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Protein expression strategies in Tobacco necrosis virus-D

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ABSTRACT

Tobacco necrosis virus (TNV-D) has a plus-strand RNA genome that is neither 5′ capped nor 3′ polyadenylated. Instead, it utilizes a 3′ cap-independent translational enhancer (3′CITE) located in its 3′ untranslated region (UTR) for translation of its proteins. We have examined the protein expression strategies used by TNV-D and our results indicate that: (i) a base pairing interaction between conserved ACCA and UGGU motifs in the genomic 5′UTR and 3′CITE, respectively, is not required for efficient plant cell infection, (ii) similar potential 5′UTR-3′CITE interactions in the two viral subgenomic mRNAs are not needed for efficient translation of viral proteins in vitro, (iii) a small amount of capsid protein is translated from the viral genome by a largely 3′CITE-independent mechanism, (iv) the larger of two possible forms of capsid protein is efficiently translated, and (v) p7b is translated from subgenomic mRNA1 by a leaky scanning mechanism.

Introduction

Betanecrovirus is a genus in the plus-strand RNA plant virus family Tombusviridae (Sit and Lommel, 2010). Species in this genus include Tobacco necrosis virus-D (TNV-D), Beet black scorch virus (BBSV) and Leek white stripe virus (LWSV); with TNV-D and BBSV being the best characterized. The genome of TNV-D is ~3.8 kb in length and it encodes five open reading frames (ORFs) (Fig. 1) (Coutts et al., 1991). The 5′-proximal p22 is an accessory RNA replication protein and readthrough of its stop codon produces the p82 RNA-dependent RNA polymerase (RdRp) (Molnár et al., 1997; Fang and Coutts, 2013) (Fig. 1). Both of these proteins are translated directly from the viral genome and readthough synthesis of p82 requires a long-range RNA–RNA interaction between a proximal readthrough element (PRTE) located near the readthrough site and a distal readthrough element (DRTE) located in the 3′ untranslated region (UTR) (Newburn et al., 2014). Additionally, it was previously determined that a ~29 kDa protein is synthesized from the genome, and it was tentatively defined as the p29 capsid protein (CP) (Fig. 1) (Newburn et al., 2014). The p7a and p7b proteins encoded in the central region of the genome are required for cell-to-cell movement and are translated from subgenomic (sg) mRNA1 (Molnár et al., 1997; Jiwan et al., 2011). Similarly, the 3′-proximal p29 CP is translated from its own message, sg mRNA2 (Jiwan et al., 2011) (Fig. 1).

Like all tombusvirids, the plus-strand RNA genome of TNV-D is neither 5′-capped nor 3′-polyadenylated (Nicholson and White, 2011; Simon and Miller, 2013). Instead, the virus relies on a 3′ cap-independent translational enhancer (3′CITE) that is located in the 3′UTR of its genome (Shen and Miller, 2004, 2007) (Fig. 1). This 3′ CITE is structurally similar to that found in Barley yellow dwarf virus (BYDV), which interacts with eukaryotic translation initiation factor (eIF) 4G to recruit ribosomes to the viral genome (Treder et al., 2008; Kraft et al., 2013; Sharma et al., 2015). In BYDV, a long-range RNA–RNA interaction between a sequence in its 3′CITE and one in the 5′UTR of the viral genome is required to reposition the recruited translational machinery to the 5′-proximal site of initiation (Guo et al., 2001). The genomes of TNV-D and the other two betanecroviruses, BBSV and LWSV, all contain short complementary conserved motifs, ACCA and UGGU, in their 5′UTRs and 3′CITEs, respectively (Fig. 1) (Shen and Miller, 2004). Moreover, these motifs are predicted to reside in the terminal loops of small hairpin structures, which would presumably facilitate their interaction (Fig. 1, **box**). These observations, along with the likeness of the betanecrovirus 3′CITEs to that of BYDV, prompted the idea that TNV-D may also require a 5′–3′ RNA–RNA interaction for translation (Shen and Miller, 2004). However, attempts to uncover evidence for this occurrence have been unsuccessful (Shen and Miller, 2004). Nonetheless, the data did not entirely exclude this

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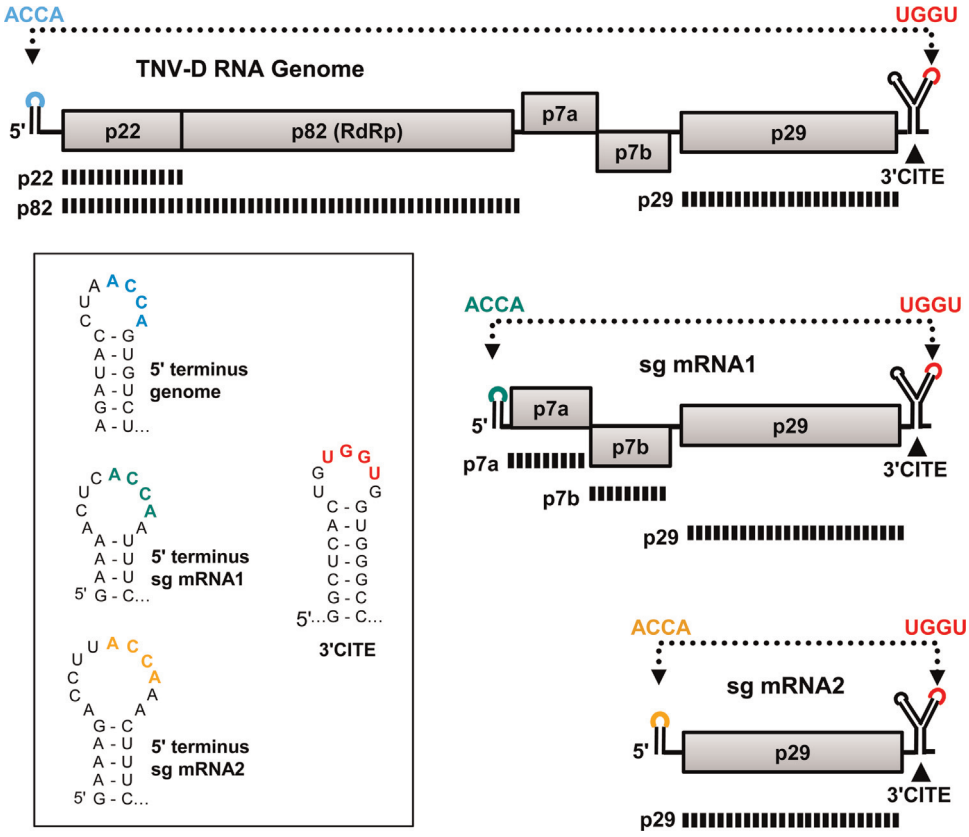

Fig. 1. Tobacco necrosis virus D (TNV-D) genomic and subgenomic mRNAs. Protein ORFs encoded in each of the viral mRNA species are shown as gray boxes with corresponding translated protein products depicted below as black lines. The 3′CITE is shown as a branched RNA structure located at the 3′ end of viral RNAs. Double-headed arrows indicate the proposed long-range RNA–RNA base-pairing interactions involving the ACCA and UGGU motifs. Color coded RNA secondary structures of the stem-loops that contain the motifs are also shown in the box.

possibility, thus, currently, a role for pairing between these motifs remains uncertain.

In this study we have examined the translational strategies used by TNV-D to express its proteins. Our results provide definitive evidence that pairing between the ACCA/UGGU motifs in TNV-D is not required for efficient infection of plant cells. We also demonstrate that a small amount of CP is produced from the viral genome, and that p7b is expressed from sg mRNA1 by a leaky scanning mechanism. These and other findings help to illuminate and clarify the varied approaches used by TNV-D for translation of its proteins.

Results

Translation of proteins from the viral genome

Previous studies established that p22 and its readthrough product p82 are translated from the TNV-D genome (Shen and Miller, 2004; Newburn et al., 2014). Additionally, another protein of ~29 kDa was identified as a low abundance genomic translation product, and it was tentatively identified as the viral CP (Newburn et al., 2014). To address the validity of this designation, we mutated the CP start codon from AUG to UUG in the context of the full-length TNV-D genome, creating gmCP-UUG1. When this genomic mutant was tested in vitro in a wheat germ extract (wge) translation system, p22 and p82 levels remained constant while the ~29 kDa band was reduced over ten-fold, consistent with it representing the CP and likely being produced by an internal ribosome entry site (IRES) (Fig. 2B). Further assessment of gmCP-UUG1 in protoplast infections revealed greater than wild-type (wt) levels of viral RNA accumulation, which is probably the consequence of greater levels of unpackaged genome being available for replication and sg mRNA transcription (Fig. 2C).

Next, we examined the role of the 3′CITE in translation of viral proteins from the TNV-D genome. The RNA structure of the TNV-D 3′CITE is comprised of a lower stem that branches into two stem-loop structures (Fig. 2A) (Shen and Miller, 2004). In genomic mutant gmΔ3′CITE, this structure was deleted precisely at the base of the lower stem (coordinates 3554–3678). In wge, this mutant exhibited over a 10-fold reduction in p22 levels, and p82 was not detectable (Fig. 2D**, lane 3**). Interestingly, the CP p29 was only reduced to ~59% that of wt, indicating that although the 3′ CITE contributes to its production, its synthesis is primarily independent of this activity (Fig. 2D). When a 5′ cap structure was added to gmΔ3′CITE, p22 was restored to greater than wt levels, however relative p82 readthough levels remained low at ~9% and p29 showed only a slight increase in accumulation (Fig. 2D, **lane 5**). Accordingly, efficient readthrough production of p82 appears to be heavily reliant on the 3′CITE, and the 3′CITE-dependent fraction of p29 translation is not efficiently compensated for by the presence of a 5′ cap. As anticipated, deleting the 3′CITE made gmΔ3′ CITE nonviable in protoplast infections, and this defect could not be rescued by capping the genomic transcript (Fig. 2E).

Previous studies showed that the introduction of a 4 nt insertion (GAUC) at a BamHI site located within the TNV-D 3′CITE reduced its activity in a luc reporter mRNA to ~35% in wge and to near undetectable levels in protoplasts (Shen and Miller, 2004). To assess the effect of this modification in the context of the full-length genome, the same 4 nt modification was introduced into the TNV-D genome, creating gmBam. This mutant exhibited a large reduction in p22, with a lesser effect on p29 and p82 readthrough