Isolation of Tomato Yellow Leaf Curl Virus, a Geminivirus

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ABSTRACT

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The tomato yellow leaf curl virus (TYLCV) was purified from infected tomato (*Lycopersicon esculentum*) and datura (*Datura stramonium*) plants. Typical twinned particles, characteristic of members of the geminivirus group, were detected in partially purified preparations obtained from TYLCV-infected source plants. Virus-containing fractions were infective as confirmed by membrane transmission tests with the whitefly vector *Bemisia tabaci*. A circular single-stranded DNA containing about 2,800 nucleotides was isolated from the infective virus preparations.

Additional keywords: electron microscopy, plant single-stranded DNA virus.

Tomato yellow leaf curl virus (TYLCV) is a severe viral disease of tomato crops in the eastern Mediterranean basin. The disease has been reported in Israel (5), Jordan (1), Lebanon (14), Sudan (11), and Tunisia (2). All commercial tomato varieties (*Lycopersicon esculentum* Mill.) are susceptible to this disease.

TYLCV is transmitted by the whitefly *Bemisia tabaci* Genn. and fails to infect plants when inoculated mechanically (6). Electron microscopic observations of thin sections from TYLCV-infected tomato leaves indicated that geminilike particles were located in the nuclei of phloem parenchymal cells (2,16).

This is the first report on the isolation of the viral agent causing the tomato yellow leaf curl disease.

MATERIALS AND METHODS

Maintenance of whiteflies and virus cultures. Virus cultures were maintained in *Datura stramonium* L. and inoculations were done using the whitefly vector *B. tabaci* (5). Whiteflies were maintained on cotton plants (*Gossypium hirsutum* L.) grown in muslin-covered cages held in an insectary greenhouse at temperatures of 26–32 C. Datura or tomato plants were inoculated at the two-leaf stage and then sprayed with Senprotathrin (Smash). All plants were grown in an insectproof greenhouse and sprayed biweekly with Smash.

Virus purification. Datura or tomato plants 30–45 days after inoculation (1–2 wk after symptoms appearance) were the source of tissue for virus extraction. Purification procedures were adapted from methods developed for other geminiviruses (4,7,8). All steps in purification were carried out at 4 C after initial homogenization of tissues.

In Method I, leaves were frozen in liquid nitrogen, crushed to a fine powder, and homogenized in a Waring blender after adding ice-cold buffer containing 100 mM trisodium citrate, 18.5 mM ascorbic acid, 60 mM sodium sulfite, 5 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, and 1% (v/v) 2-mercaptoethanol (buffer A) (2 ml/g of tissue). The homogenate was made 2.5% (v/v) in Triton X-100, stirred overnight, squeezed through four layers of cheesecloth, and clarified by a 10-min centrifugation at 8,000 g. The supernatant was centrifuged for 3 hr at 90,000 g in a SW28 Beckman rotor. Pellets were suspended in a buffer containing 10 mM trisodium citrate, 1 mM EDTA, pH 8.0, and 0.1% (v/v) 2-mercaptoethanol (buffer CEM) and submitted to a similar low-speed centrifugation followed by a high-speed centrifugation. Pellets were suspended in 10% (w/v) sucrose in CEM (1 ml of buffer per equivalent of 100 g of leaves), homogenized with a teflon-glass homogenizer, and clarified by a 10-min centrifugation at 4,000 g. The supernatant was applied to a 32-ml, linear, 10–50% (w/v) sucrose gradient in CEM (extracts obtained from 100 g of tissue were applied on each gradient). The gradients were fractionated (2 ml per fraction) after being subjected to a 14-hr centrifugation at 90,000 g in the SW28 rotor.

In Method II, leaf powder treated with liquid nitrogen was stirred overnight in a buffer containing 0.1 M sodium phosphate, pH 7.0, 2.5 mM EDTA, 10 mM sodium sulfite, 0.1% (v/v) 2-mercaptoethanol, and 1% (v/v) Triton X-100 (3 ml/g of tissue). The extract was filtered through muslin and the resulting sap was clarified by the addition of 10% (v/v) cold chloroform followed by a 10-min centrifugation at 8,000 g. The supernatants were loaded on 20% (w/v) sucrose cushions and centrifuged for 3.5 hr at 90,000 g in a Sorvall AH627 rotor. Pellets were resuspended in 0.1 M phosphate buffer, pH 7.0, containing 2 mM EDTA (resuspension buffer), held overnight, and centrifuged 5 min at 4,000 g. Sucrose gradients (10–35%, 15 ml) were prepared in the resuspension buffer. Virus suspension (1 ml) was layered on top of each gradient and centrifuged at 90,000 g for 2.5 hr in a Sorvall AH627 rotor. Gradients were fractionated (1.5 ml per fraction) using a needle connected to a peristaltic pump.

The presence of the virus in the sucrose gradient fractions was confirmed by electron microscopic observations, by the appearance of a specific nucleic acid band after agarose gel electrophoresis, and by infectivity tests. After three dilutions of virus-containing fractions, virus was concentrated by a 3-hr centrifugation at 90,000 g. Pellets were suspended in 10% sucrose-CEM (Method I) or in 0.1 mM phosphate buffer, pH 7.0 (Method II), and stored at −20 C.

Electron microscopy. Virus preparations were stained with either 2% phosphotungstic acid and 1.5% ammonium molybdate or with 2% aqueous uranyl acetate, mounted on carbon-stabilized, Formvar-coated grids, and observed in a JEOL JEM 100-C electron microscope operated at either 60 or 80 kV. Tobacco mosaic virus (18 nm in diameter) was used as size standard. The size of particles was measured on photographs using a reticle magnifier.

DNA molecules were spread using the cytochrome c technique described by Kleinschmidt (12), mounted on Parlodion-coated grids, shadowed with platinum on an Edwards E306 shadower, and examined with the JEOL microscope operated at 60 kV. Plasmid pUC13 (900 nm in size) was used as size standard.

Infectivity assays. Virus preparations were adjusted to 25% (w/v) sucrose and dyed yellow with liquid food coloring before being fed to whiteflies through membranes (3). About 100 whiteflies per cage and five cages per fraction were used for the detection of virus infectivity in sucrose density gradient fractions and in concentrated preparations of virus.

DNA extraction. Viral DNA was extracted from sucrose gradient fractions or from concentrated viral preparations by the phenol-chloroform method, following a 2-hr incubation in 0.2% (w/v) sodium dodecyl sulfate (SDS) and 50 μg/ml proteinase K (15). After ethanol precipitation, DNA was suspended in sterile deionized water and stored at -20 C.

Gel electrophoresis. Electrophoresis of nucleic acids was performed on nondenaturing horizontal gels containing either 1 or 1.5% agarose and 0.5 μg/ml ethidium bromide, in Tris-phosphate-EDTA buffer (15). An EcoRI/HindIII digest of bacteriophage lambda DNA was prepared and used as molecular weight markers. For size estimation of virus DNA, the lambda phage DNA fragments were made single stranded by a 30-min incubation at 70 C in 1.5 M NaOH; after neutralization, the nucleic acids were immediately submitted to gel electrophoresis as described above. Identical results were obtained using denaturing gels (15).

Sensitivity to nucleases. Viral nucleic acid (2 μg of DNA/30-μl reaction) was treated with 2 μg of RNAase A (Worthington), 2 μg of DNAase I (RNAase-free, Worthington) and 2 units nuclease of S1 (Pharmacia), under conditions recommended by the manufacturers.

RESULTS

Virus purification. TYLCV was purified from inoculated tomato and datura plants, according to the procedures described above. Characteristic geminate particles were detected in the middle of the sucrose gradients, in fractions 8–10 out of the 17 from the 10–50% sucrose gradient (Fig. 1A), or fractions 6, 7 out of the 10 from the 10–35% sucrose gradient (Table 1). It is likely that low concentrations of particles, undetectable by electron microscopy, were present in other fractions of the gradients as indicated by their low infectivity in whitefly-mediated transmission tests (Table 1). Geminate structures were never found in mock preparations obtained from uninfected control plants. Spherical particles (about 15 nm in diameter) similar to phytoferritin (7) were observed in the upper fractions of gradients loaded with extracts from either control or infected plants (fractions 14–15 in 10–50% sucrose gradient). The TYLCV geminate particles isolated from infected datura and tomato plants (Fig. 1, C and D, respectively) measured about 20 × 30 nm (the size of more than 60 particles from different preparations was measured).

Fractions from sucrose gradients were fed to whiteflies that were used to inoculate datura test plants. High infectivity rates, leading to typical disease symptoms, were always associated with the presence of a high concentration of geminate particles (Table 1). Preparations obtained after pooling and concentration of virus-containing fractions extracted from either datura or tomato (Fig. 1, C and D) also were infective. Fractions from mock virus preparations (from healthy tomato and datura plants) were always noninfective.

Detection of a virus-associated nucleic acid. The presence of the viral nucleic acid in the sucrose gradient fractions was visualized by gel electrophoresis, after disruption of viral particles by SDS and heat. A unique nucleic acid band appeared in fractions 8–10 of the 10–50% sucrose gradient (Fig. 2A, indicated by an arrow) and was associated with the presence of the geminate particles (Fig. 1A). This band was not detected in control preparations obtained from healthy plants (Fig. 2B) or from tomato plants infected by other viruses such as tobacco mosaic virus, cucumber mosaic virus, potato virus Y, and by an Israeli strain of cowpea mild mottle virus

(results not shown).

Smears of high-molecular-weight nucleic acids were seen in fractions obtained from virus preparations as well as from mock virus preparations, indicating their host origin (Fig. 2, A and B). A heavily stained band of molecular weight lower than that of the TYLCV-specific nucleic acid was present in gradient fractions 10–15 from both control and infected plants (Fig. 2, A and B). The
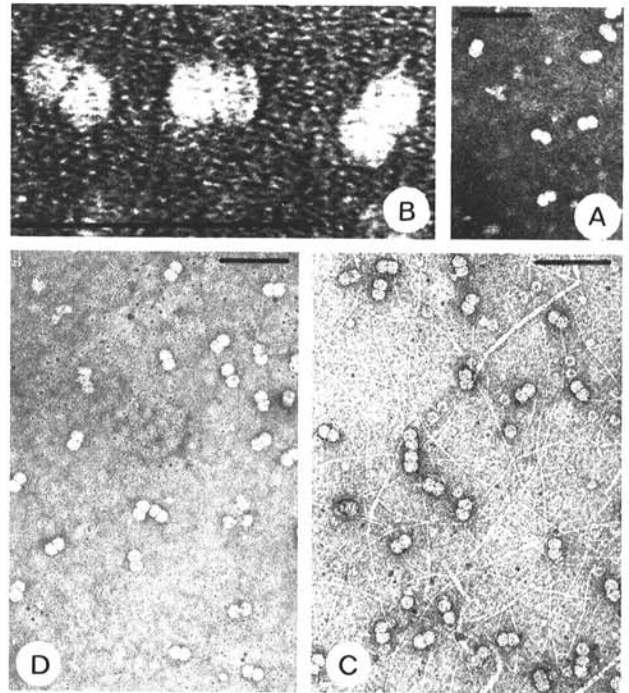

Fig. 1. Electron micrograph of tomato yellow leaf curl virus (TYLCV) particles purified from infected datura and tomato plants. **A,** Geminate particles partially purified from infected datura plants in fraction 8 from a 10–50% sucrose gradient (see Fig. 2A); **B,** Higher magnification of geminate particles; **C,** Concentrated preparation of TYLCV from datura; **D,** Concentrated preparation of TYLCV from tomato. Note the presence of rods of various diameter in C; rods are found in some preparations but are not related to TYLCV infectivity. Particles were stained in 2% uranyl acetate. Solid black line represents 100 nm.

TABLE 1. Correlation between the presence of gemini particles and infectivity in *Bemisia tabaci*-mediated transmission tests[a]

Sucrose gradient fractions (top to bottom)	Geminate particles[b]	Infectivity[c]
1	−	0/5
2	−	1/5
3	−	1/5
4	−	1/5
5	−	1/5
6	+	4/5
7	+	5/5
8	−	1/5
9	−	1/5
10	−	0/5

[a] Distribution of infectivity and tomato yellow leaf curl virus particles in a 10–35% sucrose gradient (Method II). Representative data obtained from one of five independent experiments.

[b] Electron microscope analysis. (+) represents detectable amounts of gemini particles (Fig. 1A); (−) means that particles could not be detected.

[c] Results obtained from membrane transmission tests. Samples from sucrose gradient fractions were applied to membranes (five membranes for each fraction). About 100 whiteflies were introduced to each membrane for an acquisition period of 16 hr and then transferred to *Datura stramonium* test plants for a 48-hr inoculation feeding. Symptoms were recorded 3 wk following the inoculation feeding. Numerator = number of infected plants; denominator = number of plants tested.

intensity and size of this band was considerably reduced after incubation of the fractions for 1 hr at 50 C in 0.3% SDS and 0.1 mg/ml proteinase K, suggesting protein-associated nucleic acid of host origin. The TYLCV-specific band was not affected by this treatment.

Characterization of the virus-associated nucleic acid. The virus-associated nucleic acid was extracted from concentrated virus preparations. Purified nucleic acids were treated with RNAase A, DNAase I, and nuclease S1 before gel electrophoresis. The results demonstrated the resistance of the TYLCV-associated nucleic acid to RNAase A and its sensitivity to DNAase I and to nuclease S1 (Fig. 3), indicating its single-stranded-DNA nature.
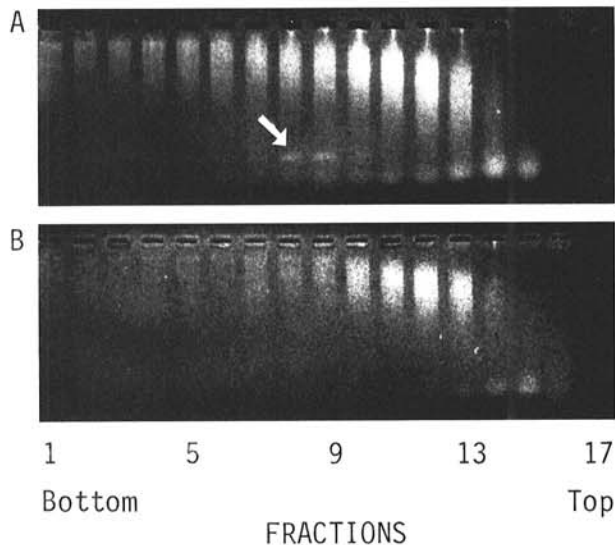
The molecular weight of the TYLCV-associated DNA was estimated by electrophoresis on agarose gels using single-stranded-DNA size markers. When the mobility of standard nucleic acids was plotted against their size, it was found that the TYLCV single-stranded DNA contained about 2,800 nucleotides (Fig. 4).

Electron microscopic spreads of DNA preparations obtained from purified TYLCV demonstrated the presence of circular and linear molecules (Fig. 5). Out of the 193 molecules examined, 89

Fig. 2. Distribution of nucleic acid associated with tomato yellow leaf curl virus (TYLCV) in 10–50% sucrose density gradient fractions. Each fraction was incubated 10 min in 0.5% sodium dodecyl sulfate at 65 C before electrophoresis in 1% agarose gel. **A,** Fractions of TYLCV viral preparations from infected datura plants; **B,** Fractions of mock virus preparations. Arrow indicates the presence of the TYLCV-specific nucleic acid band.
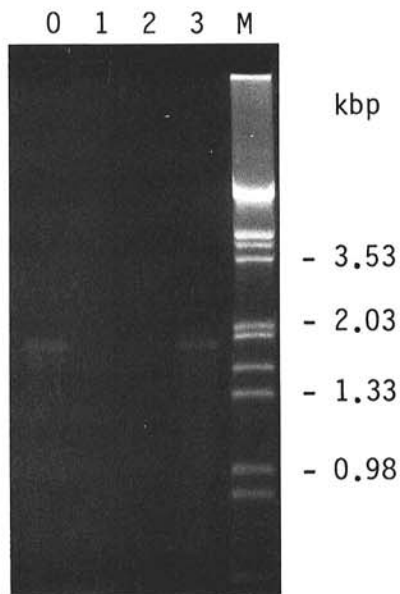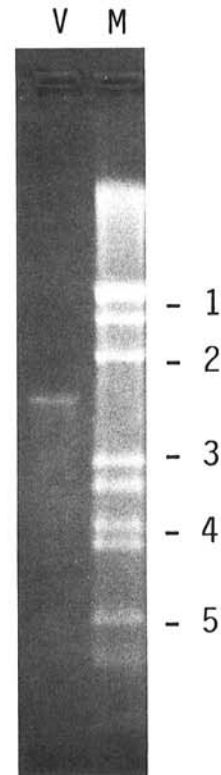

Fig. 4. Size estimation of the tomato yellow leaf curl virus (TYLCV) DNA by electrophoresis on a 1.5% agarose gel, using single-stranded-DNA markers. **V,** TYLCV DNA isolated from datura; **M,** EcoR1/Hind III phage lambda DNA digest made single stranded after incubation in alkali. Sizes are in nucleotides: 1 = 5,298; 2 = 3,530; 3 = 2,027; 4 = 1,584; 5 = 983.
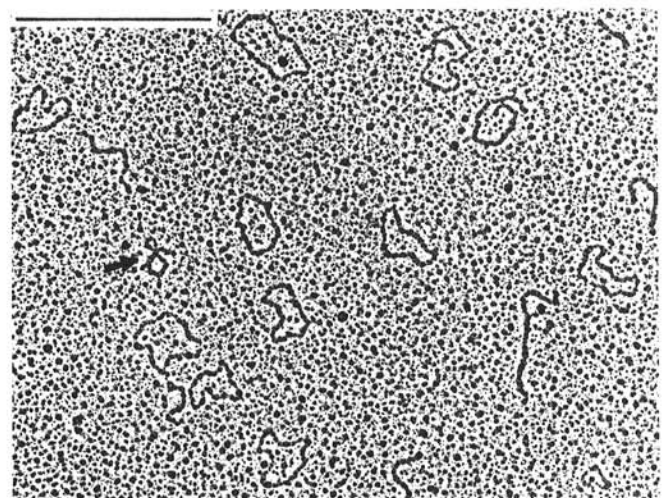

Fig. 3. Nuclease treatment of nucleic acid associated with tomato yellow leaf curl virus (TYLCV). Nucleic acids were incubated with nucleases before electrophoresis on a 1.5% agarose gel. **0,** Untreated nucleic acid preparation; **1,** DNAase I treatment; **2,** S1 nuclease treatment; **3,** RNAase treatment; **M,** Size marker consisting of an EcoR1/Hind III bacteriophage lambda DNA digest; sizes are in kilobase pairs (kbp).

Fig. 5. Electron micrograph of spread tomato yellow leaf curl virus (TYLCV) single-stranded DNA molecules extracted from TYLCV-infected datura viral preparations. Note the presence of linear and circular molecules. Arrow indicates half-size subgenomic DNA. Solid black line represents 1 μm.

were circular (46%) while 104 were linear (Fig. 6, A and B). The circular molecules appeared as two populations of defined size. Seven of the circular molecules were about 400 nm in size (about 8%), while the bulk had a size of about 850 nm (Fig. 6A). The linear molecules were more heterogeneous in size. Their length distribution suggests that a large fraction of these molecules are linearized single-stranded DNA circles (Fig. 6B).

DISCUSSION

Using the purification procedures described in this work, we found that datura plants served as a more reliable source for virus purification than tomato plants. In tomato, yields are more dependent on the age of the plant than in datura (younger plants give higher amounts of virus) although virus particles were purified from both sources (Fig. 1, C and D). Similar amounts of geminate particles were obtained by either purification procedure, as monitored by electron microscopic observation. However, viral nucleic acid yields were higher from particles purified by Method I than by Method II (15–40 vs. 3–7 µg of DNA/100 mg of leaves). Method II yielded viral preparations of higher purity, as indicated by electron microscopy. Geminate particles obtained by Method I were sometimes contaminated by host cell debris.

Our results indicate that the TYLCV is a twinned particle of 20× 30 nm (Fig. 1B) which is characteristic of the geminivirus group (see References 10 and 13 for recent reviews).

Infectivity tests of the virus-containing fractions (from both tomato and datura plants) demonstrated the association of virus particles with the typical tomato yellow leaf curl disease symptoms. The peak of infectivity (100% of test plants infected) coincided with the presence of detectable viral particles in the sucrose gradient fractions (Table 1). Low infectivity rates (no more than 20% of test plants infected) were exhibited by some fractions where particles could not be detected by either electron microscopy or nucleic acid analysis. This suggests that the whitefly vector is capable of acquiring virus from such preparations, concentrating the virus in

its body, and transmitting the disease to susceptible test plants.

A unique nucleic acid, detected by agarose gel electrophoresis, was extracted from sucrose gradient fractions containing geminate particles (Figs. 1A and 2A). This nucleic acid was not found in mock virus preparations. The TYLCV-associated nucleic acid was sensitive to DNAase I and to nuclease S1, indicating its single-stranded-DNA nature (Fig. 3). It migrated in gel electrophoresis as a single band with a mobility corresponding to a molecule 2,800 nucleotides long (Fig. 4). Electron microscopic observation demonstrated that the TYLCV-associated nucleic acid is circular (Fig. 5). All these features are shared with other viruses from the gemini family (10,13). A small fraction (about 8%) of the circular TYLCV DNA were molecules 400 nm in size, compared to 850 nm for the large molecules (Fig. 6A). A similar phenomenon has been reported for tomato golden mosaic virus (9) and cassava latent virus DNA (17). The small molecules were found to be subgenomic viral DNA derived from the large ones by a deletion process. Subgenomic viral DNA was not infective (17).

Preliminary results support the conclusion that the nucleic acid associated with the TYLCV gemini particles is indeed the TYLCV genome. When used as a probe, radiolabeled TYLCV DNA hybridized specifically with TYLCV-infected datura and tomato nucleic acid preparations, but not with DNA prepared from healthy plants (Czosnek et al, *unpublished*). Our data on the mode of TYLCV transmission, particle morphology, and properties of TYLCV nucleic acid confirm that TYLCV is a whitefly-transmitted geminivirus.

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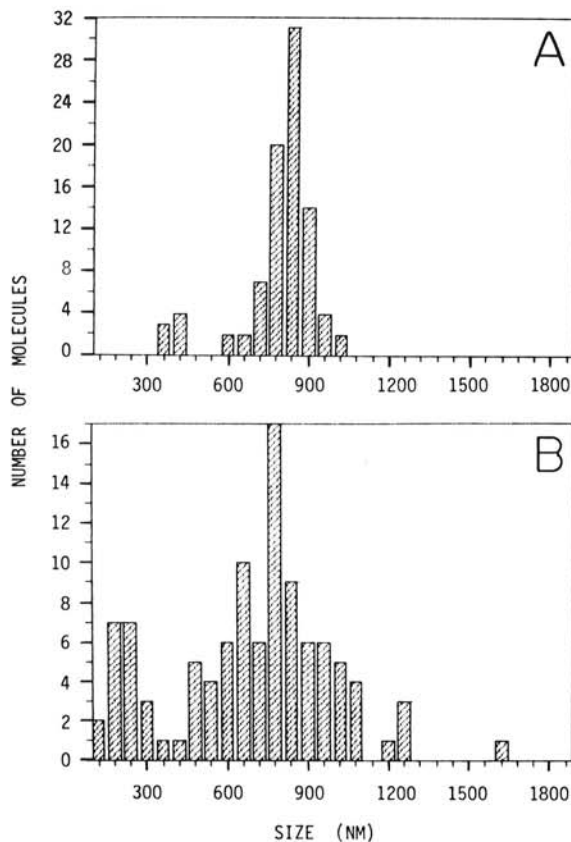
Fig. 6. Length evaluation of spread tomato yellow leaf curl virus single-stranded DNA molecules. **A,** Circular molecules. **B,** Linear molecules.

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