Review

Cis-acting RNA elements in positive-strand RNA plant virus genomes

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ARTICLE INFO

ABSTRACT

Positive-strand RNA viruses are the most common type of plant virus. Many aspects of the reproductive cycle of this group of viruses have been studied over the years and this has led to the accumulation of a significant amount of insightful information. In particular, the identification and characterization of *cis*-acting RNA elements within these viral genomes have revealed important roles in many fundamental viral processes such as virus disassembly, translation, genome replication, subgenomic mRNA transcription, and packaging. These functional *cis*-acting RNA elements include primary sequences, secondary and tertiary structures, as well as long-range RNA–RNA interactions, and they typically function by interacting with viral or host proteins. This review provides a general overview and update on some of the many roles played by *cis*-acting RNA elements in positive-strand RNA plant viruses.

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Contents

Introduction

Positive-strand RNA viruses contain single-stranded, coding-sensed RNA genomes. Most plant viruses contain this type of genome and many of them cause considerable damage to economically important crops. Accordingly, positive-strand RNA plant viruses have been the focus of intense study for many years and this has led to the accumulation of a substantial amount of information on molecular aspects of infections. The intracellular portion of infections can be broken down into several distinct steps. Following entry of a virus into a host cell, the particle must disassemble to allow for release of the RNA genome into the cytosol. This step is then followed by translation of the viral RNA genome by host ribosomes, which generates the viral proteins required for genome replication. One of these proteins is the viral RNA-dependent RNA polymerase (RdRp) that catalyzes the replication of the viral genome. For certain

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viruses, smaller viral messages termed subgenomic (sg) mRNAs are transcribed by the viral RdRp during infections, which template the translation of additional viral proteins. Following the accumulation of sufficient quantities of progeny viral RNA genomes and viral capsid protein(s), the final step in the reproductive process is packaging, in which viral genomes are encased in a particle composed of capsid protein. Each of the aforementioned steps is controlled by a combination of viral and/or host proteins and RNA elements within viral genomes. This review provides an overview and update on the different roles played by *cis*-acting RNA elements in regulating various steps in the reproduction of positive-strand RNA plant viruses.

Virus disassembly

After infecting their host cells, the genomes of plus-strand RNA viruses must shed their capsid before they can express their genetic information. This disassembly or uncoating process has been studied in plant viruses for decades, however the precise mechanisms utilized by most icosahedral or rod shaped viruses still remain elusive. Various models for disassembly have been proposed, and two of these, co-translational uncoating and co-replicational uncoating, have garnered the most attention (Brisco et al., 1986; Shaw et al., 1986; Wu et al., 1994).

Co-translational uncoating occurs when the capsid proteins (CPs) in a virion are displaced from the viral RNA genome by ribosomes translating a 5′-proximal open reading frame (ORF). Two viruses have been proposed to use this mechanism; the icosahedral Cowpea chlorotic mottle virus (CCMV) (Brisco et al., 1986) and the helical Tobacco mosaic virus (TMV) (Shaw et al., 1986; Wu et al., 1994). For CCMV, it was found that the 5′-end of the genome is released through a channel formed at a pentameric vertex, thereby allowing for ribosome binding to the 5′-cap (Albert et al., 1997). The 5′-cap structure therefore represents a key RNA element in this disassembly process. In the case of the capped TMV genome, the CP subunits in its helical capsid bind more weakly to the terminal 68 nucleotides of the 5′ untranslated region (UTR) (Mundry et al., 1991). This makes the terminus more prone to loosing CP subunits and results in exposure of the 5′-end to ribosomes (Wu et al., 1994). Thus, for TMV, the cap structure as well as the 5′-terminal sequence are important RNA elements, respectively, for ribosome binding and 5′-end access. More recently, a co-translational disassembly model, somewhat similar to that for CCMV but also involving proteolysis of CP subunits, was proposed for Turnip crinkle virus (TCV) (Bakker et al., 2012). In this case, however, the potential role of viral RNA elements is likely more complex, because the TCV genome is not capped and instead utilizes a translational enhancer in its 3′UTR for efficient translation (Stupina et al., 2008).

Co-translational uncoating provides a reasonable explanation for virion disassembly up to the end of the 5′-proximal ORF, but it does not account for how the remaining 3′-portion of a viral RNA genome is cleared of CP subunits. Consequently, a complementary mechanism, termed co-replicational uncoating, was proposed to address this issue (Brisco et al., 1986), and experimental support for the idea has been provided for TMV (Wu and Shaw, 1997). In this mechanism, once the RdRp is made, it binds to the RNA promoter in the genomic 3′UTR and displaces the remaining coat protein subunits during minus-strand synthesis (Brisco et al., 1986; Wu and Shaw, 1997). Here, the 3′-terminal promoter in the genome represents the RNA element mediating the uncoating process. Though an attractive model, this mechanism does not account for how the virus would avoid gene silencing triggered by the presence of double-stranded viral RNA in the cytosol. Collectively, the existing evidence indicates that the *cis*-acting RNA elements most relevant to disassembly reside near the 5′- and 3′-ends of viral genomes.

Translation: 5′- and 3′-proximal RNA elements

After disassembly, plus-sense RNA plant viruses use the host's translation machinery to express their proteins. These viruses use a wide variety of strategies to attract ribosomes, including utilizing both a 5′-cap and 3′-poly(A) tail, just one of the aforementioned, or neither (Dreher and Miller, 2006). Some viruses, like the potexvirus Potato virus X (PVX), contain traditional 5′-cap and 3′-poly(A) tail structures and, thus, recruit initiation factors in a similar fashion as cellular mRNAs (Huang et al., 2004; Dreher and Miller, 2006). However, PVX also contains RNA elements in its 5′UTR that contribute to translational enhancement (Huang et al., 2004; Park et al., 2013); thus, in this instance, canonical translation is augmented by these 5′-proximal viral RNA elements (Fig. 1A).

Other viruses like Turnip yellow mosaic tymovirus (TYMV) and TMV possess a 5′-cap but lack a 3′-poly(A)tail. At their 3′-termini, both of these viruses contain tRNA-like structures (TLSs) that can be amino-acylated (Gallie and Walbot, 1990; Matsuda and Dreher, 2004). The TLS of TYMV functions as a translational enhancer and this activity is linked to its amino-acylation and ability to bind eEF1A (Fig. 1B) (Matsuda and Dreher, 2004). The crystal structure of the TYMV TLS was recently solved and shows that this sequence does indeed adopt a tRNA-like conformation; however, it does so using a very different set of intramolecular interactions (Colussi et al., 2014). While the TMV TLS is structurally similar to the TYMV TLS, the former does not mediate translation enhancement, and TMV instead relies on a series of RNA pseudoknots upstream of the TLS for this activity (Leathers et al., 1993). Additionally, the omega sequence in the 5′UTR of TMV functions to promote translation by augmenting recruitment of eukaryotic initiation factor (eIF) 4F (Gallie, 2002). Thus, unconventional RNA elements at both ends of TMV act to facilitate cap-dependent translation (Fig. 1C).

Potyviruses have 3′-poly(A) tails, but no 5′-cap structures. Viral genome-linked proteins (VPgs) are attached to the 5′-end of their genomes and may contribute to translational efficiency by interacting with translation initiation factors (Khan et al., 2008; Eskelin et al., 2011). Although the role of the VPg in viral translation remains somewhat debatable in plant viruses, there is persuasive evidence for translational enhancement mediated by an internal ribosome entry site (IRES) in the 5′UTRs of potyviruses, which binds directly to eIF4G via an RNA pseudoknot (Fig. 1D) (Zeenko and Gallie, 2005; Khan et al., 2009). The activity of this IRES is further promoted by the presence of the 3′-poly(A) tail, indicating a level of cooperation between these atypical and conventional RNA elements (Gallie, 2001).

Some viral genomes, such as tombusvirids, luteoviruses, and umbraviruses, lack both a 5′-cap and 3′-poly(A) tail and instead contain a 3′ cap-independent translation enhancer (3′CITE) in their 3′UTRs (Nicholson and White, 2011; Simon and Miller, 2013). Most 3′CITEs, such as those in luteoviruses, tombusviruses and panicoviruses, function by recruiting eIF4E and/or 4G and interacting with their cognate 5′UTRs through intra-genomic RNA–RNA interactions (Fig. 1E) (Treder et al., 2008; Nicholson et al., 2010, 2013; Wang et al., 2009). The RNA-mediated joining of termini relocates the 3′CITE-bound eIFs to the 5′-end of the genome, which is the site to which ribosomes are recruited. Other 3′CITEs, such as that in TCV, bind directly to the 60S ribosomal subunit (Stupina et al., 2008) (Fig. 1F), which in turn engages the 40S subunit bound to a pyrimidine-rich sequence in the 5′UTR (McCormack et al., 2008; Stupina et al., 2011; Simon, in press).

Most viruses contain a single 3′CITE, but umbraviruses and carmoviruses harbor more than one, and this has been shown to extend host range (Miras et al., 2014; Gao et al., 2014). Structurally, most 3′CITEs have been studied at the level of RNA secondary structure, however three-dimensional reconstructions are available for two classes of 3′CITE, the T-shaped structure (TSS) in TCV
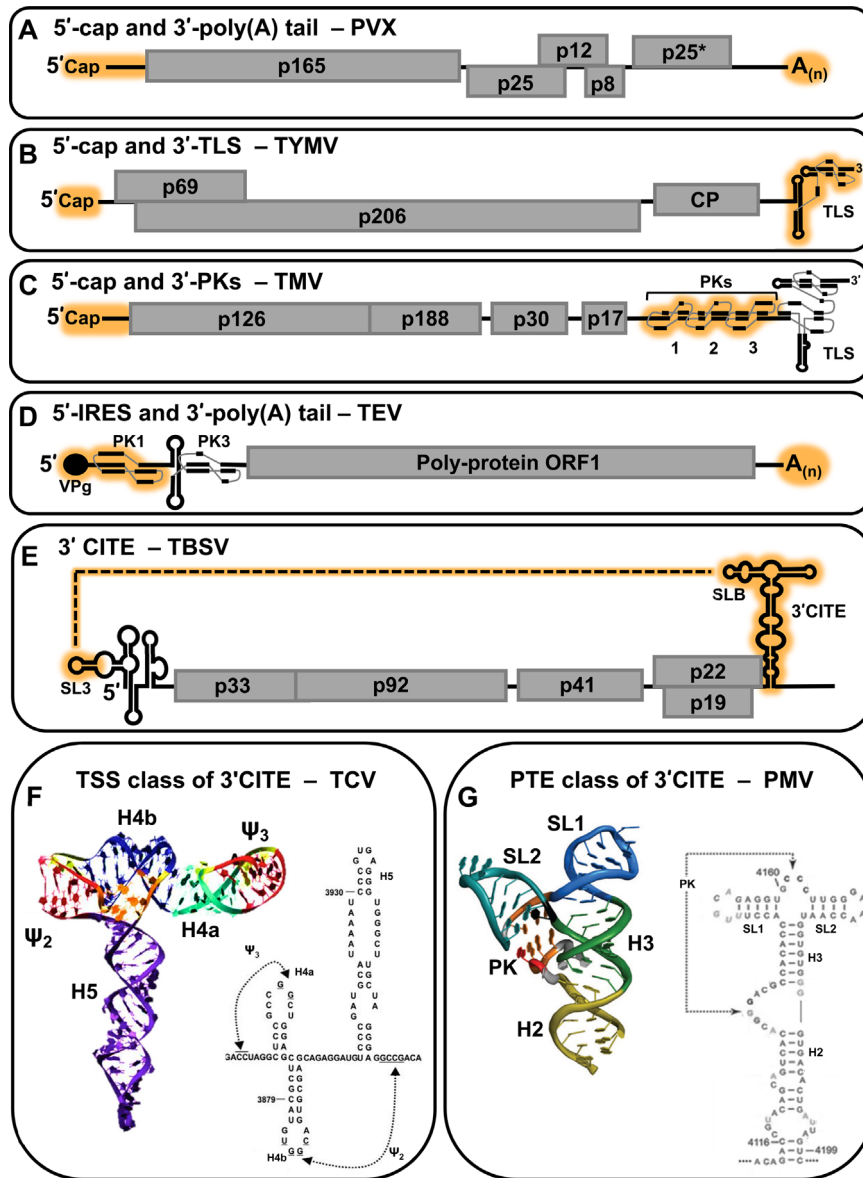
Fig. 1. Viral *cis*-acting RNA elements involved in translation. In (A)–(E), linear versions of selected viral RNA genomes are shown with coding regions represented as gray boxes. The *cis*-acting RNA sequences, structures, and long-range interactions required for efficient translation, as described in the text, are highlighted in gold. (A) PVX, showing 5′-cap, 5′UTR and 3′-poly(A) tail. (B) TYMV, showing 5′-cap and 3′-terminal TLS. (C) TMV, showing 5′-cap, 5′UTR, 3′-pseudoknots (PKs). (D) TEV, showing VPg, 5′-pseudoknot, and 3′-poly(A) tail. (E) TBSV, showing 3′CITE and its long-range RNA-RNA interaction with the 3′UTR (dotted line). (F) 3D model for the T-shaped structure class of 3′CITE in TCV. Corresponding RNA secondary structure showing tertiary interactions (dotted arrows) is shown to the right (adapted from McCormack et al., 2008). (G) 3D model of the PTE class of 3′CITE in PMV. Corresponding RNA secondary structure showing tertiary interaction (dotted arrow) is shown to the right (adapted from Wang et al., 2011).

(McCormack et al., 2008; Zuo et al., 2010) and the panicum-like translational enhancer (PTE) in Panicum mosaic virus (PMV) (Wang et al., 2011) (Fig. 1F and G). These detailed structures have provided important insights into how these RNAs engage their respective ligands and operate mechanistically (Simon and Miller, 2013; Simon, in press).

Translation: unconventional expression strategies

Due to their limited size, viruses must maximize their coding potential. Accordingly, they utilize a variety of unusual gene expression strategies to assist in this endeavor, including translational frameshifting, translation readthrough, and the utilization of IRESes. In all cases, these translational strategies involve *cis*-acting RNA elements that contribute to the function and regulation of these activities.

Frameshifting occurs when a translating ribosome shifts forward or backward into a different reading frame and continues to decode the message (Firth and Brierley, 2012). This process generally occurs at a low frequency, thus the frameshift proteins are produced in small quantities (Miller and Giedroc, 2010). Many different plus-strand RNA plant viruses employ this recoding strategy, which is mediated by RNA elements proximal and distal to frameshift sites.

Plus-strand RNA viruses, such as sobemoviruses and umbraviruses, utilize -1 frameshifting for RdRp production and this process is facilitated by a combination of primary, secondary, and/or tertiary RNA structures (Firth and Brierley, 2012). The primary element is a slippery heptanucleotide sequence at the frameshift site that is closely followed by a stem–loop (SL) or pseudoknot structure (Miller and Giedroc, 2010). It has been proposed that the downstream structure provides resistance to the translating ribosome and helps it to shift backwards into the -1 reading frame (Namy et al.,
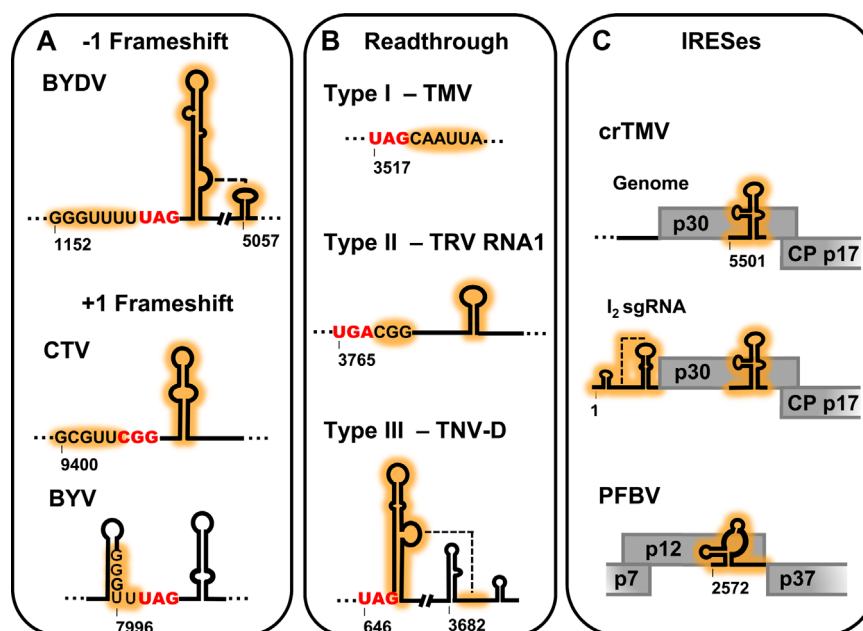
Fig. 2. Unconventional translation strategies. (A) Cartoons depicting portions of viral genomes harboring RNA sequences and structures involved in −1 frameshifting in the luteovirus BYDV and +1 frameshifting in the closteroviruses CTV and BYV. For BYDV, the structure at the frameshift site interacts with a far-downstream sequence, as indicted by the dashed line. In the examples shown the frameshift protein produced is the viral RdRp. (B) Cartoons depicting regions of viral genomes containing RNA sequences and structures involved in translation readthrough for type I–III elements in TMV, TRV and TNV-D, respectively. For TNV-D, the structure at the readthrough site interacts with a far-downstream sequence, as indicted by the dashed line. In the examples shown, the readthrough protein produced is the viral RdRp. (C) Cartoons depicting parts of viral genomes or a sg mRNA comprising RNA sequences and structures involved in mediating IRES activity in crTMV and PFBV. Viral movement or CPs are expressed in the examples shown. See text for details.

2006). In certain cases, an additional long-range RNA–RNA interaction between the shift-proximal higher-order structure and a sequence far downstream in the genome is needed for frameshifting to occur (Fig. 2A). This latter requirement is true for both luteoviruses and dianthoviruses, and the communication may help to coordinate frameshifting with minus-strand RNA synthesis (Barry and Miller, 2002; Tajima et al., 2011). The less typical +1 frameshifting has been proposed to occur in Citrus tristeza virus (CTV) and Beet yellows virus (BYV), members of the family Closteroviridae (Miller and Giedroc, 2010), but this process and its associated RNA elements are less well characterized. A slippery heptanucleotide sequence that includes a stop or "hungry" codon (i.e. CGG for Arg) has been implicated as well as a possible downstream secondary structure (Agranovsky et al., 1994; Çevik, 2001); however the precise frameshifting mechanism involved remains unclear (Fig. 2A).

Readthrough, like frameshifting, provides a way to produce small amounts of C-terminally extended proteins, most often the RdRp. In certain viruses, the stop codon of a particular gene is "leaky" and a small percentage of ribosomes are able to continue translating by incorporating an amino acid at the position of the stop codon (Firth and Brierley, 2012). Three major classes of readthrough element are associated with leaky stop codons. For type I readthrough, the stop codon UAG is followed by CARYYA, where R is a purine, and Y is a pyrimidine (Fig. 2B) (Skuzeski et al., 1991). Tobamoviruses, benyviruses and pomoviruses are known to use this class of readthrough element (Firth and Brierley, 2012). Type II readthrough elements, found in tobraviruses and furoviruses, typically involve an UGA stop codon followed by either CGG or CUA and then a large RNA stem–loop structure (Fig. 2B) (Urban et al., 1996; Firth et al., 2011). Type III readthrough occurs at an UAG stop codon followed by a guanine residue and then a secondary or tertiary structure (Firth and Brierley, 2012). This form of readthrough in tombusviruses, carmoviruses, and betanecroviruses also requires a long-range interaction between the structure at the readthrough site and the genomic 3′UTR, and in CIRV, this interaction was shown to coordinate translation of the

viral polymerase with viral genome replication (Fig. 2B) (Cimino et al., 2011; Newburn et al., 2014). Moreover, this requirement of long-range communication for readthrough appears to be a common feature of tombusvirids (Cimino et al., 2011; Newburn et al., 2014).

Another alternative translation strategy utilized by plus-strand RNA viruses is to employ IRESes, which function to recruit ribosomes independent of a 5′-cap structure or a 5′-terminus (Balvay et al., 2009). Plant virus IRESes generally correspond to poorly conserved sequences that may or may not contain significant RNA secondary structure (Dreher and Miller, 2006). The uncapped potyvirus Tobacco etch virus contains an IRES in its 5′UTR that functions by binding to eIF4G via a pseudoknot (Fig. 2D) (Zeenko and Gallie, 2005). Other VPg-containing viruses such as nepoviruses and poleroviruses also harbor IRESes in their genomes; with the nepovirus element being larger and structurally undefined, and the polerovirus element corresponding to a short run of purines (Jaag et al., 2003; Karetnikov and Lehto, 2007). In both cases, it was speculated that these RNA elements promote translation by interacting with rRNA in the ribosome.

IRESes are also located at internal genomic positions within certain viral genomes. For example, crucifer-infecting (cr) TMV has two IRESes. One is active only in the I₂ sg mRNA and directs the translation of the movement protein, while the other is active in both the genome and I₂ sg mRNA, allowing for CP production (Dorokhov et al., 2006). The latter IRES contains a bulged stem–loop structure that is flanked by two purine-rich sequences that are crucial for IRES activity (Dorokhov et al., 2002) (Fig. 2C). CP expression from viral genomes is also mediated by IRESes in two carmoviruses, Hibiscus chlorotic ringspot virus (HCRSV) and Pelargonium flower break virus (PFBV) (Koh et al., 2003; Fernández-Miragall and Hernández, 2011), as well as the betanecrovirus TNV-D (Newburn et al., 2014) (Fig. 2C). Consistent with the hard-to-define nature of these elements, these latter IRESes do not share any obvious similarities at either the sequence or predicted higher-order structure levels.

Viral genome replication

Viral genome replication of plus-sense RNA plant viruses occurs following translation of viral replication proteins. This process is executed by the RdRp-containing viral RNA replication complex (RC) and proceeds via synthesis of a genomic minus-strand RNA intermediate. *Cis*-acting RNA elements that promote and regulate this process are present in the 3′UTR, internal regions, and 5′UTR of the viral genomes. A variety of different sequences and structures have been identified and, characterized and those from some of the best studied examples are described below.

One of the most extensively investigated viruses in terms of genome replication is Brome mosaic virus (BMV), which consists of three RNA genome segments, RNA 1–3. The 3′-terminal regions of all BMV RNAs fold into a TLS in which a stem–loop, SLC, represents a key binding element for the viral RC (Fig. 3A) (Chapman and Kao, 1999; Sivakumaran et al., 2000). The atomic structure of SLC was solved and revealed a more flexible lower stem region containing an internal loop and a more rigid upper section containing an AUA

terminal loop (Kim et al., 2000). Moreover, efficient minus-strand RNA synthesis was found to require a specific conformation of the AUA loop, known as a clamped adenine motif (Kim et al., 2000; Kim and Kao, 2001). The intercistronic region of BMV RNA3 also contains an important regulatory sequence that folds into an extended structure (Fig. 3A) (Baumstark and Ahlquist, 2001). The apical stem–loop in this structure matches the box-B sequence of the RNA polymerase III promoter and interacts with the BMV protein 1a (Sullivan and Ahlquist, 1999). The B-box motif is also present in the 5′UTR of BMV RNA1 and RNA2 (Chen et al., 2001; Choi et al., 2004; Yi and Kao, 2008) and, similarly, plays important roles in 1a-dependent selection of these viral genomes for replication (Choi et al., 2004; Yi and Kao, 2008). Comparable to the BMV RNA-box-B interaction, the TMV 126-kDa RdRp protein binds to the 5′UTR of the viral genome and selects it as a template for replication. This interaction occurs during translation of the 126-kDa RdRp, thereby coupling translation and replication and promoting *cis*-preferential replication of the genome (Kawamura-Nagaya et al., 2014). For BMV, RNA elements involved in plus-strand production have also been
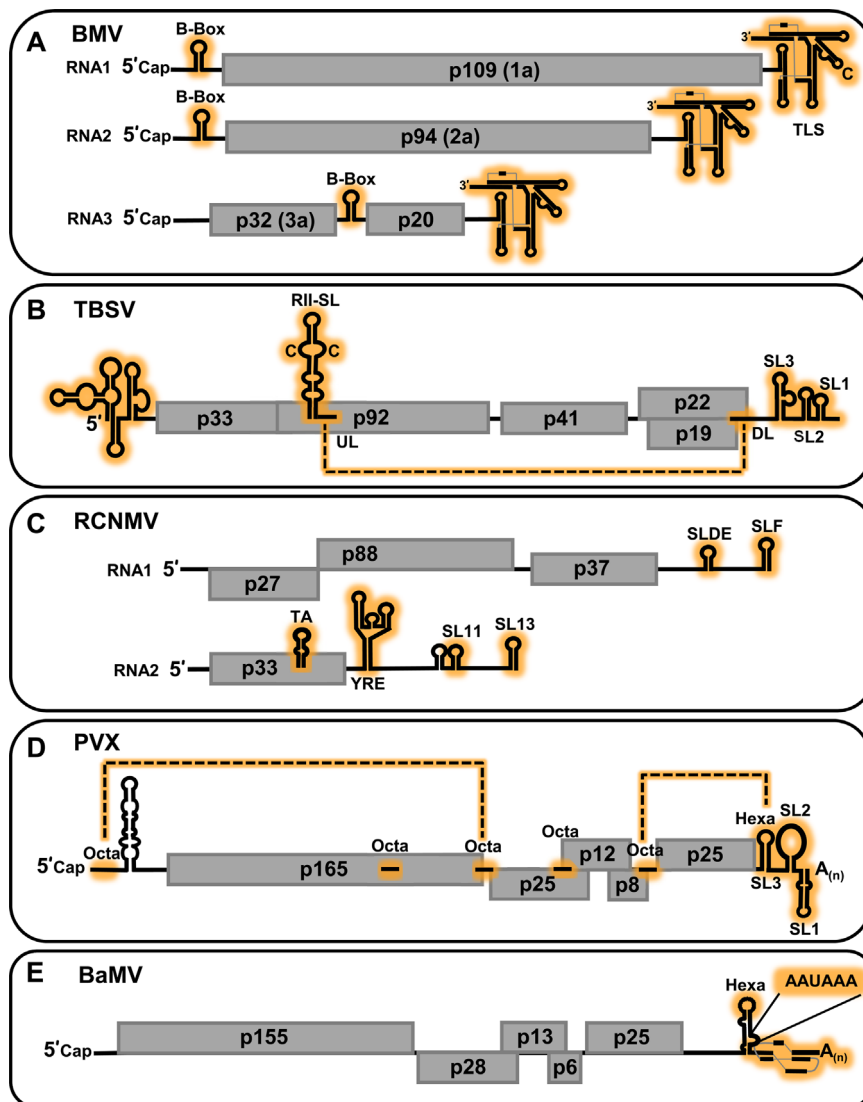

Fig. 3. Viral *cis*-acting RNA elements involved in genome replication. Linear versions of selected viral RNA genomes are shown. The *cis*-acting RNA sequences, structures, and long-range interactions involved in mediating genome replication, as described in the text, are highlighted in gold. (A) The three genomic RNA segments of BMV. Also depicted are the 5′-proximal and internal B-boxes and the 3′-terminal TLSs. (B) TBSV, showing the 5′UTR, internal RII-SL, and 3′-terminal SL1/2/3. The long-range RNA–RNA interaction between the UL and DL sequences that unite the RII-SL and SL1/2/3 is depicted by a dotted line. (C) RCNMV, showing the 3′-proximal SLs in both segments and the TA and YRE structures in RNA2. (D) PVX, showing the 5′-proximal octanucleotide (octa) in the 5′UTR and 3′-proximal hexanucleotide (hexa) in the 3′UTR. Both of these terminally-located RNA elements base pair with internal octanucleotide sequences, as shown by the dotted lines. (E) BaMV, showing the polyadenylation signal AAUAAA and 3′-terminal pseudoknot involving the 3′-poly(A) tail. The 3′-proximal hexanucleotide shown is also important for genome replication.

explored, and it was determined that a small stem–loop structure that forms at the 3′-end of the minus-strand of RNA2 serves as the core promoter for this process (Sivakumaran et al., 1999).

The tombusviruses, typified by Tomato bushy stunt virus (TBSV), have been the focus of intense studies on genome replication for many years (White and Nagy, 2004). The core promoter for minus-strand synthesis is the 3′-terminal structure, SL1 (Panavas et al., 2002), however its activity is modulated by an interaction between the 3′-terminal sequence (that includes part of SL1) and a bulge in upstream SL3 (Fig. 3B) (Pogany et al., 2003). Such short-range interactions that modulate core promoter activity also occur in the carmovirus TCV (Zhang et al., 2004) and appear to be a common feature of most tombusvirids (Na and White, 2006). In addition to these 3′-proximal structures, an internal RNA element in the p92/RdRp coding region of TBSV is also required for replication (Monkewich et al., 2005) and corresponding RNA elements are also present in other tombusvirids (Nicholson et al., 2012). The extended RNA hairpin containing a crucial CC mismatch, called RII-SL in TBSV, binds to the viral p33 auxiliary replication protein which recruits the genome for replication (Fig. 3B) (Pogany et al., 2005). RII-SL and the 3′-terminal SL1/2/3 are united by a long-range intragenomic RNA–RNA interaction between upstream and downstream linker (UL and DL) sequences, and the bipartite RNA platform that is formed (i.e. RII-SL-SL1/2/3) mediates assembly of the viral RC (Fig. 3B) (Wu et al., 2009; Nagy et al., 2012). For plus-strand synthesis, additional RNA structures in the 5′UTR, as well as in its complementary sequence, contribute to this event (Wang and White, 2007; Panavas et al., 2003). Still other RNA elements in the minus-strand are implicated in boosting genome replication by assisting in the retention and unfolding of the minus-strand genome template (Wang and Nagy, 2008; Kovalev and Nagy, 2014). Since the intermediate in tombus-virus RNA synthesis is double-stranded RNA, access to these elements in the minus strand is mediated by the recruitment of host helicases (Kovalev et al., 2014; Kovalev and Nagy, 2014).

The dianthovirus RCNMV is also a tombusvirid and represents an additional useful model system for understanding viral genome replication. RCNMV is unique among tombusvirids in that it possesses a bi-segmented RNA genome. Both genome segments contain RNA structures in their 3′UTR that are important for replication. In RNA1, the stem–loops SLDE and SLF and the intervening sequence are considered to be the core promoter for minus-strand synthesis (Fig. 3C) (Iwakawa et al., 2007). The corresponding sequence and structures in RNA2, including SL11 and SL13, represent the core promoter for this genome segment. However, RNA2 has two additional RNA elements that are also crucial for minus-strand synthesis; a Y-shaped RNA element (YRE) (An et al., 2010; Iwakawa et al., 2011), and a *trans*-activator (TA) element in the coding region (Fig. 3C) (Tatsuta et al., 2005). As RNA2 is replicated by the RdRp that is translated from RNA1, the extra RNA elements in this template likely contribute to mediating this distinctive *trans*-replication scheme (Iwakawa et al., 2011).

There have also been notable studies on the RNA elements modulating genome replication in potexviruses. PVX, the type member of the genus, contains elements important for replication in its 3′UTR, internal coding region, and 5′UTR. The 3′UTR contains three functional RNA stem–loops: SL1–SL3. The formation of SL3 is critical for minus-strand genome accumulation, while SL1 and SL2 are less important (Pillai-Nair et al., 2003). SL3 contains a hex-anucleotide sequence in its terminal loop that is conserved in all potexviruses (Bancroft et al., 1991) and, for minus-strand synthesis in PVX, this sequence must pair with one of several upstream octanucleotide elements (Fig. 3D) (Hu et al., 2007). The genomic 5′UTR contains structures that are required for plus-strand RNA accumulation (Miller et al., 1998) and an octanucleotide sequence in this region also participates in a long-range interaction with downstream internal octanucleotide elements (Fig. 3D) (Hu et al.,

2007). The precise roles of these distance-spanning interactions are not known, however they are thought to somehow position termini optimally for processes related to genome replication (Hu et al., 2007).

Bamboo mosaic virus (BaMV) is another potexvirus where RNA elements involved in genome replication have been characterized. BaMV possesses a 3′-poly(A) tail that is important for genome stability and translation (Bergamini et al., 2000) and is also involved in genome amplification (Tsai et al., 1999; Chen et al., 2005). As for PVX, the 3′UTR of the BaMV genome folds into a series of SLs, however in BaMV the 3′-poly(A) tail participates in the formation of a pseudoknot that is critical for efficient genomic minus-strand synthesis (Fig. 2E) (Cheng et al., 2002; Chen et al., 2005). Interestingly, glyceraldehyde 3-phosphate dehydrogenase binds to this pseudoknot and inhibits negative-strand synthesis, thus this structure participates in both up- and down-regulation of genome replication (Prasanth et al., 2011). In addition to the 3′-poly(A) tail, the polyadenalation signal, AAUAAA, present in the 3′UTR, is also important for minus-strand synthesis in a manner independent from its importance in the formation of the 3′-poly(A) tail (Fig. 3E) (Chen et al., 2005). Considering also that the RC isolated from BaMV-infected plants contains both replicase activity and polyadenylation activity, it appears that BaMV genome replication is intimately linked to polyadenylation (Chen et al., 2013).

Subgenomic mRNA transcription

Most plus-strand RNA viruses with polycistronic coding organization transcribe sg mRNAs that allow for translation and expression of downstream ORFs (Sztuba-Solińska et al., 2011). The majority of sg mRNAs have 5′-ends that map to internal regions of the genome, just upstream of the ORF to be translated, while their 3′-ends are coterminal with the genome. However, some sg mRNAs are 5′-coterminal with the genome and have 3′-ends mapping to internal regions. In either case, transcription of these truncated genomic messages is performed by viral RdRps, and several distinct mechanisms have been uncovered. The RdRps involved are guided by different RNA elements that control various steps in the transcription process.

The earliest transcriptional mechanism reported was that of internal initiation (Miller et al., 1985). In this mechanism, a promoter located internally in the full-length genomic minus-strand is used to initiate the synthesis of the sg mRNA, which then terminates when the RdRp reaches the 5′-end of the template (Sztuba-Solińska et al., 2011). This produces a sg mRNA that is 3′-coterminal with the genome template (Fig. 4A). BMV utilizes this mechanism to transcribe sgRNA4 from RNA3, which serves as the template for translation of the p20 CP (Miller et al., 1985). The internal initiation is guided by a core sg promoter that includes a stem–loop RNA structure in the inter-cistronic region of the minus-strand of RNA3 (Sivakumaran et al., 2004). This type of internal initiation mechanism is also used by other viruses, such as tobamoviruses and alfamoviruses (Skulachev et al., 1999; Haasnoot et al., 2000). Interestingly, BMV also produces a second sg mRNA, termed sgRNA3a, which encodes the upstream p33 movement protein (Wierzchoslawski et al., 2006). For this sg mRNA, its 5′-end is coterminal with the 5′-terminus of RNA3. The model to explain the transcription of this unconventional message is that the promoter for synthesis of plus-strand RNA3 is used to initiate transcription; however, instead of continuing copying to the end of the minus-strand RNA3 template, the RdRp terminates prematurely in the intergenic region (Fig. 4Bi) (Wierzchoslawski et al., 2006). Termination is thought to be mediated by secondary structure and a poly (U)-tract in the intergenic region of the template which would, respectively, impede replicase progression and facilitate its dissociation due to weaker base pairing (Wierzchoslawski et al., 2006).
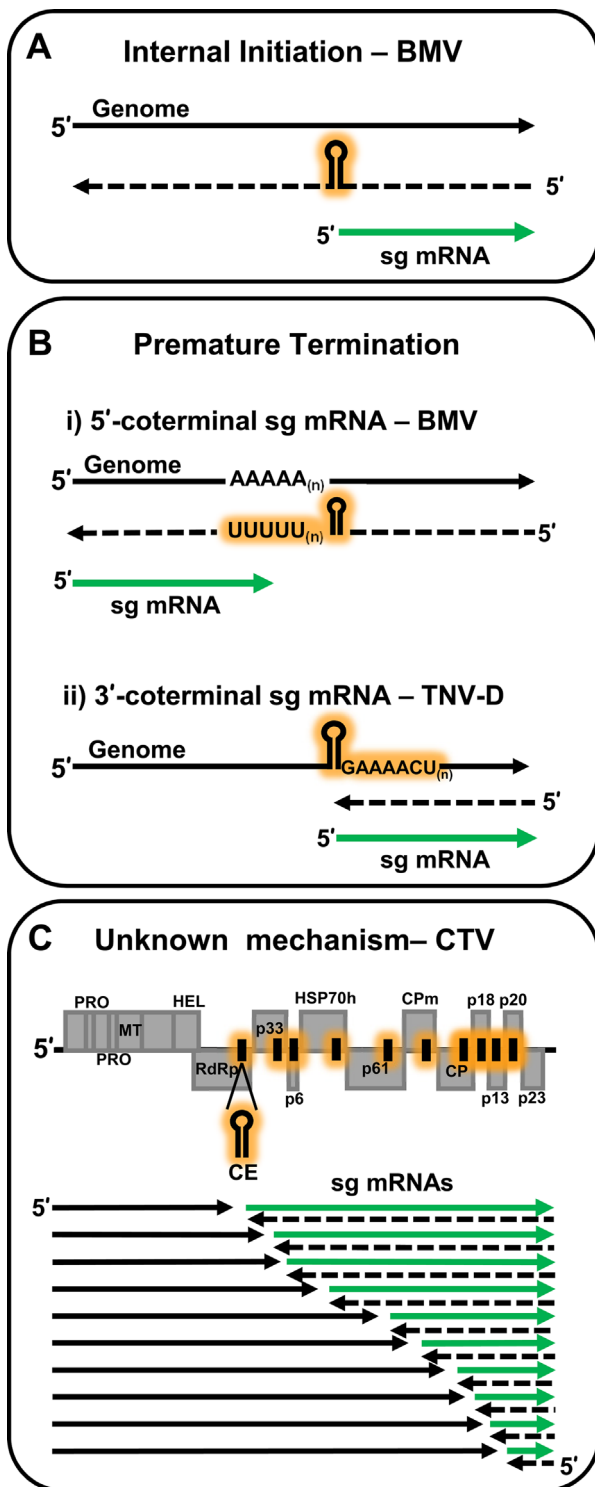
A Internal Initiation – BMV

B Premature Termination

i) 5′-coterminal sg mRNA – BMV

ii) 3′-coterminal sg mRNA – TNV-D

C Unknown mechanism– CTV

Fig. 4. Viral *cis*-acting RNA elements involved in subgenomic mRNA transcription. RNA elements that promote transcription are highlighted in gold. (A) A generic plus-strand RNA genome is depicted by the solid arrow on top. In the internal initiation model, an internally positioned promoter (i.e. the highlighted hairpin) in the full-length minus-strand genome (dotted line) directs transcription of the sg mRNA (green). An example of the virus that utilize the mechanism is listed. (B) Premature termination models. Sg mRNAs that are 5′-coterminal with the genome are produced when the RdRp terminates prematurely during plus-strand synthesis of the genome, while those 3′-coterminal with the genome are transcribed from a truncated template generated when the RdRp terminates early during minus-strand genome synthesis. In both cases, AU-rich sequences and secondary structure (highlighted) are involved in mediating the early termination events. Sg mRNAs are shown in green and examples of viruses that use these mechanisms are listed. (C) An unknown transcriptional mechanism is used by CTV to generate the sets of sg mRNA-related RNAs that emanate from the control elements (CEs). Sg mRNAs encoding viral proteins coded in the 3′-two-thirds of the genome are shown in green.

Another form of premature termination-mediated transcription of sg mRNAs occurs during minus-strand synthesis (White, 2002). In this instance, the RdRp terminates internally during minus-strand synthesis of the genome and the 3′-truncated RNA generated is used as a template for transcription of the sg mRNA (Fig. 4Bii). The result is a sg mRNA that is 3′-coterminal with the viral genome. Many tombusvirids utilize this method for creating sg mRNAs, including RCNMV, TBSV, Cucumber leaf spot virus (CLSV), TCV, and TNV-D (Jiwan et al., 2011). In these cases, a strong RNA secondary structure and associated AU-rich linear sequence are integral to promoting the termination step; which, like for production of BMV sgRNA3a, are believed to stall the RdRp in a region of weak base pairing and promote its dissociation (Jiwan and White, 2011). Interestingly, the required RNA secondary structure can be formed by either local sequences (TCV and TNV-D; Wu et al., 2010; Jiwan and White, 2011), long-range intra-genomic interactions (TBSV and CLSV; Choi and White, 2002; Lin and White, 2004; Xu and White, 2008) or even inter-genomic interactions (RCNMV; Sit et al., 1998). Moreover, in TBSV, the RNA structure required for sg mRNA2 transcription could be replaced by a hairpin RNA aptamer that binds to the small molecule theophylline. This modification allowed for dose-dependent control of sg mRNA2 transcription in vivo during infections by adding theophylline to the cell medium (Wang et al., 2008).

Sg mRNA transcription in CTV appears to involve a combination of mechanisms. The *cis*-acting RNA secondary structures upstream of each sg mRNA initiation site are referred to as controller elements (CEs), because it is unclear whether they function as promoters or terminators in sg mRNA production (Fig. 4C) (Ayllón et al., 2004, 2005). Three sets of sg-related RNAs that originate from each CE are produced in CTV infections, yet only one of the sets acts to template translation of viral proteins (Ayllón et al., 2004). The mechanism involved in producing these multiple sets of sg-related RNAs remains to be elucidated.

Virus assembly

Encapsidation of nascent viral genomes represents the last stage of virus reproduction, and here too, RNA elements can be integral. The assembly of helical and icosahedral plant viruses has been studied for many years (Rossmann, 2013); however in only a few cases have the RNA elements that initiate this process, i.e. the packaging signals or origins of assembly (OAS), been identified.

The OAS for the rod-shaped TMV was the first to be discovered and is located 900–1300 nucleotides from the 3′-end of the genome (Zimmern, 1983). Of the three stem–loops in this 3′-region, only SL1 is essential and it functions to bind to the first double-disk coat protein subunit complex to nucleate the bidirectional assembly process (Fig. 5A) (Bulter and Klug, 1971; Steckert and Schuster, 1982; Turner and Butler, 1986; Turner et al., 1988). In contrast, the OAS for the rod-shaped potexvirus PVX is located at the 5′-end of the genome and consists of ~100 nt that contain all of the regulatory elements, including a large stem–loop structure (SL1), necessary to bind the coat proteins to the genome (Kwon et al., 2005; Lough et al., 2006). Interestingly, the 5′-cap structure in this genome has also been identified as an important determinant of PVX assembly (Fig. 5B) (Petrova et al., 2013). The packaging of other potexviruses has been studied as well and their OASes also map to the 5′-ends of their genomes (Sit et al., 1994; Mukhamedzhanova et al., 2011). This OAS location may be beneficial to the assembly process, because it would preclude encapsidation of the 3′-coterminal sg mRNAs that are synthesized during infections.

For the icosahedral virus BMV, the TLSes in all BMV RNAs are necessary for packaging in vitro and are hypothesized to represent OASes (Fig. 5C) (Choi et al., 2002); however, in non-replicating assembly assays in vivo, the TLS of RNA3, but not those of RNA1
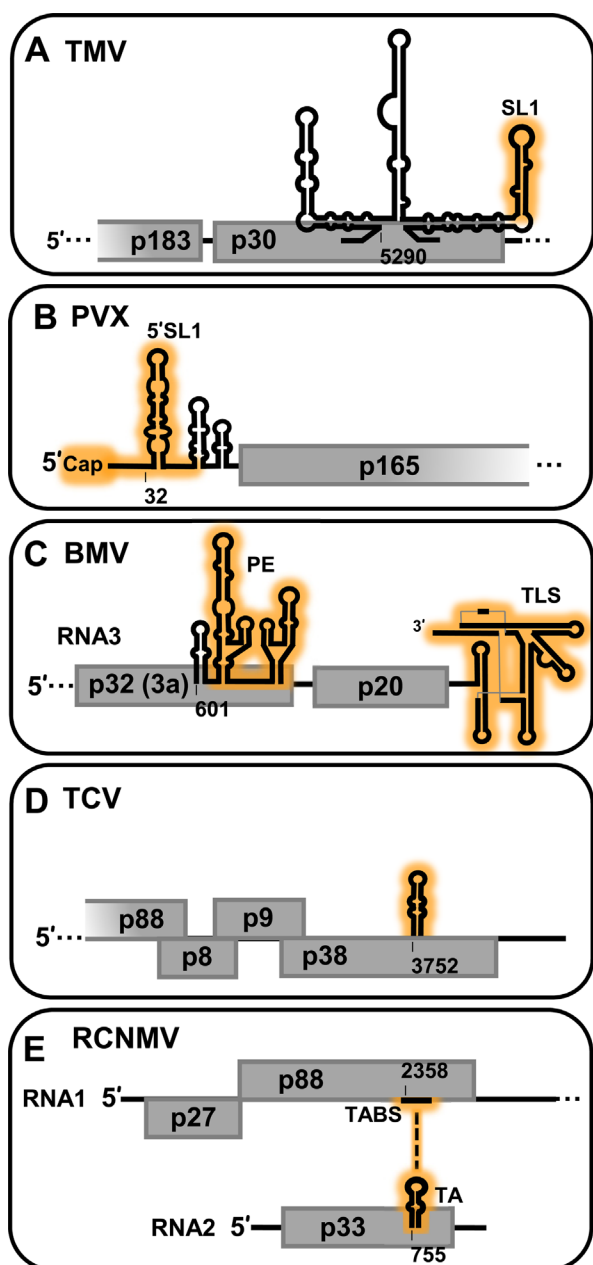
Fig. 5. Viral *cis*-acting RNA elements involved in packaging. Relevant portions of viral RNA genomes important for packaging are shown with critical elements highlighted in gold. (A) The TMV OAS, located near the 3′-end of the genome, is composed of three stem–loop structures. (B) In PVX, the 5′-cap and 5′UTR of the genome correspond to the viral OAS. (C) BMV RNA3 requires both the 3′-terminal TLS and an internal position-dependent packaging element (PE) for assembly. (D) The RCNMV OAS is the TA element in RNA2 and it base pairs with the TABS in RNA1 to mediate copackaging of both genome segments. (E) The TCV OAS is a stem–loop structure located in the CP-coding region of the viral genome.

and RNA2, is required (Annamalai and Rao, 2007). The packaging mechanism in BMV is further complicated by the fact that RNA3 and sgRNA4 are copackaged into a single particle. This latter process requires a *cis*-acting, position-dependent, packaging element (PE) in the movement protein gene in RNA3 and likely involves a sequential mode of assembly (Fig. 5C) (Choi and Rao, 2003). The packaging process in BMV has also been shown to be functionally coupled to viral RNA replication and translation, indicating that different viral processes can contribute to virus assembly (Annamalai and Rao, 2006; Chaturvedi and Rao, 2014).

The tombusvirid RCNMV is another icosahedral virus that utilizes copackaging. This virus has two RNA genome segments

and both are encapsidated into one virion. The OAS for this virus is the multifunctional *trans*-activator (TA) hairpin, which is present in the coding region of RNA2 (Fig. 5D) (Basnayake et al., 2009). Copackaging is mediated by the formation of an RNA1-RNA2 complex, which is established by the TA in RNA2 base pairing with the *trans*-activator binding sequence (TABS) in the intergenic region of RNA1 (Basnayake et al., 2009). The OAS in the non-segmented tombusvirid TCV has also been identified. For TCV, a sequence at the 3′-end of the coat protein ORF was found to be essential for genome encapsidation (Fig. 5E). This sequence forms a bulged RNA hairpin that mediates packaging of both homologous and heterologous RNAs in vivo (Qu and Morris, 1997); accordingly, this structure is both required and sufficient for capsid assembly.

Concluding remarks

Significant advances have been made in identifying and characterizing *cis*-acting RNA elements in plus-strand RNA plant viruses. These studies have revealed critical roles for these elements in essentially all steps of the virus reproductive cycle and have illuminated their diversity in both structure and function. Moreover, the discovery and characterization of novel RNA viruses and their associated RNA elements will be further facilitated by the advent of new research tools such as next generation sequencing (Barba et al., 2014) and high-throughput RNA structure probing techniques (Low and Weeks, 2010). However, having already uncovered a variety of RNA elements in many known viruses, a current challenge is to understand how these elements are integrated into the viral genome and how their structures and activities are coordinated within this complex context. Accordingly, an important future goal will be to deduce and characterize the global architectures of complete viral RNA genomes (Wu et al., 2013).

Acknowledgments

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