A stem–loop structure in the 5′ untranslated region of bean pod mottle virus RNA2 is specifically required for RNA2 accumulation

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Bean pod mottle virus (BPMV) is a bipartite, positive-sense (+) RNA plant virus of the family Secoviridae. Its RNA1 encodes all proteins needed for genome replication and is capable of autonomous replication. By contrast, BPMV RNA2 must utilize RNA1-encoded proteins for replication. Here, we sought to identify RNA elements in RNA2 required for its replication. The exchange of 5′ untranslated regions (UTRs) between genome segments revealed an RNA2-specific element in its 5′ UTR. Further mapping localized a 66 nucleotide region that was predicted to fold into an RNA stem–loop structure, designated SLC. Additional functional analysis indicated the importance of the middle portion of the stem and an adjacent two-base mismatch. This is the first report of a cis-acting RNA element in RNA2 of a bipartite secovirus.

The replication of viral genomes is a key step in the viral life cycle that depends on both virus-encoded functions and a multitude of host elements (den Boon & Ahlquist, 2010; Nagy & Pogany, 2010). For positive-sense (+) RNA viruses, this step is carried out primarily by RNA-dependent RNA polymerases (RdRPs) translated directly from viral genomic RNAs. In addition, most (+) RNA viruses also encode a second protein, designated auxiliary protein (AP), that induces the rearrangement of specific organellar membranes to form protective enclosures that shield the replication process from the cellular environment (Schwartz et al., 2002). The complete membrane-based enclosure, consisting of viral RNA, RdRp, AP, as well as various host proteins recruited to assist in this process, is often referred to as the viral replication complex (VRC).

The question of how viral RNAs access VRCs has been extensively studied for a number of viruses. Many viral genomic RNAs are also mRNAs for the translation of AP, hence may be brought to VRCs by APs in a translation-coupled manner (Yi & Kao, 2008; Wang et al., 2009). Other viral RNAs encode specific secondary structures or stem–loops (SLs) that are recognized by AP or RdRp, which in turn carries the corresponding viral RNAs to VRCs. The latter mechanism is commonly used by viruses with multipartite genomes, as some of their genome segments encode functions unrelated to genome replication, thus they have to rely on AP and RdRp encoded by other segments for their replication. This situation is best exemplified by brome mosaic virus (BMV) and red clover mosaic virus (RCNMV). The genetic information of BMV is partitioned in three RNA segments, with RNA1 and 2 encoding 1a and 2a, serving AP and RdRp functions, respectively. The smaller RNA3 encodes the cell-to-cell movement and capsid proteins (MP and CP). Accordingly, BMV RNA3 encodes a unique, cis-acting RNA structure that specifically interacts with 1a to dramatically enhance the replication of RNA3 (Baumstark & Ahlquist, 2001; and references therein). Similarly, RCNMV RNA2, which encodes solely the viral MP, is recruited to VRCs through the specific interaction between a Y-shaped RNA structure in its 3′ untranslated region (UTR) and the RNA1-encoded p27, the RCNMV AP (Iwakawa et al., 2011).

Bean pod mottle virus (BPMV) is similar to RCNMV in that all viral proteins required for genome replication are
encoded on RNA1 of its bipartite (+) RNA genome. In fact, BPMV RNA1 is known to replicate in single cells in the absence of RNA2 (Gu & Ghabrial, 2005; J. Lin & F. Qu, unpublished). However, unlike RCNMV, each of the two BPMV RNAs encodes one single polyprotein, from which multiple mature viral proteins are derived through post-translational processing by the virus-encoded protease (Pro). Specifically, the RNA1-encoded polyprotein is the precursor for five proteins: a putative protease co-factor (C-Pro), a putative RNA helicase (HEL) and possibly an AP, a small protein that covalently binds to the 5’ end of the genomic RNAs (viral protein genome-linked or VPG), Pro, and the viral RdRP (Fig. 1a). Notably, translation of BPMV RNA2 can initiate at two different start codons, leading to the production of two polyproteins that differ only at their N-termini (MacFarlane et al., 1991; Fig. 1a). The larger polyprotein is thought to be processed into three mature proteins: an N-terminal 58K protein (p58) with unknown functions, and two CP subunits (L-CP and S-CP, Fig. 1a), whereas the smaller polyprotein produces a smaller N-terminal mature protein that serves as the viral MP (Fig. 1a).

BPMV is a member of the comovirus genus in the Comovirinae subfamily of Secoviridae (Sanfaçon et al., 2009). Members of Secoviridae share a number of unique features, among them icosahedral particle structures, two or more different capsid protein subunits, and a very small VPG, which align them closely with the animal-infecting viruses of Picornaviridae (Le Gall et al., 2008). However, unlike picornaviruses, many secoviruses have bipartite RNA genomes, the replication of which is not well understood. In particular, it remains to be resolved how the replication proteins encoded by one of the genome segments replicates the other segment (van Bokhoven et al., 1993; Gaire et al., 1999). To begin to understand the replication process of BPMV, we chose to first focus on the replication requirements of BPMV RNA2, and attempted to identify cis-acting RNA sequences or structures within RNA2 that are specifically needed for its own accumulation. We report here the identification of an SL structure within the 5’ UTR of BPMV RNA2, referred to as SLC, which acts in cis to ensure the accumulation of RNA2 in infected cells. We speculate that SLC plays an essential role in shepherding RNA2 into VRCs programmed by RNA1-encoded proteins.

Our study began with the development of a reliable experimental system that allows us to monitor the accumulation of viral RNAs at both the single-cell and the intact-plant levels. The initial difficulty of developing a protoplast system amenable to BPMV infections prompted us to adopt an alternative approach. In this new approach, we placed the cDNAs of BPMV RNA1 and RNA2 between the 35S promoter and terminator (P35S and T35S; Fig. 1a) of cauliflower mosaic virus. Consequently, upon delivery of the resulting constructs into BPMV host cells, viral infection can be launched with RNAs transcribed intracellularly, thus bypassing the need for in vitro transcripts. Next, to facilitate the tracking of the infection process, we used as the RNA2 surrogate, an RNA2 derivative that contained a GFP insert between MP and L-CP (RNA2-GFP or R2G; Fig. 1a), which was shown previously to replicate to similar levels as wild-type RNA2 (Zhang et al., 2010). As a negative control, we generated a defective RNA1 cDNA, referred to as RNA1 m (Fig. 1a), which encodes a non-functional RdRP due to the replacement of the highly conserved glycine-aspartic acid-aspartic acid (GDD) motif with alanine-alanine-histidine (AAH; Fig. 1a). Finally, we used particle bombardment to deliver these constructs into lima bean cotyledons, which were shown to be well suited for particle bombardment delivery of plasmid DNA (Hernandez-Garcia et al., 2010).

Bombardment of lima bean cotyledons with RNA1 + R2G, but not with RNA1 m + R2G, resulted in rigorous BPMV replication and cell-to-cell movement as indicated by bright green fluorescent infection foci that cover multiple cells (Fig. 1b, top row, second and third panels; data not shown). Additionally, extracts of the BPMV-positive cotyledons were highly infectious to soybean plants, leading to severe BPMV symptoms and bright GFP fluorescence in the systemically infected leaves (Fig. 1b, second row, second and third panels; data not shown). The identity of R2G in both lima bean cotyledons and systemically infected soybean leaves were further confirmed with strand-specific reverse transcription-PCR (RT-PCR; Fig. 1c, d, lanes 2 and 3). In summary, our new approach permitted the observation of BPMV infection from single-cell levels to whole plants, hence is suited for our study.

We next examined the role of both 5’ and 3’ UTRs of BPMV RNA2 in RNA2 accumulation using this new procedure. To this end, we generated three R2G mutants in which the 5’ UTR, 3’ UTR, or both were replaced by their RNA1 counterparts, resulting in constructs R2G–1U5, –1U3, and –1U5/1U3, respectively (Fig. 1a). These constructs were then bombarded into lima bean cotyledons with RNA1. As shown in Fig. 1b, while replacing the 3’ UTR of RNA2 with that of RNA1 exerted no detectable impact on the infectivity of the virus in both bombarded lima bean cotyledons and systemically infected soybeans (Fig. 1b, third row, first panel), doing the same with 5’ UTRs led to a complete loss of BPMV infectivity in both types of plants (Fig. 1b, first row, last panel; data not shown). Accordingly, replacing both UTRs also caused the loss of viral infectivity (Fig. 1b, third row, second panel). These observations suggested that RNA2 5’ UTR contained critical cis-acting element(s) essential for RNA2 accumulation.

We then made additional modifications within the 5’ UTR of RNA2. Pairwise comparison revealed that the first 262 nucleotides (nts) of RNA1 and RNA2 5’ UTRs share a very high level of sequence identity (at least 91%), whereas the sequences after nt position 263 are much more divergent (J. Lin & F. Qu, unpublished; data not shown). We first determined whether the few differences within the first 262 nt could affect the replication of RNA2 by substituting
this portion of RNA2 5' UTR for its RNA1 counterpart. The resulting construct, R2G–1U5(262), was similarly competent as the wild-type R2G (Fig. 1b, third panel in third and fourth rows; Fig. 1c, d, lane 7). This result demonstrated that the first 262 nt of RNA1 and RNA2 5' UTRs are interchangeable and thus do not contain cis-acting elements unique for RNA2. Nevertheless, this section of 5' UTR contains essential cis-elements shared by RNA1 and RNA2, as its deletion from R2G led to a complete loss of BPMV infectivity (Fig. 1b, third row, last panel). Together, these results indicated that the RNA2-specific cis-acting structure does not reside in the first 262 nt of RNA2 5' UTR.

We then moved to interrogate the rest of the 5' UTR sequence (nt numbers 263–466) for a potential role in RNA2 replication. We first sought to determine the boundaries of the cis-acting elements by inserting a 36 nt non-BPMV sequence (termed 'ha' as the majority of its sequence was derived from the HA epitope tag cDNA) between nt positions 466 and 467, and 263 and 264, creating constructs R2G-466 ha and R2G-263 ha, respectively (Fig. 2a). When delivered to lima bean cotyledons and subsequently soybean plants to examine their infectivity, both of them resulted in productive infections indistinguishable from wild-type R2G (Fig. 2c, top row, last panel; and third row, first panel). Thus, the potential RNA2-specific, cis-acting element(s) must reside within the region between nt positions 263 and 466.

We then further delimited the putative cis-acting element through deletion mutagenesis. To help guide the deletion mutagenesis, we used the MFold algorithm to predict the RNA secondary structure of this region. As illustrated in Fig. 2b, this region could potentially fold into three SLs, referred to as SLA, SLB and SLC, separated by unstructured sections of varying lengths. We thus generated three different deletion mutants of R2G, namely ΔSLA, ΔSLB and ΔSLC, each removing one of the SLs together with a few flanking nts (Fig. 2a). As shown in Fig. 2c, while R2G-ΔSLA and R2G-ΔSLB caused rigorous BPMV infections essentially identical to wild-type R2G, R2G-ΔSLC was unable to cause any detectable infection even at the single-cell level (Fig. 2c, third and fourth rows, last three panels; data not shown). These results were further confirmed with strand-specific RT-PCR detection of fragments of expected size (Fig. 2d, e). Collectively, they strongly suggest that SLC constitutes a key cis-acting element for RNA2 propagation in host cells.

We next conducted a preliminary evaluation of the sequence and structural requirements of SLC. We started with the middle section of the stem because it contains the

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**Fig. 1.** RNA2 5' UTR contains key cis-acting elements required for RNA2 accumulation. (a) Schematic representation of BPMV RNA1, RNA1 m, and RNA2 (R2G) constructs, and R2G mutant with altered 5' or 3' UTRs. (b) Infectivity of mutant constructs assessed with particle-bombarded lima bean cotyledons and subsequently rub-inoculated soybean plants. The images of GFP fluorescent cotyledons were taken 48 h post bombardment, whereas those of systemically infected soybean leaves were taken two weeks post-inoculation. (c) Verification of active replication in lima bean cotyledons with (-) RNA2-specific RT-PCR. An RT-PCR product of a lima bean actin mRNA was used as controls. (d) Verification of active replication in systemically infected soybean leaves with (-) RNA2-specific RT-PCR. An RT-PCR product of a soybean actin mRNA was used as controls.
longest double-stranded stretch of SLC, with seven undisrupted base pairs (Fig. 3a). We first designed disruptions of the base pairs by changing four nts on the left arm of the stem to their complements on the right (UUCA to gggu), creating mutRR (Fig. 3a). A reciprocal mutLL mutant was similarly created by replacing UGGG on the right with acuu (Fig. 3a). In agreement with a critical importance of maintaining the stem, both mutRR and mutLL completely lost the ability to infect lima bean cotyledons (Fig. 3b, top row, last panel, and third row, first panel). However, when the two mutations were combined to create mutRL, in which the stem structure but not its original sequence was restored, wild-type infectivity was observed (Fig. 3b, third and fourth rows, middle panels; data not shown). These results indicated that the integrity of the middle section of the stem is critical for the function of SLC.

We then examined the importance of the bottom part of the SLC stem consisting of one C–U mismatch and six base pairs (Fig. 3a). We disrupted this portion of structure by inserting four nts (aucc) after nt number 461, hence creating a BamHI site in the corresponding cDNA. The resulting mutant, referred to as R2G-466ha, completely abolished the BPMV infectivity (Fig. 3b, third row, last panel). It should be noted that MFold predicted that the upper two-thirds of SLC would remain essentially undisturbed in this mutant. In addition, the six base pair stem at the bottom of SLC also remained intact despite some changes in nt identity (Fig. 3a). If this predicted structural consequence is correct, then either the two base (C–U) mismatch needs to be faithfully preserved, or the identity of certain nts within this section of SLC is critically important. In summary, our results support a critical role of SLC as a cis-acting element essential for RNA2 accumulation.
for SLC as a unique cis-acting RNA structure required for RNA2 accumulation in the host cells of BPMV.

Our study reveals the first RNA2-specific cis-acting element in a Secoviridae member. While similar elements have been identified in BMV and RCNMV, two well-studied multipartite (+) RNA viruses that adopt different coding and replication strategies than BPMV (Baumstark & Ahlquist, 2001; Iwakawa et al., 2011), such elements have not been identified in other Secoviridae members. Although cowpea mosaic virus (CPMV), another Secoviridae member with a similar genome organization as BPMV, has been subjected to UTR exchanges in a manner similar to our current study, no RNA2-specific cis-acting element was identified, as both 5′ and 3′ UTRs of CPMV RNA2 could be replaced by their RNA1 counterparts without affecting RNA2 replication (van Bokhoven et al., 1993; Rohll et al., 1993). We speculate that CPMV RNA2 could possess a cis-acting element within its polyprotein-coding sequence. Alternatively, CPMV RNA2 could be brought to VRCs using a mechanism that does not rely on a unique cis-acting RNA element.

Importantly, the conservation of SLC structure was also supported by phylogenetic analysis. As shown in Fig. 3c, among the nine BPMV isolates for which RNA2 sequences are available through GenBank, six of them contained the exact same sequence as the isolate we examined (IA-Di1). The other three isolates (two KY, one OH), while containing a number of base pair covariations, maintained the same SL structure (Fig. 3c). While it remains to be determined how SLC facilitates the accumulation of BPMV RNA2, the fact that SLC mutants were unable to accumulate even in single cells implies that SLC might help deliver RNA2 to RNA1-programmed VRCs. However, other possibilities, such as enhancing the translation or stability of RNA2, cannot be completely ruled out. Our next goals are to test if SLC interacts with RNA1-encoded proteins, and to elucidate the intra-cellular pathway through which RNA2 associates with VRCs.

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References


Fig. 3. Functionality of SLC depends on the integrity of the central portion of the stem. (a) Schematic representations of mutRR, mutLL, mutRL, and 460BamHI. The altered nts in each mutant are in lower-case letters. (b) Infectivity of mutant constructs assessed with particle-bombarded lima bean cotyledons and subsequently rub-inoculated soybean plants. (c) Conservation of SLC structure in RNA2 of various BPMV isolates. The SLC element was initially identified from an Iowa (IA-Di1) isolate, and shared with 100% identity by five additional isolates. Limited nt variations were found in two Kentucky (KY) isolates and one Ohio (OH) isolate, none of which caused significant changes in the overall structure.


