Populations of citrus tristeza virus contain smaller-than-full-length particles which encapsidate sub-genomic RNA molecules

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Plants infected by citrus tristeza virus (CTV) contain, in addition to the 2000 nm full-length thread-like virions, a heterogeneous population of smaller particles. The CTV particles and RNA extracts from purified CTV preparations were fractionated by sucrose gradient centrifugation and the RNA molecules from different fractions translated in a reticulocyte translation system. Fractions containing predominantly an RNA band of approximately 3.2 kb directed the synthesis of CTV coat protein (CP), which in SDS-PAGE had an estimated molecular mass of 28 kDa. Three additional polypeptides, with estimated sizes of 21 kDa, 23 kDa and 27 kDa, were translated from a range of RNA molecules smaller than 3.2 kb. Hybridization with cDNA to the CP gene (CTV-CPG) and with a 350 base clone complementary to the 3' and 5' termini of the genes for CTV p20 and p23.5, respectively, indicated that preparations of CTV particles contain, in addition to the genomic (20 kb) RNA, two sub-genomic RNA molecules of 3-2 kb and 2.4 kb and probably also two smaller molecules of 1.6 kb and 0.9 kb. Only the 3.2 kb RNA, its corresponding dsRNA molecule and populations of larger RNAs, including the 20 kb genomic RNA, hybridized with a CTV-CPG probe, thus conflicting with our previous assignment of the CTV-CPG to the 0.8 kbp dsRNA. Based on these results we propose that distinct populations of CTV particles encapsidate smaller RNAs which were formed as a nested set of sub-genomic RNAs. Sequence analysis of 2540 nucleotides downstream to CTV-CPG of strain VT revealed four open reading frames (ORFs) potentially encoding, in the 5' to 3' direction, 18 kDa (p18), 13 kDa (p13), 20 kDa (p20) and 23.5 kDa (p23.5) proteins. The CTV-VT ORFs showed variable but usually close levels of homology with the corresponding ORFs of CTV-T36 from Florida.

Introduction

The particles of citrus tristeza virus (CTV), a member of the closterovirus group, are 2000 nm long and 10–12 nm wide (Bar-Joseph et al., 1979). The CTV virion contains a large single positive-stranded RNA genome with an estimated size of 20 kb (Bar-Joseph et al., 1985) and a single coat protein (CP) open reading frame (ORF) of 669 nucleotides (nt) encoding a protein with a molecular mass of 25 kDa (Sekiya et al., 1991; Mawassi et al., 1992; Pappu et al., 1992). In vitro translation of RNAs prepared from two biologically distinct CTV isolates revealed translation products ranging in size from 20 kDa to 250 kDa (Nagel et al., 1982). Antiserum to the CTV-CP precipitated two polypeptides, of 26 kDa and 250 kDa (Lee et al., 1988). Other cell-free translation products of 33 kDa, 50 kDa and 65 kDa did not react with the CTV-CP antiserum. Based on these findings, it was proposed that the expression of the CTV genome involves post-translational processing (Hiebert & Dougherty, 1988).

Recent sequencing of the CTV-T36 isolate from Florida showed the presence of eight ORFs potentially coding in the 5' to 3' direction for proteins of 65 kDa (p65), 61 kDa (p61), 27 kDa (p27), 25 kDa (p25) (CP), 18 kDa (p18), 13 kDa (p13), 20 kDa (p20) and 23 kDa (p23) (Pappu et al., 1994).

Several dsRNAs, including a 20 kbp molecule corresponding to the replicative form (RF) and a number of smaller species of dsRNAs, were consistently found following electrophoresis of RNA extracts from CTV-infected plants (Dodds & Bar-Joseph, 1983; Dodds et al., 1987). Two small dsRNA segments with estimated sizes of 0.8 kbp and 1.7 kbp were isolated and translated in vitro into two polypeptides of 27 kDa and 23 kDa, respectively (Dulieu & Bar-Joseph, 1990).

In this communication we report that preparations of CTV particles encapsidate, in addition to the genomic...
(20 kb) RNA, two distinct smaller RNA molecules, of 3.2 kb and 2.4 kb, and probably also ssRNA molecules of 1.6 kb and 0.9 kb and we propose that these molecules represent a nested set of sub-genomic RNAs. Sequence analysis of 2540 nt downstream of the CTV-CP gene (CTV-CPG) of the VT strain showed the presence of four ORFs with varying levels of homology with the corresponding ORFs reported previously to make up this region in the CTV-T36 isolate from Florida (Pappu et al., 1994).

**Methods**

**Virus strain and purification.** A seedling yellows isolate (strain VT) was propagated in Alemow citrus (Citrus macrophylla) seedlings. The virions were partially purified from bark tissue using the sucrose–cesium sulphate step gradient method (Bar-Joseph et al., 1985). For further purification and fractionation of CTV particles according to size, the viral zones from two- or three-step gradient tubes were pooled and dialysed overnight against 50 mm-Tris–HCl buffer (pH 8.0). The virus suspension (3 ml) was layered on a 10–40% linear sucrose gradient which was centrifuged for 4 h at 25000 r.p.m. in a Beckman SW27 rotor. The contents of the gradient tubes were collected as 1.5 ml fractions (Bar-Joseph et al., 1985). The RNA was prepared by phenol–chloroform extraction as described by Rosner et al. (1983). The viral CP was precipitated from the phenol phase using 2 vols of acetone and the concentration of CTV-CP was estimated by Coomassie blue staining of acrylamide gels and immunoblotting. The CTV-RNA was fractionated on a 5–20% sucrose gradient as described by Karasev et al. (1989).

**dsRNA preparation.** The dsRNA was extracted from CTV-infected bark of Alemow citrus (Dodds & Bar-Joseph, 1983), separated on BAC [N,N'-bis(acryloyl)cystamine]–polyacrylamide gels (Dulieu & Bar-Joseph, 1989) and treated with methylmercuric hydroxide (Dulieu & Bar-Joseph, 1990).

**Northern blotting.** The CTV RNAs were extracted from sucrose gradient fractions and separated by electrophoresis in formaldehyde–formamide–formaldehyde denaturing agarose gels prepared in MOPS buffer, transferred to nylon membrane (Hybond N; Amersham) and hybridized according to Maniatis et al. (1982). Total RNAs were extracted from the young bark and leaves of non-infected and CTV-infected Alemow plants (Prescott & Martin, 1987) and hybridized with cDNA clones harbouring sequences of the CTV-CPG (Sekiya et al., 1991; Mawassi et al., 1992), p20 and p23.5 (Pappu et al., 1994, and the present study).

**In vitro translation of viral RNAs and immunoprecipitation of CP.** The ssRNAs that were obtained from CTV particles fractionated on sucrose gradients or from CTV RNA preparations fractionated on 5–20% sucrose gradients were translated in vitro in 25 ml of a reaction mixture containing 7 μl rabbit reticulocyte lysate (Promega), 1 mm-magnesium acetate, 150 mm-potassium acetate, 1 mm-DTT, 28 mm-HEPES (pH 7.6), a 25 μM mixture of amino acids except methionine (Promega), 8 mm-creatine phosphate, 150 μg calf tRNA (Boehringer) and 1 μCi [35S]methionine (specific activity 800 Ci/mmol; Amersham). Tubes containing the reaction mixture were incubated at 30 °C for 60 min and the reaction was stopped by further incubation for 30 min at 37 °C after adding 100 μg RNase A.

**In vitro protein synthesis from total CTV-dsRNA and from BAC-separated dsRNA fragments was carried out as in Dulieu & Bar-Joseph (1990).** The translation products were fractionated in SDS–PAGE (Laemmli, 1970) and the radioactive polypeptide bands were detected by sodium salicylate treatment of the gels prior to autoradiography as described in Dulieu & Bar-Joseph (1990). The immunoprecipitation procedure (Firestone & Winguth, 1990) was used for detecting the CTV-CP among the in vitro translation products.

**Synthesis of cDNA and cloning.** For cloning of cDNA from the CTV-VT strain, ssRNAs, separated dsRNA segments and preparations of non-separated dsRNAs were treated with DNase and 3' polyadenylated as in Ashulin et al. (1992). The synthesis of cDNA was performed according to Wexler et al. (1991) using a chimeric deoxyligonucleotide primer consisting of a polylinker and oligo(dT) (5' GCGCCGGATC-CAAGCTTTTTTTTTTTT 3') (P-dT). The cDNA strands were annealed and amplified by the polymerase chain reaction (PCR) (Mullis & Faloona, 1987) according to Wexler et al. (1991). The PCR fragments were digested with BamHI (this site was included in the P-dT primer), eluted from agarose gel and cloned into BamHI-digested Plasmid KS (Stratagene). The cloning and sequencing techniques of the amplified cDNAs were according to Maniatis et al. (1982). A clone designated pdsVT5, which was found to hybridize with the dsRNA of 0.9 kbp and with all the other larger dsRNAs (see Fig. 7), was selected.
for further analysis. Sequence information derived from pdsVT5 and the CTV-VT-CP (Mawasit et al., 1992) was used to prepare primers for reverse transcription and PCR (RT-PCR) amplification of cDNAs. For extending the cDNAs to the 3' terminus of the CTV genome, the ssRNA and the dsRNA were polyadenylated, and RT-PCR was done using P-dT as a primer and specific oligonucleotides obtained from pdsVT5 sequence for reverse PCR synthesis. The cDNA fragments were cloned in pBluescript and sequenced using the USB sequencing kit according to the manufacturer’s instructions. Sequence analyses were carried out using the (UWGCG) program (Devereux et al., 1984).

Results

Sucrose gradient fractionation of CTV particles

The CTV preparations were fractionated on a 10–40% sucrose gradient and the viral proteins were separated by SDS–PAGE, immunoblotted and probed with antibodies to CTV-CP. The CTV-CP which was recovered from the phenolic phase after RNA extraction had an estimated size of 28 kDa. The Coomassie blue staining and the immunoblotting results showed that the CTV-CP was found in the tested fractions 6–17 with an intense concentration in fractions 9 to 13 (Fig. 1). A faint band at the 27 kDa position was also detected by CTV-CP antibodies in fractions 8–13. This 27 kDa protein was found not to be detectable by using monoclonal antibodies directed against CTV-VT particles (not shown). In addition, Fig. 1 shows also several proteins (55 kDa, 82 kDa, and 110 kDa) which were visualized by Coomassie blue staining and detected by immunoblotting. The molecular masses and the reaction of these proteins with CTV-CP antibodies suggest that they represent oligomeric forms of the CP. This was also confirmed by partial digestion of these proteins by Staphylococcus aureus V8 protease (not shown).

In vitro translation

RNA extracted from CTV virions was translated in vitro using the reticulocyte lysate system. The highest level of [35S]methionine incorporation was only six to ten times

Fig. 2. Autoradiography after SDS–PAGE of the in vitro translation products and the immunoprecipitation products from CTV-ssRNA. Lane 1, translation products from CTV-VT-ssRNA. Lanes 2–4, immunoprecipitation results obtained with (2) normal rabbit serum, (3) rabbit serum to CTV particles and (4) rabbit serum to CTV particles after treating the translation products with a normal rabbit serum. Lane 5, products which were pelleted after briefly centrifuging the translation reaction mixture. Lane M, translation products from brome mosaic virus RNA used as molecular mass markers. Lane R, in vitro translation products without addition of exogenous RNA.

Fig. 3. Autoradiography after SDS–PAGE of the in vitro translation products and the immunoprecipitation products from CTV-dsRNA. Lane 1, translation products from dsRNA extracted from CTV-VT infected plants. Lane 2, immunoprecipitated products obtained with normal rabbit serum. Lane 3, immunoprecipitation results obtained with antisem prepared against CTV particles. Lane M, as Fig. 2.
Fig. 4. Autoradiography after SDS-PAGE of the in vitro translation products from ssRNA prepared from the size-fractionated virus particles. The numbers along the gels indicate the fraction numbers of the sucrose gradient (as used for Fig. 1) from the bottom (no. 1) to the top (no. 22). Lane T, translation products from non-fractionated CTV particles. Lanes M and R, as Fig. 2.

greater than the background. In vitro synthesis of polypeptides directed by total RNA extracts of the CTV-VT strain yielded several major polypeptides which in SDS–PAGE gave estimated molecular masses of 21 kDa, 23 kDa, 27 kDa, 28 kDa, 38 kDa, 54 kDa, 63 kDa and several polypeptides not exceeding 110 kDa (Fig. 2, lane 1). Between the 27 kDa and the 28 kDa polypeptides, a third band was observed in some of the in vitro translation reactions. After in vitro translation, most of the CTV reaction products were pelleted by centrifugation for a few seconds (Fig. 2, lane 5). Non-specific precipitation of CTV translation products was prevented by floating the reaction products on a sucrose cushion (Firestone & Winguth, 1990). Only the 28 kDa polypeptide was immunoprecipitated by antisera prepared against CTV particles (Fig. 2, lanes 3 and 4) and SDS–PAGE fractionated CTV-CP (not shown). The dsRNA was denatured with methyl-mercury and translated in vitro in the lysate system. The polypeptides generated resembled those obtained by translation of ssRNA and only the 28 kDa polypeptide was immunoprecipitated by CTV-antisera (Fig. 3). Two polypeptides of 27 kDa and 23 kDa were found to be generated from two separate CTV-dsRNA fragments of 0·9 kbp and 1·6 kbp, respectively, which were estimated previously by Dulieu & Bar-Joseph (1990) to be 0·8 kbp and 1·7 kbp, respectively (not shown). In contrast to the previous results (Dulieu & Bar-Joseph, 1990), the 27 kDa polypeptide was not immunoprecipitated by the CTV-Ab (Fig. 2 and Fig. 3) after incorporating a blocking step to prevent non-specific adsorption to the S. aureus cells (Firestone & Winguth, 1990).

Translation of RNAs from gradient-fractionated virus particles (Fig. 4) gave different translation products at different positions on the gradient. The high molecular mass polypeptides were generated by translation of fractions 9–10, whereas high levels of the 28 kDa CP and the 27 kDa polypeptide were observed in fractions 9–13 and 9–15, respectively. In fractions 12–13 and 14–15 the 28 kDa CP and the 27 kDa polypeptide, respectively, constituted the main translation products.

When the CTV-RNA was fractionated in a 5–20% sucrose gradient, translation of the high molecular mass RNAs (Fig. 5, fractions 1–7) resulted in mainly high molecular mass polypeptides, with only weak bands corresponding to the 23 kDa, 27 kDa and 28 kDa (CP) products. The synthesis of these smaller polypeptides probably resulted from aggregated or over-run RNAs in the gradient. Fractions 8–18 were more efficient in in vitro translation reactions and gave a range of products with mobilities similar to those from unfractonated RNA. Fractions 21–30 were translated to yield several products with estimated molecular masses of 21 kDa, 23 kDa, 27 kDa, 28 kDa, 34 kDa and 54 kDa. The yield of these polypeptides varied considerably through this part of the gradient. The 21 kDa, 28 kDa (CP) and 54 kDa polypeptides were the most pronounced products of translation of fractions 22–24; the 23 kDa polypeptide was the dominant product of fractions 24–26. Fractions 28–30 encoded mainly the 27 kDa product. The low level of translation of the RNAs in fractions 5, 6, 19 and 20 (Fig. 5) was probably the result of non-specific degradation during the preparation of the samples.

The time course of the appearance of the translation products in the in vitro translation system from unfractonated CTV-RNA (Fig. 6a) and from CTV-RNA fractionated on a 5–20% sucrose gradient (pooled fraction nos 20–30) (Fig. 6b), showed that the 23 kDa, 27 kDa polypeptides and the 28 kDa (CP) appeared after 5 min incubation, whereas the larger products appeared later. The translation of high molecular mass CTV-RNAs (fractions 1–9) generated mainly large products (Fig. 6c).
Hybridization analysis

The RNA molecules from virus particles fractionated on the sucrose gradient were separated by electrophoresis in formamide–formaldehyde denaturing agarose gels, blotted onto nylon membrane and hybridized with a $^{32}$P-labeled cDNA probe encoding the CPG (Sekiya et al., 1991; Mawassi et al., 1992). Fractions 9–13 were found to contain an RNA species with an estimated size of 3.2 kb which hybridized intensely with this probe (Fig. 7a). An RNA species of similar size was also located following electrophoresis of total RNA extracts from CTV-infected citrus plants (Fig. 7a, lane I) and in preparations of CTV-dsRNA (Fig. 6a, lane ds). A 350 bp CTV clone (pdsVT5) which was obtained by cloning the 1.6 kb CTV-dsRNA fragment and found to include 10 amino acids (aa) of the C terminus and 105 aa of the N terminus of p20 and p23.5 respectively, was labelled and used for hybridizations. The cDNA of pdsVT5 reacted with a range of CTV-dsRNA fragments of 0.9 kb, 1.6 kb, 2.4 kb, 3.2 kb and 20 kb (RF) (Fig. 7b, lane ds) and with a range of similar sized ssRNAs which were obtained from sucrose gradient-fractionated CTV particles (Fig. 7b, lanes 8–11). The 1.6 kb and 0.9 kb ssRNA molecules appeared to constitute the major bands in fractions 14 and 15, respectively. Hybridization of the pdsVT5 probe with agarose gel-separated total RNA extracts from CTV-
Fig. 6. Time course of the appearance of CTV translation products. Unfractionated CTV-RNA (a), RNA of pooled fractions 20–30 (light zone) (b) and fractions 1–9 (heavy zone) (c) from CTV-RNA fractionated on a 5-20% sucrose gradient were translated in vitro and autoradiographed after separation by SDS-PAGE. The translation reactions were stopped after incubation for 5 min (lane 1), 10 min (lane 2), 15 min (lane 3), 20 min (lane 4) and 60 min (lane 5). Lanes M and R, as Fig. 2.

Fig. 7. Northern blot analysis of CTV-RNA. The ssRNA prepared from size-fractionated CTV particles was separated on 1.1% agarose gels with denaturation conditions as described in Methods, blotted to Hybond N membrane and hybridized with [32-P]labelled cDNA probes of: (a) a cDNA clone for the CTV-VT-CP gene; (b) a cDNA clone (pdsVT5) complementary to the coding sequences of the 10 amino acids at the C terminus and the 105 amino acids at the N terminus of p20 and p23.5, respectively. The numbers along the gels indicate the fraction numbers of the sucrose gradient (as used for Fig. 1) from the bottom (no. 1) to the top (no. 16). Lanes ds, methyl-mercury-denaturated CTV-dsRNA. Lanes H and I, total ssRNAs prepared from CTV-free and CTV-infected plants, respectively. The arrow in lane I indicates a sub-genomic RNA larger than 3.2 kb. Numbers on the left indicate the estimated sizes of the RNA.

infected Alemow plants (Fig. 7b, lane I) gave the strongest signals with the 2.4 kb, 1.6 kb and 0.9 kb RNA species. The cDNA clone corresponding to ORF p23.5 was found to hybridize with dsRNAs of 0.9 kb, 1.6 kb, 2.4 kb, 3.2 kb and 20 kb (RF), whereas the cDNA clone corresponding to ORF p20 hybridized with the 1.6 kb, 3.2 kb and 20 kb (RF) dsRNAs species, but not with the 2.4 kb species (not shown).
The sequence of 2540 nt downstream of the CPG of the VT and T36 strains (Fig. 8) showed designated ORF 18 (18 kDa), ORF 13 (13 kDa), ORF 20 (20 kDa) and ORF 23'5 (23.5 kDa), similar to strain T36 Clon#lg and sequencing 14 aa, 7 aa, 8 aa and 26 aa replacements in p18, p13, p20 and p23'5, respectively. Sequencing the separated 0.9 kbp dsRNA species showed the presence of a non-translated region (ntr) of 273 nt at the 3' of ORF 23.5. The 3' ntr of CTV-VT showed 97% homology with the ntr of CTV-T36 (Fig. 9).

**Discussion**

CTV has a single-strand genomic RNA molecule of unusually large size for a plant virus (Dodds & Bar-Joseph, 1983; Bar-Joseph et al., 1985). Several limiting factors, including the restriction of the virus to the phloem tissue, a tendency of these very long virus particles to break and aggregate, and the absence of a convenient infectivity test, have all restricted the characterization of CTV at the molecular level until now (Bar-Joseph et al., 1989). Cloning of cDNA sequences coding for the CPG (Sekiya et al., 1991; Mawassi et al., 1991; Pappu et al., 1992) and other genomic sites (Pappu et al., 1994) enabled us to obtain probes for the detection and analysis of minor quantities of smaller-than-full-length (StFL) CTV particles among a heterogeneous population of virus particles. These were previously considered to consist of randomly fragmented particles resulting from the purification process (Bar-Joseph et al., 1985).

The existence of StFL particles which encapsidate small sub-genomic RNAs coding for virus CPs has been reported for different groups of elongated plant viruses (Hiebert & Dougherty, 1988). Other groups, including the potyviruses (Yeh & Gonsalves, 1985) and a capillovirus (Yoshikawa & Takahashi, 1992), use a different replication strategy whereby the genomic RNA is first translated as a large polyprotein which is then proteolytically processed to produce the CP. Our results indicate that CTV particles encapsidate full-length genomic ssRNA of 20 kb and smaller RNAs of 2.4 kb and 3.2 kb and also probably of 0.9 kb and 1.6 kb. Of these smaller RNAs, the 3.2 kb molecules were found to hybridize with the CTV-CPG. Moreover, the presence of the 3.2 kb RNA in sucrose gradient fractions was
correlated with the intensity of the CTV-CP bands observed in *in vitro* translation.

A certain discrepancy was revealed between the molecular mass of the CTV-CP (28 kDa) estimated by SDS-PAGE (low molecular mass standards, Bio-Rad) and the molecular mass calculated from sequence analysis (25 kDa) (Sekiya et al., 1993; Mawassi et al., 1993; Pappu et al., 1993). Interestingly, the molecular mass of the CTV-CP was originally estimated to be 25 ± 1 kDa (Bar-Joseph et al., 1979) using the potato virus X (PVX)-CP as a molecular standard. The PVX-CP was recently found to be glycosylated at the N and/or C terminus and reported to show similar differences between the SDS-PAGE (27–29 kDa) and sequence analysis (25 kDa) values (Tozzini et al., 1994). Studies are in progress to test for the possible glycosylation of CTV-CP.

The results of the cell-free translation and hybridization experiments of fractionated ssRNAs and dsRNAs suggest that the three RNA molecules of 0.9 kb, 1.6 kb and 3.2 kb encode the 27 kDa, 23 kDa and 28 kDa proteins, respectively, which appear to correspond to p23-5, p20 and p25 (CP), respectively (Pappu et al., 1994, and the present study). These three RNA molecules and a 2.4 kb RNA (for which the coding function is still not established) and possibly an additional RNA (Fig. 7b, arrow) are formed as a nested set of sub-genomic RNAs. These results suggest that the origin of assembly of the CTV particles is located downstream of the CTV-CPG and probably in the non-coding region, thus enabling the encapsidation of the smallest sub-genomic RNA of 0.9 kb coding for p23-5.

Cloning of the cDNA located downstream of the 3' of the CTV-CPG resulted in a group of clones which showed four ORFs, of 18 kDa, 13 kDa, 20 kDa and 23-5 kDa, similar to those described recently by Pappu et al. (1994) for T36 from Florida. Amino acid sequence comparisons of CTV-VT with CTV-T36 (Fig. 8) showed a high level of homology in p25 (CP) (96%), p20 (95%), p13 (94%) and p18 (91%), while p23-5 showed only 87% homology. Additional strains of CTV will have to be sequenced in order to determine whether differences occur between different CTV genes in the rates of conservation. Interestingly, the 3' ntr of CTV-VT showed a higher base sequence homology (97%) with CTV-T36 than the sequence of the five coding regions which were compared in this study. This high degree of base sequence conservation found in the ntr at the 3' end of the CTV genome suggests some important functional role for this sequence, probably for minus strand replication and for the CTV-CP assembly site.

RNA extracted from beet yellows virus (BYV) particles directed the synthesis of a major 250 kDa protein, covering only the 5'-terminal half of the genome (Karasev et al., 1989). Seven other genes, including the CPG, are located downstream (Agranovsky et al., 1991; Brunstedt et al., 1991) and are most likely expressed only *in planta* from non-encapsidated sub-genomic RNAs (Agranovsky et al., 1991; Dolja et al., 1991). The differences in the translation strategies between BYV and CTV probably reflect differences in the location of their origin of assembly. Agranovsky et al. (1994) found that the expression of the BYV genome includes a combination of strategies of proteolysis for the expression of the 5'-proximal replicative genes, sub-genomic RNA formation to express the 3'-proximal genes and possibly a ribosomal frameshifting. This work shows that the sub-genomic RNA strategy is also used for the expression of some CTV genes.

The authors thank Drs P. Dulieu and M. Balass for their advice with *in vitro* translation and Ms Rosa Gofman and Mr Y. Ben-Shalom for their technical help. This work was supported by grants from the US-Israel Binational Agricultural Research and Development Fund (BARID), the German-Israeli Agricultural Research Agreement (GIARA), The Biotechnology Center, ARO, and the Citrus Marketing Board, Israel.

References


CTV sub-genomic RNAs


(Received 12 September 1994; Accepted 25 October 1994)