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Dispensability of 3′ tRNA-like sequence for packaging cowpea chlorotic mottle virus genomic RNAs

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Abstract

The 3′ ends of three genomic RNAs (gRNAs) of cowpea chlorotic mottle virus (CCMV) terminate in a highly conserved tRNA-like structure (3′ TLS). To examine the intrinsic role played the 3′ TLS in packaging, the competence of each gRNA lacking the 3′ TLS (ΔTLS-gRNA) to interact with dissociated coat protein (CP) subunits and form virions was assayed in vitro. In contrast to the well established requirement for the participation of either viral 3′ TLS or host-tRNAs in the assembly of RNA-containing virions in brome mosaic virus (BMV; Choi, Y, G., Dreher, T. W., Rao, A. L. N. 2002. tRNA elements mediate the assembly of an icosahedral RNA virus. Proc. Natl. Acad. Sci. 99, 655–660), CCMV CP does not require the presence of viral TLS in cis or in trans. Similar in vitro assembly assays showed that CCMV CP subunits also packaged BMV RNAs lacking 3′ TLS as well as two other non-bromoviral RNAs although with lesser efficiency. To characterize sequences of CCMV RNA3 (C3) required for packaging, a series deletions was engineered into C3 and their effect on virus assembly was examined. It was observed that, unlike BMV RNA3 whose packaging requires a bipartite signal (Choi, Y. G., Rao, A. L. N. 2003. Packaging of brome mosaic virus RNA3 is mediated through a bipartite signal. J. Virol. 77, 9750–9757), packaging of C3 is independent of either movement protein (MP) ORF or CP ORF or 3′ non-coding regions. Based on the differential prerequisites identified in this study for the assembly of BMV and CCMV, we hypothesize that the adaptive condition for movement in monocotyledonous host has made packaging a necessary co-requirement for BMV.

Keywords: Bromoviruses; tRNA-like structure; Virus assembly; RNA packaging

Introduction

The bromoviruses, *Brome mosaic virus* (BMV) and *Cowpea chlorotic mottle virus* (CCMV), are members of a group of icosahedral plant viruses whose genomes are divided among three, separately encapsidated, messenger-sense RNAs designated RNA1 (3.1–3.2 kb), RNA2 (2.8–3.0) and RNA3 (2.1–2.7 kb). Monocistronic genomic RNAs 1 and 2 encode non-structural proteins that are required and sufficient to direct RNA replication (Kao and Sivakumaran, 2000) and share extensive amino acid similarity with nonstructural proteins encoded by animal alphaviruses and several other plant viruses (Ahlquist et al., 1985). Genomic RNA3 is dicistronic. The 5′ ORF encodes another non-structural movement protein (MP) of 30 kDa that is required for cell-to-cell movement (Mise et al., 1993; Osman et al., 1999). The 3′ half encodes the capsid protein (CP) gene that is expressed via subgenomic RNA synthesized from (−) RNA3 progeny by internal initiation (Miller et al., 1985). Although the subgenomic RNA is genetically redundant, it is efficiently packaged into virions. The three genomic and single subgenomic RNA of BMV and CCMV are distributed into three morphologically and physically identical capsids 28 nm in diameter (Rao, 2001). Genomic RNAs 1 and 2 are packaged independently, whereas genomic RNA3 and subgenomic RNA4 are co-packaged into a third capsid. Over the years, bromoviruses served as models for understanding the mechanism of virus assembly in vitro and in vivo (Fox et al., 1994; Rao, 2001).

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Genomes of BMV and CCMV have been sequenced and many events leading to efficient replication, assembly and whole plant infection have been thoroughly studied (Rao, 2001). The highly conserved aminoacylatable 3′ non-coding regions of BMV and CCMV RNAs can be folded to a multifunctional tRNA-like structure (TLS). This region harbors recognition signals for the initiation and synthesis of progeny minus strands by viral replicase (Dreher, 1999; Kao and Sivakumaran, 2000). This region is specifically amino-acylated with tyrosine during an infection in cells (Dreher, 1999). In addition, the TLS also serves as a 3′ telomere by recruiting the tRNA-specific host CCA nucleotidyltransfer-ase to maintain intact 3′ CCA termini (Kao, personal communication; Rao et al., 1989). Recently, Choi et al. (2002) observed that (i) BMV genomic and subgenomic RNAs lacking the 3′ TLS failed to assemble in vitro into virions and this defective assembly could be rescued by the addition of a 201-nt sequence encompassing the TLS to the assembly mixture; (ii) tRNAs of wheat germ and yeast supplied in trans were similarly active in promoting the assembly of truncated BMV RNAs into virions and (iii) virions assembled from truncated BMV RNAs in the presence of tRNAs or TLS-containing short sequences did not incorporate the latter molecules. Based on these observations, it was hypothesized that the highly conserved 3′ TLS serves as a chaperone, in a transient association with virion CP, functioning as nucleating element to initiate the assembly of viral RNA into BMV virions (Choi et al., 2002). Here, we report that despite having a highly conserved 3′ TLS, unlike BMV, in vitro packaging of CCMV RNAs into virions is independent of 3′ TLS. We also show that, by contrast to BMV RNA3 which requires a bipartite packaging signal consisting of 3′ TLS and a specific region within the MP ORF (Choi and Rao, 2003), packaging of CCMV RNA3 is independent of either 3′ TLS or MP or CP ORF regions.

Results

CCMV gRNAs lacking 3′ TLS are competent for assembly into virions

The structure of three CCMV gRNAs is shown in Fig. 1. Like BMV, the 3′ non-coding region of approximately 200 nt present on each CCMV gRNA is highly conserved and assumes a TLS (Fig. 1). Synthesis of TLS-less transcripts (ΔTLS) was facilitated by linearizing each wt plasmid at a unique restriction site present within the 3′ non-coding region (Fig. 1). Consequently, transcripts of C1ΔTLS, C2ΔTLS and C3ΔTLS are characterized by lacking the 3′ 158 nt, 209 nt, and 220 nt respectively and exhibited distinguishable electrophoretic mobility profile (Fig. 2A).

Crystallographically similar capsids of BMV and CCMV are assembled from 189 and 190 amino acids respectively

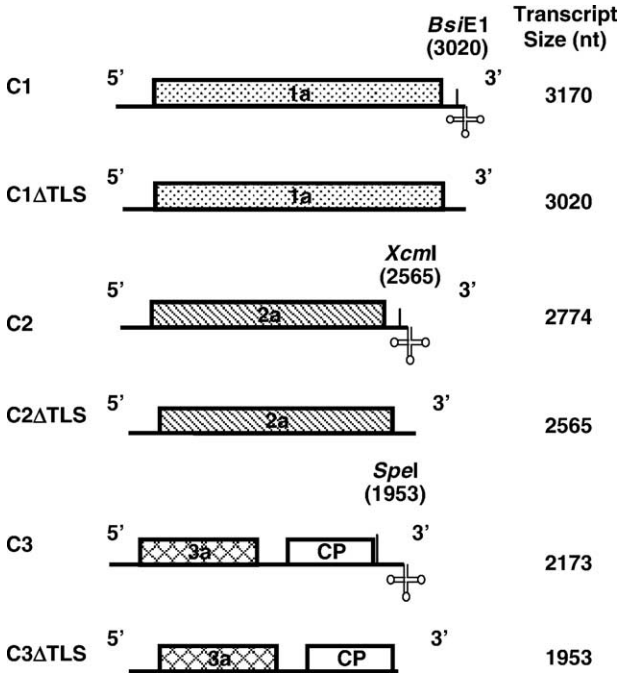

Fig. 1. Schematic representation of wt plasmid clones of CCMV genomic RNAs 1 (C1), 2 (C2) and 3 (C3) and their respective variants lacking 3′ TLS region (C1ΔTLS, C2ΔTLS and C3ΔTLS). The non-coding regions are represented as solid lines and coding regions as rectangular boxes. The clover leaf like structure at the 3′ end represents the highly conserved TLS. The lengths of wt and ΔTLS sequences are shown. The restriction enzyme shown at the 3′ end of each wt clone represents the site for linearization prior to in vitro transcription.

(Lucas et al., 2002; Speir et al., 1995). Despite only one amino acid difference in length, dissociated CPs of BMV and CCMV are readily differentiated by the respective electrophoretic mobility pattern on SDS-PAGE (Fig. 2B). Both viruses are amenable to in vitro re-assembly from dissociated CP and RNA preparations (Choi et al., 2002; Fox et al., 1994). Under low salt and neutral pH conditions, dissociated CP subunits of BMV and CCMV assembled into virions only in the presence of RNA (Fig. 2C). While mapping BMV RNA sequences required for packaging by in vitro assembly assays, we (Choi et al., 2002) observed that BMV RNAs lacking 3′ TLS are incompetent for assembly into virions; however, this defect was complemented by the TLS of either viral or host origin in trans (Choi et al., 2002). Since BMV and CCMV RNAs terminate in highly conserved TLS, we sought to examine, whether, like BMV, CCMV RNAs are also dependent on TLS for their incorporation into virions. Therefore, the packaging competence of each ΔTLS-gRNA (Fig. 1) was examined by incubating respective RNA transcripts with dissociated wt CP subunits under physiological conditions that direct in vivo packaging specificity. Assembly assays were performed with wt gRNAs as control substrates. Final products of each assembly reaction were subjected to electron microscopic (EM) examination and packaging efficiencies were determined as described under Methods. Results of these experiments are summarized in Figs. 3 and 4.

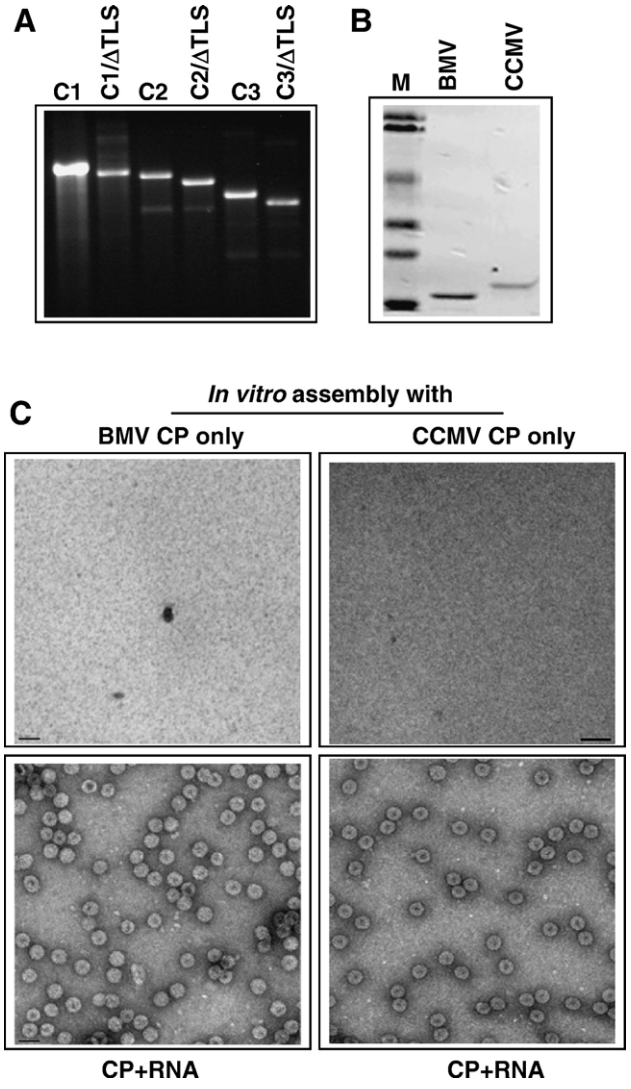

Fig. 2. (A) Agarose gel electrophoretic analysis of wt and ΔTLS-gRNAs used for in vitro assembly assays. Approximately 0.5 to 1 μg of non-capped RNA transcripts synthesized in vitro are subjected to 1% native agarose gel electrophoresis and stained with ethidium bromide prior to photography. (B) SDS-PAGE analysis of dissociated CP subunits used for in vitro assembly. Molecular weight standards (M) are shown to the left. (C) Assessment of assembly competence of dissociated CP subunits of BMV and CCMV with respective native virion RNA. Prior to EM analysis, samples assembled with RNA were diluted 1:100, whereas those containing only CP remain undiluted. Appropriately diluted virion samples were applied to glow discharged carbon coated grids and negatively stained with 1% uranyl acetate prior to examination with a FEI Tecnai12 transmission electron microscope. Scale bar = 50 nm.

EM examination revealed that the size and morphology of virions assembled with each ΔTLS-gRNA could not be distinguished from their respective wt control substrates (Fig. 3A). Furthermore, the assembly efficiency of each ΔTLS-gRNA paralleled wt control (Fig. 4A; panel I). This is in marked contrast to the data obtained with ΔTLS-gRNA of BMV (Choi et al., 2002). Northern blot analysis of RNA isolated from these in vitro assembled virions confirmed the packaging each ΔTLS-gRNA into virions (Fig. 3B).

TLS supplied in trans has no effect on the enhancement of assembly of truncated CCMV gRNAs

We (Choi et al., 2002) and others (Cuillel et al., 1979) have previously demonstrated that virion assembly in vitro is stimulated when tRNAs are added to an assembly mix containing BMV CP subunits and RNAs. Although the above presented experiments demonstrated the dispensability of TLS in promoting the assembly of CCMV RNAs into virions (Fig. 3), we examined whether tRNAs (host as well as viral origin) would stimulate virion assembly in a manner similar to that observed for BMV. Therefore RNA transcripts of 3′ 200 nt sequence encompassing the TLS of CCMV RNA3 were synthesized from pT7T3CC3t (Osman et al., 1998) and added in equimolar ratio to an assembly mixture containing transcripts of each ΔTLS-gRNA and wt CP subunits. When the assembly efficiency was determined in each case, surprisingly no significant difference was observed relative to the control assays performed without 3′ TLS (Fig. 4A, panel II). Similar results were obtained when wheat germ tRNA preparations were added to the assembly reactions containing ΔTLS-gRNA (data not shown).

Competence of wt and ΔTLS-gRNAs of BMV and CCMV assembled with homologous and heterologous CP subunits

The CPs of BMV and CCMV exhibit 70% identity in amino acid sequence and the first N-terminal 25 amino acids are basic due to the presence of a highly conserved arginine-rich RNA binding motif (Rao and Grantham, 1996). Furthermore, the two CPs are functionally interchangeable and the resulting hybrid viruses displayed neutral effects with respect to movement, RNA packaging and host range (Osman et al., 1997). Despite these conserved properties, CP subunits of CCMV, but not BMV, packaged ΔTLS-gRNAs (Fig. 3; Choi et al., 2002). Therefore, to determine whether ΔTLS-gRNAs of BMV can be assembled by CCMV CP subunits, the assembly of wt and ΔTLS-gRNAs of BMV with heterologous CP was assayed in vitro. It was observed that although CCMV CP subunits efficiently packaged wt BMV gRNAs, the packaging efficiency for each ΔTLS-gRNA was reduced by 80%, 55% and 68% respectively for B1ΔTLS, B2ΔTLS, and B3ΔTLS (Figs. 3A, 4A, panel III).

Assembly assays with CCMV CP showed that packaging of homologous gRNAs is independent of 3′ TLS (Fig. 3). To verify whether the TLS dependent assembly of RNAs is limited to BMV CP, in vitro assembly assays were performed with BMV CP subunits and ΔTLS-gRNAs of CCMV. In these assays, wt CCMV RNAs and ΔTLS-gRNAs of BMV were used as control substrates. Interestingly, BMV CP subunits efficiently packaged both wt and ΔTLS-gRNA of CCMV (Fig. 4B, panel I). Consistent with previous observations (Choi et al., 2002), only the wt BMV

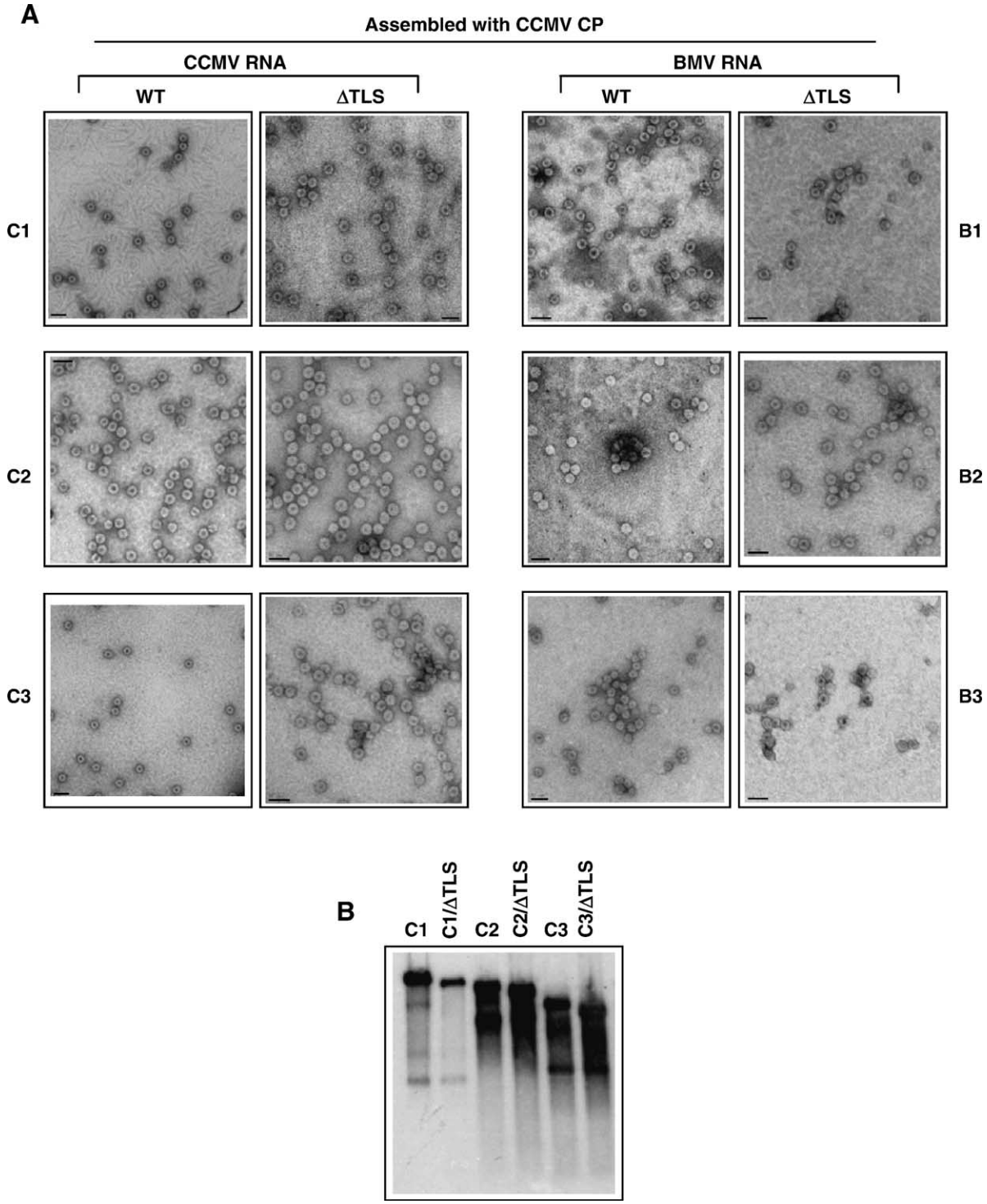

Fig. 3. In vitro assembly assays with dissociated CP subunits of CCMV. (A) Electron micrographic images showing the negatively stained virion preparations assembled in vitro from purified CP subunits of CCMV and indicated wt and ΔTLS-gRNAs of either CCMV or BMV. Samples for EM analysis are prepared as described under Fig. 1. Scale bar = 50 nm (B) Northern blot analysis of RNA isolated from in vitro assembled virions. RNA samples extracted from assembled virions were denatured and subjected to hybridization with a mixture of riboprobes specific for each genomic RNA component.

RNAs, but not ΔTLS-gRNAs, were packaged by BMV CP subunits (Fig. 4B, panel II). Collectively, these observations suggest that the interaction between CP subunits and genomic RNAs of BMV are highly specific requiring a TLS.

Characterization of CCMV RNA3 sequences required for packaging

In BMV and CCMV, genomic RNA3 and its subgenomic RNA4 are predicted to co-package into a single virion (Rao,

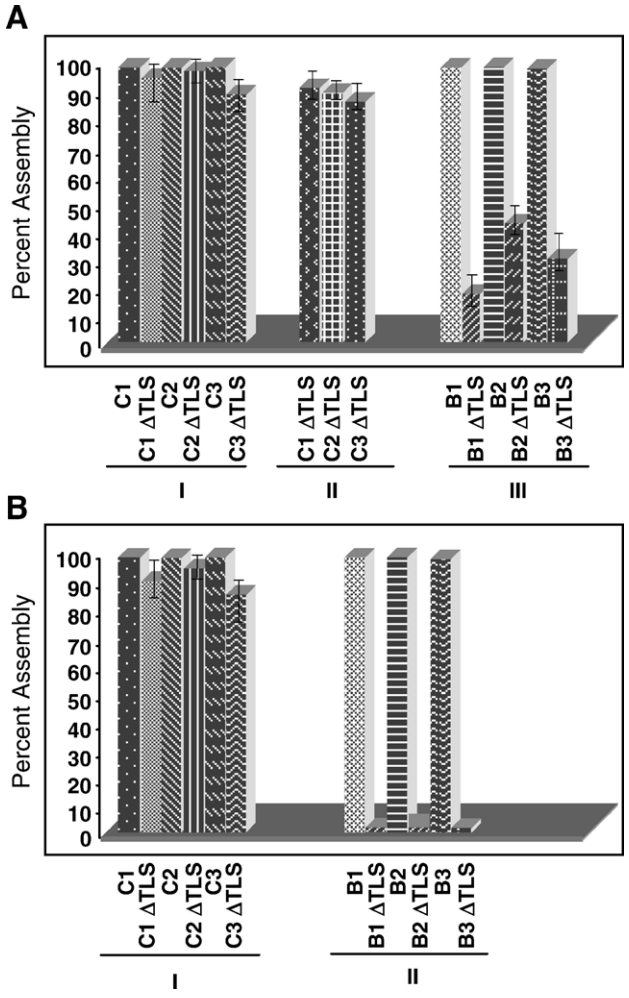

Fig. 4. Efficiency of virus assembly. Purified wt CCMV CP subunits were allowed to assemble in vitro with wt and ΔTLS-gRNAs of either CCMV or BMV. (A) Assembly assays were performed with wt CCMV CP and the following RNA preparations: Panel I, wt and ΔTLS-gRNAs of CCMV; Panel II, ΔTLS-gRNAs of CCMV and sequences encompassing the 3′ TLS added in trans; Panel III, wt and ΔTLS-gRNA of BMV. In vitro assembly of RNA containing virions was performed by mixing the dissociated CP subunits and desired RNA transcripts at a ratio (CP: RNA wt/wt) of 1:5 for RNAs1 and 2 and 1:2 for RNA3. Virus assembly efficiency was determined by measuring the concentration of virions following Centricon purification step. Assembly efficiencies of ΔTLS-gRNAs were calculated, relative to the assembly of respective control wt RNA transcripts (100%). For *trans* complementing assays, a 201-nt sequence encompassing the viral TLS region was added in equimolar ratio (Choi et al., 2002). (B) Assembly assays were performed with wt BMV CP and the following RNA preparations: Panel I, wt and ΔTLS-gRNAs of CCMV; Panel II, wt and ΔTLS-gRNAs of BMV. Assembly efficiencies were calculated as described above.

2001). Consequently, a commonly shared packaging signal is envisioned to dictate this unique packaging. In an effort to characterize this commonly shared packaging signal on BMV RNA3 (B3), it was observed that packaging of B3 into virions requires a bipartite signal constituting the 3′ TLS functioning as nucleating element and a 180-nt sequence present in the non-structural MP ORF as a packaging element (Choi and Rao, 2003). Since C3, like that of B3, is also dicistronic and identical in structural organization, we surmised that packaging of C3 is also regulated in a manner similar to that of B3. To test this, we constructed a set of C3 deletion mutants and two chimeras having EGFP sequences in the place of either the MP ORF or the CP ORF (Fig. 5A). RNA transcripts synthesized from these variant clones were assayed in vitro for their competence to assemble into virions. Unlike B3, where deletion of either 3′ TLS or MP ORF but not CP ORF affected virion assembly (Choi and Rao, 2003), deletion of neither MP ORF nor CP ORF interfered with packaging (Fig. 5A). Similarly, two C3-EGFP chimeras (C3/ΔMP-EGFP and C3/ΔCP-EGFP) were also packaged efficiently into virions (Fig. 3B). These results suggest that packaging of C3 is not dictated in sequence specific manner.

Packaging of non-bromoviral RNAs by CCMV CP

Data presented above suggested that, unlike BMV, CCMV CP can package RNA in sequence independent manner. To verify this further, two heterologous RNAs of non-bromoviral origin have been used as substrates for in vitro assembly. These are, 1800 nt RNA transcript encompassing the *Xenopus* elongation factor 1 sequence (XEF1; Figs. 5A, B) transcribed from a linearized plasmid pTRI-Xef (Ambion, Austin, TX) and a 2199 nt transcript encompassing *Cucumber mosaic virus* (CMV) RNA3 sequence (Fig. 5A) transcribed from *Bgl*II digested pT7K3 plasmid (Schmitz and Rao, 1998). The rationale for selecting these two sequences is two fold: firstly the physical size of each RNA falls within the range of CCMV gRNAs; secondly, the primary sequence of XEF1 is distinct from that of CCMV gRNA, whereas the arrangement of genes and the secondary structural folding of 3′ 200 nt of CMV RNA3 (Boccard and Baulcombe, 1993) is parallel to that of C3 (Fig. 5A). Results of in vitro assembly assays performed with these nucleic acid substrates are shown in Fig. 5. Both XEF1 and CMV RNA3 transcripts were assembled with approximately 50% efficiency (Fig. 5A) with virion morphology similar to that of CCMV (Fig. 5B).

Discussion

BMV and CCMV exhibit similar genome organization and replication mechanisms (Dreher, 1999; Kao and Sivakumaran, 2000). However, they do differ in many properties including the required virus-encoded genes for cell-to-cell spread (Rao, 1997), electrophoretic mobility patterns of respective virions and their sensitivity to RNase (Annamalai and Rao, unpublished data) and finally the interaction of highly conserved N-terminal basic arm region of the CP with respective genomic and subgenomic RNAs during encapsidation (Annamalai et al., in press; Choi and Rao, 2000). In this study, in vitro assembly assays provided another distinguishing feature for these two viruses, that is, the extent to which viral 3′ TLS is involved in packaging the

gRNAs into virions (Fig. 3; Choi et al., 2002). This study also revealed that, unlike B3 which requires a bipartite signal for packaging (Choi and Rao, 2003), assembly of C3 into virions is independent of sequences encoding either MP ORF, CP ORF or 3′ non-coding regions (Fig. 5A).

How and why do CCMV and BMV differ in the mechanism of RNA packaging?

The specific and non-specific RNA packaging displayed respectively by BMV (Choi and Rao, 2003; Cuillel et al., 1979) and CCMV (Hiebert et al., 1968; this study) raise an intriguing question concerning the mechanism of RNA packaging in these two viruses. Absence of host RNAs in the matured virions suggests that a mechanism that selectively enriches packaging of viral RNAs into virions does exist in these two viruses. The lack of host RNAs in mature virions could also be attributed to the translocation of viral RNAs to a specific location, such as membrane-enveloped, capsid like spherules induced by replicase protein 1a (Schwartz et al., 2002) that mechanistically linked to encapsidation allowing only viral RNAs to be packaged. However, it remains to be seen whether virus assembly occurs in association with spherules or not. Nevertheless, CCMV CP-driven packaging of heterologous RNAs (*Xenopus* RNA and CMV RNA3) is less efficient than for homologous RNAs (BMV and CCMV; Fig. 5). This inefficient packaging could perhaps be due to low affinity binding of CP subunits with heterologous RNAs, whereas the high affinity between viral RNA and CP subunits competitively sequester CP subunits and eliminate heterologous RNA packaging.

Selective encapsidation processes often result from the interaction of CP with RNA elements functioning as specific packaging signals encoded within the viral RNA. Such specific packaging signals have been characterized for enveloped animal viruses (Frolova et al., 1997) and non-enveloped plant viruses such as *Turnip crinkle virus* (TCV; Qu and Morris, 1997) and recently for B3 (Choi and Rao, 2003; Choi et al., 2002; Damayanti et al., 2003). Unlike BMV, which encapsidates its RNA by interacting with a specific virus-encoded packaging signal, RNA packaging by CCMV CP is non-specific (Hiebert et al., 1968; this study). This suggests that RNA packaging in CCMV is more promiscuous than in BMV. However, a mechanism inherent to CCMV, distinct from that of BMV, functioning as "selective filter" during encapsidation process must exist so that matured virions exclusively package viral RNAs. A support for this conjecture was recently provided by Johnson et al. (2003) while analyzing the interaction between CCMV CP and RNA1 leading to virion assembly. These authors

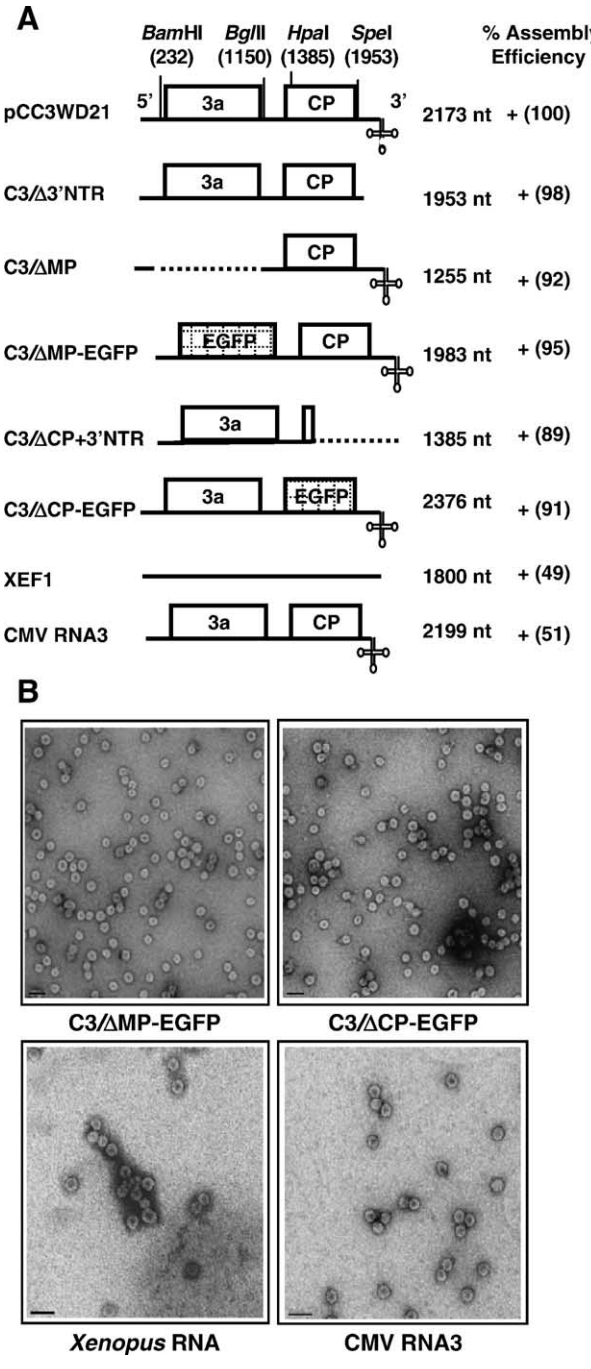

Fig. 5. Schematic representation of wt, deletion variants and chimeras of C3 used for in vitro assembly assays. (A) The genome organization of wt C3 is shown, with non-translated regions (NTR) represented as solid lines and open boxes representing the MP-coding region and the CP-coding regions. The clover leaf structure at the 3′ end represents the highly conserved tRNA-like structure (3′ TLS). Restriction sites used for constructing deletion variants of C3 are shown above the map. The lengths of wt C3, deletion variants and chimeras are shown. Broken lines in variant clones C3/ΔMP and C3/ΔCP+3′ NTR represent the extent of each deletion. Construction and characteristic features of two chimeras, C3/ΔMP-EGFP and C3/ΔCP-EGFP, are as described previously (Rao, 1997). The results of in vitro assembly assays performed between CCMV CP subunits and each C3 variant are shown to the right. The numbers shown in parentheses represent the percentage of assembly efficiency for each variant RNA sequence with respect to wt control transcripts. (B) Representative examples of electron microscopic images of negatively stained preparations of virions assembled in vitro from purified CP subunits and the indicated non-capped RNA transcripts. Samples for EM examination are prepared as described in Fig. 1. (Scale bar = 50 nm).

proposed a structure-based mechanism for packaging CCMV RNA1 that is independent of specific packaging signals. Based on the products resulting from CP-RNA interactions, it was hypothesized that binding of CP slowly folds RNA into a compact structure (C1 complex) and when CP concentration peaks during infection it preferentially binds the C1 complex with high cooperativity leading to specific encapsidation. To test this hypothesis and also to verify whether 3′ TLS contributes to the overall structure of CCMV RNA1, we performed polymerization kinetics of wt CP subunits and C1ΔTLS similar to those described by Johnson et al. (2003) and the results are summarized in Fig. 6. From these experiments, it is obvious that deletion of the 3′ TLS did not affect the formation of C1 complex and resulted in normal encapsidation (Fig. 6B). Additional experiments involving engineering mutations that disrupt the structure of CCMV RNA1 and their effect on packaging are required to be performed to further support the structure-based packaging for CCMV RNA1.

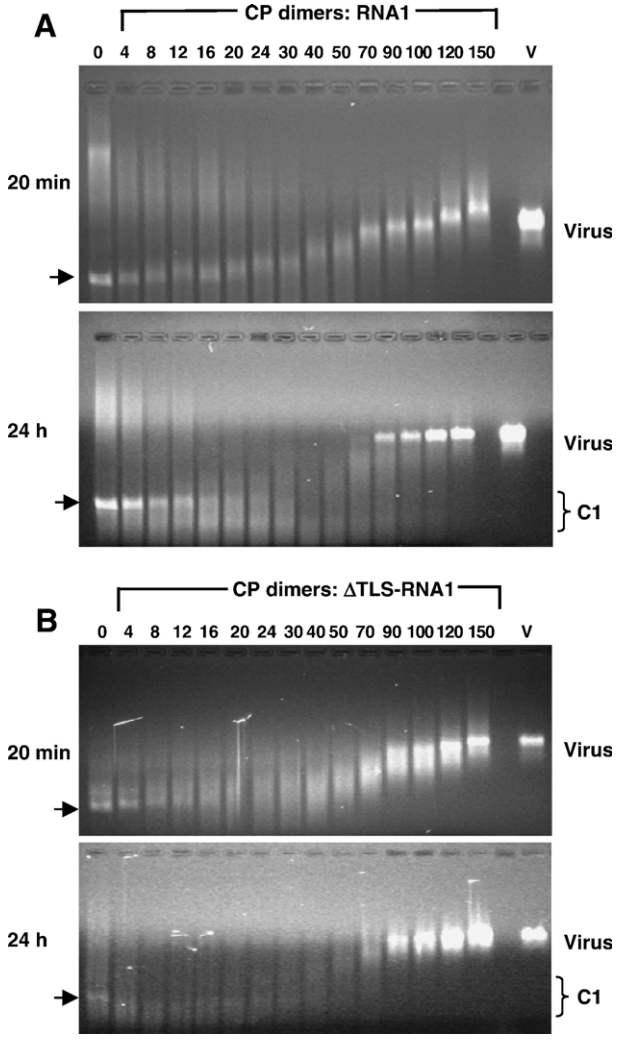

Fig. 6. Gel retardation assays. Approximately 30 nM of either wt C1 (A) or C1ΔTLS transcripts (B) was titrated with the indicated amounts of wt CP dimers for 20 min or 24 h at 20 °C. After incubation, the samples were loaded on to 1% agarose gel prepared and electrophoresed in TAE buffer. A sample of native CCMV virions (V), assembled with 90 CP dimers, purified from symptomatic cowpea leaves was shown on the right. Arrow shown to the left indicates the position of free-viral RNA. The position of C1 complex formed with RNA1 in 24 h incubation sample was indicated by a bracket on the right of each panel.

Why do BMV and CCMV employ two distinct packaging mechanisms? These viruses have evolved to infect monocotyledonous and dicotyledonous hosts respectively and the requirement of viral genes that dictate this process is also distinct. Consequently, these viruses must utilize different mechanism 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 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 (Kasteel et al., 1997; Schmitz and Rao, 1996) 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 monocot specific since encapsidation defective variants of BMV are also incompetent for cell-to-cell movement in dicotyledonous hosts such as *Chenopodium quinoa* (Schmitz and Rao, 1996) and *Nicotiana benthamiana* (Rao, 1997). Nevertheless the non-specific and promiscuous nature of assembly exhibited by CCMV CP offers unique applications in material science and nanotechnology, as exemplified by encapsulation of non-biological agents such as polyanetholesulphonic acid (Douglass and Young, 1998).

Materials and methods

Wild type and variant clones of CCMV

Full-length cDNA clones corresponding to the three genomic RNAs of CCMV, pCC1TP1, pCC2TP2 and pCC3TP4, when linearized with *Xba*I, yield wild type (wt) infectious RNAs 1 (C1), 2 (C2) and 3 (C3), respectively in vitro (Allison et al., 1988).

Full length cDNA clones corresponding to the three genomic RNAs of BMV, pT7B1, pT7B2 and pT7B3(-Tth) and respective clones lacking 3′ TLS have been described previously (Choi et al., 2002; Dreher et al., 1989). To construct variants of CCMV RNA, each cDNA clone was digested with a desired unique restriction site. Thus to synthesize each of the three CCMV genomic RNA transcripts lacking the 3′ TLS region, pCC1TP1, pCC2TP2 and pTT3TP were respectively digested with *Bsi*EI, *Xcm*I, and *Spe*I (Fig. 1). The construction and characteristic features of CCMV RNA3 variants such as, C3/ΔMP, C3/ΔMP-EGFP

and C3/ΔCP-EGFP have been described previously (Rao, 1997).

In vitro transcription

Prior to in vitro transcription, unless otherwise mentioned, all wt and variant clones of CCMV and BMV were linearized with *Xba*I and *Bam*HI, respectively. Non-capped full-length transcripts were synthesized in vitro using a MEGAscript T7 kit (Ambion Inc., Austin, TX) and DNA template was removed by LiCl precipitation.

Virus purification and coat protein preparation

Virus was purified as described previously (Rao et al., 1994). Purified virions of either wt or variants were dissociated into CP by dialyzing at 4 °C for 24 h against a buffer containing 500 mM $CaCl_2$, 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM DTT and 0.5 mM PMSF. Following a low-speed centrifugation at 12,000 × *g* for 30 min, any traces of viral RNA contaminating the supernatant was removed by dialyzing against RNA assembly buffer (see below). The dialyzed mixture was then centrifuged for 90 min at 220,000 g in a Beckman TL 100 centrifuge to pellet the assembled virions. The concentration of the dissociated CP subunits present in the supernatant was determined by a spectrophotometer.

In vitro assembly assays and Northern blot analysis

For in vitro assembly of RNA containing virions, dissociated CP subunits and desired RNA transcripts were mixed in a ratio (wt/wt) of 1:5 for RNAs1 and 2 and 1:2 for RNA3 and dialyzed at 4 °C for 24 h against RNA assembly buffer (50 mM NaCl, 50 mM Tris–HCl, pH 7.2, 10 mM KCl, 5 mM $MgCl_2$ and 1 mM DTT). The assembled virions were concentrated using Centricon-100 microconcentrators (Amicon, Beverly, MA). Virus assembly efficiency was determined by measuring the concentration of virions following Centricon treatment. In these assay, the concentration of virions assembled with RNAs that served as positive control was always taken as 100%. RNA was isolated from assembled virions using SDS-phenol method prior to ethanol precipitation in the presence of glycogen (Sigma) as a carrier. The RNA pellets were suspended in 25 μl sterile water. For Northern blot analysis desired concentration of virion RNA was dried in a microfuge tube and suspended in 10 μl of sample buffer (10× MOPS buffer/formaldehyde/formamide/water in a ratio of 1:1.8:5:2.2 respectively), heated at 65° for 10 min and electrophoresed in 1.2% agarose-formaldehyde gel (Sambrook and Russel, 2001). Following a 3-h electrophoresis, fractionated RNA was transferred to a nylon membrane with a VacuGene XL blotting unit (Phramacia Biotech). The blot was then processed for pre-hybridization and hybridization using riboprobes specific for each genomic RNA as described previously (Choi et al., 2002; Osman et al., 1997).

Gel retardation assays

The interaction between varying molar ratios of either wt or mutant CP dimers and desired genomic RNA component leading to virion assembly following a short (20 min) or long (24 h) time incubation was analyzed (Johnson et al., 2003). Approximately 0–150 CP dimers were titrated against a constant RNA concentration in a typical 20 μl reaction containing 50 mM MOPS (pH 7.2), 150 mM NaCl and 2 mM $MgCl_2$. The samples were then subjected to 1% agarose gel electrophoresis in TAE buffer (Sambrook and Russel, 2001), stained with ethidium bromide and photographed with BioRad Gel documentation system. In these assays, a sample of native wt CCMV virions purified from symptomatic leaves was always co-electrophoresed as a control.

Electron microscopy

For negative staining, purified virus preparation at a concentration of 20 to 50 μg/ml was applied to glow discharged carbon coated copper grids. Grids were washed once with water, stained with 1% uranyl acetate and air dried. Grids were examined with a FEI Tecnai12 transmission electron microscope operating at 100 kV. Images of stained samples were recorded with an electron optical magnification of 30,000×.

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