Identification of Cucumber Mosaic Virus from *Vinca minor* in New Jersey

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ABSTRACT

Smith, D. B., and Davis, R. F. 1986. Identification of cucumber mosaic virus from *Vinca minor* in New Jersey. Plant Disease 70: 1056-1059.

Mild mosaic, chlorosis, and distortion symptoms were observed on leaves of *Vinca minor* in Somerset, NJ. A virus was isolated from these plants and identified as cucumber mosaic virus (CMV) based on host reactions, aphid transmissibility in a nonpersistent manner, icosahedral particle morphology, molecular weight of 29,000 Da of coat protein subunits, and serological relationships. This isolate is designated CMV-Vi. Mouse ascites fluid produced to CMV-Vi gave specific and sensitive reactions in ELISA and Ouchterlony gel double-diffusion tests. Field surveys indicated that CMV is present throughout New Jersey in *V. minor*.

Vinca minor L., also known as trailing myrtle and common periwinkle (2), is a popular perennial ground cover. Its attractive low-growing habit, tolerance of poor conditions, and relatively few insect pests have made *V. minor* one of the three main ground cover plants in New Jersey. E. H. Varney (*personal communication*) has observed symptoms in an urban planting of *V. minor* repeatedly for several years, and Lee (12) tentatively identified cucumber mosaic virus (CMV) from these plants based on host range, morphology, and physical properties.

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New Jersey Agricultural Experiment Station Publication D-11191-1-86; supported by state and by U.S. Hatch Act funds.

Accepted for publication 7 July 1986 (submitted for electronic processing).

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CMV is an icosahedral virus with an extremely wide host range (9). This virus is distributed worldwide and is responsible for many diseases of economic importance. Wild hosts, especially perennial species, play an important role in the epidemiology of CMV by serving as reservoir hosts for aphid transmission. We hypothesized that *V. minor* serves as a primary reservoir host of CMV and is the original source of virus for CMV diseases occurring in the vicinity of this host. In support of this hypothesis, we report the identification and characterization of CMV from *V. minor*, its natural transmission from this host by aphids, and the wide distribution of infected *V. minor* in New Jersey.

MATERIALS AND METHODS

Virus isolate and maintenance. The virus isolate used in this study was obtained from leaves of *V. minor* plants showing symptoms of virus infection in Somerset, NJ. The isolate was passed through three single local-lesion transfers on squash (*Cucurbita pepo* L. 'Multipik') and subsequently maintained on the same host.

Host range and symptomatology. Inoculum for host range studies was prepared by grinding infected leaves of Multipik squash in 0.01 M sodium phosphate buffer, pH 7.0. The homogenate was applied with a cotton-tipped swab to a range of test plants dusted with 400-mesh Carborundum. Test plants were placed in an insect-screened greenhouse and observed for symptom development for 3 wk.

Purification. The CMV isolate from *V. minor* (CMV-Vi) was purified using the procedure described by Lot et al (13), with modificiations (7). Multipik squash tissue was collected 10–14 days after inoculation and homogenized in two volumes each of 0.5 M citrate buffer, pH 6.5, plus 0.1% sodium thioglycollate, and chloroform. After PEG precipitation, pellets were resuspended in 0.05 M sodium citrate buffer, pH 7.0. After differential centrifugation, 0.02 M disodium ethylenediaminetetraacetate (EDTA) buffer, pH 7.0, was used to resuspend pellets. Virus was further purified on 10–40% sucrose density gradients prepared in 0.02 M EDTA buffer, pH 7.0.

Electron microscopy. Purified virus concentrated at 1 mg/ml (9) was treated with formaldehyde to a final concentration of 1% (v/v) and applied to 400-mesh Formvar-coated copper grids stabilized with evaporated carbon. Tobacco mosaic virus (TMV) concentrated at 0.1 mg/ml was included as an internal particle size standard. Phosphotungstic acid, pH 3.0, at 2% (w/v) was used as a negative stain.

Electrophoresis. Molecular weight of the coat protein subunits was determined by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using the method of Maizel (14). The 12% resolving gel was allowed to polymerize overnight, and the

6% stacking gel was poured 1 hr before use. Purified CMV-Vi at 1 mg/ml was diluted 1:5 (v/v) and protein standards at 2 mg/ml were diluted 1:10 (v/v) in 0.06 M Tris buffer, pH 6.7, with 10% glycerol, 2% SDS, 10% mercaptoethanol, and 0.002% bromophenol blue (sample buffer). Protein was dissociated by boiling in sample buffer for 5 min at 100 C. For optimum resolution, about 0.5 μg of protein per lane was applied in a total volume of 3–5 μl of sample buffer. Low molecular weight protein standards (BIO-RAD, Rockville Center, NY) were run simultaneously with CMV-Vi to calculate molecular weight. Protein standards included: phosphorylase B (94,000 Da), bovine serum albumin (68,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (30,000 Da), and soybean trypsin inhibitor (21,000 Da). Electrophoresis was conducted in a Mini-Slab apparatus (Idea Scientific, Corvallis, OR) with gels 83 × 102 × 1 mm at 200V (constant voltage) for about 1.5 hr. Gels were stained with 0.25% Coomassie blue in methanol and water (1:1) with 9% glacial acetic acid for 2.5 hr. Gels were destained with 20% methanol plus 5.8% acetic acid.

Antisera and antibody production. Ascites against CMV-Vi was prepared in Swiss Webster mice, using sarcoma cells to increase the volume of ascites collected (5,16,17). Mice were given a weekly series of four intraperitoneal injections of purified CMV-Vi fixed with 1% formaldehyde and emulsified with Freund's incomplete adjuvant (complete adjuvant was used for the first injection). Two doses of immunogen were compared: 50 and 100 μg of virus per injection. In week five, 500 μl of freshly drained ascites cells (strain E-Erlich Lettre ascites carcinoma maintained in Swiss Webster mice) was injected in the intraperitoneal cavity. An intravenous (IV) booster of virus was administered in the tail 3 days later, followed by a second IV booster after 10 days. The fluid was then drained from the intraperitoneal cavity of each mouse about every 3 days (Table 1). Antiserum to a strain of CMV (CMV-B_{32}) originating in Spain (4) was produced previously (5). Antisera to CMV strains D, S, 9, and C (1,15,19) were provided by H. A. Scott (University of Arkansas, Fayetteville).

Enzyme-linked immunosorbent assay (ELISA). Indirect ELISA was performed as described previously (6). Plates were coated with 1:100 (w/v) dilutions of healthy and infected plant sap. Unfractionated ascites or purified IgG was tested at various dilutions. Goat antimouse IgG alkaline phosphatase conjugate (Sigma Chemical Company, St. Louis, MO) was used at 1:1,000 (v/v) dilution. Each plate contained sap from Multipik squash plants inoculated 1–2 wk previously with CMV-Vi and plants not inoculated (as positive and negative controls, respectively). Three replicates of each sample and control were tested, and results represent the average. Absorbance was read at 405 nm in a Titertek Multiskan MC Spectrophotometer (Flow Laboratories, McLean, VA) after 30 min. For all ELISA procedures in this study, average absorption values at 405 nm greater than three times the healthy control value were considered positive reactions.

IgG was purified from crude ascites fluid by affinity chromatography prepared by suspending 0.5 g of Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) in 0.02 M sodium phosphate buffer (PB), pH 7.3. Ascites fluid was diluted with an equal volume of buffer (1 ml each) and loaded onto the column. Buffer was passed through the column until the absorbance at 280 nm was 0.01 or less. The buffer was changed to 0.1 M glycine, pH 3.0, to elute the IgG. Fractions (1 ml) were collected and neutralized with 40 μl of 2 M Tris buffer, pH 8.5. Peak fractions were pooled and the IgG was precipitated with 0.9 volume of saturated ammonium sulfate, resuspended in PB, and adjusted to an absorbance of 1.4 at 280 nm (11).

Specificity of the ascites produced against CMV-Vi was tested to ensure reliability of positive results. Viruses tested included CMV-Vi from Multipik squash, alfalfa mosaic virus (AMV) from bean (*Phaseolus vulgaris* L. 'Black Turtle 2'[BT-2]), broad bean wilt virus (BBWV) from bean, potato virus X (PVX) from tabasco pepper (*Capsicum frutescens* L.), potato virus Y (PVY) from tobacco (*Nicotiana tabacum* L. 'Samsun NN'), tobacco etch virus (TEV) from tobacco, TMV from Samsun tobacco, tobacco ringspot virus (TobRSV) from bean, and tomato ringspot virus (TomRSV) from tobacco. In addition, CMV strains D, S, C, and 9 from tobacco (1), CMV-Pg from bean (7), and a local isolate from squash (CMV-NJ83-42) were tested to determine the ability of CMV-Vi ascites to recognize other CMV strains. Healthy tissues from each host were included as negative controls.

Immunodiffusion tests. Ouchterlony gel double-diffusion (3) plates were prepared with 0.7% purified agar (Code L28, Oxoid Limited, England), 0.85% sodium chloride, and 0.03% sodium azide dissolved in 0.05 M Tris buffer, pH 7.2. The well pattern consisted of one central and six outer 6-mm wells spaced equidistantly 6 mm apart. Ascites or antiserum was added to the central well without dilution. Outer wells were filled with samples prepared by grinding infected tissue 1:1 (w/v) in 0.05 M Tris buffer, pH 7.2, and straining through cheesecloth.

Aphid transmission. Green peach aphids (*Myzus persicae* Sulz.) were raised on disease-free turnip (*Brassica rapa* L.) plants. After a starvation period of 1–2 hr, aphids were placed on CMV-Vi-infected squash plants and allowed a 2-min acquisition access period. Aphids were then transferred to 10 healthy squash plants for a 10-min inoculation access period. Plants were observed for symptoms after 2 wk in the greenhouse, and leaf samples were taken for testing by an indirect ELISA.

Field survey. A survey was conducted to determine the incidence of CMV in *V. minor* in New Jersey. Thirty-one samples from 15 locations were chosen on the basis of visual symptoms such as mild mosaic, yellowing, and distortion and were tested for CMV by ELISA.

RESULTS AND DISCUSSION

Host range and symptomatology. CMV-Vi was easily sap-transmitted to 12 species in six plant families. *C. pepo*

Table 1. Volume and titer of ascites collected at indicated number of days after first injection of two Swiss Webster mice with 300 and 600 μg of cucumber mosaic virus isolated from *Vinca minor*

Mouse[a]	Day	Volume (ml)	Titer: Microprecipitin[b] (reciprocal)	Titer: ELISA[c] ($A_{405\ nm}$)
1	38	5	64	0.82
	39	7	32	0.42
	41	2	32	1.56
	43	3	16	1.68
	47	5	64	1.92
2	39	8	64	1.43
	41	2	32	1.43
	50	4	256	1.89
	54	2	64	1.21
	57	4	64	1.69

[a] Mice 1 and 2 were infected with a total of 300 and 600 μg of virus, respectively, given in six equal doses, four in the peritoneum (IP) at weekly intervals followed by an IP booster at day 32 and an intraveinous booster at day 43.

[b] The reciprocal of the highest ascites dilution that produced a visible reaction in a microprecipitin test with twofold dilutions of virus from 0.12 to 0.5 mg/ml concentration.

[c] Absorbance value ($A_{405\ nm}$) in indirect ELISA at 1:100 (w/v) dilution of CMV-Vi-infected squash and 1:1,000 (v/v) dilution of both CMV-Vi ascites and antimouse IgG alkaline phosphatase. Absorbance values of healthy were less than 0.1.

'Multipik' was very useful as a diagnostic and propagation host, developing distinct chlorotic local lesions about 1–2 mm in diameter within 5 days followed by systemic mosaic within 7–10 days. Test plants that became only locally infected, developing chlorotic local lesions, were *Beta vulgaris* L. 'Honey Red,' *Chenopodium quinoa* Willd., *Gomphrena globosa* L., and *Vigna unguiculata* (L.) Walp. subsp. *unguiculata* 'Early Ramshorn.' Plants that were infected only systemically, developing mosaic symptoms, included *Catharanthus roseus* L. (G. Dow), *Cucurbita pepo* 'Early Prolific Straightneck,' *Cucumis sativus* L. 'National Pickling Cucumber,' *Datura stramonium* L., *Lycopersicon esculentum* Mill. 'Campbells 142,' *N. tabacum* 'Samsun NN' and 'Xanthi,' *P. vulgaris* 'Black Turtle 2,' and *Physalis floridana* Ryd. Presence or absence of CMV-Vi in both inoculated and uninoculated tissues of these hosts was confirmed by ELISA.

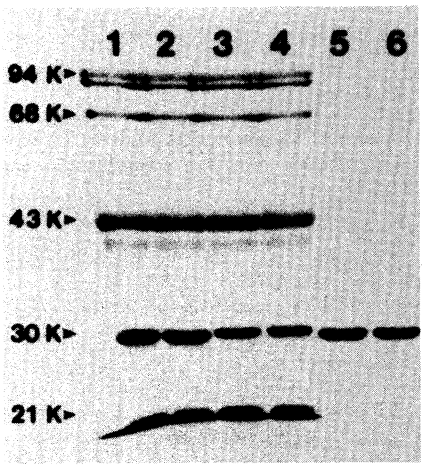

Fig. 1. Analysis of capsid protein of the *Vinca minor* isolate of cucumber mosaic virus (CMV-Vi) by discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Lanes 1 and 2 contain molecular weight protein standards with CMV-Vi, lanes 3 and 4 contain the protein standards alone, and lanes 5 and 6 contain CMV-Vi alone. Molecular weights of the standards are shown on left.

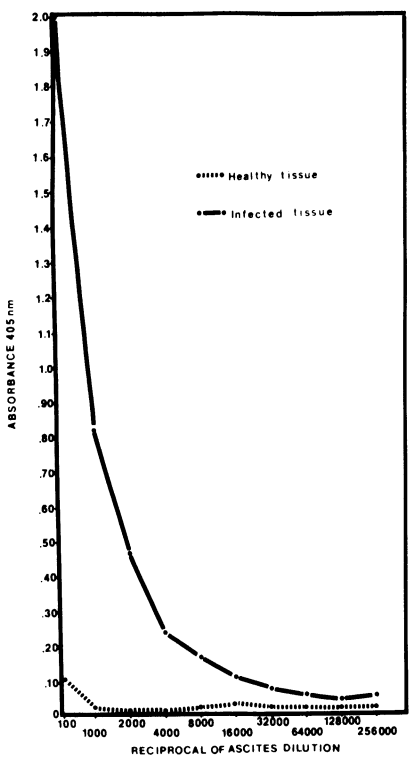

Fig. 2. Absorbance values (405 nm) of healthy and CMV-Vi-infected squash sap tested by indirect enzyme-linked immunosorbent assay using dilutions of crude CMV-Vi ascites.

Purification. Virus yields of up to 110 mg/kg of infected tissue were obtained. The ratio of absorbance at 260/280 nm was 1.78, close to the reported value of 1.7 (9). Three bands were observed after sucrose density gradient centrifugation, migrating 3.0, 3.7, and 4.2 cm from the surface of the gradient. All three bands contained infective virus particles. The middle and bottom bands probably represent dimers and trimers of the virions, respectively. We believe this aggregation resulted from overloading of the gradients.

Electron microscopy. Spherical particles typical of CMV were observed in purified virus preparations. The particle diameter was calculated as 31 nm based on the 18-nm width of TMV (21) used as the particle size standard.

Electrophoresis. CMV-Vi migrated as one band just below carbonic anhydrase in SDS-PAGE slab gels (Fig. 1). Molecular weight of CMV-Vi coat protein subunits was calculated as 29,000 Da by linear regression based on the log of the molecular weight of protein standards versus their mobility in the gel. This value is in the range of molecular weights reported for CMV coat protein subunits (9,10).

One problem encountered during SDS-PAGE was that in some gels, CMV protein did not migrate into the resolving gel, although protein standards migrated in a normal manner. This situation was corrected by mixing the buffers to the desired pH by formula. Apparently, CMV coat protein subunits are sensitive to pH in SDS-PAGE, particularly in the resolving gel, and the Ag/AgCl reference electrode on the pH meter did not provide accurate pH measurements in Tris buffer.

Antibody production. Reciprocal titers as measured by microprecipitin tests with 0.12–1.0 mg of virus per milliliter ranged from 16 to 64 and 32 to 256 for ascites drained from the mice receiving 50- and 100-μg virus injections, respectively (Table 1). Although the microprecipitin titers were higher for ascites from the mouse given 100-μg injections, the highest absorbance values obtained in indirect ELISA were not significantly different (1.92 and 1.89) for the two mice. In our opinion, the use of 100 μg of virus per injection may be an advantage if the ascites is intended for use in tests involving precipitation reactions but may not make a difference if used for ELISA. The titer in both mice increased following an intravenous injection.

ELISA. In comparing unfractionated CMV-Vi ascites fluid with IgG purified from ascites concentrated at 1 μg/ml, we found that the healthy background reaction was low with both preparations but that crude ascites reacted more strongly to CMV-Vi-infected tissue than IgG purified from ascites. The absorbance values (405 nm) for healthy tissues in this experiment were 0.02 for both ascites and IgG, but with infected tissues, the average values were 0.82 and 0.27 for ascites and IgG, respectively. Unfractionated ascites of CMV-Vi was used in all subsequent ELISA.

CMV-Vi ascites reacted with sap from plants infected with CMV strains D, S, C, and 9, representing all reported serogroups (1) and also CMV isolates from bean, CMV-Pg (7), and squash, CMV-NJ83-42. In reciprocal ELISA, CMV-D and CMV-S IgG reacted with CMV-Vi-infected sap. Further tests are in progress to determine the serogroup (1) of CMV-Vi. CMV-Vi ascites did not react with sap infected with AMV, BBWV, PVX, PVY, TEV, TMV, TobRSV, or TomRSV.

The sensitivity of the first ascites drain of the mouse given 50-μg virus injections was tested in a dilution series against healthy and CMV-Vi-infected squash sap (Fig. 2). The reaction with CMV-Vi was significant and linear at reciprocal ascites dilutions between 2,000 and 4,000.

Immunodiffusion tests. A single precipitin line was formed between CMV-Vi ascites and purified CMV-Vi but not between ascites and healthy plant extract. Clearest bands formed at a 1:8 dilution of CMV-Vi ascites with 0.5 mg/ml of purified virus. Sap from Multipik squash plants infected with CMV-Vi reacted with CMV-Vi ascites, but plants infected with CMV-D did not react, possibly because of low titers of virus. However, extracts of plants infected with CMV-Vi and CMV-D produced precipitin lines when tested against CMV-B_{32} antiserum.

Aphid transmission. With three aphids per plant, the virus was transmitted in a nonpersistent manner from CMV-Vi-infected Multipik squash to five of 20 healthy Multipik plants. With eight aphids per plant, CMV-Vi was transmitted from infected *V. minor* growing under natural conditions to one of 10 Multipik test plants. Symptoms included leaf puckering, chlorotic spots, and mild mosaic and developed within 9–12 days. These symptoms were much less severe than on plants mechanically inoculated at the same time. Virus was confirmed as CMV in all infected source and test

plants by ELISA. The efficiency of transmission was reduced when naturally infected *V. minor* was the virus source. This may have been due to low virus concentration in these plants at the time of the aphid transmission test.

Field survey. CMV was detected by ELISA in 16 of 31 *V. minor* plants collected from nine counties in New Jersey as follows (county [no. infected/no. collected]): Camden (0/3), Cumberland (0/2), Mercer (0/1), Middlesex (1/1), Monmouth (3/3), Morris (7/8), Ocean (2/2), Somerset (2/8), and Warren (1/3).

In conclusion, CMV has been identified from *V. minor* on the basis of host range, mechanical and aphid transmission, morphology, molecular weight of coat protein subunits, and serological properties. In a recent list of the CMV host range, Douine et al (8) include *V. minor* as being reported as a host of CMV by Wellman (20) and Smith (18). However, Wellman listed *V. rosea*, not *V. minor*, as a CMV host and Smith mentioned *V. minor* as a host in England but did not provide evidence to support this observation. Therefore, this is the first report supported by serological data of the natural infection of *V. minor* by CMV.

As our results have shown, CMV can be aphid-transmitted from naturally infected *V. minor*. Although no evidence was found that indicated *V. minor* was a reservoir host for economically important commercial vegetable crops, it is likely that this popular ground cover serves as an overwintering source of CMV for ornamentals, flowers, and vegetables in urban gardens. *V. minor* is widely planted as a ground cover in New Jersey, and we conclude that this common host may be serving as another natural reservoir host of CMV.

ACKNOWLEDGMENTS

We wish to thank H. A. Scott for providing antisera and P. Pogers for typing this manuscript.

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