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MINIREVIEW

Insights into the Single-Cell Reproduction Cycle of Members of the Family *Bromoviridae*: Lessons from the Use of Protoplast Systems[▽]

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The development of single-cell protoplast systems is certainly one of the milestones in the history of plant virology, allowing for the analysis of viral molecular processes at the cellular level. The use of plant cell-based systems in the study of the *Bromoviridae* family of multipartite single-stranded plant RNA viruses facilitated the discovery and dissection of viral processes engaged in the single-cell reproduction cycle: replication, transcription, protein synthesis, movement, virion assembly, and RNA recombination. This review summarizes the application of protoplast systems to the analysis of consecutive steps of the bromovirus life cycle, emphasizing their temporal and spatial patterns during virus multiplication.

Our knowledge of viral infection at the individual cell level determines our understanding of the infection at the entire plant body level. The idea of applying single-cell systems in virology was introduced with the first attempts to transfect *Escherichia coli* cells with a T4 anti-*E. coli* bacteriophage (27). E. C. Cocking was the first (1960) to enzymatically isolate plant cells (19), which were then infected with *Tobacco mosaic tobamovirus* (TMV) (20). The ensuing pioneer work showed that the infection was synchronous and that the uptake of viral particles/RNAs by protoplasts was efficient enough to support virus replication (5, 118). The first viral infection of protoplasts involved the use of poly-L-ornithine (5), but the trials that followed exploited the fusogenic polymers (24), liposomes (28), or electroporation (78, 129). By 1980, these procedures had been successfully employed to transfect protoplasts from more than six species of plants, including various members of the *Bromoviridae* family, e.g., *Brome mosaic bromovirus* (BMV; 80), *Cucumber mosaic cucumovirus* (CMV; 69), and *Cowpea chlorotic mottle virus* (CCMV; 130). The first attempts were based on the transfection of plant cells with whole-virus particles (104). Later on, viral RNA and its chemical modifications were used as the inoculum (62). Further advances in nucleic acid technology, especially the accessibility of infectious transcripts, have broadened the application of protoplast systems to the study of *Bromoviridae*.

The *Bromoviridae* constitute one of the most important families of plant RNA viruses. They are distributed worldwide, they infect an extensive range of hosts, and some of them (e.g., CMV and *Broad bean mottle bromovirus*) are responsible for major crop epidemics (111, 63). The family consists of five genera named after their most representative members: *Alfamovirus*, *Bromovirus*, *Cucumovirus*, *Ilarvirus*, and *Oleavirus*. All these viruses possess tripartite, single-stranded, positive-sense RNA genomes (Fig. 1). RNA1 and RNA2 encode the RNA-dependent RNA polymerase (RdRp) proteins 1a and 2a, respectively. The dicistronic RNA3 encodes the movement protein (MP) and coat protein (CP). The latter is translated from subgenomic RNA 4 (sgRNA4). Many of the family members, such as CMV, BMV, and *Alfalfa mosaic alfamovirus* (AMV), represent excellent model systems to shed new light on viral molecular processes.

PROTOPLAST VERSUS OTHER SYSTEMS

Before the introduction of the protoplast system, tissue cultures were commonly used to study plant viruses (73). Their advantages include the stability of the cells, the ease with which the cultures can be handled, and the availability of the system without season-dependent physiological variations (110). However, the heterogeneous sizes of the plant cells, the asynchrony of cell growth, and the presence of a rigid cell wall often hampered efficient viral transfections. Protoplasts permit one to solve these problems by representing a homogeneous population of mesophyll cells that assures synchronous and well-established viral infection. The most important advantage of this system is that the use of plant cells permits one-step virus growth experiments. It is only with an experiment of this type that the successive stages of virus replication can be identified, monitored, and analyzed. Nevertheless, during experiments, plant cells are maintained in an artificial milieu which differs from the natural environment of the leaf cells. Such changes in the environment may induce changes in the isolated cells and therefore influence virus accumulation. Thus, the possible discrepancies between virus behavior in the cell system and in plant tissue have to be considered. To verify these inconsistencies, it is useful to supplement cell-based studies with additional experiments using other well-developed systems.

The yeast system represents the optional cell-based approach, which was shown, for instance, to recapitulate all known features of BMV replication and gene expression in its natural plant host cells, including the formation of progeny virions (95). Yeast provides multiple advantages for studying

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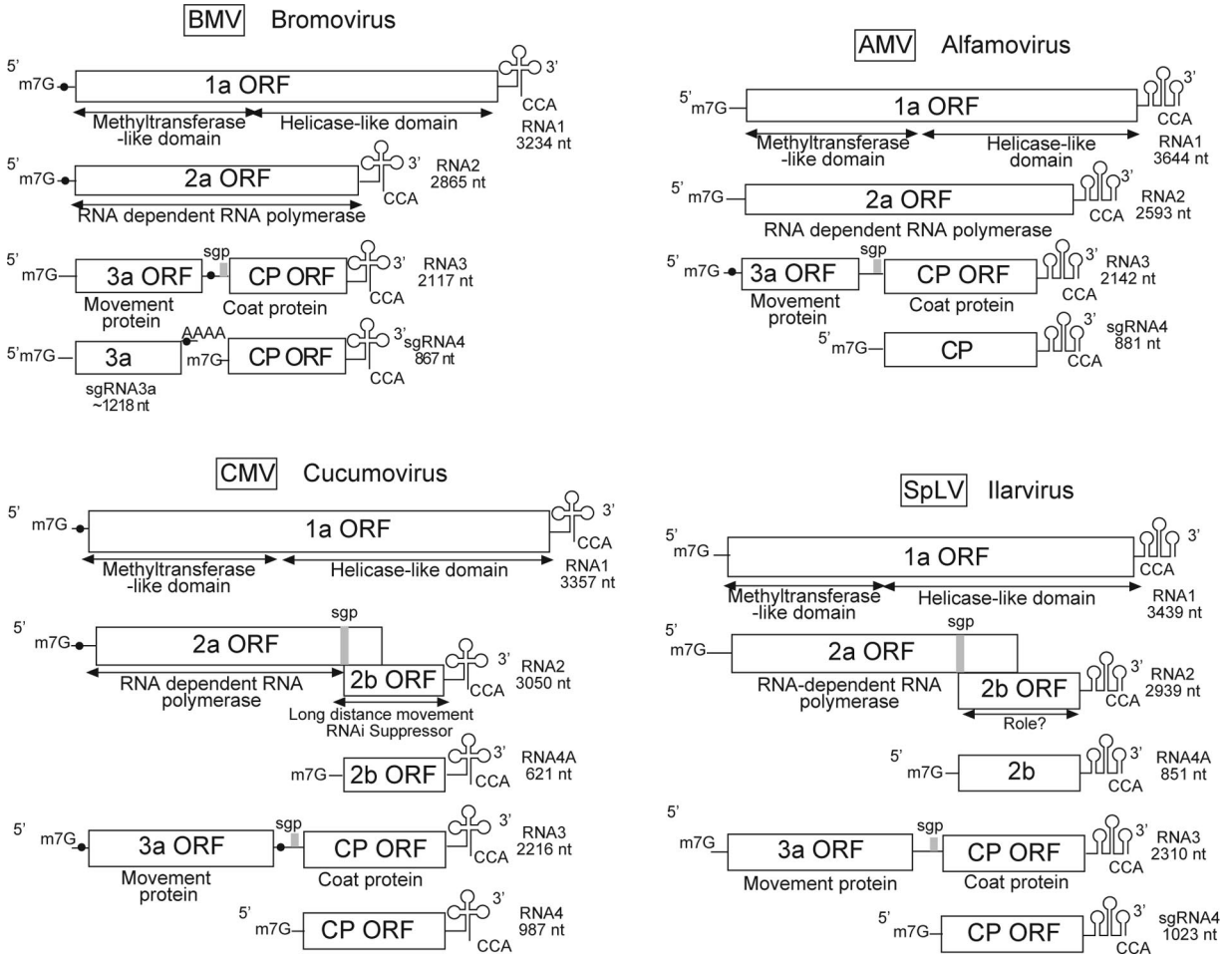

FIG. 1. Comparison of the genome organization of viruses from the family *Bromoviridae*, including BMV (51, 132), AMV (9), CMV (101), and *Spinach latent ilarvirus* (SpLV) (9). Open boxes represent ORFs, gray rectangles represent localization of sgp's, and black dots represent B-box consensus-like sequence localization. Cloverleaf-like/pseudoknotted structures represent 3′ UTRs, while 5′ m7G stands for the 7-methylguanosine cap structure. RNA1 and -2 encode two replicase polypeptides (1a and 2a), while RNA3 encodes the 3a MP and the CP. The CP gene is translated from sgRNA4 in all *Bromoviridae*. RNAi, RNA interference.

viral replication, including the abilities to review the contributions of viral and host functions (59) and to apply strong selections to large yeast populations for detecting low-frequency events, such as RNA recombination (34). However, in working with this cell-based system, one can expect some inconsistencies. Yeast cells divide every several hours and are constantly producing new RNA replication complexes, and this dynamic expansion may amplify effects associated with competition for replication factors.

Cellular expansion of the viral infection is just one of many aspects of the virus life cycle. Late-stage processes, e.g., cell-to-cell movement and systemic spread, can be analyzed only by the application of plant-based approaches. The traditional methods for introducing virions or their genomes into plants utilize manual inoculation in the presence of abrasive substances, which damage cell walls. While these methods are in common use, only a narrow number of cells become infected, and some viruses are not amenable to manual inoculation due to tissue-specific restrictions. *Agrobacterium*-directed transient gene expression (agroinfiltration), involving the delivery of the desired genes into plant cells as a liquid culture through infiltration, represents the optional method suitable for virology studies in plant-based systems. Agroinfiltration has been used widely in plant virology for the identification of disease resistance genes (7), the induction and suppression of posttranscriptional gene silencing (127), and the study of various late-stage processes, e.g., viral packaging (4) or cell-to-cell movement (50). Most importantly, agroinfiltration facilitates the delivery of several transgenes to be coexpressed into the same cell from different *Agrobacterium* transformants. These characteristics are important for studying multicomponent viruses, such as *Bromoviridae*, since the high-level accumulation of genomic RNAs and their expression is replication contingent.

Cell-free systems, although remote and different from the host environment, introduce the opportunity to investigate strictly biochemical/biophysical aspects of viral processes, e.g., recombination (131), protein interactions (1), or replication (93). Although the in vitro systems allow for the dissection of the mechanism and roles of proteins, they have been found to

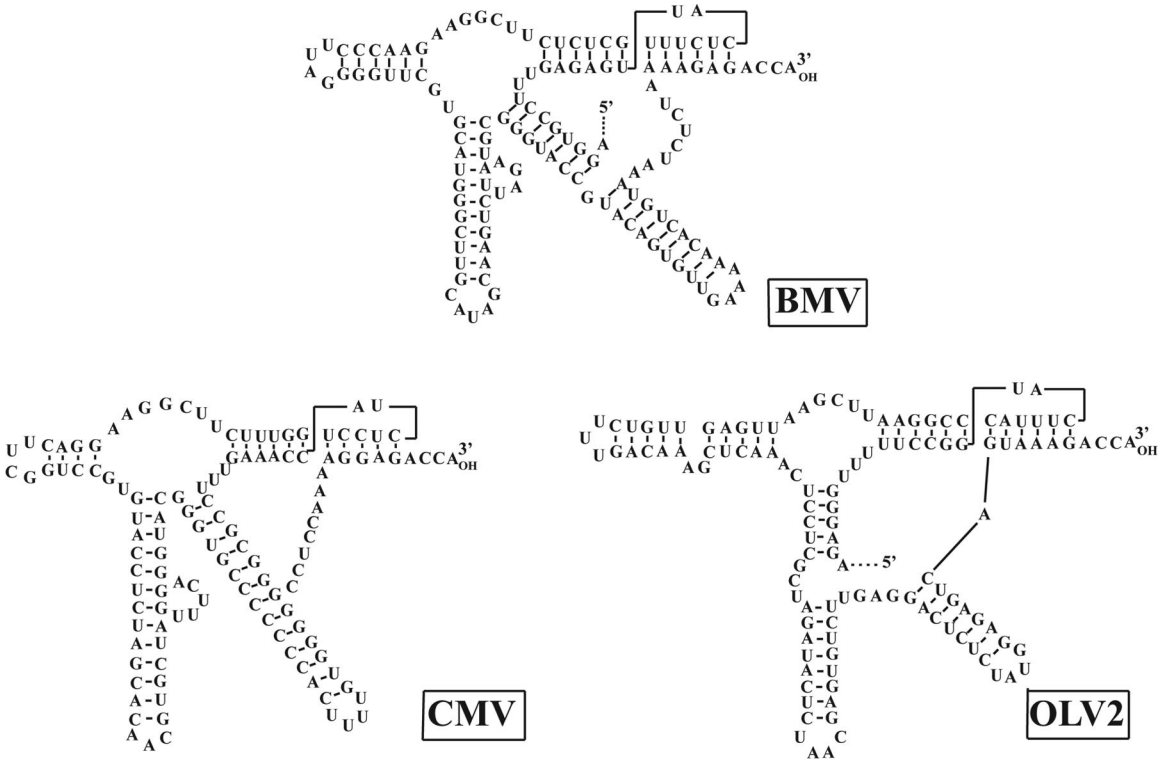

FIG. 2. Structural similarity of the secondary conformations predicted at the 3′ UTR of BMV (98), OLV-2 (37), and CMV (113) from the family *Bromoviridae*.

be difficult to obtain, probably due to the membrane association of most replication complexes. Also, in vitro systems may lack important properties found only in vivo. The results obtained by the utilization of in vivo and in vitro systems might lead to differences that reflect the synergistic benefits of various experimental designs for revealing important aspects of the viral life cycle. Plant virology will continue to benefit from integrating the complementary insights from all these approaches.

VIRUS ENTRY

Plant viruses initiate infection by penetrating the cell wall, but unlike animal viruses, there are no known receptors involved in this process (100). Electron microscopy studies have suggested that pinocytosis is involved in *Bromoviridae* entry (12, 42). An examination of fixed protoplast sections showed that BMV and CCMV induce proliferation of the endoplasmic reticulum and formation of cytoplasmic vacuoles by nuclear membranes in a process called blebbing (12). The observations of plasmalemma invaginations at the virus attachment site and virus-containing cytoplasmic vesicles supported the pinocytosis mechanism. However, in planta studies revealed that *Bromoviridae* penetrate plant tissue only via mechanical or biological damage (72).

PROTEIN SYNTHESIS

All *Bromoviridae* have RNA genomes with a 5′ cap and a non-poly(A) 3′ end that carries the tRNA-like structures (TLS) and/or pseudoknots (Fig. 2). Transfections of tobacco protoplasts with the reporter mRNAs showed that the poly(A) tail acts in synergy with the 5′ cap, serving as a translational enhancer (30). Similar functions are secured probably by the 3′ termini in all *Bromoviridae* (Fig. 2). Particularly, the aminoacylatable TLS at the 3′ termini of *Bromo-* and *Cucumovirus* RNAs were found to play a major role in translation enhancement (31, 133). The introduction of the BMV and CMV 3′ untranslated region (UTR) downstream of a reporter gene enhanced the translational efficiency of the chimeric RNAs in carrot protoplasts (31). Studies with transgenic *cum1* and *cum2 Arabidopsis thaliana* protoplasts showed that the CMV 3′ UTR interacted with translation initiation factors eIF4E and eIF4G, which might contribute to the efficient translation via RNA circularization (133).

AMV translation seems to be also enhanced by the binding of viral CP to portions of the 3′ UTR (31, 76, 77, 81). However, the CP requirement could be eliminated in carrot protoplasts by using 3′ poly(A) AMV RNAs (58), which suggested that AMV CP is also engaged in 5′-to-3′ RNA circularization (76). This idea was further supported by the discovery that AMV CP interacted with translation initiation factors in transgenic P12 tobacco protoplasts (P12 cells transformed with AMV RNA1 and RNA2, expressing replicase proteins 1a and 2a, respectively) (81). Additionally, translation of AMV RNA encoding the defective CP could be rescued by replacing the 3′ UTR with BMV 3′ UTR, suggesting that AMV translation is CP dependent, whereas BMV 3′ UTR can stimulate translation independently of CP (76, 77).

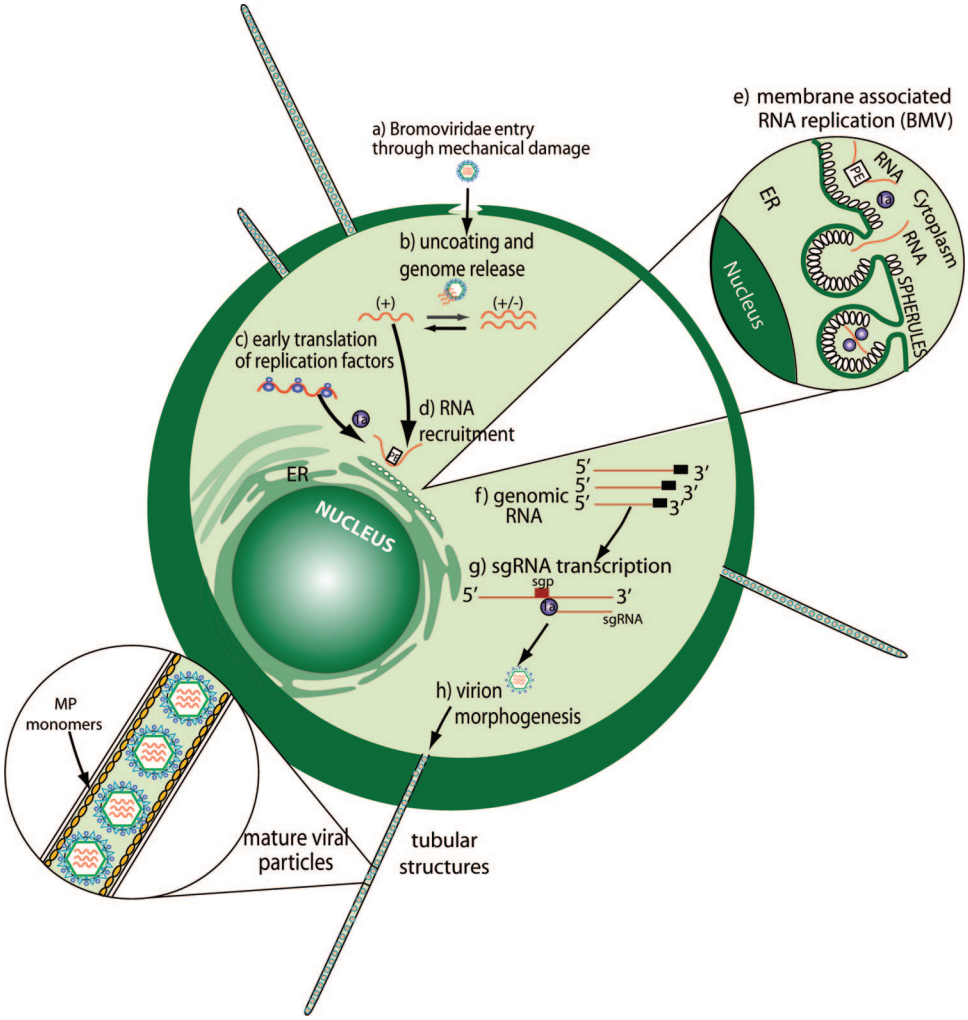

FIG. 3. Schematic illustration of the *Bromoviridae* life cycle in a protoplast cell. The viral entry (a) to the protoplast cell can be supported via polyethylene glycol or electroporation-mediated changes in membrane permeability. Following uncoating (b) and early translation (c) of viral replication proteins, the induction of spherule formation (d), where viral RNA replication (e) occurs, has been observed (6). Newly synthesized mRNAs egress into the cytoplasm for sgRNA transcription (f) and translation of other viral products, such as MP and CP, that are engaged in virion maturation (g). The presence of viral MP triggers the formation of tubular structures that mediate virion cell-to-cell transfer via a tubule-guided mechanism (99, 109, 134). ER, endoplasmic reticulum.

RNA REPLICATION

The outcome of the very first viral translations is the production of factors recruiting genomic RNAs into membrane-bound replication complexes (Fig. 3). Data from several recent studies showed that viral infection induced the formation of membrane patches called spherules, the sites of viral replication (6, 94, 120). A high-resolution immunofluorescence confocal microscopy study confirmed the localization of 1a and 2a replicase proteins in the endoplasmic reticula of BMV-infected barley protoplasts (95). In AMV-infected cowpea protoplasts, 1a and 2a colocalized at the membrane structures surrounding the vacuoles (120). Similarly, CMV 1a protein was shown to interact with a tonoplast intrinsic protein in *A. thaliana* protoplasts (54). Apparently, different *Bromoviridae* members are able to select different cellular membranes as their RNA replication sites.

The shift between early translation and replication must be synchronized to allow sufficient synthesis of RdRp proteins securing efficient RNA replication. Some elements of the 5′ UTR segments were shown to affect the transition between replication and transcription. The exchange of the RNA3 5′ UTR segments between different strains of AMV, tested with transgenic P12 protoplasts, proved that the B-box (a motif commonly present at the 5′ UTR of *Bromoviridae* RNAs, also called the ICR2-like motif due to its similarity to the internal control region of tRNA promoters [65]), might play a role in this process (122). In addition, an immunofluorescence study with yeast has suggested that 1a recruited the BMV RNAs from the translation machinery and targeted the 1a-2a viral RNA complex to the membrane replication sites (25). Particularly, the binding of 1a protein to the B-box structure recruited RNAs to the membranous replication complexes (14). These results were confirmed for barley and tobacco protoplasts by altering the B-box motif, which affected both mem-

brane association and BMV RNA replication (16, 35, 85, 86, 116).

Replication of *Bromoviridae* RNA proceeds in an asymmetric manner, with 100 positive BMV RNA strands being produced for every negative [(−)] strand in barley protoplasts (66). Apparently, the maintenance of a 100:1 ratio requires accurate coordination, as was shown by using RNA3 mutants carrying nucleotide substitutions at the RNA4 initiation site (40) or bearing frameshifts/deletions in the CP gene (43, 74). The intercistronic region of RNA3 acts as the primary determinant of asymmetric BMV replication, although the CP may be an additional factor.

The CP was shown to be involved in regulation of the balance between AMV positive [(+)]- and (−)-strand synthesis in cowpea protoplasts (43, 74). Olsthoorn et al. (81) proposed the conformational switch model explaining the switch between (+)- and (−)-strand production in AMV-infected transgenic P12 protoplasts. This model stated that the 3′ ends of AMV RNAs folded into two mutually exclusive forms playing diverse functions: the CP-free pseudoknotted structure and the CP-bound extended conformation. CP binding to the 3′ end disrupts the pseudoknot, inducing an extended conformation that is no longer capable of RdRp recognition for (−)-strand synthesis. In this way, CP binding induces asymmetric (+)-sense RNA synthesis. The alternative theory, called the 3′ organization model, proposed by Guogas et al. (84) argued that CP binding to the 3′ end compacts, rather then extends, the 3′ RNA termini. The authors proposed an organized AMV RNA-CP complex as the equivalent of a TLS, which presents a uniform population of the termini to act as templates for RNA replication. Additionally, replication assays in nontransgenic tobacco protoplasts demonstrated that the nucleotide changes proposed to both disrupt and restore the pseudoknot structure are deleterious to replication and do not support the significance of the proposed pseudoknot structure for regulating replication. These opposing theories reflect differences that may due to the use of wild-type protoplasts (84) versus transgenic P12 cells that overexpress AMV replicase (81).

Complete replication of the *Bromoviridae* genomes requires the recognition of three classes of RNA promoters, directing the synthesis of genomic (−)-strand RNAs, genomic (+)-strand RNAs, and sgRNAs (described in the next section), respectively. Experiments with tobacco and barley protoplasts have demonstrated that the 3′ UTR TLS directed the initiation of (−)-strand RNA synthesis in bromo- and cucumoviruses. The process takes place when the replicase interacts with a 3-nucleotide (nt) loop (^{67}AUA^{65}) in the TLS-terminal stem-loop C (8, 92). Any changes in the secondary structure of stem-loop C impaired the BMV RNA replication in barley protoplasts (91). However, CMV and BMV RdRps were able to direct replication from the heterologous 3′ TLS regions in the reciprocally exchanged viral RNAs (89). Also, the sequences upstream of the tRNA-like domain, including a series of stem-loops and pseudoknots, were shown to regulate BMV RNA replication in barley cells (16, 60, 85). In the case of alfamo- and ilarviruses, the interaction between CP and the 3′ UTR secondary structures was assumed to regulate the initiation of (−)-strand RNA synthesis (75), and the process was shown to be CP concentration dependent (38). Previously proposed by Olsthoorn et al. (81), the conformational switch model stated that (−)-strand RNA synthesis occurred only on CP-free viral RNAs. However, upon viral entry, viral RNAs are surrounded by CP dimers, which would create a hostile environment for (−)-strand synthesis. Further replication assays with tobacco protoplasts have noted that the AMV replication cycle depends on the CP concentration (38). The results have demonstrated that replication was activated at low CP concentrations but was gradually repressed as the ratio of the concentration of CP dimer to RNA increased. Mutations in the CP mRNA coding sequence that blocked CP translation were found to inhibit replication, suggesting that the CP was required for stimulation. Mutations in the CP RNA 3′ UTR binding domain also reduced replication, suggesting that CP binding to the 3′ UTR was required (38).

To analyze sequences required for initiation of RNA replication, BMV RNA3 3′-terminal mutants were tested with barley protoplasts, showing that BMV (+)-strand RNA synthesis could initiate from the 3′ penultimate cytidylate on the (−)-strand RNA template and that the adenylate and uridylate residues at the +2 and +3 positions were essential (40). Furthermore, the 4-nt core (CCAA) of the cB-box sequence [motif complementary to the B-box, found in the 3′ UTR of (−)-strand RNAs in all *Bromoviridae*], was found to be necessary for (+)-strand RNA replication (16). In addition, the complementary 5′ UTR B-box (65), forming cloverleaf structures in CMV, CCMV, BMV, and AMV RNAs, has been predicted to contribute to efficient genomic (+)-strand synthesis (85). In the case of AMV, replication of the (+)-strand RNA was regulated by the multifunctional CP molecule (121). E. M. Jaspars and C. J. Houwing (49) demonstrated that CP was essential for the release of viral (+)-strand RNAs from the replication complexes in cowpea protoplasts. Their "messenger release" hypothesis predicts that replication complexes liberate single-stranded viral RNAs into the cytoplasm only if CP is present.

Until now, only a yeast system has been utilized to study host factors engaged in *Bromoviridae* replication (68). Now, the results of these high-throughput studies require verification with plant cells, which could be achieved by the application of RNA silencing technology to protoplasts, followed by viral transfection and the analysis of virus-host interactions.

TRANSCRIPTION OF sgRNA

The transcription of sgRNAs is used during the late stage of infection by *Bromoviridae* to express 3′-proximal genes encoding CP. SgRNAs serve as translational templates, while genomic RNAs can be recruited for replication and encapsidation (71). Three basic mechanisms for generating sgRNAs have been tested by using cell-based systems, including the premature termination during (−)-strand RNA synthesis (61), discontinuous transcription during (+)- or (−)-strand RNA synthesis (125), and internal initiation on the viral (−)-strand template (56). The last mechanism, requiring the involvement of *cis*-acting subgenomic promoter (sgp), is widely used by *Bromoviridae*.

The sgp of BMV has been characterized by using protoplast-based assays (55, 115). The promoter sequence includes an upstream AU-rich enhancer, a poly(U) tract, a 20-nt core promoter with the core hairpin, and the +1C initiation site,

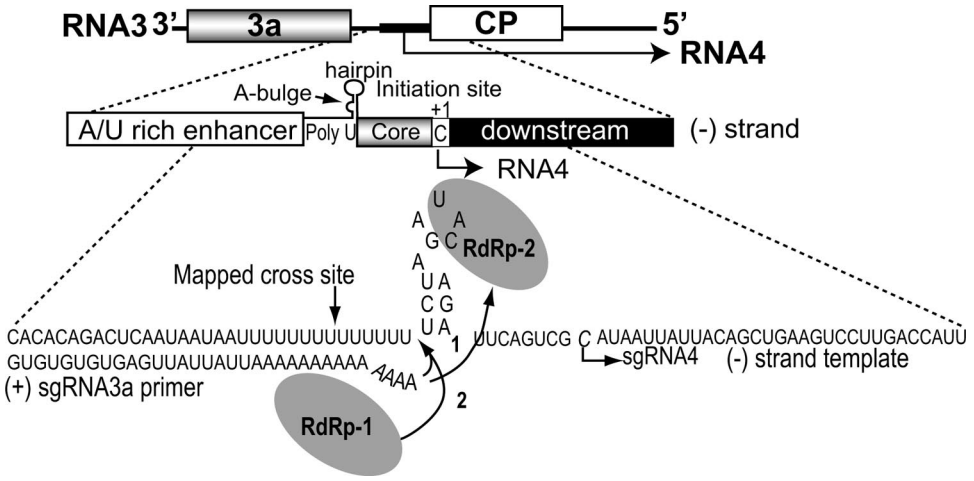

FIG. 4. Two models of the crossing-over mechanism within the sgp region (27, 132). The top part shows the organization of sgp, while the sgp sequence is shown on the bottom. For clarity, only one recombining RNA3 molecule is shown, so the bowed arrows appear to depict crossovers toward sequences on the same template; however, more RNA3 templates might be engaged in the process. The ovals represent RdRp enzymes; one is leaving the template (RdRp-1), while another one (RdRp-2) is bound to the core hairpin of another (−)-strand template (for clarity, one template is shown). According to mechanism 1, the predetached 3′ poly(A) end "snatches" the RdRp-2 on another (−) strand, and the (+)-strand synthesis continues (represented by bow arrow 1). Mechanism 2 predicts that RdRp-1 detaches along with the sgRNA3a and rehybridizes to the poly(U) tract on another (−) strand to continue the (+)-strand synthesis (bowed arrow 2).

followed by a downstream AU-rich sequence (3, 29, 64). In order to synthesize sgRNA4, the replicase enzyme recognizes the core promoter sequence by an induced-fit mechanism (115). Recently, a novel 5′ sgRNA3a in BMV that arose by premature termination of genomic RNA3 synthesis has been discovered (132). Studies with barley and *A. thaliana* protoplasts have shown that both the length of the oligo(A) tract and the stability of the sgp core hairpin affected sgRNA3a synthesis (J. Sztuba-Solińska and J. J. Bujarski, unpublished data). In the case of AMV, data from transgenic P12 tobacco protoplasts have revealed a similar mechanism (121, 123). The nucleotides in the positions −26 to +1 relative to the transcription initiation site were found to control the wild-type level of sgRNA production (123). Other important elements included an enhancer (nt −136 and −94), the sgp hairpin, and the MP C terminus (123). Moreover, an RNA binding sequence on the CP could control the synthesis of AMV sgRNA (97). Two sgp's were found to be involved in the transcription of CMV sgRNAs in vitro (83, 114). The CMV sgp responsible for sgRNA4 synthesis in tobacco protoplasts has involved a 60-nt intercistronic region in RNA3 that mapped to nt −30 to nt +30 relative to the initiation cytidylate (8). However, the promoter sequence of the sgRNA4A synthesis still remains to be characterized. A potential difficulty here is that its sequences overlap in part with the coding region for replicase protein 2a (114).

GENETIC RECOMBINATION OF *BROMOVIRIDAE*

The multipartitism of the *Bromoviridae* genome facilitates genetic recombination of this family of viruses. The first recombination event was reported for BMV (in 1986), and since then, recombination processes have been studied extensively with this model bromovirus (10). The most popular model of RNA recombination is the template switching (copy choice) mechanism, which predicts that the viral replicase enzyme (RdRp) switches templates during RNA synthesis (91). Based on the pairing between the acceptor and the nascent RNAs, one can distinguish between homologous, aberrant homologous, and nonhomologous recombination events (11). Nonhomologous recombination was described for tobacco protoplasts after inoculation with BMV RNA1 and -2, and an RNA3 derivative lacking the 3′ noncoding region. It has been shown that 1 per 10^5 inoculated protoplasts acquired a replicating RNA3 that arose by nonhomologous recombination with RNA1 or RNA2 (45). Data obtained from protoplast systems have demonstrated that some regions of the BMV genome could support higher recombination frequencies than the others due to the presence of recombination signals, e.g., the insertion of the BMV AU-rich region into the *Tomato bushy stunt virus* supported frequent recombination in *Nicotiana benthamiana* protoplasts (112). Recently, the assistance of a novel 5′ sgRNA3a in the BMV RNA3-RNA3 homologous recombination has been tested with barley protoplasts (A. Dzianott, J. Sztuba-Solińska, and J. J. Bujarski, unpublished results), suggesting that prematurely detached sgRNA3a could prime recombination events on (−)-strand RNA3 templates within the sgp region (Fig. 4).

RNA recombination can salvage the damaged or mutated bromoviral RNAs and/or can contribute to the genome variability (91, 92, 124). Studies of barley protoplasts have shown that RNA recombination could be a rapid and frequent phenomenon (39, 88). New viruses or strains may emerge via recombination. A study using a tobacco protoplast system with chimeric BMV and TMV RNAs that carried the exchanged 3′ UTR segments revealed their efficient replication but also showed the formation of new RNA recombinant species (45). Replication efficiency assays of viral reassortants between CMV and *Tomato aspermy cucumovirus* (TAV) in tobacco protoplasts (26, 105) and *Cassia yellow blotch bromovirus* (CYBV), BMV, *Spring beauty latent bromovirus*, and CCMV in

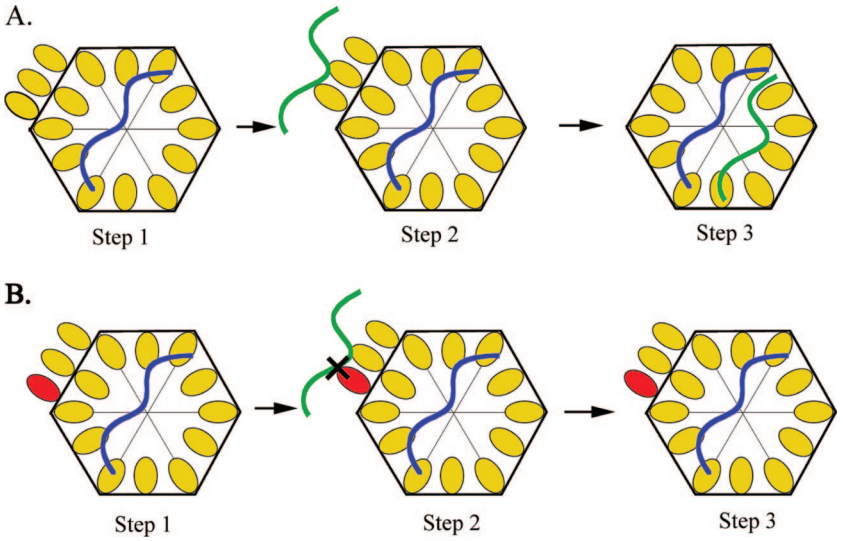

FIG. 5. The sequential packaging model suggests that RNA3 and sgRNA4 copackage chronologically (87). Yellow ovals represent wild-type CP, the red oval symbolizes mutated CP, and blue and green ribbons indicate RNA3 and sgRNA4, respectively. (A) Assembly with wild-type coat protein subunits. Step 1, binding of wild-type CP subunits to the bipartite signal (3′TLS NE and MP NE) of RNA3 results in prior packaging of RNA3 into a virion. Step 2, the N-terminal arginine-rich motif of CP is displayed on the surface of the virion, allowing interaction of sgRNA4 with the arginine residues. Step 3 promotes copackaging of RNA3 and sgRNA4 into a virion. (B) Assembly with mutant coat protein subunits. Step 1, a mutation in the N-terminal arginine-rich motif of CP did not disrupt the assembly of RNA3. However, the sgRNA4 interaction is rigorously affected (Step 2). As a result, the virion will contain only the prepacked RNA3 (Step 3). (Adapted and reprinted from the *Annual Review of Phytopathology* [87] with permission of the publisher.)

N. benthamiana protoplasts, e.g., between BMV and CYBV or between CMV and TAV (48, 26), have demonstrated the replication advantage of the chimeric viruses.

Replicase errors and RNA recombination are also responsible for the formation of defective RNAs, the deleted forms of viral RNAs that contain portions of the parental virus genome (21). For instance, the ability of defective RNA to replicate and to interfere with genomic BMV RNA replication has been demonstrated for barley (21, 22) and for zucchini (52) protoplasts.

ASSEMBLY OF *BROMOVIRIDAE*

The segmented genomes of *Bromoviridae* are assembled within separate viral particles (87). Encapsidation studies with protoplasts allowed for the mapping of the genomic signals securing the specificity of RNA encapsidation, as well as factors stabilizing virions inside the cell (21, 23, 97). BMV RNA3 constructs carrying modifications in both the 3′ UTR and 3a gene allowed identification of two signals required for correct viral assembly in barley protoplasts: the 3′ TLS nucleating element (NE) for CP subunits and a *cis*-acting, position-dependent packaging element of 187 nt that was present within the MP open reading frame (ORF) (18, 22, 23). The lack of the packaging element made the sgRNA4 incompetent for autonomous assembly, whereas prepackaging of RNA3 was a prerequisite for sgRNA4 copackaging (Fig. 5) (18).

The encapsidation effectiveness was shown to be dependent on the specific RNA secondary structure (23). The disruption of the stem of the 3′-proximal hairpin 1 or mutation of AUGC motif 2 in the AMV 3′ UTR inhibited CP binding to the 3′ termini, which affected both RNA replication and assembly encapsidation in barley protoplasts (97).

The selectivity of the virion assembly was proposed to be assured by the specific interaction between RNA and CP. Studies in barley protoplasts pinpointed the highly conserved N-terminal arginine-rich motif of BMV CP as being responsible for both RNA binding and RNA packaging (17, 90, 103). Likewise, the C-terminal region of the CP, especially Phe-184 and the corresponding sequence, affected both the encapsidation and the stability of the virus particles in barley and in *N. benthiamaina* protoplasts (79). In the case of AMV, the infection of transgenic P12 tobacco protoplasts with viral RNAs confirmed that CP molecules were required in *trans* for both replication and encapsidation of RNA1 and RNA2 but in *cis* for replication and encapsidation of RNA3 (75). Again, the role of CP C-terminal domain during interaction with viral RNA was shown to be critical for AMV encapsidation (119). The virion assembly and protection of the (+)-sense viral RNA in protoplasts seemed also to rely greatly on the CMV CP, since even a single amino acid change (Leu-129 to Phe-129) disrupted both processes (8, 117).

Furthermore, encapsidation assays of *N. benthamiana* protoplasts emphasized the importance of RNA-CP interaction for this process. The tested CP chimeras between BMV and CMV were unable to direct efficient RNA encapsidation (82). In contrast, the exchange of CP ORFs between AMV and *Tobacco streak ilarvirus* (TSV) showed that the heterologous CP sequence supported the encapsidation of TSV in P12 tobacco protoplasts (96), confirming the similarities between alfamo- and ilarvirus encapsidation.

VIRUS MOVEMENT

Although the process of cell-to-cell movement in protoplasts cannot be addressed directly by this system, some

useful conclusions about the molecular mechanisms can be gained. The structural phenotype of the MPs of AMV and BMV, studied with cowpea, *Hordeum vulgare*, and *N. benthamiana* protoplasts, have shown that the MPs of these viruses did assemble into tubular structures at the surfaces of the protoplast cells (53). Electron microscopy and immunogold analyses confirmed the presence of both MP and virus particles in the tubules, suggesting that AMV and BMV can move from cell to cell as virions through tubule-like structures (53, 79, 108, 134). CMV MP also induced tubules in transfected protoplasts, but since the RNA3 tubule-defective mutants were capable of cell-to-cell spread in the infected tissue, these structures seem not to contribute to CMV movement (13). Thus, CMV is assumed to move as a ribonucleoprotein complex. Also, MPs of other members of *Bromoviridae*, TSV (67), and *Olive latent virus 2* (OLV-2) (36), were reported to be the structural elements inside the tubules.

To identify the region of MP that is dispensable for protoplast protrusions, a set of deletion mutants within the AMV MP-GFP construct has been transfected onto tobacco protoplasts (44, 107, 134). The removal of MP amino acids 1 to 77, 84 to 142, and 226 to 300 (134), the introduction of point mutations at positions 25, 53, 123, and 138, and both N- and C-terminal deletions (107) all have affected tubule formation.

Studies in planta confirmed the role of microtubules during viral spread. These structures were shown to traverse the cell wall through modified plasmodesmata, and they mediate virion transfer via a tubule-guided mechanism (13, 41, 99, 109, 134). Recent studies performed with *N. benthamiana* plants and protoplasts have shown that host factors were engaged in the regulation of the BMV MP localization to the plasmodesmata (50).

Movement of the *Bromoviridae* sometimes requires compatibility between the MP and CP, manifested by the fact that not all chimerical MP and CP combinations are capable of cell-to-cell movement. The study of biological properties of some pseudorecombinants between CMV and TAV has shown that, even though they retained the ability to replicate in protoplasts, they were incapable of cell-to-cell movement (105, 106). An analysis of the recombinant clones suggested a requirement for compatibility between the C-terminal 29 amino acids of the MP and the C terminus of the CP (106). Following this discovery, computer analyses of the AMV CP constructs that were first tested with transgenic P12 plants and protoplasts have identified differences in the charge distribution of the CP/MP contact zone. Mutations in the N-terminal arm of AMV CP affected the cell-to-cell movement, whereas mutations at both the N and C termini affected the movement through the vascular system (119). Recent studies with barley protoplasts have confirmed interactions between BMV CP and MP that are regulated by the phosphorylation of a serine residue(s) by cellular protein kinase(s) (2).

WHAT'S NEXT?

Protoplasts will continue to serve as multipurpose systems for further lessons about the *Bromoviridae* single-cell reproduction cycle. We do not possess unambiguous answers concerning viral and cellular determinants that recruit genomic RNA into a membrane-bound replication complex (94, 120). The analysis of protoplast cells expressing viral proteins will help to identify key viral factors. On the other hand, the use of fluorescent tags in plant cells and small interfering RNA technology will be useful to characterize signals important for interactions between cellular and viral components. The use of the latest methods for three-dimensional analysis of cellular structures, such as electron tomography (70), will assist in our understanding of the architecture of these unique structures and in explaining such key aspects as how *Bromoviridae* replication and assembly get functionally connected or how viral replicase distinguishes between replication and transcription.

The use of protoplast systems for the analysis of host mutants will expand progress in identifying host factors that are crucial to the viral life cycle. A well-known illustration of this approach from *Arabidopsis* protoplasts has been the identification of the *tom1* and *tom2A* host factors that did not support TMV replication (46, 47). Enhanced knowledge in this area would shape the groundwork for plant breeding efforts to develop virus resistance in crops. For instance, with the discovery of the eIF(iso)4E interaction with *Turnip mosaic potyvirus* VPg protein, the eIF4E was confirmed as a resistance gene and is now widely used by breeders to protect crops against potyviruses (33, 102). The cellular resistance characterized by the usage of protoplast systems along with RNA silencing technology will address questions about the limits of the involvement of host gene products in virus replication before the cell-to-cell movement occurs. By switching off the host genes followed by viral transfection, one will be able to recognize the pathogen-associated molecular patterns of innate immune responses, which will enhance our understanding of virus-host interactions. One well-known example of such a response includes the resistance controlled by the *Rx* locus against *Potato virus Y* (*Potyvirus*) in potato protoplasts (57).

The use of cell-based systems for studying molecular and biological properties of viruses that are considered new *Bromoviridae* family members might provide reliable answers concerning their membership and etiology. Differences of opinion exist concerning the taxonomy of OLV-2 within the family, whether *Raspberry bushy dwarf virus* and *Pelargonium zonate spot virus* should be considered members of the *Bromoviridae*, and even the accurate clustering of viruses within the *Ilarvirus* genus (32). Therefore, plant cell systems might help us to set up proper evolutionary relationships between *Bromoviridae* and to define the mechanisms of their life cycle or of the emergence of new viral strains.

Protoplast systems will open up new possibilities toward establishing cell culture assays, similar to bacterial, yeast, or animal monolayer cultures. Besides determining the molecular activities of RNA viruses in new hosts, they would help to define exact host-range determinants or to predict the role of molecular factors in virus evolutionary adaptation. For example, Chen et al. (15) have reported that sequences of lily strains of CMV were highly similar to one another, in spite of their origins, and that they became adapted to lily plants in their evolutionary history. It would be of utmost interest to employ protoplast system for tracing the host factors that increase the fitness of CMV isolates. Since the frequency of recombination is an obvious factor that determines viral fitness, the study of

the new RNA arrangements in protoplasts will be essential to enhance our understanding of the *Bromoviridae* evolutionary dynamics and to determine what, if anything, limits their diversification.

Finally, the molecular strategies of viral gene expression used by plant viruses are also used by animal RNA viruses (126, 128). Thus, progress in our understanding of the *Bromoviridae* single-cell replication cycle might lead to the discovery and dissection of factors controlling the major agents of worldwide viral epidemics.

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