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Replication-independent expression of genome components and capsid protein of brome mosaic virus in planta: A functional role for viral replicase in RNA packaging

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Abstract

To begin elucidation of the relationship between Brome mosaic virus (BMV) replication and encapsidation, we used a T-DNA-based *Agrobacterium*-mediated transient expression (agroinfiltration) system in *Nicotiana benthamiana* leaves to express either individual or desired pairs of the three genomic RNAs. The packaging competence of these RNAs into virions formed by the transiently expressed coat protein (CP) was analyzed. We found that in the absence of a functional replicase, assembled virions contained non-replicating viral RNAs (RNA1 or RNA2 or RNA3 or RNA1 + RNA3 or RNA2 + RNA3) as well as cellular RNAs. By contrast, virions assembled in the presence of a functional replicase contained only viral RNAs. To further elucidate the specificity exhibited by the functional viral replicase in RNA packaging, replication-defective RNA1 and RNA2 were constructed by deleting the 3′ tRNA-like structure (3′ TLS). Co-expression of TLS-less RNA1 and RNA2 with wt RNA3 resulted in efficient synthesis of subgenomic RNA4. Virions recovered from leaves co-expressing TLS-less RNA1 and RNA2 and either CP mRNA or wt RNA3 exclusively contained viral RNAs. These results demonstrated that packaging of BMV genomic RNAs is not replication dependent whereas expression of a functional viral replicase plays an active role in increasing specificity of RNA packaging.

Keywords: Agroinfiltration; Bromoviruses; RNA packaging; Transient expression; Viral replicase

Introduction

Brome mosaic virus (BMV), a plant infecting member of alphavirus-like super family, is the type member of the genus *Bromovirus* of the family Bromoviridae (Rao, 2001). BMV comprises of a tripartite genome of messenger sense polarity (Rao, 2001). Viral RNA replication is dependent on efficient interaction between two non-structural proteins, 1a and 2a, encoded by monocistronic RNAs1 (B1) and 2 (B2), respectively (Kao and Sivakumaran, 2000). Non-structural protein 1a (109 kDa) contains a methyltransferase/guanylyl-transferase domain in its N-terminal half and a helicase domain in its C-terminal half while non-structural protein 2a (94 kDa) contains central polymerase-like domain (Kao and Sivakumaran, 2000). These replication proteins share amino acid sequence similarity with proteins encoded by many morphologically and genetically diverse (+) stranded RNA viruses of plants and animals (Ahlquist et al., 1985). The genomic RNA3 (B3) encodes a 5′ non-structural movement protein (MP) of 32 kDa and a 3′ capsid protein (CP) of 19 kDa. Only the 5′ proximal MP gene, but not 3′ proximal CP gene, is translated directly from the genomic B3. Instead, the CP is translated from a subgenomic mRNA (sgB4), which is synthesized by internal initiation on negative strand B3 (Miller et al., 1985). The two gene products encoded by the dicistronic B3 are dispensable for viral replication but are required for infection of whole plants (Mise et al., 1993; Rao, 2001).

In BMV, the three genomic and a single subgenomic RNA are packaged into three physically and morphologi-

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cally indistinguishable icosahedral virions (Fox et al., 1994; Rao, 2001) with $T = 3$ symmetry and are assembled from 180 identical subunits of a single CP (Lucas et al., 2002; Speir et al., 1995). Based on physical and biochemical analysis of BMV virions, it was hypothesized that genomic B1 and B2 are packaged independently into two virions whereas genomic B3 and the sgB4 are co-packaged into a third virion. The rationale and the mechanism by which the CP regulate this balanced distribution of four BMV RNAs into three individual virions remain elusive. This limitation can be largely attributed to existing in vivo systems that are unable to accomplish high level expression of individual genome components and their assembly into virions without enduring complete replication cycle.

One attractive option to further advance our knowledge in elucidating the mechanism of RNA packaging in BMV is to develop a DNA-based transient expression system in planta that will result in efficient expression of desired set of BMV mRNAs independent of viral replication. *Agrobacterium*-mediated transient gene expression (hereafter referred to as agroinfiltration) involves delivery of desired genes of interest into plant cells as liquid culture through infiltration. This process results in transfer of transgenes from the T-DNA region of the bacterial Ti plasmid into the plant cells and the T-DNA copies remain transiently present in the nucleus, which can be transcribed, leading to transient expression of T-DNA genes. Agroinfiltration has been widely used in plant biology for identification of disease resistance genes (Bendahmane et al., 2000) and induction and suppression of post transcriptional gene silencing (PTGS; Johansen and Carrington, 2001; Voinnet et al., 2003). Most importantly, agroinfiltration facilitates delivery of several transgenes to be co-expressed into the same cell from different *Agrobacterium* transformants (Marillonnet et al., 2004). These traits are particularly attractive for studying multicomponent viruses like BMV since high level accumulation of genomic RNAs and their expression is replication contingent. Here we applied this agroinfiltration approach for high level autonomous transient expression in plant cells of desired set of genome components of BMV for understanding the intimacy between replication and RNA packaging.

Results

Characteristics of BMV constructs used for agroinfiltration

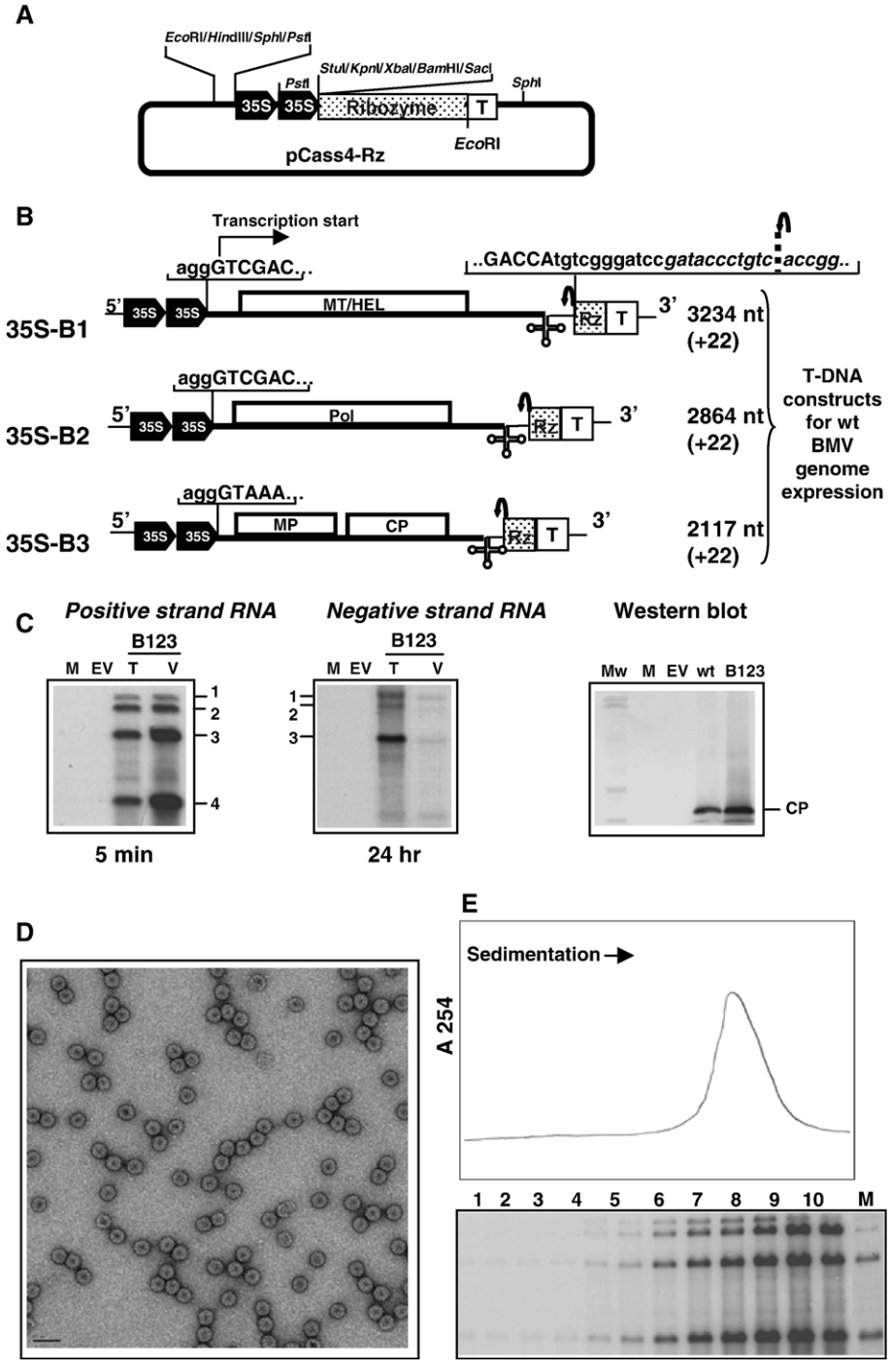
The characteristic features of pCass4-RZ (Fig. 1A), a T-DNA plasmid amenable for agroinfiltration of plant cells and its derivatives harboring full-length cDNAs of BMV genomic RNAs are shown in Fig. 1B. Presence of a natural 5′ sequence is an essential prerequisite for efficient replication and wild-type (wt) biological activity of each of the three BMV genomic RNAs (Boyer and Haenni, 1994; Janda et al., 1987). Unlike natural 5′ end, the effect of 3′ non-viral nucleotide (nt) extensions on the biological activity of BMV RNAs varied significantly among the expression systems tested (Dreher et al., 1989; Dzianott and Bujarski, 1989; Ishikawa et al., 1997). Keeping these critical requirements in perspective, cloning of each of the three full-length cDNA copies of BMV genomic RNAs into pCass4-Rz binary vector (Fig. 1A) was designed to initiate the transcription by the cauliflower mosaic virus 35S promoter precisely at the authentic viral 5′ end (Fig. 1B). However, the possibility of having heterologous 5′ end sequence in transiently synthesized mRNAs due to the presence of double 35S promoter is not excluded. The 3′ end of each of the three de novo synthesized RNA transcripts will terminate with + 22-nt extensions beyond the natural 3′ CCA_{OH} due to the presence of a self-cleaving ribozyme (Fig. 1B).

To verify the biological activity of BMV mRNAs transiently expressed in vivo (Fig. 1B), *Nicotiana benthamiana* leaves were co-infiltrated with a mixture of *Agrobacterium* cultures containing all three wt T-DNA constructs. Four days post infiltration (dpi), leaves were divided into two lots. One lot was used to extract total RNA preparations and the other for purifying virions and isolation of packaged RNA. Northern blot analysis of total and virion RNA preparations revealed the presence of an RNA profile similar to that of native wt BMV infections (Fig. 1C). Production of sgB4 (Fig. 1C, left panel) and detection of progeny (−) strands (Fig. 1C, middle panel) confirmed that all three BMV RNAs generated from their respective T-DNA plasmids are biologically active, faithfully mimicking complete replication behavior similar to that observed in plants infected with wt BMV (Dreher et al., 1989). Western blot analysis (Fig. 1C, right panel) and examination of purified virions under EM (Fig. 1D), respectively, confirmed the identity of parental lineage of CP and morphology of the assembled virions. Sucrose-gradient sedimentation of partially purified virions revealed a single faster sedimenting peak (Fig. 1E, top panel), characteristic of wt BMV virions purified from symptomatic barley plants (Rao et al., 1994). Gradient fractions encompassing the single major peak predominantly contained an RNA profile indistinguishable from that of wt BMV (Fig. 1E, bottom panel). Furthermore, wt BMV virions were also recovered from uninoculated symptomless systemic leaves (characteristic of BMV infections in *N. benthamiana*; Rao, 1995) (data not shown). Collectively these observations suggested that BMV mRNAs transiently expressed by agroinfiltration are biologically active and duplicated all events of replication, cell-to-cell and long distance spread.

Evidence showing agroinfiltration delivers multiple T-DNAs to a single cell

The major objective of this study is to identify a system that facilitates autonomous assembly of individual or a pair of desired BMV mRNAs by the CP expressed independent

of replication. Consequently, this requires co-delivery and expression of two or more T-DNAs to the same cell. To quantify this requirement, we subcloned a cell-to-cell movement-defective variant sequence of B3 into pCass4-Rz vector yielding 35S-B3/ΔCP-EGFP (Fig. 2A). This variant clone is characterized by the substitution of EGFP reporter gene sequence for the 3′ CP ORF (Rao, 1997). *N. benthamiana* leaves were infiltrated with a mixture of *Agrobacterium* cultures containing 35S-B1 + 35S-B2 + 35S-B3/ΔCP-EGFP. Leaves infiltrated with only 35S-B3/ΔCP-EGFP served as a negative control. The rational in performing this assay is that expression of EGFP will result in those cells that receive all three T-DNAs since initiation and synthesis of subgenomic ΔCP-EGFP mRNA (sgΔCP-EGFP) will occur only when B3/ΔCP-EGFP enters replication cycle to yield minus-strand progeny. Furthermore, cell-to-cell movement of BMV requires MP as well as encapsidation competent CP (Rao, 1997; Schmitz and Rao, 1996) and therefore progeny accumulated in initially infected cells will not be transported to the adjacent cells.



Consequently, only those cells that receive all three T-DNAs are competent for EGFP expression via subgenomic RNA. Northern blot analysis of total RNA isolated from leaves infiltrated with 35S-B1 + 35S-B2 + 35S-B3/ΔCP-EGFP confirmed the replicative nature of infiltrated inoculum by the detection of sgΔCP-EGFP of expected size (Fig. 2B).

To verify the efficacy of agroinfiltration in delivering multiple T-DNAs to the same cell, EGFP expression was monitored by two methods in leaves harvested at 3 dpi. First, the whole leaf was scanned through Typhoon 9410 (Amersham) equipped with appropriate filter set to enhance visualization of EGFP fluorescence. Cells encompassing the infiltrated zone with *Agrobacterium* cultures containing 35S-B1 + 35S-B2 + 35S-B3/ΔCP-EGFP appeared distinctly brighter green than uninfiltrated area (Fig. 2C, panel 1). By contrast, leaves infiltrated with *Agrobacterium* culture containing only 35S-B3/ΔCP-EGFP did not show any green fluorescence (Fig. 2D, panel 1). To monitor the EGFP expression more closely, infiltrated leaves were subjected to confocal laser scanning microscopy. Optical scans were obtained for two major sites: one within the infiltrated zone (demarked by a zone of white cells in Fig. 2C, panel 1) and the other distant from primary infiltration site. Representative examples of this analysis are shown in Fig. 2C (panels 2–4). It was observed that in leaves infiltrated with all three T-DNAs, majority of cells (epidermal and mesophil) expressed EGFP (Fig. 2C, panels 2–4), whereas control leaves infiltrated only with 35S-B3/ΔCP-EGFP appeared red due to autofluorescence throughout the leaf (Fig. 2D, panels 2–4). Collectively, these observations suggested that agroinfiltration efficiently co-delivered multiple T-DNAs to the same cell.

Transient expression of BMV capsid protein

In BMV, although the CP gene is encoded by the 3′ ORF of B3 (Fig. 1B), it is not translated directly from this RNA component. Instead, an sgB4 of 886 nt sequence serving as a CP mRNA, corresponding to the 3′ half of B3, is synthesized de novo by internal initiation on minus-strand progeny B3. Therefore, synthesis of sgB4 and its gene product is replication contingent. Thus, to examine autonomous packaging of transiently expressed BMV RNAs, we sought to express CP independent of B3 replication. Consequently, a cDNA copy of 886 nt sequence encompassing wt sgB4 was amplified by PCR and subcloned into pCass4-Rz, yielding 35S-B4 (Fig. 3A). It is anticipated that delivery of 35S-B4 into plant cells by agroinfiltration will result in the production of B4 mRNA with 5′ and 3′ ends similar to BMV genomic RNAs synthesized from respective T-DNA constructs (Fig. 1B).

Northern blot analysis of total RNA preparations isolated from *N. benthamiana* leaves infiltrated with 35S-B4 revealed the presence of B4 mRNA transcripts of expected size (Fig. 3B, left panel). However, this mRNA failed to translate in vivo (Fig. 3B, bottom panel) perhaps due to the lack of a correct 3′ terminus which appears to be a prerequisite for efficient translation (Gallie, 1998). The problem, however,

Fig. 1. Physical and biological characteristics of T-DNA constructs of BMV genomic RNAs. (A) Schematic representation of T-DNA vector, pCass4-Rz that was used to clone the cDNAs of BMV genomic RNAs. The vector contains a double CaMV 35S promoter (35S) followed by a ribozyme sequence cassette derived from *satellite tobacco ring spot virus* (Rz) and a *Nos* terminator (T). A flanking region of five restriction enzyme recognition sites located between the 35S promoter and the ribozyme cassette is shown. Note: Since the *Stu*I site is methylation sensitive, it is necessary to propagate recombinant plasmids in *dcm*$^-$ strains of *E. coli*. (B) Characteristics of T-DNA plasmids harboring BMV genomic RNAs used for transient expression in plants. The 35S-B1, 35S-B2, and 35S-B3 constructs contain full-length cDNA copies of BMV genomic RNAs 1 (B1), 2 (B2), and 3 (B3), respectively. Single lines and open boxes represent non-coding and coding regions, respectively. Monocistronic B1 with methyltransferase-like domain (MEL), helicase-like domain (HEL), and monocistronic B2 with polymerase-like domain (POL) are indicated. The location of the movement protein (MP) and coat protein (CP) genes on the dicistronic B3 is shown. At the 5′ junction, nucleotide sequence of the 35S promoter (indicated by lower case) and the 5′ sequence of each genomic cDNA (indicated by upper case) are shown. The transcription start site was indicated by a bent arrow. At the 3′ end of each construct the clover leaf-like conformation represents a tRNA-like motif conserved among all three BMV genomic RNAs and the 3′ nucleotide sequence commonly shared between each cDNA (indicated by upper case) and the non-viral sequences (indicated by lower case) left after self-cleavage by the ribozyme is shown. The predicted self-cleavage site is indicated by a bent arrow. The length of wt BMV genomic RNAs and the number of non-viral nucleotides left after self-cleavage by ribozyme (shown in bracket) are indicated. (C) Progeny analysis: Northern blot analysis of viral RNAs accumulated in *N. benthamiana* leaves infiltrated with *Agrobacterium* cultures containing either empty vector pCass4-Rz (EV) or a mixture of 35S-B1 + 35S B2 + 35S B3 (B123). A total RNA preparation extracted from uninfiltrated *N. benthamiana* leaves (M) was also used in this analysis. Approximately 5 μg of total nucleic acid preparations (T) from agroinfiltrated leaves or 100 ng of RNAs extracted from purified virions (V) was denatured with formamide/formaldehyde and subjected to 1.2% agarose electrophoresis prior to vacuum blotting to a nylon membrane. The blot was hybridized with ^{32}P-labeled riboprobes probes complementary to the homologous 3′ non-coding region present on all four BMV RNAs. Note that in BMV, negative strand RNAs (middle panel) accumulate 100-fold less than the positive strand RNAs (left panel; Marsh et al., 1991); therefore, autoradiographic image shown for positive and negative strands required a 5-min and 24-h exposure times, respectively. The position of BMV RNAs is indicated between the panels. Western blot analysis of viral coat protein (right panel): detection of BMV CP from total protein extracts of uninfiltrated leaves (M), leaves agroinfiltrated independently with empty vector (EV), and a mixture of all three wt T-DNA constructs of BMV (B123) were fractionated by SDS–PAGE followed by transferring to PVDF membrane and probed with antibodies prepared against purified BMV. Dissociated CP preparation from wt BMV (wt) obtained from barley plants was used as a marker. The position of the CP is indicated. (D) Electron microscopy of purified virions. Virions were purified from B123 agroinfiltrated leaves, applied to glow discharged carbon coated copper grids, and negatively stained with 1% uranyl acetate prior to viewing under EM. Scale bar = 50 nm. (E). Analysis of virions purified from B123 agroinfiltrated leaves. Top panel: Sucrose density gradient profile. Partially purified virions were centrifuged through 10–40% linear sucrose gradient and gradient fractions were analyzed by UV absorption at 254 nm. Bottom panel: RNA analysis of sucrose gradient fractions. Virion RNA from each fraction was extracted and subjected to Northern blot hybridization as described in the legend of panel C. Wt BMV RNA was used as a marker (M).

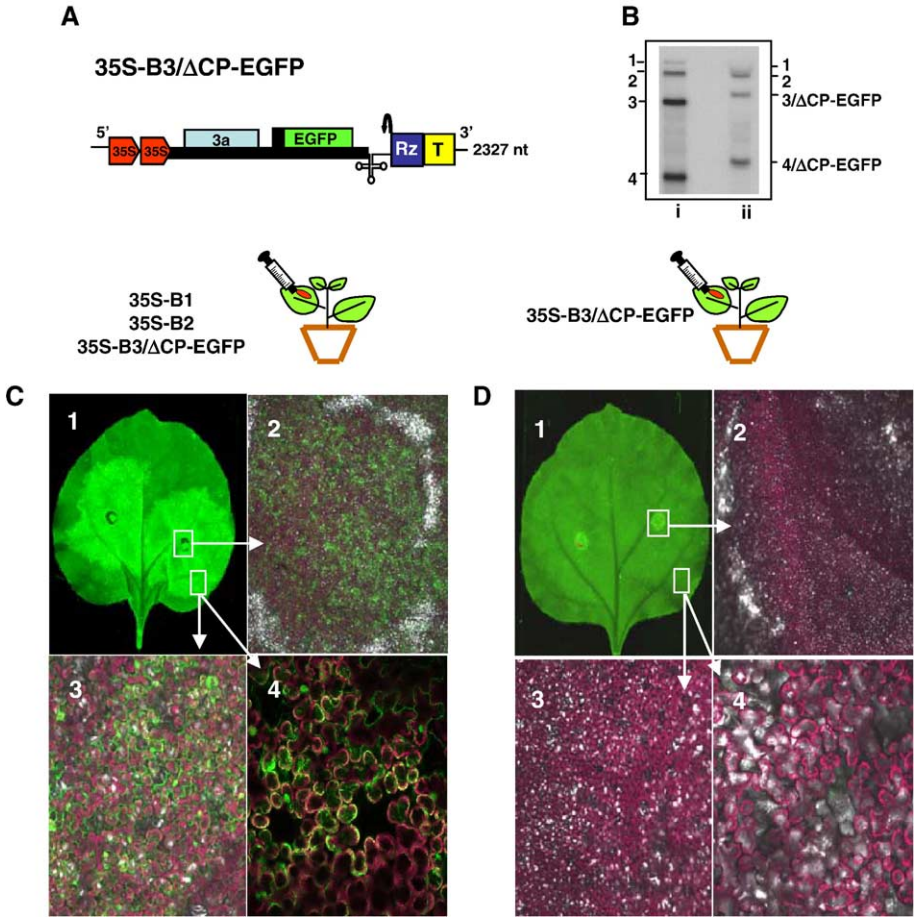


Fig. 2. Evidence for co-delivery of multiple T-DNAs to the same cell. (A) Schematic representation of a B3 variant T-DNA construct, 35S-B3/ΔCP-EGFP, used for transient expression of EGFP via subgenomic RNA synthesis. The characteristic features of 35S-B3/ΔCP-EGFP are identical to that of 35S-B3 (Fig. 1B), except a sequence encompassing EGFP was fused to the second AUG codon of BMV CP ORF (Rao, 1997). (B) Replication of B3/ΔCP-EGFP in leaves delivered using *Agrobacterium*. *Agrobacterium* cultures containing a mixture of 35S-B1 + 35S-B2 + 35S-B3/ΔCP-EGFP were infiltrated into *N. benthamiana* leaves, total RNA was isolated 3 dpi and subjected to Northern blot analysis as described in the legend of Fig. 1C. Replicated progeny resulting form agroinfiltration of all three wt BMV plasmids (lane 1) and those with wt B1 and B2 and B3/ΔCP-EGFP (lane 2) are shown. The relative positions of four wt BMV RNAs and those corresponding to B3 variant are shown to the left and right, respectively. (C) Visualization of EGFP. Panel 1: macroscopic image of *N. benthamiana* leaf infiltrated with *Agrobacterium* cultures containing 35S-B1 + 35S-B2 + 35S-B3/ΔCP-EGFP. The infiltrated leaf harvested at 3 dpi was scanned through Typhoon 9410 equipped with emission filter 670 bp 30–633 nm (red laser) and emission filter 520 BP–488 nm (blue laser). Images shown in panels 2–4 represent subcellular localization of EGFP in selected areas of the infiltrated leaf analyzed by confocal laser scanning microscopy. In panel 2, agroinfiltrated leaf, scanned at lower magnification, displays the expression of EGFP at the site of infiltration. Note that the boundary of infiltration site is represented by dead cells appearing as white and either non-transformed or those receiving a pair of T-DNA constructs incompetent to express EGFP exhibit reddish auto-fluorescence. Panels 3 and 4 represent higher magnification confocal images of an area distant from the site of infiltration. (D) Confocal images of a leaf infiltrated with 35S-B3/ΔCP-EGFP alone serving as a negative control. Viewing conditions of images shown in panels 1–4 are similar to those described under C.

was circumvented by constructing a variant of 35S-B4, referred to as 35S-B4.1 (Fig. 3A) in which a 149-nt fragment encompassing the translation enhancer sequence from *tobacco etch potyvirus* (Carrington and Freed, 1990) was fused to the 5′ end of CP-ORF (Fig. 3A). As result of these cloning manipulations, the size of the CP mRNA generated in vivo was anticipated to be 1035 nt. Infiltration of *Agrobacterium* cultures with 35S-B4.1 into *N. benthamiana* leaves resulted in the generation of mRNA of expected size (Fig. 3B, right panel). Since 35S-B4.1 lacks a self-cleaving ribozyme, heterogeneous population of mRNAs due to the differential processing of polyadenylation signal (Huang and Carmichael, 1996) were also detected (indicated by arrowheads in Fig. 3B, right panel). By contrast to B4 mRNA, Western blot analysis confirmed that B4.1 mRNA translated efficiently to yield wt BMV CP (Fig. 3B, bottom panel). Consequently, 35S-B4.1 was co-infiltrated as a source vector for the transient expression of CP.

Replication-independent expression of CP exhibits virion polymorphism

Under in vivo conditions sgB4 is predicted to co-package with genomic B3 (Rao, 2001) and evidence for its autonomous packaging into separate virions is lacking due to the physical and morphological homogeneity of the

virions. However, assembly of sgB4 into virions, independent of B3, was observed in vitro (Choi and Rao, 2000a, 2000b; Choi et al., 2002; Zhao et al., 1995). To verify the assembly competence of transiently expressed CP mRNA, virions were purified from 35S-B4.1 infiltrated leaves. Although the yield of the purified virus was significantly lower than wt (250 μg/g compared to 1 mg/g for wt), EM examination revealed the presence of two virion populations: one with an average diameter similar to that of wt BMV virions (i.e., 28 nm) and the other approximately 10% smaller (i.e., 26 nm; Fig. 3C, top panel). These two virion populations are respectively segregated into heavy (H) and lighter (L) sedimenting peaks by sucrose density-gradient centrifugation (Fig. 3C, middle panel). Western blot analysis revealed that the CP of these two virion populations is of BMV origin (Fig. 3C). Northern blot analysis of RNA recovered from various fractions revealed that virions constituting the light sedimenting peak predominantly contained fully processed CP mRNA (1035 nt), whereas those constituting the heavy sedimenting peak contained heterogeneous larger size RNAs generated due to differential processing of polyadenylation signal (Huang and Carmichael, 1996; Fig. 3C, bottom panel). Furthermore, detection of these RNA bands required longer exposure times of the Northern blot indicating that the transiently expressed CP mRNA is inefficiently packaged.

Packaging competence of B3/ΔCP-EGFP and its subgenomic RNA by transiently expressed CP

Previous characterization of B3 sequences required for packaging revealed that a 180-nt sequence of MP ORF and the 3′tRNA-like structure (3′TLS) of 200 nt constitute the core packaging signal (Choi and Rao, 2003). Furthermore, packaging of B3 is a prerequisite for co-packaging of sgB4 since deletion of the required region from MP ORF to exclusively block B3 assembly into virions also abolished sgB4 packaging (Choi and Rao, 2003). Nevertheless, the above-described agroinfiltration experiments demonstrated that CP mRNA, though inefficient, is competent for autonomous assembly (Fig. 3C). Therefore, to uncouple the intimacy between B3 and B4 packaging/co-packaging, the following experiment was performed. *N. benthamiana* leaves were infiltrated with a mixture of *Agrobacterium* culture containing the following four components: 35S-B1 + 35S-B2 + 35S-B3/ΔCP-EGFP + 35S-B4.1. Three additional inocula 35S-B1 + 35S-B2 + 35S-B3, 35S-B3/ΔCP-EGFP + 35S-B4.1, and 35S-B1 + 35S-B2 + 35S-B3/ΔCP-EGFP were also infiltrated as controls. At 4 dpi, leaves were harvested, total and virion RNAs isolated and subjected to Northern blot hybridization. Results are summarized in Fig. 4.

As shown above (Fig. 1C), infiltration of all three wt plasmids generated mRNAs that are competent for complete replication exemplified by the presence of sgB4 in total (Fig. 4A; lane 1) and virion preparations (Fig. 4B; lane 1). Similar Northern blot analysis revealed that even in the absence of genomic wt B1 and B2 RNAs that are required for sustained replication, infiltration of 35S-B3/ΔCP-EGFP + 35SB4.1 resulted in the accumulation of B3/ΔCP-EGFP mRNA to detectable levels (Fig. 4A; lane 2). In these leaves, wt BMV CP translated from transiently expressed B4.1 mRNAs efficiently packaged B3/ΔCP-EGFP (Fig. 4B; lane 2), confirming the dispensability of CP ORF for B3 packaging (Choi and Rao, 2003). As demonstrated above (Fig. 2B), replicase proteins 1a and 2a amplified B3/ΔCP-EGFP to yield sgΔCP-EGFP mRNA (Fig. 4A, lane 3). To examine the packaging/co-packaging of sgΔCP-EGFP mRNA, CP was transiently expressed by supplementing 35S-B4.1 (Fig. 4, lane 4). Analysis of total RNA preparations showed that the levels of B3ΔCP-EGFP and its sgB4/ΔCP-EGFP were higher due to stabilization by CP than in its absence (compare in Fig. 4A, lanes 3 and 4). Interestingly, in these leaves, CP up-regulated packaging of genomic B1 and B2 RNAs while down-regulating B3ΔCP-EGFP and its sgB4/ΔCP-EGFP packaging (Fig. 4, lane 4). This differential regulation could be due to efficient interaction of CP with wt B1 and B2 whereas absence of CP ORF sequences on B3 variant competitively down-regulated its packaging (Fig. 4, lane 4). This assumption was supported by efficient packaging of B3 variant in the absence of wt B1 and B2 RNAs (Fig. 4, lane 2). Although CP ORF sequences are not required for B3 packaging (Fig. 4B; Choi and Rao, 2003) its presence might provide a regulatory mechanism for efficient co-packaging of B3 and B4 into a single virion.

Stabilization of transiently expressed non-replicating BMV RNAs by capsid protein

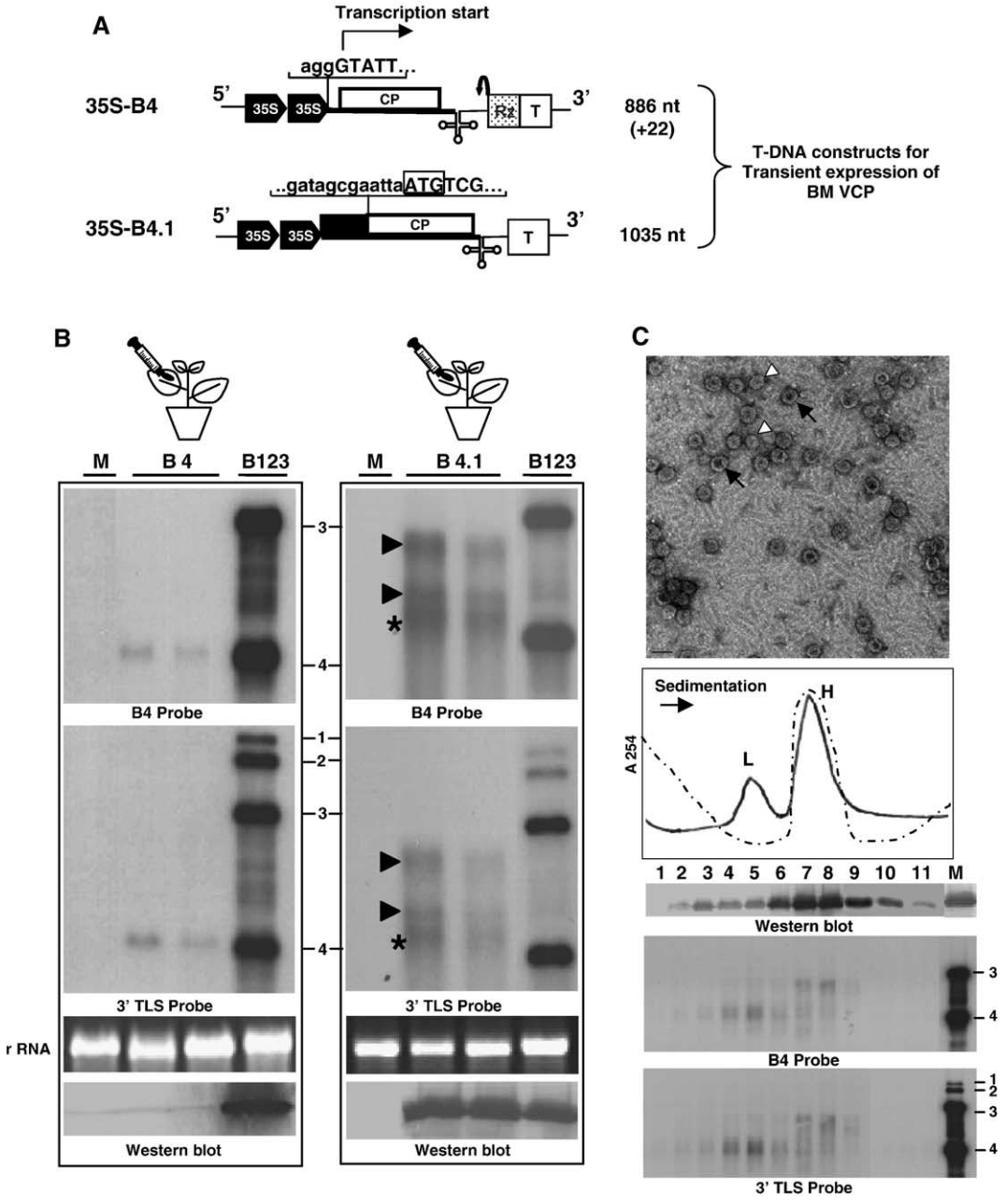
To verify the expression and accumulation of each of the three BMV genomic RNAs independent of replication, *N. benthamiana* leaves were infiltrated with *Agrobacterium* cultures transformed with 35S-B1 or 35S-B2 or 35S-B3. A mixture of all three wt plasmids was infiltrated as a positive control. After 4 dpi, total RNA was isolated from the infiltrated leaves and subjected to Northern blot hybridization. As shown in Fig. 5A (left panel), even in the absence of replicase proteins 1a and 2a required for sustained replication, infiltration of leaves with 35S-B1 or 35S-B2 or 35S-B3 resulted in the accumulation of mRNAs to detectable levels (Fig. 5A, left panel). In most experiments, the processing of mRNA by the sToRSV ribozyme was complete resulting in a major mRNA product of expected size.

Results of Northern blot hybridization of total RNAs from infiltrated leaves expressing individual BMV genomic RNAs in the presence of transiently expressed CP are shown in Fig. 5A (right panel). The relative levels of individual non-replicating BMV genomic RNA accumulation in the absence and presence of CP were quantitatively analyzed. Consistent with previous observations (Krol et al., 1999),

co-expression of CP increased the accumulation of each of the three genomic RNAs by 20-fold (Fig. 5B) suggesting that CP stabilized the transiently expressed genomic RNAs due to encapsidation (see below).

Expression of functional viral replicase blocks packaging of cellular RNAs into virions

In BMV it is impractical to physically separate native virions into three individual components due to their physical homogeneity. Furthermore, the factors that regulate this homogeneity are currently obscure owing to our inability to separate replication from packaging. Thus, one of the major objectives in using the agroinfiltration approach is to examine the autonomous assembly of BMV RNAs without having to replicate and analyze whether factors such as viral replicase would contribute toward assembly of infectious virions. To determine the involvement, if any, of functional viral replicase in RNA packaging, the competence of transiently expressed CP to package a single or a pair of BMV RNAs in agroinfiltrated leaves was examined by purifying virions and analyzing their RNA content. Northern blot hybridization of each virion RNA preparation revealed expected RNA profile (Figs. 6A and B). Western blot analysis confirmed the identity of the CP (Figs. 6A and B).



Interestingly, an unexpected RNA profile was observed when each of the above virion RNA sample was subjected to native agarose gel electrophoresis (Fig. 6C). In addition to the expected viral RNA species corresponding to a single or a pair of non-replicating BMV RNAs (Fig. 6C, lanes 2–6), at least one additional prominent RNA species of unknown origin with an electrophoretic mobility close to one of the cellular RNAs was consistently detected. This cellular RNA species was not seen in virion samples recovered from leaves co-expressing functional replicase (Fig. 6, lanes 7 and 8). To authenticate the identity of this unidentified RNA species, a Northern blot containing the virion RNA samples was hybridized with a 5′ end labeled total cellular RNA probe. The specificity of this riboprobe was exemplified by its hybridization to at least three prominent cellular RNAs but not to BMV virion RNA (Fig. 6C, bottom panel). A most striking observation emerged from this experiment is that only those virions recovered from leaf samples expressing a functional replicase did not contain any cellular RNA (Fig. 6C, bottom panel compare lanes 2–6 and 7–8).

Evidence that expression of a functional replicase enhances specificity of RNA packaging

The data presented above suggested that functional viral replicase is likely increase the specificity of BMV RNA packaging. To examine this possibility directly, we constructed replication-defective B1 and B2 by deleting the highly conserved 3′ 200 nt region (i.e., 3′ TLS) encompassing the minus strand promoter. Transient expression of replication-defective B1 (B1ΔTLS) and B2 (B2ΔTLS) mRNAs was confirmed by Northern blot analysis (Fig. 7A). To verify whether sufficient amounts of biologically active replication factors 1a and 2a could be translated, respectively, from the TLS-less B1 and B2, *N. benthamiana* leaves were co-infiltrated with a plasmid expressing wt B3. As shown in Fig. 7A, efficient accumulation of sgB4 mRNA confirmed that sufficient amount of replication proteins 1a and 2a were, respectively, expressed from TLS-less B1 and B2 mRNAs. In these assays the accumulation levels of wt B3 and its sgB4 was stimulated up to 45 fold (Fig. 7A) since only the wt B3 contained the minus strand promoter resulting in efficient amplification.

As surmised above, if the functional replicase has a selective role in enhancing specificity of viral RNA packaging, virions recovered from leaves expressing B1ΔTLS and B2ΔTLS should only contain viral RNAs. To verify this, virion RNA was isolated from *N. benthamiana* leaves infiltrated with a mixture of *Agrobacterium* cultures containing 35S-B1ΔTLS + 35S-B2ΔTLS + 35S-B4.1 or 35S-B1ΔTLS + 35S-B2ΔTLS + 35S-B3 and subjected to native agarose gel followed by multiple Northern blots hybridized with desired set of riboprobes (Figs. 7B and C). Even in the absence of replication, TLS-less B1 and B2 were efficiently packaged by the CP (Fig. 7B, top panel), demonstrating that packaging of genomic RNAs is not dependent on replication. However, their encapsidation into virions were effectively out competed by wt B3 and its sgB4 (Figs. 7B and C). As shown in Fig. 6C (top panel, lanes 7 and 8) virions isolated from leaves expressing functional viral replicase from TLS-less B1 and B2 did not package any cellular RNA (Fig. 7B). Taken together these results conclusively showed that expression of a functional viral replicase enhances specificity of BMV RNA packaging. At this stage we also want to emphasize that although in vitro assembly of BMV RNAs requires a virus encoded 3′ TLS either in *cis* or in *trans* (Choi et al., 2002), this requirement can be functionally substituted in *trans* by host tRNAs (Choi et al., 2002). Therefore, we hypothesize that the observed encapsidation of TLS-less B1 and B2 by transiently expressed CP in agroinfiltrated leaves is medi-

Fig. 3. Replication-independent in vivo expression of BMV CP. (A) Characteristics T-DNA constructs of BMV subgenomic RNA4 (sgB4) used for transient expression of CP in plants. In 35S-B4, the position of 35S promoter at the 5′ and the position of ribozyme (Rz) and *Nos* terminator (T) at the 3′ end and the nucleotide sequences at respective junctions are identical to those of T-DNA cassettes of wt BMV genomic cDNAs shown in the legend of Fig. 1B. The length of wt sgB4 and the number of non-viral nucleotides left after self-cleavage by ribozyme (shown in bracket) are indicated. In 35S-B4.1, a 149-nt translational enhancer leader sequence from *tobacco etch potyvirus* (TEV) was fused to the 5′ end of BMV CP ORF. Nucleotide sequence around the junction of TEV leader (lower case) is shown. The first AUG codon of BMV CP is boxed. The length of wt sgB4.1 generated from this modified plasmid was indicated. (B) Analysis of CP mRNA and its translation product. *N. benthamiana* leaves were infiltrated with *Agrobacterium* cultures containing either 35S-B4 (left panel) or 35S-B4.1 (right panel) and the accumulation of mRNA in two independently infiltrated leaves were analyzed on duplicate Northern blots and hybridized with indicated riboprobes. Asterisks in the right panel mark the completely processed B4.1 mRNA and those with arrow heads denote incompletely processed B4.1 mRNAs due to the absence of a self-cleaving ribozyme cassette and differential 3′ end processing by polyadenylation signal. In each blot, total RNA preparations isolated from leaves infiltrated with a mixture of *Agrobacterium* culture containing all three wt T-DNA constructs (B123) represent positive control and those extracted from uninfiltrated leaves (M) as negative control. The position of BMV RNAs is shown between the blots. CP analysis: Infiltrated leaves were homogenized and total protein extracts were subjected Western blot analysis as described under legend Fig. 1C. (C) Characterization of virions recovered from plants infiltrated with 35S-B4.1. Top panel: Electron microscopic image of virions purified from leaves infiltrated with 35S-B4.1. Procedure used to prepare the sample for viewing under EM was same that described in the legend of Fig. 1D. Arrows and white arrow heads indicate normal and smaller size virions, respectively. Scale bar = 50 nm. Middle panel: Sucrose density gradient profile of B4.1 virions. Partially purified virions were centrifuged through 10–40% linear sucrose gradient and gradient fractions were analyzed by UV absorption at 254 nm. The position of faster (H) and slower (L) sedimenting peaks relative to the sedimentation of wt BMV virions purified from symptomatic barley plants (indicated by dotted line) is shown. CP analysis: The procedure used to analyze the CP of sucrose gradient fractions by Western blot as described in the legend of Fig. 1C. Bottom panels: Northern blot analysis of sucrose gradient fractions. RNA extracted from each fraction was subjected to duplicate Northern blots and hybridized with the indicated riboprobes. Wt BMV RNA was used as a marker (M). Conditions of hybridization are as described in the legend of Fig. 1C. The position of BMV RNAs is indicated to the right.

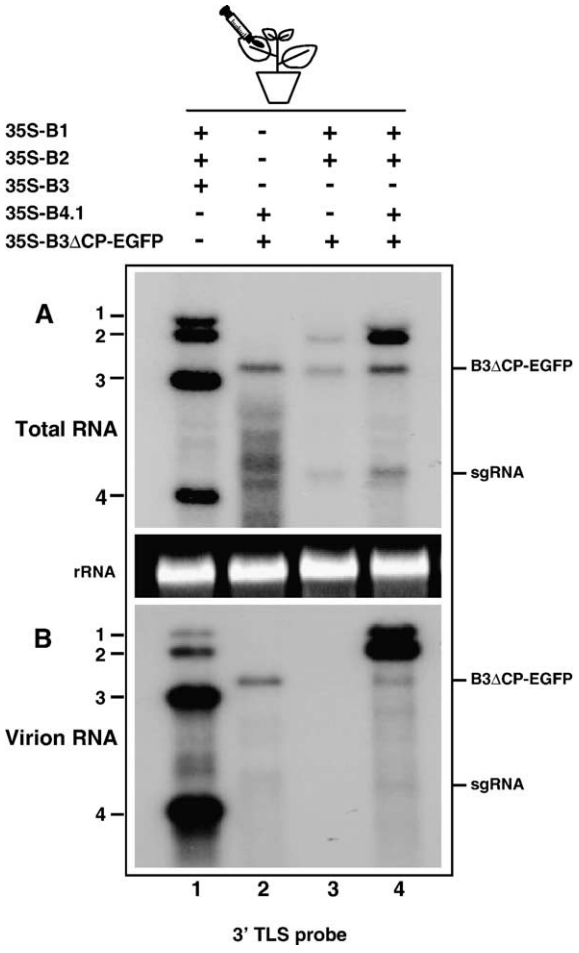


Fig. 4. Packaging of CP-defective B3 variant progeny by transiently expressed CP. *N. benthamiana* leaves were infiltrated with the following *Agrobacterium* cultures: 35S-B1 + 35S-B2 + 35S-B3 (lane 1), 35S-B3/ΔCP-EGFP + 35S-B4.1 (lane 2), 35S-B1 + 35S-B2 + 35S-B3/ΔCP-EGFP (lane 3), and 35S-B1 + 35S-B2 + 35S-B3/ΔCP-EGFP + 35S-B4.1 (lane 4). Total (A) and virion RNA (B) was isolated from each infiltrated leaf and subjected to Northern blot hybridization with the indicated riboprobes as described in the legend of Fig. 1C. The position of four BMV RNAs is shown to the left whereas the position of B3/ΔCP-EGFP and its sgB4 is shown to the right.

ated by host tRNAs functioning as efficient nucleating agents of viral CP subunits.

Discussion

Agroinfiltration as a tool for rapid and efficient transient expression of multicomponent genomes of BMV

Here we describe a simple, efficient, user-friendly agroinfiltration system for synchronized delivery of DNA-based genomic components of BMV to *N. benthamiana* plants. The inherent salient features of the system include the following: (i) transiently expressed mRNAs are biologically active resulting in efficient expression of viral gene products required for sustained replication (Fig. 1C); (ii) synchronized delivery of multiple plasmids to same cell offering the advantage of examining the replication and gene expression of movement-defective variants of B3 (Fig. 2C); (iii) abundant amounts of viral mRNAs, independent of replication, are continuously generated from the T-DNA transformed cells (Figs. 5–7); and (iv) most importantly, high level transient expression of encapsidation-competent CP generated independent of replication will permit in vivo packaging studies with ease (Figs. 6 and 7).

DNA-mediated in vivo transient expression of viral RNAs was established in non-plant systems such as *Sacchromyces cerevisiae* for BMV (Ishikawa et al., 1997)

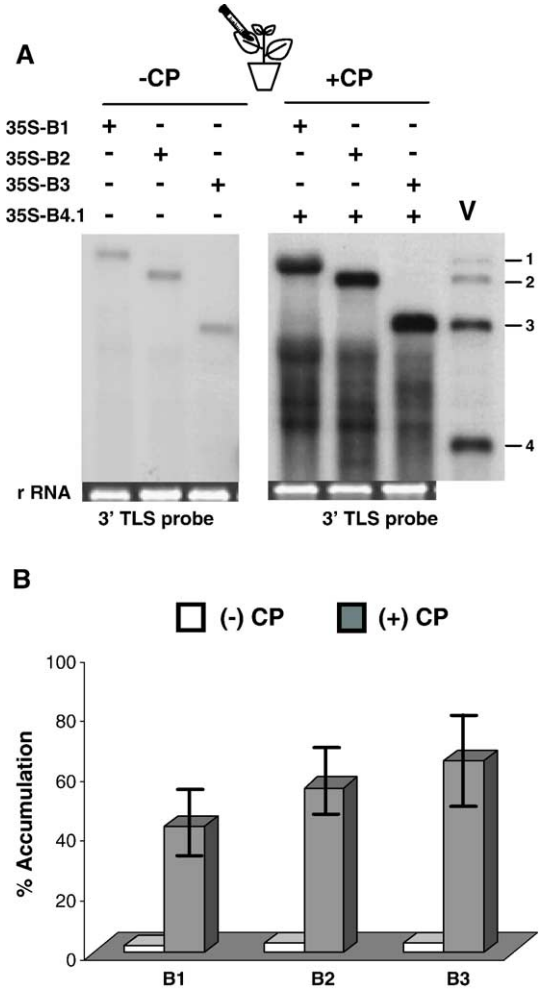


Fig. 5. (A) Replication-independent expression of individual BMV genomic RNAs in the absence and presence of CP. *N. benthamiana* leaves were infiltrated with indicated *Agrobacterium* cultures and the accumulation of mRNA was analyzed by Northern blots hybridized with the indicated riboprobes. Conditions of hybridization are as described in the legend of Fig. 1C. Approximately 100 ng of wt BMV virion RNA (V) was used as internal marker. (B) Relative accumulation of individual BMV genomic RNAs in absence (−) and presence (+) of CP. The histogram summarizes results from three independent agroinfiltration assays and the data were normalized against RNA1 component of wt BMV virion RNA.

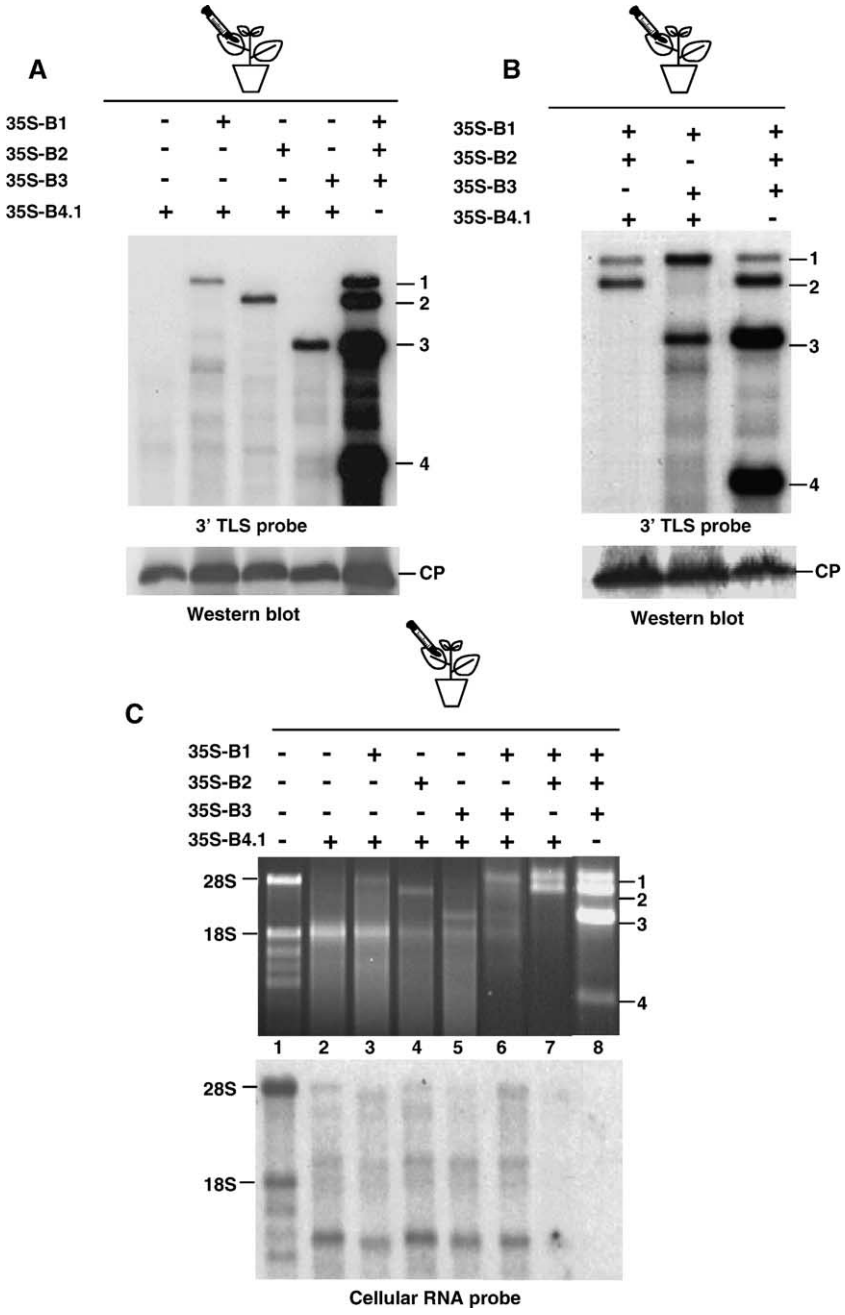


Fig. 6. In vivo packaging of a single or a desired pair of BMV RNAs in to virions. Packaging of a single (A) or a pair of BMV RNAs (B). *N. benthamiana* leaves were co-infiltrated with the indicated *Agrobacterium* cultures. Virions were purified from leaf tissue infiltrated with each sample and their RNA content analyzed by Northern blot hybridization with indicated riboprobes as described in the legend of Fig. 1C. The position of BMV RNAs is indicated to the right. Bottom panel: CP analysis by Western blot, as described under Fig. 1C. The position of the CP is indicated. (C) Top panel: Agarose gel electrophoretic analysis of virion RNA. Virion RNA isolated from indicated samples was subjected to 1% non-denaturing agarose gel electrophoresis and stained with ethidium bromide prior to photography. The positions of 28S and 18S cellular RNAs and the four BMV RNAs are indicated to the left and right, respectively. In each lane, RNAs recovered from virions purified from 7 g of infiltrated leaf material was subjected electrophoresis. Bottom panel: Northern blot analysis of virion RNA samples shown in the top panel. The blot was hybridized with a 5′ end labeled cellular RNA probe. Conditions of hybridization are as described in the legend of Fig. 1C. The position of 28S and 18S cellular RNAs is indicated to the left.

and *tomato bushy stunt tombusvirus* (TBSV; Panavas and Nagy, 2003). Although yeast has proven to be an excellent genetically tractable model system for analyzing the role of viral proteins in virus-host interaction studies (Ishikawa et al., 1997), agroinfiltration is becoming increasingly popular for studying various events leading to establish plant virus infection cycle in their natural hosts (Lee et al., 2005; Marillonnet et al., 2004; Volt et al., 2001). Once established, agroinfiltration of genetically tractable host plants such as *Arabidopsis thaliana* will help unraveling in identifying host factors involved in replication and other virus-host interactions.

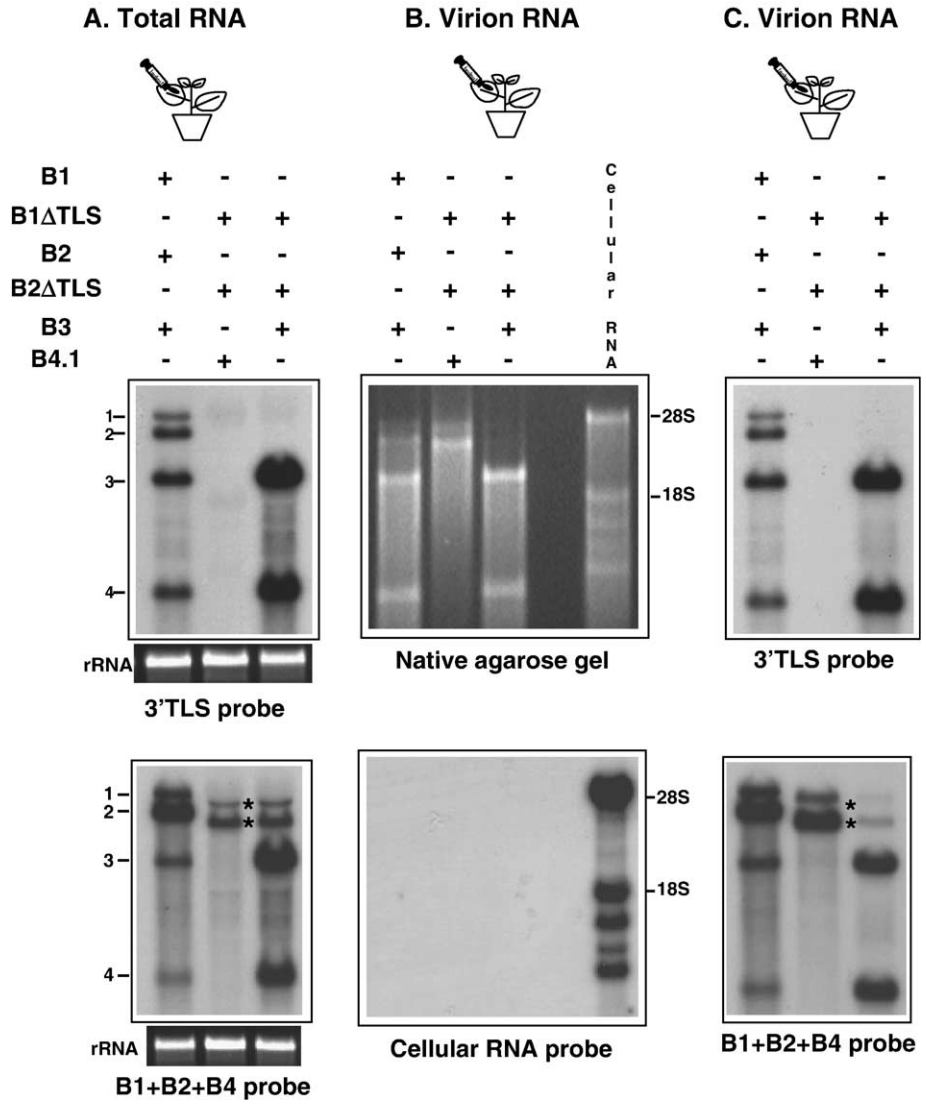


Fig. 7. In vivo expression and packaging of replication-defective B1 and B2 mRNAs. *N. benthamiana* leaves were co-infiltrated with the indicated *Agrobacterium* cultures. (A) Total RNA preparations were isolated from indicated agroinfiltrated leaves and subjected to Northern blot hybridization with the indicated riboprobes as described in the legend of Fig. 1C. (B) Virion RNA analysis: top panel: Ethidium bromide-stained native agarose gel showing the profile of virion RNA isolated from the indicated samples. Note that the intensity of RNA bands corresponding to TLS-less B1 and B2 in samples co-infiltrated with wt B3 was weaker; however, their presence was confirmed by Northern blot hybridization (see panel C). Bottom panel: Northern blot analysis of virion RNA samples shown in top panel. The blot was hybridized with a 5′ end labeled cellular RNA probe. The positions of 28S and 18S host RNAs are indicated to the right. (C) Northern blot analysis of virion RNA isolated from the indicated infiltrated samples. The blots were hybridized with the indicated riboprobes. The asterisk in the bottom panels indicates the position of faster migrating TLS-less B1 and B2. Conditions of hybridization are as described in the legend of Fig. 1C.

Agrobacterium-derived BMV mRNAs having extended 3′ non-viral bases are biologically functional

In BMV, initiation of negative strand synthesis by viral replicase occurs at the penultimate C residue of the 3′ CCA_{OH} terminus (Miller et al., 1986), and therefore authentic 3′ termini are considered to be crucial for efficient initiation of viral replication. However, Dreher et al. (1989) showed that inoculation of whole plants with in vitro transcripts derived from BMV cDNAs having + 11-nt 3′ extensions induced infectivity that was comparable to that of wt BMV RNAs. Mori et al. (1991) directly compared the relative efficiency of initiation of BMV replication from RNA transcripts to that of DNA templates fused to 35S promoter in mechanically inoculated plants. It was observed that the infectivity induced by DNA generated RNAs was 10,000-fold lower than in vitro transcribed RNAs. This lower efficiency of infection was attributed to the presence of several non-viral bases at the 5′ and 3′ ends. Similar studies involving DNA-based expression of BMV RNAs in yeast system revealed that initiation of BMV RNA replication from DNAs transformed into yeast cells is highly sensitive to 3′ extensions (Ishikawa et al., 1997). These authors observed that in vivo transcripts terminating

with either authentic 3′ ends (+ 0) or + 3-nt extensions, but not + 19-nt extensions, were found to be efficient templates for negative strand synthesis (Ishikawa et al., 1997). By contrast to the yeast system, in this study, *Agrobacterium*-generated BMV RNAs terminating with + 22-nt 3′ extensions appear to have no effect on initiating negative strand synthesis (Fig. 1C). One possible explanation for the observed discrepancy could be due to the inherent limitation of the yeast system since, consistent with the data presented here (Fig. 1C), Dzianott and Bujarski (1989) also reported that in vitro transcripts of BMV terminating with + 19-nt 3′ extensions are also highly infectious to whole plants. Taken together, the data suggested that the 3′ extensions required for initiation of RNA synthesis are more relaxed in plant system than in yeast. Alternatively, the nature of 3′ non-viral sequences of BMV RNAs generated in yeast may lack the secondary structure that exposes the 3′ CCA_{OH} terminus for recognition by the viral replicase.

Mechanism of RNA packaging in BMV

The mechanism that regulates controlled distribution of four BMV RNAs into three physically and morphologically indistinguishable virions is not known. Furthermore, absence of cellular RNAs in BMV virions purified from infected plants suggests that packaging is a highly coordinated process involving specific sequence and/or structure dependent interactions between CP and viral RNAs. Alternatively it is possible that the specificity of RNA packaging in BMV, as proposed for poliovirus (Nugent et al., 1999), could be directly coupled to replication so that only the actively replicated genomes can be packaged. As discussed below, analyses of packaging of replication-competent and replication-defective BMV RNAs by transiently expressed CP suggested that RNA packaging in BMV involves both replication-independent and dependent mechanisms.

RNA packaging independent of replication

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 with defined mutations in the 2a gene, capable of efficient replication in protoplasts, failed to support systemic infection in whole plants (Traynor et al., 1991). Because of the intrinsic involvement of viral replicase at various steps in BMV infection cycle, previous in vivo approaches such as whole plant infections or protoplast transfections are not suitable for separating replication from other functions such as RNA packaging. The transient expression system described here has proven to be ideal for dissecting the relationship between replication and assembly. This study revealed that packaging of BMV genomic RNAs is not replication contingent since replicating (Fig. 7C) as well as autonomously expressed full-length BMV genomic RNAs or replication-defective TLS-less B1 and B2 are efficiently packaged into virions by CP expressed in trans (Figs. 7B and C). This scenario contrasts with the situation that was described for polio virus replicons (Nugent et al., 1999), Kunjin virus (a member of the family Flaviviridae; Khromykh et al., 2001), and flock house virus (Venter et al., 2005) in which only the replicated RNAs were found to be packaged by the CP. However, our observation that virions assembled in the absence of a functional replicase also contained cellular RNAs in addition to the expected genomic RNAs (Fig. 6C, top panel, lanes 2–5) suggests that some sort of coupling exists between functional viral replicase and specificity of RNA packaging.

It was observed in yeast cells that BMV replication complex is associated with characteristic ER membrane invaginations, referred to as spherules (Schwartz et al., 2002). These specialized structures are induced by replicase protein 1a and have been shown to be the actual sites of RNA synthesis (Schwartz et al., 2002). Following its synthesis, 2a interacts with 1a and co-localizes to spherules to initiate viral replication and the necks connecting spherules and cytoplasm serve as channels to export newly synthesized progeny RNA to the cytoplasm for translation and virion assembly. Since CP has been found to co-purify with active replicase complex (Bujarski et al., 1982), our results might be rationalized by hypothesizing that CP must be synthesized near the necks of spherules. Consequently, transient association of functional viral replicase with CP can be envisioned to increase specificity of BMV RNA packaging as observed in this study (Figs. 6C and 7B, top panels). Absence of a functional replicase decreases CP specificity resulting in non-specific packaging of cellular RNAs (Fig. 6C, top panel, lanes 2–5). Alternatively it is possible that 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. Additional replication-independent assembly assays involving co-expression of 1a mutants exclusively defective in spherules formation coupled with immunocytological localization of replicase proteins and CP are likely to provide more information concerning the intimate relationship between viral replicase and RNA packaging.

Replication-dependent packaging

Results of this study also invite discussion concerning packaging of sgB4 into virions. Although sgB4 can be packaged autonomously in vitro, previous assays involving several B3 variants revealed that only B3, but not sgB4, is competent for efficient autonomous packaging in vivo (Choi and Rao, 2003). Consistent with these observations, unlike genomic RNAs, transient expression of CP mRNA independent of replication failed to exhibit efficient autonomous packaging or co-packaging with its progenitor genomic B3 (Fig. 6A). This packaging inefficiency of sgB4 is not due to the presence of non-viral sequences at the 5′ and 3′ end (Fig. 4A) since recently we also found that a transiently expressed B4 mRNA with natural 5′ and 3′ ends is also incompetent

for efficient packaging with B3 (Annamalai and Rao, unpublished data). It has been consistently observed that despite having a minus-strand promoter region there is no evidence for B4 to enter replication cycle and therefore the transcription of sgB4 is entirely dependent on the replication of B3 (Dreher et al., 1989; Miller et al., 1985). In addition, prior packaging of B3 appears to be a required prerequisite for B4 to co-package since mutations debilitating B3 packaging also blocked B4 packaging in vivo (Choi and Rao, 2003). Based on these intrinsic requirements and results of this study we hypothesize that unlike BMV genomic RNAs, newly transcribed sgB4 can only be packaged into virions. Therefore, a mechanism that couples replication and packaging in a fashion similar to that observed for polio and Kunjin viruses (Khromykh et al., 2001; Nugent et al., 1999) is also likely to regulate co-packaging of sgB4. The agroinfiltration system described here is ideal for finding such links between RNA synthesis and packaging.

Materials and methods

Construction of T-DNA-based plasmids for agroinfiltration

Plasmids pT7B1, pT7B2, and pT7B3 contain full-length cDNA clones corresponding to BMV genomic RNAs1 (B1), 2 (B2), and 3 (B3), respectively (Dreher et al., 1989), and characteristic features of plasmid pT7B4 containing cDNA of BMV CP mRNA were as described previously (Choi et al., 2000). To facilitate construction of T-DNA-based plasmids, a general vector pCass4-Rz (Fig. 1A), a variant of pCass2 (Shi et al., 1997), was constructed by amplifying a PCR product encompassing multiple cloning site consisting of five restriction sites and a 68-nt fragment encompassing the ribozyme (Rz) sequence of a satellite RNA of tobacco ring spot virus (sTobRV; Bruening et al., 1988) with a 5′ forward primer d(AGGGAATTC**GGTACC***TCTAGA*GGATCCGATACCCTGTCACCGGAT; boldface, italics, and underline sequences, respectively, represent *Kpn*I, *Xba*I, and *Bam*HI sites) and a 3′ reverse primer d(AGCGGGGTACCCGGAGCTCCTGCAGGACAGTC; *Sac*I site is underlined). Following digestion of the PCR product with *Kpn*I and *Sac*I, the product was ligated into similarly treated pCass4, a binary vector derived from pCAMBIA 1301. Each full-length cDNA sequence corresponding to the three BMV genomic RNAs and subgenomic RNA4 was amplified by PCR with 5′ primers d(CGACTCACTGCAGTAGACCACGGAACGAGG; *Pst*I site is underlined); d(CGACTCACTTACGTAACCACGGAACGAGG; *Sna*BI site is underlined); d(CGACTCACTTACGTAAAATACCAACTAATT; *Sna*BI site is underlined), d(TGTCCTAATTCTACGTATTAATAATGTC; *Sna*BI site is underlined), respectively, for B1, B2, B3, and B4 and a common 3′ reverse primer d(CAGGATCCCGACATGGTCTCTTTTAGAGATTTACAGTG3′; *Bam*HI site is underlined). The resulting PCR products were digested with either *Pst*I (for B1) or *Sna*BI (for B2 and B3), treated with T4 DNA polymerase to create blunt-ended products followed by digestion with a commonly shared *Bam*HI and finally subcloned into *Stu*I/*Bam*HI digested pCass4-Rz vector. 5′ sequences cloned into *Stu*I site of pCass4-Rz will contain no vectors sequences after in vivo transcription. The resulting plasmids, 35S-B1, 35S-B2, 35S-B3 (for genomic RNA expression), and 35S-B4 (for CP subgenomic mRNA expression), contains, in sequential order, a double 35S promoter, cDNA complementary to respective full-length BMV RNAs, Rz sequence, and *Nos* terminator (Fig. 1B). Another B4 construct, referred to as 35S-B4.1 (Fig. 3A), was constructed initially by amplifying a 886-nt fragment encompassing the full-length B4 sequence by PCR with a 5′ primer d(CTATGTCCTAATTTCGCGAATTAATAATGTCGA; *Nru*I site is underlined) and a 3′ primer d(CAGGATCCCGACATGGTCTCTTTTAGAGATTTACAGTG; *Bam*HI site is underlined), digested with *Nru*I and *Bam*HI and fused to *tobacco etch potyvirus* (TEV) translation enhancing sequence and subcloned in to a similarly treated pRTL2 vector to yield pRTLBCP. Finally, a *Hin*dIII fragment of pRTLBCP was subcloned into similarly treated pGEM 482 binary vector. The resulting construct 35S-B4.1 contains, in sequential order, a double 35S promoter, TEV enhancer element, BMV RNA4 sequence, and a *Nos* terminator (Fig. 3A). A variant of 35S-B3, referred to as 35S-B3ΔCP-EGFP (Fig. 2A), was constructed by amplifying the entire B3 sequence from pT7B3/ΔCP-EGFP (Rao, 1997) and subcloning into pCass4-Rz as an *Sna*BI–*Bam*HI fragment. Replication-defective B1 and B2 cDNAs, precisely lacking the 3′ TLS region (Duggal et al., 1992; Rao and Hall, 1990), were amplified in a PCR reaction and subcloned into pCass4-Rz vector as *Sna*BI–*Bam*HI fragments. The presence of the engineered deletion was confirmed by sequencing.

Agroinfiltration procedure

The agroinfiltration procedure was performed as described by Guo and Ding (2002). Briefly, the *Agrobacterium* strain EHA105 containing desired transformant was initially streaked on LB plate containing the antibiotics (kanamycin 50 μg/ml, rifampicin 10 μg/ml) and incubated at 28 °C. A single colony was inoculated into a 2-ml LB medium with above antibiotics, and grown at 28 °C for 48 h with vigorous shaking. One microliter of the culture was transferred to 50 ml LB medium containing the above antibiotics, 10 mM MES (pH 5.6) and 40 μM acetosyringone. After incubation at 28 °C for 16 h with vigorous shaking, OD_{600} of the culture reached 1.0. The bacteria were spun down at 2000×*g* for 10 min, the pellet was resuspended in 50 ml 10 mM $MgCl_2$, and then 125 μl 100 mM acetosyringone was added. To maintain a uniform concentration of *Agrobacterium*, co-infiltration experiments involving two or more *Agrobacterium* cultures, the OD_{600}

of the final mixture was adjusted to 1.0–1.2. The bacteria were kept at room temperature for at least 3 h without shaking. These cultures were infiltrated into abaxial surface of the fully expanded *N. benthamiana* leaves using 1-cc syringe without a needle.

EGFP imaging in whole leaves

Whole leaves of *N. benthamiana* infiltrated either with EGFP construct or empty vector were scanned with Typhoon 9410 fluorescence imager using 488 nm excitation with a 520-bp emission filter, and 633 nm excitation with 670/30 bp emission filter. Infiltrated leaves were also examined with a Zeiss 510 confocal laser scanning microscope, using 488 nm blue excitation and two sets of emission filters, 500–550 nm for EGFP and 560LP for chlorophyll. Images were arranged using Adobe Photoshop.

Progeny analysis

Total RNA was isolated from 3 to 4 days post agroinfiltrated (dpi) or uninfiltrated leaf samples as described by Verwoerd et al. (1989) with minor modifications. Briefly, 0.5 g leaf tissue was ground to a fine powder in liquid nitrogen. Then 1 ml hot (kept at 80 °C) extraction mixture containing buffer and phenol (100 mM Tris pH 9.0, 10 mM EDTA, 100 mM LiCl, 1% SDS, and equal volume of phenol) and 0.5 ml chloroform–isoamyl alcohol (24:1, V/V) were added and continued to ground until the mixture was completely homogenized. Each sample was transferred to a microfuge tube, vortexed for 5 min, and centrifuged at 12,000 rpm for 5 min at 4 °C. The supernatant was collected into a fresh microfuge tube and an equal volume of 4M LiCl was added and kept at −80 for 2 h. The sample was centrifuged at 12,000 rpm for 15 min at 4 °C and the pellet was resuspended in sterile distilled water. Total RNA was precipitated by adding 1/10 volume of 3M NaOAC (pH 5.2) and 2.5 volume of 100% ethanol. The tubes were kept at 4 °C overnight and centrifuged at 12,000 rpm for 15 min at 4 °C. The RNA pellet was washed with 70% ethanol, vacuum dried, and finally resuspended in desired volume of sterile distilled water. The concentration of total RNA was estimated by measuring the OD_{260} of the sample with a spectrophotometer.

Virions were purified from agroinfiltrated leaves as described previously (Rao et al., 1994). For Northern blot analysis (Annamalai and Rao, 2005) virion RNA (0.5 μg) or plant total RNAs (5 μg) were dried in a microfuge tube and suspended in 10 μl of sample buffer (10× MOPSbuffer/formamide/formaldehyde/H_2O 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 Russell, 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 prehybridization and hybridization using ^{32}P-labeled riboprobes corresponding to either 3′ conserved region (Rao et al., 1989), CP ORF, or sequences specific for B1 and B2 as described previously (Choi et al., 2000). CP samples were analyzed by Western blots according to Osman et al. (1997). For the preparation of a cellular RNA probe, total nucleic acids were isolated from healthy leaves using hot-phenol and LiCl precipitation (Verwoerd et al., 1989). RNAs were de-capped with tobacco acid pyrophosphate (Epicenter, Madison, WI) followed by dephosphorylation with alkaline phosphatase prior to end labeling with γ-[^{32}P]ATP using T4 polynucleotide kinase (Sambrook and Russell, 2001).

Electron microscopy

For negative staining, purified virus preparation was applied to glow discharged carbon-coated copper grids (Annamalai et al., 2005). Grids were washed once with water, stained with 1% uranyl acetate, and air dried. Grids were examined with an FEI Tecnai12 transmission electron microscope operating at 100 kV.

Acknowledgments

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