in the MP sequences of *Caulimovirus* species. A, Schematic representation of CaMV MP wild type and mutants depicted by boxes with predicted YXXΦ signals (gray boxes) and mutated signals (white boxes). B, Alignment of MPs from distinct species of the genus *Caulimovirus*. Shown are virus acronyms (listed below) and the number of residues separating amino acid blocks and the protein N and C termini, predicted Tyr-based sorting signals (boxes), amino acids identical to MP (boldface), and amino acids conserved in all MPs (highlighted in gray). FMV, *Figwort mosaic virus*; CERV, *Carnation etched ring virus*; MMV, *Mirabilis mosaic virus*; DMV, *Dahlia mosaic virus*; HrLV, *Horseradish latent virus*.

three Tyr-based sorting signals to traffic through the endocytic pathway.

YXXΦ Signals Play Redundant, But Essential, Roles in CaMV Infectivity

When fused to GFP, MP cannot facilitate virus movement, probably because the fusion protein is sterically hindered in tubule assembly within the PD pore (Thomas and Maule, 2000; Amari et al., 2010). Therefore, a role for MP endocytosis in virus activity was examined by testing the effect of Tyr domain mutations on CaMV viability and infectivity. To this aim, we replaced wild-type MP with all Tyr mutants in the infectious viral clone pCaΔ2 and inoculated turnip plants. Results from three separate experiments showed symptom appearance on all inoculated plants, with no significant delay for the MP YXXΦ mutants. Sequencing analysis of the MP coding sequence revealed that at least one of the motifs was restored in all virus mutants, and after one passage to new turnip plants, exclusively wild-type sequence was found in all extracted viral DNA (Supplemental Table S1). The high level of reversion of mutations strongly suggests the importance of the YXXΦ signals for CaMV viability, but it hampers the unequivocal conclusion that a virus containing MPts1-2-3 is not infectious. To further support this inference, we next tested the ability of wild-type CaMV to infect the BFA-visualized endocytic trafficking defective1 (BEN1) and BEN2 Arabidopsis mutants, which are both defective in TGN/EE trafficking (Tanaka et al., 2009, 2013). BEN1 carries a mutation in the Sec7 domain protein ADP ribosylation factor guanine nucleotide exchange factor/Brefeldin A-inhibited guanine nucleotide-exchange protein5 that prevalently affects the process from the EE to the recycling endosome, whereas BEN2 bears a modified vacuolar protein sorting-associated protein45 protein, which functions in the vacuolar targeting pathway.

The absence of symptoms and viral DNA (Supplemental Fig. S2) in systemic leaves of both BEN1 and BEN2 provides strong and conclusive support for the hypothesis that endocytosis is essential for CaMV infectivity.

Tyr-Based Sorting Motifs Are Essential for Tubule Formation But Not for MP Targeting to the PM

To gain more insight into the role of YXXΦ motifs in MP function, we examined MP mutant performance in tubule assembly, which is an essential function for MP and, in turn, for infectivity of CaMV. GFP fusions of these seven mutants were expressed in protoplasts and observed at 30 hpt. Mutations of the conserved motifs one by one or in pairs did not suppress the capacity of MP to induce tubule formation at the protoplast surface (Fig. 4, A–F). Results obtained from six independent experiments indicated that tubules produced by these mutants appear at the same time as those formed by the wild-type MP but in significantly reduced numbers (1%–20% of total fluorescent protoplasts, corresponding to about 50% of all transfected protoplasts). Tubules were thinner than the wild type (50%–70% of normal diameter). Only a few extended to a length (60–100 μm) similar to the average size of wild-type tubules, whereas most of the mutant-formed tubules did not grow longer than 20 μm, and in no case were

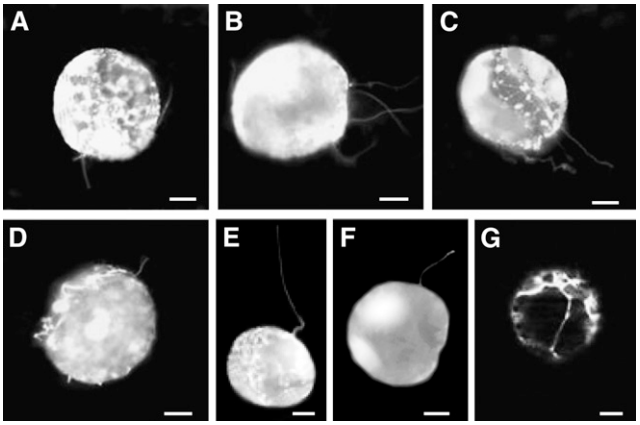

Figure 4. Transient expression of GFP-MP Tyr mutants in *N. benthamiana* protoplasts. Tubules are visible in GFP-MPts1 (A), GFP-MPts2 (B), GFP-MPts3 (C), GFP-MPts1-2 (D), GFP-MPts1-3 (E), and GFP-MPts2-3 (F) but not in GFP-MPts1-2-3 (G). Note that images A through F are overexposed to allow better resolution of the thin tubules. Bars = 20 μm.

extra-long tubules, normally formed in limited amounts by wild-type MP, observed. Upon simultaneous mutation of all three YXXΦ motifs, the MPts1-2-3 mutant failed to form tubules and diffused in the cytoplasm similar to free GFP (Fig. 4G).

We reasoned that the absence of tubules upon the expression of GFP-MPts1-2-3, besides depending on the loss of tubule-assembling function, could also be an indirect consequence of the inability of MP to target the PM. To verify this hypothesis, we monitored the localization of GFP-MPts1-2-3 in transfected protoplasts at different time points between 4 and 30 hpt. At 8 hpt, when the majority of wild-type GFP-MP is accumulated in foci, the triple mutant was diffused prevalently at the cell periphery of protoplasts, with early colabeling with FM4-64 confirming this location to be the PM (Fig. 2F). However, after this time point and within 18 to 20 hpt, GFP-MPts1-2-3 was completely diffused in the cytoplasm (Fig. 4G). These results demonstrate that Tyr signals are not essential for sorting MP to the PM.

To investigate whether GFP-MP conserved the ability to enter PD upon mutation of the three Tyr signals, we next transiently expressed in intact *N. benthamiana* cells the seven GFP-MP mutants together with tobacco mosaic virus (TMV) red fluorescent protein (DsRed)-tagged MP, which shows strong targeting to PD in newly infected cells (Lucas, 2006). Colocalization with TMV-tagged MP confirmed that all Tyr mutants accumulate in PD except MPts1-2-3 (Supplemental Fig. S3).

The evidence that MPts1-2-3 can target the PM but lacks the capacity to accumulate in PD and to form tubules provides support for the notion that the three YXXΦ signals might be required for targeting MP to PD.

Within PD, CaMV MP interacts with PD-located protein1 (PDLP1), a protein with receptor-like properties involved in the assembly of MP tubules required for virus movement (Amari et al., 2010). To exclude that mutation of Tyr signals changed the structural properties of MP, causing the failure of such interaction and thus explaining the loss of MP tubule assembly function, we tested the ability of the MPts1-2-3 mutant to bind PDLP1. To this aim, we produced recombinant PDLP1 in fusion with an influenza virus hemagglutinin epitope (HA) tag (Fig. 5, lane 4), which we used to challenge glutathione *S*-transferase (GST)-MPwt and GST-MPts1-2-3 in a pull-down assay.

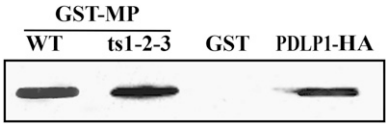

Figure 5. MPts1-2-3 mutant interacts with PDLP1. GST-MPwt (WT), GST-MPts1-2-3, and control GST were subjected to pull-down reaction in the presence of recombinant PDLP1-HA. GST alone was used to detect nonspecific protein interactions. The samples were fractionated by SDS-PAGE and analyzed by western blotting with an anti-HA antibody.

Western-blot analysis with anti-HA antibody revealed that the MPts1-2-3 mutant efficiently binds to PDLP1-HA (Fig. 5), thus confirming that the structure of MP is not affected upon mutation of all three MP Tyr signals. As the interaction with PDLP1 is required for the assembly of viral MPs into tubules, this result also suggests that MPts1-2-3 is potentially able to form tubules in PD. The evidence that MPts1-2-3 interacts with PDLP1 but does not accumulate in PD supports the hypothesis that lack of tubule assembly in protoplasts might relate to an inability of MPts1-2-3 to target PD rather than to assemble into tubules.

Effect of Tyrphostin A23 and BFA on the Internalization of MP from the PM

To characterize in more detail the vesicle-mediated pathway of CaMV MP, we tested the effect of two inhibitors on MP localization in protoplasts transfected with GFP-MP. In plants, BFA inhibits recycling from endosomes back to the PM (Geldner et al., 2003; Dhonukshe et al., 2007). Treatment of transfected protoplasts with BFA for 1 h led to the accumulation of GFP-MP in large patches (Fig. 2G), reminiscent of BFA-induced compartments where rapidly recycling proteins accumulate upon the treatment (Murphy et al., 2005). The patches, which were not observed in protoplasts transfected with free GFP (Supplemental Fig. S4), were also stained with internalized FM4-64 (Fig. 2G). This result suggests that MP behaves like those proteins cycling between PM and endosomes and accumulating in BFA compartments upon the inhibition of recycling.

To further confirm the notion that CaMV MP traffics in the endocytic pathway, we tested the pharmacological interference of tyrphostin A23 with MP internalization from the PM. Tyrphostin A23 inhibits endocytosis by interfering with the μ2 recognition of Tyr signals (Banbury et al., 2003; Ortiz-Zapater et al., 2006; Dhonukshe et al., 2007). In transfected protoplasts, treatment with tyrphostin A23 led to the reorganization of GFP-MP distribution, which concentrated almost exclusively at the cell surface and no longer in foci (Fig. 2H; Supplemental Fig. 5), but had no effect on free GFP localization (Supplemental Fig. S4). Colocalization with the PM marker H^+-ATPase expressed in fusion with DsRed further confirmed the PM localization of GFP-MP (Supplemental Fig. S6). FM4-64-labeled endosomes remained visible upon the same treatment, but no colocalization with GFP-MP was observed (Fig. 2H, overlay).

CaMV MP Interacts with Arabidopsis μA-Adaptin

The results presented here suggest that CaMV MP can traffic in the endocytic pathway, probably via recruitment of specific PM-located adaptins. μA-Adaptin (μA) from Arabidopsis is restricted to the TGN and mediates trafficking to the vacuole; nevertheless, it shows the highest similarity to mammalian PM μ2-adaptin

(Happel et al., 2004). Arabidopsis is a natural host of CaMV, and μA has been shown previously to bind YXXΦ motifs located in the cytoplasmic tail of both plant and human receptors (Happel et al., 2004; Ortiz-Zapater et al., 2006). Therefore, we tested μA as a possible binding partner for CaMV MP by transiently expressing CaMV MP in fusion with the GST in Arabidopsis (AtGST-MP) and pulling it down on glutathione-Sepharose 4B beads. Western-blot analysis with anti-μA antibody revealed the presence of μA pulled down together with GST-MP from leaf extract (Fig. 6A, lane 2). In addition, as CaMV MP accumulates very poorly in the cytosolic fraction, we used large amounts of Arabidopsis leaf extract to treat 4B beads previously saturated with GST-MP expressed in *Escherichia coli* (EcGST-MP) and confirmed by western blot with anti-μA antibody that GST-MP interacts with Arabidopsis μA (Fig. 6A, lane 3).

To examine the functionality of the three Tyr signals, we tested direct interaction of the six mutants of MP expressed in bacteria (Fig. 6B, first row) with GST-μA.

Since expression of the full-length μA adaptin in fusion with GST in our hands generated very low levels of fusion protein, we cloned the C-terminal receptor-binding region of μA, which conserves its functionality when expressed separately (Happel et al., 2004), in fusion with GST to obtain a GST-μA fusion protein of 64 kD (Fig. 6B, first row). This protein was challenged with the seven MP mutants in a pull-down assay. Figure 6B (third row) shows that wild-type CaMV MP binds the μA subunit, thus confirming that it can act as a cargo protein for vesicle transport. Mutation of any one of the three MP YXXΦ signals (single or pairs in any combination) did not affect the interaction, whereas GST-μA was unable to pull down MP upon mutation of all three YXXΦ domains together (Fig. 6B, third row).

However, CaMV MP is a multifunctional protein with low tolerance of sequence modification. To rule out that the triple mutation of Tyr signals changed the structural properties of MP, causing failure of binding, we challenged the seven MP YXXΦ mutants in a pull-down assay against the CaMV-encoded virion-associated protein (GST-VAP), whose interaction with MP is essential for virus movement (Stavolone et al., 2005). We show that GST-VAP can efficiently pull down all seven mutants (Fig. 6B, bottom row). This result, together with the evidence of the interaction with PDLP1 (Fig. 5), confirms the unchanged structural properties of the MPts1-2-3 mutant.

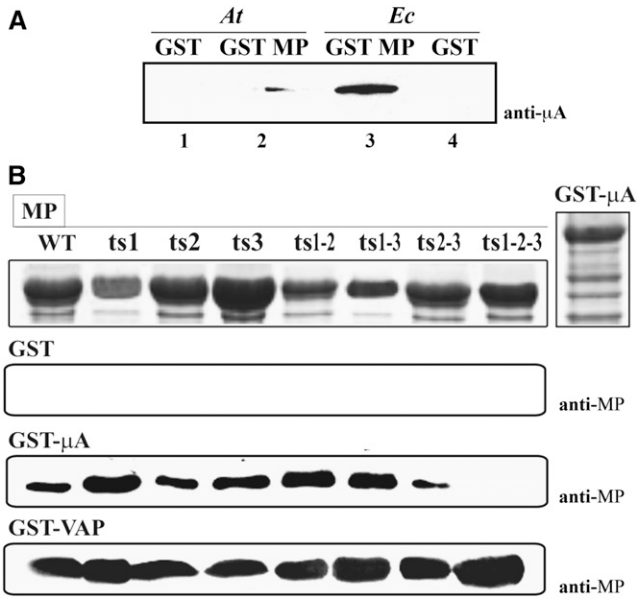

Figure 6. CaMV MP interacts with Arabidopsis μA. A, GST (lane 1) and GST-MP (lane 2) expressed in Arabidopsis (*At*) and pulled down with (or without) μA using glutathione-Sepharose beads and GST-MP (lane 3) and GST alone (lane 4) expressed in *E. coli* (*Ec*) and subjected to pull-down reaction in the presence of Arabidopsis leaf extract. Pulled-down protein complexes were separated by SDS-PAGE and analyzed by western blotting with an antibody raised against μA. B, GST-μA and CaMV MP wild type (WT) and its YXXΦ mutants expressed and separated by SDS-PAGE (first row). GST pull-down assay was carried out using glutathione-Sepharose beads to collect GST alone (second row), GST-μA (third row), and GST-VAP (fourth row) and test their interaction with CaMV MP wild type and its ts mutants. Interacting MP wild-type and mutant proteins are indicated at the top. Pulled-down protein complexes were separated by SDS-PAGE and analyzed by western blotting with an antibody raised against the CaMV MP coiled coil.

MP Is Retrieved in the Late Endocytic Pathway

Our findings provide strong evidence that CaMV MP traffics in the endocytic pathway with the help of its YXXΦ signals, binding a yet uncharacterized PM μ2-like plant adaptin. Nevertheless, these same signals mediate MP interaction with μA, which resides mainly at the trans-Golgi rather than at the PM (Happel et al., 2004). As Tyr signals can interact with several adaptor complexes, these results are clearly not in conflict and suggest their possible involvement in additional functions. Consistent with our findings, it is very intriguing that CaMV MP also binds Prenylated Rab acceptor1 (PRA1), a Rab GTPase receptor that localizes to prevacuolar compartments (PVCs; Alvim Kamei et al., 2008). To investigate in more detail the identity of the endosomal vesicles labeled by CaMV MP, we used fluorescently tagged Arabidopsis ARA7/RabF2b GTPase (GFP-ARA7) as a marker for endosomal compartments/PVCs and analyzed the colocalization with CaMV MP expressed in fusion with red fluorescent protein (DsRed-MP). In protoplasts extracted from plants bombarded with GFP-ARA7 and DsRed-MP, we observed that a fraction of the endosome and vesicle population labeled with CaMV MP also contained GFP-ARA7 (Fig. 2I). Vesicles labeled with GFP-ARA7 appeared very polymorphic, particularly in size (Fig. 2J). This result indicates that CaMV MP is also present in PVCs.

DISCUSSION

The fundamental role of MPs is to mediate viral genome transport across the plant cell wall through PD

channels. Some viruses, including CaMV, have developed surprisingly complex mechanisms of transport, which require significant anatomic modification of PD. The evolutionary and functional meaning of the tubule-mediated virus movement strategy is not yet understood, and very little is known about the underlying mechanism or the host factors involved. Our results draw connection lines between the plant endosomal system and tubule-mediated virus movement, both yet poorly understood topics.

Previous work on CaMV MP has shown that neither the formation nor the maintenance of foci and tubules requires normal functioning of the cytoskeleton, whereas interference of BFA (Huang et al., 2000) and interaction of CaMV MP with secretory cargos, such as PRA1 (Huang et al., 2001), pectin methylesterase (Chen et al., 2000), and PDLP1, support a possible role of endomembrane transport in virus infection. Consistent with its virus transport function, CaMV MP localizes to the PM, and while not a typical transmembrane protein, it harbors three domains fitting the consensus YXXΦ motif, each of which binds specifically and with significant affinity to μA. The presence of three Tyr-based sorting signals in genus *Caulimovirus* MPs is intriguing because, in most cargo proteins, only one signal occurs together with a transmembrane domain, and this arrangement is functionally sufficient. Indeed, the presence of only one of the three motifs supports MP tubule formation and CaMV viability and infectivity, suggesting a compensatory/redundant function. On the other side, evidence that also single and double Tyr CaMV mutants, even though infectious, undergo an extremely high rate of back mutation in infected plants supports the essential role of each of the three signals. Therefore, we do not exclude that, under normal conditions, the MP YXXΦ signals may act specifically in the distinct steps of MP post-Golgi trafficking (i.e. endocytosis and degradation) and can carry out more than one function as required. Our data demonstrate, to our knowledge for the first time, that a plant viral protein interacts with the μ-subunit of an AP complex and that vesicle carrier activity is essential for MP tubule formation and, in turn, virus movement.

Endocytosis was demonstrated in protoplasts more than 25 years ago. However, it is only in the last few years, with the invaluable help of amphiphilic styryl dyes (particularly FM4-64), that internalization of cargo molecules has been discovered and a number of PM receptors identified. On the other side, while orthologs of the endocytic machinery in mammalian cells have been detected in plants, an AP-2-like complex, specifically involved in the formation of endocytic vesicles at the PM, has not yet been characterized (Robinson et al., 2008). With endocytosis established as a fundamental plant transport process, it is conceivable that, due to their obligate nature, plant viruses use such mechanisms to support essential functions like replication and movement. Although a few plant viral proteins have been shown to interact or colocalize with components of the endocytic pathway (Haupt et al., 2005; Lewis and Lazarowitz, 2010), the crucial interaction with an AP-2-like complex, which mediates protein internalization from the PM, remains to be proved not only for plant virus proteins but for any protein in plants.

We observed that, shortly after labeling the PM, the endocytosis marker FM4-64 colocalizes with CaMV MP in endosomes and PM-derived vesicles. Based on this result, and on the evidence that the localization of MP to endosomes is hampered upon treatment with the inhibitor of endocytosis tyrphostin A23, we conclude that CaMV MP traffics in the endocytic pathway. In animal systems, tyrphostin A23 interferes specifically with the μ2 recognition of Tyr signals (Banbury et al., 2003). The evidence that tyrphostin A23 suppresses the internalization of MP and that this is dependent on functional YXXΦ cargo signals suggests strongly that interaction with the μ-subunit of a plant AP-2 complex is required for the internalization of this protein, lending support to the hypothesis invoking a direct link between endocytosis and the clathrin machinery in plants. Taken together, these results suggest that MP can traffic in PM-derived vesicles, probably via the recruitment of cytosolic adaptins.

Virus transport to the next cell entails that, after reaching the PM, MP targets PD specifically and then

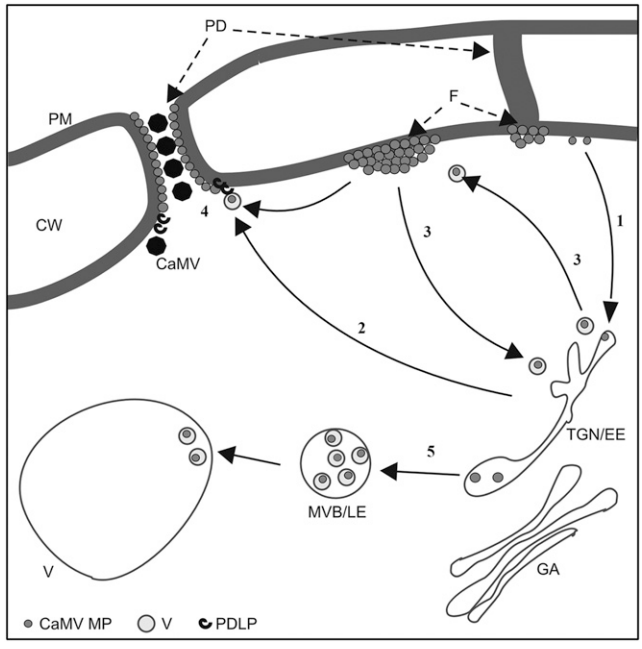

Figure 7. Model of intracellular traffic of CaMV MP through the post-Golgi endomembrane system. CaMV MP localizes to the PM quite early in the infectious cycle. Three Tyr signals mediate protein recycling to EEs via PM-derived vesicles (1). From there, MP probably enters a continuous cycling between PM and TGN/EE (2) that regulates the correct uptake of the protein to PD for tubule formation (3). Protein homeostasis at the PM is regulated via cycling breaking and targeting excess CaMV MP for degradation via MVBs/late endosomes (LE; 4). CW, Cell wall; F, foci of accumulation of MP; GA, Golgi apparatus; V, vesicles.

starts to assemble into tubule structures. How CaMV MP targets the PM remains to be demonstrated. While we do not exclude that initial sorting of MP to the PM could be mediated by some other type of transport vesicles, here we demonstrate that Tyr sorting signals are essential for foci and tubule formation but not to target MP to the PM, thus connecting directly endocytosis and tubule assembly. The evidence we provide that the MPtyr1-2-3 mutant can efficiently interact with PDLP1 (Fig. 5), a PD protein involved in the assembly of CaMV MP into tubules (Amari et al., 2010), suggests that this mutant is potentially competent to form tubules and that its failure to accumulate in PD (Supplemental Fig. S3) and to form tubules (Fig. 4) more probably depends on its inability to target PD. A recent study demonstrated that MPs from TMV and *Cabbage leaf curl virus* sequester a synaptotagmin to target EE and, from there, via a recapture pathway, to dock at PD for cell-to-cell transport (Lewis and Lazarowitz, 2010). Interestingly, the human transferrin receptor, which, like CaMV MP, binds μA, also cycles between PM and endosomal compartments in both animal cells and Arabidopsis protoplasts (Ortiz-Zapater et al., 2006). In our system, mutation of the three Tyr signals blocks endocytosis and PD localization but does not prevent the targeting of newly synthesized MP to the PM, at least in the early stages of infection. This suggests that, after targeting the PM (via an as yet unknown strategy), MP might use a recycling pathway (via direct interaction with a plant AP-2-like complex) for specific targeting of PD via constitutive cycling between EEs and PD. As the constitutive cycling of PM proteins is blocked by BFA (Geldner et al., 2003; Richter et al., 2009), recycling of MP is supported here by the demonstration that the formation of foci (and tubules) is inhibited upon treatment of protoplasts with BFA. In plants, constitutive cycling between endosomes and the PM controls polar targeting of the auxin efflux carrier PIN-FORMED1 (PIN1) that determines the direction of auxin flow (Wisniewska et al., 2006). MP bears striking similarity to PIN1 in its localization and traffic. Similar to MP, PINs are first targeted to the PM, and the subsequent endocytic step is crucial for the generation of polarity (Dhonukshe, 2009). Interestingly, polar transport of auxin regulates the polarized growth of tip-growing plant cells (such as pollen tubules and hair tips), which in several respects share similarities with viral MP tubules. Therefore, it is intriguing to speculate that endocytosis may promote a polar transport of MP regulating tubule extension.

Tyr signals can interact with several μ-adaptins and help the same protein to traffic in different compartments of the endomembrane system (Robinson, 2004). In Arabidopsis, μA does not localize to the PM and binds the YXXΦ motifs of the pea (*Pisum sativum*) vacuolar sorting receptor PS1, which mediates the secretion of cargo proteins to the vacuole via multivesicular bodies (MVBs; Happel et al., 2004). Consistently, colocalization with ARA7 provides us with a line of evidence for CaMV MP entering a degradation pathway via multivesiculate PVCs. GFP-ARA7 resides on MVBs (Reichardt et al., 2007), which serve as PVCs and are usually referred to as late endosomes (Richter et al., 2009). A very recent study has demonstrated that MVBs mature from the TGN, which serves as a landing station for proteins arriving from the PM (Scheuring et al., 2011). Therefore, if endocytosed PM proteins do not enter the recycling pathway, they become ubiquitinated and are targeted for degradation. It is conceivable that, to maintain homeostasis, a fraction of the CaMV MP molecules located in large excess at the PM would be not recycled further and would become part of MVBs en route for degradation. Indirect support for this hypothesis comes also from the evidence that Arabidopsis PRA1, the small Rab GTPase interacting with MP (Huang et al., 2001), resides entirely in endosomal compartments/PVCs and colocalizes perfectly with ARA7 (Alvim Kamei et al., 2008). Taken together, these results suggest that CaMV MP becomes part of MVBs on the way to the vacuole, and evidence that Potato mop-top virus (PMTV) triple gene block2 also colocalizes in vesicles with ARA7 (Haupt et al., 2005) supports the hypothesis that viral proteins can exploit the endomembrane system for degradation.

Our findings allow us to form a hypothesis on how CaMV uses the endomembrane system for different steps of viral transport occurring after the newly synthesized protein has targeted to the PM (via an as yet unknown strategy), namely, constitutive recycling and degradation (Fig. 7). Interaction with the AP complex at the PM directs, via endocytosis, MP onto EEs and, via a recapture pathway, to dock at PD. CaMV movement certainly requires large amounts of MP at the cell periphery; thus, MP would cycle between the PM and endosomes to specifically target PD and to be readily available in the amounts required for tubule formation. As CaMV MP and virions have never been found in close vicinity anywhere other than in the PD channel (Stavolone et al., 2005), we hypothesize that, at or near the PD, MP encounters virus particles and with the help of PDLP proteins (Amari et al., 2010) assembles in tubules to move virions through the plasmodesmal pore. To maintain homeostasis at the cell periphery and/or to prevent permanent gating of PD and the consequent deleterious cytopathic effects for host (and, in turn, virus) survival, excess MP molecules at the PM are targeted for degradation via MVBs.

MATERIALS AND METHODS

Cloning and Ectopic Expression of MP Wild Type and Tyr Mutants

All expression plasmids were based on pCKGFP (Reichel et al., 1996), with GFP replaced with the enhanced GFP coding sequence in pCKEGFP and with DsRed in pCKDsRed. The MP open reading frame was amplified from pCa37 (Stavolone et al., 2005) and cloned in frame with the fluorescent proteins. MP mutants were derived from pCKEGFP-MPwt using the QuickChange XL Site-Directed Mutagenesis Kit (Agilent Technologies). Plasmids (0.5 μg per shot) were used to coat 0.6-μm gold particles. Working with a 900-p.s.i. rupture disk, they were introduced into leaf epidermal cells of *Nicotiana benthamiana* plants at the two- to three-true leaf stage by particle bombardment using the helium-driven Biolistic PDS-1000/Helium Particle Delivery System and disposable components supplied by Bio-Rad. *N. benthamiana* protoplasts were

isolated and transfected as described (Goodall et al., 1990). Fluorescent proteins were examined with a Nikon Eclipse 80i microscope equipped with video confocal technology with excitation at 488 nm and emission at 510 to 560 nm. For GFP images, the ET-GFP filter set (Chroma 49002; Nikon) was used, with the G-2A filter (Nikon) being used for DsRed and FM4-64.

Chemical Treatments

FM4-64 (Molecular Probes) was used at a final concentration of 50 μM, tyrphostin A23 at 50 to 150 μg mL^{-1}, and BFA at 50 μg mL^{-1} for the times indicated. All samples were washed before fluorescence microscopy analysis.

Infectious CaMV Clones and Plant Inoculation

The CaMV pCa540 clone (Howarth et al., 1981) was modified by the addition of two unique restriction sites: *Spe*I at the 5′ end of the MP coding sequence and *Sac*II exactly before the coiled-coil domain to generate an infectious CaΔ2 clone. Into the same restriction sites, the MP derivative Tyr mutants were cloned. CaMV wild type and derivative plasmids were linearized by *Sal*I and inoculated in *Brassica rapa* var. Just Right grown in a greenhouse at 21°C to 25°C with a 16-h photoperiod. At the three- to four-leaf stage, plants were inoculated mechanically with 20 μg of DNA each. For further passages, 500 μg of leaf tissue collected from four different leaves of the same plant was ground with 5 mL of phosphate-buffered saline and inoculated as described above. For Arabidopsis (*Arabidopsis thaliana*) inoculation, plants were grown in a growth chamber at 20°C to 22°C with a 10-h photoperiod. Four leaves of 4-week-old plants were inoculated with ground leaf tissue of CaMV-infected *B. rapa* plants.

GST Pull-Down Assay

CaMV MP wild type and derivative Tyr mutants were amplified from the corresponding pCKEGFP plasmids and cloned into the pET-3d expression vector (Novagen). The μA subunit receptor-binding region (residues 156–435) was amplified from an Arabidopsis complementary DNA (clone BX830242; Institut National de la Recherche Agronomique-Centre National de Ressources Genomiques Vegetales; http://cnrgv.tolouse.inra.fr) and cloned into the pGEX-2TK expression vector (GE Healthcare) in an in-frame fusion with the GST coding sequence. MP (wild type and Tyr mutants) and GST-VAP (Stavolone et al., 2005) expression was induced by growing the cell cultures at 37°C for 3 h with 0.4 and 0.2 mM isopropylthio-β-galactoside, respectively. GST-μ2 was induced with 2 mM isopropylthio-β-galactoside for 4 h at 25°C. The pull-down assay was performed essentially as described in Stavolone et al. (2005). *Escherichia coli* GST-MP was challenged with leaf extract from Arabidopsis for 3 h at 4°C. For the in vivo test, leaf extract from Arabidopsis expressing GST-MP under the control of the 35S promoter (AtGST-MP) was incubated for 3 h at 4°C with glutathione-Sepharose 4B beads. The presence of proteins in the bound fractions was detected by western blotting using anti-MP coiled-coil, anti-VAP coiled-coil antibody (Stavolone et al., 2005), anti-HA antibody (Sigma-Aldrich), and rabbit antibody raised against Arabidopsis μA.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. GFP-MP colocalizes with FM4-64 in epidermal cells of *N. benthamiana*.

Supplemental Figure S2. CaMV infectivity in BEN1 and BEN2 Arabidopsis mutants.

Supplemental Figure S3. Localization of GFP-MP and Tyr mutants in PD.

Supplemental Figure S4. Tyrphostin A23 and BFA have no effect on the subcellular localization of GFP in *N. benthamiana* protoplasts.

Supplemental Figure S5. Effect of tyrphostin A23 on GFP-MP distribution in *N. benthamiana* protoplasts.

Supplemental Figure S6. Tyrphostin A23 directs GFP-MP to the PM in *N. benthamiana* protoplasts.

Supplemental Table S1. Reversion of MP Tyr signal mutants after inoculation of CaMV in turnip plants.

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