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Differential use of 3'CITEs by the subgenomic RNA of Pea enation mosaic virus 2

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ABSTRACT

The genomic RNA (gRNA) of *Pea enation mosaic virus 2* (PEMV2) is the template for p33 and −1 frameshift product p94. The PEMV2 subgenomic RNA (sgRNA) encodes two overlapping ORFs, p26 and p27, which are required for movement and stability of the gRNA. Efficient translation of p33 requires two of three 3' proximal cap-independent translation enhancers (3'CITEs): the kl-TSS, which binds ribosomes and engages in a long-distance interaction with the 5'end; and the adjacent eIF4E-binding PTE. Unlike the gRNA, all three 3'CITEs were required for efficient translation of the sgRNA, which included the ribosome-binding 3'TSS. A hairpin in the 5' proximal coding region of p26/p27 supported translation by the 3'CITEs by engaging in a long-distance RNA:RNA interaction with the kl-TSS. These results strongly suggest that the 5' ends of PEMV2 gRNA and sgRNA connect with the 3'UTR through similar long-distance interactions while having different requirements for 3'CITEs.

1. Introduction

Positive-sense, monopartite RNA viruses that infect eukaryotic hosts encode multiple viral proteins on their single genomic (g)RNA. However, eukaryotic canonical translation is nearly always monocistronic, with translation initiating near the 5' end of the mRNA and then proceeding in a 5' to 3' direction until reaching a termination codon (Aitken and Lorsch, 2012; Jackson et al., 2010). To circumvent this limitation, polycistronic RNA viruses must use one or more non-canonical mechanism to synthesize their proteins, including internal ribosome entry, re-initiation, leaky scanning and translational recoding (Firth and Brierley, 2012; Miras et al., 2017). In addition, with the exception of viruses in the *Potyviridae* and *Secoviridae* that employ a polyprotein expression strategy for gRNA translation (Zaccomer et al., 1995), many RNA viruses produce at least one subgenomic RNA (sgRNA), which repositions downstream ORFs proximal to a 5' end (Firth and Brierley, 2012; Miras et al., 2017). The majority of sgRNAs are 3' co-terminal with the gRNA, although sgRNAs that are 5' co-terminal are associated with some viruses (Gowda et al., 2003; Tatineni et al., 2009; Vives et al., 2002).

Many plant RNA viruses lack 5' caps and 3' poly(A) tails, and efficient translation relies on 3' cap-independent translation enhancers (3'CITEs) (Nicholson and White, 2011; Simon and Miller, 2013). 3'CITEs are located wholly or partially within 3'UTRs and therefore are present in both the gRNA and any 3'co-terminal sgRNAs. Based on their structures, 3'CITE have been placed into several categories including: translation enhancer domain (TED), which were originally discovered in *Satellite tobacco necrosis virus*; Y-shaped structure (YSS), which are found exclusively in tombusviruses; I-shaped structure (ISS), present in a subset of tombusviruses, aureusviruses and carmoviruses; T-shaped structure (TSS), present in several carmoviruses and umbraviruses; Panicum mosaic virus-like translational enhancer (PTE), found in panicoviruses and a subset of aureusviruses, carmoviruses and umbraviruses; and Barley yellow dwarf virus (BYDV)-like element (BTE), found in luteoviruses, dianthoviruses, alphanecroviruses, betanecroviruses and some umbraviruses (Nicholson and White, 2011; Simon and Miller, 2013). 3'CITEs mainly facilitate translation by recruiting translation initiation factor eIF4F, *via* binding to its eIF4E and/or eIF4G subunits, followed by attraction of 40S subunits (Das Sharma et al., 2015; Gazo et al., 2004; Nicholson et al., 2010, 2013; Treder et al., 2008; Wang et al., 2009). In addition, some 3'CITEs can directly bind ribosomes or ribosomal subunits (Das Sharma et al., 2015; Gao et al., 2012, 2014; Stupina et al., 2008). Recruited translation elements are usually delivered to the 5' end of the gRNA *via* a long-distance RNA:RNA interaction between the terminal loop of a hairpin associated with the 3'CITE and accessible sequences in the 5'UTR or nearby coding region (Chattopadhyay et al., 2011; Fabian and White, 2004, 2006; Gao et al., 2012; Nicholson and White, 2008; Nicholson et al., 2010; Simon and Miller, 2013; Wu et al., 2009). 3'CITEs are also assumed to enhance translation of any 3' co-terminal

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sgRNAs, and complementary sequences allowing for long-distance interactions between sgRNA 5' regions and 3'CITE hairpins have been predicted and in a few cases validated (Chattopadhyay et al., 2014). However, sgRNA sequences suggested as potential pairing partners for the BTE of *Tobacco necrosis virus D* are not required for efficient translation of viral proteins *in vitro* (Chkuaseli et al., 2015).

While most viruses contain a single 3'CITE, one virulent isolate of *Melon necrotic spot virus* (MNSV-N) contains a second, previously unobserved 3'CITE that was acquired through interfamiliar recombination with a polerovirus and permits infection of otherwise resistant melon varieties (Miras et al., 2014). Another virus with multiple 3'CITEs is the umbravirus *Pea enation mosaic virus 2* (PEMV2), which has three 3'CITEs in its unusually long (703 nt) 3'UTR (Gao et al., 2014). These 3'CITEs are: (1) the kissing-loop T-shaped structure (kl-TSS), which binds 80S ribosomes and 40S and 60S ribosomal subunits and engages in a long-distance RNA:RNA interaction with a 5' end hairpin (5H2) in the coding region of the 5' proximal ORF (Gao et al., 2012); (2) the PTE, which is located just downstream of the kl-TSS and binds to eIF4E (Wang et al., 2009); and (3) the 3'TSS, which is also capable of binding to 80S ribosomes and 60S ribosomal subunits and is similar to the well-studied TSS of carmovirus *Turnip crinkle virus* (Gao et al., 2014; Stupina et al., 2008) (Fig. 1B). Only the kl-TSS and PTE were required for translation of full-length PEMV2 gRNA in wheat germ extracts (WGE) (Du et al., 2017), or reporter constructs containing the entire 3'UTR of PEMV2 (Gao et al., 2014) and thus the functional role of the 3'TSS is unknown.

The umbravirus genus within the *Tombusviridae* is unusual in that its member viruses do not code for a coat protein (CP) (Adams et al., 2015). While fully capable of replicating in plant cells and establishing a systemic infection within a host plant, umbraviruses require a helper virus (commonly a polerovirus or enamovirus) for encapsidation and aphid transmission. The helper virus for PEMV2, the enamovirus PEMV1, is incapable of systemic movement in the absence of the PEMV2-encoded movement proteins (MP). PEMV2, whose positive-sense genome (4252 nt) contains no 5'cap or 3' poly(A) tail (Demler et al., 1993), has 4 ORFs that encode: (1) p33, a protein likely associated with replication (when compared with similar proteins in other tombusvirids); (2) p94, the RNA-dependent RNA polymerase (RdRp), whose expression requires −1 ribosomal frameshifting to bypass the termination codon at the end of the p33 ORF (Demler et al., 1993; Gao and Simon, 2016); (3) p26; and (4) p27, whose ORF nearly completely overlaps with the p26 ORF (Fig. 1A). p26 does not share sequence similarity with any non-umbravirus proteins and serves as both a long-distance MP and as a stabilizing protein, functionally substituting for the TMV CP in long-distance movement of TMV gRNA (Ryabov et al., 2001). In addition, the p26 orthologue of umbravirus *Groundnut rosette virus* (GRV) redistributes nucleolar protein fibrillarin to the cytoplasm, which facilitates long-distance movement through the phloem (Kim et al., 2007a, b). As a stabilizing protein, p26 forms ribonucleoprotein particles with viral RNA, likely protecting it from the host RNA silencing machinery and degradation by other cellular nucleases (Taliansky et al., 2003). The GRV p27 orthologue is a cell-to-cell MP, which can functionally replace the MP of unrelated *Potato virus X* and *Cucumber mosaic virus* (Ryabov et al., 1998, 1999). Whereas p33 and p94 are related to their counterparts in carmoviruses, the origin of p26 and p27 is more obscure. p27 shares 26% amino acid identity and 50% nucleotide sequence identity with a putative MP encoded by the 5' proximal ORF of unclassified *Japanese holly fern mottle virus* (JHFMoV) RNA2 (Valverde and Sabanadzovic, 2009), and thus may have been acquired by a recombination event. However, no overlapping ORF corresponding to a p26-type protein in JHFMoV is discernable.

p33 and p94 are translated from the PEMV2 gRNA, and p26 and p27 are translated from an as yet uncharacterized sgRNA (Fig. 1A). The recent finding that only two of the three PEMV2 3'CITEs (the kl-TSS and PTE) are necessary for efficient translation of the gRNA (Du et al., 2017) suggested that the 3'TSS may function in translation of the sgRNA. In the current study, we investigated this possibility by mapping the PEMV2 sgRNA transcription start site, which allowed for translation of full-length sgRNA transcripts in WGE and for the generation of reporter constructs to assay for translation *in vivo*. We determined that efficient translation *in vitro* and *in vivo* required the PTE and a long-distance RNA:RNA interaction between the kl-TSS and an sgRNA coding region hairpin. In addition, we determined that, unlike translation of the gRNA, the 3'TSS is as important as the PTE for translation of the sgRNA in WGE. Furthermore, all three 3'CITEs enhanced translation of reporter constructs carrying sgRNA 5' and 3' sequences *in vivo*. These data suggest that PEMV2 gRNA and sgRNA have different translation mechanisms, which could be an effective strategy for fine-tuning viral gene expression at various stages of the viral infection cycle.

2. Results

2.1. Mapping the transcription start site of PEMV2 sgRNA

As previously reported (Gao and Simon, 2016), inoculation of Arabidopsis protoplasts with *in vitro*-transcribed PEMV2 gRNA results in the accumulation of gRNA and a putative sgRNA of approximately 1.5 kb (see Fig. 1E, lane 2). To map the precise transcription start site of the putative sgRNA, primer extension reactions were performed using total RNA isolated from PEMV2-infected protoplasts at 24 h post-inoculation (hpi) and a primer complementary to positions 2845–2869. A sequence ladder was generated using the same primer and *in vitro*-transcribed gRNA as template. PEMV2 containing a GDD mutation in the RdRp active site that eliminates enzyme activity was used as a negative control.

As shown in Fig. 1C, reverse transcription reactions using wt PEMV2 generated a strong-stop product corresponding to a guanylate at position 2772 (G2772), which would correspond to a 3' co-terminal sgRNA of 1481 nt. The PEMV2 sequence beginning at position 2772 is 5'-GGGAAAUAU, which is similar to the sequence at the 5' end of the gRNA (5'GGGUAUUUA). Genomic RNA and sgRNA of related carmoviruses are known to begin with a "Carmovirus Consensus Sequence" or CCS, which consists of one to three guanylates (usually two or three) followed by a short stretch of A/U residues (Guan et al., 2000). Umbravirus gRNAs, with the exception of *Carrot mottle virus* (CMoV), *Tobacco bushy top virus* (TBTV) and *Carrot mottle mimic virus* (CMoMV), also have a canonical CCS at their 5' ends. The presence of a CCS at position 2772 in PEMV2 supports the designation of G2772 as the 5' terminus of the sgRNA. Examination of other umbraviruses revealed that, with the exception of CMoV and CMoMV, each has a CCS in an equivalent position upstream of their p26-corresponding ORFs (Fig. 1D). The PEMV2 sgRNA has a short 9-nt 5'UTR followed by p26 and p27 overlapping ORFs, with 16 nt separating the two initiation codons (AUG^{26} and AUG^{27}). A 16-nt spacer between AUG^{26} and AUG^{27} is also present in other umbraviruses with the exception of CMoV and CMoMV, where the spacer is slightly longer.

To determine the importance of p26 and p27 for PEMV2 accumulation in protoplasts, gRNA transcripts containing point mutations in AUG^{26} and/or AUG^{27} (AUG to CAG) were inoculated onto protoplasts and gRNA levels were examined by Northern blots at 24 hpi. Altering AUG^{26} (m1) reduced gRNA accumulation by over 4-fold (Fig. 1E). In contrast, mutations in AUG^{27} (m2) resulted in a 1.7-fold increase in gRNA levels. Combining both mutations (m1 + m2) reduced gRNA levels to a similar extent as m1 alone. This result indicates that p26, but not p27, is important for robust PEMV2 gRNA accumulation in protoplasts. Since no movement is associated with protoplast infection, it is likely the stabilizing property of p26 that is required for efficient gRNA accumulation in single cells.