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Brome Mosaic Virus (*Bromoviridae*)

Guijuan He, Virginia Tech, Blacksburg, VA, United States
Zhenlu Zhang, Shandong Agricultural University, Tai'an, China
Preethi Sathanantham, Virginia Tech, Blacksburg, VA, United States
Arturo Diaz, La Sierra University, Riverside, CA, United States
Xiaofeng Wang, Virginia Tech, Blacksburg, VA, United States

This is an update of X. Wang, P. Ahlquist, Brome Mosaic Virus, In Encyclopedia of Virology (Third Edition), edited by Brian W.J. Mahy and Marc H.V. Van Regenmortel, Elsevier Ltd., 2008, doi:10.1016/B978-012374410-4.00560-4.

Nomenclature

aa Amino acid(s)
CAT Chloramphenicol acetyltransferase
CP Coat protein or capsid protein
CPR5 Constitutive expresser of Pathogenesis-Related genes 5
ER Endoplasmic reticulum
ESCRT Components of the endosomal sorting complex required for transport
GFP Green fluorescent protein
kb Kilobases; the size of a ssDNA or ssRNA molecule
kDa Kilodaltons; the size of a protein
mRNA Messenger RNA
NCR Non-coding region
nm Nanometer(s)
nt Nucleotide(s)
ORF Open reading frame
RdRp RNA-dependent RNA polymerase
RNAi RNA interference
tRNA Transfer RNA
UTR Untranslated region
VIGS Virus-induced gene silencing

Glossary

Host factor Host proteins, lipids, and metabolites subverted by the virus at multiple stages of the life cycle required for successful viral infection.
Spherule Single-membrane vesicular invaginations of host membranes induced by positive-strand RNA viruses in which viral replication proteins, RNA templates, and specific host factors are enriched and viral genome replication takes place. Other names used to refer to RNA replication competent spherules are viral replication compartments, viral replication complexes, or viral replication mini-organelles.
Sub-genomic RNA A messenger RNA produced during viral replication to express downstream viral genes from a viral genomic RNA expressing multiple genes.
Tripartite RNA genome The viral genome is composed of three pieces of RNAs, which are all required for a successful systemic infection, may differ in length and in viral proteins encoded but may share similar sequences and/or structures of untranslated regions as well as 5′ and/or 3′ ends.

Introduction

Brome mosaic virus (BMV) is a positive-strand RNA virus that infects cereal plants, causing mosaic symptoms and stunting. BMV is the type member of the genus *Bromovirus* in the family *Bromoviridae*. BMV is also a representative member of the alphavirus-like super-family that include viruses infecting human, animal, and plants that share similar genome structures, gene expression strategies, and protein functions. BMV has been used as a model to study gene expression, RNA replication, virus-host interactions, recombination, and encapsidation by positive-strand RNA viruses. Major contributions have revealed insights and principles extending to many other viruses and to general cell biology. Among numerous advances, research in BMV defined the first ribosome binding sites in eukaryotic mRNAs, produced the first eukaryotic viral RNA-dependent RNA polymerase (RdRp) extract that was template specific, the first infectious transcripts from cloned cDNA, the first engineered RNA virus expression vector expressing foreign genes, the first definition of sub-genomic mRNA synthesis pathways and determinants, and the first demonstration of higher eukaryotic viral replication in yeast. BMV has contributed many insights into virion assembly, virus-host interactions, RNA recombination, and RNA replication, including parallels in the replication of positive-strand RNA, reverse-transcribing and dsRNA viruses.

Virion Structure and RNA Encapsidation

BMV forms non-enveloped virions ~28 nm in diameter. The outer capsid is composed of 180 copies of coat protein (CP) arranged with $T = 3$ quasi-icosahedral symmetry. Cryo-electron microscope reconstructions and subsequently x-ray crystallography showed that the BMV capsid structure is extremely similar to that of the related bromovirus Cowpea chlorotic mottle virus (CCMV). Intriguingly, some features of these capsids are dissimilar from other known isometric RNA virus capsids, but similar to capsids of the DNA-

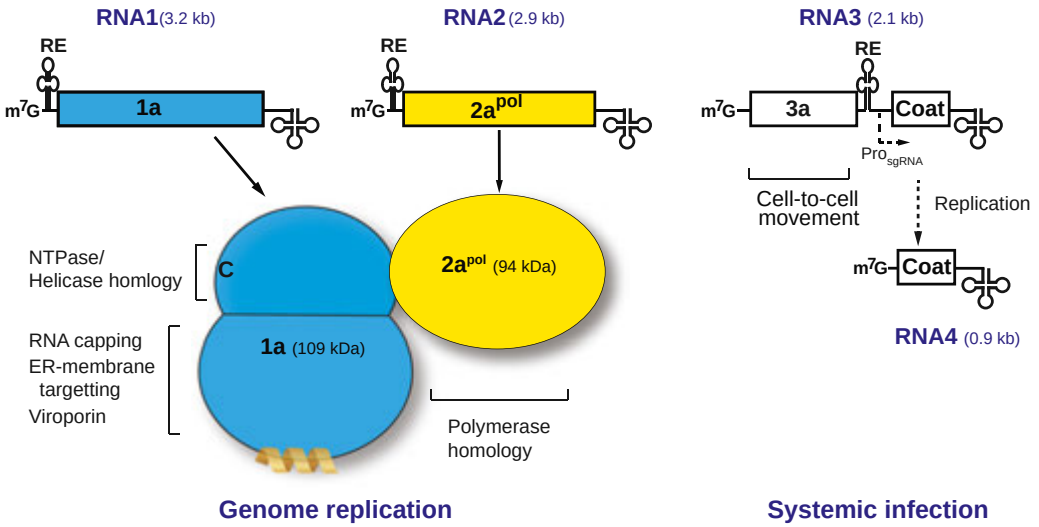

Fig. 1 Organization of the Brome mosaic virus (BMV) RNA genome. BMV has three genomic RNAs, RNA1, RNA2, and RNA3, and a sub-genomic mRNA, RNA4, encoded by the 3′ portion of RNA3 as shown. Each of these four BMV positive-strand RNAs bears a 5′ m^7G cap and 3′ tRNA-like structure (cloverleaf). The recruitment element (RE) in each viral genomic RNA and the promoter of sub-genomic RNA4 (Pro $_{sgRNA}$) in RNA3 are shown. Open boxes represent the genes for the viral proteins 1a and $2a^{pol}$, which are required for RNA replication, and 3a and coat protein, which are required for systemic movement in host plants. Some specific functions and features of each viral protein are listed, including the amphipathic α-helices at the N-terminus of 1a. The N-terminus (bottom half) and C-terminus (top half) of 1a are labeled as N and C, respectively. BMV 1a interacts with $2a^{pol}$ via the C-terminus of 1a and the N-terminus of $2a^{pol}$.

containing papovaviruses. These features include orientation of the core β-barrel nearly perpendicular to the capsid surface to form distinctively prominent hexameric and pentameric capsomeres, and linking of adjacent capsomere clusters by exchange of invading C-terminal arms. These shared features suggest that BMV CP and the CPs of polyoma- and papillomaviruses likely share a common ancestor. BMV virion RNA is arranged as an interior subshell inside the capsid, leaving a hollow virion center. The N-terminal 26 aa of the CP, which are highly basic and required for RNA packaging, interact with the RNA to neutralize its charge.

In addition to the predominant 180-subunit capsid, BMV CP can also assemble *in vivo* a 120-subunit capsid, composed of 60 CP dimers, first discovered in yeast and subsequently confirmed in infected plants. This assembly polymorphism is controlled *in vivo* by the RNA packaged, with BMV RNA2 packaged in 180-subunit capsids, while a small chimeric mRNA containing the CP open reading frame (ORF) as its only BMV sequence is packaged in 120-subunit capsids. Structural features shared by 120- and 180-subunit capsids imply that a common pentamer of CP dimers is an important intermediate in BMV virion assembly.

In vitro and *in vivo* encapsidation identified portions of the BMV 3a coding region whose deletion blocked RNA3 encapsidation and interfered with normal co-encapsidation of RNA3 and RNA4. Studies also implicated the 3′ tRNA-like structure of BMV RNAs as a facilitator of encapsidation in *cis* or *trans*, possibly by nucleating CP interactions into productive assemblies such as pentamers of dimers, and showed that BMV RNA replication promotes selective encapsidation of viral RNAs, possibly by inducing coupled synthesis of viral RNA and CP in close proximity.

In 1971, buoyant density gradients were used to separate BMV virions into three classes, all three have identical capsids but package different RNAs (Fig. 1). Heavy virions contain a single copy of RNA1 (3.2 kb), medium density virions contain one copy each of RNA3 (2.1 kb) and RNA4 (0.9 kb), and light virions contain a single copy of RNA2 (2.9 kb). The three classes of viral particles are indistinguishable by electron microscopy, however, distinct capsid-RNA contacts influence the stability of the virions, viral RNA release, and BMV infection. Mapping of the contacts made between CP and the viral RNAs showed that RNA1 contacts CP differently than RNA2 or RNA3/4. Virions containing RNA1 were more sensitive to protease digestion and treatment of virions with RNase A revealed that RNA1 was preferentially degraded, while RNA2 or RNA3/4 were more resistant to RNase A digestion. The more facile interaction between RNA1 and the capsid proteins could ensure earlier and higher expression of BMV 1a, which is responsible for the formation of the viral replication compartments (see below in the Section of "Properties and Functions of Viral Gene Products"). The interaction of the viral RNAs with capsid is also influenced by the phosphorylation state of the N-terminal arm of the capsid protein. Interestingly, virions produced from different plant hosts had different post-translational modifications that impacted the thermostability of virions as well as the capsid-RNA interactions.

Genome Organization

Genome Structure, Gene Expression and Sequences

Interest in characterizing the protein(s) encoded by each of the four BMV virion RNAs motivated early *in vitro* translation studies with purified BMV virion RNAs. RNAs 1, 2, and 4 were characterized as monocistronic while RNA3 is dicistronic. Although early

infectivity studies showed that RNA3 encodes CP, tryptic analysis showed that the principle translation product of RNA3 was unrelated to CP, while RNA4 served as an excellent template for CP. Moreover, when mixtures of all BMV RNAs were added to wheat germ extracts, increasing amounts of RNA4 inhibited translation of genomic RNAs 1–3. These results showed that RNA4 is a sub-genomic CP mRNA derived from RNA3, and implied an elegant system of gene regulation by translational competition: early in infection when viral RNA concentrations are low, all viral proteins including nonstructural RNA replication factors are translated, while after the virion RNAs are sufficiently amplified, CP is preferentially translated to encapsidate these RNAs.

By the early 1970s, RNA bacteriophage studies had provided valuable information on prokaryotic translation initiation sites, including the finding that the first AUG initiation codon usually was 100 or more nucleotides from the RNA 5′ end. In 1975, the first eukaryotic translation initiation site was characterized by isolating two fragments of BMV RNA4 that were efficiently bound by wheat germ ribosomes. RNA sequencing (a challenging, chromatography-based process at the time) revealed that the two fragments were 5′ terminal, overlapping, and encoded the first 4 and 14 aa of CP, respectively. The most distinguishing feature was that the initiating AUG codon for CP began only 10 nucleotides from the $m^7G^{5'}ppp^{5'}$Gp-capped RNA4 5′ end, presaging the now well-known mechanistic linkage between most eukaryotic translation initiation and 5′ mRNA caps.

The ~8.2 kb BMV genome sequence was completed in 1984. In good agreement with *in vitro* translation results, RNA1 and RNA2 encode single proteins 1a (109 kDa) and 2a (94 kDa), respectively, the 5′ half of RNA3 encodes protein 3a (35 kDa), and the 3′ half of RNA3 encodes for RNA4, the sub-genomic mRNA for CP (20 kDa) (**Fig. 1**). Later work discussed below showed that 3a is required for infection movement in plants. Comparisons with other emerging viral RNA and protein sequences quickly revealed that the BMV 1a and 2a proteins, already implicated in RNA replication by protoplast experiments, shared extensive amino acid sequence similarities with proteins encoded by a diverse set of plant and animal positive-strand RNA viruses. These similarities initially were recognized between BMV, Alfalfa mosaic virus, Tobacco mosaic virus and Sindbis virus, and subsequently were found to extend to many other viruses now grouped together as the alphavirus-like super-family. Similarities shared by these viruses include a polymerase domain in BMV 2a (hereafter $2a^{pol}$) and RNA capping and RNA helicase-like domains in 1a (**Fig. 1**).

Noncoding Regions, *Cis* Signals, and Sub-Genomic Promoter

The first region of BMV RNAs to attract attention as a potential regulatory element was the 3′ end. Synergistic work by multiple groups showed that the 3′ non-coding regions (NCR) of BMV RNAs are highly conserved, contain multifunctional domains that direct negative-strand RNA synthesis, contribute to RNA encapsidation, translation and stability, and possess multiple tRNA-like features and functions.

Limited early sequence data showed that BMV RNAs 1–4 share a tRNA-like CCA_{OH} 3′ end. All four BMV RNAs are selectively amino-acylated *in vitro* with tyrosine. Further studies showed that tyrosylated BMV RNAs interacted with translation elongation factor 1a and that BMV RNAs were tyrosylated *in vivo* during infection of barley protoplasts. BMV RNA 3′ ends were also found to interact with (ATP, CTP):tRNA nucleotidyl transferase, which can add 3′ CCA_{OH} ends to mature BMV RNAs or maintain incomplete BMV RNAs, thus acting as a primitive telomerase. Beginning in the 1970s, further sequencing, enzymatic structure probing, and three-dimensional computer modeling showed that the 3′ ~200 nt of all BMV RNAs were strongly conserved and folded into an extended, tRNA-like structure with at least two alternate forms that differed in pairing nucleotides near the 3′ with local or distal partners. Similarly conserved, highly structured 3′ regions with related alternate forms were also found in other members of the family *Bromoviridae*.

In vitro and *in vivo* studies using RNA fragments and mutations showed that sequences within the 3′-terminal tRNA-like structure direct negative-strand RNA initiation for RNA replication. Recent results have also implicated the tRNA-like sequence in translation and encapsidation. Involvement in all of these functions led to early and continuing suggestions that the 3′ region mediates co-regulation of the varied uses of BMV positive-strand RNAs to minimize conflicts between multiple essential processes.

A second class of elements combining tRNA-like features, replication signals and possible interaction with translation are the BMV template recruitment element (**Figs. 1** and **2**). Deletion analysis revealed that, in addition to 3′ and 5′ sequences required for negative- and positive-strand RNA initiation, BMV RNA3 replication *in vivo* requires a segment of ~150 nt in the 5′ half of the intergenic non-coding region between the 3a and CP genes (**Fig. 1**). Subsequent studies showed that this region is required for a step prior to negative-strand RNA synthesis, and is necessary and sufficient for 1a to recognize and recruit an RNA to a membrane-associated, translationally inaccessible, nuclease-resistant state that appears to be the interior of the replication compartments, also known as spherules (see the Section "BMV 1a and Viral Replication Compartments"). Structure probing studies show that this intergenic RNA3 sequence folds into a long, bulged stem-loop, which presents at its apex the invariant sequence and structure of tRNA TΨC stem-loops. In plant and yeast cells, the appropriate BMV residues in this consensus are modified to T and Ψ, showing that, like the 3′ end, this sequence interacts *in vivo* with tRNA-specific enzymes. Moreover, any mutations to this TΨC stem-loop mimicry abolish 1a-mediated template recruitment. Similar stem-loops with apical TΨC stem-loop regions are found at the extreme 5′ ends of BMV RNA1 and RNA2, where they similarly direct 1a-mediated template recruitment (**Fig. 1**).

A common approach to express downstream gene(s) in a viral genomic RNA encoding more than one gene is to produce sgRNA(s) efficiently. *In vivo* and *in vitro* analyses of the BMV sub-genomic mRNA promoter have complemented well to reveal a core promoter within the 20 nt immediately upstream of the RNA4 start site, which directs low level but accurate initiation of sub-genomic mRNA. *In vivo*, the activity of this core promoter is greatly enhanced by upstream sequences that include an oligo(A) tract of variable, ~16–22 nt in length in the viral population as well as upstream, partially conserved repeats of core promoter

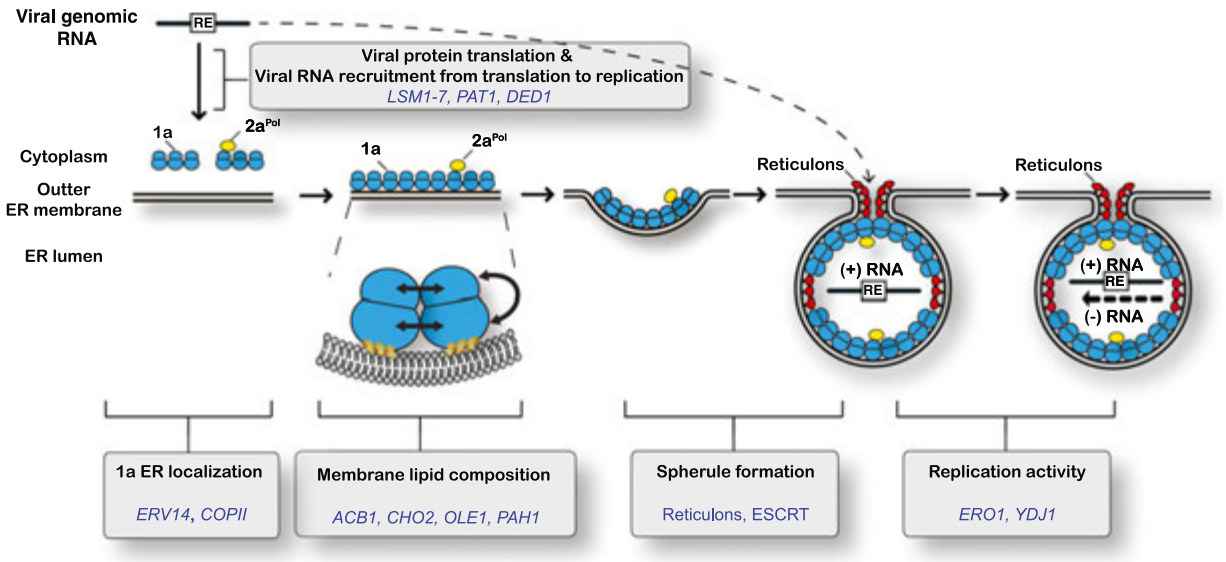

Fig. 2 Host factors required for the formation of functional BMV spherules. Host genes are involved in every step of BMV genome replication, including viral protein synthesis, recruitment of the viral RNA from ribosomes to the interior of spherules, targeting 1a to the nuclear ER membrane, membrane lipid composition and 1a-membrane interactions, spherule formation, and activation of replication activity. Host genes and pathways whose involvement in viral replication has been well characterized are listed.

sequences. The important role of the oligo(A) tract suggests that while negative-strand RNA can serve as a sub-genomic mRNA template *in vitro*, the natural *in vivo* template might be a dsRNA within which the oligo(A) provides a melting site to facilitate internal initiation.

As one important aspect of *cis* signals in BMV replication, minimal core promoters were defined and dissected for the synthesis of negative-strand, positive-strand, and sub-genomic RNA, using a variety of approaches. Among other results, mutational studies imply that the BMV RdRp-promoter interaction has the characteristics of an induced fit, wherein the RdRp has some tolerance to adjust its binding to a range of promoter variants as long as some key sequence features remain. This model potentially reconciles the specificity of BMV RNA synthesis with the ability of the RdRp to synthesize different forms of viral RNA from separate promoters with distinct primary sequences and secondary structures. DNA or DNA/RNA hybrid templates containing the key BMV promoter sequences can be recognized *in vitro* by BMV RdRp extracts and copied into RNA. Although the efficiency of copying DNA templates is ~15-fold lower than for BMV RNA templates, these results have significant potential implications for virus evolution.

Replication and Propagation

In Vitro and *In Vivo* Replication Studies

Positive-strand RNA viruses, like BMV, replicate their genomes in a completely RNA-dependent manner, producing a negative-strand RNA replication intermediate for each genomic RNA. Studies on BMV RNA replication were greatly advanced by the development and subsequent use of such tools as *in vitro* RdRp extracts and cultured plant protoplasts. In 1979, a virus-specific *in vitro* RdRp extract was developed from BMV-infected barley leaves that synthesized full-length negative-strand RNAs using added BMV virion RNAs as templates. This was the first eukaryotic *in vitro* RdRp preparation exhibiting a high level of template specificity, with other viral RNAs having less than 20% of the template activity of BMV RNAs. As noted in part below, this and similar BMV *in vitro* systems have been utilized to make many advances regarding promoters for positive- and negative-strand RNA synthesis, initiation mechanisms, and other issues.

In parallel to *in vitro* systems, cultured plant protoplasts provided a valuable substrate for *in vivo* replication studies due to their ability to be infected or transfected with nearly 100% efficiency with virions, virion RNAs, or *in vitro* transcripts from cloned viral cDNAs. For BMV, barley protoplast systems have allowed examining all aspects of BMV RNA replication, sub-genomic RNA synthesis, progeny RNA encapsidation, and the like. The highly synchronized infections obtained also allow detailed kinetic studies.

In the early 1990s, it was shown that BMV also would direct RNA replication, sub-genomic RNA synthesis, selective viral RNA encapsidation, RNA recombination and the like in the well-studied yeast, *Saccharomyces cerevisiae*. This host provides some of the same advantages as plant protoplasts together with rapid growth, a particularly small genome, well-characterized gene functions and cell biology, and powerful classical and molecular genetic tools including genome-wide arrays of isogenic yeast strains with each gene systematically modified by deletion, GFP-tagging, etc. Yeast cells expressing BMV RNA replication proteins 1a and $2a^{pol}$ support the

replication of BMV genomic RNAs introduced by transfection or DNA-dependent transcription from plasmids or chromosomally integrated expression cassettes, duplicating all major features of replication in plant cells. To facilitate yeast genetic studies, BMV RNA replicons can express selectable or screenable markers and are transmitted to yeast daughter cells during cell division with 85%–90% efficiency, rivaling the transmission of yeast DNA plasmids. Some key findings identified and characterized in the BMV-yeast system have been confirmed in plants, including the enrichment of phosphatidylcholine at the viral replication sites in BMV-infected barley cells and an inhibition of BMV replication in BMV-infected *Nicotiana benthamiana* by overexpressing host gene *PAH1* (Phosphatidic Acid Hydrolase 1) or a dominant-negative mutant of host *SNF7* (Sucrose Non Fermenting 7)(see below in the Section "Host Factors in RNA Replication"). However, it should be noted that there are some differences in the replication features between plant and yeast cells as further discussed in the Section "BMV 1a and Viral Replication Compartments".

Sub-Genomic mRNA Synthesis

Early observations that the nature of CP in BMV infections is dictated by RNA3 rather than RNA4, and that RNA4 was regenerated when omitted from BMV inoculum, were partially explained when sequencing revealed that RNA4 was encoded by the 3′ portion of RNA3. Nevertheless, whether RNA4 was produced from RNA3 by cleavage or any of several possible RNA synthesis pathways remained uncertain. In 1984, it was shown that a BMV RdRp extract produced sub-genomic RNA4 *in vitro* when supplied with negative-strand RNA3 templates, and that the product RNA4 could be labeled by γ-^{32}P-GTP incorporation, demonstrating *de novo* initiation. This first elucidation of a pathway for sub-genomic mRNA synthesis appears to provide a meaningful precedent for similar sub-genomic mRNA synthesis by many positive-strand RNA viruses, and an important foundation for understanding the diversity of alternate mechanisms that has begun to emerge with the demonstration of distinctly different sub-genomic mRNA synthesis pathways used by coronaviruses, nodaviruses and some other positive-strand RNA viruses.

RNA Recombination

In 1986, BMV was used to provide an early demonstration of RNA recombination in a plant virus. Subsequent work demonstrated many forms and features of inter- and intra-molecular, homologous and non-homologous RNA recombination in BMV. Mutations in BMV RNA replication proteins 1a and 2a^{pol} could alter the frequencies and distributions of crossover sites, implying that at least a significant portion of such recombination was a byproduct of RNA replication, as by template switching. These and other results show that RNA recombination is a major force for repairing BMV genomes damaged by the high mutation rates of viral RNA replication and other events, thereby contributing to BMV survival and adaptability. Subsequently, it was further demonstrated that the recruitment of parental RNAs to a shared replication compartment is a limiting step in intermolecular RNA recombination. These results support a model in which BMV genomic RNAs are individually recruited into replication compartments, and intermolecular recombinants arise only in replication compartments receiving multiple viral RNAs.

Properties and Functions of Viral Gene Products

BMV RNA3 encodes two proteins, 3a and CP, that are dispensable for RNA replication but are required for systemic spread (**Fig. 1**). Disruption of the 3a gene blocks cell-to-cell movement, limiting infection to individual, directly inoculated cells. The 3a protein shares multiple properties with cell-to-cell movement proteins of other viruses, including cooperative binding to single-stranded RNA, localization to the plasmodesmatal connections, and induction of virion-containing tubules from the surface of BMV-infected protoplasts. Disruption of the CP gene stops virus spread to uninoculated leaves. Whether local cell-to-cell spread occurs in the absence of CP depends on several factors including the 3a allele and the host plant.

Protoplast studies showed that BMV RNA replication and sub-genomic RNA4 synthesis require 1a and 2a^{pol} but not 3a or CP. The conserved central domain of 2a^{pol} shows similarity to RdRps encoded by picornaviruses and many other RNA viruses. The N-terminal 1a domain is related to alphavirus protein nsp1 and contains m^{7}G methyltransferase and m^{7}GMP covalent binding activities required for capping viral RNA *in vivo*. The C-terminal 1a domain has sequence similarity to super-family I NTPase/helicases and NTPase activity that is required for RNA template recruitment and RNA synthesis. The N- and C-proximal domains are separated by a short proline-rich sequence that may serve as a flexible spacer. As shown in several systems, the C-terminus of BMV 1a interacts with the N-terminus of 2a^{pol} and this interaction is required for the recruitment of 2a^{pol} into the viral replication compartments.

BMV 1a and Viral Replication Compartments

In yeast cells replicating BMV RNAs, the outer perinuclear endoplasmic reticulum (ER) membrane is invaginated towards the lumen to form numerous 50–80 nm spherular invaginations, which are referred to as spherules. Similar membrane invaginations are seen in plant cells infected by viruses from *Bormoviridae*, *Tombusviridae*, and *Tymoviridae* families, and in animal cells infected by viruses in and beyond the alphavirus-like super-family. However, in BMV-infected *N. benthamiana* cells, there are three types of polymorphic vesicles that are associated with cytoplasmic ER membranes but are absent from the perinuclear ER region. Type 1 vesicles are ~66 nm in diameter with some spherule-like structures that remain connected to the ER through neck-like structures and as such, type 1 vesicles resemble those formed in yeast cells. Type 2 vesicles are ~359 nm in diameter and likely are induced

by CP. Type 3 vesicles are most probably formed by fusing type 1 and type 2 vesicles. Some possible reasons for the differences in BMV-induced membrane rearrangements between yeast and *N. benthamiana* are likely due to the organization and lipid composition of the ER membranes in yeast and *N. benthamiana* or possible variation in relative protein expression levels and replication efficiency in each system.

Using immunogold-labeling and electron microscopy in yeast, replication proteins 1a, $2a^{pol}$, and nascent BMV RNAs were found to be localized to the spherule interiors, which remain connected to the cytoplasm by a narrow neck that likely allows for the import of ribonucleotides required for replication and the export of offspring RNAs (Fig. 2). Immunogold and biochemical studies show that each spherule contains one or a few hundred 1a proteins and ~25-fold fewer $2a^{pol}$ proteins along with specific host proteins (Fig. 2 and see the Section on "Host Factors in RNA Replication" below). The structure, assembly, and operation of these spherular replication complexes have functional and perhaps evolutionary links to the replicative cores of retrovirus and dsRNA virus virions, which sequester viral RNA replication templates and their polymerases in a protein shell.

BMV 1a serves as the primary organizer to form active replication compartments: it invaginates the outer ER membranes into the ER lumen to form spherules, recruits $2a^{pol}$ via 1a-$2a^{pol}$ interactions, recruits RNA templates into the interior of preformed spherules by recognizing the *cis*-element RE present only in viral genomic RNAs, and also interacts with and recruits specific host factors to the site of viral replication (Fig. 2, see below in the Section of "Host Factors in RNA Replication"). Spherular invaginations are formed when 1a is expressed in yeast cells in the absence of any other viral proteins or RNA templates. Localization of 1a to the perinuclear ER membrane requires an N-terminal amphipathic α-helix, termed Helix A (amino acids 392–407). NMR analysis shows that three leucine residues are deeply immersed into SDS micelles that mimicked a lipid bilayer. Substitutions of the three leucine residues with alanine prevent 1a from associating with the perinuclear ER membrane, and block spherule formation. In addition, Helix A plays critical roles in regulating the size and frequency of spherules as several individual substitutions in Helix A lead to similar viral replication defects including the formation of spherules that are more numerous in number but smaller in size.

A second amphipathic α-helix, Helix B (amino acids 415–433), plays a crucial role in 1a's function as a viroporin to permeabilize ER membranes to release oxidizing potential, which requires host thiol oxidase Ero1p (ER Oxidation 1), from the ER lumen to promote the formation of disulfide-bound 1a multimers, 1a's RNA capping activity, and active genome replication (See Ero1p in the Section "Host Genes Required for Viral Replication Activity").

Host Factors in RNA Replication

As for many other viruses, the small size of BMV genome relative to the complexity of BMV replication suggests that many, if not most, steps in BMV RNA replication involve contributions from host factors. Indeed, genetic and systematic genome-wide screens have identified more than 100 genes that, when deleted, inhibited or enhanced BMV RNA replication by at least 3-fold. The mechanisms by which BMV usurps host factors to complete the various steps of its life cycle are illustrated in Fig. 2.

Host Genes That Regulate Lipid Metabolism and Membrane Composition

Since positive-strand RNA viruses replicate on intracellular membranes, cellular lipid synthesis and appropriate lipid composition are crucial for their replication. BMV replication increases total fatty acids per yeast cells by 33%. Mutation or deletion of the *OLE1* (OLEic acid requiring 1) gene, which encodes for the Δ9 fatty acid desaturase that converts saturated fatty acids to unsaturated fatty acids (UFAs), revealed that BMV RNA replication requires UFA levels five times higher than those required for normal yeast growth. Moreover, the gene products of *DOA4* (Degradation Of Alpha 4), *SPT23* (SuPpressor of Ty 23), and *MGA2* (Multicopy suppressor of GAm 2), which are involved in regulating the expression of lipid metabolism genes, including *OLE1*, are also critical for proper BMV RNA replication.

As major structural constituents of membranes, phospholipids are particularly important for the formation and activation of viral spherules. Phosphatidic acid is a precursor for both phospholipids and storage lipids. phosphatidic acid can be used to make phospholipids for membrane synthesis during active cell growth, or it can be converted to the storage lipid precursor diacylglycerol by Pah1p when cell growth reaches the stationary stage. Deleting *PAH1* led to a 2-fold increase in total phospholipids levels, resulting in twice as many spherules and a 3-fold increase in viral replication. Further studies showed that BMV replication leads to a 25% increase in the levels of phosphatidylcholine, which is predominantly enriched at the sites of BMV replication in both yeast and barley cells. At least in yeast, BMV 1a interacts with and recruits Cho2p (Choline requiring 2), an enzyme involved in the conversion of phosphatidylethanolamine into phosphatidylcholine, to the viral replication sites to promote phosphatidylcholine synthesis, and facilitates BMV replication. In addition, deleting *ACB1* (Acyl-CoA-Binding 1), another gene that regulates general lipid transport and synthesis, reduced BMV replication by up to 30-fold and it led to the formation of smaller but more abundant spherules compared to those in wild-type cells.

Host Genes That Induce/Stabilize Spherules

Host factors are also essential to form and/or maintain BMV-induced spherules. The reticulons are a family of proteins that stabilize highly curved tubules in the peripheral ER. Through an interaction with BMV 1a, the reticulons are relocalized from the

peripheral ER into the interior of the spherules. The reticulons were shown to stabilize the membrane curvature in the neck of the spherules and to also regulate the size and number of spherules. Components of the endosomal sorting complex required for transport (ESCRT) are also essential for spherule formation. One group of ESCRT genes regulate BMV RNA replication and the frequency of spherule formation while a second group of genes regulate RNA replication independent of spherule formation. In particular, deleting *SNF7* significantly reduced viral replication and abolished detectable spherule formation. The current model suggests that BMV 1a initiates the invagination of the perinuclear ER, followed by recruitment of Snf7p and other ESCRT components to constrict the wide membrane rim, allowing the formation of the spherule body and neck. The reticulons in turn stabilize the curvature in the neck and regulate the size of the spherules.

Host Genes That Regulate Translation of Viral Proteins and 1a Targeting

Until recently the mechanism by which BMV 1a is targeted to the perinuclear ER was unclear. It was shown that Erv14p (ER-vesicle protein of 14 kDa), a cargo receptor in COPII (COat Protein complex II) coated vesicles, not only interacts with 1a but helps localize it to the perinuclear ER. Deletion of *ERV14* disrupts the proper distribution of 1a and significantly inhibits BMV RNA replication.

Host ribosomes recognize the genomes of positive-strand RNA viruses to initiate translation of the viral proteins. Once sufficient viral replication proteins accumulate, viral genomic RNAs are recruited out of the cellular translational machinery and into spherules to serve as templates for viral replication. For BMV, control of these two functions is tightly regulated by the host complex consisting of Pat1p (Protein associated with topoisomerase 1) and 7 members of the Lsm (Like SM) family: Lsm1p to Lsm7p. Lsm1–7-Pat1 complex binds to the 5′ and 3′ untranslated regions (UTR) of BMV RNAs to circularize the RNAs to facilitate translation. When BMV 1a is present, 1a binds to the viral RNA and interacts with the Lsm1–7-Pat1 complex, likely disrupting Lsm1–7-Pat1 mediated 3′-5′ circularization, thus repressing translation to allow recruitment of the viral RNA to the spherules. Moreover, classical yeast genetic approaches identified *DED1* (Defines Essential Domain 1), an RNA helicase required for translation of all yeast mRNAs, to regulate translation of $2a^{pol}$ relative to other replication factors.

Host Genes Required for Viral Replication Activity

Host factors are also essential to activate the viral replication proteins after spherules have been assembled. Ydj1p (Yeast dnaJ 1), a J-protein co-chaperone of Hsp70 and Hsp90, is required to activate the BMV RNA replication complexes. In yeast with a mutation in the *YDJ1* gene, negative-strand RNA synthesis is inhibited even though 1a, $2a^{pol}$, and RNA3 were recruited to ER membranes. The results suggest that the Ydj1p chaperone likely modulates $2a^{Pol}$ folding or assembly into the complex. BMV RNA replication also depends on the ER luminal thiol oxidase Ero1p. 1a can act as a viroporin by permeabilizing ER membranes to release the Ero1p-generated oxidizing potential from the ER lumen to activate 1a's capping function via a new state of 1a multimerization involving oxidized linkages and potential conformational changes within the protein. 1a mutants lacking the viroporin function were able to recruit and stabilize positive-strand RNA3 templates but did not support significant RNA replication.

Transmission and Host Range

BMV transmission in plants is not well characterized. BMV can be transmitted in the field by human activities involved in agricultural production, such as machinery trampling. In laboratory settings, plants can be easily infected by mechanical inoculation using sap prepared from BMV-infected tissues, purified virions or virion RNAs, or *in vitro* transcripts from cloned viral cDNAs. Vectors transmitting BMV in the field are unknown. Spotted cucumber beetles and nematodes in the genus *Xiphinema* have been reported to be able to transmit BMV in the laboratory.

BMV replicates and encapsidates its RNAs in directly inoculated cells from a wide variety of plants, but has a fairly restricted host range for systemic infection of whole plants beyond monocotyledonous plants. The effective host range for BMV infection thus appears to be determined at the level of initiating or sustaining infection spread from the sites of primary infection.

Exchanging genomic RNAs, individual genes and gene segments among BMV strains and between BMV and other viruses shows that adaptation for infection spread in particular host plants depends not only on 3a and CP but also on features of RNA1 and RNA2. Host adaptation of 3a generally exerts the predominant effects on infection spread, and only a few amino acid changes in 3a are required to extend BMV host range from monocotyledonous to dicotyledonous plants. However, changes modulating the efficiency of systemic spread also map to 1a and $2a^{pol}$. Such changes may alter systemic spread through host-specific effects on RNA replication, as by influencing the ability of the virus to replicate and spread faster than host defense responses. Alternatively, 1a and $2a^{pol}$ may possess additional functions, as for some C-terminal $2a^{pol}$ sequences that are dispensable for RNA replication but required for efficient systemic infection.

BMV primarily infects monocotyledonous cereal crops such as barley, maize, rice, wheat, and sorghum, as well as model plants *Brachypodium distachyon* and *Setaria viridis*. While *N. benthamiana* is a dicotyledonous host for BMV, *Arabidopsis thaliana* is generally considered as a non-host even though it allows BMV to infect in rare conditions. Screening numerous accessions and mutants of *Arabidopsis*, CPR5 (Constitutive expresser of Pathogenesis-Related genes 5) was identified as a key restriction factor for BMV systemic infection in *Arabidopsis*. Although *Arabidopsis cpr5* mutant plants show an enhanced resistance to multiple bacterial and oomycete

pathogens and several positive-strand RNA viruses compared to wt plants, they all support efficient systemic BMV infection. BMV RNA replication increased at the single-cell level by 6-fold in protoplasts prepared from *cpr5* mutant plants compared to wt. The allowance for BMV replication in various *cpr5* mutants is independent of host defense-related salicylic acid, jasmonic acid, and ethylene. *Arabidopsis* CPR5 is a component of nuclear pore complexes and serves as a selective barrier to restrict nuclear access of signaling cargos. However, the mechanisms whereby *Arabidopsis cpr5* mutants allow BMV to replicate efficiently in single cells and promote systematic infection are unclear. The role of CPR5 might be direct, or it could indirectly regulate specific downstream effectors that might be responsible for the non-host resistance in *Arabidopsis* and possibly other dicotyledonous plants.

BMV Application in Biotechnology

BMV was used to produce the first infectious transcripts from cloned RNA virus cDNA in 1984. Specially designed BMV cDNA clones were transcribed to produce capped *in vitro* transcripts of genomic RNAs 1–3, each with the natural viral RNA 5′ end and only a few extra nucleotides at the 3′ end. Mixtures of all three BMV RNA transcripts, but not their parent cDNA clones, were infectious to barley plants, a natural BMV host.

This ability to engineer the expression of infectious transcripts provided a means to manipulate the viral RNA genome at the cDNA level using recombinant DNA technology, which has subsequently proved applicable to many other RNA viruses. In one of the first applications of these new reverse genetics approaches, it was successfully demonstrated that foreign genes could be inserted into the viral genome while retaining the ability to replicate and express genes. Using a transcribable BMV RNA3 cDNA clone, the CP gene was replaced with the bacterial reporter gene chloramphenicol acetyltransferase (CAT). When *in vitro* transcribed and inoculated onto barley protoplasts with RNA1 and 2 transcripts, this RNA3 derivative was replicated and produced CAT activity at higher levels than previously achieved by DNA-based transformation. This first demonstration that RNA viruses can be engineered at the cDNA level showed that the viral RNA genome functions in a sufficiently flexible and modular fashion to tolerate even large changes such as whole gene replacements without substantial optimization, which has significant implications for virus evolution, biotechnology research and applications such as additional gene expression vectors.

RNA interference (RNAi) or gene silencing has been widely employed as a powerful tool to reveal gene functions in plants by degrading gene-specific transcripts. Virus-induced gene silencing (VIGS) knocks down gene expression of targeted cellular genes during virus infection, without a need to make transgenic plants. A BMV strain (fescue strain) isolated from tall fescue has been engineered as a VIGS vector by inserting a fragment of the target genes into the 3′ UTR of RNA3. These BMV vectors have been successfully used in barley, maize, rice, and sorghum to understand gene function otherwise hard to achieve.

Future Perspectives

Through a variety of intrinsic features and the work of many investigators, research on BMV not only has advanced understanding of bromoviruses, but also has contributed significantly to general virology and molecular biology. Some of the challenges for the future include improved definition and analysis of distinct substeps in viral RNA synthesis including initiation, elongation, termination and capping; better characterization at molecular, cellular and tissue levels of the pathways and mechanisms involved in infection spread and the interplay of virus-directed processes and host defenses; and improved understanding of the linkages between different infection processes including regulated gene expression, RNA replication, encapsidation and spread. Since the mechanism of action has been characterized for several host genes involved in BMV replication, the manipulation of host genes via knockout, knockdown, targeted substitutions, or overexpression could potentially be used as a way to achieve viral control in crop plants.

See also: Alfalfa Mosaic Virus (*Bromoviridae*). Bromoviruses (*Bromoviridae*). Cucumber Mosaic Virus (*Bromoviridae*). Ilarviruses (*Bromoviridae*)

Further Reading

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