The ER in Replication of Positive-Strand RNA Viruses

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Abstract All eukaryotic positive-sense single-strand RNA viruses, (+)ssRNA, replicate their genome in association with membranes of host cells. The presence of a replicating virus frequently induces proliferation and rearrangement of the host membranes into various cytopathic structures, including invaginations, vesicles, spherules or membranous webs. Such structures are considered to be virus-induced organelles specialized in replication functions. Virtually all membranes are able to be rearranged to support replication. Thus, membranes from peroxisomes, endosomes, lysosomes, vacuoles, mitochondria, and chloroplasts are used for (+)ssRNA virus replication, but the endoplasmic reticulum (ER) is by far the preferred organelle. The specific type of membrane system utilized in assembling the viral replication complex is strictly dependent on individual viruses and is likely to be genetically determined. The various molecular interactions that govern ER targeting of plant viruses highlight how viruses can exploit the diversity of interactions that occurs between proteins and membrane or lipid structures.

1
Introduction

Positive-sense single-stranded RNA [(+)ssRNA] viruses represent the largest class of viruses. They infect both animal and plants and encompass 23 families, which represent over one third of all virus genera (www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm). (+)ssRNA viruses replicate their genomes through negative-strand RNA intermediates and encapsidate messenger-sense genomic RNAs. Numerous well-known human and animal pathogens belong to this class, including *Hepatitis C virus* (HCV), *Yellow fever virus*, *Poliovirus*, *Foot-and-mouth disease virus*, and *Coronavirus*. The latter becoming well known recently as being responsible for severe acute respiratory syndrome (SARS). Remarkably, the replication of all known eukaryotic (+)ssRNA viruses occurs in association with host cell membranes. These viruses modify the intracellular membranes to create a compartment, with defined components and functions, often considered as a virus-specific organelle dedicated to RNA replication.

Although the strict requirement for membranes in viral RNA synthesis is not well understood, multiple properties have been attributed to membranes. Thus, membranes may provide a physical support on which the RNA

replication complex can anchor (Lyle et al. 2002). Membranes may also offer the possibility to locate and concentrate cellular and viral replication factors (Ahola et al. 1999; Schwartz et al. 2002). In addition, the flexibility of membranes might allow the replication complex to be compartmentalized into a specific virus-induced organelle, thereby sequestering RNA replication factors and genomic RNAs from competing RNA templates and competing processes such as translation (Schwartz et al. 2002). This "caging" mechanism could also offer adequate protection against host antiviral defence mechanisms such as RNA interference, or interferon-induced responses (Baulcombe 2004; Pelham 1996). Finally, evidence also exists for the involvement of membranes in tethering viral RNA during unwinding (Egger et al. 2002).

Depending on the virus, replication may occur on altered membranes derived from very diverse organelles. Thus, *Turnip yellow mosaic virus* (TYMV) is probably the best studied virus whose replication occurs in association with chloroplasts. TYMV infection is accompanied by the induction of characteristic membranous vesicles at the periphery of the chloroplast envelope. Cytological abnormalities include the swelling and clumping of the chloroplasts and the appearance of peripheral structures consisting of membrane vesicles, 50–100 nm in diameter, that are likely to result from the invagination of the chloroplast envelope into the organelle (Hatta et al. 1973; Prod'homme et al. 2001, 2003). Membranes from mitochondria support the replication of the tombusvirus *Carnation Italian ringspot virus* (CIRV). Studies of CIRV infections have identified vesiculated structures (multivesicular bodies [MVBs]), made up of a main body surrounded by many spherical to ovoid vesicles 80–150 nm in diameter, resulting from proliferation of the limiting mitochondrial membrane (Rubino and Russo 1998; Rubino et al. 2001; Weber-Lotfi et al. 2002). Similar MVBs are also observed upon infection with the closely related tombusvirus *Cymbidium ringspot virus* (CymRSV). However, here membranes from peroxisomes rather than mitochondria were shown to support the biogenesis of the MVBs (Bleve-Zacheo et al. 1997; Burgyan et al. 1996; Navarro et al. 2004; Rubino and Russo 1998). With *Alfalfa mosaic virus* (AMV) and *Cucumber mosaic virus* (CMV), it is the vacuolar membrane or tonoplast that appears to participate in the replication process (Cillo et al. 2002; Van Der Heijden et al. 2001).

The endoplasmic reticulum (ER) can represent up to 50% of the intracellular membrane surface. It is the most versatile, adaptable, and largest organelle in eukaryotic cells (Staehelin 1997). It is also the most common organelle for the replication of viruses infecting animals and plants. Thus, the membranous web observed during HCV infection of human cells is found in close association with rough ER (for review see Moradpour et al. 2003; Moriishi and Matsuura 2003). For *Poliovirus*, it is believed that the translation of viral proteins and replication takes place at dispersed sites on the ER, resulting in membrane protrusions and the budding of vesicles from the ER (Egger et al. 2000; Egger and Bienz 2005). The emerging individual vesicles grow

rapidly into ER-associated small clusters dispersed throughout the cytoplasm and are engaged in RNA synthesis (Bolten et al. 1998; Egger and Bienz 2005). Although consensus is emerging indicating that ER membranes contribute to the origin of the *Poliovirus*-induced vesicles, the exact mechanism underlying their generation remains controversial. The data available indicates that different pathways, controlled by host cellular processes and enhanced by the virus, may be involved. Thus, it was initially suggested from the sensitivity of *Poliovirus* to Brefeldin A, that the viral-induced vesicles might be derived from the COPI pathway (Belov et al. 2005; Cuconati et al. 1998). However, Rust et al. (2001) provided evidence that vesicles could form at the ER by subversion of the cellular COPII budding machinery. More recently, Jackson et al. (2005b) highlighted the role of autophagy in *Poliovirus* replication. In particular, they showed that autophagy marker proteins colocalize in PV-infected cells, a phenomenon that otherwise occurs only upon induction of autophagy. In addition, they established that stimulation of autophagy by tamoxifen or rapamycin, increased *Poliovirus* yield, whereas inhibition of the autophagosomal pathway decreased *Poliovirus* yield (Jackson et al. 2005).

In plants, more than 90% of all known viruses possess a (+)ssRNA genome. Based on their genome organization, they have been classified into three main superfamilies: alphavirus-like, picornavirus-like, and flavivirus-like (Koonin and Dolja 1993). All contain members whose replication occurs in association with the ER (for a recent review see Salonen et al. 2005). We will present in some detail relevant studies on plant viruses belonging to the alphavirus-like and picornavirus-like superfamilies that replicate on ER membranes.

2
Plant Viruses Belonging to the Alphavirus-like Superfamily

Plant viruses belonging to the alphavirus-like superfamily can target different types of membranes for their replication as exemplified above with TYMV and AMV. *Brome mosaic virus* (BMV) and *Tobacco mosaic virus* (TMV), probably two of the best-studied members of the alphavirus-like superfamily, exploit ER membranes for their replication.

2.1
Brome Mosaic Virus

BMV is the type-member of the genus *Bromovirus* within the family *Bromoviridea*. Its replication cycle has been carefully reviewed in recent years (Noueiry and Ahlquist 2003). Its genome consists of three positive-sense RNAs designated RNA 1, RNA 2, and RNA 3. All RNAs possess a "cap" structure at their 5′ extremities and contain a conserved 200-nucleotide tRNA-like

structure that can be acylated by tyrosine at their 3′ends (Ahlquist 1992; Ahola and Ahlquist 1999). The monocistronic RNAs 1 and 2 encode the nonstructural proteins 1a and 2a, respectively, which are essential for viral RNA replication. The 109-kDa 1a protein contains a N-proximal domain with m7G methyltransferase and covalent GTP-binding activities required for viral RNA capping in vivo, and a C-proximal RNA helicase homology domain (Ahola and Ahlquist 1999; Ahola et al. 2000; Kong et al. 1999). The 94 kDa 2a protein contains a large central RNA-dependent RNA-polymerase-like domain (Sivakumaran et al. 2000). RNA 3 is dicistronic and dispensable for replication. It encodes the 3a cell-to-cell movement protein and the coat protein that is required for systemic movement during virus infection (Mise and Ahlquist 1995). The coat protein is translated from the subgenomic RNA 4 synthesized from the negative-strand RNA 3 during replication.

Remarkably, proteins 1a and 2a can direct replication of all BMV RNAs not only in plant cells, but also in the yeast *S. cerevisiae*, indicating that the essential host features required for BMV replication are widely conserved (Janda and Ahlquist 1993). RNA replication, transcription, and persistence of BMV RNA replicons in *S. cerevisiae* have been reported (Ishikawa et al. 1997). In both organisms, RNA replication occurs on 50- to 70-nm spherular vesicles or spherules made from the invagination of the outer nuclear envelope or perinuclear ER membrane. The interior of these spherules remains connected to the cytoplasm via a narrow membranous neck contiguous with the ER membrane (Ahola and Ahlquist 1999; Restrepo-Hartwig and Ahlquist 1999; Schwartz et al. 2002). Such compartmentalization of BMV replication into ER-derived spherules was shown to protect viral RNA from nucleases (Schwartz et al. 2002) and is thought to preserve double-stranded viral replication intermediates from dsRNA-induced host defence responses such as RNA interference (Ahlquist 2002).

A combination of genetic, biochemical, and cell biology approaches has revealed that 1a is a multifunctional protein and the primary viral determinant for the subcellular localization of the BMV RNA replication complex. Thus, 1a localizes to the cytoplasmic face of the perinuclear ER independently of other viral factors and is sufficient for spherule formation (Schwartz et al. 2002). 1a expression increases total membrane lipids but not composition in yeast cells (Lee and Ahlquist 2003). 1a self-interacts and its binding domain has been mapped to the N terminus of the protein (O'Reilly et al. 1998). This self-interaction property is most likely responsible for spherule biogenesis, into which up to several hundred molecules of protein 1a can accumulate (Schwartz et al. 2002). In the absence of the polymerase, protein 1a also interacts with the genomic RNA resulting in a dramatic increase of stability, but reduced translatability (Janda and Ahlquist 1998). BMV RNA replication protein 1a dramatically increases in vivo stability but not translation of viral genomic RNA 3 (Sullivan and Ahlquist 1999). Finally, 1a recruits the RNA-dependent RNA polymerase 2a into the spherules (Schwartz et al. 2002). This

recruitment of 2a protein was shown to be driven by direct interaction between the *C* terminus of 1a and the *N* terminus of 2a (Kao and Ahlquist 1992; O'Reilly et al. 1995, 1997; Schwartz et al. 2002). In a recent study, the group of Ahlquist established by modulating the relative levels and interactions of BMV replication factors 1a and 2a, that spherules are dispensable for RNA replication. Thus, double ER membranes (termed karmellae) induced upon expression of 1a plus higher than normal levels of 2a supported RNA replication and protected viral RNA as efficiently as spherules (Schwartz et al. 2004). The mechanism that triggers the change from ER-derived spherules to karmellae is still not well understood, but involves protein–protein and protein–membrane interactions.

The capacity of yeast cells to duplicate all known features of BMV replication in plant cells has been exploited to identify cellular factors and functions required for BMV RNA replication. Thus, classical yeast genetics has been used to identify host genes involved in (i) BMV translation, (ii) selection of RNA templates, (iii) activation of the replication complex, and (iv) association of replication complexes with membranes (for a review see Noueiry and Ahlquist 2003). More recently, a high-throughput approach performed on 4500 yeast deletion strains, representing approximately 80% of all yeast genes, revealed nearly 100 genes whose absence inhibited or significantly stimulated BMV RNA replication (Kushner et al. 2003).

Not surprisingly, a number of the identified genes encode proteins whose function is related to host cell membranes (Table 1). Thus, BMV RNA replication was increased in *DRS2, RCY1, PBS2, NEM1, SPO7* and *GIT1* knock-out yeast strains, whose functions involve phospholipid translocation, membrane recycling and MAP kinase activities. Conversely, mutations in *OLE1, ACB1, SEL1* and *SCS2*, whose involvement in membrane functions are also very diverse, resulted in mild to severe inhibition of BMV replication (Table 1). The mechanisms by which these genes contribute to BMV replication are still unknown except maybe for *OLE1*. This gene encode a delta9 fatty acid desaturase Ole1 involved in the conversion of saturated fatty acids (SFA) into unsaturated fatty acids (UFA) in yeast. UFAs are incorporated into membrane lipids and are major determinants of membrane fluidity and plasticity. Interestingly, Ole1 is an integral ER membrane protein and thus is present at sites of BMV RNA replication. However, (Lee et al. 2001) established that the Ole1 protein was not required for BMV RNA replication, but inhibition was rather linked to membrane composition. Thus, a 12% decrease in UFA levels resulted in a 95% or more reduction in BMV RNA replication. Interestingly, none of the above-described 1a properties (1a-induced membrane synthesis, 1a localization to the ER, recruitment of 2a polymerase, spherule formation) were altered in *OLE1* mutant yeast. However, membrane staining of spherules revealed that they are locally depleted in UFAs (Lee et al. 2001). This implies that the membrane, rather than Ole1 protein itself, is an essential functional component of the RNA replication complex. At this stage, the precise nature of the replica-

Table 1 Genes encoding membrane proteins involved in BMV replication (Adapted from Kushner et al. 2003, and Lee et al. 2001)

Genes	Protein name	Function/phenotype	References	Localization	Effect on BMV replication
OLE1	9 Fatty acid desaturase (Oleic acid linoleic acid)	Synthesis of unsaturated fatty acid	(Lee et al. 2001)	ER	– 50 X
ACB1	Acyl-CoA-binding protein	– synthesis of long-chain acyl-CoA esters – Intracellular transporter of acyl-CoA – Vesicles trafficking	(Rasmussen et al. 1993) (Rasmussen et al. 1994) (Faergeman et al. 2004)	Mitochondria + microsomes	– 11.1 X
SEL1	S-uppressor and/or enhancer of lin-12	– Secretion regulation – Regulate ER stress response – Protein-protein interaction	(Grant and Greenwald 1997) (Sundaram and Greenwald 1993)	Cytoplasm – intracellular vesicles	– 4.8x
SCS2	Suppressor of Ca^{2+} sensitivity VAP homolog SCS2 (vesicle-associated-membrane protein-associated protein)	– Myoinositol metabolism – Regulate phospholipid metabolism (serine palmitoyltransferase activity)	(Zhao et al. 1994) (Loewen and Levine 2005)	ER	– 3.6x
DRS2	Deficient in ribosomal subunits	Phospholipid-translocating ATPase	(Natarajan et al. 2004) (Ripmaster et al. 1993)	trans-Golgi network (TGN)	+ 4x
NEM1	Nemaline myopathy	Regulate nuclear membrane/ ER morphology	(Santos-Rosa et al. 2005) (Siniossoglou et al. 1998)	nuclear/ ER membrane	+ 4.1x
RCY1	Recycling1 (F-box protein)	Mediate endosome-to-Golgi transport	(Chen et al. 2005) (Wiederkehr et al. 2000)	Golgi and endosomes	+ 3.7x
GIT1 SPO7	Gastrointestinal tract injuries Sporulation-defective	Glycerophosphoinositol uptake Regulate nuclear membrane/ ER morphology	(Hughes 1999) (Santos-Rosa et al. 2005) (Siniossoglou et al. 1998) (Klapholz and Esposito 1980)	nuclear/ ER membrane	+ 3.5x + 3.2x
PBS2	Polymyxin B sensitivity	– Kinase activity – Osmoregulation – Scaffold protein	(Boguslawski 1992) (Bansal et al. 2001)		+ 3x

tion block remains to be determined. It is suggested that membrane fluidity or plasticity may be needed for the 1a or 2a polymerase to function correctly or to interact properly with each other or with host factors (Lee et al. 2001). Interestingly, *OLE1* gene is well conserved throughout kingdoms and the Ole1 protein from yeast OLE1 shares 31% identity with its *Arabidopsis thaliana* ortholog (accession number AK119146.1). Whether plant Ole1 protein acts on BMV replication similarly to it yeast counterpart needs to be determined.

2.2
Tobacco Mosaic Virus

The 6.3-kb (+)ssRNA genome of TMV encodes at least four proteins, of which the 5′ proximal open reading frame (ORF) encodes a 126 kDa protein terminated by an amber stop codon that when suppressed yields a read-through 183 kDa protein (Goelet et al. 1982; Pelham 1978). Both the 126 kDa and the 183 proteins represent essential replicase components, containing methyltransferase and helicase domains as well as a polymerase domain on the read-through portion of the 183 kDa ORF (Buck 1999; Koonin and Dolja 1993). The two additional proteins, the 30 kDa movement protein (MP) and the 17.5 kDa capsid protein are dispensable for replication but essential for cell–cell spread of infection and RNA encapsidation, respectively (for review, Heinlein 2002).

For TMV, the virus replication occurs on amorphous proliferations of membranes, previously termed viroplasms or X-bodies, which were initially observed in infected plant cells by Ivanowski (1903). Electron microscopy revealed that these inclusion bodies contained ribosomes, viral RNA, tubules, and 126/183 kDa replication proteins (Esau and Cronshaw 1967; Hills et al. 1987; Martelli and Russo 1977; Saito et al. 1987). Additionally, membrane fractions from TMV-infected cells also contained active replicase complexes capable of synthesizing both plus- and minus-strand viral RNAs in a template-dependent fashion (Osman and Buck 1996).

The use of GFP later revealed that TMV replication complexes localized into large, irregularly shaped, ER-derived structures (especially the cortical ER) containing viral RNA and also the MP (Heinlein et al. 1998; Mas and Beachy 1999; Reichel and Beachy 1998). Mas et al. (1999) further established that the MP was not required for association of the viral RNA with the ER, but was required for the formation of the large irregular bodies. Structural studies recently confirmed the membrane-binding properties of the MP (Brill et al. 2000, 2004). It has been suggested that two highly hydrophobic regions conserved among tobamovirus MPs, the domains I (residues 56–96) and II (residues 125–164) are responsible for the behavior of the MP as an integral membrane protein (Moore et al. 1992; Reichel and Beachy 1998), including its association with cortical, cytoplasmic, and perinuclear ER (Heinlein et al. 1998; Mas and Beachy 1999).

As was the case for the MP, homology searches of the 126 kDa protein failed to identify any potential ER retention motifs or obvious membrane-spanning domains (dos Reis Figueira et al. 2002). However, the TMV 126/183 kDa replicase proteins encode a predicted bipartite nuclear localization signal (NLS) at amino acids 29 to 47 that is conserved within the genus *Tobamovirus* as well as a potential 21 amino acid amphipathic helix between residues 708–728 (dos Reis Figueira et al. 2002). Interestingly, results from transient expression studies have demonstrated that:

1. The NLS present within the 126/183 kDa protein could function to nuclear localize large polypeptides
2. The NLS domain is necessary for TMV replication
3. The NLS is required but not sufficient for the formation of ER-associated inclusion bodies
4. The full-length 126 kDa protein associates with the ER in the absence of other viral proteins and components (dos Reis Figueira et al. 2002)

Whether the 21 amino acid amphipathic helix is involved in the retention of the 126 kDa protein to the ER is still not known. It has been proposed that a phenylalanine at one end of the helix could function in membrane attachment in a fashion similar to the alphavirus *Semliki forest virus* nsP1 protein (Lampio et al. 2000). Alternatively, the membrane retention of the 126 kDa could be conferred via a membrane-bound host protein.

In view of the retention of the TMV replication complex by host proteins, Ishikawa and colleagues have identified three Arabidopsis genes, *TOM1*, *TOM2A* and *TOM3*, that are required for efficient tobamovirus replication (Tsujimoto et al. 2003; Yamanaka et al. 2000, 2002). *AtTOM1* and *AtTOM3A* share a high degree of similarity (56% identical). They are predicted to be seven-pass transmembrane proteins and are likely to share a parallel and essential function in tobamovirus multiplication (Yamanaka et al. 2002). *AtTOM2A* is predicted to be a four-pass transmembrane protein. Remarkably, neither of these *TOM* proteins possesses well-known sorting signals to specific organelles (Tsujimoto et al. 2003; Yamanaka et al. 2000). *AtTOM1* and *AtTOM3* have been shown to interact with *AtTOM2A* and with the helicase domain of the 126/183 kDa proteins (Tsujimoto et al. 2003; Yamanaka et al. 2000), suggesting that *TOM* proteins are constituents of the replication complex of tobamoviruses and play important roles in the formation of the complex on ER membranes where they colocalize. In support of this hypothesis, subcellular fractionation patterns revealed that the viral RdRp activity coincided well with that of both the membrane-bound 126/183 kDa proteins and *TOM1*–*TOM2A* proteins (Hagiwara et al. 2003). However, confocal microscopic analyses of the GFP-tagged *TOM* proteins showed that *TOM1* and *TOM2A* were predominantly targeted to the vacuolar membranes and not to the ER (Hagiwara et al. 2003). Therefore, whether TMV replication complex is

associated with the tonoplast, as strongly suggested by the latter findings, or with the ER still needs to be determined.

2.3
Plant Viruses Belonging to the Picornavirus-like Superfamily

Viruses belonging to this superfamily bear strong resemblance to animal picornaviruses both in gene organization and in the amino acid sequence of replication proteins. In plants, the genome of picorna-like viruses can be monopartite such as in *Potyviridae* or distributed over two RNAs as in members of the *Comoviridae* family. The RNAs of these viruses are characterized by a covalently genome-linked protein (VPg) at the 5' end, a unique open reading frame encoding a single polyprotein and a poly (A) tail at the 3'-terminus. Within the *Potyvirida*, the *Tobacco etch virus* 6-kDa protein has been shown to localize specifically to ER membranes when expressed as a fusion with GFP or glucuronidase and was proposed to cause the ER modifications that are observed during *Tobacco etch virus* infection (Schaad et al. 1997). Here, the analysis has been restricted to some of the best-studied *Comoviridae* members such as *Cowpea mosaic virus* (CPMV), the type member of the genus *Comovirus*, and two nepoviruses, the *Tomato ringspot virus* (ToRSV) and the *Grapevine fanleaf virus* (GFLV).

2.3.1
Cowpea Mosaic Virus

CPMV RNA1 and RNA2 express large polyproteins, which are proteolytically cleaved into different proteins by the RNA1-encoded viral 24-kDa proteinase (24 K). The proteins encoded by RNA1 are necessary and sufficient for virus replication. The function of RNA1-encoded proteins functions has been attributed as follows. The 32-kDa protein (32 K) is a hydrophobic protein which does not contain a motif common to other positive-strand RNA viruses outside the *Comoviridae*. It is involved in the regulation of processing of RNA1-encoded polyprotein and is required as a cofactor in the cleavage of the RNA2 polyprotein (Peters et al. 1992). The 58 K is able to bind ATP via a conserved nucleotide-binding motif and has been proposed to be a viral helicase (Peters et al. 1994). The 28 amino acid VPg is linked to the 5' end of the RNA via a serine residue and is likely involved in initiation of viral RNA synthesis (Carette et al. 2001). The 24 K protease is structurally similar to the trypsin-like family of serine proteases with residues His40, Glu76, and Cys166 representing the catalytic triad of the active site (Dessens and Lomonossoff 1991). The 87 K has a domain specific to RNA-dependent RNA polymerases; however, the 110 K (87 K plus 24 K) is the only viral protein present in highly purified "replication complexes" capable of elongating nascent viral RNA chains, suggesting that fusion to 24 K is required for replicase activity (Eggen et al. 1988).

Upon infection of cowpea plants with CPMV, a typical cytopathic structure is formed, often adjacent to the nucleus and consisting of an amorphous matrix of electron-dense material that is traversed by rays of small membranous vesicles (De Zoeten et al. 1974). Both subcellular fractionation experiments of CPMV-infected leaves and autoradiography, in conjunction with electron microscopy, on sections of CPMV-infected leaves treated with [^{3}H] uridine revealed that the membranous vesicles are closely associated with CPMV RNA replication (De Zoeten et al. 1974; Eggen et al. 1988; Zabel et al. 1974). Curiously, more detailed analysis later showed that the bulk of the replication proteins in CPMV-infected cells immunolocalized not to the vesicles, but to the adjacent electron-dense structures, suggesting that only a small part of the RNA1-encoded proteins contributes to the active replication complexes (Wellink et al. 1988). The accumulation of replication proteins into the electron structure is likely to depend on interactions between the 32 K and the 60 K precursor (VPg + 58 K) since deletion of the 32 K coding region, single amino acid substitution within the VPg, or the NTP binding of the 58 K protein all abolished formation of the dense bodies (Carette et al. 2001; Peters et al. 1994; van Bokhoven et al. 1993). The significance of these electron-dense structures for viral replication remains unclear as a mutation in the VPg was identified that prevented the formation of the cytopathic structures, without abolishing viral replication (Carette et al. 2001). They may simply represent deposition of inactive nonstructural protein or sites where overproduced proteins accumulate (Carette et al. 2001).

In an attempt to identify the membrane that contributes to CPMV replication, it was demonstrated through the use of transgenic *Nicotiana benthamiana* plants expressing ER- or Golgi-targeted GFP that CPMV infection causes a strong proliferation of ER membranes without affecting the structure of the Golgi stacks (Carette et al. 2000). ER modifications start at the cortical ER network and culminate with the apparition of a large region of densely packed ER membranes, often near the nucleus (Carette et al. 2000). Treatment with cytoskeleton inhibitor further revealed that intracellular trafficking of replication complexes to the large juxtanuclear structure occurs via association with the actin cytoskeleton and not the microtubular network (Carette et al. 2002a). The combined use of fluorescent in situ hybridization (FISH) and ER-GFP marker also showed that during the course of an infection, CPMV RNA colocalizes with the 110 K viral polymerase and other replication proteins and is always found in close association with proliferated ER membranes, supporting the view that ER membranes act as a source for the small membranous vesicles (Carette et al. 2002a). Not surprisingly, changes in ER morphology could be attributed to RNA1 alone. Thus, expression of RNA1-encoded proteins in insect cells, by using a baculovirus expression system, showed that 60 K, but not 110 K, is able to induce and associate with small membranous vesicles in the cytoplasm in these cells (van Bokhoven et al. 1992). More significantly, expression of individual viral proteins in *N. benthamiana* epi-

dermal cells using a viral vector revealed that both 32 K and 60 K, when fused to GFP, associate to membranes and induce rearrangement of the ER (Carette et al. 2002b). The alterations of the ER morphology resembled the proliferations that occur in CPMV-infected cells, although some differences could be observed. In particular, the GFP-32 K was present mainly in the cortical ER, whereas GFP-60 K was found mainly in the nuclear envelope, the plastidial membrane, and aggregates presumably derived from the ER. Other RNA1-encoded proteins, the 110 K polymerase and the *N*-terminal cleavage product 24 K proteinase, behaved as freely soluble proteins when expressed in isolation (Carette et al. 2002b). The localization signals that target the 32 K and 60 K, and probably also the replication complexes to ER membranes remain to be identified. It has been suggested that the three stretches of hydrophobic amino acids at the *C*-terminal end of the 32 K contribute to the membrane attachment (Carette et al. 2002b). Similarly, the 60 K contains two hydrophobic domains that are conserved among the comoviruses: an amphipathic helix at the *N* terminus (amino acids 45 to 61) and a 22-amino-acid stretch of hydrophobic residues (amino acids 544 to 565) at the *C* terminus immediately upstream of VPg (Carette et al. 2002b; Peters et al. 1992).

How ER membranes contribute to the formation the CPMV-induced vesicles is still a matter of debate. Immunogold labelling experiments on CPMV-infected tobacco leaf cells showed that they contained a relatively low amount of the luminal GFP-ER marker protein, indicating that luminal ER proteins are excluded (Carette et al. 2000) during generation of these vesicles. Additionally, it seems that the vesicle formation in CPMV-infected cells involves de novo membrane synthesis rather than a modification of preexisting membranes, as it was found that cerulenin, a fungal antibiotic which prevents de novo phospholipid biosynthesis and exerts its action by specifically inhibiting document β-ketoacyl-acyl carrier protein synthase (Moche et al. 1999), proved to be a strong inhibitor of CPMV RNA replication (Carette et al. 2000). Carette et al. (2002a) suggested that the vesicles may form as a result of the unfolded-protein response (UPR). This response can occur after various biochemical and physiological stimuli that affect ER homeostasis and impose stress to the ER, subsequently leading to accumulation of unfolded or misfolded proteins in the ER lumen (Shen et al. 2004). However, attempts to demonstrate the up-regulation of BiP that serves as a hallmark in the UPR remained unsuccessful (Carette et al. 2002a). Interestingly, a yeast two-hybrid search of an Arabidopsis cDNA library showed that the *C*-terminal domain of 60 K protein interacts with two vesicle-associated proteins (termed VAP27-1 and VAP27-2) that belong to the VAP33 family of SNARE-like proteins (Carette et al. 2002c). These host proteins also localized with the 60 K in CPMV-infected protoplasts. Carette et al. (2002c) suggested that VAP27-1 and VAP27-2 could play an important role in vesicular transport to or from the ER and may also act as a membrane anchor for the virus replication complex. Alternatively, interference of 60 K with VAP function could contribute

to the ER vesiculation process seen during infection. However, experimental evidence demonstrating the involvement of VAP27-1 and VAP27-2 in CPMV replication is still missing.

2.3.2
Grapevine Fanleaf Virus and *Tomato Ringspot Virus*

Grapevine fanleaf virus (GFLV) and *Tomato ringspot virus* (ToRSV) are very closely related nepoviruses belonging to the subgroups a and c, respectively. Their bipartite genome encodes two polyproteins P1 and P2 that are cleaved into final maturation products by the RNA1-encoded proteinases (Hans and Sanfaçon 1995; Margis et al. 1994). Similarly to CPMV, the polyprotein P1 contains the domains for proteins likely to be involved in replication, including a putative helicase (Hel), the VPg, the proteinase (Pro), and the RNA-dependent RNA polymerase (Pol) (Ritzenthaler et al. 1991; Rott et al. 1995). For GFLV, five final products referred to as 1A, 1B[Hel], 1C[VPg], 1D[Pro] and 1E[Pol] are generated by P1 processing, whereas ToRSV P1 is matured into six proteins in vitro due to the presence of an additional cleavage site within protein 1A (Andret-Link et al. 2004; Wang et al. 1999; Wang and Sanfaçon 2000). Similarly, the processing of polyprotein P2 of GFLV generates three proteins termed 2A, 2B, 2C, whereas an additional *N*-terminally located protein is produced from the maturation of polyprotein P2 from ToRSV (Carrier et al. 2001; Margis et al. 1993). Protein 2B and 2C are involved in virus movement, transmission by nematode vectors and encapsidation (Andret-Link et al. 2004; Laporte et al. 2003; Ritzenthaler et al. 1995; Wang and Sanfaçon 2000).

Initial studies on the cytopathology of nepovirus-infected cells have revealed the presence of diffuse inclusions, often near the nuclei, which consist of complex membranous structures, some of which form vesicles (Francki et al. 1985). Further studies performed in our laboratory using infectious full-length transcripts of GFLV showed that RNA1 is able to self-replicate in protoplasts and therefore encodes all the functions needed for its own replication (Viry et al. 1993). However, analysis of a set of deletion mutants in the P2-coding sequence demonstrated that protein 2A is necessary but not sufficient for RNA2 replication (Gaire et al. 1999). Remarkably, 2A protein, when fused to GFP, distributed to punctate structures throughout the cytoplasm when expressed alone, and accumulated in a juxtanuclear area together with other RNA1-encoded proteins upon infection. This perinuclear area (Fig. 1a), also termed the viral compartment and initially described by Franski et al. (1985), could be defined as the site of viral RNA replication where newly synthesized RNA molecules, double-stranded replicative intermediates, and RNA1-encoded protein accumulate (Gaire et al. 1999; Ritzenthaler et al. 2002). It was hypothesised from these results that protein 2A enables RNA2 replication through its association with the replication complex assembled from RNA1-encoded proteins (Gaire et al. 1999).
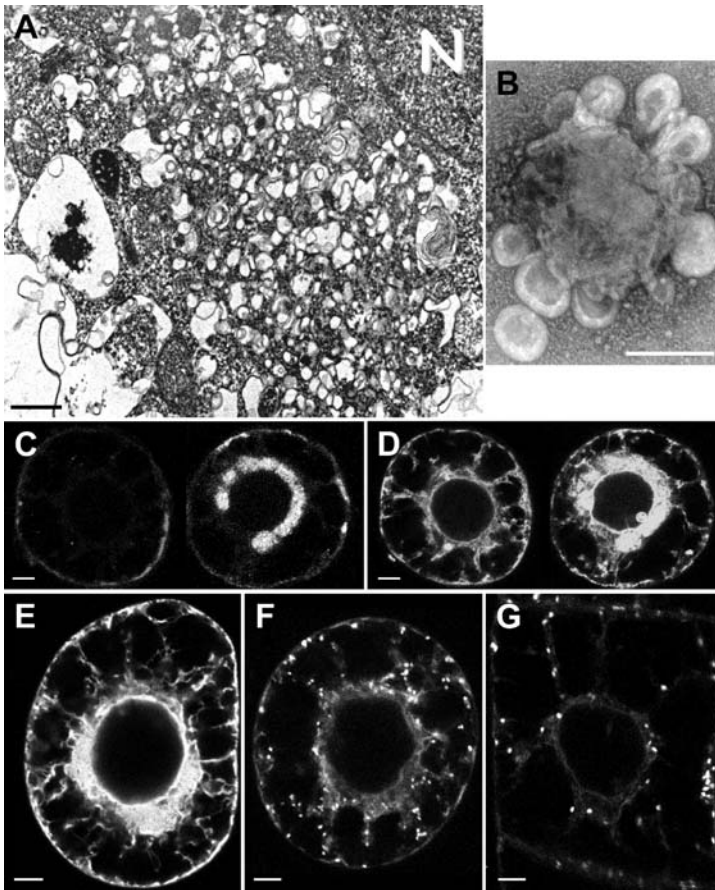
Fig. 1 Effects of GFLV replication on the endomembrane system of tobacco BY-2 cells. **a** Ultrastructural analysis of the viral compartment made of numerous vesicular structures and proliferated membranes. **b** GFLV "rosette" isolated from infected BY-2 cells after anti-VPg affinity-purification and negative-staining. **c** Detection of VPg in GFLV-infected protoplasts (*right cell* infected, *left cell* healthy). The protein specifically accumulates in the viral compartment where replication occurs. **d** The ER is visualised in the same cell as in **c**. Note that the ER is highly condensed in the viral compartment. **e** Another example of modified ER in GFLV-infected protoplast. Golgi distribution in **f** an infected and **g** a healthy cell. Note the regularly-shaped Golgi in healthy cells compared to the modified ones observed in infected cells. *Bars* = 5 μm except in **a** (1 μm) and **b** (0.5 μm)

How does the replication complex assemble, what type of intracellular membranes are used, and which are the viral and host proteins involved in the formation of the viral compartment, are questions that have been recently addressed for nepoviruses. By similarity with other picorna-like viruses, it was suggested that the 1B^{Hel} could act to anchor the replication

complex to membranes. In view of this hypothesis, stretches of hydrophobic residues were identified within the 1B^{Hel} from both GFLV and ToRSV (Rott et al. 1995; Ritzenthaler, unpublished results). Thus, the 1B^{Hel} from ToRSV, also named nucleoside triphosphate-binding (NTB) protein, contains a hydrophobic region at its *C* terminus consisting of two adjacent stretches of hydrophobic amino acids separated by a few amino acids. Sanfaçon and colleagues recently showed that 1B^{Hel}VPg associates with canine microsomal membranes in the absence of other viral proteins in vitro and with ER membranes in planta (Wang et al. 2004). Analysis performed on truncated proteins fused to GFP confirmed the presence of a functional transmembrane domain within the 60 amino acids at the *C* terminus of protein 1B and also revealed the presence of a putative amphipathic helix within the *N*-terminal 80 amino acids of 1B (Zhang et al. 2005). In agreement with the replication of ToRSV on ER-derived membranes (Han and Sanfaçon 2003), both domains of the fusion proteins were sufficient to promote partial association with ER membranes, supporting the hypothesis that the HEL–VPg polyprotein acts as a membrane anchor for the replication complex of ToRSV (Zhang et al. 2005).

Similary to ToRSV, in silico analyses have revealed the presence of four stretches of 21 hydrophobic residues, predicted to correspond to transmembrane domains (TMD), situated near the *N* terminus (amino acids 518–538 and 547–567) and almost at the *C* terminus of protein 1B^{Hel} of GFLV 1B (amino acids 1168–1188 and 1191–1211). Transient expression of individual RNA1-encoded proteins as GFP fusions in vivo confirmed that only protein 1B was targeted to the ER and often led to the apparition of a highly modified ER network (R. Elamawi & C. Ritzenthaler, unpublished results). These 1B^{Hel}-induced modifications are very reminiscent of those observed in vivo upon infection with GFLV (Fig. 1c–e) (Gaire et al. 1999; Ritzenthaler et al. 2002). Whether the 1B^{Hel} alone will also allow the formation of rosette-like structures as shown during viral infection (Fig. 1b) still needs to be determined. Nevertheless, they are perfectly in agreement with the replication of the virus on ER membranes (Ritzenthaler et al. 2002). Remarkably, transient expression of protein 1B^{Hel} in tobacco BY-2 cells also prevented the export of the resident Golgi protein α-1,2 mannosidase I (R. Elamawi & C. Ritzenthaler, unpublished results). Together with the facts that GFLV replication (i) is inhibited by brefeldin A (Ritzenthaler et al. 2002) and (ii) may affect the number and structure of Golgi stacks within infected cells (compare the distribution of Golgi stacks in Figs. 1f and 1g), it is suggested that the virus could exploit the cellular COPII transport pathway for the assembly of the replication complex and the biogenesis of the perinuclear viral compartment. Current research in our laboratory is aimed at testing this hypothesis.

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