Isolation and Characterization of a Rod-Shaped, Whitefly-Transmissible, DNA-Containing Plant Virus

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ABSTRACT

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A whitefly-transmissible virus, cucumber vein yellowing virus (CVYV), was purified and characterized. The virus is unstable, forms various aggregates, and its infectivity and particle integrity depend greatly on the ionic strength of the medium used in extraction and purification. The virus particles are rod-shaped (740–800 × 15–18 mm) and have a sedimentation coefficient of about 220 S. CVYV consists of double-stranded DNA and a single proteinaceous subunit (39,000 daltons). These characteristics indicate that CVYV is a type of virus that has not been described previously.

The cucumber vein yellowing disease severely affects plants of the family *Cucurbitaceae,* inflicting great economic losses (4). The causal agent is transmitted in a semi-persistent manner by the whitefly *Bemisia tabaci* Gennadius (3,10) and also is transmissible by sap inoculation. Both types of transmission are inefficient. The viral nature of the causative agent was assumed on the basis of this data, and it was designated cucumber vein yellowing virus (CVYV).

The present work was initiated to verify the viral nature of the causative agent of the cucumber vein yellowing disease, to isolate the virus and to characterize it. Our results showed that CVYV is a new type of plant virus, and probably a new type of virus in general.

MATERIALS AND METHODS

Virus cultivation. Cucumber seedlings were inoculated mechanically when cotyledons were fully expanded, but before expansion of the first true leaf. Symptoms appeared after 10–12 days at 25 C and usually were visible on the second true leaf.

Infectivity. Frozen infected leaves were stored at −20C and retained their infectivity for a few weeks. The infectivity of sap in various buffers, and of purified virus preparations, either frozen or at 4 C never persisted more than a few days, and usually was lost in a few hours. Levels of infectivity were expressed as the percentage of at least 50 inoculated plants that became infected.

Electron microscopy. CVYV, at various stages of purification, was mounted on carbon coated collodion-filmed grids and negatively stained with 2% potassium phosphotungstate (PTA). The integrity of the particles and the quality of the electron micrographs were greatly dependent on the pH. The best results were obtained when the sedimented virus was resuspended in 0.1 M acetate buffer, pH 6.0, and stained with PTA at the same pH. Particle lengths were determined with TMV as an internal standard.

Polyacrylamide gel electrophoresis. The CVYV-protein was electrophoresed in cylindrical gels of 5% polyacrylamide in 36 mM tris-phosphate buffer pH 7.3 containing 0.2% SDS. Electrophoresis was performed for 3 hr at 5 mA/gel. The molecular weight of the CVYV protein was determined from the linear-log relationship between the distance of electrophoretic migration of proteins and their molecular weight. The following proteins served as molecular-weight markers: TMV capsid-protein (17,500), elastase (25,000), pepsin (35,000) and reduced bovine serum albumin (64,000). Gels were stained for proteins with Coomassie brilliant blue according to Fairbanks et al (6).

The nucleic acid of CVYV was electrophoresed in slab-gels composed of 0.5% agarose and 1.7% polyacrylamide and stained with "stains all" according to Dahlberg et al (5).

RESULTS

Some properties of CVYV and procedures for its purification. When CVYV-infected leaves were ground in water and centrifuged at 5,000 *g* for 10 min, most of the infectivity was found in the pellet. However, when the leaves were homogenized as described below in 0.01–0.2 M buffers (tris, glycine, and borate) at pH values around 9.0, the 5,000 *g* supernatant was infective, and viruslike particles, resembling those in Fig. 1, were detected in drops of the supernatant fluids. These preparations, however, were unstable and infectivity was lost within a few hours at 4 C. Moreover, upon further centrifugation of the supernatant fluid at 30,000 *g* for 10 min, infectivity was pelleted almost entirely. This result indicated

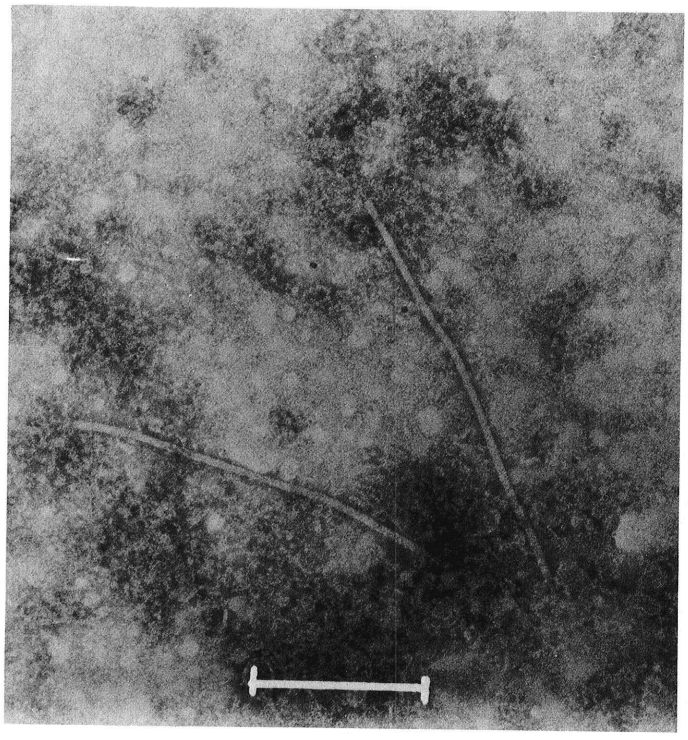

Fig. 1. Electron micrograph of a cucumber vein yellows virus preparation purified by two cycles of differential centrifugation. The preparation was negatively stained with 2% sodium phosphotungstate, pH 6.0. The bar represents 0.25 μm.

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another form of aggregation. Indeed, scans of these preparations after sedimentation into sucrose gradients (10–40%, 50,000 g; 4 hr) showed polydispersity and were variable.

The effect of various buffers and additives on the infectivity and homogeneity of CVYV was tested. The best medium found for purification was 0.04 M tris-HCl buffer, pH 8.7 containing 0.05 M EDTA and 0.1% 2-mercaptoethanol (purification buffer). When prepared in the purification buffer CVYV did not pellet rapidly at 30,000 *g*, exhibited size-homogeneity (Fig. 2), and retained its infectivity for several days at 4 C. The infectivity of CVYV preparation reduced rapidly with an increase in the ionic strength of the buffer. For example, in 0.08 M tris-HCl, the virus sedimented at 30,000 *g* and lost infectivity in 24 hr at 4 C. In 0.3 M NaCl, infectivity was lost in 3 hr. Neither infectivity nor virus particles were detected in any fraction following CsCl gradient centrifugation. CVYV was precipitated by 0.6% or higher concentrations of polyethylene glycol (PEG) without the addition of NaCl. However, it was impossible to resuspend CVYV from PEG-pellets, and it did not form a band in the PEG-sucrose gradient technique of Clark and Lister (2).

On the basis of these results, the following differential centrifugation method was employed for purification. Two hundred grams of symptomatic leaves were homogenized in a Waring Blendor in 400 ml of purification buffer, and squeezed through four layers of cheesecloth. The expressed sap was centrifuged at 5,000 *g* for 10 min. The supernatant fluid was diluted with an equal volume of purification buffer and centrifuged at 30,000 *g* for 10 min. The 30,000-*g* supernatant fluid was then centrifuged at 100,000 *g* for 60 min. The pellet was covered with a small volume of purification buffer and left overnight at 4 C. After gentle shaking, the resuspended virus was centrifuged again at 5,000 *g*, and the cycle of differential centrifugation was repeated. The final preparation was analyzed on sucrose gradients and exhibited a reasonable size-homogeneity as shown in Fig. 2.

Electron microscopy. Freshly purified virus was required for electron microscopy, because the virus disintegrated upon storage. Particles resembling those shown in Fig. 1 were readily visible at all stages of purification. With the use of TMV as internal standard, the length of most virus particles at pH 6.0 was found to be between

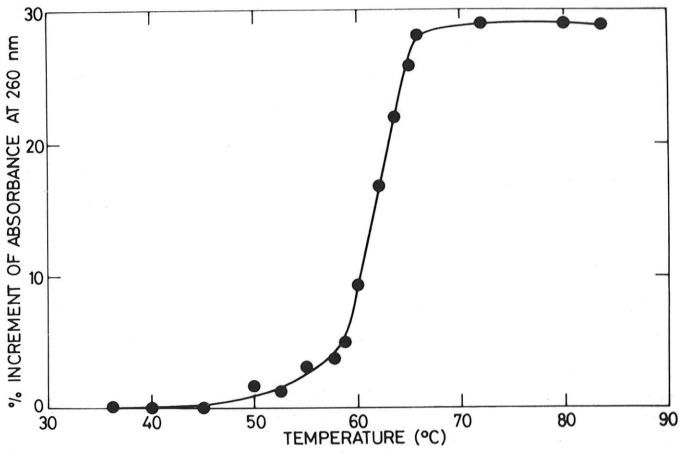

Fig. 4. Temperature melting curve of the nucleic acid of cucumber vein yellowing virus in 0.01 × SSC.

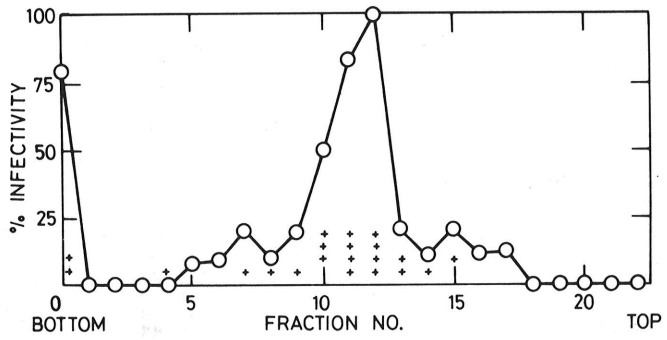

Fig. 2. Distribution of cucumber vein yellows virus (CVYV) infectivity in a sucrose density gradient. A preparation of CVYV purified by two cycles of differential centrifugation was centrifuged for 4 hr at 50,000 *g* at 4 C on a density gradient column of 100 to 400 mg of sucrose per milliliter in 0.04 M tris-HCl buffer, pH 8.7, containing 0.05 M EDTA and 0.1% 2-mercaptoethanol. After centrifugation, the gradient was fractionated into 0.5-ml fractions and the infectivity of each fraction was determined. Sedimentaion is from right to left. The numbr of plus signs indicate the relative abundance of virus particles observed in each fraction in the electron microscope.

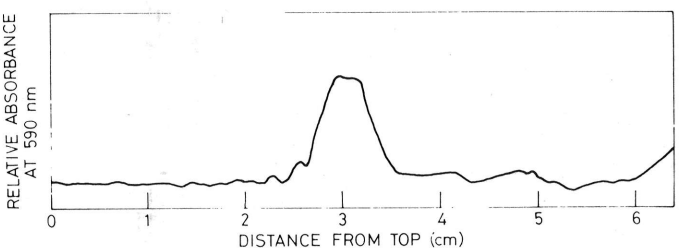

Fig. 3. Absorbance scan at 590 nm of a 5% polyacrylamide gel after electrophoresis of cucumber vein yellows virus dissociated in 0.2% SDS, 6 M urea, and 0.1% 2-mercaptoethanol. The gel was stained with Coomassie Blue and destained in 10% acetic acid: 10% iso-propanol.

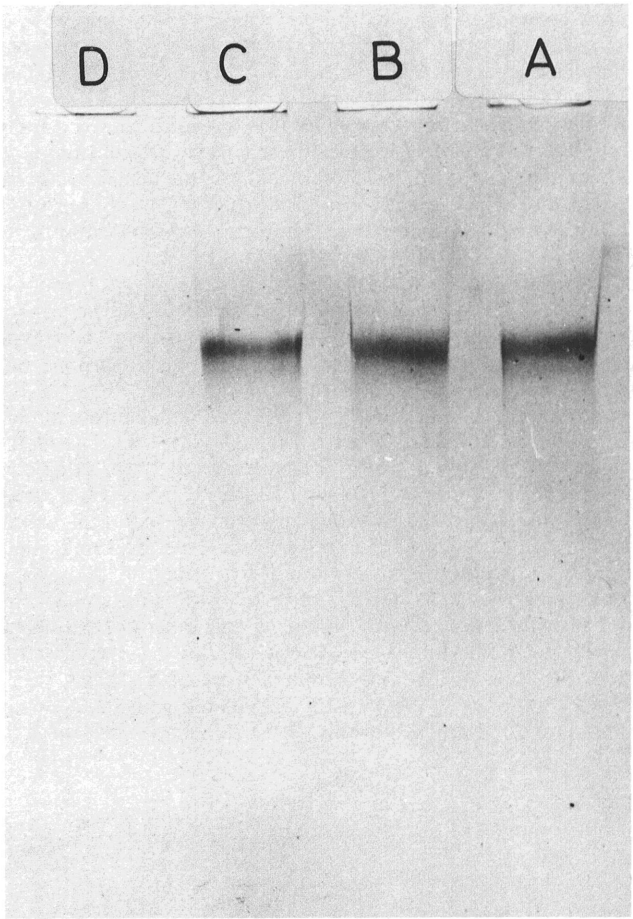

Fig. 5. Agarose-polyacrylamide slab gel electrophoresis of the nucleic acid of cucumber yellows vein virus. **A** and **B,** Untreated preparation at 3 and 1 μg, respectively. **C,** Ribonuclease-treated preparation. **D,** Desoxyribonuclease-treated preparation.

740–800 nm, but particles as long as 900 nm were observed. The diameter of the particles was 15–18 nm.

The virus and its components. When CVYV was centrifuged in sucrose gradients as in Fig. 2 with TMV (180 S) and tobacco ribosomes (80 S) as internal standards, the sedimentation coefficient of the virus was estimated to be 220 S, by assuming a linear relationship between distance of sedimentation and sedimentation coefficient.

When purified CVYV was brought to 0.2% with SDS, 6 M urea, and 0.1% 2-mercaptoethanol, the opalescent solution cleared immediately. A sample of the cleared disrupted virus solution was electrophoresed in 5% polyacrylamide gels. Protein molecular-weight markers were electrophoresed on sister gels. After electrophoresis, proteins were stained with Coomassie blue and the gels were scanned at 590 nm. With CVYV a single protein band of about 39,000 daltons was observed (Fig. 3).

Another portion of the purified virus preparation was made 1% with SDS and extracted with an equal volume of phenol. When ethanol was added to the aqueous phase of the phenol extract, a sieve-like material formed immediately. This material was wound on a thin glass-rod, gently transferred to another test tube, washed three times with 70% ethanol, and redissolved in water. The solution obtained exhibited an ultraviolet spectrum typical of nucleic acids.

Melting temperature curves were obtained with solution (A_{260} = 0.6) of the nucleic acid in 0.01 × SSC (1.5 mM NaCl; 0.15 mM Na-citrate). Absorbance readings were taken in a temperature cell in a Perkin-Elmer spectrophotometer. The melting curve of the nucleic acid of CVYV (Fig. 4) is typical of a double-stranded nucleic acid structure. The T_m in 0.01 × SSC was 61 C.

The nucleic acid of CVYV reacted positively in the diphenylamine test (14) and negatively with orcinol test (1).

The DNA nature of the nucleic acid of CVYV was verified as follows. Samples of the nucleic acid were incubated in 0.01 M tris-HCl, 0.01 M $MgCl_2$, pH 7.6 with 10 μg/ml of pancreatic ribonuclease or deoxyribonuclease (both from Sigma) for 30 min at 37 C. An untreated control sample of the preparation was incubated similarly but without enzyme. Control and enzyme-treated preparations were then electrophoresed in agarose-polyacrylamide slab gels and stained with "stains-all". The nucleic acid band stained blue like a double-stranded structure, was insensitive to ribonuclease but was destroyed completely by deoxyrobonuclease (Fig. 5).

Infectivity of CVYV-DNA. Many attempts to inoculate cucumber seedlings with CVYV-DNA failed. However, when the method of Graham and Van der Eb (9) for assaying adenovirus-DNA-infectivity was employed, with some modifications, some inoculated plants were successfully infected.

The DNA of CVYV (A_{260} = 10) was dialysed against a modified TBS buffer (0.8 NaCl; 0.04% KCl; 0.012% $Na_2HPO_4 \cdot 2\,H_2O$; 0.1% glucose; 0.2% $MgCl_2$, 0.3% tris-base, pH 8.5). A sample was incubated for 30 min at 37 C with 10 μg/ml DNase, and another sample was incubated without the enzyme. After incubation, calcium chloride was added to 0.1 M, and the resulting calcium phosphate precipitate was allowed to settle for 10 min. The suspensions were shaken well, and while still being shaken, were applied with a wide-mouth pipette to cucumber cotyledons, and rubbed gently onto the leaves with a sterile glove. At the second-leaf stage, 8 of the 45 plants inoculated with CVYV-DNA became infected, but none of the 48 plants inoculated with DNase-treated material showed any symptoms.

DISCUSSION

The results presented here show that CVYV is a rather unstable rod-shaped virus, containing double-stranded DNA. It is, therefore, unique among plant viruses, since the other DNA-containing plant viruses exhibit spherical symmetry (8,11,13). Only a few whitefly-transmitted viruses have been purified to date. The sweet potato mild mottle virus, described by Hollings and Stone (12), is rod-shaped. However, this virus apparently contains RNA and certainly differs from CVYV in host-range (17 plant species, including several *Nicotiana* spp.). Golden bean yellow mosaic virus occurs as small paired spherical particles and apparently contains circular, single-stranded DNA (8,11).

The tentative cryptogram of CVYV (15) is D/2;-/-;E/E;S/Al. A search in virus catalogues (7,13) revealed no bacterial, invertebrate, plant, or animal virus with the properties of CVYV. Therefore, CVYV is probably a new type of virus.

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