In vitro translation of the citrus tristeza virus coat protein from a 0.8 kbp double-stranded RNA segment

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Several dsRNA segments, ranging in size from 0.8 to 19.5 kbp, have been isolated from citrus plants infected with the VT and ST strains of citrus tristeza virus (CTV) and translated, after denaturation, in a reticulocyte lysate cell-free system. Only two small dsRNA segments of 0.8 and 1.7 kbp were efficiently translated and showed a consistent pattern of products. The major translation product of the 0.8 kbp dsRNA was a 27K polypeptide, immunoprecipitated by antiserum to the CTV coat protein. The major translation product of the 1.7 kbp dsRNA segment was a 21.5K peptide. The possibility that the CTV coat protein may be translated from a subgenomic viral messenger RNA is discussed.

Plants infected with citrus tristeza virus (CTV) contain several dsRNA segments, including a 19.5 kbp molecule corresponding to a full-length replicative form (RF) of this unusually large ssRNA virus (Dodds & Bar-Joseph, 1983). In addition to this, several dsRNAs of a smaller size than the RF are also present, which vary in size and in their relative amounts depending on the virus strain, hosts and temperature (Dodds et al., 1987). Two of these dsRNAs with estimated sizes of 1.2 to 1.7 kbp and 2.8 to 3.0 kbp were present in all CTV strains examined. One or more other dsRNAs with sizes varying between different CTV isolates are also present, and it was suggested that their electrophoretic patterns could be used as markers to identify specific CTV strains on a given host (Dodds et al., 1987). The dsRNAs smaller than the RF are CTV-specific, as CTV-specific probes have been shown to hybridize with them (Dodds et al., 1987; Rosner et al., 1986). The functions of any CTV dsRNA are unknown. We have recently developed a method for separating and isolating dsRNA segments from N,N'-bis(acryloyl)cystamine (BAC) polyacrylamide gels (Dulieu & Bar-Joseph, 1989), which allowed analysis of the messenger activity of dsRNA segments appearing in CTV-infected tissues and to assign the CTV coat protein messenger activity to a dsRNA segment of 0.8 kbp.

The biological properties and experimental procedures for transmission and propagation of the CTV isolates VT and ST have been previously described (Bar-Joseph et al., 1979). For the isolation of dsRNA, frozen bark tissue from graft-inoculated Citrus macrophylla was ground to a powder in liquid nitrogen. The nucleic acids were extracted twice with phenol and once with chloroform, the dsRNA was isolated by two cycles of chromatography on CF11 (Whatman) columns (Morris & Dodds, 1979; Dodds & Bar-Joseph, 1983). The dsRNAs were electrophoresed in 5% disulphide cross-linked polyacrylamide gels, prepared as described by Hansen (1981). After ethidium bromide staining the dsRNA segments were isolated from the gel using a method recently described by Dulieu & Bar-Joseph (1989) with the following modifications. Pieces of gel containing PAGE-fractionated dsRNA segments were dissolved in STE buffer (10 mM-Tris–HCl pH 8, 100 mM-NaCl, 1 mM-EDTA) containing 5% 2-mercaptoethanol. The solution was loaded onto a DEAE-52 mini-column (prepared in a 1 ml micropipette tip), pre-equilibrated in the same buffer. The column was then centrifuged in an Eppendorf tube at 500 r.p.m. Depending on the amount of dissolved acrylamide loaded the column was then washed with 3 to 5 ml of buffer. The dsRNA was eluted with 4 x 100 μl of Tris buffer (10 mM-Tris–HCl pH 8, 1.5 M-NaCl). After the volume had been increased to 1–1.1 ml with TE buffer (10 mM-Tris–HCl pH 8, 1 mM-EDTA), the solution was adjusted to 22% ethanol and loaded on an STE buffer–22% ethanol-equilibrated CC41 (Whatman) mini-column (constructed as before). The column was washed with 3 ml of the same buffer and the purified dsRNA segment was finally eluted with 4 x 50 μl of ultra-pure water and precipitated with ethanol. The dsRNA (8 μl) was denatured by incubation at room temperature for 10 min with 10 mM-methylmercuric hydroxide (Shelbourn et al., 1988).

After 2 μl of 700 mM-mercaptoethanol had been added, the solution was mixed with 24 μl of cell-free
translation mixture, containing 11 µl of nuclease-treated reticulocyte lysate (Promega) and 13 µl of translation buffer (4.7 mM-magnesium acetate, 310 mM-potassium acetate, 80 μM amino acid mix without methionine, 90 mM-HEPES pH 7.4, 25 mM-creatine phosphate, 0-5 mg/ml calf transfer RNA and 1.5 mCi/ml of L-[35S]methionine). The mixture was incubated at 30 °C for 105 min and the reaction stopped by RNase A treatment at a final concentration of 100 µg/ml (37 °C, 30 min), to digest peptidyl-tRNA complexes.

For immunoprecipitation, 5 to 20 µl of the reaction mixture was adjusted to 100 µl with NP40 buffer (10 mM-sodium phosphate pH 7.2, 150 mM-NaCl, 1% NP40) and 1 µl of a polyclonal antiserum to SDS PAGE-separated CTV coat protein (kindly provided by M. Mawassi) were mixed and the solution was incubated at 4 °C overnight. After the addition of 20 µl of Protein A-Sepharose (50% in NP40 buffer) the mixture was kept on ice for 30 min and the Sepharose phase was separated by centrifugation. The pellet was washed four times with NP40 buffer and twice with ultra-pure water. Finally, the Sepharose pellet was resuspended in 40 µl of SDS sample buffer. For PAGE analysis (Laemmlli, 1970) samples of 5 to 20 µl of the total translation products were diluted threefold in SDS sample buffer, heated at 100 °C for 5 min and loaded on a 1 mm thick, 12-5% polyacrylamide gel, which was then run for 1 h at 20 mA and for 3 h at 30 mA. Before autoradiography the gel was fixed and dehydrated in DMSO for 1 h and soaked in DMSO containing 22%, 2,5-diphenyloxazole (PPO) for 30 min at 68 °C. The PPO was precipitated in the gel by several washes in water and the gel was dried and exposed to Kodak X-OMAT film at -80 °C.

Several dsRNAs were readily detected by gel electrophoresis of preparations from C. macrophylla bark tissue infected with VT, a seedling yellows isolate and ST, an ordinary CTV isolate. Fig. 1 (lanes 2 and 3) shows typical dsRNA profiles obtained from extracts of 2 g of tissue infected with the ST and VT strains, respectively. Four segments with estimated sizes of 19.5 kbp (the larger segment corresponding to the RF; Dodds & Bar-Joseph, 1983), 3-0, 1.7 and 0-8 kbp were present in both strains. The 3-0 kbp segment was usually present at a higher concentration in ST-infected plants, whereas the 0-8 kbp segment was more evident in the VT-infected plants. The two strains also differed in two other major segments, of 2.6 and 5.1 kbp, found in the ST and VT strain, respectively. A range of dsRNA segments (indicated by arrows in Fig. 1) was selected for in vitro translation analysis. Total dsRNA extracts obtained from 200 g of bark tissue were separated on 5% BAC-polyacrylamide gels and the selected segments were isolated as described above. The efficiency of segment isolation was evaluated by running samples containing 5% of the recovered segments on PAGE gels (data not shown). The dsRNA concentrations of the different segments were estimated by comparing the intensity of ethidium bromide fluorescence and finally adjusted to approximately 20 ng/µl.

The dsRNA segments did not stimulate polypeptide synthesis by the rabbit reticulocyte lysate system, unless they had been denatured by methylmercuric hydroxide. Only a twofold stimulation of incorporation was obtained with any of the denatured dsRNA segments, compared to 20- to 40-fold incorporation with the ssRNAs of brome mosaic virus (BMV). Only the small
dsRNA segments of 0.8 and 1.7 kbp were efficiently translated. The polypeptides obtained by translating the different dsRNA segments from the two CTV strains after SDS-PAGE analysis and fluorography are shown in Fig. 2. The major translation product of the 0.8 kbp segment from both strains was a 27K polypeptide (Fig. 2a, lane 6 and 2b, lane 7) comigrating with the CTV coat protein (not shown). In addition a minor peptide of 20K was also observed. Two polypeptides were translated from the 1.7 kbp dsRNA. The major translation products of the 1.7 kbp dsRNAs from both strains was found to be the 21.5K polypeptide, although a low intensity 27K peptide was also observed. The other dsRNA segments of the CTV-VT, including the 19.5 kbp segment, have not been translated efficiently (Fig. 2a, lanes 3 and 4). The efficiency of translation of the 19.5 kbp dsRNA segment from CTV-ST varied considerably between different dsRNA batches. Only the 20K to 22K and 27K polypeptides were observed at varying intensities in most translations of the 19.5 kbp dsRNA (Fig. 2b, lane 3, indicated by arrows).

Translation products from all segments were treated with an antiserum (R14) raised against electrophoretically separated viral coat protein; Fig. 3(a) shows immunoprecipitated polypeptides from translation products of the four CTV-VT dsRNA segments. The 27K, 20K and 21.5K polypeptides were precipitated by the antiserum to CTV coat protein. In some cases the translation of the CTV-ST dsRNA segments showed two to three closely banded peptides of 20K to 22K and 26K to 28K (Fig. 3b), the reason for which is unknown. All of these polypeptides were immunoprecipitated by CTV coat protein antiserum (Fig. 3c). Immunoprecipitation tests with R14 antiserum for the BMV RNA translation products were negative. Recent experiments indicate that the CTV translation products tend to adsorb nonspecifically to Protein A-Sepharose and only the 27K polypeptide is specifically immunosorbed to polystyrene wells coated with CTV coat protein antibodies (M. Mawassi & M. Bar-Joseph, unpublished).

Compared with most other filamentous plant viruses (Hiebert & Dougherty, 1988) very little genomic characterization has been carried out on members of the closterovirus group (Bar-Joseph & Murant, 1982; Lee et al., 1988; Karasev et al., 1989). The main reason for this is the difficulty in purifying and isolating sufficient quantities of intact nucleic acid from viruses with large (14 to 19.5 kb) single-stranded genomic RNAs. However, surprisingly plants infected by a number of closteroviruses contain relatively large concentrations of several distinct dsRNAs (Dodds & Bar-Joseph, 1983). Assignment of coding functions from in vitro translation analysis was previously obtained using genomic dsRNAs (Smith et al., 1980; Ghabrial et al., 1987; Accotto et al.,
Recent developments in the isolation of dsRNA segments from total dsRNA extracts (Dulieu & Bar-Joseph, 1989) and the currently described modifications enabled us to analyse the putative translation functions of dsRNAs associated with CTV infection. Only the two smaller dsRNAs of 0.8 and 1-7 kbp were efficiently translated and the main translation products were the 27K and the 21.5K peptides, respectively. The 27K polypeptide, which comigrated with the CTV coat protein, was specifically immunoprecipitated by antibodies to the CTV coat protein. Reasons for the inefficient translation of the larger dsRNAs, including the 19.5 kbp RF, have not yet been elucidated. Only minor amounts of 20K to 21K and 21.5K polypeptides were regularly observed when the 19.5 kbp dsRNA was translated. This does not necessarily imply translation of the coat protein from the RF dsRNA, since a certain amount of trapping or carryover of smaller dsRNAs in preparations of larger dsRNAs was observed (Fig. 4). It should be noted that the coat protein of beet yellows virus was not translatable from the 14.5 kb genomic ssRNA (Karasev et al., 1989).

Assuming an average Mr of 110 per amino acid residue the 27K polypeptide corresponds to 92% of the coding capacity of the 0.8 kbp segment.

The role of dsRNA segments in viral RNA replication is unknown. However, when it is known that subgenomic transcripts serve as messengers for specific viral proteins, e.g. the coat protein genes in the tobamoviruses or cucumoviruses, the infected plants were often found to contain dsRNA segments with Mr twice the size of the coat protein messengers (Bar-Joseph et al., 1983; Dawson & Dodds, 1982; Kaper & Diaz-Ruiz, 1977). Interestingly, whereas the 1.7 kbp segment is consistently present in all CTV strains, the 0.8 kbp one was prominent in some CTV seedling yellows isolates but much less abundant or even undetectable in many
ordinary Californian CTV isolates (Dodds et al., 1987). Since the ordinary (ST) and the seedling yellows (VT) strains produce comparable levels of viral antigen (Bar-Joseph et al., 1979) it will be appropriate to determine whether the relative concentrations of their corresponding ssRNAs follow the same pattern.

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References


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