Double-Stranded RNA from Plants Infected with Closteroviruses

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ABSTRACT

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Expected double-stranded RNA replicative forms (RFs) and unexpected smaller dsRNAs were isolated by chromatography on CF-11 cellulose from extracts of plants infected with closteroviruses. Molecular weights of RFs were estimated to be 13.3×10^6 for citrus tristeza virus (CTV) by electron microscopy, 8.4×10^6 for beet yellows virus (BYV) and carnation necrotic fleck virus (CNFV), and 4.6×10^6 for apple chlorotic leafspot virus (ACLSV) by gel electrophoresis. The patterns of dsRNA segments resolved by polyacrylamide gel electrophoresis were distinct for each virus, but were sufficiently similar for BYV, CNFV, and CTV to be diagnostic for closteroviruses. There was little variation in the pattern of dsRNA segments detected in extracts from groups of plants of a single host, from different tissue sources of a single host, from different hosts, and from the first pellet normally discarded from a CTV and BYV purification scheme. A higher yield of dsRNA was recovered from bark or from recently expanded leaves than from other sources. Sufficient amounts of dsRNA were routinely obtained from only 0.2 to 7.0 g of tissue.

Additional key words: disease diagnosis.

The closteroviruses are an unusual group of flexuous rod-shaped plant viruses because, unlike the other groups, the lengths of the particles of its members are not similar (1). Three subgroups with particle lengths of approximately 700 nm (eg, apple chlorotic leafspot virus [ACLSV]), 1,250–1,400 nm (eg, beet yellows virus [BYV]), and 2,000 nm (eg, citrus tristeza virus [CTV]) have been included in this group. Two of the viruses are known to contain undivided single-stranded (ss) RNA genomes with molecular weights of 2.3×10^6 for ACLSV (3) and 4.6×10^6 for BYV (7). The CTV genome has been assumed to be undivided and to have a molecular weight of $6.3–6.9 \times 10^6$ on the basis of CTV particle length (1,2). This assumption has recently been confirmed by agarose gel electrophoresis of CTV-RNA (J. A. Dodds, *unpublished*). These values are still in need of careful evaluation. The CTV genome is nevertheless one of the largest of all ssRNA plant virus genomes.

An objective of this study was to investigate the recovery and distribution of double-stranded (ds) RNA replicative forms (RFs) from plants infected with closteroviruses. This approach has confirmed the expectation that dsRNA molecules approximately twice the size of genomic ssRNAs are present in plants infected with closteroviruses, has implicated numerous other dsRNAs in the replication of closteroviruses, and has demonstrated the diagnostic value (17) of the RF and the other dsRNAs detected. Assessing the value of dsRNA analysis as a diagnostic tool was the other objective of this study. A gratifying observation has been the ease with which RF and other dsRNAs have been isolated from small (0.2–7.0 g) amounts of tissues infected with a group of viruses that are characteristically limited to phloem tissues and are difficult to purify.

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MATERIALS AND METHODS

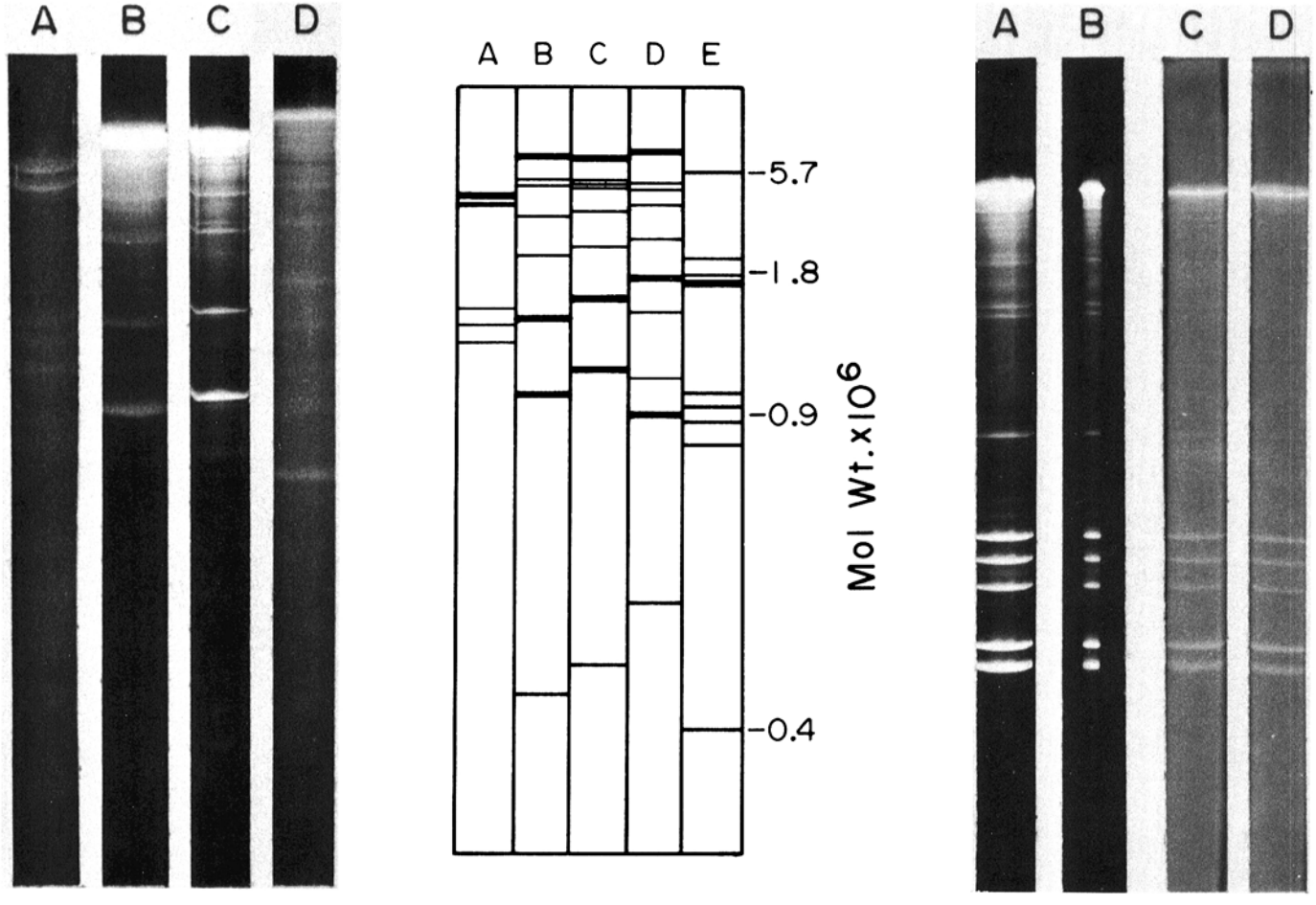
The closteroviruses examined and the hosts in which they were propagated were ACLSV in *Chenopodium quinoa*; carnation necrotic fleck virus (CNFV) in *Saponaria vaccaria*; BYV in *S. vaccaria*, *Claytonia perfoliata*, *Beta vulgaris* and *Chenopodium quinoa*; and CTV in *Citrus aurantifolia* (Mexican lime), *C. medica* (citron), *C. sinensis* (sweet orange), and a new host, *Passiflora gracilis* (C. N. Roistacher, *unpublished*). ACLSV was transmitted mechanically, BYV and CNFV were transmitted with *Myzus persicae*, and CTV was transmitted with *Aphis gossypii* and by bud grafting. Two biologically different isolates of BYV, BYV-B, and

BYV-5 (5) were analyzed. Tobacco mosaic virus (TMV) was propagated in *Nicotiana tabacum* 'Turkish.'

Virus concentrations of BYV and CTV in tissue extracts were estimated by electron microscopy and by ELISA (4). Fresh or frozen leaf or bark tissue (7 g) was ground to a powder in liquid N_2 in a mortar with a pestle. The powder was stirred for 30 min with 14 ml of double-strength TSE buffer (0.05 M tris(hydroxymethyl)aminomethane (tris), 0.1 M NaCl, 0.001 M ethylenediaminetetraacetic acid (EDTA) (pH 7.0), 2.0 ml of 10% sodium dodecyl sulfate (SDS), 20 ml of water-saturated phenol, and 16 mg of bentonite. Alternatively, each 1 g of the powder was suspended in 5 ml of 0.1 M tris (pH 7.8) and the liquid extract was expressed through four layers of cheesecloth and one layer of paper wipes. The extract was centrifuged for 5 min at 6,000 *g* and then, without removing the tubes, for a further 10 min at 8,000 *g*. The pellets were extracted with TSE buffer, SDS, and phenol as described above. The solution was centrifuged (10,000 *g* for 20 min) and the supernatant was collected, adjusted to 15% (v/v) ethanol, and stored overnight at 4 C. The solution was centrifuged (10,000 *g* for 20 min) and the supernatant was subjected to two cycles of chromatography on small columns (2.5 g dry powder, 10 ml bed volume when wet) of CF-11 (Whatman, Clifton, NJ 07014) cellulose designed to purify dsRNA (13,15,17). Each cycle consisted of loading the column with 20 ml of sample in 85% TSE buffer: 15% ethanol (v/v), washing the column with 80 ml of 85% TSE buffer: 15% ethanol (v/v), and eluting the column with 15 ml of TSE buffer. The final samples were adjusted to 66% ethanol (v/v) and stored at −20 C. Samples were centrifuged (5,000 *g* for 30 min) and prepared for electrophoresis by resuspending pellets in 200 µl of electrophoresis buffer (0.04 M tris, 0.02 M sodium acetate, 0.001 M EDTA pH 7.8). Electrophoresis was on cylindrical 5% polyacrylamide or 0.8% agarose gels (9 cm × 6 mm) in electrophoresis buffer for either 9 hr at 6 mA/gel (polyacrylamide gels) or 2.5 hr at 3 mA/gel (agarose gels).

The dsRNAs from three fungal viruses, Hm9 ($MW = 5.7 \times 10^6$), PcV ($MW = 1.8 \times 10^6$), and PsV ($MW = 0.9 \times 10^6$, 0.4×10^6), were used as molecular weight markers (7). Gels were stained in ethidium bromide (10 ng/ml) in water, destained in water, and analyzed for fluorescing dsRNA bands on a transilluminator (302 nm transmission). Gels were photographed with lengthy exposures (5–20 min, f4.5, Polaroid type 52 black and white film, 400 ASA, Wratten 9 [yellow] and 23A [red] gelatin filters) to maximize detection of minor dsRNAs. Short exposures (1 min or less) were used when only the major RF dsRNA was to be recorded. Stained gels were soaked in water with or without ribonuclease (type 1A, 10 µg/ml, Sigma, St. Louis, MO 63178) or 0.3 M NaCl with or without ribonuclease for 2–3 hr (17). If digestion occurred, it was complete in the outer half of the gel, but a core of undigested dsRNA remained (Fig. 3).

Purified dsRNA molecules from CTV- and TMV-infected plants were spread and shadowed by the method of Davis et al (10). Images of dsRNA molecules on electron micrograph negatives were projected and traced. Their lengths were measured with a map measurer.



Figs. 1–3. 1, Electrophoretic visualization of double-stranded RNA segments isolated from 4.0, 0.2, 0.2, and 2.0 g, respectively, of tissue from **A,** leaves of *Chenopodium quinoa* infected with ACLSV; **B,** leaves of *Saponaria vaccaria* infected with CNFV; **C,** leaves of *Saponaria vaccaria* infected with BYV; and **D,** bark of *Citrus aurantifolia* infected with CTV. All dsRNA recovered from a sample was loaded on a single gel. Samples were electrophoresed for 9 hr at 6mA/gel on 5% acrylamide gels in 0.04 M tris, 0.02 M sodium acetate, and 0.001 M EDTA (pH 7.8). Gels were stained in ethidium bromide (10 ng/ml). **2,** Relative electrophoretic mobility and intensity of dsRNAs consistently detected in samples prepared from plants infected with **A,** ACLSV; **B,** CNFV; **C,** BYV; and **D,** CTV compared with **E,** segments of dsRNA of known molecular weight. Electrophoresis as in Fig. 1. **3,** Effect of ribonuclease on stained electrophoresis gels containing dsRNAs from *Beta vulgaris* infected with BYV. Stained gels were soaked in either water **B,** with or **A,** without ribonuclease or in 0.3 M NaCl **C,** without or **D,** with ribonuclease. Electrophoresis and staining as in Fig. 1.

RESULTS

Healthy plants. No distinct or sharp fluorescent bands resembling those described in the following sections were detected in gels on which extracts from 2 g of uninoculated *C. medica*, *C. aurantifolia*, *S. vaccaria*, *C. perfoliata*, and *C. quinoa* were electrophoresed. Extracts from *B. vulgaris* were usually free of dsRNA. Samples from uninoculated *B. vulgaris* that contained dsRNA are described in another section. After prolonged photographic exposure, indistinct broad bands migrating between the 0.9 and 0.4×10^6 dalton dsRNA markers were detected in gels used to analyze extracts from *S. vaccaria*, *C. quinoa*, and *B. vulgaris*.

Typical dsRNA segments. An example of the distinct and sharp bands detected in gels on which dsRNA samples from plants infected with four closteroviruses (ACLSV, CNFV, BYV, and CTV) were electrophoresed is shown in Fig. 1. Single gels do not give an adequate representation of the results obtained from many analyses. Figure 2 shows the bands consistently detected in three to 20 separate analyses of plants individually infected with the four viruses.

The intense band with the slowest mobility near the top of each gel is believed to be the double-stranded form (replicative form or RF) of the ssRNA genome of each virus. The relative mobility of the four RFs was in the same order as the relative sizes of the viral genomes (ACLSV < CNFV = BYV < CTV).

The average length of the projected magnified images of 20 full length molecules of CTV and TMV RFs was 57.7 cm (range = 57.5–58.0 cm) and 18.7 cm (range = 18.5–19.0 cm), respectively. This corresponds to a molecular weight of 13.3×10^6 for CTV RF, assuming a value of 4.3×10^6 for TMV RF (11). This value is approximately twice that predicted for the CTV ssRNA genome (1,2). A full length molecule of CTV dsRNA is shown in Fig. 4.

This value was used, together with those of the marker dsRNAs, to graphically estimate (7) the molecular weights of the RFs of ACLSV (4.6×10^6) and BYV (8.4×10^6). These values are approximately twice those reported for ACLSV (2.3×10^6) and BYV (4.6×10^6) ssRNA genomes (3,8). The smooth curvilinear relationship that existed between log molecular weight and distance migrated on 5.0% polyacrylamide gels and 0.8% agarose gels is shown in Fig. 5. Neither gel system is optimal for estimating molecular weights. Sharper and thinner bands were obtained in polyacrylamide gels than in agarose gels, but the curve generated from the data was much steeper and more difficult to draw.

Multiple bands of various mobilities (sizes) and intensities were detected in addition to the RF in stained gels on which dsRNA samples from plants infected with CNFV, BYV, and CTV were electrophoresed. Two of these were next in intensity to the RF and had molecular weights of about 2.0 and 1.0×10^6. Minor dsRNA with mobilities between those of the 1.8 and 5.7×10^6 dalton dsRNA markers and a minor dsRNA with a mobility corresponding to a molecular weight of about 0.5×10^6 were among the other distinctive dsRNAs. The absolute mobilities of these unexpected dsRNAs segments were consistently similar for a single virus. The relative mobilities were different and diagnostic for CNFV, BYV, and CTV (Fig. 2B–D, respectively).

The result for ACLSV did not follow this pattern. Two dsRNA segments with similar mobilities (MV approximately 4.0×10^6) were readily detected (Figs. 1A and 2A). Three minor dsRNA segments were detected in one of three preparations. The amount of dsRNA recovered from plants infected with ACLSV was less than that from plants infected with the other three viruses (see below).

Ribonuclease and LiCl treatments. The dsRNA samples used for these experiments were from *B. vulgaris* infected with BYV (Fig. 3) or *C. medica* infected with CTV. Treatment with RNAse in water resulted in the gradual loss of fluorescence associated with all the diagnostic bands in stained gels (Fig. 3B). Treatment with RNAse in 0.3 M NaCl increased background fluorescence (a 0.3 M NaCl

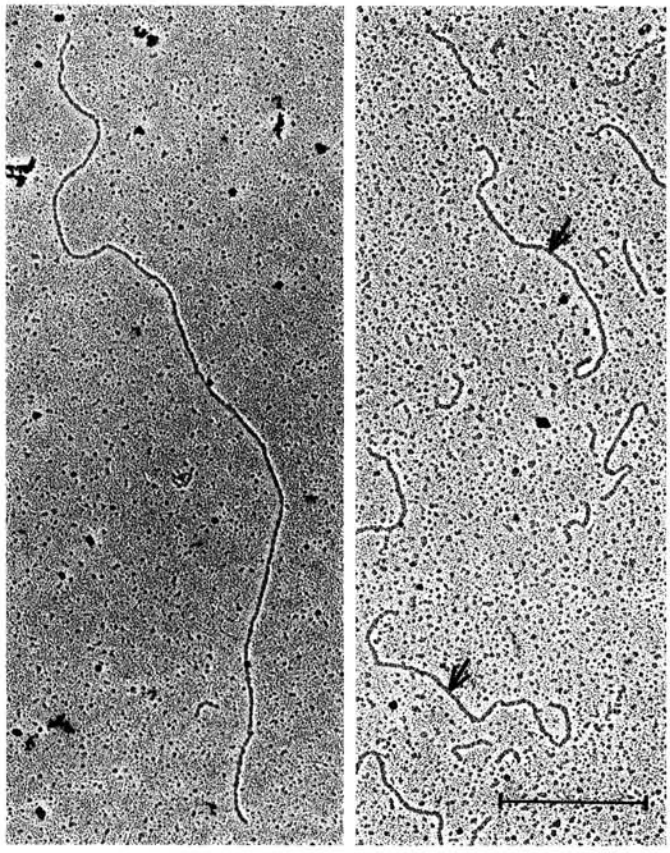


Fig. 4. A full length molecule (6.2 μm) of CTV RF dsRNA (MW = 13.3 $\times 10^6$ daltons) and two full-length molecules (2.0 μm) of TMV RF dsRNA (arrowed). Bar represents 1 μm.

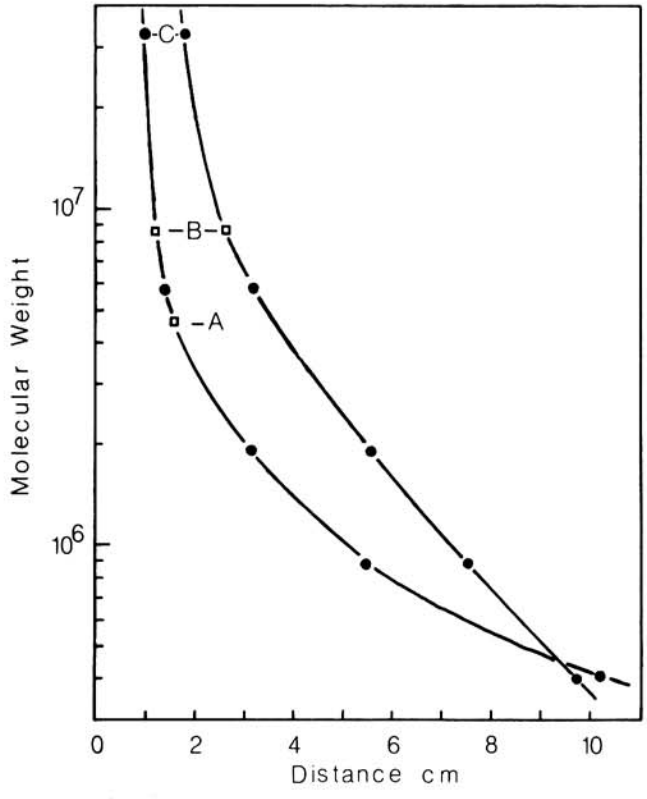


Fig. 5. Relative electrophoretic mobility of the RF dsRNAs from plants infected with either ACLSV (**A**) or BYV (**B**), compared to CTV RF dsRNA (MW = 13.3×10^6, [**C**]) and dsRNA standards (solid circles) with molecular weights of 5.7, 1.8, 0.9, and 0.4×10^6. Electrophoresis was on either 5% acrylamide gels (lower curve) as in Fig. 1 or on 0.8% agarose gels (upper curve) electrophoresed for 2.5 hr at 3 mA/gel.

effect) to the point where minor bands could no longer be resolved. No enzyme-related loss of fluorescence was observed from the stained bands that could still be resolved. These results confirm that all fluorescing bands contained RNA and most, if not all, contained dsRNA. All the dsRNA segments from *C. medica* infected with CTV were soluble in 2 M and 3 M LiCl, but were precipitated by 4 M LiCl. This is the expected result for dsRNA (9).

Sources and variability of dsRNA. Different sets of plants of *C. medica* infected with CTV were examined. Seedlings bud-inoculated with one isolate of CTV were divided into three blocks (25 seedlings in each) and bark was sampled from each block. Gel electrophoresis was used to analyze the dsRNA in aliquots of the extracts from 7 g of each bark sample and no quantitative or qualitative differences in RF and faster migrating dsRNAs were observed between samples.

Several different plant species infected with CTV and BYV were analyzed. The dsRNA segments illustrated in Fig. 2 were detected in dsRNA samples from *C. sinensis*, *C. aurantifolia*, *C. medica*, and *P. gracilis* infected with CTV and in dsRNA samples from *C. perfoliata*, *S. vaccaria*, *C. quinoa*, and *B. vulgaris* infected with BYV-5 (Fig. 6). The only obvious variation was seen in *B. vulgaris*. Four prominent dsRNA segments (MW $\sim 1.0 \times 10^6$ daltons) that were not present in other hosts (Fig. 6E) were detected in *B. vulgaris* infected with BYV-5. Two of these were also observed in a second set of *B. vulgaris* plants inoculated at a different time with BYV-5 (Fig. 6F). The other two additional segments of dsRNA were not detected in the second experiment, but were detected in the uninoculated plants of *B. vulgaris* used as controls for the second inoculation (Fig. 6H). These results suggest that some of the uninoculated plants of *B. vulgaris* and those inoculated with BYV may have contained a latent virus, but this does not entirely explain the detection of prominent additional dsRNA segments in this host compared to other hosts infected with BYV.

The dsRNAs recovered from *C. perfoliata* infected with two isolates of BYV (BYV-B and BYV-5) were the same (Fig. 6A and B). Results for these two strains in *B. vulgaris* were not the same, (Fig. 6F and G). Extracts from *B. vulgaris* infected with either strain did, however, contain all the dsRNAs illustrated in Fig. 2C. The pattern of dsRNAs expected for CTV infections was detected in extracts from *C. sinensis* bud inoculated with each of five different isolates of CTV that had been isolated by aphid transmission and designated "typical" based on reactions in grapefruit, lemon, sour orange, and sweet orange seedlings (C. N. Roistacher, *personal communication*).

The effect of harvest time on the quality and quantity of dsRNAs recovered was not critically examined. There was no need to harvest soon after inoculation, however. The results in Figs. 1, 6, and 7 were from plants infected for 1–3 mo (except *C. quinoa*).

The effect of tissue source on the dsRNAs isolated from *C. quinoa* and *B. vulgaris* infected with BYV and *C. medica* infected with CTV was examined. Qualitatively similar results were obtained from all sources tested. Results for BYV were the same in *C. quinoa* from mechanically inoculated leaves with local lesions and from leaves with systemic symptoms produced in plants infected by aphids. The highest yield of dsRNA from *B. vulgaris* infected with BYV was from the youngest elongated leaves (Fig. 7C). The highest yield of dsRNA from *C. medica* infected with CTV was from the bark. The next best source was the youngest elongated leaves (Fig. 7K). These results were paralleled by a similar quantitative distribution of virus particles and antigen. Relative

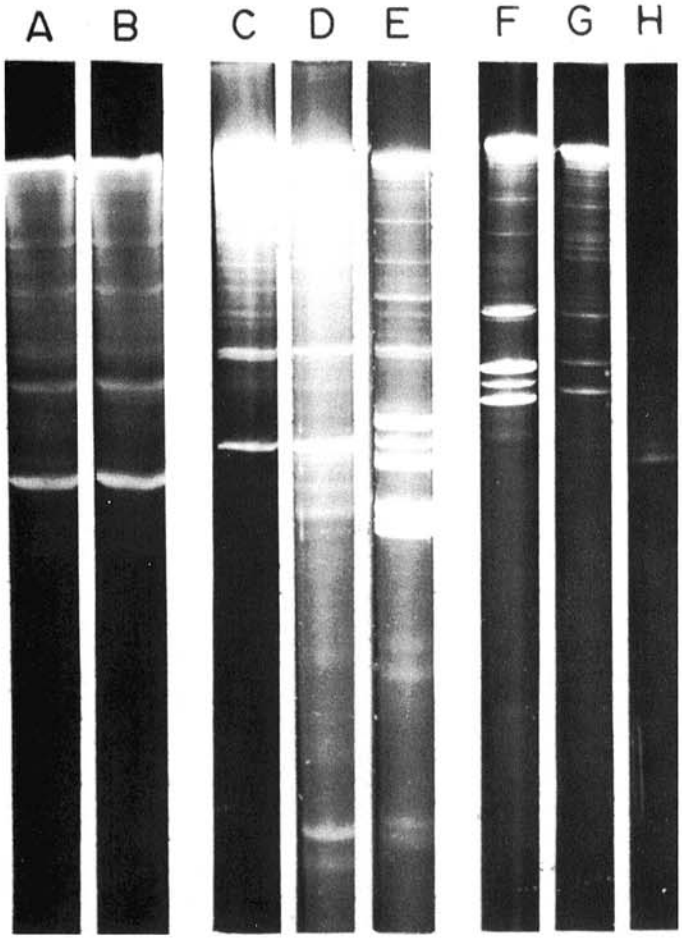


Fig. 6. Effects of host and virus strain on electrophoretically analyzed dsRNAs from plants infected with BYV. *Claytonia perfoliata* infected **A**, with BYV-5 and **B**, with BYV-B. **C**, Saponaria vaccaria, **D**, *Chenopodium quinoa* and **E**, *Beta vulgaris* infected with BYV-5. *Beta vulgaris* infected **F**, with BYV-5 and **G**, BYV-B, and **H**, uninfected. Electrophoresis and staining as in Fig. 1 except that mobility on gels **F–H** was slower than on gels **A–E**.

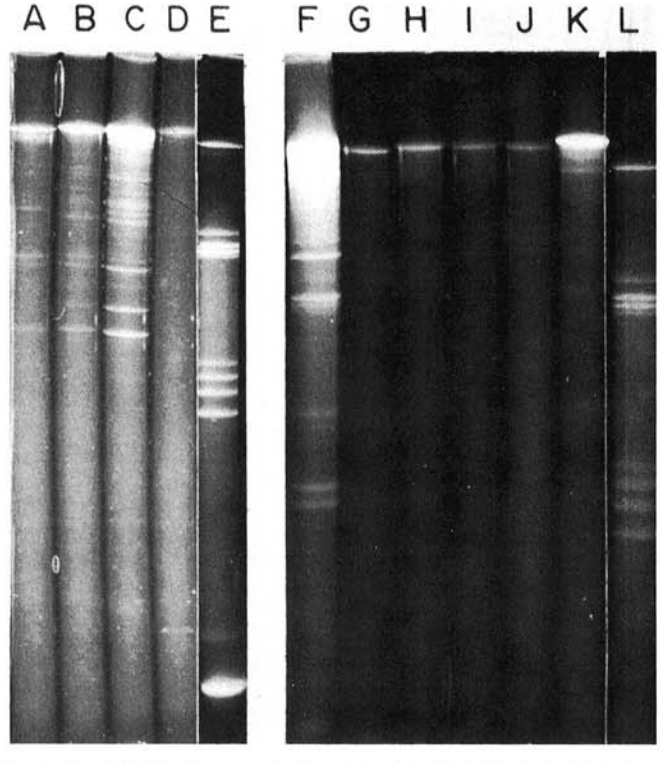


Fig. 7. Effect of tissue source on electrophoretically analyzed dsRNAs from plants of *Beta vulgaris* and *Citrus aurantifolia* infected with BYV-B (channels A–D) and CTV (channels F–K), respectively. Gels contain all the dsRNA recovered from 2.0 g of tissue. The tissues sampled were bark (channel F), the immature leaves (channels D and G), and the fully elongated leaves that were sampled in sets of two (channels A–C) or four (channels H–K), the youngest sets being C and K. Numerical values for the molecular weight standards electrophoresed in channels E and L are as given in Fig. 2. Electrophoresis and staining as in Fig. 1.

recovery of dsRNA from equal weights of tissue (2 g) was assessed by comparing the intensity of fluorescence in stained gels. Alternatively, recovery of dsRNA was assessed by determining the amount of tissue required to produce a sample that did not underload or overload a single polyacrylamide gel. The least amount of tissue required was leaves of *S. vaccaria* infected with either BYV or CNFV (0.2 g required). The following infections required 2.0 g of tissue: CTV in bark of *Citrus* spp. and leaves of *P. gracilis*, and BYV in leaves of *C. quinoa*, *B. vulgaris*, and *C. perfoliata*. The poorest source was ACLSV in *C. quinoa*. Insufficient dsRNA was obtained from 7 g of leaves (the largest amount tested) to analyze optimally for unexpected dsRNA segments of ACLSV.

The RF and other dsRNA segments from leaves of *S. vaccaria* infected with BYV or CNFV and from bark of *C. medica* infected with CTV could also be recovered from the pellet obtained after the first low-speed centrifugation of the virus purification scheme (see Materials and Methods) and which are normally discarded. Recovery from pellets or bark was quantitatively and qualitatively similar.

DISCUSSION

The main objective of this research was to describe the RFs of closteroviruses. It was conceivable that the three best estimates of the genome molecular weights of closteroviruses, 2.3×10^6 (ACLSV), 4.6×10^6 (BYV) and 6.5×10^6 (CTV) were members of a polymeric series (x, 2x, 3x, ...) and that replication could have proceeded through a 4.6×10^6 RF for all members of the group. This is clearly not the case and the RFs are of predictable size, twice that of the ssRNA genomes. The RF from CTV-infected tissue is twice as large as the next largest characterized dsRNAs, those from hypovirulent strains of *Endothia parasitica* (13) and from the VLP Hm9 from *Helminthosporium maydis* (6). It will be valuable as a size marker in future studies of dsRNA.

An unexpected result was the detection of multiple dsRNAs with molecular weights lower than that of the expected RF. These dsRNAs were diagnostic for each virus. Unexpected dsRNAs have been detected in tissues infected with several plant viruses (9,11,12,14,16,18). Their role in replication is as yet unclear. None have been as numerous as those detected for closteroviruses. These dsRNAs could be templates for transcription of viral messenger subgenomic ssRNAs (9,16). Were this true, it could logically be expected that many segments would be found in tissues infected with the plant viruses having the largest genomes, the closteroviruses.

The second objective of this study was to assess the value of dsRNA analysis as a diagnostic tool, in this case for a group of viruses that are generally difficult to purify and to transmit mechanically. If the minor dsRNAs are templates for subgenomic ssRNAs, then they are a "fingerprint" of the entire genome. This concept has novel diagnostic significance at a time when much of plant virus diagnosis is based on serology, which exploits only the variation encountered in the part of the genome that codes for the capsid protein.

The detection of a single RF with a molecular weight greater than 8.0×10^6 and a similar, but different, diagnostic pattern of lower molecular weight dsRNAs for three closteroviruses (CNFV, BYV, and CTV) implies that this approach has potential diagnostic value. This conclusion is reinforced by two other observations: the small effects of host and tissue sources on the quality of the result and the ease with which the dsRNAs were purified from small tissue weights (0.2–7.0 g).

The failure of results with ACLSV to resemble the pattern apparent for the other three closteroviruses implies that either some caution has to be exercised in using this approach for diagnosis, or further improvement of the techniques used with ACLSV are still needed, or the inclusion of ACLSV in the closterovirus group is not justified. Indeed, the lack of characteristic vesicles in infected cells introduced a question mark (1) in grouping ACLSV as a typical closterovirus.

The similarity of results from tissue or from the low-speed pellets normally discarded during this virus purification procedure suggests that the dsRNAs detected are associated with a particulate fraction, which could be further purified, but this is not the fraction that contains virus. This observation makes it possible in the case of closteroviruses to purify both the virus and the dsRNA from a single tissue source.

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