Genome Packaging by Spherical Plant RNA Viruses

A.L.N. Rao

Department of Plant Pathology, University of California Riverside, Riverside, California 92521-0122; email: arao@ucr.edu

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Key Words

viral genome, RNA packaging, packaging signals, Arginine-rich RNA-binding motif, coat protein-RNA interactions, RNA-RNA interactions

Abstract

The majority of positive-strand RNA viruses of plants replicate and selectively encapsidate their progeny genomes into stable virions in cytoplasmic compartments of the cell where the opportunity to copackage cellular RNA also exists. Remarkably, highly purified infectious virions contain almost exclusively viral RNA, suggesting that mechanisms exist to regulate preferential packaging of viral genomes. The general principle that governs RNA packaging is an interaction between the structural CP and a specific RNA signal. Mechanisms that enhance selective packaging of viral genomes and formation of infectious virions may involve factors other than CP and nucleic acid sequences. The possible involvement of replicase proteins is an example. Our knowledge concerning genome packaging among spherical plant RNA viruses is still maturing. The main focus of this review is to discuss factors that have limited progress and to evaluate recent technical breakthroughs likely to help unravel the mechanism of RNA packaging among viruses of agronomic importance. A key breakthrough is the development of in vivo systems and comparisons with results obtained in vitro.

INTRODUCTION

Genome: the infectious component of the virus

Packaging or encapsidation: assembly of nucleic acid sequence into a virion

Capsids: empty virus particles

Mono-, bi-, and tripartite viruses: one, two, or three genome components, respectively, required to initiate infection

Virions: nucleic acid–containing virus particles

Packaging, or encapsidation, of the viral genome by structural protein components leading to the assembly of infectious progeny virions is an essential step in the life cycle of RNA viruses infecting plants and other eukaryotic organisms. At present, recognized plant viruses are classified into 80 genera (28); of these, viruses belonging to 25 genera exhibit helical symmetry (rigid rod or flexuous rods), 2 have unknown or no capsid structure, and the remaining 53 genera have icosahedral symmetry (spherical). Whatever the ultimate architecture of the virus, assembly of infectious virions is a carefully orchestrated process maintaining a high degree of precision and requires protein subunits that interact with each other and with viral nucleic acids. Some aspects of the assembly process are common to all viruses with icosahedral symmetry, although the mechanisms might differ widely. Once viruses have been assembled, the resulting virion architecture has three primary roles: (*i*) to be stable enough to survive as an infectious particle in the external environment required for transmission between host individuals; (*ii*) to be flexible enough to disassemble upon entering uninfected host cells facilitating release of the infectious genome; and (*iii*) to provide an optimal interaction with the host machinery for the inception of successful infection. Most important, in several plant virus genera assembly into infectious virions is obligatory for cell-to-cell and/or long-distance spread (64, 79, 91), as well as for acquisition and dissemination to new hosts by a wide range of vectors such as aphids, leaf hoppers, nematodes, mites and fungi (14, 15, 39). Thus, knowledge of the detailed mechanism by which viruses assemble and package their genomes into structurally stable virions is an important prerequisite for understanding the overall biology of plant viruses.

This review aims to update readers on how plant viral genomes with icosahedral symmetry are selectively encapsidated and the mechanisms that regulate this process, rather than describing virus structure and assembly per se (for reviews see 43, 71, 73). Space limitations confine our focus to exciting recent discoveries about the genome packaging for a few well-studied spherical viral systems that are likely to pave the way for similar studies with other plant viruses. In this review, unless otherwise stated, the terminology "capsid" and "virion," respectively, refers to empty and nucleic acid-containing virus particles.

PRINCIPLES REGULATING GENOME PACKAGING

Of the many strategies for packaging viral genomes, the most commonly employed options include either filling of the preformed capsid structures with previously synthesized nucleic acids or the nucleation of capsid assembly around viral nucleic acids. Most plant viruses adopt the second strategy. Principles that govern RNA packaging in spherical plant viruses have been derived from studies on monopartite *Tombusviruses*, *Tymoviruses*, *Sobemoviruses*, bipartite *Dianthoviruses*, and tripartite *Bromoviruses*, *Cucumoviruses*, and *Alfamovirus*. The replication of all plant viruses with ss (single stranded), positive-sense RNA genomes is entirely cytoplasmic. During the infection process, perturbation of internal membrane structures leads to vesicle formation; in several cases these vesicles have been shown to be associated with replication complexes (**Figure 1**) (11, 31). However, the final accumulation of progeny virions appears to occur in the cytoplasm (**Figure 1**) (40). Since infectious virions predominantly contain viral RNAs, viruses may have evolved mechanisms that filter out RNAs other than those associated with the virus itself. Even though protein-protein and protein-RNA interactions play a crucial role in assembly and packaging (29, 30), as discussed below, several other factors are also likely key players that contribute either directly or indirectly to selective packaging.

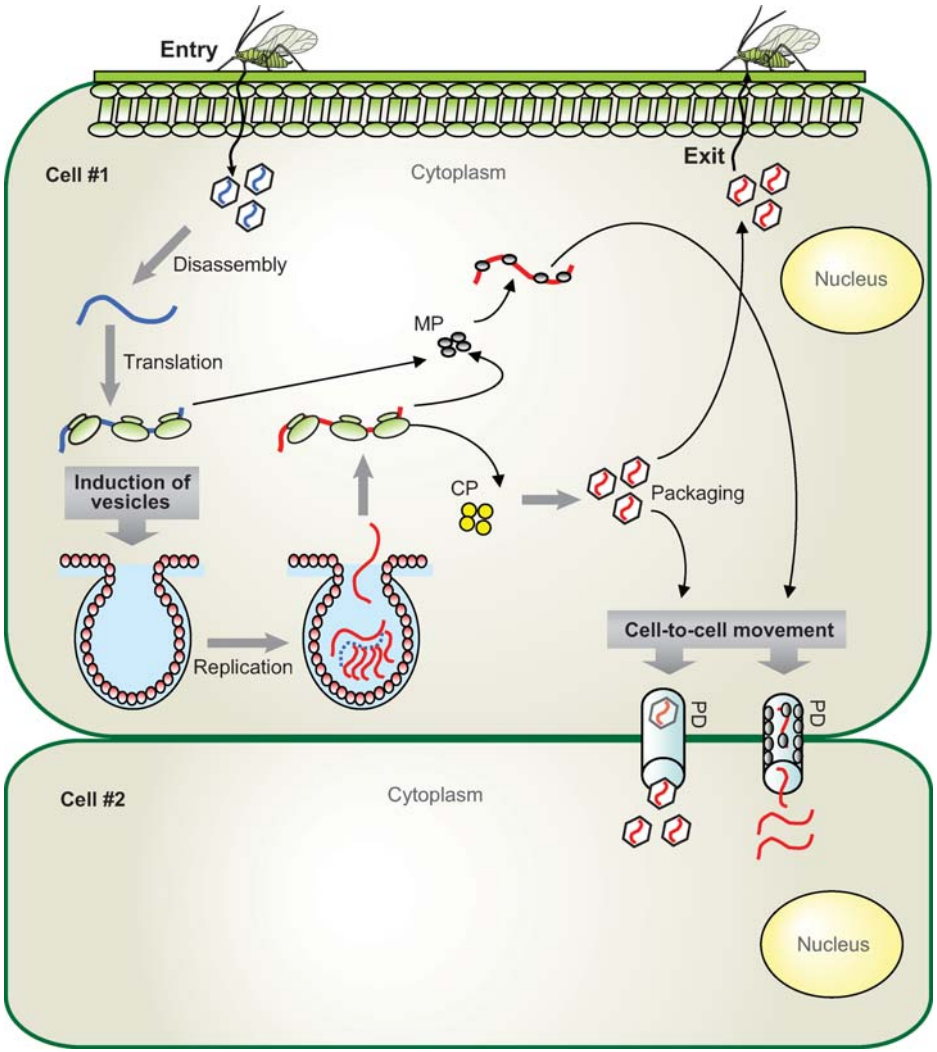

Figure 1

Schematic illustration of replication, assembly and cell-to-cell movement characteristics of plant viruses with ss, (+) RNA genomes. In nature, one method of initial entry of plant viruses is achieved through insect vectors. Following disassembly and translation of viral replicase proteins, induction of a wide range of cytopathic effects such as vesicle-like structures, where viral RNA replication occurs, has been observed for many plant RNA viruses (31, 82). Normally, these vesicles measure about 50–70 nm in diameter (31, 82) but for clarity their size is enlarged. Newly synthesized mRNAs egress into cytoplasm for translation of other viral products such as MP (movement protein) and CP (coat protein). Gating of plasmodesmata (PD) results in transportation of viral progeny either in the form of a ribonucleoprotein complex or intact virions (51, 65).

Genome Configuration

Viruses exhibit a wide range of genome configurations, and a given plant RNA virus can be either nonsegmented or segmented (40). **Table 1** summarizes characteristic features and packaging profiles of spherical plant RNA viruses, and representative examples are schematically shown in **Figure 2**.

Table 1 Packaging profiles of spherical plant RNA viruses

Family/Genus	No. RNAs accumulated during replication			Packaged RNAs		
	Genomic	sgRNA	DI/Sat	Genomic	sgRNA	DI/Sat
Bromoviridae						
Bromovirus (T)	3	1	+/0	3	1	+/0
Cucumovirus (T)	3	3	+/+	3	3	+/+
Alfamovirus (T)	3	1	0/0	3	1	0/0
Ilarvirus (T)	3	1	0/0	3	1	0/0
Oleavirus (T)	3	1	0/0	3	1	0/0
Ourmiavirus (T)	3	0	0/0	3	0	0/0
Comoviridae						
Fabavirus (B)	2	0	2	2	0	0/0
Nepovirus (B)	2	0	0/+	2	0	0/0
Tombusviridae						
Tombusvirus (M)	1	2	+/+	1	1	+/+
Carmovirus (M)	1	2	+/+	1	2	+/+
Dianthovirus (B)	2	1	0/0	2	0	0/0
Sequiviridae						
Sequivirus (M)	1	0	0/0	1	0	0/0
Waikavirus (M)	1	0	0/0	1	0	0/0
Luteoviridae						
Luteovirus (M)	1	2	0/+	1	0	0/+
Polerovirus (M)	1	1	0/0	1	0	0/0
Enamovirus (M)	1	1	0/0	1	0	0/0
Floating genera						
Tymovirus (M)	1	1	0/0	1	1	0/0
Sobemovirus (M)	1	1	0/+	1	1	0/+

M, monopartite; B, bipartite; T, tripartite. Packaging profiles shown are for the type member of the respective genus.

Some of the thoroughly investigated plant virus genera with monopartite genomes include *Tymovirus*, *Luteovirus*, *Sobemovirus*, and *Tombusvirus*. In the genus *Tymovirus* the gRNA and sgRNA are packaged into separate virions (**Figure 2*a***). In the genus *Luteovirus* only the gRNA is packaged whereas the members of the genera *Sobemovirus* and *Tombusvirus* package their gRNA and sgRNA into virions (**Table 1**). Viruses belonging to genera *Comovirus*, *Nepovirus*, and *Dianthovirus* exhibit bipartite genomes consisting of two RNA molecules packaged either separately into two distinct virions (*Comovirus* and *Nepovirus*) or into the same virion (*Dianthovirus*; **Figure 2*b***). The genomes of plant viruses belonging to the genera *Bromovirus* (icosahedral), *Cucumovirus* (icosahedral), and *Alfamovirus* (bacilliform) are divided among three RNA segments (**Figure 2*c***). In each of these genera, the genomic and a single subgenomic RNA (sgRNA) are distributed into separate particles and their size and number vary with genera. For example, in the genera *Bromovirus* and *Cucumovirus*, the largest two genomic RNAs are packaged individually into two virions and the third genomic RNA and its sgRNA are copackaged into a third virion (**Figure 2*c***) (34, 48, 70). In these two genera, the assembled virions do not display any heterogeneity in either size or appearance. By contrast, in the genus *Alfamovirus*, the three genomic and the sgRNA are packaged individually into four distinctly sized virions with

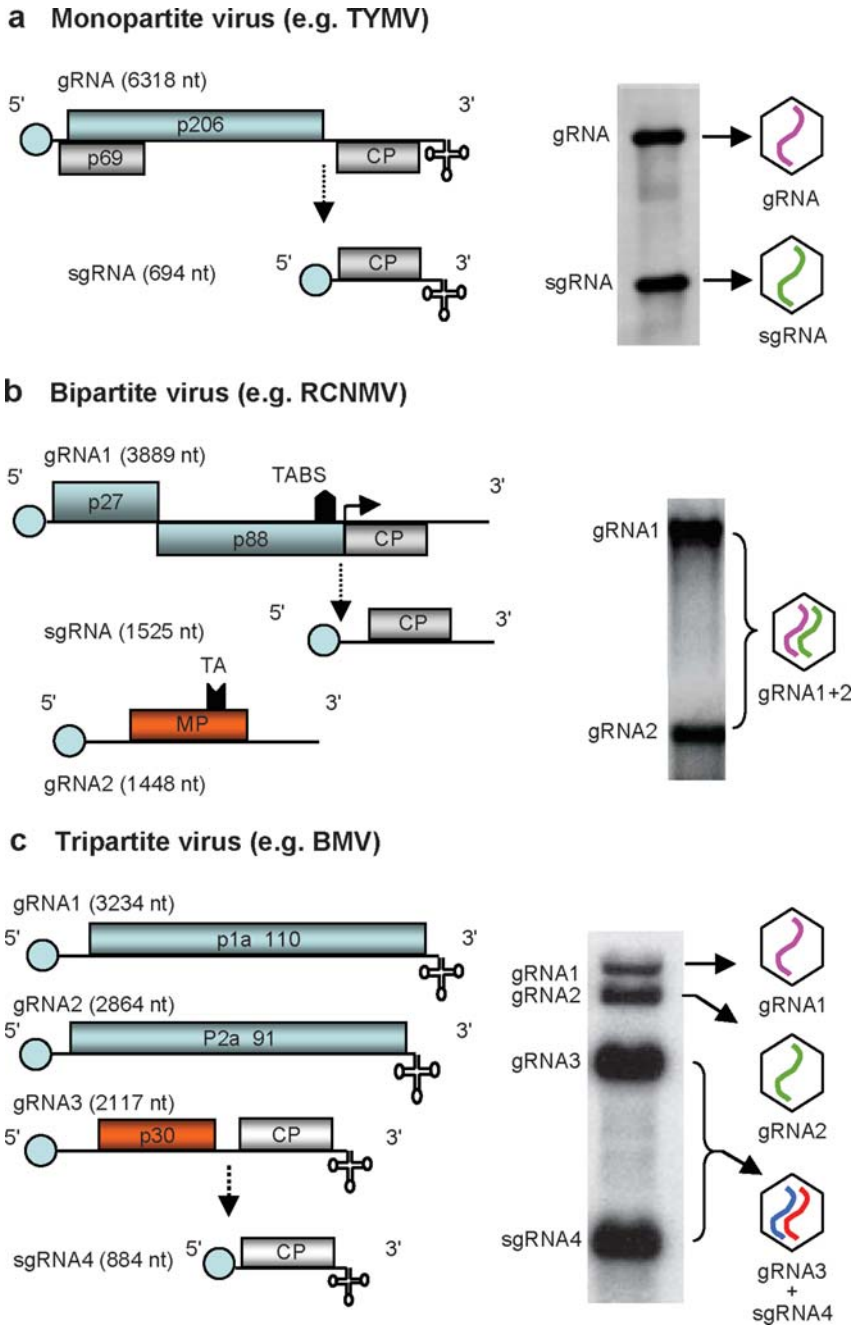

Figure 2

Schematic illustration of genome organization and virion packaging profiles representing configuration of (*a*) monopartite virus (TYMV); (*b*) bipartite virus (RCNMV); TA depicts the *trans*-activator element and TABS the TA binding site and (*c*) tripartite virus (BMV). Note that in monopartite TYMV and tripartite BMV, gRNA and sgRNA are packaged whereas in bipartite RCNMV only gRNAs are packaged. Solid lines and rectangular boxes, respectively, represent UTR and ORF regions. A filled circle at the 5′ end represents cap structure and a 3′ clover leaf represents TLS.

DI-RNA: defective interfering RNA

gRNA: genomic RNA

sgRNA: subgenomic RNA

ARM: arginine-rich RNA-binding motif

satRNA: satellite RNA

OAS: origin of assembly sequence

Packaging signal: an RNA sequence required to specifically interact with CP to mediate packaging of that sequence into a virion

BMV: *Brome mosaic virus*

TMV: *Tobacco mosaic virus*

TCV: *Turnip crinkle virus*

bacilliform morphology (8). In addition to genomic RNAs (gRNAs), secondary RNAs such as sgRNA, satRNA or DI-RNA copackaged into virions are characteristic features of certain virus groups (**Figure 2**; **Table 1**). sgRNA, sat-RNA, and DI-RNAs are not required to initiate infection but are, however, dependent on the parental virus for replication and packaging (83). The rationale for their packaging (either independently or together with gRNA) is not well understood.

Molecular Interactions

Four types of molecular interactions may aid in precise assembly and packaging of viral genomes. (*i*) Protein-protein interactions play a major role in virion assembly, but their contribution relative to RNA-protein interactions varies among different plant virus genera. For example, members of the genera *Tymovirus* and *Comovirus* are predominantly stabilized by protein-protein interactions and therefore form capsid shells in the absence of RNA. (*ii*) RNA-protein interactions dictate assembly in plant virus genera such as *Bromovirus*, *Cucumovirus*, and *Alfamovirus*. In these viruses virion formation requires RNA functioning as a nucleating agent and therefore capsids are never found in vivo. The physical interaction between viral capsid protein and RNA ensures that progeny virions exclusively contain viral RNA. Recent experimental evidence suggests that other virus-encoded proteins such as replicases may also contribute significantly to this process (3). (*iii*) Sequence-independent RNA-protein interactions are envisioned to stabilize encapsidated RNAs within the virus particle. Basic N-terminal ARM (arginine-rich RNA-binding motif) found in the CP of several RNA viruses is not visible in the electron density map of capsid structure but is thought to interact with viral RNA inside the capsid shell (43, 94). Following encapsidation, the interaction between basic residues of the N-ARM region and RNA phosphates stabilizes the assembled infectious virions (94). (*iv*) Finally, sequence-dependent RNA-protein interactions are critical for initiating the viral assembly process. Specific sequence- and/or structural-dependent interactions between RNA and CP are envisioned to ensure that the majority of assembled virions contain exclusively viral RNA. Specific recognition between viral RNA and CP is thought to lead to the formation of a complex that nucleates the binding of additional CP molecules, resulting in the assembly of a complete particle.

Despite these well-defined interactions, heteroencapsidation or transencapsiadtion or genome masking, a mechanism in which the genome of one virus is packaged by the CP of another, has also been observed (40). However, such a phenomenon usually occurs only between related viruses of the same taxonomic group (21, 86) and rarely between taxonomically distinct viruses (24).

Packaging Signals

Genome packaging is considered to be a highly specific process. During packaging, viral nucleic acids must be distinguished from other cellular RNA molecules present in the compartment where assembly takes place. Such discrimination is the result of specific recognition of sequences or structures unique to viral nucleic acids, often termed OAS (origin of assembly sequence) or packaging signals. In eukaryotic RNA viruses, the most thoroughly characterized packaging signal is that of TMV (*Tobacco mosaic virus*), a helical plant RNA virus in which the specific interaction between the CP and a 69-nt region of the MP ORF (open reading frame) leads to the specificity of virion assembly (102). Among plant RNA viruses with icosahedral symmetry, sequence elements that act as specific packaging signals have been characterized only for monopartite TCV (*Turnip crinkle virus*) (63) and tripartite BMV (*Brome mosaic virus*) (20, 23). However, the presence of a packaging signal alone does not guarantee packaging of RNA into virions; several factors of the packaging signal such as secondary structure,

position with respect to genomic context and location (*cis* or *trans*) also affect packaging efficiency (20, 23).

Physical Size Constraints

The genome sizes of spherical plant viruses vary significantly among genera, but virions lie in the same size range (25–35 nm). In addition to specific sequences that promote packaging, another important parameter is the dimension of the capsid, which imposes an upper limit on the size/amount of viral nucleic acid that can be accommodated. Consequently, nucleic acids larger than a wild-type genome cannot be packaged, even when they contain appropriate packaging signals. This size limitation is illustrated by packaging studies performed with TCV. TCV has a 30-nm diameter virion with 180 copies of a single CP arranged in T = 3 icosahedral symmetry. Purified virions of TCV encapsidate the gRNA of 4054 nt but not sgRNAs (63). While analyzing the size limits for TCV virion, Qu & Morris (63) observed that virions of TCV isolated from protoplasts cotransfected with wild-type TCV and a *Tomato bushy stunt virus* (TBSV)-TCV chimera contained only the 2044-nt sgRNA of the chimera but not the larger 4665 nt genomic RNA of TCV-TBSV chimera. Similar cotransfection assays performed with a truncated version of the TBSV-TCV chimera resulted in packaging of 4133-nt gRNA. Collectively, these observations suggested that, despite having the required packaging signal, TCV virions cannot accommodate RNA larger than 4600 nt.

By contrast to the TCV situation, packaging of RNA larger than wild type was observed in vitro for *Cucumber mosaic virus* (CMV), another icosahedral RNA virus whose capsid is 29 nm in diameter (62). The packaging scheme for CMV RNAs is identical to that of BMV (**Figure 2*C***): RNA1 (~3300 nt) and RNA2 (~3000 nt) are packaged separately, whereas RNA3 (~2200 nt) and its sgRNA4 (~1010 nt) are copackaged into a third virion (62). In vitro assembly assays demonstrated that the dissociated CP subunits of CMV are competent to package TMV RNA (~6500 nt) into icosahedral virions with increased diameter of 34 nm (14). If we extrapolate this scenario to in vivo, several configurations (e.g., one molecule each of CMV RNA1 and RNA2 can be copackaged) can be envisioned. Furthermore, depending on the serogroup or strain, additional RNAs such as satRNA or other sgRNAs (RNA4A, RNA5) are also copackaged with genomic RNAs (62). Despite these envisioned variations, CMV displays remarkably uniform-sized virions of 29-nm diameter (34), which suggests its packaging in vivo is a tightly regulated process.

Virion size is also regulated by RNA size for BMV. When the BMV CP mRNA was expressed either autonomously in yeast cells (47) or via a TMV expression vector (19), different sizes of virus-like particles (VLPs) were formed. Physical and molecular characterization revealed that VLPs smaller than wild type corresponded to 120 CP subunits with T = 1 symmetry exclusively packaged CP mRNA (47). The fact that wild-type BMV infections never resulted in the assembly of virions with T = 1 symmetry suggests that physical homogeneity is maintained either by virus-related proteins or some other factors yet to be identified (see below).

RNA Structure

A variety of structural conformations assumed by mRNAs contribute to their specific recognition by proteins (13). In the context of virology, secondary and tertiary structural folding of viral RNAs has been shown to be crucial for replication, translation, and packaging. For example, intramolecular base-pairing of the 3′ nontranslated regions of a wide range of plant viral RNAs results in the formation of aminoacylatable tRNA-like structures (TLS) (25). Mutational analysis of the 3′ TLS in several viruses demonstrated that maintenance of TLS conformation is important for initiation of minus-strand synthesis by viral replicase (25). Likewise, simple

TBSV: *Tomato bushy stunt virus*

CMV: *Cucumber mosaic virus*

VLPs: virus-like particles

TLS: tRNA-like structure

EMV: *Eggplant mosaic virus*

FHV: *Flock house virus*

stem-loop structures of a given viral RNA have been shown to be important for viral replication (95) and translation (4). However, information concerning the relationship between the secondary structural conformations of the RNA regions involved in packaging is limited. In BMV and TCV, sequences specifying RNA packaging have been identified and shown to assume stem-loop structures (20, 23, 63). In addition to these localized structural features, the overall structure of a given ssRNA molecule could also be important for interaction with CP prior to packaging. For example, CMV satRNA is ~300 nt and is efficiently copackaged with the genomic RNAs. Lack of any sequence homology with the genomic RNAs of the helper virus suggests that packaging of CMV satRNA is regulated by the overall RNA structure rather than any specific sequence. Therefore, studying the structural features of ssRNA of many eukaryotic viruses is essential to understand the relationship between the 3D shape of an RNA molecule and its relationship to a specific function such as translation, replication, and packaging. Recent advances in biophysical techniques such as small-angle X-ray and neutron scattering allow for direct visualization of size and shape of viral RNA molecules and their interaction with capsid protein and other substrates.

Auxiliary Factors Influencing Genome Packaging

Transfer RNAs. Among many cellular RNAs, tRNAs act as adapter molecules that translate codon sequences in mRNA into the amino acid sequence of a protein. In addition, they participate in other cellular activities such as biosynthesis of chlorophyll and heme (41). tRNAs or tRNA-like activities have been shown to be associated with a variety of RNA viruses including several plant viruses and retroviruses. Host tRNAs found in the virions of retroviruses function as primers for RNA-directed DNA synthesis (53). The TLS found at the 3′ end of the genomes of some plant virus genera such as *Tobamovirus*, *Tymovirus*, *Bromovirus*, and *Cucumovirus* (25) serve as efficient origins of replication and as primitive telomeres to ensure that the 3′-terminal CCA nucleotides are not lost during replication (37, 66).

Cellular tRNAs have in some instances been reported in the icosahedral virions of plant RNA viruses. Two to three molecules of RNA with the properties of $tRNA^{Lys}$, with smaller amounts of other tRNAs, were reported in the top component particles of *Eggplant mosaic virus* (EMV) (10). Virion preparations from other *Tymoviruses* also contain tRNAs capable of accepting a range of amino acids other than valine, which is bound by the viral RNA (25). However, it is not known in these cases whether tRNAs are accidentally copackaged or whether their presence reflects active selection by the viral CP. The ability of tRNAs of viral and host origin to play a transient but crucial role in virion assembly was first demonstrated experimentally for BMV using in vitro assembly assays (16). Because BMV RNAs devoid of TLS do not replicate in vivo (69), the active participation of host tRNAs in the assembly of BMV virions cannot be confirmed in vivo. However, the feasibility of expressing viral mRNA in a replication-independent mode using *Agrobacterium*-mediated transient assays (see below) is likely to advance our understanding of the role of host tRNAs and viral TLS in the packaging of viral genomes.

Viral replicase. In addition to participating in the synthesis of progeny minus and plus sense RNAs, viral replicase has been implicated in many roles during the establishment of virus infection. The involvement of viral replicase in packaging has been demonstrated for nonplant viruses such as poliovirus (58), *flock house virus* (FHV) (92), and Kunjin virus (KUNV) (44). Until recently there has been no evidence for the involvement of plant virus replicase in genome packaging. Although in vitro assembly systems allow study

of RNA packaging independent of other viral functions (such as replication), the results must ultimately be validated in vivo. Progress to this end has been severely hindered by the lack of suitable in vivo systems that allow study of RNA packaging independent of replication. However, a recent breakthrough in extending the in vivo transient expression system to RNA packaging studies suggested a direct and active role for viral replicase in RNA packaging (3).

Accessory proteins. Virus assembly is a tightly regulated process because multiple identical CP subunits must undergo conformational switching to correctly determine the size of the viral capsid. As each identical CP subunit is added to the capsid, a choice must be made as to which conformation the subunit will adopt. Various mechanisms exist to regulate the conformational switching such as structural transitions in the regulatory regions of CP subunits and interactions of CP subunits with ordered nucleic acid within the capsid (42). In some complex viruses such as herpesviruses and adenoviruses, auxiliary proteins referred to as scaffolding proteins are transiently required to direct the correct assembly of procapsids (72). In the absence of these scaffolding proteins, aggregation of CP subunits yielding procapsids is slower and also results in the assembly of either aberrant spiral structures or smaller-than-normal capsids that are incapable of genome packaging. No evidence exists for the involvement of any accessory proteins in the assembly of plant viruses. However, such a possibility should not be ruled out because unsuccessful in vitro reassembly for many plant viruses may be attributed to the transient involvement of accessory proteins in the pathway of virus assembly. Therefore it is possible that virus-encoded replicase proteins or host proteins associated with the compartment where assembly occurs can regulate packaging of plant viruses similar to the mechanism observed in herpesviruses and adenoviruses. The involvement of accessory proteins in the assembly of plant viruses has, to date, been technically difficult to demonstrate. However, the agroinfiltration approach for analyzing virus assembly independent of replication directly in whole plants is likely to shed light on factors other than structural proteins required in promoting virus assembly and RNA packaging pathways.

MECHANISMS REGULATING SELECTIVE PACKAGING OF VIRAL RNA

The mechanisms that regulate selective packaging of mRNAs of plant viruses are not well understood. The observation that a given viral RNA is not usually packaged by the CP of an unrelated virus upon coinfection of the same cell (22, 63) suggests that viral CP can distinguish between different viral RNAs to enhance selective packaging. Packaging in monocomponent viruses is considered to be relatively simple. By contrast, viruses with segmented genomes have evolved mechanism(s) to balance packaging of the constellation of required genome components among multiple virions. In most cases, the capsid protein has to discriminate gRNA and sgRNAs prior to their segregation into individual virions. Therefore, unlike in monopartite viruses, for those with divided genomes selective packaging is asssumed to be a multifaceted event. This involves RNA-RNA interactions and CP-RNA interactions where the RNA-binding property of the CP stabilizes RNA-RNA interactions or alters the structure of the viral mRNAs in a manner that makes RNA packaging signals distributed on each genome segment sterically accessible. Even though knowledge concerning CP as well as RNA sequences is essential to determine the mechanism of genome packaging, much of the progress to this end was made in the genus *Bromovirus* because of its reliable and reproducible in vitro and in vivo systems. In other genera, technical constraints limited the progress either to CP or RNA but not both.

SeMV: *Sesbania mosaic virus*

SCPMV: *Southern cowpea mosaic virus*

COAT PROTEIN MOTIFS INVOLVED IN SELECTIVE PACKAGING

A Case Study of Protein-RNA Interactions: Arginine-Rich RNA-Binding Motif

A major factor that influences selectivity in the packaging of viral genomes from a large pool of cellular RNAs is the specific interaction between the CP and RNA. Viral CPs exhibit both specific and nonspecific RNA-binding activity (27, 98). The affinity and specificity with which a given protein binds the RNA is determined by different RNA-binding motifs. In eukaryotic cells, characterization of RNA-binding proteins has led to the identification of a variety of RNA-binding motifs that play key roles in posttranscriptional gene expression (13), and one such motif is the ARM. The identifying feature of ARM is the preponderance of arginine residues without exhibiting any sequence similarity within regions of short 10 to 20 amino acids. ARMs also display a variety of conformational preferences such as α-helices, β-hairpins, and probably extended chains. The ARM was first identified in bacteriophage λ (49) and subsequently in human immunodeficiency virus (HIV) Tat (96) and Rev (45). Similar motifs are also found in CPs of several RNA viruses that infect plants and insects (**Figure 3**). In a given cellular environment, if a viral protein (such as CP) were to utilize a common cellular RNA-binding motif such as the ribonucleoprotein motif (13), the possibility of inadvertent cross-talk between viral genomes and cellular circuits would be maximized. The fact that RNA-binding proteins with ARM are by and large viral proteins (13, 49, 68) suggests that they must have evolved to specifically recognize their target, viral RNAs themselves, within the context of high backgrounds of cellular RNAs.

The fundamental importance of the ARM in RNA interaction leading to genome packaging in plant virus genera *Sobemovirus* and *Bromovirus* is well established. The members of the genus *Sobemovirus* (87) are characterized by isometric virions of 30 nm that encapsidate one copy each of gRNA (~4 kb) and sgRNA (~1 kb). *Sobemoviruses* also encapsidate a viroid-like satRNA of size 220–390 nucleotides (87). The *Sobemovirus* capsids exhibit T = 3 symmetry. The CP is generally considered to be made of two domains, a random (R) domain comprised of the N-terminal 71 residues and a shell (S) domain that forms the jelly-roll motif commonly found in most viral CPs. The R domain of the CP is rich in positively charged amino acid residues thought to be essential for RNA encapsidation and that are not structurally ordered in all of the known structures. The flexible nature of this domain is believed to be essential for enhancing the probability of protein-RNA interaction and probably for RNA encapsidation (17). *Sobemoviruses* are stabilized by RNA-protein, protein-protein, and calcium-mediated protein-protein interactions (77). *Sobemoviruses* do not form capsids in vivo. *Sobemoviruses* can assemble in vitro into smaller T = 1 icosahedral capsids in the absence of RNA. Partial tryptic digestion of EDTA-treated virus results in the cleavage of the R domain and subsequent assembly into smaller T = 1 capsids devoid of RNA (78). The crystal structures of trypsin-treated T = 1 capsids from native *Southern cowpea mosaic virus* (SCPMV) and recombinant T = 1 capsids formed by N-terminal 65 amino acid-deleted *Sesbania mosaic virus* (SeMV) CP (75) have been determined. The overall fold of SeMV CP in T = 1 capsids is identical to that in T = 3 capsids even though it lacks the R domain. Thus, the R domain controls the size of the assembled particles and is also responsible for RNA encapsidation. The essential region in the R domain can be narrowed down to two important motifs, the ARM and amino acid residues that form a β-annulus structure. The β-annulus is considered to be the switch that controls the assembly into T = 3 capsids because it is not present in the T = 1 capsids. However, deletion analysis of SeMV CP showed that removal of ARM alone (36 amino acid residues from the N terminus)

Peptide	Sequence	% α-helix
CPs of plant viruses		
BMV (7-25)	K M T R A Q R R A A A R R N R W T A R	23
CCMV (7-25)	K L T R A Q R R A A A R K N K R N T R	18
BBMV (7-25)	K A L N R K Q R R A L N R S N R L R K E	-
CMV (7-25)	N A S R T S R R R R P R R G S R S A S	-
TAV (7-25)	G G S R R P R R G R R N N N N N N S T	-
SeMV (26-42)	A G R R R N R R R Q R S A V Q Q L	-
SBMV (25-41)	S R R P R N R R R R R S A A R Q P	-
Non-plant viruses		
HIV (34-50)	T R Q A R R N R R R R W R E R Q R	28
λN (1-22)	T R R R E R R A E K Q A Q W K A A	35
Yeast PR6 (129-144)	T R R N K R N R I Q E Q L N R K	22
FHV (35-49)	R R R R N R T R R N R R R V R	-

Figure 3

Arginine-rich RNA-binding motif (ARM) found in plant and nonplant viral CP. The boxed region represents the highly conserved ARM region of each protein. The numbers in the parenthesis refer to amino acid positions within the protein. Data on % helical content is from Tan & Frankel (88) and Choi & Rao (18).

could prevent T = 3 assembly even though the β-annulus was retained (50). Recently, the deletion of the entire β-annulus motif (amino acid residues 48–59) was shown not to affect T = 3 assembly in SeMV CP (76). Thus, the RNA encapsidation mediated by ARM might be an essential prerequisite for assembly into T = 3 capsids.

In the genus *Bromovirus*, the first 25 N-terminal amino acid region of CP is highly basic and is believed to play an important role in RNA recognition during encapsidation (29, 74, 94, 101). The basic environment is due to the presence of a conserved N-terminal ARM (68). The ARM is hypothesized to recognize specific regions in RNA when the peptide is in α-helical form. Vriend et al. (94) suggested that the N-terminal ARM of *Bromovirus* CP is flexible prior to interacting with viral RNA. This flexibility of the N-terminal ARM is of great importance because it provides a mechanism to enhance the probability of interaction between protein and RNA. The large numbers of basic residues within the first 25 N-terminal amino acids in *Bromovirus* CPs [7 arginines and 1 lysine for BMV and 6 arginines and 3 lysines in *Cowpea chlorotic mottle virus* (CCMV)] suggest that interaction

CCMV: *Cowpea chlorotic mottle virus*

between the negative phosphate groups in the RNA and the positive basic amino acids is important for nucleoprotein assembly. Based on in vitro analysis, it has been suggested that many nucleic acid binding proteins provide the best nucleic acid-protein interaction when the protein adopts an α-helical conformation (88).

The importance of the N-ARM of BMV CP in binding to RNA1 in vitro was demonstrated by band shift and UV-cross-linking experiments (27). In these assays strong nonspecific cooperative binding was observed between wild-type CP and RNA1, whereas deletion of first 25 N-terminal amino acids failed to bind RNA1. Analysis in vivo of a comprehensive set of deletion mutants of BMV CP encompassing the N-ARM demonstrated that variants lacking the N-proximal 7, but not 19, amino acids are biologically active and assembled into RNA-containing virions (67, 68, 74). Comparative analysis of total and virion RNA recovered from symptomatic leaves infected with a mutant of BMV RNA3 (Δ919) expressing a defective CP lacking the ARM (located between amino acids 9 through 19) revealed that the N-ARM specifically interacts with the 5′ 943 nt of RNA1, a result consistent with the in vitro binding data of Duggal & Hall (27). In addition, the N-ARM also contains crucial amino acids required for sgRNA4 packaging (18). Evidence that determinants for selective packaging of sgRNA4 are localized within the N-ARM was more apparent when the CP bearing proline substitutions for arginine residues located at positions 10, 13, or 14 were allowed to package in vivo and in vitro (18). Although these studies demonstrate a specific interaction between the ARM region and the RNA during encapsidation in vivo, in vitro binding studies proved otherwise. Studies with model peptides of HIV Rev and Tat proteins have shown that the ARM enables RNA-binding proteins to recognize specific regions in RNA when they are in α-helical conformation (88). However, no correlation between α-helical conformation and RNA binding was observed when wild-type and mutant peptides of BMV CP bearing proline substitutions (expected to have an α-helical conformation) were allowed into bind each of the four BMV RNAs. Furthermore, both wild-type and mutant peptides also effectively bound to heterologous RNAs, suggesting that interaction between BMV CP and RNA is nonspecific (17, 27). One possible explanation for this differential interaction is that under in vivo conditions the binding specificity between the ARM and the RNA is likely to be enhanced by other proteins (e.g., replicase), which results in selective packaging (see below).

The CPs of BMV and CCMV share 70% identity at the amino acid level (85, 101), and hybrid viruses engineered to express heterologous CPs exhibited neutral effects with respect to the host range and packaging (61). The N-proximal region of CCMV CP also contains the N-ARM like BMV CP (**Figure 3**) and is envisioned to be neutral with respect to RNA packaging. However, experimental evidence showed that the ARMs of BMV and CCMV are not functionally analogous with regard to RNA packaging. For example, when a deletion mutation similar to Δ919 was engineered in CCMV CP, the mutation down-regulated packaging of all four viral RNAs and the packaging effect was more pronounced on gRNA1s and gRNA2s than gRNA3 and its CP mRNA (1). Self-assembly kinetics between Δ919 mutant CP subunits and each of the three CCMV genomic RNA accentuated the role of interaction between the N-ARM region and the RNA during packaging (1).

The critical involvement of ARM in viral RNA packaging is also evident from mutational analysis of the CMV CP. *Cucumoviruses* are genetically very similar to *Bromoviruses*, and the N-terminal regions of their CPs also contain a stretch of arginines resembling ARMs (**Figure 3**). Deletion of either the 19 N-terminal amino acids or the cluster of 6 arginine residues completely inhibited packaging, whereas deletion of the first 12 N-terminal amino acids (retaining the cluster of

6 arginine residues) has no effect on packaging (80). Like *Bromovirus*, α-helical conformation is not required for the ARM of *Cucumovirus* CP in order to promote RNA binding because helix-destabilizing proline residues are inherent in these proteins (**Figure 3**).

RNA SEQUENCES INVOLVED IN SELECTIVE PACKAGING

A Monopartite Model: TCV

As discussed above, discrimination of viral genomes from cellular RNA is achieved by the presence of a defined sequence referred to as a packaging signal. Once identified, it is imperative to establish the functionality of the packaging signal by fusing the sequence to an unrelated RNA and testing its competence to package into virions by the homologous CP. Evidence for the existence of a packaging signal in a spherical plant RNA virus was first demonstrated for TCV (63). Initial identification of sequences that are likely to be the packaging signals was based on the assumption that CP subunits bind to a specific RNA sequence to form a complex leading to selective packaging. Consequently, characterization of high-affinity CP-binding sites on TCV RNA identified two distinct regions: one in the polymerase gene about 700 nt from the 5′ end and the other in the CP gene of about 700 nt from the 3′ end (97). Based on these observations, Wei et al. (97) suggested that the efficiency of encapsidation into a relatively small volume requires the participation of two distinct binding sites. Furthermore, the fact that the two distinct sgRNAs of 1.7 kb and 1.45 kb, originating from the 3′ terminus of the genome lacking the 5′ proximal region, are not packaged supports the contention that the binding sites identified at the 5′ region of the TCV genome are involved in packaging. Following this initial in vitro characterization, Qu & Morris (63) performed a series of protoplast experiments demonstrating that a 186-nt fragment at the 3′-end of the TCV CP-coding region is responsible for specific packaging of viral RNA. Further delineation of the region encompassing the packaging signal revealed a bulged hairpin loop of 28 nt to be the most essential element of the packaging core. Confirmatory evidence for the functionality of the TCV packaging signal was established by demonstrating the assembly of a chimeric virus genome consisting of TCV CP coding region and the genetically unrelated RNA genome of TBSV (63). The efficient packaging of TCV satellite RNA (satRNA, sat-C) and DI-RNA, which lack the 5′ end but contain the 16 nt of the critical 28-nt packaging core, argues that the actual sequence element involved in packaging could be smaller than 28 nt. However, surrounding sequences could play a role in stabilizing the functional structure since the 186-nt sequence containing the packaging core failed to promote packaging of heterologous TBSV RNA *in trans* (63). Collectively, these results suggest that selective packaging in TCV is dictated by interaction between the CP and the specific packaging signal.

Two other viruses with monopartite genomes in which the packaging signals are tentatively identified include *Southern bean mosaic virus* (SBMV) and *Turnip yellow mosaic virus* (TYMV). The CP-binding site in the genomic RNA of SBMV was mapped to the position corresponding to the nucleotides 1410–1438 (35). This region was also predicted to form a stem-loop structure, an essential feature required for specific interaction. Likewise in TYMV, packaging of genomic RNA is initiated at two hairpins in the 5′ untranslated region (UTR) (7). However, in these two instances the ability of the predicated sequence to act as an OAS site similar to that of TMV or TCV has not been demonstrated experimentally.

A Bipartite Model: RCNMV

The *Dianthovirus* genus is distinct by virtue of its bipartite RNA genome (**Figure 2*B***). RNA1 encodes for the viral polymerase components (p27 and p88) and the CP (100), whereas

SBMV: *Southern bean mosaic virus*

TYMV: *Turnip yellow mosaic virus*

UTR: untranslated region

RCNMV: *Red clover necrotic mosaic virus*

CRSV: *Carnation ringspot virus*

SCNMV: *Sweet clover necrotic mosaic virus*

RNA2 codes for the MP required for cell-to-cell spread of the infection (99). The CP is expressed from RNA1 by way of a sgRNA whose transcription is induced by the base-pairing of RNA1 to RNA2. This interaction occurs between the *trans*-activator (TA) element found on RNA2 and the TA base-pairing site (TABS) within the subgenomic promoter on RNA1 (**Figure 2*B***) (84). This genome arrangement and CP expression mechanism makes both RNAs interdependent for a productive infection. Pseudorecombinant studies involving the three members of the *Dianthovirus* genus, *Carnation ringspot virus* (CRSV) (the type species), *Red clover necrotic mosaic virus* (RCNMV), and *Sweet clover necrotic mosaic virus* (SCNMV), revealed that virions could be formed with all combinations of RNA1 and RNA2 (59; T. Sit & S.A. Lommel, unpublished data), suggesting that the encapsidation mechanism is highly conserved.

Unlike in monopartite viruses, the situation is more complex when two or more RNAs are involved (**Figure 4**). The CP must be able to recognize and encapsidate each RNA into either a single virion (**Figure 4**, *Model A*) or into separate virions containing each RNA (**Figure 4**, *Model B*). The situation for the dianthoviruses is complex because there is physical evidence to support both models. Purified RCNMV virions sediment as a single peak when subjected to CsCl density centrifugation (33, 38) suggesting that the virion population is physically homologous as shown in Model A. However, viral RNA purified from CRSV and RCNMV virions contains RNA2 in molar excess to RNA1 (~3:1) (36); S. A. Lommel, unpublished data) favoring Model B.

Cross-link formation by UV irradiation has been used to determine the RNA complement within several icosahedral virions (46, 54, 57). Like RCNMV, the genome of FHV is also bipartite, and heating and UV irradiation of FHV virions showed that both genomic RNAs are copackaged into a single virion (46). Heating of RCNMV virions indicated that the majority of genomic RCNMV RNAs are converted into high-molecular-weight complexes upon virion heating at 65°C; these complexes consist of RNA1:RNA2 heterodimers as well as RNA2 multimers (6). Similar results were also obtained when virions were subjected to UV irradiation but the degree of complex formation was much less than that observed from heat treatments (6). The formation of RNA2 homomeric complexes suggests that a percentage of the RCNMV virion population is composed of RNA2 only virions. Based on these observations, hybrid-packaging Model C (**Figure 4**) most likely represents the scheme employed by RCNMV, which satisfies all of the existing data. The difference in total RNA content between virions containing RNA1 + RNA2 and those containing RNA2 only is 455 nucleotides or ~8%. This difference would not be resolvable on CsCl density gradients based on the inability to separate the

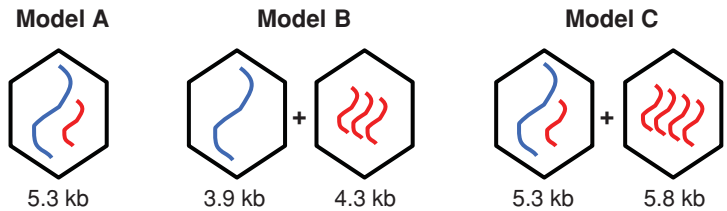

Figure 4

Various schemes for the packaging of RCNMV genomic RNAs into virions. Model A displays only one virion type containing a copy of each RNA together in a single virion. Model B displays two virion types: one with a single copy of RNA1 and the other with three copies of RNA2. Model C, a hybrid of Models A and B, displays two virion types: one with a copy of each of RNA1 and RNA2 and the other with four copies of RNA2.

three distinct virions of BMV, which differ by as much as 11% total RNA per virion (48). In order to maintain the observed ~3:1 ratio of RNA2:RNA1, virions containing both genomic RNAs would need to be present in two thirds of the population, whereas the other third would consist of virions containing four copies of RNA2.

Origin of assembly. Based on observations of the RNA content in virions, RNA2 would seem most likely to contain the OAS for RCNMV since both virion populations contain RNA2. However, the presence of an OAS element on RNA1 cannot be dismissed. To uncouple the requirement of RNA2 for expression of the CP (84), the RCNMV CP ORF was expressed from a heterologous TBSV vector, pHST2 (81). Co-inoculation experiments with this CP-expressing construct (pHST2-RCP) and RCNMV RNA1 were performed to rule out the possibility that RNA1 contains an independent OAS element for producing virions; absence of virion formation suggested that RNA2 contains the minimal OAS element for RCNMV (6).

During the original characterization of the TA element, a TBSV vector construct referred to as pHST2-ΔBX (6) expressing a 209-nt region of RCNMV RNA2 was shown to induce CP expression when coinoculated with RCNMV RNA1 (84). Surprisingly, this coinoculation produced virions, albeit at only 15% of the wild-type level (100 μg/g leaf tissue) (6). RT-PCR on purified virion RNAs revealed the presence of both RNA1 and the sgRNA derived from pHST2-ΔBX. The pHST2-ΔBX construct was shortened to produce truncated sgRNAs that were more similar to the actual size of RNA2 in an attempt to increase the efficiency of virion production. Construct pHST2-ΔBP, which most closely resembled RNA2 in size, gave the best yield of virions at ~60% of wild-type RCNMV (6). This finding establishes that the RCNMV virions have been tailored to accept a certain amount of RNA for optimal packaging and that deviation above or below this level decreases the packaging efficiency.

Further dissection of the 209-nt region expressed from pHST2-ΔBP was performed directly on RCNMV RNA2 because this was the optimal template for assembly. Subtle changes in packaging efficiency would be more apparent with the wild-type RNA2 now that the region involved had been delineated. This 209-nt region of RNA2 contains several stem-loop structures as well as the TA element involved in CP expression (84). Deletion mutagenesis of each structural element individually or in combinations had no appreciable effect on virion formation (5). The only mutation that had a drastic effect was the deletion of the TA element itself which abolished virion formation completely even in the presence of pHST2-RCP supplying CP *in trans*. However, it could not be ruled out that this was not a direct consequence of the TA element's *cis*-acting role in the replication of RNA2 itself (89). To uncouple this replication requirement, the TA element was expressed from pHST2 as a shortened sgRNA (pHST2-SLΔNA) (6), which was then able to copackage with RCNMV RNA1 into virions. This demonstrated that the minimal specific OAS element for RCNMV was the TA element and that copackaging of RNA1 with RNA2 is most likely due to the TA-TABS interaction between the genomic RNAs. Furthermore, the presence of RNA2 only virions could be rationalized since these RNAs contain the OAS element.

A Tripartite Model: BMV

The BMV life cycle is typical of (+) strand, eukaryotic RNA viruses and its replication is entirely cytoplasmic. Virions purified from *Bromovirus*-infected plants measure 28 nm in diameter with icosahedral morphology and contain three gRNAs and a single sgRNA (**Figure 2*C***) (70). Estimated molecular weights of CP and the encapsidated viral RNAs together with the sedimentation values of purified virions suggested that physically

all four RNAs cannot be encapsidated into a single virion. Therefore a logical configuration accommodating all four RNAs would be that gRNA1 and gRNA2 are packaged independently, whereas genomic RNA3 and its sgRNA4 are copackaged into a third virion (**Figure 2*C***) (70). Despite variation in RNA size, it is remarkable to note that all three virions are morphologically indistinguishable and physically inseparable. Consequently, the distribution of four RNAs into three virions is tightly regulated during infection, and the mechanism that controls this encapsidation phenomenon is currently obscure.

TLS-mediated assembly. In BMV, the division of function among three different RNA species together with our ability to reconstitute the virus in vitro from dissociated CP subunits and RNA under a variety of physiological conditions has helped determine the mechanism of genome packaging in an RNA virus. All four BMV RNAs contain a highly conserved 200-nt 3′ UTR sequence that can be folded to mimic TLS. In addition to functioning as a minus-strand promoter, the 3′ UTR is also an active template for tRNA-associated activities such as aminoacylation and adenylation by nucleotidyltransferase (25). Even though the highly conserved 3′ TLS of the four BMV RNAs would be a likely candidate for harboring sequence elements involved in packaging, no experimental evidence supports this possibility. However, the intrinsic involvement of TLS in packaging BMV RNA became apparent when transcripts of each genomic and sgRNA lacking the 3′ TLS were used as substrates for in vitro assembly (16). A series of in vitro assembly assays revealed that (*i*) BMV RNAs lacking the 3′ TLS were incompetent for assembly into virions; (*ii*) addition of a 200-nt sequence encompassing the TLS *in trans* restored the assembly of truncated RNAs; (*iii*) TLS-mediated assembly is dependent on its structure and independent of sequence since TLS from two other plant viruses, CMV and TMV, as well as tRNAs from wheat germ and yeast, were also active in promoting BMV assembly; and finally, (*iv*) *trans*-complementing homologous and heterologous TLS are not encapsidated (16). The ability of tRNA to support the encapsidation of BMV RNA *in trans* raises the possibility that host RNAs play a role in virus assembly. The fact that no capsids are found in vivo favors the hypothesis that plant or viral TLS acts as a scaffold for nucleating CP dimers to form pentamers of dimers, the building blocks of icosahedral virions (103). Furthermore, the nonspecific interaction between the CP and the viral TLS (as well as host tRNAs) may serve to stabilize these pentamers or productive complexes of pentamers in favor of nonproductive aggregates that fail to associate with a genomic RNA and progress to full virions.

The requirement of TLS in the promotion of RNA packaging appears to be distinct among the members of the *Bromoviridae*. For example, in vitro assembly studies with dissociated CP subunits of CCMV revealed that unlike BMV RNA3, which requires a bipartite signal for packaging (20), assembly of CCMV RNA3 into virions is independent of sequences encoding either MP ORF, CP ORF, or 3′ noncoding regions (2). Why do BMV and CCMV employ two distinct packaging mechanisms? BMV and CCMV have evolved to infect monocotyledonous and dicotyledonous hosts, respectively, and the requirement of viral genes that dictate this process is also distinct (64). Consequently, these viruses must utilize different mechanisms to transport their genomes across different cell types. Analysis of movement characteristics of CCMV revealed that it can be transported efficiently between cells without CP (64), suggesting that assembly of RNA-containing virions is neither a priority nor a prerequisite for CCMV. By contrast, BMV is transported between cells only in virion form (79), and therefore BMV has evolved to display specialized mechanisms of RNA packaging different from that of CCMV. Under this scenario, a mechanism involving the

recognition of virus-coded sequence-specific packaging signal by CP ensures that assembled virions exclusively contain viral progeny prior to cell-to-cell transportation. However, this packaging requirement for movement of BMV is not monocotyledonous specific since encapsidation-defective variants of BMV are also incompetent for cell-to-cell movement in dicotyledonous hosts such as *Chenopodium quinoa* (79) and *Nicotiana benthamiana* (64).

Earlier studies showed that a 3′ 145-nt region of all three gRNAs of *Alfalfa mosaic virus* (AMV) shows >80% sequence similarity and can adopt stem-loop structures but not a TLS (8). However, recent biochemical and structure probing studies suggested that the 3′ 112-nt region can be folded into two alternate structures: a CP-binding conformer consisting of a series of stem-loop structures and a TLS. Available evidence indicates that the CP-binding conformer and TLS represent a molecular switch that regulates the transition from translation to replication (60). Because of the sequence similarity and the TLS conformation, the 3′ end can be envisioned to participate in packaging similarly to BMV. However, expression of AMV RNA1 and RNA2 devoid of the 3′ UTR together with full-length RNA3 resulted in the packaging of the truncated RNAs in agroinfiltrated leaves (93). The 3′ TLS of RNA3 likely plays a *trans*-acting role during packaging, in a fashion similar to that of BMV.

It is hard to reconcile the specific distribution of four BMV RNAs into three morphologically indistinguishable virions, if viral 3′ TLS or tRNAs were the only elements that interact with BMV CP and promote packaging. It would make more sense if gRNA1 and gRNA2 had their own OAS. By contrast, the entire sequence of the sgRNA4 is located in the 3′ half of genomic RNA3, and RNA3 and RNA4 are copackaged into a single virion. This suggests that the commonly shared CP ORF region may contain the packaging signal. But in vitro assembly assays showed that deletion of CP ORF has no significant effect on RNA3 packaging (20). A finer deletion analysis of other regions of RNA3 revealed that in addition to 3′ TLS, a *cis*-acting, position-dependent 187-nt region present in the MP ORF is also an essential constituent of the packaging core (20). As demonstrated for TCV (63), the functionality of the packaging signal in BMV RNA3 was established by assessing the packaging competence of a genetically engineered chimeric RNA molecule. When the 3′ TLS and the 187 nt from MP ORF are fused to the *Xenopus* sequence, BMV CP subunits packaged the heterologous sequences with the same efficiency as control wild-type BMV RNA3 (20). These observations were extended to the in vivo level by transfecting protoplasts with a variant of BMV RNA3 that lacks a portion of the MP ORF encompassing the packaging signal. Despite efficient replication and synthesis of CP mRNA, packaging of variant progeny was prevented (20).

AMV: *Alfalfa mosaic virus*

Even though RNA3 and RNA4 are predicted to copackage, virions containing only RNA4 can be assembled efficiently in vitro (16). Analysis of RNA distribution in purified virions obtained from natural infections revealed that 94% of virions contained one molecule each of RNA3 and RNA4, whereas only 6% contained three molecules of RNA4 (39). This suggested that RNA4 is incompetent for efficient autonomous assembly in vivo. However, recall the observation that BMV RNA3 packaging is mediated by a signal specifically encoded within the MP ORF that is absent in RNA4; its deletion debilitated packaging not only of RNA3 but also of RNA4 when analyzed in vivo (20), raising the question of how RNA3 and RNA4 are copackaged into a single virion.

Based on existing information, two models can be proposed to explain copackaging of RNA3 and RNA4. The first model, referred to as the sequential packaging model (**Figure 5**), envisages that RNA3 and RNA4 copackage sequentially in a fashion similar to that proposed for bacteriophage Φ6 (56). In this model, binding of wild-type CP subunits to the bipartite signal (i.e., 3′ TLS and the

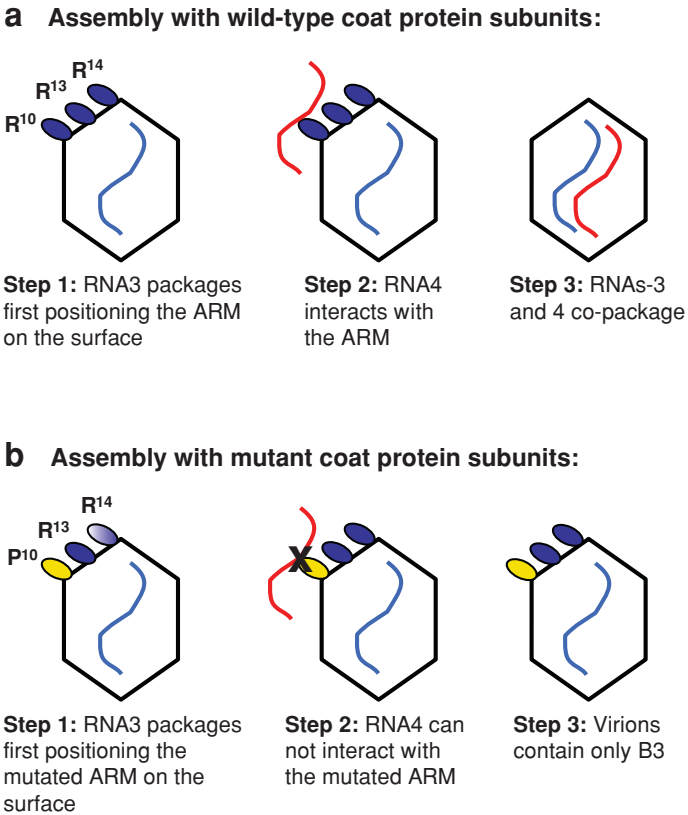

Figure 5

A schematic model for sequential packaging of BMV RNA3 and RNA4 into a single virion. For details see Choi & Rao (20). [Image was redrawn from (20) with permission.]

MP packaging signal) of RNA3 results in prior packaging of RNA3 into a single virion (**Figure 5*a***). This provides an architectural structure to the virion such that the basic N-ARM of the CP is displayed on the surface of the virion (**Figure 5*a***), similar to that observed for FHV (9). Such a surface conformation allows specific interaction of RNA4 with the required arginine residues (**Figure 5*a***), promoting copackaging of RNA3 and RNA4 into a single virion (**Figure 5*a***). As observed previously in vivo (18), a mutation in the N-ARM (e.g., R10→P10; **Figure 5*b***) does not affect prior packaging of RNA3 but the RNA4 interaction is severely disrupted (**Figure 5*b***). As a consequence, the virion will contain only RNA3 (**Figure 5*b***).

The underlying principle of an alternate concerted packaging model derives from our observations that deletion of the packaging signal blocked not only RNA3 packaging but also packaging of RNA4 (20). Since RNA4 has no specific packaging signal (P. Annamalai & A. L. N. Rao, unpublished data), the packaging signal of RNA3 could be acting *in trans* to form a complex with RNA4 resulting in co-packaging. We predict that formation of this initiation complex is mediated either through RNA-RNA interactions or the CP subunits (upon binding to the packaging signal) functioning directly or indirectly as chaperones to promote RNA3 and RNA4 copackaging.

Role of viral replicase in RNA packaging. Most in vivo packaging studies performed with Bromoviruses relied heavily on either whole plants or protoplasts. The inherent limitation of these in vivo approaches is the inability to dissect replication from packaging because high-level expression of individual genome components and their assembly into virions cannot be accomplished without involving complete replication. This limitation, however, can be circumvented by agroinfiltration (3, 32, 52), a transient gene expression strategy mediated by Agrobacterium. Delivery of cultures of Agrobacterium transformants into whole leaves by infiltration (Agroinfiltration) results in transfer of transgenes from the T-DNA region of the bacterial Ti plasmid into the plant cells. The non-integrated T-DNA copies remaining transiently in the nucleus can be transcribed, leading to transient expression of T-DNA genes (**Figure 6*a***). Most important, agroinfiltration facilitates synchronized delivery of several transgenes to be coexpressed into the same cell from different Agrobacterium transformants (**Figure 6*b***) (3, 52, 93). As discussed below, transient expression of BMV RNAs and CP subunits in *N. benthamiana* leaves (3) revealed that RNA packaging in BMV is likely to be regulated by replication-independent and -dependent mechanisms.

In addition to participating in the synthesis of genomic negative and positive RNAs, BMV replicase has been implicated in other roles. For example, selected variants of BMV

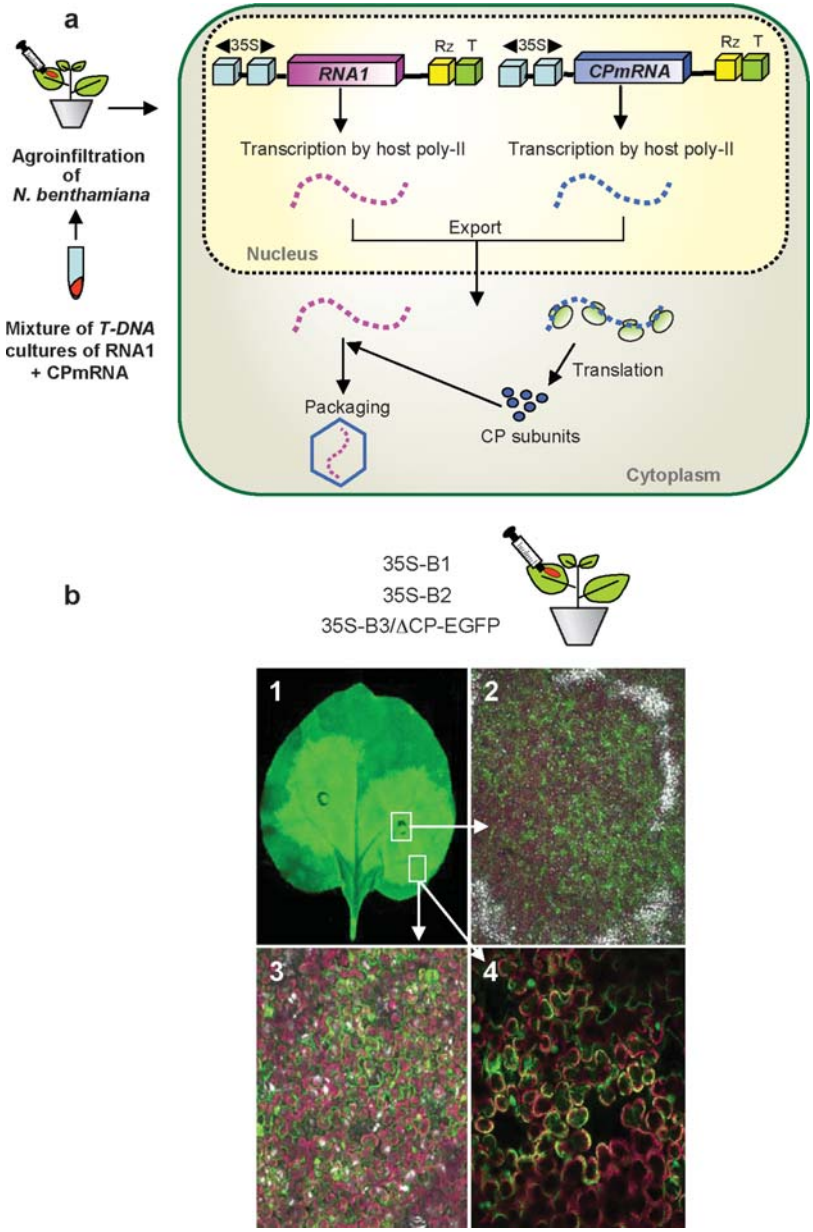

Figure 6

(*a*) Schematic illustration of agroinfiltration concept for studying genome packaging in RNA viruses. 35S, CaMV double 35S promoter; Rz, ribozyme; T, *Nos*-terminator. (*b*) Evidence showing multiple plasmids can be delivered to a single cell. In this experiment, *N. benthamiana* leaves were coinfiltrated with a mixture of wt BMV RNA1 and RNA2 and an RNA3 variant containing the EGFP gene in the place of CP; EGFP expression is contingent on BMV replication. Since BMV infection cannot spread cell-to-cell without CP, EGFP expression demonstrates that the majority of cells received all three BMV plasmids. (*Panel 1*) Macroscopic image of infiltrated leaf showing EGFP expression; (*Panel 2*) microscopic image of EGFP expression at the infiltrated site; (*Panels 3* and *4*) EGFP expression in an area distant from the infiltrated site. For details see Annamalai & Rao (3). [Images are reproduced from (3) with permission.]

with defined mutations in the 2a gene, capable of efficient replication in protoplasts, failed to support systemic infection in whole plants (90). Analysis of a series of packaging assays performed using the transient expression system revealed two critical roles for viral replicase. First, it functions as a "specificity filter" by blocking host RNA packaging such that the assembled virions are enriched in viral RNA. Packaging of BMV genomic RNAs is not replication contingent because autonomously expressed full-length BMV genomic RNAs or replication-defective TLS-less RNA1 and RNA2 are efficiently packaged into virions by CP expressed *in trans* (3). However, virions assembled in the absence of a functional replicase also contained cellular RNAs, in addition to the expected genomic RNAs (3); this suggests that some sort of coupling exists between functional viral replicase and the specificity of RNA packaging. A scenario similar to these observations was reported in two other systems. Expression of either SeMV CP in *Escherichia coli* or FHV CP in baculovirus led to the assembly of VLPs that encapsidated host RNAs in addition to expected CP mRNA (50, 92). A second function of viral replicase during in vivo assembly is to maintain the physical homogeneity among progeny virions. When *N. benthamina* leaves were infiltrated with either BMV RNA4 alone or coinfiltrated with either RNA1 or RNA2 or RNA3, the assembled virions exhibited polymorphic forms (3). By contrast, assembly in the presence of a functional replicase, i.e., RNA4 coinfiltrated with RNA1 and RNA2 or all three wild-type BMV RNAs, resulted in a physically homogeneous virion population (P. Annamalai & A. L. N. Rao, unpublished data). Similar virion polymorphism was observed when SeMV or FHV CP was autonomously expressed in heterologous systems (50, 92).

How do replicase and CP interact to selectively package viral RNAs? The cytopathological studies of bromoviruses revealed the induction of vesicles (31) or spherules (82) in the perinuclear spaces of infected cells. These specialized structures are induced by replicase protein 1a and have been shown to be the actual sites of RNA synthesis (82). Replicase protein 2a interacts with 1a and recruits RNA to spherules to initiate viral replication (82); the necks connecting spherules serve as channels to export newly synthesized progeny RNA to the cytoplasm for translation. Because CP has been found to copurify with active replicase complex (12), it is reasonable to hypothesize that CP must be synthesized near the necks of spherules. Consequently, a transient association of functional viral replicase with CP can be envisioned to increase specificity of BMV RNA packaging. Absence of a functional replicase decreases CP specificity, resulting in nonspecific packaging of cellular RNAs (3). Alternatively, CP could have been translated elsewhere in the cell and transported to the vicinity of spherules by the functional replicase to increase specificity of RNA packaging.

In BMV, sgRNA4 is synthesized from negative-strand RNA3 by internal initiation (55). Even though sgRNA4 contains the minus-strand promoter region, absence of negative strand RNA4 (26, 66) suggests that it never enters the replication cycle. Consequently, the transcription of sgRNA4 is entirely dependent on the synthesis of (−) RNA3 (55). Although BMV RNA3 and RNA4 are copackaged into a single virion in 1:1 ratio, RNA4 is competent for autonomous packaging in vitro (16, 18). However, in vivo packaging of RNA4 is contingent on RNA3 packaging (20). Recent agroinfiltration experiments also revealed that only newly transcribed RNA4 is competent for efficient packaging (P. Annamalai & A. L. N. Rao, unpublished data). A similar situation was encountered with TYMV.

Expression of wild-type TYMV genomic RNA in *N. benthamiana* via agroinfiltration resulted in normal infections. The typically observed combination of virions and capsids of 30-nm was seen by electron microscopy, and a ribonuclease protection assay indicated that most of the viral gRNAs and sgRNAs present in the leaf tissue were encapsidated.

To study the requirements for TYMV RNA encapsidation, TYMV CP was expressed *in trans* from a specialized mRNA, while a nonreplicating gRNA with authentic 5′ and 3′ termini was separately expressed. The nonreplicating genome had a mutation in the active site of the RNA-dependent RNA polymerase. Although the nonreplicating gRNA was present at much lower levels (1 μg/g leaf tissue) than the replicating genome (1 mg/g leaf tissue), most of the RNA present in leaves was encapsidated. Truncation of the gRNA upstream of the 3′ TLS did not alter efficiency of encapsidation. These results demonstrate that efficient encapsidation of the TYMV genomic RNA does not require a coupling to replication and that the TLS is not needed for encapsidation. The coat protein mRNA expressed separately via agroinfiltration in these studies was not encapsidated. This mRNA differed from authentic subgenomic RNA by the addition of a translational enhancer derived from TMV RNA in the 5′-untranslated region. At present, it is not known whether the failure to encapsidate is due to the altered 5′-end or a requirement that the subgenomic RNA be produced by replication (T-J Cho & T. W. Dreher, unpublished data). Collectively, these observations suggest that unlike the genomic RNAs of BMV and TYMV, the sgRNAs in these two viral systems are packaged only when they are newly transcribed from the gRNA. A scenario parallel to these observations was described for polio virus replicons (58), Kunjin virus [a member of the family *Flaviviriade* (44)], and FHV (92) in which only the replicated RNAs were found to be packaged by the CP. Therefore a mechanism that couples replication and packaging in a fashion similar to that observed for polio and Kunjin viruses is also likely to regulate packaging of sgRNAs in BMV and TYMV.

SUMMARY POINTS

1. Genome packaging into stable virions involving protein-protein and protein-RNA interactions is an important phase in the viral infection cycle since the majority of plant viruses are disseminated in assembled form by insect vectors.
2. Genome packaging in vivo is a carefully orchestrated process in that viral RNAs are selectively distinguished from other cellular RNA molecules present in the compartment in which assembly takes place.
3. Packaging of viral genome involves CP and sequence-dependent and sequence-independent RNA interactions.
4. A highly conserved ARM of viral CP has been found to contain determinants that specifically interact with viral RNA during packaging.
5. The requirement of TLS-mediated assembly appears to be distinct between BMV and CCMV and this may have parallels in the mechanism of cell-to-cell movement.
6. Recent in vivo studies showed that expression of a biologically active replicase enhances selective packaging of viral progeny RNA by functioning as a specificity filter.
7. Viral replicase also plays an important role in maintaining particle homogeneity in multicomponent *Bromoviruses*.

FUTURE ISSUES

1. The relationship between replication and packaging should be dissected for monopartite and multipartite viruses with the help of the agroinfiltration approach.

2. The biochemical interaction between viral replicase and CP subunits and their localization at the subcellular level is likely to be an important future research avenue to elucidate the extent of replicase involvement in packaging.
3. The relative packaging competence between CP subunits expressed in replication-independent and replication-dependent mode needs to be examined to resolve the intimacy between replication and packaging.

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