Subgenomic mRNA transcription in tobacco necrosis virus

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Abstract

Tobacco necrosis virus-D (TNV-D), a positive-strand RNA Necrovirus in the family Tombusviridae, transcribes two subgenomic (sg) mRNAs during infections. We have investigated the strategy used by TNV-D in this process and uncovered evidence that it employs a premature termination (PT) mechanism for the transcription of its sg mRNAs. Structural and mutational analysis of the TNV-D genome identified local RNA structures upstream from transcriptional initiation sites that functioned in the plus-strand as attenuation structures and mediated the production of sg mRNA-sized minus-strands. Other evidence in support of a PT mechanism included the ability to uncouple minus-strand sg RNA production from plus-strand sg mRNA synthesis and the sequence similarities observed between the sg mRNA promoter and that for the viral genome. Accordingly, our results indicate that the necrovirus TNV-D, like several other genera in the family Tombusviridae, uses a PT mechanism for transcription of its sg mRNAs.

Introduction

Tobacco necrosis virus-D (TNV-D) is an icosahedral plus-sense RNA plant virus from the genus Necrovirus in the family Tombusviridae (Russo et al., 1994). Necroviruses can be further divided into two main subgroups, strain A-like and strain D-like, based on the similarity of their RNA-dependent RNA polymerases (RdRps) to those of either carmoviruses or tombusviruses, respectively (Castano and Hernandez, 2005). However, despite their tombus-like RdRps, the genome organization of the strain D-like subgroup is more similar to that of carmoviruses (Coutts et al., 1991) (Fig. 1A). Sequenced members of the strain D-like subgroup include: TNV-D (Coutts et al., 1991), Beet black scorch virus (BBSV; Cao et al., 2002), and Leek white stripe virus (LWSV; Lot et al., 1996).

The ~3.8 kb RNA genome of TNV-D encodes at least five proteins (Coutts et al., 1991; Molnar et al., 1997), p22 and p82 (the RdRp), which are translated from the genome, are required for viral RNA replication, p7a and p7b are necessary for cell-to-cell movement, while the coat protein (CP), p29, is needed for particle assembly and systemic movement (Molnar et al., 1997; Offei and Coutts, 1996). p7a has single-stranded nucleic acid binding activity and has been suggested to assist in the transportation of viral RNA to plasmodesmata (Offei et al., 1995). Additionally, a third small ORF, p71, predicted in two different isolates of TNV-D, has been implicated in cell-to-cell movement (Molnar et al., 1997) (Fig. 1A). Like many plus-strand RNA viruses, the more 3′-proximal ORFs encoded in the TNV-D genome are expressed via the production of subgenomic (sg) mRNAs (Miller and Koev, 2000; Sztuba-Solifska et al., 2011).

Lacking both a 5′-cap and a 3′-poly(A) tail, TNV-D genome translation relies on the use of a Barley yellow dwarf virus (BYDV)-like translation element, or BTE, that is located in its 3′-untranslated region (UTR) (Shen and Miller, 2004) (Fig. 1A). The sg mRNAs that are transcribed during TNV-D infections are 3′-coterminal with the genome (Fig. 1A) and thus also contain, and presumably utilize, BTEs (Shen and Miller, 2004). Primer extension-mediated mapping of the sg mRNA 5′-termini in TNV-D indicated that sg mRNA1 (~1.5 kb) templates translation of p7a and p7b, while sg mRNA2 (~1.2 kb) mediates CP translation (Offei and Coutts, 1996) (Fig. 1A). Similar mapping results were also obtained from analyses of the sg mRNAs in TNV-A (Meulewaeter et al., 1992) and BBSV (Yuan et al., 2006). Although the coding capacities of necrovirus sg mRNAs have been well defined, the mechanism of sg mRNA transcription and the RNA elements that regulate these events have not been investigated.

Detailed analyses of members of several different genera in Tombusviridae (i.e. Dianthovirus, Tombusvirus, Aureusvirus and Carmovirus) have revealed that they use a premature termination (PT) mechanism for the production of their sg mRNAs (Jiwan and White, 2011). In this mechanism, a higher-order RNA structure in the viral genome, termed the attenuation RNA structure, serves as stop signal for the RdRp during (−)-strand synthesis and causes it to terminate. The 3′-truncated (−)-strand generated is complementary to a sg mRNA and contains, at its 3′-end, a promoter that is used by the RdRp to initiate and transcribe sg mRNAs (White, 2002). There are several positive indicators for viruses
that use a PT mechanism for sg mRNA transcription, these include: (i) the presence of sg RNA-sized minus-strands in infections, (ii) the ability to uncouple minus-strand sg RNA production from plus-strand sg mRNA synthesis, (iii) an attenuation RNA structure positioned just 5′ to the initiation site that functions in the plus-strand of the viral genome and (iv) a 3′-terminal promoter sequence in the minus-strand intermediate that is similar in sequence identity to that for the viral genome. The presence of all four indicators provides strong support that a PT mechanism is used by a virus. In this study we have investigated the process of sg mRNA transcription in TNV-D and our data suggest that this virus utilizes a PT mechanism, with some features that are distinct from those previously reported.

Results

Accumulation profile of TNV-D RNAs

Time-course analysis in cucumber protoplasts was performed to determine the timing of appearance and relative levels of TNV-D RNAs during infections. Following transfection with in vitro-generated RNA transcrips of the TNV-D genome, total nucleic acids were extracted over a time period spanning 32 h. Northern blot analysis of plus-strand viral RNAs showed that viral genomic RNA levels began to increase at ~6 h post infection (p.i.) and continued to increase over the course of the experiment (Fig. 1B). Sg mRNA1 was first clearly detected at ~4 h p.i. and its rate of accumulation increased until ~8 h p.i., after which its level stabilized (Fig. 1B). In contrast, sg mRNA2 was not readily detectable until ~16 h p.i. and its accumulation appeared to stabilize at ~20 h and beyond at a level that was lower than that observed for sg mRNA1 (Fig. 1B). Corresponding minus-strand analysis of viral RNAs revealed detectable increases of genomic and sg mRNA1 complements at ~5 and ~6 h, respectively (Fig. 1C). The short delay in appearance of minus-strands versus their plus counterparts is likely a result of the detection limits of the Northern

blots. The presence of minus strands of sg mRNA1 is consistent with it being transcribed by a PT mechanism. Minus-strands of sg mRNA2 were not detectable (Fig. 1C), a finding in accordance with the very low levels of plus-strand sg mRNA2 observed (Fig. 1B).

Identification of the sg mRNA initiation sites for TNV-D

To determine the sg mRNA initiation sites in TNV-D, primer extension was performed on total nucleic acids extracted from protoplasts transfected with TNV-D transcripts. The results revealed that the 5′-termini of sg mRNA1 and 2 corresponded to guanylates at positions 2216 (Fig. 2A, lane 1; Fig. 2B) and 2557 (Fig. 2C, lane 1;
Fig. 2D), respectively, in the TNV-D genome. Conversely, in control reactions, no corresponding primer extension products at these positions were observed when (i) nucleic acids from mock-infected protoplasts were analyzed (Figs. 2A and C, lanes 2), (ii) the initiating guanylates were mutated to adenylates in TNV-D mutants isg1A and isg2A (Figs. 2A and C, lanes 3) or, (iii) when in vitro transcriptions of the wt TNV-D genome (t) were used directly as the templates for primer extension (Figs. 2A and C, lanes 4). The position mapped for sg mRNA1 (coordinate 2216) corresponds to the same position previously mapped by Offei and Coutts (1996) (Fig. 2B), but the sg mRNA2 initiation site mapped in our analysis (coordinate 2557) was different in that it was located 4 nt upstream from the previously mapped position (Fig. 2D).

Uncoupling sg mRNA minus- and plus-strand accumulation

We next wanted to further confirm the importance of the proposed start sites for the production of the sg mRNAs and to determine if sg mRNA minus-strand accumulation could be uncoupled from plus-strand production. TNV-D genomic transcripts with the initiating guanylate substituted with an adenylate for sg mRNA1 (mutant isg1A), or an adenylyl (isg2A), cytidylate (isg2C), or uridylyl (isg2U) for sg mRNA2, were constructed and tested in protoplast infections. The relative sg mRNA1 level was greatly reduced for TNV-D mutant isg1A containing a G-to-A substitution at the sg mRNA1 initiation site (Fig. 3A, lane 3). Similarly, changing the initiating G for sg mRNA2 to any other nucleotide caused notable decreases in sg mRNA2 accumulation levels (Fig. 3A, lanes 4–6). Analysis of the corresponding viral minus-strand accumulation showed that, contrary to its plus-strands, the sg mRNA1 minus-strand level did not decrease for mutant isg1A (Fig. 3B, lane 3). This ability to uncouple minus- from plus-strand sg mRNA1 accumulation is consistent with a PT model of sg mRNA1 transcription. As before (Fig. 1C), minus-strands of the poorly accumulating sg mRNA2 were not detectable by Northern blotting (Fig. 3B).

An RNA secondary structure is necessary for sg mRNA2 accumulation

Mfold RNA secondary structure analysis (Zuker et al., 1999) of the sequence surrounding the sg mRNA2 start site predicted the formation of a large RNA structure, located 5′-proximal to the initiation site (Fig. 4A). The lower stem was predicted to be maintained when compared with corresponding stems in other necroviruses and included one covarying base pair in BBSV (Fig. 4A). This conservation of RNA secondary structure adjacent to the site of sg mRNA2 initiation suggested a possible role in mediating sg mRNA transcription.

To test whether this RNA structure was important for sg mRNA2 transcription, the conserved lower stem of the structure was targeted for mutational analysis that altered its stability (Fig. 4A). Changes in mutant 1sg2 and 2sg2 predicted to disrupt a base pair in the stem resulted in greatly reduced levels of sg mRNA2 accumulation (~5%) in protoplast infections (Figs. 4A–C). Restoring the RNA pairing in 3sg2 resulted in recovery of sg mRNA2 accumulation to ~40% of that of wt levels. Replacement of the more stable GC pair with an AU pair in 6sg2 essentially abolished sg mRNA2 accumulation (Figs. 4A–C). Similar results were observed when strand-preferential destabilization was attempted in 4sg2 and 5sg2 (Figs. 4A–C), thus no inference could be made in terms of the strand in which this RNA structure functions (see next section for explanation of strand preferential destabilization).

A smaller RNA structure is involved in sg mRNA1 accumulation

Mfold analysis of the sequence surrounding the sg mRNA1 start site also predicted the formation of an RNA secondary structure, however it was significantly smaller than that predicted for sg mRNA2 (Fig. 5A). This structure was closed by a 13 bp long stem (when the two internal GU pairs are included) and comparison of this stem with corresponding predicted stems in other necroviruses revealed a high level of base identity, as well as conservation of the stem through base mono- and co-variations (Fig. 5A). The structure was also assessed by selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE) (Merino et al., 2005), which identifies flexible and likely single-stranded nucleotides in a sequence-independent manner (Fig. 5A). The low reactivity of residues in the closing stem is consistent with formation of a helix. Similarly, the nucleotides in the two predicted stems in the intervening region were generally less reactive. In contrast, predicted loop, budge, junction and non-structured regions were more reactive, which is also in agreement with their proposed unpaired nature (Fig. 5A).

To test whether this predicted RNA structure was important for sg mRNA1 transcription, substitutions were made in the closing stem. Since this structure was located in the p82 coding region, changes were made at degenerate codon positions that maintained amino acid identity of the viral RdRp (Fig. 5A). Single nucleotide substitutions in mutants 1sg1 and 2sg1 that were predicted to destabilize the stem resulted in decreased levels of sg mRNA1 accumulation, while the combined substitutions in 3sg1, which would restore canonical base pairing, acted to revive levels of sg mRNA1 accumulation in protoplast infections (Figs. 5A–D). When the CG pair was replaced with UA in 6sg1, sg mRNA1 accumulation was also readily detectable (Figs. 5A–C). Additionally, strand preferential destabilization of the stem was carried out by substituting the CG pair with either a CA mismatch or UG wobble base pair. The UG pair in the plus-strand stem would correspond to a less stable AC mismatch in a potential minus-strand stem, thus the minus-strand stem would be preferentially destabilized. Conversely, the opposite would be true for a CA mismatch introduced into the plus-strand stem. Mutant 5sg1 containing the GU wobble pair yielded notably higher levels of sg mRNA1 accumulation than 4sg1 containing the AC mismatch (Figs. 5A–D), implicating the plus-strand RNA stem as being important for sg mRNA1 transcription. This plus-strand activity is consistent with this sequence functioning as an attenuation structure in a PT transcriptional mechanism. Moreover, the large reduction of both
plus- and minus-strands when this structure was disrupted is also consistent with this concept, as in the PT mechanism this attenuation structure is responsible for generating the minus-strand intermediates.

**Ectopic expression of sg mRNA1**

The smaller size of the RNA structure associated with sg mRNA1 and the more robust accumulation of sg mRNA1 made it an attractive candidate for further analysis. However, because this structure overlaps with the p82 ORF, many potential modifications at this natural location were precluded. To deal with this impediment, this structure along with the 7 nt downstream of the initiation site was duplicated and inserted at a more 3′-proximal location within the coding region for the coat protein, creating Δsg1 (Fig. 6A). At this new location, the transcriptional elements could be freely mutated without affecting the coding of the replication protein p82. Additionally, to avoid possible effects related to interference with CP production, the genome with the inserted transcriptional segment, Δsg1, also contained an upstream frame-shift mutation in the coat protein ORF that prevented its translation into and across the inserted sequence. When Δsg1 was transfected into protoplasts, an additional small viral sg RNA, termed sg RNA1* (sg1*), accumulated (Fig. 6B). Extending the 7 nt segment downstream of the transcription initiation site by 6 additional wt TNV-D nucleotides did not result in increased levels of sg RNA1* from mutant Δsg1pL2, suggesting that the 8 nt long sequence (including the initiation site) in Δsg1 was sufficient to direct efficient transcription.

**Promoter and spacer analysis of sg mRNA1**

If TNV-D is using a PT mechanism, as the preceding data suggest, then the residues at the 3′-end of the minus-stand intermediate...
is one nucleotide (Fig. 7B). However, the low levels of sg1*(+) did not affect translation in TNV-D, the one nucleotide long uridylate spacer in the plus-strand was either deleted or extended by up to 6 U’s (Fig. 7B). Studies carried out on Tomato bushy stunt virus (TBSV), a member of the genus Tombusvirus (Wang et al., 2008; Xu and White, 2009). The stable hairpin was functional in both TBSV and Cucumber leaf spot aureusvirus (CLSV) when it was inserted upstream of cognate promoter and spacer sequences in each of these viruses. To determine whether the same small stable RNA hairpin could also function in necrovirus transcription, the wt attenuation structure upstream of the sg RNA1* initiation site was replaced by this RNA hairpin (Figs. 8A and B). In contrast to the results observed for TBSV and CLSV, the hairpin introduced into TNV-D in mutant U1p severely reduced levels of sg RNA1* accumulation to near undetectable levels (Fig. 8C, compare lanes 1 and 2). To test whether this result was related to the specific location in the viral genome, hairpin-containing and wt transcriptional units were also inserted at a more 3′-proximal location, thereby creating U1p-3′ and Δsg1-3′, respectively (Fig. 8A). At this new position a smaller sg RNA, termed sg1**, was produced. Transcription of sg1** from Δsg1-3′ containing the wt transcriptional unit was efficient, however that from U1p-3′ with the artificial hairpin was very inefficient (Fig. 8C, compare lanes 4 and 3, respectively).

One possible explanation for this result was that, during the infection, the artificial hairpin was deleted from the genome. To determine if this was the case, reverse transcription-PCR of this region was performed on the total nucleic acids extracted from virus-transfected protoplasts. Sequencing results confirmed that the RNA hairpins were still present in
progeny from U1p and U1p-3', thus the artificial transcription units were maintained, but were weakly functional.

Results with the wt sg mRNA1 transcriptional unit showed that a spacer of one uridylate was optimal for sg RNA1* accumulation (Fig. 7B). However, the artificial hairpin attenuator structure with a spacer of one uridylate did not give high levels of accumulation, possibly because this more stable RNA structure had a different spacer requirement (Fig. 8B). To determine whether the low level of sg RNA1* accumulation from the stable hairpin mutant U1p was related to suboptimal spacing, the spacer length was adjusted from no spacer up to six uridylicates (Fig. 8B). Changes in spacer length did not affect the levels of sg RNA1* accumulation, with all exhibiting low levels of sg RNA1* accumulation (Fig. 8D). This inability to efficiently utilize an artificial stable RNA hairpin distinguishes the TNV-D transcriptional units were designed to test some of these differences (Fig. 9A). Deletion of the intervening sequence between the closing stem of the wt attenuator structure and replacement of it with a GAAA loop sequence in mutant Δsg1-t gave reduced levels of sg RNA1* accumulation (~50%), indicating that the intervening sequence in the wt structure is important for efficient transcription (Figs. 9A and B). Increasing the stability of the closing stem in the wt structure by introducing GC pairs near its top, middle or bottom in mutants Δsg1-3, Δsg1-2 and Δsg1-1, respectively, led to increased, similar and decreased levels of sg RNA1* relative to Δsg1 (Figs. 9A and B). Making the wt attenuation structure more similar to the artificial RNA hairpin by both deleting the intervening region and stabilizing the closing stem in mutant Δsg1–t–1–2–3 yielded low levels of transcription (Figs. 9A and B). Collectively, these results suggest that the intervening sequence between the closing stem in the wt structure is important, but not essential, for transcription. Additionally, stabilization of the upper and middle sections of the closing stem is well tolerated while stabilization of the lower portion is not. Finally, modification of the wt attenuation structure into a more stable hairpin-like structure rendered it highly defective, which is consistent with the low activity observed when the artificial hairpin structure was tested (Fig. 8).

Discussion

Coding and accumulation of sg mRNAs

The positions of the transcriptional initiation sites mapped indicate that the ORFs encoding p7a and p7b movement proteins could be translated from sg mRNA1, while p29, the viral coat protein, could be translated from sg mRNA2. Interestingly, based on sg mRNA1 5'-end mapping results, p71 ORF cannot be translated from this sg mRNA, as the G in the initiation codon for p71 corresponds to the transcriptional start site (Fig. 2B). Additionally, the genomic mutation that was made previously to test the function of p71 in TNV-D replaced the G of the start codon with A to inactivate translation initiation from this site (Molnar et al., 1997). However, this same mutation (present in isg1A) also greatly inhibits sg mRNA1 accumulation (Fig. 3A, lane 3). Thus, it remains unclear as to whether p71 is important for plant infections, because the mutant tested to assess this function (Molnar et al., 1997) both inactivates the p71 initiation codon and inhibits sg mRNA1 transcription (Fig. 3A, lane 3). If p71, if indeed functional, it would have to be translated from either a larger sg mRNA (yet to be identified) or the genomic message (possibly by a coupled termination–reinitiation strategy) (Powell, 2010).

Our mapped position for the transcription initiation site for sg mRNA2 is located 4 nt upstream from the site determined previously (Fig. 2D) (Offei and Coutts, 1996). Regardless of this difference, either 5'-end would still allow the sg message to encode the CP ORF. The reason for the discrepancy in the sites mapped is not clear, however our position is consistent with the corresponding site mapped for sg mRNA2 of BBSV (Yuan et al., 2006). Moreover, the more upstream position yields a 5'-terminal sequence [sg2: (+)5'-GAAAAGACC...] that is more similar to that of the 5'-end of the genome [(+5')-AGAUACCU...] and sg mRNA1 [(+5')-GAAAACUC...]. The complements of these sequences represent the 3'-terminal promoters for plus-strand transcription and genome replication, and such similarity is typical for viruses that use a PT mechanism (Jiwan and White, 2011; White, 2002).

The accumulation profile of the two sg mRNAs indicates that sg mRNA1 is produced earlier and in greater amounts than sg mRNA2 (Fig. 1). This profile is similar to what was observed when TNV-A sg mRNA levels were assessed over time, where sg mRNA1 also appeared...
first (Meulewaeter et al., 1992). However, in that study, later time points showed that sg mRNA2 increased to levels similar to sg mRNA1 (Meulewaeter et al., 1992). Such differences may be related to genetic differences that were acquired as the two strains diverged or the different experimental hosts used (tobacco versus cucumber). Regardless, the early appearance of sg mRNA1 followed by sg mRNA2 is consistent with an earlier requirement for the cell-to-cell movement proteins, which are able to mediate intercellular transmission independent of CP (Molnar et al., 1997; Offei and Coutts, 1996). The later expression of CP from sg mRNA2 would then allow for assembly of accumulated progeny viral genomes and systemic invasion. This temporal regulation is likely important for promoting successful plant infections.

The sg mRNA1 attenuation RNA structure

The data gathered from several different studies suggest that a key component required by the PT mechanism for sg mRNA transcription is some form of higher-order RNA structure in the viral genome positioned just ahead of the RdRp stall site (Jiwan and White, 2011; White, 2002). Such structures can involve inter-molecular RNA–RNA interactions, as in Red clover necrotic mosaic virus (Sit et al., 1998), long-distance intra-molecular RNA–RNA interactions spanning one to two thousand nucleotides, as in TBSV (Choi and White, 2002; Lin and White, 2004; Zhang et al., 1999) and CLSV (Xu and White, 2008), or short-range RNA–RNA interactions that form local RNA structures, as in Turnip crinkle virus (TCV; Wu et al., 2010) and CLSV (Xu and White, 2009). In the case of TNV-D, the RNA attenuation structure associated with sg mRNA1 production involves 91 nt (Fig. 10) and, in terms of size, is most similar to the attenuation structure for TCV sg mRNA2, which encompasses 89 nt. However, for TCV, wt activity was maintained when the internal sequence between the closing stem was deleted (Wang et al., 1999; Wu et al., 2010). In contrast, removal of the intervening sequence between the closing stem in the TNV-D structure led to a 50% drop in sg RNA1* transcription (Fig. 9). Also, a stable RNA hairpin that functionally substituted for wt long-range interactions in TBSV and CLSV was not able to mediate efficient transcription when introduced into TNV-D, even when different contexts were tested (Fig. 8). Thus, TNV-D differs from TBSV and CLSV in that it cannot effectively utilize a generic RNA hairpin for an attenuation structure. Collectively, these results indicate fundamental differences in the role of the attenuation structure for TNV-D and suggest that the intervening sequence and/or identity of the closing stem region in this structure serve additional roles beyond the presumed function of acting as a physical barrier for the RdRp.

Interestingly, deletion of the intervening sequence led to a sizable reduction in sg1*(+), while having little visible effect on minus-strand accumulation (Fig. 9; Δsg1-t). A possible explanation for this is...
that the intervening sequence may be involved in recruitment of a host or viral protein(s) or be involved in an RNA–RNA interaction that assists in plus-strand synthesis. In contrast, replacing the UA pair at the bottom of the stem severely debilitated both minus- and plus-strand production, indicating a role in efficient RdRp termination (Fig. 9; Δsg1-1). This defect may be related to a requirement for specific nucleotides at these positions or affect the effective spacer distance, either of which could alter termination efficiency. Further detailed studies are planned to dissect the specific roles of the stem and intervening regions in the termination and initiation steps.

The sg mRNA1 promoter and spacer elements

In the PT model, the promoter and spacer elements play important roles in initiation and termination, respectively. The spacer element can modulate the efficiency of stalling and thus the abundance of minus-strand templates. Subsequently, the promoter sequence determines the roles of the stem and intervening regions in the termination and initiation steps. Consistent with an important role for the +1 to +8 residues in both the transcription promoter spanned ~8 nt (Lin et al., 2007) into which a SmaI restriction site was added to allow position the initiating nucleotide in the minus-strand template at the 3′-end, the preferred position for initiation by the RdRp (Panavas et al., 2003). In TBSV the optimal spacer length is 2–3 nt and in CLSV it is 3 nt (Wu and White, 2007; Xu and White, 2008). Our results suggest that a spacer of 1 nt is optimal for sg mRNA1 transcription in TNV-D (Fig. 10). Decreasing or increasing the length of the spacer from the wt length of 1 nt led to severely reduced levels of plus-strand sg RNA1 accumulation (Fig. 7A). In contrast, minus-strand levels were mildly affected or tended to increase with the longer spacer sequences. A possible explanation for the increase in minus-strand accumulation is that the uridylicate nucleotides, which would more weakly pair with the nascent minus-strand, could further promote termination. However, the minus-strands produced may have extra 3′-terminal sequences that could inhibit efficient use of it as a template for transcription (Panavas et al., 2003), and this could explain the corresponding low levels of plus-strands. Indeed, this appeared to be the case for some sg mRNA minus-strand templates in TBSV (Wu and White, 2007). The importance of the spacer length for transcription in TNV-D is consistent with results from other viruses that utilize a PT mechanism.

TNV-D and a premature termination mechanism

The results of this study have generated data that support sg mRNA1 transcription in TNV-D through a PT mechanism (Fig. 10). The lines of evidence include: (i) the detection of sg mRNA1-sized minus-strands in infections, (ii) the ability to uncouple minus-strand sg mRNA1 production from plus-strand sg mRNA synthesis, (iii) the identification of an attenuation RNA structure that functions in the plus-strand of the genome and (iv) the delineation of a sg mRNA1 transcription promoter that is similar in sequence identity to that of the genome. Based on these observations, we propose that TNV-D, like four other genera in Tombusviridae (Jiwan and White, 2011), utilizes a PT mechanism for transcription for its sg mRNAs (Fig. 10).

Materials and methods

Plasmid construction

All mutants were derivatives of the full-length cDNA copy of TNV-D in pUC19 plasmid (a gift from Robert Coutts, Imperial College London) (Coutts et al., 1991) into which a Smal restriction site was added to allow generation of an authentic 3′-terminus (Jiwan, 2010). PCR-derived
regions that were introduced into constructs were sequenced completely to ensure that only the desired modifications were present. The CP ORF in Δsg1 was inactivated by a +1 frame-shift at nt position 2558, corresponding to an EcoRI restriction enzyme site, by filling in the single-stranded 5′-overhangs followed by religation. For mutant Δsg1, the sg mRNA1 transcriptional elements were inserted between positions 3032 and 3172 corresponding to the restriction sites EcoRV and PflMI, whereas in Δsg1-3′ the elements were inserted between nt 3032 and 3439 using EcoRV and AgeI restriction sites. The relevant modifications in other mutant TNV-D genomes are shown in the figures of this report.

**In vitro transcription and protoplast transfection**

TNV-D clones used for in vitro transcription were prepared by linearizing the plasmids with SmaI. Genomic RNA transcripts were prepared using the AmpliScribe T7-Flash Transcription Kit (Epicentre Technologies). Protoplasts used for viral transfections were prepared from cucumber cotyledons and ~3×10^5 protoplasts were transfected with 5 μg of each viral RNA transcript using the PEG-mediated method as described previously (White and Morris, 1994). Transfected cells were incubated in a growth chamber under fluorescent lighting at 22 °C for 22 h, or up to 32 h for the time course experiment.

**Northern blot analysis**

Total nucleic acids were isolated from protoplasts as described previously (White and Morris, 1994). For plus-strand viral RNA detection, one-fifth of the total nucleic acids isolated was separated in 1.4% agarose gels and subjected to Northern blot analysis using three [32P]-5′-end-labeled oligonucleotides (pdjr1, pdjr2, and pdjr3) complementary to segments 3731–3762, 3671–3704 and 3616–3654, respectively, of the TNV-D genome.

Minus-strand RNAs were analyzed by Northern blotting following electrophoretic separation of glyoxal-treated samples (Lin and White, 2004). Detection of minus-strand viral RNAs was carried out as described previously (Choi et al., 2001) using α-[32P]-labeled in vitro-generated RNA transcripts corresponding to the 3′-proximal 334 nucleotides of the TNV-D genome. For both plus- and minus-strand RNAs, radioanalytical quantification of Northern blots was performed using a PhosphorImager Molecular Imager (Bio-Rad). All experiments were repeated at least three times and the means for relative sg mRNA accumulation (with standard deviations) are presented.

**SHAPE analysis and RNA secondary structure prediction**

SHAPE analysis was performed on in vitro-transcribed TNV-D genomic RNA as described previously by Mortimer and Weeks (2007). Briefly, TNV-D genomic RNA transcripts (4 pmol) in 24 μl of 0.5X TE was refolded by heating at 95 °C for 4 min., incubating on ice for 2 min., adding 12 μl of 3.3X folding buffer (333 mM HEPES pH 8.0, 16.5 mM MgCl2, 333 mM NaCl) and incubating at 37 °C for 30 min. 20 μl of the refolded RNA was treated with 4 μl of 50 mM of 1-methyl-7-nitroisatoic anhydride (1M7) in dimethylsulfoxide (DMSO) at 37 °C for 4 min. Another 20 μl of refolded RNA was treated with only DMSO under the same conditions, and served as a negative control. RNA was recovered by ethanol precipitation (with 200 mM NaCl, 2 mM EDTA, and 40 μg of glycogen). The precipitated RNA was dissolved in 20 μl of 0.5X TE. Detection of 2′-O-adducts by primer extension was achieved using a procedure modified from Wilkinson et al. (2008). Briefly,
fluorescently-labeled primers (6 μl) were added to the 1M7-treated
(4 μM WellRED D4) and untreated (4 μM WellRED D3) RNAs, and
primer-template solutions were incubated at 65 °C for 5 min and
37 °C for 5 min. Primer extension was initiated by addition of enzyme
mix (6 μl; 250 mM KCl; 167 mM Tris–HCl [pH 8.3]; 1.67 mM each
dATP, dCTP, dGTP, and dTTP; 10 mM MgCl₂; 52 °C, 1 min) and
Superscript III (1.5 μl) were added. One set of primers were used that were complementary to TNV-D positions
2323–2353 (5’-GCTGTACCATCGTTTATCCACTC). 4 μl of 3 M NaOAc pH
5.2 was added to each of the three reactions to terminate them, after
which they were combined and precipitated on ice for 10 min with
240 μl ethanol, 2 μl glycogen. Pellets were washed twice with 70%
ethanol, dried for 15 min at room temperature, and resuspended in
45 μl of deionized formamide. cDNA samples were separated in a 33-
cm × 75-μm capillary using a Beckman CEQ 800 DNA sequencer. Raw
fluorescence intensity versus elution time profiles were analyzed using
a draft software suite called ShapeFinder (Vasa et al., 2008).

First, the average for the top 10 peak intensities was calculated. Next,
all reactivities were divided by this average and then multiplied by 2.
This normalization procedure places all absolute reactivities on a scale
of 0 to approximately 2.5. Each nucleotide was color coded according
to its reactivity score and mapped onto the lowest free energy RNA
structure predicted by MFOLD version 3.0 (Zuker et al., 1999).

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