Cellular Remodeling During Plant Virus Infection

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Abstract

This review focuses on the extensive membrane and organelle rearrangements that have been observed in plant cells infected with RNA viruses. The modifications generally involve the formation of spherules, vesicles, and/or multivesicular bodies associated with various organelles such as the endoplasmic reticulum and peroxisomes. These virus-induced organelles house the viral RNA replication complex and are known as virus factories or viroplasms. Membrane and organelle alterations are attributed to the action of one or two viral proteins, which additionally act as a scaffold for the assembly of a large complex of proteins of both viral and host origin and viral RNA. Some virus factories have been shown to align with and traffic along microfilaments. In addition to viral RNA replication, the factories may be involved in other processes such as viral RNA translation and cell-to-cell virus transport. Confining the process of RNA replication to a specific location may also prevent the activation of certain host defense functions.

INTRODUCTION

Viruses induce the appearance of symptoms in the plants that they infect. These symptoms can be wide ranging in their manifestations—severe or mild; mosaics, yellowing, or necrotic lesions on leaf surfaces; or stunting and deformation of the whole plant. Conversely, some viruses can replicate to high titer without provoking any apparent symptoms. Moreover, strains of the same virus can produce different symptoms on the same plant, and these may vary depending on the plant cultivar and environmental conditions. The molecular mechanisms responsible for symptom induction are yet to be deciphered. However, these altered phenotypes must be related to the interaction of viral components (proteins and nucleic acids) with host factors (proteins, nucleic acids, carbohydrates, lipids, and metabolites) that affect the plant physiology and development. These interactions are required for the fulfillment of specific virus functions (e.g., RNA synthesis), or they can lead to the induction of host responses that are designed to fight off infecting pathogens. Over the past years, a large number of investigations have been devoted to understanding virus-host interactions at the molecular level. For instance, genome-wide analyses have revealed that several host gene transcripts are either up- or down-regulated during virus infection (reviewed by 144). These effects range from nonspecific changes in gene expression related to the accumulation of viral proteins to responses that are initiated by the specific interactions between virus and host proteins. Several plant proteins have now been identified that interact specifically with viral proteins or with defined region of the genome (reviewed by 13). Such studies have been facilitated by genomic and proteomic studies using *Saccharomyces cerevisiae* as a surrogate model host for bromovirus and tombusvirus infections (reviewed by 78).

Concurrent to these molecular studies, an avenue of investigation at the interface of molecular virology and cell biology has emerged. These studies have been made possible with the development of novel techniques that allow for quick and easy expression of proteins in plants (agroinfiltration in *Nicotiana benthamiana*), their visualization and tracking in individual cells (fluorescent protein fusions and confocal microscopy), and powerful reverse genetic tools (infectious cDNA clones of RNA viruses). Experiments performed with these methodologies have shed new light on old data—the observation made several decades ago using electron microscopy (EM) that plant viruses induce substantial cellular remodeling during infection (see 68 for example). Virus particles were detected frequently, and when present in sufficient numbers, could form crystalline arrays (143). Certain viruses were found to induce the production of protein inclusions, which have become useful diagnostic features for these infections (18). In other cases, organelles or membranes showed altered morphologies during infection (20, 34, 48, 107). Finally, in some instances, tubules containing virus-like particles were identified in or near the cell walls of infected cells (11, 126, 129). These early studies were descriptive, and the exact origin, composition, and role of these cellular alterations were restricted to conjecture as a result of the technical limitations of that period. These virus-induced membrane structures house the RNA replication complex and have been designated as virosomes, virus inclusions, virus factories or viroplasms (82, 86). Other investigations have shown that the intracellular movement of viruses is also accompanied by morphological changes.

A recent focus of plant virology has been to identify the molecular requirements for the formation of these virus-induced structures. It is thought that virus factories function to (*a*) increase the local concentration of components required for replication, (*b*) provide a scaffold for anchoring the replication complex, and (*c*) confine the process of RNA replication to a specific location that prevents the activation of host defense functions. Current questions center on the membrane origins that give rise to the virus-induced factories and the molecular motors that are involved in their trafficking from their site of origin to their final

destination. Another area of investigation is the content of the vesicles and the role of each individual component in virus replication. Accordingly, the acquisition of high definition structures of the different vesicle architecture will be necessary to better understand the interplay between virus replication and associated cellular processes. This review endeavors to provide a comprehensive overview of our current understanding of this field based on significant advances that have been made in recent years.

PLANT VIRUS REPLICATION CYCLE

Before discussing the biogenesis of virus-induced alterations, a brief description of the replication cycle of the viral players within the host cell is warranted. Plant viruses are small, obligate, intracellular parasites. Genetic information coded by their genomes is limited, thus they depend entirely on host cells to replicate their genome and produce infectious progeny. Plant viruses, like animal viruses, can be classified according to the type of nucleic acid making up their genome. The vast majority of plant viruses have positive-sense (+) RNA genomes (i.e., the RNA genome has the same polarity as cellular mRNA), although negative-sense (−) RNA and double-stranded RNA genome viruses also exist. Other plant viruses have a DNA genome, which can be double-stranded (caulimoviruses) or single-stranded (geminiviruses).

Because most investigations on cellular remodeling have been conducted using (+) RNA viruses, this review mainly focuses on this class of viruses. Despite differences in genome organization and expression, virion morphology, and host range, (+) RNA viruses have fundamentally similar strategies for genome replication. Genome replication involves the copying of the (+) RNA into a complementary (−) RNA strand, which then serves as template for the generation of multiple (+) copies. The RNA-dependent RNA polymerase (RdRp) is the core protein that catalyzes the nucleotide polymerization step. The reaction also requires the participation of several factors of both viral and host origin that collectively form replication complexes (reviewed by 87, 109).

ANIMAL VIRUS–INDUCED MEMBRANE MODIFICATIONS

Although beyond the scope of the present chapter, it is important to note that cellular remodeling also takes place during animal virus infections. Induction of membrane rearrangements has been described for virtually all groups of animal viruses (reviewed by 73, 82, 86, 108). In some cases, EM micrographs of (+) RNA virus infections revealed the presence of heterogeneously-sized vesicles that are derived from the endoplasmic reticulum (ER) and often present in clusters around the nucleus. However, other organelles are also targeted during infection. For example, Rubella virus modifies lysosomes into cytopathic vacuoles (64) whereas *Flock house virus* (FHV) assembles its replication complex on mitochondrial membranes (71).

Electron tomography has recently been used for the generation of three-dimensional imaging of virus-induced membrane alterations at high resolution. The 3D portrait revealed the presence of FHV replication factor A and genomic RNA inside 50-nm vesicles (spherules) localized between the inner and outer mitochondrial membranes (51). The spherules are outer mitochondrial membrane invaginations with interiors connected to the cytoplasm by a necked channel approximately 10-nm in diameter, which is a size sufficient for ribonucleotide import and progeny RNA export. It has been calculated that one spherule contains, on average, three RNA replication intermediates. In another investigation, coronavirus-induced alterations resulted in a reticulovesicular network of modified ER that integrates convoluted membranes, numerous interconnected double membrane vesicles (DMVs) (diameter 200–300 nm) and vesicle packets apparently arising from the merging of DMVs (49). A similar network was also observed for *Dengue virus* (141), except that it contained

RdRp: RNA-dependent RNA polymerase

FHV: *Flock house virus*

Electron tomography: an EM specimen is tilted over a range of ±65° in 1° increments and recorded images are used for calculating a 3-D representation

DMV: double membrane vesicle

Brome mosaic virus **(BMV):** the genome comprises three RNAs (RNA1-3). RNA1 and RNA2 encode proteins 1a and 2a, which are required for RNA replication

Supplemental Material

neck-like connections between the outer layers of DMVs, which differs from the apparently sealed versions in coronavirus. The reader is invited to view the **Supplemental Movie** clips showing three-dimensional renderings of the *Dengue virus* membrane network at the publisher's Web site (follow the **Supplemental Material link** from the Annual Reviews home page at **http://www.annualreviews.org**). These beautiful reconstitutions provide a spatio-temporal platform for the virus replication cycle and suggest that not only RNA replication but also translation and virion assembly are associated with these virus-induced structures.

The biogenesis and chemical properties of animal virus–induced cellular alterations have many parallels with what is observed during plant virus infection. This shows the fundamental, universal nature of virus replication, which can be illustrated by the fact that certain plant viruses can replicate in insects and in yeast. The characterization of plant virus factories is thus intertwined with that of animal virus factories, and important discoveries in one sector impact the other.

MORPHOLOGY OF PLANT VIRUS–INDUCED CELLULAR ALTERATIONS

Just as for animal viruses, different plant virus groups induce the formation of diverse cellular structures, both in terms of architecture and membrane/organelle origin. These virus-induced cellular alterations are required for viral genome replication or for virus cell-to-cell movement. The modifications generally involve the formation of spherules, vesicles, and/or multivesicular bodies, which may be bound by a double-layer membrane and are often connected by a narrow channel to the surrounding cytosol. Essentially, every organelle found in a plant cell is targeted by one virus or another. The specific organelle targeted varies among viruses from different families or genera and also among viruses within a genus. The significance of this organellar diversity is unknown, but specific membrane targeting appears not to be a strict requirement for efficient viral infection as replication complexes can be redirected to an alternate subcellular localization (41, 72). Below we describe a selection of well-studied examples of cellular alterations induced by plant viruses. **Supplemental Table 1** (follow the **Supplemental Material link** from the Annual Reviews home page at **http://www.annualreviews.org**) provides a more comprehensive list of membrane/organelle modifications by plant viruses.

Modification of the Endoplasmic Reticulum for Viral Replication and for Host Defenses

The structural changes induced by *Brome mosaic virus* (BMV) were among the first investigated in detail. The powerful genetic tools available for the host surrogate *S. cerevisiae* were of great utility for these studies. When expressed in yeast, proteins 1a and 2a can direct BMV RNA replication and duplicate all known features of BMV replication in plant cells (40). Protein 1a was found to associate with the cytoplasmic face of the outer ER membrane, interact with specific lipids within the membrane, and induce invaginations of this membrane into the ER lumen to form spherules or vesicles, whose interiors are connected through narrow necks with the cytoplasm (**Figure 1*a***) (112). These spherules have a single, bounding lipid bilayer and contain condensed or fibrillar material. The diameters of spherule sections vary from 30 nm to 70 nm (112). In addition to protein 1a, the spherules contain protein 2a and viral RNA, which is protected from nuclease degradation (112). Similar spherules have been observed in bromovirus-infected plant cells (48, 101).

Proliferation of ER membranes in infected cells leading to the formation of viral factories is also observed for several other viruses including potyviruses (111, 148), nepoviruses and comoviruses (9, 32, 102), potexviruses (1), tobamoviruses (45, 84), and reoviruses. In the case of potyvirus infections, the biogenesis of the replication vesicles occurs at ER Exit Sites in a COPI- and COPII-dependent manner (139), which might stabilize the vesicles.

Concurrently, the secretion of a soluble marker targeting the apoplast is arrested at the level of the ER, and this inhibition may contribute to vesicle accumulation. Hijacking components of the cellular secretory pathway has been noted during poliovirus infection, and it has been suggested that vesicle formation results from the inhibition of an intracellular protein transport pathway (6). Interestingly, reoviruses, which have a double-stranded RNA genome and are

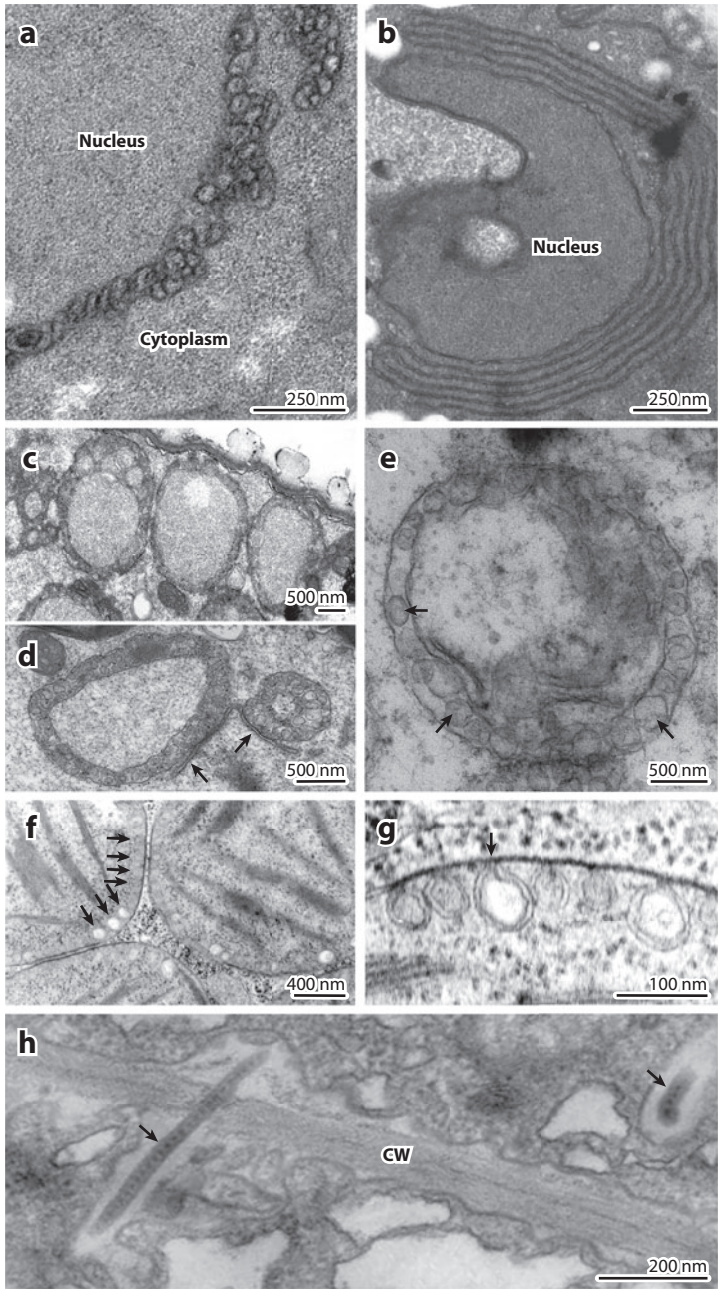

Figure 1

Electron microscopy images of cellular alterations that are induced by different plant viruses. (*a*) 50–70 nm diameter spherular vesicles invaginated from the outer perinuclear endoplasmic reticulum (ER) membrane into the ER lumen in a yeast cell expressing *Brome mosaic virus* (BMV) replication factor 1a in the absence of other viral components. Indistinguishable spherules occur in cells expressing 1a and low levels of BMV 2a, and replicating BMV RNA3. Similar spherules are seen in bromovirus-infected plants. (*b*) Double membrane layers induced from the outer perinuclear ER membrane in cells expressing BMV 1a plus elevated levels of BMV 2a, and replicating BMV RNA3. (*c*) and (*d*) Electron micrographs of *Nicotiana benthamiana* leaves systemically infected with Tomato bushy stunt virus (TBSV) showing individual peroxisomal multivesicular bodies (MVBs) in infected mesophyll cells. Arrows in (*d*) highlight a portion of tube-like ER adjacent to two peroxisomal MVBs. (*e*) Mitochondria-derived MVBs in mesophyll cells of *Chenopodium quinoa* leaves infected with *Carnation Italian ringspot virus* (CIRV). Arrows denote examples of distinct vesicle/spherule-like structures located in the intermembrane space of the mitochondria-derived MVB that are proposed to be derived by invaginations of the outer mitochondrial membrane and serve as the sites for CIRV RNA replication. (*f*) and (*g*) Chloroplasts in *Turnip yellow mosaic virus* (TYMV)–infected Chinese cabbage leaves. Arrows in (*f*) indicate vesicles at the chloroplast periphery, and the arrow in (*g*) indicates a vesicle in which an open channel is apparently connecting the interior of the vesicle to the cytoplasm. (*h*) Tubular structures containing virus-like particles (*arrows*) in *Tomato ringspot virus* (ToRSV)–infected *Nicotiana clevelandii* tissues. The tubules are seen traversing the cell wall (CW) or in close proximity to the cell wall. (*a*) is adapted, with permission, from (112) © 2002, Elsevier; (*b*) is adapted, with permission, from (113) © 2004 by the National Academy of Sciences; (*c*) and (*d*) are adapted, with permission, from (69) © 2005 American Society of Plant Biologists. (*e*) is adapted, with permission, from (39) © 2008 Hwang et al.; licensee BioMed Central Ltd.; (*f*) and (*g*) are adapted, with permission, from (99) © 2001, Elsevier.

Persistent infection: viruses are transmitted in a nonpersistent, or in a persistent manner by invertebrate vectors. In the latter case, viruses are ingested by and may replicate in the vector

TMV: *Tobacco mosaic virus*

CymRSV: *Cymbidium ringspot virus*

TBSV: *Tomato bushy stunt virus*

CIRV: *Carnation Italian ring spot virus*

transmitted in a persistent manner by their insect vector, replicate not only in plants but also in insects. Just as in plant cells, these viruses induce cell remodeling in insect cells, forming ER-derived multivesicular compartments, which likely represent replication factories (135, 136, 138). Taken together, these results suggest that the formation of virus factories requires interactions between viruses and highly conserved cellular factors present in plants, yeasts, and insects.

ER modification has also been implicated in host defense responses. Upon inoculation with *Tobacco mosaic virus* (TMV), *Nicotiana* plants that carry the *N* resistance gene mount a hypersensitive response that induces cell death. In this case, production of autophagosomes (119), which are double-membrane bound structures derived from the ER, is induced. The autophagosomes sequester TMV and subsequently fuse with the central vacuole, where the contents are degraded by hydrolytic enzymes (63). Thus, the ER is modified by both viruses and their hosts, and these modifications determine the outcome of the infection: virus replication or in some cases, virus degradation.

Replication in Association with Peroxisomal or Mitochondrial Membranes

Tomato bushy stunt virus (TBSV), *Cymbidium ringspot virus* (CymRSV), and *Cucumber necrosis virus* (CNV) are tombusviruses that induce the formation of multivesicular bodies derived from peroxisomes (69, 80, 89, 107; reviewed by 77) (**Figure 1*c,d***). These intracellular structures form initially by a progressive inward vesiculation of the boundary membrane of preexisting peroxisomes, resulting in the organelle's interior (matrix) housing up to several hundred spherical to ovoid vesicles 80 nm to 150 nm in diameter. Occasionally, multivesicular bodies were found in close association with tubular membranous structures that resembled the ER. The modified peroxisomes contain the replication proteins and viral RNA, and there is EM evidence that the spherules have channels/necks that connect them to the cytosol. The morphology and distribution of other subcellular organelles in infected cells, including mitochondria, ER, plastids, and Golgi, are unaltered (69). However, the tombusvirus CIRV and related *Melon necrotic spot carmovirus* (MNSV) induce the formation of multivesicular bodies from the mitochondrial outer membrane (75, 134) (**Figure 1*e***). This mitochondrial damage appears to translate into necrotic spots on MNSV-infected leaf tissue (75). Some isolates of CIRV target peroxisomes rather than mitochondria probably as the result of a recombination event that transferred peroxisome-targeting signals from a peroxisome-targeted tombusvirus into the CIRV replication proteins (50). Finally, *Red clover necrotic mosaic virus* (RCNMV), a member of the family Tombusviridae that has a bipartite RNA genome, induces perinuclear ER proliferation, accompanied by thickening of ER tubules (124). Although these viruses target different organelles for their replication, the type of organelle does not appear to be of prime importance. This concept is supported by the observation that the ER can substitute for peroxisomes as replication sites for TBSV. As with BMV, some tombusviruses can replicate in *S. cereviseae* (80, 89, 90; reviewed by 78). It was shown that in a yeast strain genetically deficient for peroxisome biogenesis, the viral replication proteins and RNA were retargeted to the ER, which became the site of RNA replication (41, 79). Additionally, when hybrids between the mitochondrial targeting signal located within p36 of CIRV and the peroxisomal targeting signal of p33 of CymRSV were made, multivesicular bodies were derived from both peroxisomes and mitochondria (105). These examples illustrate that some viruses have remarkable flexibility in terms of the membrane source used to assemble their replication complex.

Replication in Association with Chloroplast Membranes

Turnip yellow mosaic virus (TYMV) infection induces the formation of chloroplastic

membrane vesicles (34). The chloroplasts become swollen, rounded and clumped together (**Figure 1*f*,*g***). The virus-induced vesicles are likely to result from invaginations of the chloroplast envelope, with some of them having an open channel that connects the interior of the vesicle to the cytoplasm. TYMV replication proteins are associated with these structures, suggesting that they represent TYMV replication factories (98, 99). Additionally, chloroplast amalgamation and chloroplast membrane invaginations are observed during *Turnip mosaic virus* (TuMV) infection (137).

Replication in Association with Nuclear Membranes

EM data revealed that large numbers of bacilliform particles are observed in the perinuclear spaces and viroplasms of different shapes are present in the swollen nuclei of tissue infected with the (–) RNA nucleorhabdovirus *Sonchus yellow net virus* (67). The viroplasms contain replication proteins as well as viral RNA (27, 67) and result from the invagination of the inner nuclear membrane, which remains contiguous with the endomembrane system (28). In contrast, *Potato yellow dwarf virus*, which is another nucleorhabdovirus, induces only perinuclear viroplasms (27). Thus, rhabdoviruses constitute another example in which, similarly to the tombusviruses, members of a single genus can differ in their specific interactions with intracellular membranes.

Modification of Plasmodesmata and Plasma Membrane for Viral Cell-to-Cell Movement

Viral movement proteins have been reported to modify the plasmodesmata and increase their size exclusion limit. These studies have been reviewed in detail elsewhere and are not discussed here (36, 81). However, in some cases, distinct cytopathological structures are observed in association with the plant cell wall. *Lettuce infectious yellow virus*, from the family Closteroviridae, induces the formation of plasmalemma deposits in the vicinity of plasmodesmata. Such deposits may be important for orienting virus particles near the plasmodesmata for systemic transport (117). Similarly, fibrillar structures that have been observed in the plasmodesmata of potexvirus-infected cells may correspond to viral movement complexes (127). Several icosahedral viruses (e.g., nepoviruses, comoviruses, caulimoviruses) induce tubular structures containing virus-like particles in or near the cell wall (**Figure 1*b***). The viral movement protein is a structural component of the tubules. Expression of the movement protein alone is sufficient to induce the formation of tubules that extend from the surface of plant protoplasts (38, 103, 140, 142). However, these tubules are empty in the absence of the coat protein. The formation of tubules involves several steps including the transport of the movement protein to focal sites in the plasma membrane and the formation of the tubules through polymerization of the movement protein (97).

Formation of Inclusion Bodies

Some viruses induce the formation of inclusion bodies that are often composed of a single viral protein. Potyvirus infections are characterized by the presence of cytoplasmic inclusions that are composed of a putative RNA helicase and appear as bundles if cut longitudinally and as scrolls and pinwheels if cut transversely (148). Nuclear inclusion bodies consisting of the VPg-proteinase (NIa) and/or the RdRp (NIb) are also present for a limited group of potyviruses (31). The DNA virus *Cauliflower mosaic virus* (CaMV) induces two types of inclusion bodies in infected cells. Electron-dense inclusion bodies consist of a matrix of the viral p6 protein, a multifunctional protein, and also include virus particles and the virion-associated protein (pIII). Electron-translucent inclusion bodies contain the aphid transmission vector protein (pII). The function of inclusion bodies is not clear. It has been suggested that they may represent a means for the virus to inactivate excessive concentrations of potentially toxic

TYMV: *Turnip yellow mosaic virus*

TuMV: *Turnip mosaic virus*

VPg: The 5′ end of genomic RNA of some viruses is covalently linked to a viral protein known as VPg

soluble viral proteins in the cytoplasm (110). In the case of CaMV, the separation of viral proteins into different bodies may be essential for aphid transmission (47). Kinetic studies of the formation of these inclusion bodies revealed that pIII and pII first localize to electron-dense inclusion bodies, which are thought to be the site of translation and replication, before pII is redirected to electron-lucent inclusion bodies (66).

In conclusion, intracellular changes conveyed by plant viruses are widespread and are required for either viral RNA replication or virus transport. Although EM photographs provide fine details on the modifications induced by viral infections, more information is needed on the overall organization of the spherules/vesicles within the endomembrane system, and whether other viral functions (e.g., translation and encapsidation) are associated with them.

VIRAL PROTEINS AND MEMBRANE TARGETING

For a given virus, membrane and organelle alterations are attributed to the action of one or two viral proteins (3, 14, 39, 69, 79, 98, 111, 134, 139). The responsible factors are integral membrane proteins, but there are instances in which peripheral proteins are implicated. When expressed alone, these viral proteins induce similar membrane modifications to those observed in infected cells. However, in some instances, structures induced by a single viral protein differ from the ones observed in infected cells. For example, association of the BMV membrane–targeted 1a protein with 2a (the other viral component of the replication complex) modifies the architecture of the virus-induced structure. Modulating the relative levels and interactions of la and 2a shifts the membrane rearrangements from small invaginated spherules to large multilayer stacks of appressed double membranes (**Figure 1*b***) (113). This suggests that an intricate network of factors and conditions are necessary for proper membrane modification.

Viral integral membrane proteins are firmly attached to membranes. They may have one or several transmembrane domains that consist of stretches of approximately 20 hydrophobic amino acid residues. In addition to these transmembrane domains, some proteins also have amphipathic helices. Such helices usually lay flat at the surface of the membrane with the hydrophobic side of the helix embedded in the membrane and the hydrophilic side exposed at the surface. Oligomerization of proteins containing amphipathic helices can allow these helices to traverse the membrane by creating an aqueous pore. Such proteins, termed viroporins, can affect the stability and permeability of the membrane and can enhance the passage of ions or other small molecules through the membrane (25).

In the case of potyviruses, the membrane anchoring protein is well defined. $6K_2$, a 6-kDa protein with apparently no other function, is responsible for vesicle formation. Characteristic green fluorescing vesicles are produced when the $6K_2$ protein of *Tobacco etch virus* (TEV) is fused to GFP and expressed in *N. benthamiana* (111, 139). In the case of TuMV, the $6K_2$-VPg-Pro polyprotein, through its hydrophobic $6K_2$ domain, was shown to be responsible for the formation of cytoplasmic vesicles derived from the ER (3), similar in structure to those observed during TEV and TuMV infections (12, 111, 139). The $6K_2$ protein is characterized by the presence of a central hydrophobic α-helix domain of 19 amino acids flanked by charged residues. This domain is required for vesicle production (111). It is not known whether the hydrophobic residues traverse the membrane or constitute a hydrophobic patch at the surface.

For other viruses, the determinant for organelle targeting and membrane alteration is a subdomain of a longer viral protein, which has additional functions. The nepovirus nucleoside triphosphate binding (NTB) protein is found in association with ER-derived membranes active in viral replication in *Tomato ringspot virus* (ToRSV)–infected cells (32). Two hydrophobic domains direct the membrane association: a C-terminal transmembrane domain and an

N-terminal amphipathic helix (32, 131, 150). The topological analysis of the protein within the membrane suggests that both ends of the protein are translocated to the lumen of the ER, whereas the central region, which possesses the NTB activity and is a putative helicase, is exposed to the cytoplasmic face of the ER membrane (150). Translocation of the N-terminal amphipathic helix may occur via the formation of an aqueous pore after polymerization of the protein. The ToRSV X2 protein is another ER-targeted multipass transmembrane protein. It has two transmembrane helices at its C-terminus and another less well-defined ER targeting domain at its N-terminus (possibly also an amphipathic helix) (149).

The tombusviridae 33–36 kDa protein contains targeting signals for its subcellular localization and is responsible for spherule/vesicle production (39, 69, 79, 106, 134). As mentioned above, tombusviruses target different organelles for replication. CymRSV and CIRV induce the formation of multivesicular bodies that develop from peroxisomes or mitochondria, respectively. By exchanging small portions of the ORF 1 sequence between infectious clones of the two viruses, it was found that the N-terminal hydrophilic region and transmembrane segments of the 33–36 kDa protein specify which organelle is involved in the synthesis of multivesicular bodies (105). In the case of CIRV, the mitochondrial sorting signal was further dissected, and two hydrophobic transmembrane domains of approximately 20 amino acids and a 45 amino acid amphipathic helix located within the intervening loop sequence were found to be critical for proper targeting (39). This targeting signal is similar to those found within mitochondrial membrane proteins (39).

Specific organelle targeting can also be achieved through the interaction of viral proteins with a host transporter protein. The yeast Pex3p protein is involved in transport to peroxisome membranes and was shown to play a role in peroxisomal localization of TBSV replication factories through interaction with the integral viral membrane protein p33 (93).

Several viral movement proteins have also been shown to be integral membrane proteins that associate with ER membranes and possess one or several transmembrane domains. Well-studied examples include two carmovirus movement proteins, a closterovirus movement protein, the tobamovirus movement protein, and the potexvirus TGBp2 and TGBp3 proteins (21, 65, 95, 127, 128).

Peripheral proteins are loosely associated with membranes and require interactions with intrinsic components of the membrane (often but not always a host membrane protein) to promote their association. The 1a protein of BMV interacts with ER membranes and induces membrane invaginations known as spherules. However, the 1a protein does not have a transmembrane domain, and it resides on the cytoplasmic side of the ER membrane (14). The domain responsible for membrane attachment and spherule formation is an amphipathic α-helix of 18 amino acids (62). The transport of BMV replication proteins to ER membranes is affected by the Lsm1-7p/Pat1p/Dhh1p complex (5). This complex has been suggested to facilitate the preassembly of the BMV replicase complex into processing bodies (P-bodies), prior to their retargeting to the ER. As with BMV, the TMV RdRp is a peripheral membrane protein. Association of the replication complex with ER membranes is dependent on interaction of the TMV RdRp with two transmembrane ER-resident proteins, Tom1 and Tom2 (84, 123, 146).

How individual viral proteins promote membrane alterations remains largely unknown. The formation of spherules, vesicles, or multivesicular bodies involves membrane bending. There are several mechanisms, likely working in concert, that generate curvature (reviewed by 70). First, transmembrane proteins that have a conical shape or attain it upon oligomerization can influence membrane shape. Additionally, proteins containing amphipathic helix domains have the ability to associate with one of the two leaflets of a membrane, thereby creating asymmetry and membrane bending. Finally, the cytoskeleton,

Processing bodies (P-bodies): regions within the cytoplasm consisting of proteins involved in mRNA turnover

as well as changes in lipid composition, influence membrane shape changes. As mentioned above, the membrane-targeting viral proteins have transmembrane and/or amphipathic helix domains, and many interact with themselves. Additionally, active lipid biosynthesis is needed for replication of many plant viruses (1, 9, 102), although there are cases in which it is not a requirement (16). Lipid composition changes are associated with BMV infection (55).

Eukaryotic initiation factor 4E (eIF4E): plants possesses two isomeric forms for eIF4E, eIF4E and eIF(iso)4E. They are functionally interchangeable, but appear to have distinct roles in vivo

Another important feature of the membrane-targeting signal is that it is a subdomain of a larger protein entity. Many of these anchoring viral proteins are multifunctional and can harbor enzymatic functions, such as RdRp activity. Furthermore, they can self associate and interact with host and other viral proteins, as well as RNA. Consequently, the membrane-targeting viral proteins not only induce membrane rearrangements but also act as a scaffold for the assembly of a large complex of proteins (of viral and host origins) and viral RNA. This is well illustrated by the $6K_2$-VPg-Pro polyprotein of TuMV: $6K_2$ induces the formation of cytoplasmic vesicles, and VPg-Pro interacts with the viral RdRp (15), the host translation eukaryotic initiation factor 4E (eIF4E) (3) and elongation factor 1a (eEF1a) (121), the polyA binding protein (PABP) (4), and an RNA helicase-like protein (37). Moreover, the heat shock cognate 70 (HSc70) protein interacts with RdRp and consequently with $6K_2$-VPg-Pro (15). Finally, VPg is covalently linked to the viral RNA (116). All these interactions take place within the replication factories (3, 4, 121). A similar scaffold of protein complexes has also been uncovered for tombusvirus replication factories (114, 115).

Supplemental Material

Although one or two viral proteins induce the formation of spherules/vesicles, the process is undoubtedly complex. Host proteins regulating the size and fine architecture of replication factories need to be identified. Furthermore, the presence of neck-like openings suggests that a filtering complex may be at work, sorting what goes in and what goes out.

VESICLE TRAFFICKING: INVOLVEMENT OF THE CYTOSKELETON

There are numerous examples of intracellular transport of viral components. These studies have essentially dealt with the trafficking of virus entry or release (reviewed by 29). For example, small vesicles induced by viral movement proteins have been shown to move rapidly along actin filaments (24, 43). In other cases, it is the secretory pathway rather than the cytoskeleton that has been implicated in virus cell-to-cell movement. For example, the transport of the movement protein from a nepovirus and a caulimovirus to the cell periphery and the subsequent assembly of tubules traversing the cell wall are dependent on the secretory pathway (54). However, in the case of a comovirus, the induction of tubules and the intracellular movement of the movement protein are independent of the secretory pathway and the cytoskeleton (97).

It is only recently that transport of replication factories has been addressed, especially those that are ER derived. TuMV- and TMV-induced replication vesicles are motile (**Supplemental Movies 1** and **2**) (12, 61), and in the latter case it has been suggested that they move from one cell to another through plasmodesmata (45). Movement is unidirectional and accompanied with stop-and-go activity. Although the exact destination is not known, occasional fusion with perinuclear vesicles is observed. It was also found that CaMV P6 forms highly motile cytoplasmic inclusion bodies (33). Whether replication factories that are located within organelles (e.g., peroxisomes, mitochondria, etc.) are motile is not known.

Given that the protein content and organized nature of the cytoplasm restrict diffusion of large molecular complexes, movement of replication factories is likely to require cytoskeletal elements (35). The above virus-induced structures have been shown to align with microfilaments (**Figure 2**) (12, 33, 61). Additionally, microfilament-depolymerizing compounds such as Latrunculin B (LatB)

inhibit movement and significantly reduce virus yield (12, 33). Actin may also have a role in establishing the large central cytopathic structure induced during CPMV infection (9). Movement of replication-associated vesicles is not restricted to plant viruses. *Hepatitis C virus* (HCV) replication complexes are associated with two types of factories. Large factories, representing membranous webs, show limited motility. In contrast, small replication factories show fast saltatory movement that is microtubule dependent (145).

It is not yet known how the replication factories are tethered to the microfilaments. In the case of HCV, a direct interaction between two replication viral proteins and either tubulin or actin has been shown (53). Direct interaction between the membrane-targeting viral proteins and components of the cytoskeleton has not been reported for plant viruses. eEF1A binds and bundles actin (30) and is a component of replication factories (60, 84, 121). It will be interesting to see if eEF1A can act as an intermediate between plant replication factories and microfilaments.

Actin filaments are major determinants for the generation of membrane tension and curvature (70) and may consequently be involved in the formation of virus-induced structures. However, the molecular reasons for the trafficking of replication factories within the plant cell are not known. One possible raison d'être is to maintain widespread distribution of replication vesicles within the plant cell (**Figure 2**). Distribution of viral replication factories might preserve physiologic cell structure and function. Moreover, independent replication sites within a given cell may increase the chances of highly adapted genomes to establish productive infection and at the same time limit the detrimental effects of deleterious mutations (145). Trafficking may also help in the coalescence of small nascent replication factories into larger ones (9, 45). Three-dimensional tomographic reconstitutions indicate a possible spatio-temporal relationship among the different components of the reticulovesicular network making up replication factories of coronaviruses and the

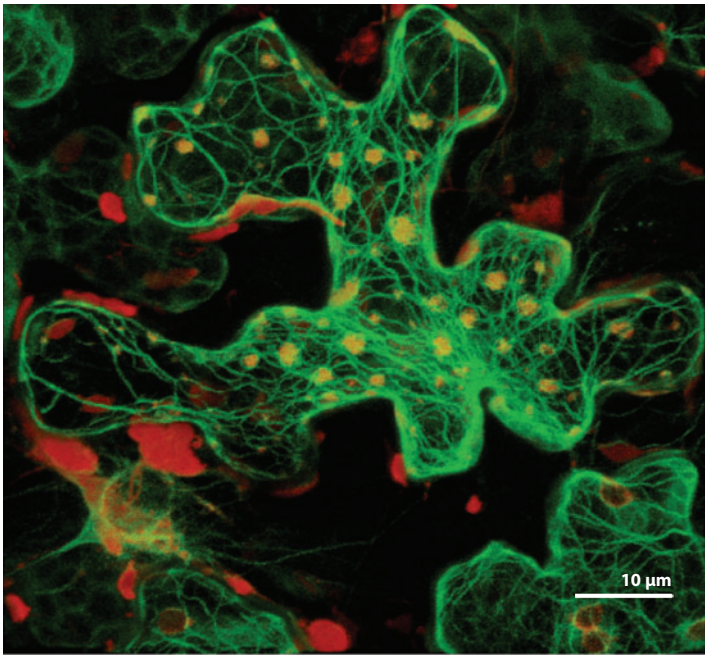

Figure 2

Co-alignment of *Turnip mosaic virus* (TuMV) replication factories with microfilaments. *Nicotiana benthamiana* cells expressing $6K_2$mCherry-tagged TuMV-induced replication factories and the actin domain of fimbrin fused to GFP observed by confocal microscopy at 4 days postagroinfiltration. Photograph is a three-dimensional rendering of 40 1-μm thick slices that overlap by 0.5 μm. Reproduced, with permission, from (12) © 2009 American Society for Microbiology.

Dengue virus (49, 141). Maturation of each of these components may require their movement along the cytoskeleton. Finally, cell-to-cell movement of replication factories, as shown for TMV (45), may require the cytoskeleton.

TOPOLOGY OF VESICLES

A prime function of virus-induced membrane rearrangement is to enclose the virus replication complex. One feature that characterizes viral RNA synthesis is the generation of double-stranded RNA intermediates that colocalize with viral RdRp and with accessory viral proteins involved in replication, all being enclosed within the virus-induced membrane structures (12, 16, 69, 102, 112).

In addition to viral replication proteins, host proteins have been found within replication factories. One such protein is eIF4E, an important

Bimolecular fluorescence complementation (BiFC): a method for viewing the association of proteins inside cells. Fusing two non-fluorescent fragments to two putative interacting partners leads to restoration of fluorescence within a cell when the two parts of the split fluorophore become associated

regulatory protein involved in the initiation of translation that recognizes the 5′ cap structure of mRNAs (8). eIF4E interacts with the VPg of potyviruses (58) and caliciviruses (animal viruses) (26) and with the VPg-Pro polyprotein of ToRSV (57). eIF4E plays an important role in potyvirus replication. Knockout *Arabidopsis thaliana* plants for *eif(iso)4E* are resistant to several potyviruses (17, 56). Additionally, naturally occurring potyviral resistance has been mapped to the genes coding for either eIF4E or eIF(iso)4E (reviewed by 104). The virulence determinant toward these recessive resistances is VPg, and failure of the eIF4E isomer to bind VPg generally correlates with resistance. Despite the demonstrated importance of this interaction for virus replication, it is not yet known for which specific step it is required, although a participation in viral RNA translation is likely (46). Using bimolecular fluorescence complementation (BiFC), VPg-eIF(iso)4E interaction has been shown to take place in TuMV-infected, 6K-VPg-Pro-induced vesicles (3). Besides eIF(iso)4E, other factors involved in protein synthesis and folding such as PABP, eEF1a, and Hsc70-3 have been found within TuMV-induced replication factories (4, 12, 15, 121).

Other proteins of cellular origin that are present in plant virus factories have been identified. Subunits of eIF3 have been found in highly purified replication complexes of BMV and TMV (88, 100). eEF1a and chaperones, such as the heat shock protein (Hsp) 70 or the yeast DNAJ protein, copurify with the replication complexes of BMV, TMV, and TBSV (60, 84, 114, 122). Hsp70 promotes the subcellular localization of TBSV replication proteins to membranes and facilitates replication complex assembly (96, 133). Components of the ubiquitin pathway of protein modification/degradation, including the Nedd4-type Rsp5p ubiquitin ligase and the Cdc34 ubiquitin-conjugating enzyme, interact with TBSV replication proteins and copurify with virus factories (2, 59). Based on the accepted functions of these proteins, they are presumed to regulate the stability of the viral replication proteins and modulate their activity. Finally, glyceraldehyde 3-phosphate dehydrogenase selectively binds and retains (−) RNA within the TBSV replication complex, thereby allowing asymmetric synthesis of (+) RNA (132).

This picture of the complex composition of virus factories and of the important role of host factors within these factories is only beginning to emerge. Indeed, proteomic and genomic studies using TBSV and BMV have revealed a large number of host proteins that can interact with viral replication proteins or that are essential for viral replication (52, 89, 114, 115).

VIRAL RNA TRANSLATION AND SYNTHESIS

The presence of protein translation factors within virus replication factories prompts the question of the spatial relationship between viral RNA translation and synthesis. Although both are obviously important for infection, viral RNA translation and synthesis are seemingly conflicting processes. Ribosomes translate the viral RNA in a 5′→3′ direction, whereas the replication complex transcribes the template viral RNA in the opposing 3′→5′ direction. Thus, a collision is predicted if both processes were to occur simultaneously on the same template. Consequently, one longstanding question in virology is what controls the switch between translation and RNA synthesis. In the case of (+) RNA viruses, virology textbooks generally depict viral RNA translation and synthesis as physically separated processes. In this model, viral RNA is translated on ribosomes distributed randomly in the cytoplasm, and the resulting viral proteins necessary for viral RNA replication are exported to vesicle-enclosed replication complexes. In the case of poliovirus, it was even suggested that the viral RNA intended to be translated is structurally different (i.e., it does not have a VPg) from the RNA found associated with the replication complex (85). However, there are reports indicating that viral RNA translation and replication are tightly coupled events. This is the case for picornaviruses (23) and ambisense viruses

(83), where viral replication and/or transcription necessitate continuous viral protein synthesis. Preformed poliovirus vesicles do not incorporate viral RNA and replication proteins when supplied in *trans*, and it was concluded that vesicle formation, viral RNA translation, and replication are *cis*-linked events (19), conceivably on the same assembly line. Inefficient complementation activity of poliovirus proteins for the rescue of lethal mutations in the viral genome further indicates that poliovirus RNA replication shows a marked preference for proteins contributed in *cis* (120). There is also an indication that constituents of the Dengue virus translation and replication machineries colocalize (91, 92). Poxviruses are large DNA viruses that replicate in cytoplasmic DNA factories. Translation factors are found within these factories, and there is some evidence that translation might also take place there (44, 130).

Tight coupling between viral RNA translation and synthesis may also occur for plant viruses. Efficient replication of RNA1 of BMV requires 1a synthesis from RNA1 in *cis* (147). Coupling between translation and replication of RNA2 occurs in cells infected with RCNMV (74). Finally, a TuMV-induced vesicle was shown to originate from a single viral genome (12), implying a *cis*-acting mechanism that incorporates the proteins resulting from the translation of the viral RNA into the same vesicle. One must also consider the coupling of translation with replication of viral genomes having multiple components. Because only one of the genomic RNAs encodes the RdRp, the replication of multipartite genome implies a *trans*-acting activity of the RdRp. However, even in these cases, a coupling between translation and RNA synthesis is not excluded. As has been elegantly demonstrated for BMV, interaction between the membrane-anchor protein 1a and the RdRp protein 2a occurs during the process of translation at a time when they are partially synthesized proteins that are still associated with their cognate RNAs (10). This mechanism could conceivably allow the recruitment of other multipartite viral RNAs into the replication complex. It is interesting to note that in the case of the bipartite como- and nepoviruses, although RNA1 codes for the core replication proteins, the N-terminal region of the RNA2-encoded polyprotein is necessary for replication of RNA2 (22, 125). It is not known whether the nascent polyprotein can act in the recruitment of RNA2 to the replication complex during translation.

One possible mechanistic explanation for the coupling of viral RNA translation with viral RNA synthesis may come from the studies examining the coronavirus- and *Dengue virus*-induced reticulovesicular network of modified ER (49, 141). This network integrates convoluted membranes, numerous interconnected double-membrane vesicles, and vesicle packets. Ribosomes are present on the exterior surface of the double-membrane vesicles that contain dsRNA. Hypothetically, upon entry in the cell, viral RNA translation takes place on ribosomes affixed to the ER. After a few rounds of translation, enough membrane-targeted viral proteins are synthesized for vesicle production. The translating ribosomes remain apposed to their respective vesicles, and the newly synthesized viral proteins are directly imported into them. Similarly, newly replicated viral RNA can be translated on neighboring ribosomes. This is in agreement with the view that cellular mRNAs are translated at the final destination site of their encoded protein (7). Examination of plant virus-induced vesicles by electron tomography may reveal a similar assemblage of membrane structures and vesicles.

Another question that will need to be resolved is the dynamic state of exchange between the content of the replication factories and the rest of the plant cell. It is not yet known how the vesicles are filled with viral and host proteins, supplied with small molecules (e.g., nucleotides) and energy, and cleansed of deteriorated proteins. Is it a passive system, or is there a specific transport system that controls what goes in and what goes out? Most of the described virus-induced vesicles appear to have an opening that would link the inside of the vesicle with the cytoplasm. But in the case of HCV, fluorescence recovery

after photobleaching (FRAP) has shown that the factories have a static internal architecture (145), suggesting that there is limited protein exchange between the inside of the vesicles and the outside world. This goes against what one might expect of replication sites, which would require active and constant exchange and reorganization of viral material. Apparently, HCV RNA replication sites have a fixed complement of viral and host proteins that allow them to function as autarchic viral factories for producing viral RNA (42). These questions have yet to be addressed for plant viruses.

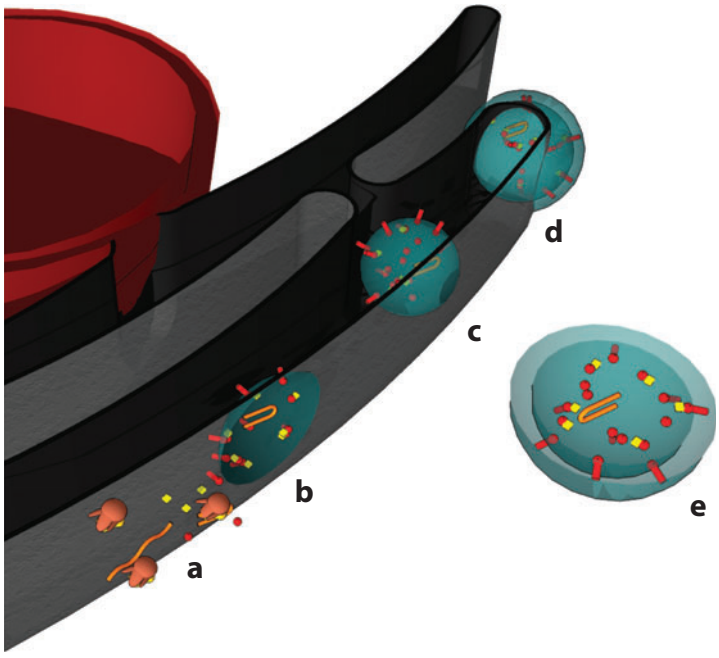

Figure 3

Model for the formation of virus-induced vesicles. The large red sphere and gray structure represent the nucleus and the ER, respectively. Partially transparent virus-induced vesicles are shown in blue. Orange ribbons and small red spheres or rods depict viral RNAs and proteins, respectively. Host proteins are shown by the yellow cubes, and the brown structures represent the ribosomes. (*a*) Upon release of the genomic RNA into the cytoplasm, production of viral proteins takes place on ER-associated ribosomes. (*b*) During viral RNA translation, membrane-targeting viral proteins accumulate in patches on the outer ER membrane and initiate membrane curvature. (*c*) Membrane curvature increases with the accumulation of replication components, which ultimately leads to the formation of single-membrane spherules/vesicles within the organelle lumen, which may or may not have a pore-like connection to the exterior. (*d*) For some viruses, the spherule/vesicle produced after a first budding event within the ER lumen undergoes a second budding event, acquiring a second membrane, and (*e*) detaches itself from the ER to give rise to a double membrane vesicle (DMV).

BIOGENESIS OF PLANT VIRUS REPLICATION FACTORIES: A MODEL

Although a parallel between the replication complexes of (+) RNA viruses and the formation of budded retrovirus particles has been proposed (112), formulating a unifying model that explains how replication factories are generated for all plant viruses is difficult. Despite accumulating experimental data on the membrane/organelle origin of replication factories and on the viral proteins and host factors involved in their formation, the state of our present knowledge is nevertheless rudimentary. Additionally, although a basic scheme may be at work, biogenesis of ER-derived factories is likely to be different from those that are associated with non-ER organelles such as peroxisomes, mitochondria, and chloroplasts. However, by incorporating the experimental data common to plant viruses and integrating what is known about replication factories for animal viruses, a tentative model for replication factory biogenesis can be proposed. We acknowledge that this model is incomplete and a simplification. It cannot address all aspects of a particular plant virus replication factory. For instance, it does not explain the formation of double-membrane vesicles derived from the outer chloroplastic membrane during TYMV infection (**Figure 1*f*,*g***). Moreover, this model is pertinent only for (+) RNA viruses.

The sequential steps can be schematized as follows (**Figure 3**). Upon release of the genomic RNA into the cytoplasm, the host protein synthetic machinery is usurped for the production of viral proteins, very likely on ER-associated ribosomes (**Figure 3*a***). After several rounds of viral RNA translation, membrane-targeting viral proteins accumulate in patches on the outer ER membrane, initiating membrane curvature (**Figure 3*b***). For non-ER derived factories, direct connections exist between the ER and these organelles

(76, 118), and the viral proteins would be translocated to their final destination through some sort of piggyback transport system or organelle-addressing signal. The newly formed viral protein patches then (or concurrently) initiate the assembly of the viral replication complex through protein-protein and protein-RNA interactions involving viral and host factors. Membrane curvature increases with the accumulation of replication components, which ultimately leads to the formation of single-membrane spherules/vesicles within the organelle lumen, which may or may not have a pore-like connection to the exterior (**Figure 3*c***). For some viruses (e.g., BMV, CIRV, TBSV, etc.), the spherules/vesicles appear to remain inside the lumen of the targeted organelle, and the biogenesis process would stop at this point. For many other viruses, clusters of (large) vesicles are observed within the cytoplasm. Although the resolution of EM photographs is often insufficient to confirm if cytoplasmic vesicles produced during plant virus infection contain one or two layers of membranes, these cytoplasmic vesicles may correspond to the DMVs often reported for animal viruses. There are two mechanisms that could explain the formation of DMVs. In one model (shown in **Figure 3**), the spherule/vesicle produced after a first budding event within the ER lumen (**Figure 3*c***) undergoes a second budding event, thereby acquiring a second membrane (**Figure 3*d***), and detaches itself from the ER to give rise to a DMV (**Figure 3*e***). In an alternative model, DMVs could originate from the ER by a protusion-and-detachment mechanism (94) (not shown). In this case, part of an ER cisterna bends, and the two lipid bilayers become tightly apposed. The curved cisternal membranes then pinch off and seal to form a DMV. It must be reiterated that many virus factories are likely made up of several spherule/vesicle units (e.g., multivesicular bodies) assembled into a large network of connecting membranous structures, where in addition to viral RNA synthesis, other viral processes would be taking place. Regardless of their unique features, such intracellular assemblies represent exquisite biological structures that provide viruses with favorable environments for their reproduction.

SUMMARY POINTS

1. A prime function of virus-induced membrane rearrangement is to enclose the virus replication complex. It is thought that these virus factories are needed to increase the local concentration of components required for viral RNA replication, to provide a scaffold for anchoring the replication complex, and to confine the process of RNA replication to a specific location for preventing the activation of certain host defense functions.
2. Viruses have targeted specific organelles for their replication, and the virus-induced modifications involve the formation, through invagination of the targeted organelle membrane, of spherules, vesicles, and/or multivesicular bodies. These bodies may be bound by a double-lipid layer membrane and connected by a narrow channel to the surrounding cytoplasm. Specific membrane targeting does not appear to be a strict requirement for efficient viral replication as viral replication complexes can be redirected to an alternate subcellular membrane.
3. The responsible viral factors for membrane and organelle alterations are integral or peripheral membrane proteins. These viral proteins are also multi-functional and interact with host and other viral factors. Consequently, these viral proteins not only induce

membrane rearrangements but also act as a scaffold for the assembly of the RNA replication complex. Several host proteins are redirected to the interior of virus factories, and many are related to protein synthesis. They act as accessory factors for virus replication.
4. Cellular remodeling is likely important for other functions, such as cell-to-cell movement of the virus.

FUTURE ISSUES

1. A more refined three-dimensional view of virus factories is needed in order to better understand the interplay between virus RNA replication and various viral processes, such as translation and encapsidation.
2. A corollary is to determine the full content of host proteins and the mechanistic role for their presence in virus factories.
3. Trafficking and the fate of virus factories in cell-to-cell and long distance transport needs to be investigated.
4. Future studies will also be directed at examining the mechanism(s) (i.e., invagination or membrane wrapping) involved in the release of ER-derived vesicles.

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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