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Transcriptional Strategy of Closteroviruses: Mapping the 5′ Termini of the Citrus Tristeza Virus Subgenomic RNAs†

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Citrus tristeza virus (CTV) induces formation of a nested set of at least nine 3′ coterminal subgenomic RNAs (sgRNAs) in infected tissue. The organization and expression of the 19,296-nucleotide (nt) CTV genome resembles that of coronaviruses, with polyprotein processing, translational frameshifting, and multiple sgRNA formation, but phylogenetically the CTV polymerase, like polymerases of other closteroviruses, belongs to the Sindbis virus-like lineage of RNA virus polymerases. Both positive-strand RNA virus supergroups, coronaviruses and Sindbis-like viruses, utilize different mechanisms of transcription. To address the mechanism of CTV transcription, 5′ termini for the two most abundant sgRNAs, 1.5 and 0.9 kb, respectively, were mapped by runoff reverse transcription. The two sgRNAs were demonstrated to have 48- and 38-nt 5′ untranslated regions (5′-UTRs), respectively. The 5′-UTR for the 1.5-kb RNA was cloned, sequenced, and demonstrated to be colinear with the 48-nt genomic sequence upstream of the initiator codon of the respective open reading frame 10, i.e., to be of continuous template origin. The data obtained suggest that the sgRNA transcription of CTV is dissimilar from the coronavirus transcription and consistent with the transcriptional mechanism of other Sindbis-like viruses. Thus, the Sindbis virus-like mechanism of transcription of the positive-strand RNA genomes might be successfully utilized by the closterovirus genome of up to 19.3 kb with multiple sgRNAs.

Citrus tristeza virus (CTV) is a member of the closterovirus group, which is composed of filamentous, insect-transmitted plant viruses having messenger-sense single-stranded RNA (ssRNA) genomes (4, 5, 11). The remarkably large CTV genome, 19,296 nucleotides (nt), encodes 12 open reading frames (ORFs) (13), and its mode of expression includes proteolytic processing of the viral polyprotein, translational frameshifting, and formation of at least nine 3′-coterminal subgenomic RNAs (sgRNAs) ranging in size from 0.9 kb to more than 8 kb (12). In CTV-infected plants, a set of double-stranded RNAs (dsRNAs), "replicative forms," is present that correspond to the genomic RNA and nine sgRNAs (12). The overall genome organization of CTV (13, 23), as well as those of other characterized closteroviruses, beet yellows virus (BYV) (2), lettuce infectious yellows virus (16), and beet yellow stunt virus (BYSV), (15), resembles those of animal coronaviruses having genomes of up to 31 kb (18). Both groups of viruses encode a single large polyprotein translated directly from the genomic RNA, and the C terminus of the polyprotein is expressed via a translational frameshifting mechanism, while 3′-proximal genes are expressed through formation of a nested set of multiple subgenomic RNAs (cf. references 11 and 18). The unique feature of coronaviruses is an unusual mechanism of sgRNA transcription, a discontinuous leader-primed transcription resulting in sgRNAs with identical 5′-terminal leader sequences of about 70 nt. The leader sequence is a derivative of the 5′ terminus of the genomic RNA, and in sgRNAs is not colinear with the sequences upstream of the respective 5′-proximal ORFs. This unique transcription mechanism is believed to be used by coronaviruses to stably maintain and express their unusually large, single-component RNA genomes, which are up to 31 kb in size (reviewed in reference 18).

Phylogenetic reconstructions, based on amino acid sequences of RNA-dependent RNA polymerases and other replication-associated proteins, placed closteroviruses into the alphavirus-like (or Sindbis virus-like) supergroup of positive-strand RNA viruses, in contrast to coronaviruses, which grouped with picorna-like viruses (cf. references 11 and 17). The mechanism of transcription in alpha-like viruses is different from the coronavirus transcription, because it involves initiation of the 5′ ends of plus-strand sense sgRNAs on internal regions of the full-length genomic minus strand called subgenomic promoters (19, 24, 26). The genomes of alpha-like viruses are generally much shorter than those of closteroviruses, with the number of sgRNAs rarely exceeding two or three (cf. references 7 and 9), which might, in principle, imply some inherent problems in transcription, i.e., temporal or quantitative regulation of gene expression of large genomes (more than six sgRNAs) with this alphavirus-like transcription mechanism (11). To address the transcriptional strategy of closteroviruses, we initiated mapping of the 5′ termini of sgRNAs of CTV.

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Two genetically distinct Florida CTV isolates, T3 and T36, with different biological characteristics (11a, 25) were maintained under greenhouse conditions in Mexican lime (*Citrus aurantifolia* (Christm.) Swing) or sweet orange (*Citrus sinensis* (L.) Osb.) plants. Total RNAs were isolated from the young sweet orange bark tissue of infected plants by the guanidine-HCl procedure of Logemann et al. (20) with modifications described previously (12). Separation of the virus-specific RNAs in agarose gels, and Northern blot hybridizations with the digoxigenin labelling kit (Boehringer Mannheim, Indianapolis, Ind.) were performed essentially as described previously (12).

The two 3′-terminal ORFs of CTV, ORF 10 and ORF 11, encode proteins with sizes of 20 and 23 kDa, respectively, and

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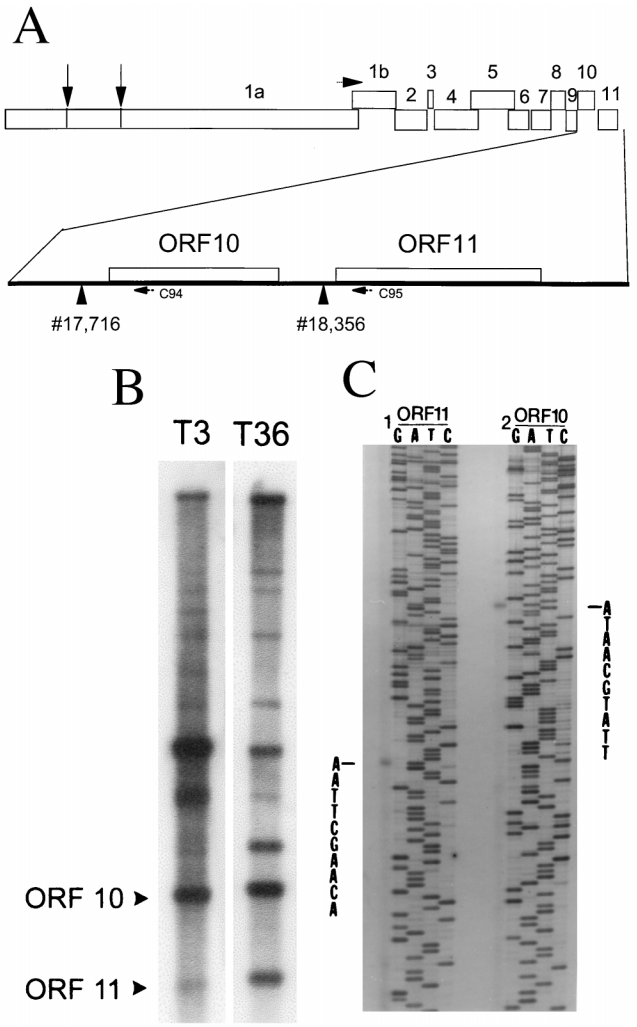

FIG. 1. (A) Schematic diagram of the CTV genome, based on data from reference 13, showing the 3′-terminal ORFs 10 and 11 in detail. Boxes indicate ORFs with the respective numbers, the vertical arrows mark the protease cleavage sites in the polyprotein, the horizontal arrow marks the translational frameshift between ORFs 1a and 1b, and the upward arrowheads on the enlarged 3′-terminal diagram indicate the mapped transcription start sites. The positions of the C94 and C95 primers are also indicated on the enlarged 3′-terminal diagram. (B) Northern blot hybridization analysis of the virus-specific dsRNAs. The isolate source is indicated at the top of each lane. The two lanes represent similar but separate agarose electrophoresis and hybridization events. The positions of the two sgRNAs corresponding to ORFs 10 and 11 are indicated by arrows. (C) Analysis of the runoff reverse transcription products in a 6% polyacrylamide gel side by side with the sequencing ladders obtained on the cloned fragments of the CTV genome. Sequencing reactions were performed with the same primers used for the runoff transcription reaction: ORF 11, primer C95 (lane 1); and ORF 10, primer C94 (lane 2). The respective sequences of the 5′-UTRs starting with the first nucleotide of the sgRNA are presented to the left (ORF 11) and to the right (ORF 10): the 5′-to-3′ direction is from top to bottom.

are thought to be expressed from the highly abundant 1.5- and 0.9-kb sgRNAs, respectively (12). No specific functions have yet been assigned to either protein: the ORF 10-encoded 20-kDa protein has similarly sized and positioned homologs in genomes of BYV and BYSV (15), and the ORF 11-encoded 23-kDa protein might possess RNA-binding properties and so far is unique among characterized closteroviruses (11). Both ORFs have extended nontranslated regions upstream of the initiator codons (Fig. 1A), which were suggested to contain the respective subgenomic promoters (12). Figure 1B illustrates the relative abundance of the two sgRNAs in the infected sweet orange bark tissue for both CTV isolates T3 and T36, as revealed by Northern hybridization. The RNAs from infected plant tissue were hybridized with the probe synthesized on the dsDNA fragment containing the entire ORF 11, with Klenow fragment, random hexamers, and the digoxigenin labeling kit (Boehringer-Mannheim) as described previously (12). Runoff transcriptions were performed as previously described (13). Briefly, two primers, C94 (5′-CGTCCATCACTTCCGAAGA-3′) and C95 (5′-GTTCGACGTCAGTGGTCGCTGTGTT-3′), complementary to positions 17,853 to 17,872 and 18,448 to 18,473 of the CTV genome, respectively, were 5′ labelled with 10 pmol of each primer, 150 μCi of [γ-^{32}P]ATP (New England Nuclear [6,000 Ci/mmol]), and polynucleotide kinase (Promega) in a 70 mM Tris-HCl (pH 7.6)–10 mM $MgCl_2$–5 mM dithiothreitol (DTT) buffer for 30 min at 37°C. Labelled primers were added to the total RNAs isolated from the CTV-infected plants, denatured with 20 mM methylmercury hydroxide, and annealed for 20 min at 60°C in 16 mM Tris-HCl buffer containing 16 mM KCl, 3 mM $MgCl_2$, 3 mM DTT, and 0.2 mM spermidine. Extensions were performed in 50 mM Tris-HCl (pH 8.3)–50 mM KCl–10 mM $MgCl_2$–0.5 mM spermidine–10 mM DTT–10 U of RNasin (Promega)–125 μM each deoxynucleoside triphosphate–10 U of avian myeloblastosis virus reverse transcriptase (Promega) at 42°C for 1 h. The synthesized transcripts were analyzed on sequencing gels side by side with sequencing reactions performed with the same primers and respective fragments of the CTV genome cloned into pBluescript SK (Stratagene).

The primer-extension experiments performed with total RNA isolated from the CTV-infected sweet orange bark tissue with primer C95 complementary to the coding region of ORF 11, 78 nt downstream of the AUG codon, gave a strong single band indicating a 38-nt leader upstream of the AUG codon (Fig. 1C, lane 1). The same experiments, performed with primer C94 complementary to the coding region of ORF 10, 109 nt downstream of the initiator codon, produced a single band indicating a 48-nt leader upstream of the AUG codon (Fig. 1C, lane 2). Assuming colinearity of these leader sequences to the CTV genome sequence upstream of the respective ORFs, in both cases, the transcription start site corresponded to an A, suggesting that both sgRNAs, like the genomic CTV RNA (13), start with the A (Fig. 1C).

To determine the sequence of the 5′-UTR of the ORF 10-encoding 1.5-kb sgRNA directly, we amplified the 5′-terminal region of this sgRNA from both T3 and T36 isolates by using primer C94 and an oligo(dT)-containing primer and cloned and sequenced it. Total dsRNA isolated from the CTV-infected plants was used as a template for the reverse transcription-PCR. dsRNAs were isolated according to the procedure of Valverde et al. (27). The 5′-UTRs of the 1.5-kb sgRNA species for the CTV isolates T36 and T3 were cloned by the dsRNA polyadenylation technique utilized previously for the cloning of the CTV genome 5′ and 3′ termini (13). Briefly, dsRNAs isolated from bark tissue of T36- or T3-infected sweet orange plants were denatured with 20 mM mercury hydroxide, and the 3′ termini of both plus and minus strands were polyadenylated for 20 min at 30°C with the yeast poly(A) polymerase (U.S. Biochemicals) according to the manufacturer's instructions. Following the phenol-chloroform extraction, the RNAs were reverse transcribed with Superscript II (Bethesda Research Laboratories) and random hexamers for priming (Promega). The resulting cDNAs were extracted with phenol-chloroform and used in PCR with two primers, M111 [5′-GG TCTCGAG$(T)_{18}$-3′] as a plus-strand primer and C94 as a minus-strand primer. Amplification was by 30 cycles of 1 min at

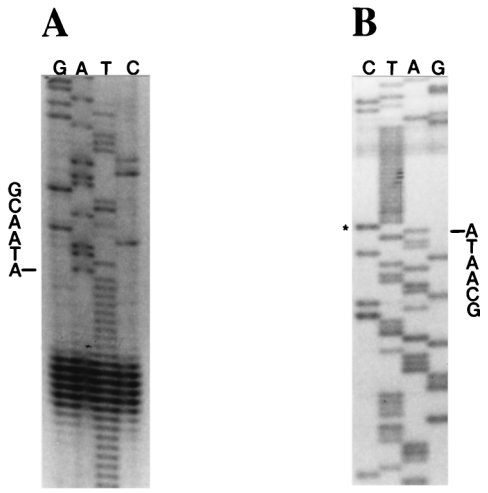

FIG. 2. Sequencing analysis of the 5′-UTRs for the CTV 1.5-kb sgRNA from isolates T3 (A) and T36 (B) upstream of ORF 10. Reading (5′ to 3′) is from the bottom to the top for T3 (A) and from the top to the bottom for T36 (B). The asterisk on panel B marks a nontemplate C, presumably a complement of an extra G present in the minus strand of the subgenomic dsRNA. (C) Alignment of the 5′-UTRs for the 1.5-kb sgRNA from isolates T3 and T36. Identical positions are indicated by dashes, the sgRNA start site is marked by an arrow, and the initiator codon is underlined.

94°C, 1 min at 50°C, and 1 min at 72°C, followed by the final extension at 72°C for 10 min. The resulting PCR products were treated with T4 DNA polymerase to create blunt ends, digested with *Xho*I restriction enzyme, and cloned into pBluescript (Stratagene) between the *Xho*I and *Eco*RV sites.

The virus-specific inserts were sequenced with the Sequenase 2.0 kit (U.S. Biochemicals) and an outside, plasmid primer in the case of the T3-specific sequence and the C94 primer in the case of the T36-specific insert, according to the manufacturer's instructions. This direct cloning and sequencing gave the exactly matching transcription start site for the 1.5-kb sgRNA for two CTV isolates, T3 (Fig. 2A) and a genetically distinct, prototype isolate, T36 (13) (Fig. 2B), and matched the transcription start site determined by an independent runoff transcription technique with the ssRNA templates (Fig. 1C). The 5′-UTRs for the same sgRNA from two CTV isolates apparently had identical sizes and transcription start points, although the nucleotide sequence difference in this region between T36 and T3 did not seem negligible: 5 substitutions per 48 nt, or more than 10% (Fig. 2B). This complete correspondence between the runoff data, and the direct cloning and sequencing of the 5′-UTR apparently provided evidence of colinearity of the 5′-UTR of the 1.5-kb sgRNA and the respective genomic sequence. This colinearity (Fig. 1 and 2) indicated that the transcription of the 1.5-kb sgRNA, and perhaps that of all other CTV sgRNAs, occurred by a mechanism dissimilar from the coronavirus transcription, which produces sgRNAs having identical, nontemplate 5′ termini derived from the 5′ terminus of the virion genomic RNA (18). Two sgRNAs of another closterovirus, BYV, coding for the coat protein and a 24-kDa diverged duplicate of the coat protein were reported to have 52- and 105-nt-long 5′-UTRs (1).

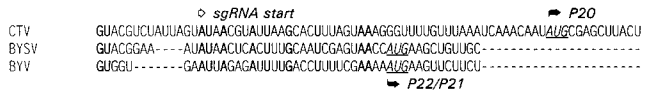

FIG. 3. Alignment of the nucleotide sequences preceding ORF 10 (CTV), ORF 9 (BYSV), and ORF 8 (BYV) generated by the MACAW program. The 1.5-kb CTV sgRNA start site is indicated by an open arrow; initiator codons for P20 (CTV), P22 (BYSV), and P21 (BYV) are italicized and underlined.

However, colinearity of these UTRs to the corresponding genomic sequences was not addressed, and, thus, the distinction between alphavirus and coronavirus types of transcription could not be made.

Although the 5′-UTR of the ORF 11-encoding 0.9-kb sgRNA was not sequenced directly, based on the 1.5-kb sgRNA data, we might assume that it is also colinear to the sequence upstream of the respective AUG codon. The 48- and 38-nt 5′-UTRs upstream of ORFs 10 and, presumably, 11 are very AT rich: 36 A+T bases out of 48 and 26 A+T bases out of 38, respectively. Inspection of the 5′-UTRs of the 1.5- and 0.9-kb sgRNAs suggests that they probably lack extensive secondary structure (data not shown). Analysis of the 200-nt region upstream of the ORF 10 and 11 initiator codons with the MACAW program (3) did not reveal statistically significant similarities to regions preceding other CTV ORFs, thus indicating the possible existence of different transcription signals. On the other hand, analysis of the sequence preceding ORF 10 and sequences preceding related genes of two other closteroviruses, BYSV and BYV, revealed an interesting resemblance (Fig. 3). In BYSV and BYV genomes, the respective ORFs 9 and 8 encode protein products with sizes of 22 and 21 kDa, respectively, which demonstrated modest sequence similarity to the ORF 10-encoded 20-kDa protein of CTV (15). The alignment of the three sequences (Fig. 3), although not significant statistically, indicates possible conservation of some elements of the respective UTRs, especially between CTV and BYSV. For CTV and BYSV, a 6-nt block which includes the start of the CTV sgRNA seems to be conserved. This finding may suggest that the corresponding BYSV sgRNA also starts with the A; however, this resemblance in the UTRs could be accidental, and the actual sgRNA start site needs to be determined experimentally.

The 3′ end of the minus strand for the double-stranded equivalent of the ORF 10 sgRNA from the T36 isolate had an extra G which had no complement in the genomic sequence. In contrast, the T3 isolate had no extra G (Fig. 2). The presence of extra nucleotides at 3′ termini of the replicative dsRNA forms of positive-strand viruses is believed to be a feature necessary for RNA replication. This hypothesis is supported by the fact that the genome-size replicative forms of cucumber mosaic virus (6, 28), potato virus X (10), and barley stripe mosaic virus (8) had an extra G at the 3′ termini of the minus strand, while double-stranded sgRNA equivalents did not. The genome-size dsRNA replicative form of CTV (prototype isolate T36) was also demonstrated to have an extra G at the 3′ terminus of the minus strand (13). Whether the presence of an extra G at the 3′ terminus of the minus strand in the ORF 10 sgRNA double-stranded equivalent plays a role in the CTV replication remains to be determined. The presence of an extra G was also recorded for some ORF 11 sgRNA double-stranded equivalents in another CTV isolate, VT (29), where it is perhaps involved in recombination and defective RNA formation (21, 22).

Positive-strand alpha-like viruses employ a transcription strategy which utilizes minus-strand templates for the internal

initiation of the sgRNA synthesis, resulting in messenger RNAs with different 5′ termini which are colinear to the corresponding genomic sequence (19, 24). Transcription in the coronaviruses, on the other hand, utilizes the so-called leader-primed mechanism resulting in production of sgRNAs with identical 5′ termini derived from the 5′ end of the genomic RNA (18). The latter transcriptional strategy was hypothesized to evolve as a consequence of the increased genome length that caused some inherent problems for the alphavirus-like mode of transcription in large positive-strand RNA genomes (11, 18). Indeed, most of the alpha-like viruses usually have only two or three sgRNAs derived from the same genomic RNA (7, 9). However, the data presented here indicate that the alphavirus-like mechanism of transcription and regulation of gene expression may be successfully employed by a virus with a genome as large as 19.3 kb and may produce multiple, at least nine, sgRNAs, i.e. even more than are produced by the coronaviruses. This observation suggests that the evolutionary pressure in favor of the coronavirus type of transcription comes from forces in addition to the mere increase in genome length and gene number.

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REFERENCES

1. **Agranovsky, A. A., R. Koenig, E. Maiss, V. P. Boyko, R. Casper, and J. G. Atabekov.** 1994. Expression of the beet yellows closterovirus capsid protein and p24, a capsid protein homologue, *in vitro* and *in vivo*. J. Gen. Virol. **75:**1431–1439.
2. **Agranovsky, A. A., E. V. Koonin, V. P. Boyko, E. Maiss, R. Frötschl, N. A. Lunina, and J. G. Atabekov.** 1994. Beet yellows closterovirus: complete genome structure and identification of a leader papain-like thiol protease. Virology **198:**311–324.
3. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
4. **Bar-Joseph, M., S. M. Garnsey, and D. Gonsalves.** 1979. The closteroviruses: a distinct group of elongated plant viruses. Adv. Virus Res. **25:**93–168.
5. **Candresse, T., and G. P. Martelli.** 1995. Genus *Closterovirus*, p. 461–464. *In* F. A. Murphy, C. M. Fauquet, D. H. L. Bishop, S. A. Ghabrial, A. W. Jarvis, G. P. Martelli, M. A. Mayo, and M. D. Summers (ed.), Virus taxonomy. Sixth Report of the International Committee on Taxonomy of Viruses. Springer-Verlag, Vienna, Austria.
6. **Collmer, C. W., and J. M. Kaper.** 1985. Double-stranded RNAs of cucumber mosaic virus and its satellite contain an unpaired terminal guanosine: implications for replication. Virology **145:**249–259.
7. **Dawson, W. O.** 1992. Tobamovirus-plant interactions. Virology **186:**359–367.
8. **Dolja, V. V., and J. G. Atabekov.** 1987. The structure of barley stripe mosaic virus double-stranded RNAs. FEBS Lett. **214:**313–316.
9. **Dolja, V. V., and J. C. Carrington.** 1992. Evolution of positive-strand RNA viruses. Semin. Virol. **3:**315–326.
10. **Dolja, V. V., D. P. Grama, S. U. Morozov, and J. G. Atabekov.** 1987. Potato virus X-related single- and double-stranded RNAs: characterization and identification of terminal structures. FEBS Lett. **214:**308–312.
11. **Dolja, V. V., A. V. Karasev, and E. V. Koonin.** 1994. Molecular biology and evolution of closteroviruses: sophisticated build-up of large RNA genomes. Annu. Rev. Phytopathol. **32:**261–285.

11a. **Hilf, M. E., A. V. Karasev, W. O. Dawson, and S. M. Garnsey.** Unpublished observations.

12. **Hilf, M. E., A. V. Karasev, H. R. Pappu, D. J. Gumpf, C. L. Niblett, and S. M. Garnsey.** 1995. Characterization of citrus tristeza virus subgenomic RNAs in infected tissue. Virology **208:**576–582.
13. **Karasev, A. V., V. P. Boyko, S. Gowda, O. V. Nikolaeva, M. E. Hilf, E. V. Koonin, C. L. Niblett, K. Cline, D. J. Gumpf, R. F. Lee, S. M. Garnsey, D. J. Lewandowski, and W. O. Dawson.** 1995. Complete sequence of the citrus tristeza virus RNA genome. Virology **208:**511–520.
14. **Karasev, A. V., M. E. Hilf, S. M. Garnsey, and W. O. Dawson.** 1995. Transcriptional strategy of the citrus tristeza virus: mapping 5′ termini of the subgenomic RNAs, abstr. W4-9. *In* Abstracts of the 14th Annual Meeting of the American Society for Virology, Austin, Tex., 8 to 12 July 1995.
15. **Karasev, A. V., O. V. Nikolaeva, A. R. Mushegian, R. F. Lee, and W. O. Dawson.** 1996. Organization of the 3′ terminal half of beet yellow stunt virus genome and implications for the evolution of closteroviruses. Virology **221:** 199–207.
16. **Klaassen, V. A., M. L. Boeshore, E. V. Koonin, T. Tian, and B. W. Falk.** 1995. Genome structure and phylogenetic analysis of lettuce infectious yellows virus, a whitefly-transmitted, bipartite closterovirus. Virology **208:**99–110.
17. **Koonin, E. V., and V. V. Dolja.** 1993. Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. Crit. Rev. Biochem. Mol. Biol. **28:**375–430.
18. **Lai, M. M. C.** 1990. Coronavirus: organization, replication and expression of genome. Annu. Rev. Microbiol. **44:**303–333.
19. **Levis, R., S. Schlesinger, and H. V. Huang.** 1990. The promoter for Sindbis virus RNA-dependent subgenomic RNA transcription. J. Virol. **64:**1726–1733.
20. **Logemann, J., J. Schell, and L. Willmitzer.** 1987. Improved method for the isolation of RNA from plant tissues. Anal. Biochem. **163:**16–20.
21. **Mawassi, M., A. V. Karasev, E. Mietkiewska, R. Gafny, R. F. Lee, W. O. Dawson, and M. Bar-Joseph.** 1995. Defective RNA molecules associated with the citrus tristeza virus. Virology **208:**383–387.
22. **Mawassi, M., E. Mietkiewska, M. E. Hilf, L. Ashoulin, A. V. Karasev, R. Gafny, R. F. Lee, S. M. Garnsey, W. O. Dawson, and M. Bar-Joseph.** 1995. Multiple species of defective RNAs in plants infected with citrus tristeza virus. Virology **214:**264–268.
23. **Mawassi, M., E. Mitkiewska, R. Gofman, G. Yang, and M. Bar-Joseph.** 1996. Unusual sequence relationships between two isolates of citrus tristeza virus. J. Gen. Virol. **77:**2359–2364.
24. **Miller, W. A., T. W. Dreher, and T. C. Hall.** 1985. Synthesis of brome mosaic virus subgenomic RNA *in vitro* by internal initiation on (−)sense genomic RNA. Nature **313:**68–70.
25. **Rosner, A., R. F. Lee, and M. Bar-Joseph.** 1986. Differential hybridization with cloned cDNA sequences for detecting a specific isolate of citrus tristeza virus. Phytopathology **76:**820–824.
26. **Strauss, J. H., and E. G. Strauss.** 1994. The alphaviruses: gene expression, replication, and evolution. Microbiol. Rev. **58:**491–562.
27. **Valverde, R. A., S. T. Nameth, and R. Jordan.** 1990. Analysis of double-stranded RNA for plant virus diagnosis. Plant Dis. **74:**255–258.
28. **Wu, G. C., and J. M. Kaper.** 1994. Requirement of 3′-terminal guanosine in (−)-stranded RNA for *in vitro* replication of cucumber mosaic virus satellite RNA by viral RNA-dependent RNA polymerase. J. Mol. Biol. **238:**655–657.
29. **Yang, G., R. Gofman, M. Mawassi, R. Gafny, and M. Bar-Joseph.** Submitted for publication.