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Siddarame Gowda
University of Florida

Satyanarayana Tatineni
USDA-ARS, Satyanarayana.Tatineni@ars.usda.gov

Svetlana Y. Folimonova
University of Florida

Mark E. Hilf
USDA-ARS, mark.hilf@ars.usda.gov

William O. Dawson
University of Florida, wodtmv@lal.ufl.edu

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Accumulation of a 5′ proximal subgenomic RNA of *Citrus tristeza virus* is correlated with encapsidation by the minor coat protein

Siddarame Gowda [a], Satyanarayana Tatineni [a,1], Svetlana Y. Folimonova [a], Mark E. Hilf [b], William O. Dawson [a,*]

[a] Citrus Research and Education Center, University of Florida-IFAS, 700 Experiment Station Road, Lake Alfred, FL 33850, USA
[b] U.S. Horticultural Research Laboratory, 2001 South Rock Road, Fort Pierce, FL 34945, USA

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ABSTRACT

During replication, *Citrus tristeza virus* (CTV) produces large amounts of two unusual subgenomic (sg) RNAs that are positive-stranded and 5′ coterminal. Although these RNAs are produced in similar amounts and are similar in size, with LMT1 (~750 nt) only slightly larger than LMT2 (~650), we found that the similar sgRNAs are produced differently. We previously showed that the LMT1 RNA is produced by premature termination during genomic RNA synthesis. However, LMT2 production was found to correlate with virion assembly instead of RNA replication. The time course of accumulation of the LMT2 RNA occurred late, coinciding with virion accumulation. The long flexuous virions of CTV contain two coat proteins that encapsidate the virions in a polar manner. The major coat protein encapsidates ~97% of the virion, while the minor capsid protein encapsidates the remainder of the genome beginning in the 5′ non-translated region with the transition zone at ~630 nucleotides from the 5′ end. The section of the virion RNA that was encapsidated by CPm was identical in size to the LMT2 RNA, suggesting that the LMT2 RNA represented a portion of the viral RNA protected by CPm encapsidation. Mutations that abrogated encapsidation by CPm also abolished the accumulation of LMT2 RNA. Thus, these two unusual but similar RNAs are produced via different pathways, one from RNA replication and one processed by the virion assembly process. To our knowledge, this represents the first evidence of a viral RNA processed by the assembly mechanism.

Introduction

Citrus tristeza virus, a member of the Closteroviridae, genus *Closterovirus*, has a ~20 kb single-stranded, positive sense RNA genome (Pappu et al., 1994; Karasev et al., 1995). Yet, this virus whose genome resides in a single RNA molecule produces an unusually large number of less-than-full-length viral RNAs during replication. There are ten genes in the 3′-portion of the genome that are expressed via 3′-coterminal sg mRNAs (Hilf et al., 1995; Pappu et al., 1994; Karasev et al., 1995). For each of the mRNAs, two additional less-than-full-length RNAs are produced: a negative-stranded RNA with sequence complementary to the sg mRNA, plus a 5′-terminal positive-stranded sgRNA produced by termination near the controller element upstream of the start of the mRNA, apparently during genomic RNA synthesis (Gowda et al., 2001). Thus, CTV produces 30 sgRNAs associated with its ten 3′-terminal genes. However, the most unusual sgRNAs are two small 5′-coterminal positive-stranded RNAs that have been referred to as 'low molecular weight tristeza RNAs' (LMT) (Mawassi et al., 1995; Che et al., 2001). LMT1 and LMT2 RNAs are ~750 and 650 nt, respectively (Gowda et al., 2003). Both accumulate to high amounts, at molar levels higher than that of the virion RNA. We previously dissected the production of the LMT1 RNA, which is produced during replication by termination upstream of a previously unknown controller element that produces only minute amounts of a 3′-terminal mRNA-like sgRNA, but with no known function (Gowda et al., 2003; Ayllon et al., 2004). The mechanism of production of the smaller LMT2 was unknown.

The CTV genome consists of 12 open reading frames (ORFs). The replicase related proteins are translated from the genomic RNA into a large polyprotein containing two papain-like proteases plus methyltransferase- and helicase-like domains, and by +1 ribosomal frameshift a larger polyprotein containing an additional RNA-dependent RNA polymerase-like domain (Karasev et al., 1995). The 3′ genes, which are not required for replication (Satyanarayana et al., 1999), are thought to be involved in host interactions, virion assembly, insect vector specificity, and movement within the host. The two coat proteins partition the flexuous virions in a unique manner: the major coat protein (CP) and the minor coat protein (CPm) encapsidate the genomic RNA in regular arrays at opposite ends. The CP encapsidates most (>97%) of the genome while the CPm encapsidates the

* Corresponding author. Fax: +1 863 956 4631.
E-mail address: wodtmv@ufl.edu (W.O. Dawson).
[1] Present address: U.S. Department of Agriculture-Agriculture Research Service and Department of Plant Pathology, University of Nebraska, Lincoln, NE 68583, USA.

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remainder of the genome (Febres et al., 1996; Satyanarayana et al., 2004). Two other virus encoded proteins, HSP70h and p61, apparently in unison, augment virion assembly (Satyanarayana et al., 2000; Napuli et al., 2003). Together with p6, a small hydrophobic protein proposed as a membrane anchor, the HSP70h, p61, CPm, and CP constitute a signature 'quintuple gene module' characteristic of all closteroviruses. The p20 gene encodes the amorphous inclusion body protein, and the p23 gene regulates the ratio of plus-to-minus RNA (Gowda et al., 2000; Satyanarayana et al., 2002), and are identified as components of the suppression of RNA silencing system of CTV (Lu et al., 2004). Of the remaining genes, the p13 has not been associated with any known function; the p33 and p18 genes are suggested to function in differential movement of CTV in citrus hosts (S. Tatineni, unpublished).

In this study, we examined the kinetics of accumulation of LMT2 RNA in protoplasts and found that it is produced much later than that of the LMT1 RNA. Previously, we showed that the origin of assembly (OA) of CPm resides in the 5′ non-translated region (NTR) and encapsidation by CPm is limited to ~630 nt (Satyanarayana et al., 2004). In the presence of HSP70h and p61, and in the absence of CP, CPm encapsidates and protects 5′ terminal RNA of ~630 nucleotides which is close to the CPm–CP transition boundary of the bipolar virion. Here we show that the LMT2 RNA was the same size as the CPm encapsidated RNA and independent of the accumulation of LMT1 RNA. Changes that affect the accumulation of LMT2 RNA also affect the encapsidation by CPm. Thus, our results suggest that the LMT2 RNA was derived from the assembly process, in contrast to the LMT1 RNA, which was produced from the RNA replication process.

Results

Accumulation of LMT2 RNA by different isolates of CTV

Most strains of CTV are conserved in the 3′-portion of the genome, differing in nucleotide sequence by about 10% or less as expected for strains of the same virus, but the 5′-portion of the genomes are progressively dissimilar with as little as 42% homology in the 5′ end (Mawassi et al., 1996; Lopez et al., 1998; Albiach-Marti et al., 2000). The divergence in the 5′ portion applies to T36 but not necessarily to other isolates except for the 5′ NTR. The two small 5′-coterminal sgRNAs were observed in plants infected with the Israeli strain, VT (Che et al., 2001; Mawassi et al., 1995) and later found to be produced by the Florida decline strain, T36, in plants and protoplasts (Gowda et al., 2003). We next examined whether production of both of these 5′-terminal sgRNAs was a general phenomenon among CTV strains. *Nicotiana benthamiana* protoplasts were inoculated with crude sap expressed from the bark tissue of small citrus trees infected with CTV isolates that represent the diversity available in Florida (T3, T5, T66, T68, FL43, and FL674). T66 and FL43 are independent isolates of the T36 strain, and FL674 is a Florida isolate of the VT strain. T3 and T68 are independent strains that have been introduced into Florida. The biological diversity of these isolates was assessed by biological indexing and RT-PCR markers (Hilf and Garnsey, 2000). Four days post inoculation (dpi), total RNA was extracted from the infected protoplasts and analyzed by Northern blot hybridization. Because of the sequence diversity of the 5′ ends of the different strains, the same hybridization probe does not hybridize to all strains. We grouped the isolates into two groups of more related strains and chose two 5′ probes and conditions to allow cross hybridization to the heterologous isolates to be able to visualize the LMT RNAs of all isolates. The T36 probe was used to hybridize to the total RNA from T36, T66 and FL43 infected protoplasts, and the VT probe was used for the RNA from T3, T5, T68 and FL674 infected protoplasts. The probes used correspond to 700 nt of the 5′ end of the genomic RNA of T36 and VT isolates of CTV. The results shown in Fig. 1 demonstrated that all of these isolates accumulated both LMT1 and LMT2 RNAs, although in different ratios.

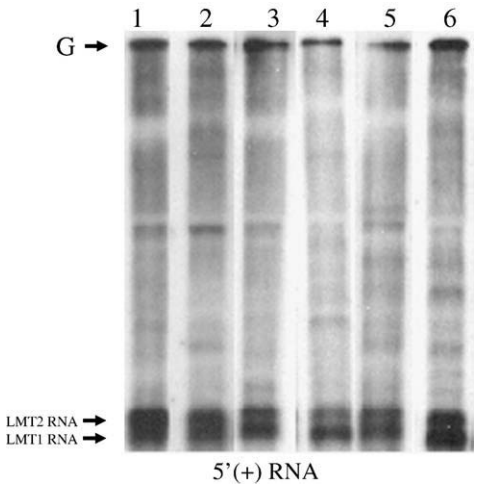

Fig. 1. Accumulation of LMT1 RNA and LMT2 RNA by different isolates of CTV. Northern analysis of the total RNA from *N. benthamiana* protoplasts transfected with the sap prepared from the bark tissue of citrus plants infected with different CTV isolates: T36 (lane 1), T66 (lane 2), FL43 (lane 3), T3 (lane 4), T68 (lane 5), and FL 674 (lane 6). The blot was hybridized with 5′ positive-stranded RNA-specific digoxigenin-labeled riboprobes prepared from the 5′ end of the VT (lanes 4–6) and T36 (lanes 1–3) isolates of CTV. Arrows indicate the positions of the genomic (G), LMT1 RNA and LMT2 RNA.

Kinetics of accumulation of LMT1 and LMT2 RNAs

To examine the kinetic relationship of the two small 5′-coterminal sgRNAs, we examined the time course of their accumulation after inoculation of *N. benthamiana* mesophyll protoplasts with CTV strain T36. We used virions present in the crude sap from the bark tissue of infected *Citrus macrophylla* trees as the inoculum to transfect protoplasts, which result in infection of a much greater proportion of protoplasts than the use of RNA transcripts from the infectious clone (Satyanarayana et al., 2000). Total RNA was isolated from the protoplasts at 1–6 dpi and analyzed by Northern blot hybridization with 5′ plus- or minus-stranded RNA-specific riboprobes. Accumulation of positive- and negative-stranded genomic RNAs generally increased in parallel and reached a maximum at 5 dpi (Fig. 2). The LMT1 RNA also accumulated essentially in parallel with the genomic RNA. However, LMT2 RNA accumulated substantially later (Fig. 2A, lanes 1–6). Negative-stranded RNAs corresponding to the LMT RNAs were not observed at any time points (Fig. 2B).

Production of LMT2 RNA was correlated with the expression of 3′ genes

A mini-replicon of CTV consisting of only the 5′ and 3′ NTRs, plus ORF1 replicates efficiently in *N. benthamiana* protoplasts (Satyanarayana et al., 1999). This replicon produces abundant amounts of LMT1 RNA and was used to show that this sgRNA was produced by termination during replication (Gowda et al., 2001), but in those experiments we noticed the absence of LMT2 RNA. There are ten 3′ genes in CTV. Four of these genes are involved in efficient assembly of virions: HSP70h, p61, CPm and CP (Satyanarayana et al., 2000). We examined if the deletion of these genes along with other 3′ genes affected the accumulation of the LMT2 RNA. Replicons were engineered that contained deletions of p18; p18 and p13; or p18–p20 genes in addition to p33 and the genes of 'quintuple gene module' (p6, HSP70h, p61, CPm, and CP). RNA transcripts of the mini-replicons that contained: p18, p13, p20 and p23; p13, p20 and p23; p20 and p23; or only p23 gene (Fig. 3A, mutants 1–4) — were used to inoculate protoplasts, and at 4 dpi RNA was extracted and analyzed by Northern blot hybridizations. All of these constructs produced normal amounts of LMT1 RNA but failed to produce LMT2 RNA (Fig. 3B, lanes 1–4), demonstrating that the p18, p13, p20 and p23 genes were not sufficient for the production of the LMT2 RNA.

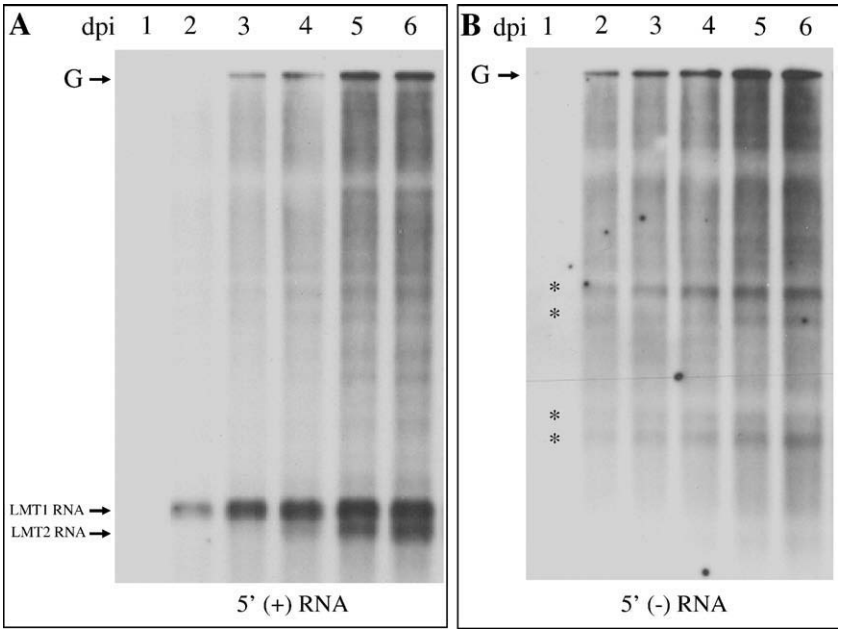

Fig. 2. Kinetics of accumulation of LMT1 and LMT2 RNAs. Northern analysis of the total RNA isolated from *N. benthamiana* protoplasts (1–6 dpi, lanes 1–6, respectively) transfected with the sap from the bark tissue of citrus plants infected with T36 isolate of CTV and hybridized with 5′ positive-stranded RNA-specific (A) or 5′ negative-stranded RNA-specific (B) digoxigenin labeled riboprobes. Positions of the genomic (G), LMT1 RNA and LMT2 RNAs are indicated by arrows.

All of the above constructs that failed to produce LMT2 RNA, also failed to produce virions. To examine the role of the assembly related genes in the accumulation of LMT2 RNA, we generated constructs that contained all of the assembly related genes: HSP70h, p61, CPm, CP plus p23 that is required to make an excess of genomic plus strands, but with deletions of all of the other 3′ genes (p33, p18, p13, and p20) (Fig. 3A, mutants 5–8). Since these larger replicons are less efficient in infecting protoplasts and because virions are much more efficient in infecting protoplasts (Satyanarayana et al., 2000), we passaged these constructs in protoplasts and used virions for inoculum as described (Satyanarayana et al., 2000) to increase the proportion of infected protoplasts to levels comparable to that of the smaller replicons described above. Northern blot hybridization analysis of the total RNA isolated from the protoplasts hybridized with 5′ plus-stranded RNA-specific riboprobe showed accumulation of both LMT1 and LMT2 RNAs by all of the replicons that contained the assembly gene block (Fig. 3B, lanes 5–8), suggesting that the assembly related functions plus the replicase genes and p23 gene are sufficient for the accumulation of the LMT2 RNA.

LMT2 RNA was the same size as the CPm encapsidated portion of the virion

We previously found that assembly by the minor coat protein (CPm) begins at the 5′ end of the genomic RNA, and in the presence of both Hsp70h and p61, the encapsidation is restricted to the 5′ ~630 nt (Satyanarayana et al., 2004). In the absence of the major coat protein (CP), the unencapsidated RNA was degraded during extraction resulting in a 5′-coterminal positive-stranded RNA ~630 nt long (Satyanarayana et al., 2004). By comparison to the ~750 nt LMT1 RNA, LMT2 RNA appeared to be ~650 nt long, which was similar in size to the CPm-protected component of the virion. In order to determine the precise size of the LMT2 RNAs, the sequence of the 3′ terminus of the LMT2 RNA was determined by the addition of a polyA tail to the total RNA isolated from *N. benthamiana* protoplasts transfected with the virions in crude sap extracted from T36 infected citrus plants. Under these conditions both LMT1 and LMT2 RNAs were produced (Figs. 1 and 2). The polyA tailed RNA was reverse-transcribed using an oligo dT primer and PCR products were amplified using oligo dT primer and a CTV specific primer corresponding to nts 383–405, resulting in amplification of two products of ~400 and ~300 nt, corresponding to LMT1 and LMT2 RNAs, respectively (Fig. 4A). The smaller product corresponding to LMT2 RNA was cloned and sequenced. Of ~65 sequenced clones, 55% had 3′ termini at nts 627/628/629; 28% at nts 630/631; one each at nt 603/604 and 605/606; 2 at nts 560 and 626; and, one each at nts 558, 559, 624, 637, 638 and 646. The ambiguity of the 3′-terminal nucleotide with some sequences occurred when the apparent 3′ terminus was adjacent to an adenylate residue in the CTV genome (Fig. 4B). In this event we were unable to distinguish if the adenylate residue(s) represented the terminal nucleotide or part of the polyA tail. Most of the RNAs isolated from CPm encapsidated particles ended between 627–631 (Satyanarayana et al., 2004) and 83% the LMT2 RNAs terminated between 627–631, demonstrating that the LMT2 RNA was essentially identical in size to the RNA encapsidated by CPm in the presence of Hsp70h and p61.

Changes in the LMT2 RNA termination region affect LMT2 RNA accumulation

The above experiments suggested that the 3 termini of most (83%) of the LMT2 RNAs were restricted to a region of 5 nucleotides between nts 627–631. Mutations were introduced in the area surrounding this region in an effort to determine if the changes affected accumulation of LMT2 RNA and consequent encapsidation by CPm. The mutations included changes in the sequence of nucleotides or the deletion of nucleotides (Fig. 5A). Since the LMT2 RNA region of the genomic RNA encodes the 5′ terminus of the ORF1 polyprotein only in-frame deletions were introduced so as not to alter the polyprotein reading frame (Fig. 5A). The mutants contained the deletion of 24 nucleotides (nts 609–632; mutant 1); 6 nucleotides (nts 609–614; mutant 4) and 8 nucleotides (nts 652–659; mutant 6). The changes in the nucleotide sequence included: change of CCCCA into aattc (nts 610–614 in mutant 2); CCAAGA into aagctt (nts 626–631 in mutant 3); and ACCCGG into gagctc (nts 640–645 in mutant 5). Total RNA, and RNA from the nucleocapsids isolated from infected *N. benthamiana*

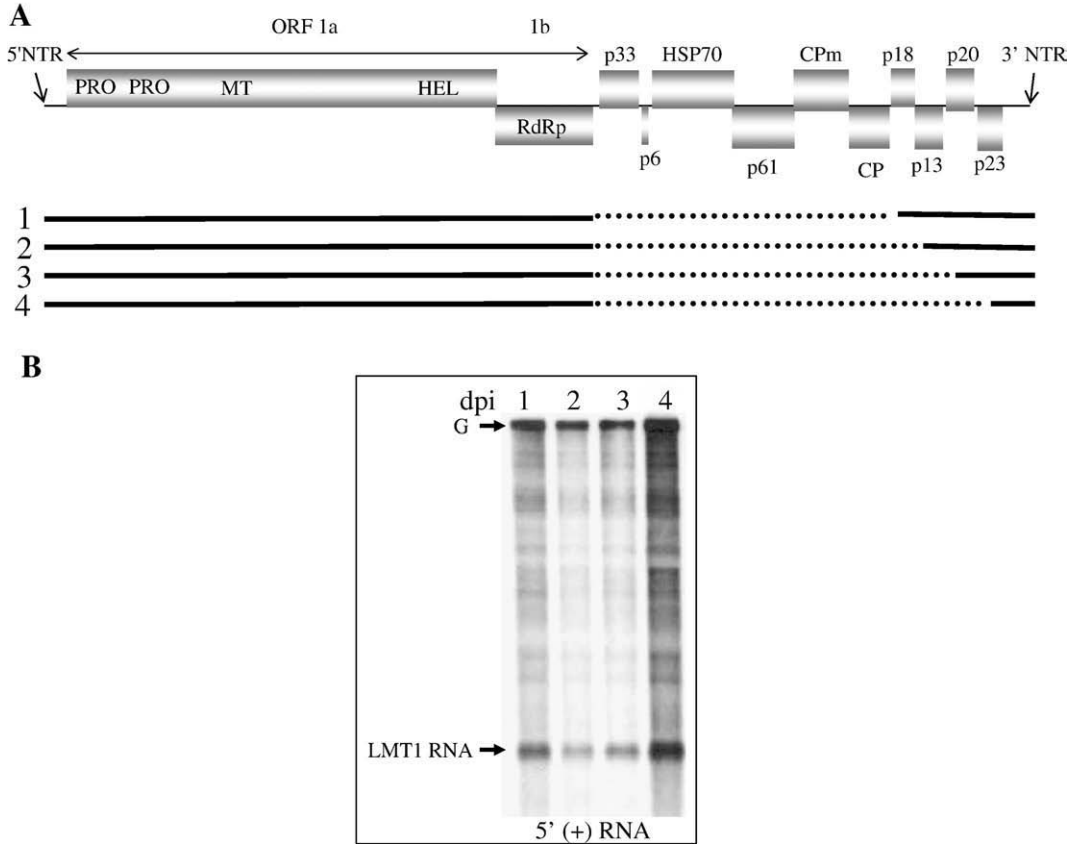

Fig. 3. Accumulation of LMT1 and LMT2 RNAs in protoplasts by CTV deletion mutants. (A) A diagrammatic representation of the CTV genome showing the positions of different genes and their putative protein products and mutant replicons of CTV containing deletion in 3′ genes; deletion of p33-CP, p33–p18, p33–p13 and p33–p20 (mutants 1–4, respectively); and deletion of p18, p18–p13 and p18–p20 in addition to p33 and p6 (mutants 6–8, respectively), and p33–p6 (mutant 5). The deleted regions are shown as broken lines. (B) Northern analysis of the total RNA isolated from *N. benthamiana* protoplasts at 4 dpi, transfected with *in vitro* generated transcripts from CTV mutants (lanes 1–4), the virions from the protoplasts were transfected to a new batch of protoplasts to increase concentration (lanes 5–8), and hybridized with 5′ positive-stranded RNA-specific digoxigenin labeled riboprobe. Arrows indicate the positions of the genomic (G) and LMT1 RNA and LMT2 RNA.

protoplasts were hybridized with 3′ plus-stranded RNA-specific (Fig. 5B) and 5′ plus-stranded RNA-specific (Fig. 5C) riboprobes, respectively. Double-stranded RNA of CTV was included in the Northern blot served as marker for 3′ sgRNAs and 5′ LMT1 RNA. All mutants replicated in protoplasts and accumulated 3′ sgRNA almost at similar levels (Fig. 5B), but mutants 1 (larger deletion of 24 nt) and 3 (altered nts in the predominant LMT2 RNA termination region) did not accumulate or accumulated barely detectable LMT2 RNA (Fig. 5C).

Mutations in the 5′ NTR that affect the encapsidation by CPm also affected the accumulation of LMT2 RNA

The origin of assembly (OA) of CPm was shown to encompass a conserved double hairpin structure in the 5′ NTR of the genomic RNA (Satyanarayana et al., 2004). Certain mutations in the stems and loops allowed continued replication, but abrogated the initiation of CPm encapsidation. To confirm the association of encapsidation by CPm with the accumulation of LMT2, mutations known to prevent encapsidation by CPm were introduced into the 5′ NTR of CTV-HSP70h-p61-CPm (Satyanarayana et al., 2004) and their ability to accumulate LMT2 RNA was examined. The changes included: 1) deletion of nts 1–9; 2) changes in the primary sequence with compensatory changes in the top stem of stem loop 1 (SL1); 3) similar changes to the bottom stem of stem loop 2 (SL2); and 4) contained deletion of the top loop (nts 69–79) of SL2 (Fig. 6A; the mutations are highlighted in the predicted secondary structure of the 5′ NTR). Mutations 1 and 4 were shown to prevent encapsidation by CPm while mutations 2 and 3 had no effect on encapsidation by CPm (Satyanarayana et al., 2004). *N. benthamiana* protoplasts were transfected with transcripts from these replicons and the total RNA and encapsidated RNA from the protoplasts were analyzed by Northern hybridizations using 3′ plus-stranded RNA-specific (Fig. 6B, lanes 1–4), and 5′ plus-stranded RNA-specific (Fig. 6C; right panel, lanes 1–4) riboprobes, respectively. The results indicated that all mutants replicated equally, as evidenced by the accumulation of the genomic and 3′-coterminal sgRNAs (Fig. 6B, indicated by arrows), while the RNA hybridized with the 5′ plus-stranded RNA-specific probe showed the accumulation of LMT2 RNA in protoplasts infected with mutants 2 and 3 and the absence of LMT2 RNA in protoplasts infected with mutants 1 and 4 (Fig. 6B). The total RNA from virion inoculated protoplasts (Fig. 6B, S) is included in the Northern blot as a positive control to show LMT1 and LMT2 RNAs. These data suggest that the mutations that prevented CPm encapsidation also interfered with the accumulation of LMT2 RNA.

Immuno-selection of virions with CPm antibodies

To confirm that the LMT2 RNA was encapsidated by CPm, we immuno-selected virions using CPm antiserum and examined the encapsidated RNA by Northern blot hybridization analysis. *N. benthamiana* protoplasts transfected with the crude sap containing wild type T36 virions were harvested at 4 dpi. A plus-stranded 5′-terminal sgRNA of ~650 nt resulted from the immuno-selected virions (Fig. 7, lane 2). No hybridization was observed in immuno-selection of mock inoculated protoplasts (Fig. 7; lane 4). Lane 1 represented the hybridization of the total RNA from the virion inoculated protoplasts,

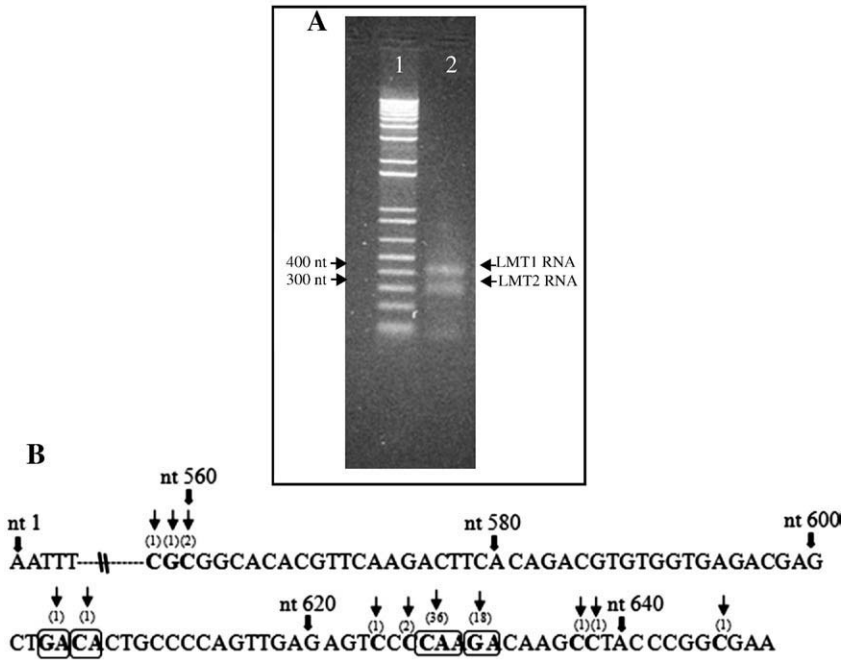

Fig. 4. (A) Analysis of the 3′ terminus of the LMT2 RNA. (A) Ethidium bromide stained 1% agarose gel showing the RT-PCR amplified product from the polyA tailed total RNA isolated from the CTV virion inoculated *N. benthamiana* protoplasts. Lane 1 contains DNA molecular weight markers. Lane 2 contains the DNA amplification products obtained using 5′ CTV specific and oligo (dT) primer pairs. Positions of the LMT1 and LMT2 RNA-specific RT-PCR amplified products (400 and 300 bp) are indicated by the arrows. (B) Nucleotide sequence of the genomic RNA near the 3′ terminus of the LMT2 RNA and the corresponding nucleotide numbers (thick arrows) are indicated. The number of clones obtained (in parentheses) with the corresponding 3′ terminal nucleotides (thin arrows) are also shown.

which showed the accumulation of LMT1 and LMT2 RNAs. The immuno-selection by CPm antiserum would select the full-length virions and the CPm encapsidated LMT2 RNA, and as expected the full-length genomic RNA was observed in the Northern blots (Fig. 7, lane 3). Because the amount of full-length virions immuno-selected by CPm antiserum were considerably less than the CPm encapsidated LMT2 RNAs, the Northern hybridization blot was exposed for a longer time to visualize the full-length genomic RNA (Fig. 7, lane 3). Total RNA from the virion inoculated protoplasts is included in the Northern blot to show the accumulation of LMT1 RNA and LMT 2 RNA (Fig. 7, lane 1).

LMT2 RNA accumulation was independent of LMT1 RNA accumulation

In the above experiments the accumulation of LMT2 RNA was always observed with the accumulation of LMT1 RNA. The LMT1 RNA accumulates in copious amounts and has no identified function. Since the LMT1 RNA contains the core origin of assembly for CPm, one possibility is that LMT1 RNA is encapsidated by CPm: since it would not have an origin of assembly for CP, it would be expected to stop encapsidation at ~650 nt. The unprotected ~100 nt could be degraded *in vivo* or during extraction to produce LMT2 RNA. To examine this possibility, we generated replicons that were defective in the accumulation of LMT1 RNA. We previously described mutations in the LMT1 RNA controller element (5′ CE) that abolish LMT1 RNA accumulation, with no significant effect on the replication of the replicon (Gowda et al., 2003). We showed above that the small replicon, CTV-HSP70h-p61-CPm, produced both LMT1 and LMT2 RNAs. We introduced into this replicon mutations in the LMT1 5′-CE that included changes in the primary sequence shown to prevent LMT1 RNA production with (CTV-HSP70h-p61-CPm/716 and CTV-HSP70h-p61-CPm/736) and without (CTV-HSP70h-p61-CPm/738) amino acid changes in the ORF 1a polyprotein (Fig. 8A, lanes 1–4). The parent replicon, CTV-HSP70h-p61-CPm, and the mutants replicated efficiently in *N. benthamiana* protoplasts as evidenced by the accumulation of plus-stranded genomic and 3′ coterminal sgRNAs (Fig. 8B, lanes 1–4). The parent replicon produced LMT1 RNA but the mutants failed to accumulate detectable amounts of LMT1 RNA as shown previously (data not shown; Gowda et al., 2003). In contrast, all of the replicons accumulated LMT2 RNA (Fig. 8C; lanes 1–4), demonstrating that the accumulation of LMT2 RNA was independent of LMT1 RNA accumulation.

No evidence that LMT2 RNA resulted from breakage of virions

Another possibility was that LMT 2 RNA results from breakage of virions at the transition between regions encapsidated by CPm and CP. The long flexuous virions are very labile and could be even more fragile at the interfaces between the coat proteins, which have been proposed to be occupied by a different structural protein, Hsp70h (Peremyslov et al., 2004). Thus, if virions break resulting in a 5′-coterminal sgRNA of ~650 nt, there should be a corresponding fragment containing the 3′ terminus but missing ~650 nt of the 5′ end. A large number of LMT2 molecules suggest that there would be an abundant amount of the 3′-terminal sgRNA. The difference between the 3′-terminal sgRNA (~18640 nt) and the genomic RNA (19293 nt) would be too small to be resolved as distinct bands during Northern blot hybridization analysis in agarose gels. Therefore, we used the 5′ RACE reaction with the total RNA from the protoplasts transfected with wild type virions followed by PCR with a 5′ primer downstream of the CPm–CP junction site to identify 5′ termini. The minus primer (complementary to nts 1564–1545) was used to synthesize the cDNA, and a nested minus primer (complementary to nts1465–1442) was used for amplification of the tailed cDNA in the 5′ RACE reaction. If LMT2 RNA was the result of the breakage of the genomic RNA, the 5′ RACE reaction would show the amplification of an ~815 nt product, in addition to a 1465 nt product from the full-length genomic RNA. However, the result of 5′ RACE reaction on a 1.5% agarose gel resolved only a product of 1465 nt, which resulted from unbroken virions (Fig. 9, lane 3). This result suggested that the 5′-coterminal LMT2 RNA that accumulated during

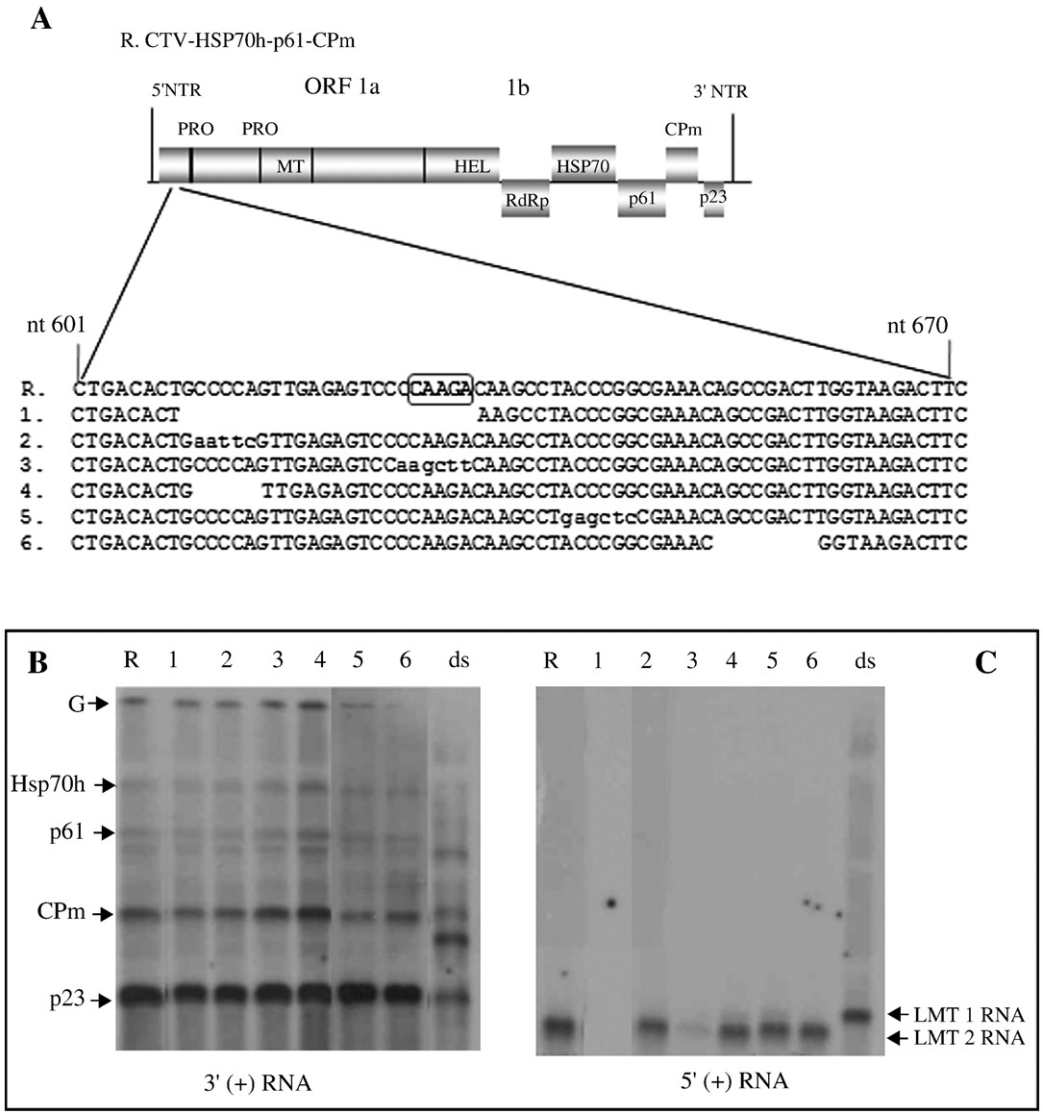

Fig. 5. Mutational analysis of the LMT2 RNA region in the replicon CTV-HSP70h-p61-CPm (R) (Satyanarayana et al., 2004). (A) Schematic representation of the CTV replicon showing the nucleotides 601 to 670 (located in the LMT2 RNA region). The sequences in the lowercase font represent changes in the nucleotide sequence. Blank regions indicate the deleted nucleotides. The boxed nucleotides indicate the nucleotides that contained most of the LMT2 RNA 3′ ends. (B) Northern analysis of the total RNA hybridized with the 3′ positive sense RNA-specific riboprobe. (C) RNA from the nucleocapsids hybridized with 5′ positive sense RNA-specific riboprobe. Arrows indicate the positions of the sgRNAs, LMT1 and LMT2 RNAs. ds indicates double stranded RNA control.

replication of CTV in protoplasts was not a product of the breakage of the genomic RNA in virions.

Discussion

CTV produces large amounts of two unusual RNAs, both of which are small, positive-stranded, and 5′ coterminal. Abundant production of both RNAs appears to be a general characteristic of different CTV strains. These RNAs were observed with the VT strain (Mawassi et al., 1995; Che et al., 2001) and later we observed them with our main laboratory strain, T36. We then examined citrus tissues infected with representatives of all of the strains that are available to us in Florida (we cannot import CTV isolates from other states or countries), and found that both RNAs were produced, although the ratios of the two sgRNAs varied among the different strains.

We previously found that production of the larger (~750 nt) LMT1 RNA was associated with an unusual controller element located in ORF1 near the 5′ terminus (Gowda et al., 2003). This controller element produces minute amounts of an ~18.5 kb sgRNA that is near genomic-length — it is minus only the 5′ 750 nt. We characterized this controller element by duplicating it near the 3′ terminus of a replicon with most of the 3′ genes deleted, which simplified characterization because promoter elements are much more active when moved closer to the 3′ terminus. In this position, this controller element initiated large amounts of the 3′-terminal sgRNA. Even though CTV has ten other controller elements for the 3′ genes, the 5′ controller element was different (Gowda et al., 2003). It appeared to function by promotion on the minus strand genomic RNA, because in contrast to the 3′ controller elements, which produce large amounts of complementary negative-stranded sgRNAs, the 5′ controller element did not produce a complementary negative strand. The LMT1 RNA apparently initiated at the 5′ terminus, probably the same as progeny genomic strands, but was terminated immediately downstream of this 5′ controller element (Ayllon et al., 2004), perhaps as a replicase complex attempting to initiate the 3′-terminal sgRNA. In contrast to the production of LMT1 RNA, we found no controller element activity associated with the region of the genome near nt 650, where LMT2 RNA terminates. This was particularly obvious when sequences near

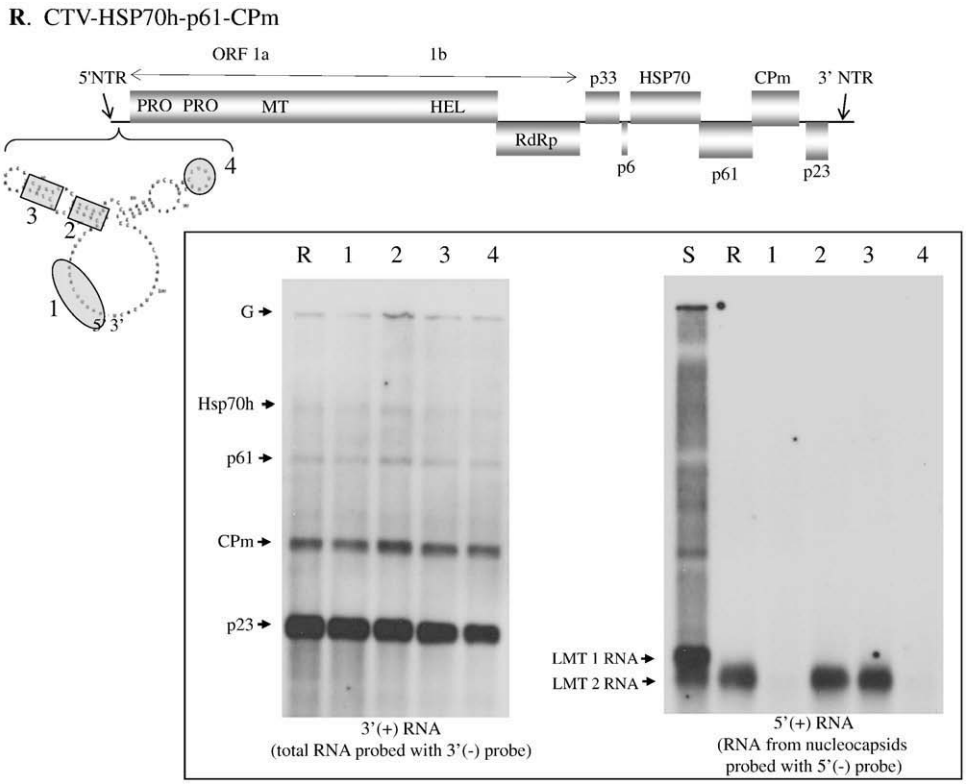

Fig. 6. Accumulation of genomic, 3′ and 5′ sgRNAs of CTV replicon, and its 5′ NTR mutants. (A) Schematic representation of the replicon, pCTV-HSP70-p61-CPm (Satyanarayana et al., 2004). The replicon, CTV-HSP70h-p61-CPm and mutants, 1–4, with mutations (shaded regions) introduced in the 5′ NTR of the replicon are also shown. (B) Northern analysis of the total RNA and the nucleocapsid RNA isolated from the protoplasts of *N. benthamiana* inoculated with SP6 RNA polymerase generated transcripts from the mutants, 1–4, and hybridized with 3′ positive-stranded RNA-specific and 5′ positive-stranded RNA-specific digoxigenin labeled riboprobes, respectively. The arrows indicate the positions of the genomic (G) and different 3′ and 5′ sgRNAs.

nt 650 were substituted into the mini-replicon near the 3′ terminus, the process that amplified the production by the LMT1 controller element (Gowda et al., 2003).

We first noticed the difference in production of the LMT RNAs from examining the replication of the mini-replicon. During replication in protoplasts, the mini-replicon produced large amounts of LMT1 RNA, but LMT2 RNA was not produced. When replication of the full-length virus was examined in protoplasts, both LMT RNAs were produced, but their kinetics of accumulations was different. The time course of accumulation of LMT1 RNA was similar to that of the genomic and sg mRNAs. In contrast, production of the LMT2 RNA occurred later. Thus, the time course of LMT2 RNA production was correlated more with virion assembly than RNA synthesis.

Not only was production of the LMT2 RNA correlated with the time course of virion assembly, its size was identical to the region of the genomic RNA that is encapsidated and protected by the minor coat protein. The origin of assembly for the minor coat protein is within the 5′ NTR, and in the presence of two assembly accessory proteins, Hsp70h and p61, encapsidation stops near nt 630 (Satyanarayana et al., 2004). Mutations or conditions that prevented virion assembly also prevented production of the LMT2 RNA. Thus, it appears that the LMT2 RNA is produced as a consequence of the CTV assembly process.

So, if LMT2 RNA is a product of assembly, how is this RNA produced? One obvious possibility was that virions are more fragile at the interface between the major and minor coat proteins and often break at this point. However, if so, we should have been able to find an equal amount of an RNA beginning at the break point and continuing to the 3′ end of the genomic RNA. We were unable to find such an RNA. Another possibility was that all 5′-terminal sgRNAs could be assembled by the minor coat protein. The most abundant 5′-terminal sgRNA is LMT1 RNA. It could be assembled by the minor coat protein up to the junction point at ~650 nt, and the remaining 100 nt could be unprotected and degraded. However, we found that LMT2 RNA production continued normally when production of LMT1 RNA was prevented, and mutants that prevented assembly did not markedly increase the amount of LMT1 RNA. Although the origin of assembly of

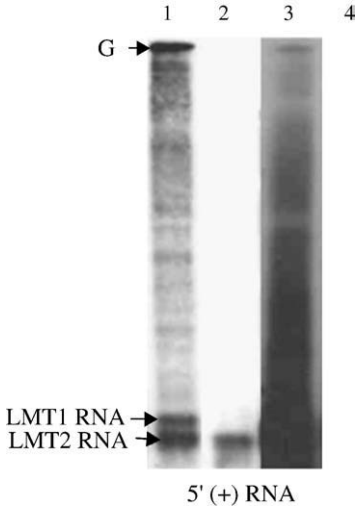

Fig. 7. Immunoselection of virions and encapsidated LMT2 sgRNA. Northern analysis of the total RNA (lane 1) isolated from *N. benthamiana* protoplasts transfected with CTV T36 virions (lane 1) and the RNA from the virions generated in *N. benthamiana* protoplasts and immunoselected using magnetic beads coated with CPm specific antiserum (lanes 2 and 3) with the 5′-plus RNA-specific riboprobe. The blot was hybridized with 5′ positive-stranded RNA-specific riboprobe. Lane 4 represents similarly processed mock inoculated protoplasts. Lane 3 represents over exposed lane 2. Positions of the genomic (G) and LMT sgRNAs are shown by arrows.

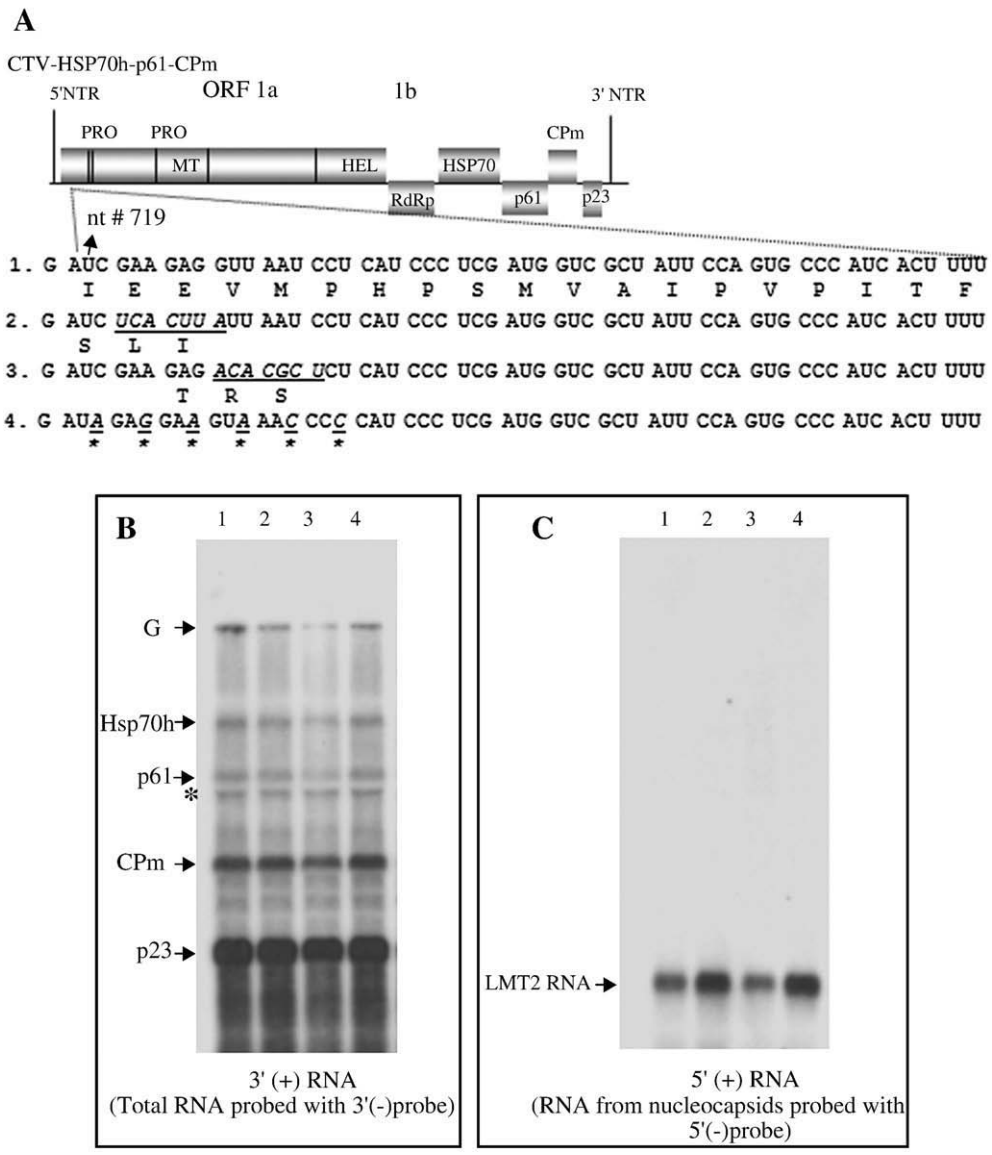

Fig. 8. (A) Mutational analysis of the 5′ TR element in the replicon CTV-HSP70h-p61-CPm (Satyanarayana et al., 2004). Schematic representation of the CTV replicon showing the nucleotides 719–776 (located in the LMT1 RNA TR region) in triplets and the amino acids they encode (below the nucleotide sequence). The underlined sequences in italics represent changes in the nucleotide sequence and corresponding change of amino acids in the mutants 2–4. The asterisks indicate changed nucleotide sequence (underlined) in the wobble position that did not result in an amino acid change. (B) Northern analysis of the total RNA and RNA from the nucleocapsids (bottom panel, right) isolated from *N. benthamiana* transfected protoplasts with the SP6 RNA polymerase generated transcripts. The blots were hybridized with 3′ positive-stranded RNA-specific and 5′ positive-stranded RNA-specific riboprobe.

CPm is in the 5′ NTR, regions approximately 0.5–1.0 kb downstream also affect assembly (S. Tatineni, unpublished). Thus, the LMT1 RNA may not be capable of assembly. However, there are longer 5′ terminal sgRNAs that contain the downstream sequences associated with initiation of assembly. Each of the ten 3′ genes produces small amounts of a 5′-terminal RNA that terminates upstream of the controller element. Each of these sgRNA would have the origin of assembly for the minor coat protein. We have not mapped the origin of assembly for the major coat protein, but preliminary experiments have suggested that it is somewhere near the 3′ end (S. Tatineni, unpublished). If these 5′-terminal positive-stranded sgRNAs lack the origin of assembly for the major coat protein, but retain the origin of assembly for the minor coat protein, the 5′ 630 nt could be encapsidated and protected, but the rest of the RNA could be degraded resulting in LMT2 RNA. Even though we are not sure of the origin of the RNAs, the abundance of evidence suggests that the LMT2 RNA is processed by the assembly process. As far as we are aware, this is the first evidence of a viral RNA processed by the assembly mechanism.

We still do not know whether there is a function for the LMT2 RNA. It appears not to be needed for replication. Its production is correlated with assembly, but there is no evidence that it is needed for virion assembly. So far, we have identified 33 sgRNAs produced during CTV replication. These include for each of the ten 3′ genes a sgRNA messenger RNA, its negative-stranded complement, and the 5′-terminal RNA that terminates near the controller element, which adds to 30 sgRNAs, plus LMT1 RNA and its corresponding 3′-terminal sgRNA, and LMT2 RNA. In fact, we have identified functions for none of the other eleven 5′-terminal sgRNA. It is possible that all of these sgRNAs are excess RNAs with no functions — that they could be considered an inefficiency cost of this replication strategy. It should be kept in mind that in order to survive in a host, viruses must adapt their replication and gene expression strategies to the molecular rules of the host. For example, the polyprotein strategies of viruses that produce the same amounts of each protein even though some proteins are needed in larger amounts than others, have to produce excess amounts of some proteins. The production of excess amounts of some

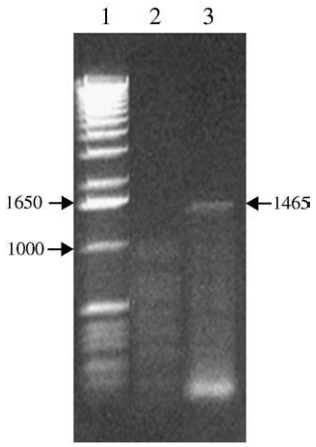

Fig. 9. Determination of integrity of the genomic RNA by 5′ RACE. Agarose gel electrophoresis of the 5′ RACE reaction product amplified by 5′ RACE anchor primer and CTV specific minus oligonucleotide (lane 3) from the total RNA isolated from *N. benthamiana* protoplasts transfected with the virions from the bark tissue of CTV infected citrus. Lane 1, the DNA size markers; lane 2, total RNA from vector transfected protoplasts used for reverse transcription, anchor ligation and amplification. The arrows indicate the size of the 5′ RACE product (lane 3) and the size of the bands of the DNA molecular weight markers (lane 1).

proteins is the cost of that strategy. It is possible that the cost of the closterovirus replication strategy is the production of excess RNAs.

Materials and methods

Virus isolates and transfection of N. benthamiana protoplasts

Isolates of CTV used in this investigation were maintained under greenhouse conditions on Alemow (*C. macrophylla* Wester) or Valencia sweet orange on sour orange root stocks at the University of Florida-CREC, Lake Alfred. The preparation of sap from the bark tissue of CTV infected citrus for use as a source of virions for transfection of protoplasts, preparation of mesophyll protoplasts of *N. benthamiana*, synthesis of SP6 RNA polymerase-derived transcripts from CTV replicons and mutants, and polyethylene-glycol mediated transfection of protoplasts were carried out as outlined earlier (Navas-Castillo et al., 1997, Satyanarayana et al., 1999).

Construction of mutants

The full-length cDNA clone of CTV, CTV9R (Satyanarayana et al., 1999; 2003), was used as the parent plasmid in the design of primers in the generation of replicons and their mutants. The replicons; CTV-Δp33–p20, and CTV-Δp33–p18, CTV-Δp33–p23 have been described earlier (Satyanarayana et al., 2002). The mutants that contained deletion of the p33 gene with additional deletions of p13, p13–p20 or p13–p23 genes have been described (Satyanarayana et al., 2000). Mutations in the LMT1 controller region, and mutations in the 5′ NTR in the replicon, CTV-HSP70h-p61-CPm, were introduced using the primers and the protocols described earlier (Gowda et al., 2003; Gowda et al., 2003b). Mutations in the LMT2 RNA region were carried out by overlap extension PCR technique (Ho et al., 1989) using CTV specific primers containing in-frame deletions of 24 (nts 609–632), 6 (nts 610–615) and 9 (nts 651–659) nts in mutants 1, 4 and 6, respectively. Similarly, nucleotide changes were introduced in mutants 2, 3 and 5.

Northern blot analysis of the total and encapsidated RNA from nucleocapsids

Isolation of the total RNA from the sap or transcript transfected *N. benthamiana* protoplasts, isolation of nucleocapsids from transfected protoplasts, isolation of RNA from the nucleocapsids, and subsequent hybridizations using 3′ plus-stranded and 5′ plus- and/or minus-stranded RNA-specific digoxigenin labeled riboprobes were carried out as described earlier (Satyanarayana et al., 1999, 2004).

Determination of the 3′ end of LMT2 RNA

Total RNA extracted from *N. benthamiana* protoplasts infected with T36 virions was used to determine the 3′ end of RNA. Total RNA was denatured at 90 °C and 3′ polyadenylated using yeast polyA polymerase (US Biochemicals), reverse transcribed using SuperScript II (Invitrogen) using an oligo (dT) primer M-111 (5′ GGTCTCGAG(T) 18). The RT-PCR product was amplified with the primer M-111 and a CTV specific positive sense oligonucleotide (5′ CAGATGCGTTGA-TAACGGG, corresponding to nts 383–401). The amplified product corresponding to LMT2 RNA was gene cleaned, cloned into pGEM-T Easy vector (Promega) and the clones were sequenced at the Interdisciplinary Center for Biotechnology Research DNA sequencing core facility of the University of Florida (Gainesville, FL).

Immuno-selection of virions

Virions produced in *N. benthamiana* protoplasts inoculated with the crude sap from the bark tissue of citrus infected with T36 isolate of CTV were immuno-selected using the polyclonal antiserum developed against CPm. The virions were extracted from the infected protoplasts by the procedure described earlier (Satyanarayana et al., 2004), and immuno-selected on CPm antiserum coated magnetic beads as outlined earlier (Lee et al., 2005). Mock-infection of protoplasts represented transfection of protoplasts with transcripts generated using the empty vector.

5′ RACE

Total RNA isolated from the protoplasts transfected with the sap from the bark tissue of *C. macrophylla* infected by the T36 isolate of CTV was used for the amplification of CTV specific 5′ coterminal RNAs using the 5′ RACE kit (Invitrogen). CTV specific minus-strand primer, (5′-CCACCAACTAAATGATGGTT, complementary to nts 1564–1545), was used for the synthesis of first strand cDNA and tailed with nucleotide clamp sequences following manufacturer's recommendations. The tailed cDNA was used for amplification of the 5′ RACE product using the plus-stranded primer supplied by the manufacturer and a nested CTV specific minus-stranded primer (5′-GAACCTTAA-GAGAGTCGCGACCG, complementary to nts 1465–1442).

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