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Characterization of the 5′- and 3′-terminal subgenomic RNAs produced by a capillovirus: Evidence for a CP subgenomic RNA

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ABSTRACT

The members of *Capillovirus* genus encode two overlapping open reading frames (ORFs): ORF1 encodes a large polyprotein containing the replication-associated proteins plus a coat protein (CP), and ORF2 encodes a movement protein (MP), located within ORF1 in a different reading frame. Organization of the CP sequence as part of the replicase ORF is unusual in capilloviruses. In this study, we examined the capillovirus genome expression strategy by characterizing viral RNAs produced by *Citrus tatter leaf virus* (CTLV), isolate ML, a *Capillovirus*. CTLV-ML produced a genome-length RNA of ~6.5-kb and two 3′-terminal sgRNAs in infected tissue that contain the MP and CP coding sequences (3′-sgRNA1), and the CP coding sequence (3′-sgRNA2), respectively. Both 3′-sgRNAs initiate at a conserved octanucleotide (UUG**A**AAGA), and are 1826 (3′-sgRNA1) and 869 (3′-sgRNA2) nts with 119 and 15 nt leader sequences, respectively, suggesting that these two 3′-sgRNAs could serve to express the MP and CP. Additionally, accumulation of two 5′-terminal sgRNAs of 5586 (5′-sgRNA1) and 4625 (5′-sgRNA2) nts was observed, and their 3′-termini mapped to 38–44 nts upstream of the transcription start sites of 3′-sgRNAs. The presence of a separate 3′-sgRNA corresponding to the CP coding sequence and its cognate 5′-terminal sgRNA (5′-sgRNA1) suggests that CTLV-ML produces a dedicated sg mRNA for the expression of its CP.

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Introduction

Single-stranded positive-sense RNA viruses utilize different strategies to express their genomes including ribosomal frameshifting or read-through proteins, polyprotein processing, multipartite genomes, and expression of internal genes by subgenomic messenger RNAs (sg mRNAs) (Buck, 1996). Viruses can make use of a single or a combination of expression strategies for efficient expression of their genomes. The sg mRNAs are 3′-coterminal, and possess one to several ORFs, but generally with only the 5′ most ORF translated. Thus, even though sg mRNAs may be polycistronic in nature, functionally they are monocistronic with few exceptions (Rochon and Johnston, 1991; Dinesh-Kumar et al., 1992; Zhou and Jackson, 1996). The mechanisms of synthesis of 3′-terminal sgRNAs have been studied for several viruses and two models have been proposed for their synthesis (Sawicki and Sawicki, 1990; van Marle et al., 1999; Miller and Koev, 2000). In the first model a viral replicase complex recognizes the sgRNA promoters on the complement of the genomic RNA and synthesizes the sg mRNA (Miller et al., 1985; Miller and Koev, 2000). The second model envisages that during the synthesis of genomic complementary strands, the replicase complex terminates at the promoter region generating a negative-strand 3′-sgRNA, which acts as a template to amplify 3′-sg mRNAs (Sawicki and Sawicki, 1990; Sit et al., 1998; Price et al., 2000; White, 2002).

The sgRNA promoters of internal genes in several families of positive-stranded RNA viruses also produce 5′-terminal sgRNAs (5′-sgRNAs) in addition to 3′-sg mRNAs (German et al., 1992; Wielgosz and Huang, 1997; Che et al., 2001; Gowda et al., 2001; 2003; Vives et al., 2002; Galiakparov et al., 2003; Wierzchoslawski et al., 2006). Although no known functions have been ascribed for these sgRNAs, Wierzchoslawski et al. (2006) proposed that they function to separate RNAs designated for translation from those involved in replication. However, this hypothesis remains to be tested for other viruses that produce 5′-sgRNAs.

The genus *Capillovirus* of the family *Flexiviridae* includes two definitive species, *Cherry virus* A and *Apple stem grooving virus* (ASGV) (Yoshikawa et al., 1992; Jelkmann, 1995; Martelli et al., 2007). Based on amino acid sequence homology, *Citrus tatter leaf*

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virus (CTLV) isolates from Kumquat (GenBank accession D16681), lily (Ohira et al., 1995) and Meyer lemon (Tatineni et al., in press) have been considered strains of ASGV with 13–15% differences in amino acid identity between replication-associated proteins of ASGV and CTLV isolates. The single-stranded RNA genomes of capilloviruses contain two open reading frames (ORFs) (Fig. 1A) (Martelli et al., 2007). ORF1 encodes a polyprotein which possesses replication-associated domains for a methyltransferase function, a papain-like protease, a helicase function and an RNA-dependent RNA polymerase, followed by a 24–27 kDa coat protein (CP) (Fig. 1A) (Yoshikawa et al., 1992; Jelkmann, 1995). ORF2 encodes a protein similar to the tobamovirus 30 kDa-like movement protein (MP) (Melcher, 2000) and is expressed from a different reading frame within the 3′ end of ORF1. This genome organization is in contrast to that observed with other genera of the *Flexiviridae*, which encode the CP as a separate ORF, and is expressed through a dedicated 3′-terminal sgRNA (Martelli et al., 2007). The organization of the CP sequences as part of the replicase ORF is unusual. There is no information on how capilloviruses express the CP, whether through a polyprotein as a contiguous protein with replication-associated proteins, which is subsequently processed, or through a separate sg mRNA dedicated for the expression of CP as in other genera of the *Flexiviridae*.

CTLV was first reported from California as a latent infection in Meyer lemon trees [*Citrus limon* (L.) Burm. f. hyb.] (Wallace and Drake, 1962), with subsequent reports of the virus from this host in Florida (Garnsey, 1964). Experimental inoculations and reports from China showed that CTLV induces a budunion incompatibility of citrus trees grafted on trifoliate orange [*Poncirus trifoliate* (L.) Raf.] or on its hybrid rootstocks (Miyakawa and Ito, 2000), which are widely used citrus rootstocks worldwide. The genome of CTLV derived from the original Meyer lemon source (CTLV-ML) has been determined (Tatineni et al., in press). CTLV-ML contains a single-stranded RNA genome of 6495 nts and a poly (A) tail at the 3′-terminus with two overlapping ORFs. ORF1 and ORF2 possess 87% and 95% amino acid identity, respectively, with the corresponding proteins of ASGV and therefore is considered a strain of ASGV. The CP, a 27 kDa protein of 237 amino acids, is encoded by ORF1 (nt 5641–6354) contiguous with replication-associated proteins (Tatineni et al., in press).

Viral RNAs produced in capillovirus-infected tissues have not been characterized, so it is not clear which expression strategy is utilized by CTLV and other capilloviruses *in planta*. In the present study, we characterized CTLV-ML-specific RNAs found in total RNA and in double-stranded RNA (dsRNA)-enriched preparations from infected tissue. In addition to the genomic RNA, accumulation of two 5′-terminal sgRNAs (5′-sgRNA1 and 2) and two 3′-terminal sgRNAs (3′-sgRNA1 and 2) was observed. Mapping the transcription start sites of 3′-sgRNAs and 3′ termini of 5′-sgRNAs revealed that the 3′-sgRNAs initiated at a conserved octanucleotide (UUG**A**AAGA, +1 nt in bold) with 119 (3′-sgRNA1) and 15 (3′-sgRNA2) nt leader sequences, and the 3′ termini of the 5′-sgRNAs mapped to 38–44 nts upstream of the transcription start sites of 3′-sgRNAs. We provide evidence for a separate 3′-sgRNA corresponding to the CP coding sequence (3′-sgRNA2) and its cognate 5′-terminal sgRNA (5′-sgRNA1), which would suggest that capilloviruses produce a dedicated sg mRNA for the expression of CP.

Results

CTLV-ML infected tissue contains two 5′-terminal and two 3′-terminal sgRNAs

The CTLV-ML RNAs from dsRNA-enriched preparations were analyzed by Northern blot hybridizations using five different

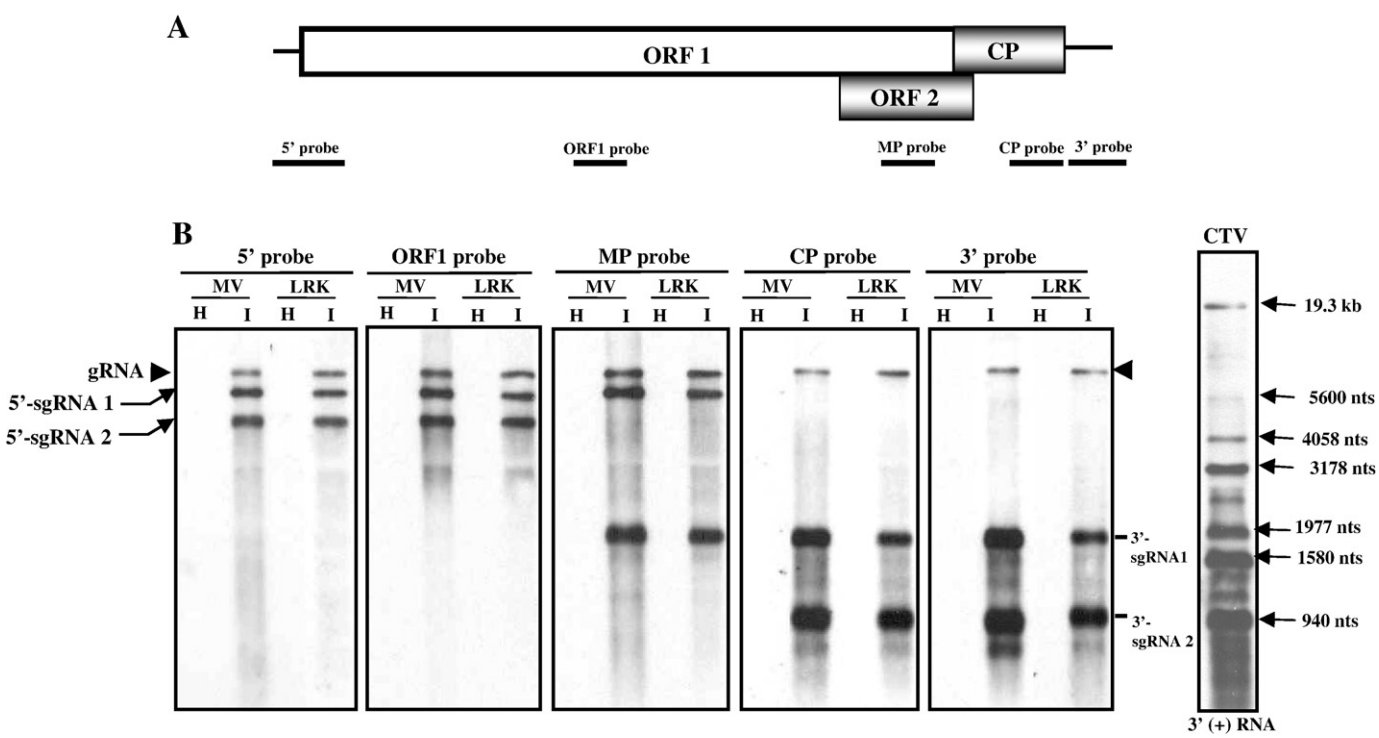

Fig. 1. Northern blot hybridization analysis of dsRNA-enriched preparation from CTLV-ML-infected and healthy tissues. (A) Schematic diagram of the genomic organization of CTLV-ML. Boxes represent open reading frames (ORF). Location of positive-stranded RNA-specific probes used for Northern blot hybridizations are shown with solid lines below the genome organization. (B) Northern blot hybridization of dsRNA-enriched preparations from Madam vinous sweet orange (MV) and *Phaseolus vulgaris* cv. Light Red Kidney bean plants (LRK) infected with CTLV-ML. DsRNA isolated from healthy citrus and bean leaves (H) included as negative controls. The size of CTLV-specific RNAs was estimated by using *Citrus tristeza virus* (CTV) RNAs with a size range of 0.94 to 19.3 kb as molecular size markers (Hilf et al., 1995) and the size of CTV RNAs are shown on the right side of the figure. gRNA, genomic RNA; MP, movement protein; CP, coat protein.

positive-stranded RNA-specific probes corresponding to different regions of the genomic RNA (Fig. 1A). The following riboprobes were used in Northern blot hybridizations: the 5′ probe (nts 1–643), the ORF1 probe (nts 2365–2966), the MP probe (nts 4733–5308), the CP probe (nts 5688–6199), and the 3′ probe (nts 6172–6495). All these riboprobes hybridized with a ~6.5-kb RNA in Northern blots, with size equivalent to the genomic RNA (Fig. 1B). Two less-than-genome sized RNAs hybridized with probes specific to the 5′ end and ORF1, but not with probes specific to the CP or 3′ end, suggesting that these sgRNAs are 5′-terminal (Fig. 1B), and consequently were designated 5′-sgRNA1 and 5′-sgRNA2, with estimated sizes of 5.6 and 4.6 kb, respectively. The CP- and 3′ end-specific probes hybridized to two small sgRNAs with estimated sizes of 1.8 and 0.9 kb, and were designated 3′-sgRNA1 and 3′-sgRNA2. These sgRNAs were 3′-terminal, since they hybridized with a 3′-specific probe, but failed to hybridize with probes specific to the 5′ end and ORF1 (Fig. 1B). The MP-specific probe hybridized with both 5′-sgRNA1 and 3′-sgRNA1, while the CP-specific probe did not hybridize with either 5′-sgRNA (Fig. 1B), indicating that the 3′ end of the 5′-sgRNA1 extends to the region between the MP- and CP-specific probes. The MP-specific probe failed to hybridize with the 3′-sgRNA2, which would suggest that this RNA extends from the 3′ end of the genomic RNA only to the region between the MP- and CP-specific probes. The 5′-sgRNA2 hybridized only with the 5′ end and ORF1 probes suggesting that its 3′ end does not extend into the MP-probe region (Fig. 1B).

These results demonstrate that CTLV-ML-infected tissue contains two nested sets of sgRNAs. The first set of sgRNAs consists of two 5′-sgRNAs of 5.6 (5′-sgRNA1) and 4.6 (5′-sgRNA2) kb, which are 5′-terminal with the genomic RNA, but terminate at different positions. The second set of sgRNAs consists of two 3′-sgRNAs of 1.8 (3′-sgRNA1) and 0.9 (3′-sgRNA2) kb, which are 3′-terminal with the genomic RNA, but with variable 5′ termini. Based on Northern hybridization results, the 3′ ends of 5′-sgRNA2 and 5′-sgRNA1 were estimated to be between nts 2966 and 4773, and 5308 and 5688, respectively. The 3′-sgRNA1 extends from the 3′ end through the MP probe region with its 5′ end between nts 2966 and 4773 and most likely contains the MP ORF as its 5′-proximal ORF. The 3′-sgRNA2 extends from the 3′ end through the CP probe region with its 5′ end between nts 5308 and 5688 and probably contains the CP coding sequence as its 5′ most ORF.

Analysis of positive- and negative-stranded RNAs from CTLV-infected tissue

To investigate the accumulation of plus- and minus-sense genomic and sgRNAs, total RNA preparations were analyzed by Northern blot hybridization with positive- and negative-stranded RNA-specific probes corresponding to the 5′ or 3′ end of the genomic RNA (Fig. 2). The genomic plus- and minus-stranded RNAs accumulated at approximate ratio of 60–80:1 in the total RNA extracted from citrus and *Phaseolus vulgaris* cv. Light Red Kidney (LRK) bean leaves, a ratio characteristic of positive-stranded RNA viruses (Buck, 1996). The positive-stranded 5′- and 3′-sgRNAs were readily detected in total RNA preparations (Fig. 2), but the complementary minus-strands of these sgRNAs were not at detectable levels. These data suggest that the positive-stranded RNA molecules accumulate in infected cells to a much higher level than the negative-stranded RNAs, similar to other plus-strand RNA viruses (Buck, 1996). However, the kinetics of accumulation of genomic RNA and sgRNAs in CTLV-ML-infected tissues remain to be elucidated.

The 3′-terminal sgRNAs initiate at a conserved octanucleotide sequence

The transcription start sites of 3′-sgRNAs were determined by amplifying the 5′ ends of sgRNAs with 5′RACE system (Fig. 3), followed by cloning into pGEM-T Easy vector. We sequenced twenty 5′RACE derived clones for 3′-sgRNA1, and found that the 5′ end sequence of all clones started at an adenylate at nt 4669 (Fig. 4A). These data suggest that the 3′-sgRNA1 is 1826 nts, extends from 119 nts upstream of the MP ORF, which is the 5′ proximal ORF, to the 3′ end of the genomic RNA (Figs. 4A and 5). We used MFOLD (Zuker et al., 1999) to predict a secondary structure around the transcription initiation site of 3′-sgRNA1 by analyzing a 67 nt region, which extended from positions −18 to +48 in the minus sense. This sequence was predicted to fold into two stem-and-loop (SL) structures, with the 5′ most SL2 predicted to be more stable and which also included the transcription initiation site at nt 4669 (Fig. 4C). Similar SL structures were observed even when a larger region (−200 and +100 nts) around the transcription start site was analyzed by MFOLD (data not shown).

We sequenced sixteen 5′RACE clones of 3′-sgRNA2. All sixteen clones initiated at an adenylate residue at nt 5626, indicating 3′-sgRNA2 is 869 nts long with a 15 nt leader sequence preceding the CP

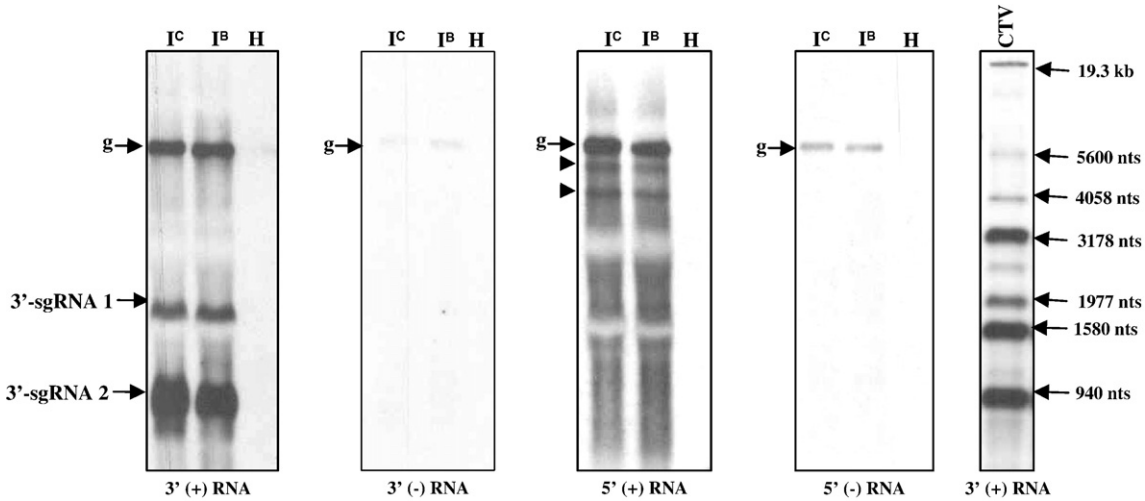

Fig. 2. Detection of positive- and negative-stranded genomic and sgRNAs of CTLV-ML. Northern blot hybridization of total RNA extracted from Madam vinous sweet orange (I^C) and *P. vulgaris* cv. light red kidney bean (I^B) leaves infected with CTLV-ML. The Northern blot of total RNA with negative-stranded RNA-specific probes was exposed 3× times longer than corresponding positive-stranded RNA-specific probes. The position of two 5′-terminal sgRNAs are indicated with arrow heads. CTV RNAs were used as molecular size markers to estimate the sizes of CTLV-ML specific RNAs and the size of CTV RNAs are shown on the right side of the figure. g, genomic RNA; H, total RNA from healthy Madam vinous or healthy LRK bean leaves. Northern blots were hybridized with positive- and negative-stranded RNA-specific probes corresponding to the 5′ or 3′ end of the genomic RNA.

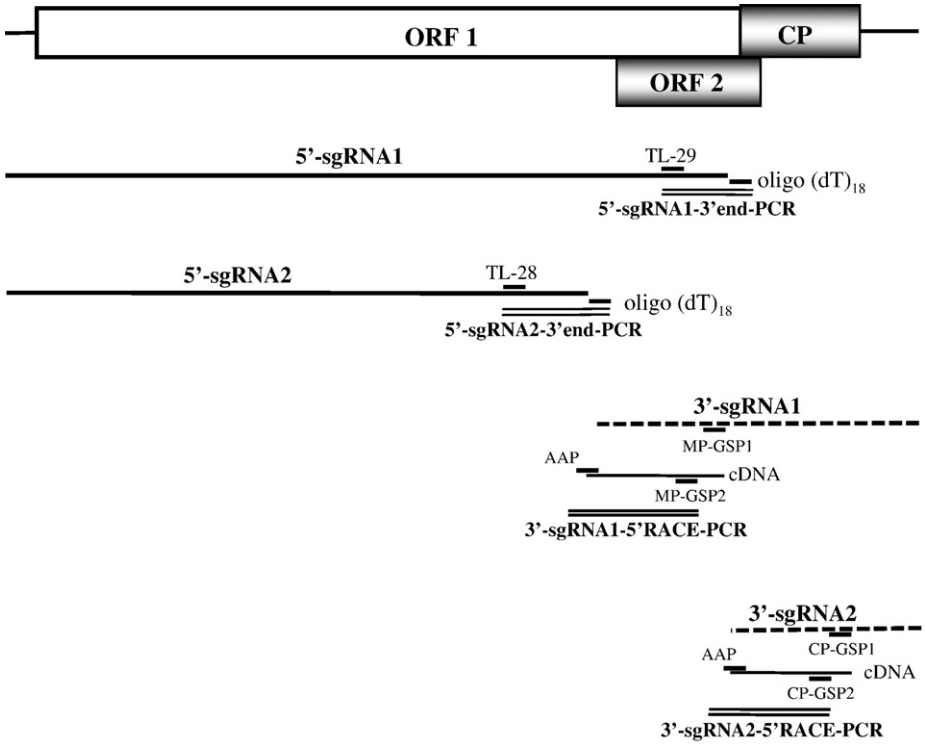

Fig. 3. Schematic representation of the strategy followed to amplify the 5′ ends of 3′-terminal sgRNAs and the 3′ ends of 5′-terminal sgRNAs. Genome organization of CTLV-ML is shown with open reading frames (open boxes), and the 5′ and 3′NTRs (solid lines). The 5′-terminal (5′-sgRNA1 and 5′-sgRNA2) and 3′-terminal (3′-sgRNA1 and 3′-sgRNA2) sgRNAs are shown below the genome organization with solid and broken lines, respectively. Polyadenylated 5′-sgRNAs were reverse transcribed with oligo(dT)$_{18}$ primer, followed by PCR with oligo(dT)$_{18}$ and TL-29 (for 5′-sgRNA1), and oligo(dT)$_{18}$ and TL-28 (for 5′-sgRNA2). The 3′sgRNAs were reverse transcribed with oligonucleotides MP-GSP1 (3′-sgRNA1) and CP-GSP1 (3′-sgRNA2), 3′ end of cDNAs were C-tailed and PCR amplified using Abridged Anchor Primer (AAP, supplied with kit) and oligonucleotides MP-GSP2 (for 3′-sgRNA1) or CP-GSP2 (for 3′-sgRNA2). Double lines represent the RT-PCR products of 3′ and 5′ ends of 5′-sgRNAs and 3′-sgRNAs, respectively.

start codon (Fig. 4B) and that the 3′-sgRNA2 encodes the CP coding sequence (Fig. 5). The secondary structure of a 61 nt region around the transcription initiation site (−15 and +45 nts) was predicted to fold into two SL structures, SL1 and SL2, with the transcription initiation site located in the loop region of SL2 (Fig. 4D). The predicted secondary structures were maintained even with extended sequences of −100 and +200 nts from the transcription start site (data not shown).

These results suggest that in infected cells CTLV-ML synthesizes a sgRNA associated with expression of the MP (3′-sgRNA1), and a second sgRNA associated with expression of the CP (3′-sgRNA2) (Fig. 5). Both the sgRNAs contained a conserved octanucleotide sequence around the transcription start site (−3 and +4 nts, UUG**A**AAGA) (Figs. 4A and B), which might play a critical role in the regulation of transcription of 3′-sgRNAs. The conserved octanucleotides around the transcription start sites of both 3′-sgRNAs are located in the loop region of predicted SL2 (Figs. 4C and D).

Determination of the 3′ ends of 5′-terminal sgRNAs

The 5′-sgRNAs were further characterized by mapping their 3′ ends. The 3′-termini were amplified by reverse transcribing the polyadenylated dsRNA-enriched preparation, followed by PCR amplification with oligo (dT)$_{18}$ paired with either TL-29 (for 5′-sgRNA1) or TL-28 (for 5′-sgRNA2) (Table 1 and Fig. 3). Oligonucleotides TL-29 and TL-28 are located 359 and 509 nts upstream of translation start codons of CP and MP ORFs, respectively. Amplification with primer pairs oligo (dT)$_{18}$/TL-29 and oligo (dT)$_{18}$/TL-28 yielded a PCR product with each pair with estimated sizes of 300 and 360 bp, respectively. This suggests that the 3′ ends of 5′sgRNA1 and 2 would each map to ~50 and ~150 nts upstream of the translation start codons of CP and MP ORFs, respectively. The RT-PCR products were cloned in pGEM-T Easy vector and inserts were sequenced.

Sequence analysis of twenty five clones of the oligo(dT)$_{18}$/TL-29 PCR product (corresponding to 3′ of 5′-sgRNA1) showed that twenty clones had 3′-termini at position 5586 and five clones had 3′-termini at position 5588, which are located 55 and 53 nts, respectively, upstream of the translation start codon of the CP ORF, and 40 and 38 nts, respectively from the transcription initiation site of 3′-sgRNA2 (Figs. 4B and 5). These data suggest that 5′-sgRNA1 synthesis terminates at nts 5586 or 5588, which are located upstream of the predicted secondary structures around the transcription initiation site of 3′-sgRNA2 (Fig. 4D).

Sequence analysis of sixteen clones of the oligo (dT)$_{18}$/TL-28 PCR product (corresponding to 3′ of 5′-sgRNA2) showed that eight clones had their 3′ ends at nt 4625, six clones at nt 4628 and one clone each at nt 4630 and 4631, positions located 157–163 nts upstream of the translation start codon of the MP, and 38–44 nts from the transcription initiation site of MP sgRNA (3′-sgRNA1) (Figs. 4A and 5). These results suggest that the 3′ ends of majority of 5′-sgRNA2 molecules (>80%) terminate at nts 4625 and 4628 in the predicted SL1, located 44 and 41 nts from the transcription initiation site of 3′-sgRNA1, respectively (Fig. 4C).

The 5′-sgRNAs are coterminal with the genomic RNA 5′ end

The exact nature of the 5′-termini of 5′-sgRNAs is not known. The 5′ ends of genomic and 5′-sgRNAs of CTLV-ML were determined using the 5′RACE system, and obtained a single RT-PCR product, which was cloned into pGEM-T Easy vector. We sequenced fifteen 5′RACE clones and all fifteen clones contained identical 5′ ends, suggesting that the 5′ termini of 5′-sgRNAs are identical with the genomic RNA 5′ end (data not shown; Tatineni et al., in press). The 5′RACE system would have amplified the heterogeneity among the genomic and 5′-sgRNAs, if any existed, since dsRNA-rich preparation from CTLV-ML-infected

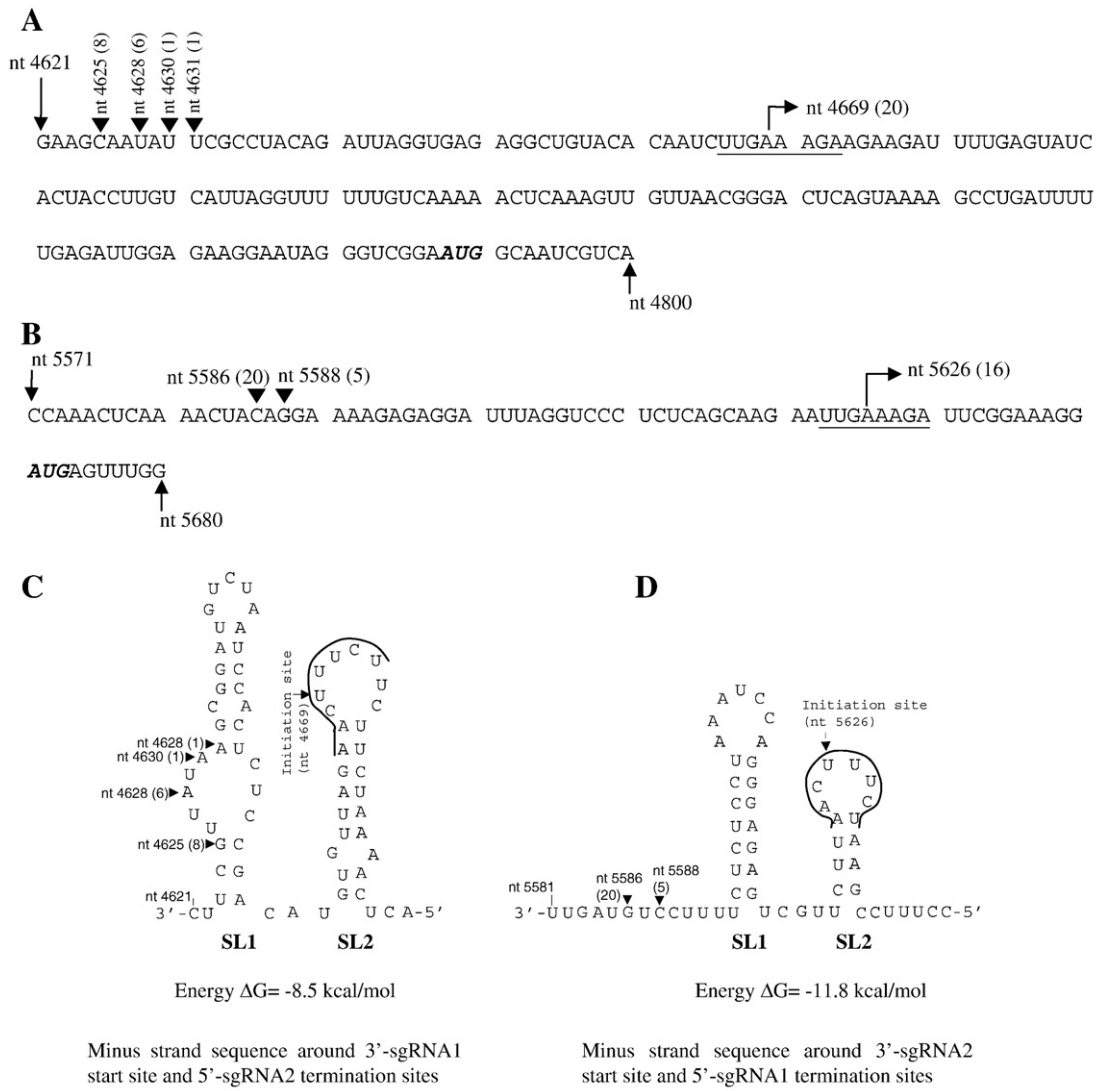

Fig. 4. Nucleotide sequence of the portion of the genomic RNA showing the transcription termination and transcription initiation sites of 5′-sgRNA2 and 3′-sgRNA1 (A), and 5′-sgRNA1 and 3′-sgRNA2 (B), respectively. The bent arrows indicate the transcription start sites; the translation initiation codon is shown in bold *italics*. The conserved octanucleotide sequence around the transcription start sites of 3′-sgRNAs is underlined. Numbers indicate the nucleotide coordinates with the genomic RNA, and numbers in parentheses indicate the number of independent clones sequenced. The predicted secondary structures of the minus-strand sequences around the transcription termination and initiation sites of 5′-sgRNA2 and 3′-sgRNA1 (C) and 5′-sgRNA1 and 3′-sgRNA2 (D). The 3′ ends of minus-strand sequence are indicated in each structure. The transcription initiation sites are indicated; conserved octanucleotide sequence in the loop region is indicated with a line around the sequence; arrow heads indicate the termination sites of 5′-sgRNAs.

tissue was used, which contained approximately equal concentrations of the genomic and 5′-sgRNAs. These data suggest that the 5′-sgRNAs are coterminal with the genomic RNA 5′ end.

Discussion

The mechanism of capillovirus gene expression is poorly understood. Within the *Flexiviridae* family, only members of the *Capillovirus* genus do not encode CP as a separate ORF; instead the CP is located at the C-terminal region of a polyprotein encoded by ORF1 (Martelli et al., 2007). In general, CP is the most abundantly expressed viral protein, since large quantities are required for virion formation (Callaway et al., 2001). How then do capilloviruses express the CP in abundant amounts from a polyprotein? Capilloviruses would have to over produce replicase-related proteins in order to provide enough CP for virion formation. One possibility is that the surplus replication-related proteins accumulate as inclusion bodies as observed with the members of the potyvirus group (Dougherty and Carrington, 1988). However, there is no available evidence of significant amounts of inclusion bodies in CTLV-ML and other capillovirus-infected tissues.

In the present study, we showed that the CTLV-ML produces two 3′-terminal sgRNAs with estimated sizes of 1.8 (3′-sgRNA1) and 0.9 (3′-sgRNA2) kb, and two 5′-terminal sgRNAs of 5.6 (5′-sgRNA1) and 4.6 (5′-sgRNA2) kb. The plus polarity of the 5′- and 3′-terminal sgRNAs were readily detected in total RNA preparations from two different hosts, citrus and red kidney beans, while their complementary minus-strands were not at detectable levels. In contrast, Magome et al. (1997) reported ASGV-specific sgRNAs with estimated sizes of 5.5, 4.5, 2.0 and 1.0 kb from dsRNA-enriched preparations, but failed to detect similar sgRNAs from total RNA preparations.

Synthesis of 3′-coterminal sgRNA(s) is the strategy most commonly used by positive-stranded RNA viruses to express their internal genes (Buck, 1996; Miller and Koev, 2000). Two 3′-terminal sgRNAs were found in large amounts in total RNA and dsRNA-enriched preparations from CTLV-ML-infected tissue, which would suggest that CTLV-ML (and possibly other capilloviruses) could express the MP and CP from these abundantly transcribed 3′-terminal sgRNAs. The 1826 nts long 3′-sgRNA1 initiates at nt 4669 and contains the MP gene as its 5′ most ORF, whereas the 3′-sgRNA2 is 869 nts long, initiates at nt 5626 and has the CP coding sequence (Fig. 5). The

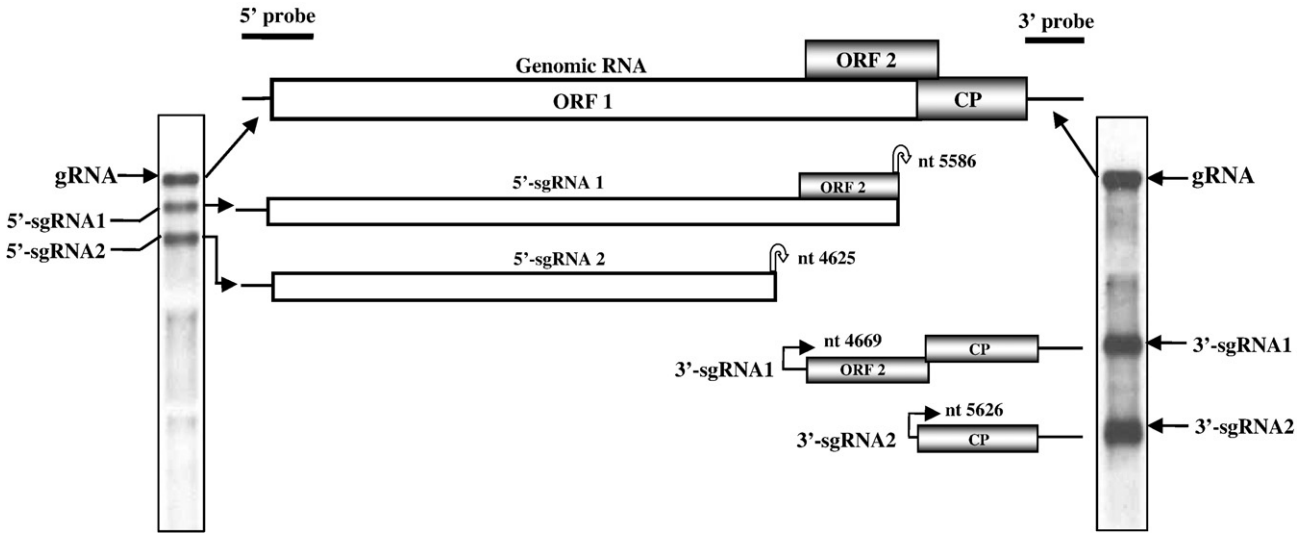

Fig. 5. Schematic diagram of the genomic and sgRNAs of CTLV-ML. Organization of the genomic RNA, 5′-terminal sgRNAs and 3′-terminal sgRNAs are aligned with RNA bands from the Northern blots of dsRNA-enriched preparation from CTLV-ML-infected tissue, probed with 5′- and 3′-positive-stranded RNA-specific probes. The 3′ ends of 5′-sgRNAs and the 5′ ends of 3′-sgRNAs are indicated with u-turn and bent arrows, respectively. Position of probes used for Northern blot hybridizations are shown above the genomic organization. The 5′-sgRNA-1 and 5′-sgRNA2 contain parts of ORF1 and ORF2, and part of ORF1, respectively.

conserved octanucleotide (UUG**A**AAGA) sequence at the transcription start sites of 3′-sgRNAs located in the loop of predicted SL structures might play a regulatory role in recognition by the viral replicase complex on minus strand genomic RNA for the synthesis of plus-strand sgRNAs. Identical nucleotides at the 5′ ends of 3′-terminal sgRNAs and genomic RNAs have been reported for several other viruses (Marsh and Hall, 1987; van der Kuyl et al., 1991; Boccard and Baulcombe, 1993; Miller et al., 1985; Hacker and Sivakumaran, 1997; Scheets, 2000; Vives et al., 2002). Although ASGV and its strains possess the conserved octanucleotides, −3 to +4 nts from the transcription start sites of 3′-sgRNAs, no homology was observed with the genomic RNA 5′ end sequence (Yoshikawa et al., 1992; Jelkmann, 1995; Ohira et al., 1995; Shim et al., 2004; Tatineni et al., in press). The 5′ terminal nucleotides of both 3′-terminal sgRNAs are adenylates, similar to that of the genomic RNA (Tatineni et al., in press). The leader sequences of 3′-sgRNAs are colinear with the corresponding genomic RNA sequence. The relative lengths of the leader sequences observed with CTLV-ML, 119 and 15 nts for 3′-sgRNA1 and 2, respectively, are similar to those of *Tobacco mosaic virus* MP and CP sgRNAs with 115 and 9 nts (Grdzelishvili et al., 2000).

In addition to two 3′-terminal sgRNAs, CTLV-ML-infected tissue contain two 5′-terminal sgRNAs with estimated sizes of ~5.6 (5′-sgRNA1) and ~4.6 (5′-sgRNA2) kb. As with CTLV-ML, 5′-sgRNAs, one corresponding to each 3′-terminal sgRNA, have been reported in tissues infected with *Citrus tristeza virus* (CTV) (Che et al., 2001; Gowda et al., 2001), *Grapevine virus* A (GVA) (Galiakparov et al., 2003), *Citrus leaf blotch virus* (CLBV) (Vives et al., 2002), *Apple chlorotic leaf spot virus* (ACLSV) (German et al., 1992), and *Brome mosaic virus* (BMV) (Wierzchoslawski et al., 2006). While the mechanism of synthesis of 5′-sgRNAs is not known, these sgRNAs appear to be produced by termination of synthesis of genome length positive-strand RNAs near the *cis*-acting promoter elements of 3′-sgRNAs (Gowda et al., 2001; Galiakparov et al., 2003). In this model, the replicase complex first pauses and then releases the nascent genomic plus-strand near the *cis*-acting elements of 3′-sgRNAs, since another replicase complex already occupies that site to initiate 3′-sgRNA synthesis (Gowda et al., 2001; Galiakparov et al., 2003). In support of this model, the 3′ ends of 5′-sgRNAs of CTLV-ML were mapped to upstream of, or in the predicted SL structures, which possess the conserved octanucleotide sequence with transcription initiation sites of 3′-sgRNAs. It is possible that the SL structures, plus the sequence between these structures and 3′ termini of 5′-sgRNAs might contain the *cis*-acting elements for 3′-sgRNAs. Perhaps these *cis*-acting elements would involve in termination of synthesis of the genomic RNA as suggested by Galiakparov et al. (2003). As evidence in support of this model, Gowda et al. (2001, 2003) reported that mutations that abolished the production of 3′-terminal sgRNAs also abolished the production of 5′-terminal sgRNAs. Moreover, evidence supports the 5′-sgRNAs in CTLV-ML not being derived from breakage of the genomic RNA, as their 3′ termini were mapped to 38–44 nts upstream of the 5′ ends of 3′-sgRNAs. Thus a gap of 38–44 nts was observed between the 3′ ends of 5′-sgRNAs and the 5′ ends of 3′-sgRNAs, suggesting that a mechanism other than specific cleavage might be involved in generation of 5′-sgRNAs. Similar results were also reported for CTV, GVA and CLBV, in which the 3′ ends of 5′-sgRNAs are

Table 1
The nucleotide sequence of oligonucleotides used to amplify CTLV-ML-specific fragments to generate riboprobes

Probe to	Primer	Sequence of oligonucleotide (5′ to 3′)[a]	Primer polarity	Location of the primer in CTLV-ML sequence[b]
5′ end	TL-1	CCT*ATTTAGGTGACACTATAG* AAATTTAACAGCGCTTAATTTCCGCGC	+	1–26
	TL-24	CCT**TAATACGACTCACTATAG** GGAACACAGTTGTCGCAACAAC	−	643–625
ORF1	TL-30	CCT*ATTTAGGTGACACTATAG* GGCTTAAGGCTGGGTTTTGCTGGTTCTGG	+	2365–2393
	TL-31	CCT**TAATACGACTCACTATAG**GG TTTGCTACTAGAATGAGCCCATACTCCTC	−	2966–2938
MP	TL-32	CCT*ATTTAGGTGACACTATAG* AGGAATAGGGTCGGAATGGCAATCGTCAAT	+	4773–4802
	TL-33	CCT**TAATACGACTCACTATAG**GG AGTTCTGTGTCCTGTTCATAGTGTGGGCAG	−	5308–5279
CP	TL-34	CCT*ATTTAGGTGACACTATAG* AGTATATCTGTGGAAGACCCATATAGACCC	+	5688–5717
	TL-35	CCT**TAATACGACTCACTATAG**GG CCCAAGGGCTCTTTTCGAAAGCTTTGGGCC	−	6199–6170
3′ end	TL-7	CCT*ATTTAGGTGACACTATAG* CCCAAAGCTTTCGAAAAGAGCCCTT	+	6172–6196
	TL-8	CCT**TAATACGACTCACTATAGG** AAATGGATAAACTCCAGATACTAG	−	6495–6471

[a] Sequences in italics and bold represent the SP6 RNA polymerase and T7 RNA polymerase promoter, respectively.
[b] Nucleotide numbering according to CTLV-ML sequence (GenBank accession EU553489).

30–50, 35–69 and 35–49 nts upstream of 3′-sgRNAs initiation sites, respectively (Vives et al., 2002; Galiakparov et al., 2003; Ayllón et al., 2004). Furthermore, difference in hybridization intensity of the 5′- and 3′-sgRNAs of CTLV-ML does not support the involvement of specific cleavage of the genomic RNA in the production of 5′-sgRNAs, which would result in equimolar amounts of 5′- and 3′-sgRNAs.

Even though there is evidence for a 3′-terminal sgRNA for the expression of the CP, a feature of numerous other positive-stranded RNA viruses (Miller and Koev, 2000), we cannot exclude the possibility that capillovirus CP is expressed through a combination of 3′-sgRNA and as a component of polyprotein as suggested by the genomic sequence (Yoshikawa et al., 1992; Jelkmann, 1995; Ohira et al., 1995; Shim et al., 2004; Tatineni et al., in press). The CP of closely related ASGV, the type member of the *Capillovirus* genus, was not detected from *in vitro* translated products of virion RNA, while the *in vitro* translated polyprotein was selectively immuno-precipitated by ASGV CP antiserum (Yoshikawa and Takahashi, 1992). Furthermore, Yoshikawa and Takahashi (1992) readily detected the CP from ASGV-infected tissue similar to purified virions, and a polyprotein of ~200 kDa could not be detected. In contrast, the presence of abundant levels of 3′-sgRNA2 with the CP coding sequence in CTLV-ML-infected tissues would suggest that the CP is expressed through a sg mRNA. Taken together, the presence of a 3′-terminal sgRNA with CP coding sequence and failure to detect an expected cleavage product of CP from *in vitro* translated products of ASGV virion RNA (Yoshikawa and Takahashi, 1992) would suggest that the CP might have dual functions: one in replication as a polyprotein along with replication-associated proteins and the second is for virion formation, which is expressed through a 3′-sg mRNA. It would be novel for a virus producing a 3′-terminal sgRNA for the expression of CP to also express CP as a component of larger polyprotein, and it is possible that capilloviruses employ a combination of both strategies for the expression of their CP.

Materials and methods

Virus source and maintenance

CTLV-ML used in this study was originally obtained from commercial Meyer lemon trees in the early 1960's and subsequently subjected to several single lesion passages in red kidney bean plants prior to its re-introduction into citrus plants by mechanical inoculation (Garnsey, 1964). Subsequently, CTLV-ML was maintained in the greenhouse in citrus plants by periodical graft inoculations.

RNA isolation

Total RNA was isolated from 100 to 200 mg of CTLV-ML infected Madam vinous sweet orange [*Citrus sinensis* (L.)] or systemically infected young leaves of LRK bean plants using Trizol reagent (Invitrogen) as per the manufacturer's instructions. The final RNA pellet was suspended in 25–50 μl of sterile water.

Virus-specific dsRNA-enriched preparations were obtained from total nucleic acid extracts by CF-11 cellulose column chromatography in the presence of 16% (v/v) ethanol as per the procedure of Moreno et al. (1990).

Preparation of riboprobes and Northern blot hybridization of CTLV RNAs

The following riboprobes were prepared to target specific regions of the genomic RNA: the positive- and negative-stranded RNA-specific probes for nts 1–643 (5′ positive- and negative-stranded RNA-specific probes) and nts 6172–6495 (3′ positive- and negative-stranded RNA-specific probes); and positive-stranded RNA-specific probes for nts 2365–2966 (ORF1 probe), nts 4773–5308 (MP probe), and nts 5688–6199 (CP probe) (Fig. 1A). DNA templates for riboprobe synthesis were amplified from pCTLV-81 (Tatineni et al., in press) using primers specific for these sequences (Table 1). SP6 and T7 RNA polymerase promoter sequences were included in the forward and reverse primers, respectively, to generate negative- and positive-stranded RNA-specific probes. Agarose-gel eluted PCR products were used as templates to generate negative- or positive-stranded RNA-specific probes using either SP6 or T7 RNA polymerase and nucleotides containing digoxigenin-labeled UTP as described in Satyanarayana et al. (1999).

Total RNA or dsRNA isolated from CTLV-ML-infected LRK beans and Madam vinous sweet orange leaves were separated in 1.0% agarose gels containing formaldehyde-formamide, and transferred onto a nylon membrane using electrotransfer as described in Lewandowski and Dawson (1998). Prehybridization at 65 °C for 1–2 h and hybridization at 65 °C for overnight were performed in a hybridization incubator with positive- or negative-stranded RNA-specific probes. The nylon membranes were washed with 2 X SSC containing 0.1% SDS for 10 min at room temperature, followed by two washes with 0.1 X SSC containing 0.1% SDS for 30 min at 65 °C. Membranes were developed as per the manufacturer's instructions (Roche), followed by image development by exposing the nylon membrane to X-ray film (Kodak). The relative amounts of genomic positive- and negative-stranded RNAs accumulated were quantified using different exposures of Northern blots by scanning and densitometry with the OS-SCAN program (Oberlin Scientific, Oberlin, Ohio). Northern blot results presented represent at least three to four independent isolation of total RNA or dsRNA from CTLV-ML-infected LRK beans and Madam vinous sweet orange leaves.

Mapping the transcription start sites of 3′-sgRNAs

CTLV-ML-specific dsRNA was used to map the transcription start sites of two 3′-sgRNAs using the 5′RACE system (Invitrogen). The gene specific primers, MP-GSP1 (5′-GATTCCAACCAAGATGACTCC ATAGTG-3′, complementary to nts 5072–5046) and CP-GSP1 (5′-CCACCTTT-CATGAAGAAATTCACGAGC-3′, complementary to nts 6135–6109) were used to synthesize the first-strand cDNAs (Fig. 3). The first-strand cDNAs were column purified and 'C' tailed at the 3′ end using terminal deoxynucleotidyl transferase, and amplified using Taq DNA polymerase (2.5 U) and an abridged anchor primer (supplied with kit) together with gene specific nested primer MP-GSP2 (5′-TCTAATGGAGTCGATTT-CAGTGTCATC-3′, complementary to nts 5018–4992) or CP-GSP2 (5′-GGTACTATATTTGACAAGTCAATTCTC-3′, complementary to nts 6020–5994) (Fig. 3). The following conditions were used for PCR: 1 cycle at 94 °C for 2 min, followed by 40 cycles at 94 °C for 20 s, 55 °C for 20 s and 72 °C for 1 min, and one cycle at 72 °C for 10 min. The 5′RACE products were ligated into pGEM-T Easy vector (Promega) and the inserts were sequenced at the USDA-USHRL Genomics Facility, Fort Pierce, FL using an Applied Biosystems 3730 model sequencer.

Mapping the 3′ ends of 5′-sgRNAs

CTLV-ML-specific dsRNA was used to determine the termination sites of 5′-sgRNAs. The denatured dsRNA was 3′ polyadenylated by yeast poly (A) polymerase (US Biochemicals) and reverse transcribed using oligo $(dT)_{18}$ and SuperScriptIIRT (Invitrogen) (Fig. 3). Using this cDNA as template, the 3′ ends of the 5′-sgRNAs were determined by amplifying PCR fragments with oligo $(dT)_{18}$ and virus-specific forward primers TL-28 (GGTTCACCGGAGAATTCTGTACCT, corresponding to nts 4280–4303) and TL-29 (CCCACAGTATGAACAGGACACAGA, corresponding to nts 5282–5305) (Fig. 3). The following conditions were used for PCR amplification: 1/10th of reverse transcription reaction (2 μl) was used in a 40 μl reaction volume with final concentrations of 1 X buffer with 2 mM $MgCl_2$ (TAKARA, Buffer FB II), 0.2 μM of each oligonucleotide [oligo $(dT)_{18}$ and TL-28 or TL-29], 0.2 mM dNTPs, and 5 U SpeedSTAR HS DNA polymerase (TAKARA) for one cycle at 94 °C for 2 min, followed by 9 cycles at 94 °C for 20 s, 58 °C for 20 s and 72 °C for

30 s, and 29 cycles at 94 °C for 20 s, 60 °C for 20 s and 72 °C for 30 s, followed by one cycle at 72 °C for 3 min. The amplified products were cloned into pGEM-T Easy vector (Promega) and the inserts were sequenced at the USDA-USHRL Genomics Facility, Fort Pierce, FL.

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