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Identification of Three Distinct Classes of Satellite RNAs Associated With Two *Cucumber mosaic virus* Serotypes from the Ornamental Groundcover *Vinca minor*

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Abstract

Cucumber mosaic virus (CMV) is a cosmopolitan virus which may also have small satellite RNAs (satRNA) associated with it affecting symptom development. *Vinca minor* (periwinkle) plants exhibiting subtle mosaic symptoms tested positive for CMV by enzyme linked immunosorbent assay (ELISA). Double-stranded ribonucleic acid (dsRNA) analysis of CMV-Vinca field isolates in *Nicotiana tabacum* 'Glurk' suggested two sizes of putative satRNA associated with CMV. Immunocapture RT-PCR, cloning, and sequencing of the movement protein, coat protein, and satRNAs demonstrated serogroup 1A and serogroup 2 CMV helper strains and three distinct classes of satRNAs of four sizes. Further, two classes of satRNAs could be distinguished by their necrosis domains. Previously CMV was reported in *V. minor* in New Jersey. This is the first report of CMV in *V. minor* in Ohio and the first report of satRNA associated with CMV in *V. minor* in the United States.

Introduction

Cucumber mosaic virus (CMV) is a cosmopolitan virus with a worldwide distribution and very broad host range, infecting more than 1000 weed, crop, and ornamental hosts in over 85 families (11). The virus is the type member of the genus *Cucumovirus* of the family *Bromoviridae* with spherical particles, tripartite positive sense, single-stranded RNA genome encoding one structural (capsid) and four non-structural proteins (1,8,11). Occasionally CMV isolates have small satellite (sat) RNAs [~330 to 390 nucleotides (nt)] associated with them whose nucleotide sequences are unrelated to the CMV genome and non-essential to CMV replication but are dependent upon CMV for replication and encapsidation (2,4,12). These satRNAs often have profound effects on CMV symptoms ranging from lethal necrosis to disease attenuation (6,18), and can even alter the disease phenotype on a particular host (5,9,14,18) and among various hosts (9).

Vinca minor L. (common periwinkle) is an evergreen ground cover in temperate climates (3). Previously, virus-like symptoms have been attributed to CMV isolated from *V. minor* in New Jersey (16). In early 2010, *V. minor* samples exhibiting subtle mosaic symptoms (Fig. 1) were submitted to the Ohio Plant Diagnostic Network for analysis. The samples tested positive for CMV by triple antibody sandwich (TAS) enzyme-linked immunosorbent assay (ELISA) using commercial antibodies (Agdia Inc., Elkhart, IN). Additionally, satellite RNA was detected in several of the samples by immunocapture (IC) reverse transcription (RT) polymerase chain reaction (PCR). Here we report the identification and characterization of three distinct classes of satellite RNAs supported by CMV serogroup 1A and 2 helper strains from *V. minor*. To our knowledge, this is the first report of CMV in *V. minor* in Ohio and satellite RNA associated with CMV in *V. minor* in the United States. These findings have

important implications for commercial ornamental and vegetable growers in proximity to *V. minor* plantings which may serve as an overwintering reservoir for CMV and the satRNAs associated with it. The CMV helper strains and associated satRNAs described here currently appear to be benign on tobacco and tomato, but very little is known about the disease impact they may have on other ornamental and vegetable crops.

Fig. 1. Subtle mosaic symptom observed on *Vinca minor* leaves.

Characterization of CMV-Vinca Isolates and Satellite RNAs

Five CMV-Vinca field isolates (designated N1-05, N1-04, N1-03, N1-02, and 0100) were used for this project. Two isolates (N1-05 and 0100) were used for IC-RT-PCR directly from source plants. Three isolates (N1-02, N1-03, and N1-04) were passaged to *Nicotiana tabacum* 'Glurk' by triturating Vinca tissue (1:5 ratio) in 100 mM sodium phosphate buffer (pH 7.0) containing 1% (w/v) celite and rubbing on plants at the four leaf stage. CMV in symptomatic inoculated tobacco was confirmed by TAS-ELISA. Additionally, CMV-Vinca N1-03 in *Vinca* and tobacco 'Glurk' was arbitrarily screened by ELISA for impatiens necrotic spot, tomato spotted wilt, tobacco ring spot, tobacco mosaic, alfalfa mosaic, arabis mosaic, and tomato ring spot viruses using commercial antibodies (Agdia Inc.). Test samples and controls were loaded in duplicate wells and evaluated at 405 nm (MRX Revelation plate reader, Dynex Technologies, Chantilly, VA). A sample was considered positive if the OD was twice the mean of the negative controls and a plate was valid only if the positive controls reacted. Healthy tissue and extraction buffer alone were used as negative controls. None of these other viruses were detected.

Asymptomatic *V. minor* was pre-screened for CMV prior to back-inoculation from tobacco infected with the N1-03 isolate. Four healthy *Vinca* plants were inoculated with CMV-Vinca N1-03 and one plant mock inoculated with extraction buffer. Three of the four experimental plants developed the subtle mosaic symptom observed on the original samples, and the mock inoculated plant remained symptomless. All plants were tested for CMV by ELISA 28 days post inoculation. The three symptomatic plants tested positive. The asymptomatic experimental plant and mock inoculated plant tested negative. These results satisfy Koch's postulates for the CMV-Vinca N1-03 isolate and demonstrate with some certainty that CMV is the causal agent of the mosaic symptom observed on *V. minor* and not due to a mixed infection with another common virus.

Double-stranded ribonucleic acid (dsRNA) was purified from CMV-Vinca infected tobacco (17), analyzed on 1% or 1.5% ethidium bromide-stained agarose gels, and results recorded (Gel Logic 112, Eastman Kodak, Rochester, NY). DsRNA analysis of CMV-Vinca N1-02, N1-03, and N1-04 infected tobacco 'Glurk' produced banding profiles typical for CMV, with major genomic bands corresponding to approximately 3.3 Kb, 3.0 Kb, 2.2 Kb, and a sub-genomic band at approximately 1.0 Kb (1,11), but with apparent size variation among the genomic RNAs as well as several additional lower MW RNAs in the 0.5 to 0.75 Kb range (Fig. 2). N1-03 and N1-04 also had an additional low molecular weight (~0.3 to 0.4 Kb) molecule that N1-02 apparently lacked, and the low MW species present in the N1-03 and N1-04 isolates did not co-migrate (Fig. 2). This result suggested the N1-03 and N1-04 isolates possessed a putative satellite RNA absent in N1-02, and that the N1-04 satRNA was of greater MW than the N1-03 satRNA.

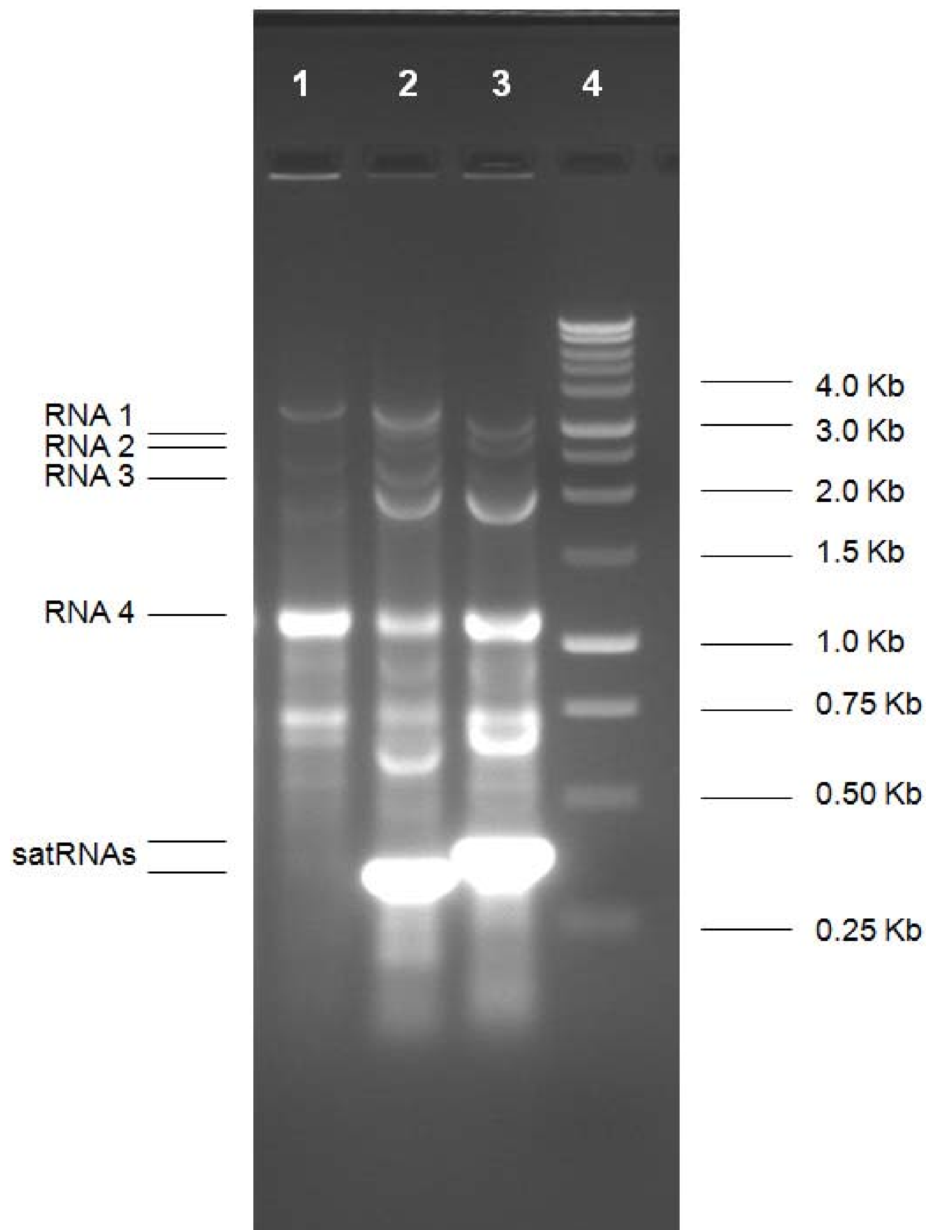

Fig. 2. DsRNA analysis of CMV-Vinca isolates N1-02 (lane 1), N1-03 (lane 2), and N1-04 (lane 3) in *N. tabacum* 'Glurk.' Approximate locations of dsRNAs 1, 2, 3, 4, and putative satRNAs indicated on left. 1 Kb DNA ladder (lane 4). 1% agarose gel, 100 volts, 2 hours.

Tomato (*Lycopersicon esculentum* Mill.) cv 'Rutgers' was used as a lethal necrosis bioassay (19). Seed was sown in sterile potting mix (Miracle-Gro Lawn Products, Marysville, OH) and grown to the fully expanded cotyledon stage. CMV-Vinca infected *N. tabacum* 'Glurk' inoculum was prepared as above. Inoculum or buffer alone was rubbed on cotyledons using sterile gauze pads and the plants maintained in indoor growth rooms (25°C, ~12 h day/night) and monitored for symptoms over a 28-day period. All plants were screened for CMV by TAS-ELISA. A minimum of ten seedlings was inoculated with each isolate with a further five plants mock inoculated with buffer, and the experiment was repeated three times. ELISA positive tomato 'Rutgers' inoculated with CMV-Vinca N1-04 never developed symptoms, and those inoculated with N1-03 and N1-02 developed a fern leaf symptom and were stunted compared to buffer mock inoculations. None of the seedlings developed a lethal necrosis symptom. These results demonstrate that none of the tested satellite RNAs (N1-04, N1-03, and N1-02) induces lethal necrosis on tomato.

For IC-RT-PCR magnetic beads conjugated with sheep anti-rabbit IgG (Dynabeads M-280, Dynal Biotech/Invitrogen, Carlsbad, CA) were incubated with polyclonal rabbit anti-CMV IgG (Agdia Inc.). Tissue was ground in phosphate sucrose buffer (116.1 ml 1M $Na_2H_2PO_4$, 33.9 ml 1M NaH_2PO_4, 50g sucrose; pH 7.4; 1L) at a 1:10 ratio and 10- to 20-µl antibody-coated beads incubated for two hours with shaking. Beads were pulled out of suspension on a magnetic rack (Dynal/Invitrogen), washed four times, and used for first strand synthesis by addition of 20 µl MMLV-RT cocktail [10 µl H_2O, 4 µl 5X MMLV buffer, 2 µl 5 mM dNTPs, 0.5 µl 0.5 µg/µl random hexamers, 1 µl 1 mg/ml BSA, 1 µl 0.1 mM dithiothreitol, 0.5 µl 40 units/µl RNAsin, 1 µl 200 units/µl MMLV-RT (Promega Inc., Madison, WI)] and incubating for one hour in a 42°C water bath. 5 µl cDNA or sterile water was used as template for separate PCR reactions using three primer pairs (Integrated DNA Technologies Inc., Coralville, IA) to amplify the movement protein (MP), coat protein (CP), and satRNA (Table 1) in 25 µl reactions [1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.2 µM primer pair, 0.625 units Taq polymerase (Promega Inc.)]. Amplification was done in an Eppendorf Mastercycler (Hamburg, Germany) with the cycling parameters: 94°C (2 min), 40 cycles of 94°C (30 sec), 59°C (30 sec), 55°C (30 sec), 52°C (30 sec), 72°C (45 sec), final extension 72°C (10 min). PCR products were analyzed on 0.8% agarose gels, DNA cut from the gel, purified (QIAquick gel extraction kit, Qiagen, Inc., Valencia, CA), ligated into pGEM-T Easy plasmid (Promega Inc.), the ligation used to transform competent *E. coli* JM109 cells, and colonies screened for insert by PCR using M13 primers. Selected colonies were grown in LB broth containing ampicillin and plasmid purified using the Wizard Plus SV mini-prep kit (Promega Inc.). DNA was quantified (Nanodrop 2000, Thermo Scientific, Waltham, MA) and sequenced (Plant Microbe Genomics Facility, The Ohio State University, Columbus, OH) in both directions using M13 primers. Vector was trimmed from raw sequences (Chromas v. 2.33, Technelysium Pty Ltd., Australia), contigs assembled, pairwise and multiple sequence alignments with reference sequences performed (Contig Express, ClustalW, Vector NTI 11, Invitrogen), sequences edited and open reading frames translated (Genedoc v. 2.6.001, 2000, Karl Nicholas). Known CMV strains C, Fny, K, LS, My17, Q, Rb, Sn, SO, WL, Y, Z1, and peanut stunt virus PSV-ER (GenBank Accession numbers D00462.1, D10538.1, AF127977.1, AF127976.1, AF103993.1, J02059.1, GU327365.1, U22822.1, AF103992.1, D00463.1, M57602.1, GU327368.1, and U15730.1, respectively) were used as reference sequences for movement and coat protein comparison. Known satRNAs D, E, G, B1, B2, B3, Fny1, Fny2, Ky, WL, WL2, S, T43, Y, OY2, and D27 (GenBank Accession numbers M20350.1, M20844.1, M16585.1, M16586.1, M16587.1, M16588.1, X54065.1, X54066.1, DQ975201.1, M30589.1, M16590.1, M14934.1, D10039.1, D00542.1, M20845.1, and U31661.1, respectively) were used as reference sequences for satRNA comparison. Sixteen CMV-Vinca isolate sequences were deposited in GenBank under Accession numbers JF918960-918963 (MP), JF918964-918967 (CP), and JF918968-918975 (satRNA).

Table 1. CMV coat protein, movement protein, and satRNA PCR primers.

Primer	**Region amplified**	**Sequence (5'-3')**	**Expected product size** (bp)
CMV-MPfwd	movement protein	GCTYTCCAAGGTMCCAGYAGGAC	1331
CMV-MPrev	movement protein	GGASCGRGAACCWCKACGCGGRC	1331
CMV-CPfwd	coat protein	CCTTACTTTYTCATGGATGCTTC	1005
CMV-CPrev	coat protein	CCTTCCGAAGAAAYCTAGGAGRTG	1005
CMV-satRNAfwd	satellite RNA	GTTTTGTTTGWTRGAGAAYTGCGTRG	339
CMV-satRNArev	satellite RNA	GGGTCCTGBWRRGGWATGWRTARAC	339

The CMV-Vinca MP open reading frame (ORF) is 840 nt and encodes a predicted 279 amino acid (aa) protein. CMV-Vinca isolates N1-05, N1-04, and N1-03 have the greatest nt and aa identity to CMV serogroup 1A strain Fny, while CMV-Vinca isolate 0100 has the greatest percent nt and aa identity to CMV serogroup 2 strain LS (Table 2). The CMV-Vinca CP ORF is 657 nt and encodes a predicted 218 aa protein. CMV-Vinca isolates N1-05, N1-04, and N1-03 have the greatest percent nt identity to CMV-Fny and the greatest percent aa identity to CMV-Fny and MY17. CMV-Vinca isolate 0100 has the greatest percent nt and aa identity to CMV-LS, although not significantly different from CMV-Sn and WL (Table 3). No clones were obtained from the MP and CP PCR products of the CMV-Vinca N1-02 isolate. Phylogenetic analysis of the MP and CP ORF nt sequences (maximum likelihood phylogeny estimation with 1000 bootstrap replicates; RAxML ver. 7.0.4, A. Stamatakis, San Diego, CA) grouped CMV-Vinca N1-05, N1-04, and N1-03 isolates with CMV serological subgroup 1A strains, and CMV-Vinca 0100 isolate with serological subgroup 2 strains (Figs. 3 and 4), demonstrating that CMV from *Vinca minor* are actually a subgroup 1A strain not significantly different from CMV-C, Fny, and MY17, and a subgroup 2 strain not significantly different from CMV-LS, SN, and WL. Amino acid 129 of the CP gene has been implicated in chlorosis induction; a proline residue in mosaic-inducing strains and a serine or leucine residue in chlorosis-inducing strains (13). CMV-Vinca N1-05, N1-04, N1-03, and 0100 all have a proline residue at amino acid 129 which is consistent with the mosaic symptom observed on N1-02, N1-03, and N1-04 inoculated tobacco.

Table 2. Movement protein ORF percent nucleotide (top panel) and predicted amino acid (bottom panel) sequence identities between CMV-Vinca isolates and known CMV strains.

CMV isolate	**Fny**^{x}	**Y**^{x}	**SO**^{x}	**MY17**^{x}	**Z1**^{x}	**Rb**^{y}	**K**^{y}	**LS**^{z}	**Q**^{z}
N1-05^{t}	99.3	98.1	97.7	97.2	96.6	97.2	94.4	78.4	65.4
N1-04^{u}	98.0	97.0	96.7	96.2	95.8	96.3	93.5	78.1	64.8
N1-03^{v}	98.6	97.4	97.1	96.6	96.0	96.5	93.6	78.3	65.2
0100^{w}	78.6	78.6	79.3	78.7	79.5	77.9	78.3	98.8	81.3
CMV isolate	**Fny**	**Y**	**SO**	**MY17**	**Z1**	**Rb**	**K**	**LS**	**Q**
N1-05^{t}	99.4	99.1	99.1	99.1	98.7	98.8	95.7	87.7	ND
N1-04^{u}	98.9	98.5	98.5	98.5	98.2	98.9	94.3	82.7	ND
N1-03^{v}	98.6	98.2	98.2	98.2	97.9	97.9	95.0	86.7	ND
0100^{w}	83.8	84.2	84.2	84.2	86.0	83.8	83.1	98.9	ND

Mean of 5^{t}, 1^{u}, 2^{v}, and 3^{w} clones. CMV subgroup 1A^{x}, 1B^{y}, and 2^{z} reference sequences. ND=not determined. Shaded cells indicate highest percent identity to CMV reference sequences.

Table 3. Coat protein ORF percent nucleotide (top panel) and predicted amino acid (bottom panel) sequence identities between CMV-Vinca isolates and known CMV strains.

CMV isolate	Fny[x]	C[x]	Y[x]	MY 17[x]	Z1[x]	SO[x]	Rb[y]	K[y]	LS[z]	Sn[z]	WL[z]
N1-05[t]	99.2	98.7	97.2	97.8	96.6	96.3	97.7	94.0	76.5	76.3	76.3
N1-04[u]	98.2	97.8	96.6	97.2	96.2	95.9	97.6	93.7	76.9	76.6	76.8
N1-03[v]	99.3	99.0	97.4	98.0	96.8	96.4	97.8	94.2	76.5	76.5	76.3
0100[w]	77.0	76.6	77.5	77.2	77.6	77.8	77.2	78.5	99.3	98.8	99.1
CMV isolate	**Fny**	**C**	**Y**	**MY 17**	**Z1**	**SO**	**Rb**	**K**	**LS**	**Sn**	**WL**
N1-05[t]	98.2	97.0	96.3	98.2	97.7	97.7	98.0	97.7	81.9	81.6	81.9
N1-04[u]	98.8	97.8	97.8	98.8	98.3	98.3	98.3	99.2	83.2	83.1	83.1
N1-03[v]	98.9	98.0	96.1	98.9	97.5	97.5	98.4	97.5	80.1	80.1	80.1
0100[w]	84.0	83.5	83.0	84.0	83.5	83.5	83.5	84.0	99.8	99.5	99.5

Mean of 2[t], 8[u], 2[v], and 2[w] clones. CMV subgroup 1A[x], 1B[y], and 2[z] reference sequences. Shaded cells indicate highest percent identity to CMV reference sequences.

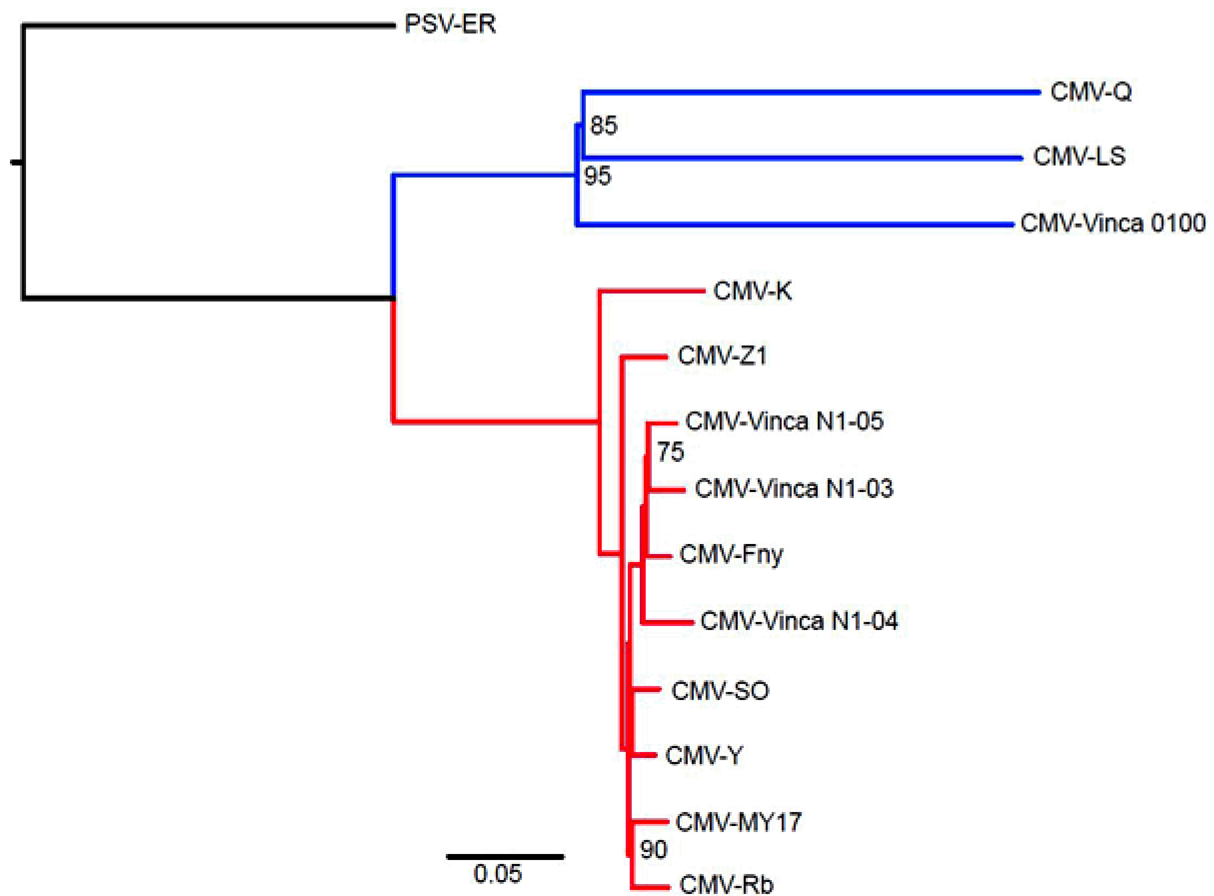

Fig. 3. Rooted phylogram of CMV-Vinca isolate MP open reading frame nucleotide sequences. Peanut stunt virus-ER strain used as the outgroup. 1000 bootstrap replicates were performed and bootstrap values ≥ 70% are shown.

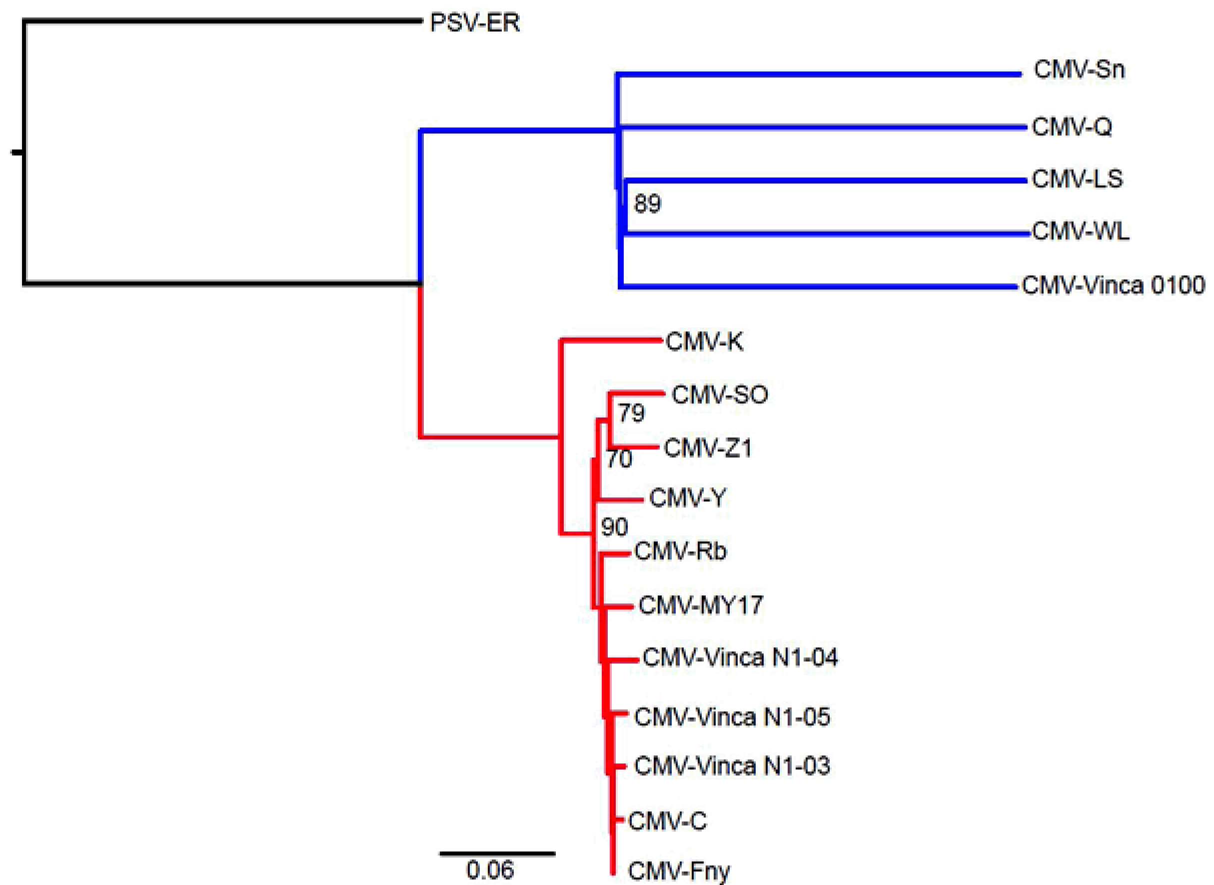

Fig. 4. Rooted phylogram of CMV-Vinca isolate CP open reading frame nucleotide sequences. Peanut stunt virus-ER strain used as the outgroup. 1000 bootstrap replicates were performed and bootstrap values ≥ 70% are shown.

Four size classes of satRNA clones were obtained from CMV-Vinca: 339 and 340 nt species from isolates N1-03 and N1-05, respectively; 390 nt species from N1-04; 339 and 389 nt species from N1-02; and 339, 340, and 389 nt species from 0100. When compared to reference satRNA sequences, N1-05 (340 nt), N1-03 (339 nt), N1-02 (339 nt), and 0100 (340 nt) satRNAs had the greatest identity to chlorosis-inducing WL2 satRNA, and the 389 and 390 nt satRNAs from N1-04 (390 nt), N1-02 (389 nt), and 0100 (389 nt) had the greatest identity to T43 satRNA (Table 4).

Table 4. Percent nucleotide sequence identities between CMV-Vinca satRNAs and known CMV satRNAs.

CMV/sat isolate (# nt)	N1-05[s] (340)	N1-04[t] (390)	N1-03[u] (339)	N1-02[v] (339)	N1-02[w] (389)	0100[x] (339)	0100[y] (340)	0100[z] (389)
WL2	96.2	73.4	89.8	90.1	73.3	88.7	94.8	72.6
G	92.6	71.0	87.6	87.5	70.7	87.1	91.5	71.3
B1	95.4	72.8	88.8	89.2	72.2	87.7	93.8	72.6
B2	95.1	73.2	88.4	88.7	73.3	87.4	93.5	73.1
S	94.6	73.2	89.1	89.3	73.7	83.3	93.2	72.9
Fny2	93.9	72.5	88.7	89.0	72.9	88.0	92.6	72.4
Fny1	93.6	72.8	88.6	88.9	73.0	88.0	92.4	72.4
E	93.0	72.2	88.8	88.3	72.7	88.0	93.5	72.1
B3	92.8	72.1	87.9	88.2	71.7	87.4	91.5	72.3
WL1	90.4	69.9	88.6	89.1	70.6	87.0	90.6	69.7
Ky	90.2	69.9	89.7	90.0	70.6	88.8	89.7	69.4
D	87.1	70.3	89.3	89.7	70.1	88.5	86.8	69.2
Y	74.9	71.8	74.5	75.0	72.5	73.1	74.7	71.6
T43	71.1	87.0	70.6	71.0	88.1	70.4	70.4	86.6
D27	72.3	86.9	69.2	68.2	86.8	68.8	72.0	86.2
OY2	67.7	84.4	67.5	67.1	85.0	68.6	68.4	84.1

Mean of 10[s], 9[t], 6[u], 3[v], 2[w], 1[x], 2[y], and 1[z] clones. Shaded cells indicate highest percent identity to satRNA reference sequences.

The domain responsible for chlorosis on tobacco and tomato has been localized to the 5' half of the molecule between nt 97-191 (7), and nucleotides 148, 149, 153, and 170 have been shown to play roles in symptom development (15,20). The 389 and 390 nt CMV-Vinca satRNA chlorosis domains are very different from the 339 and 340 nt satRNAs (Table 5). N1-05 (340 nt), N1-03 (339 nt), N1-02 (339 nt), and 0100 (340 nt) satRNAs all have chlorosis domains suggestive of a non-chlorosis phenotype (Table 5) and in fact CMV-Vinca N1-02, N1-03, and N1-04 did not cause chlorosis on tobacco or tomato (0100 isolate was not inoculated onto tobacco), but rather all three isolates caused a mosaic symptom on tobacco.

Table 5. Chlorosis and necrosis domains of CMV-Vinca satRNAs compared to necrogenic (Dsat), ameliorative (WL1), and chlorosis-inducing (WL2, B1) satRNAs.

SatRNA	Phenotype[s]	nt 148-170 chlorosis domain	nt 290-309 necrosis domain
		148[t] |153[v] 170[w] ||149[u] |	290 293[x] 299[y] 304[z] | | | |
Dsat	N	ACTCT-CAGTACTACACTCTCA-	AAGG-CTTAT---GCTATGC
N1-03 (339 nt)	NN, NC	ACTCT-CAGCACTACGCACTCA-	AAGG-CTTAAT--GCTATGC
N1-02 (339nt)	NN, NC	ACTCT-CAGCACTACGCACTCA-	AAGG-CTTAAT--GCTATGC
0100 (339nt)	not tested	ACTCT-CAGCACTACGCACTCA-	AAGG-CTTAAT--GCTATGC
WL1sat	NN, A	ACTCT-CAGCACTACGCGCTCA-	AAGA-CTTAG---GTTATGC
T43sat	N, NC ?	GAACTGGCGCCGGAGGCCCTCAG	AAGG-CTTAT---GCTATGC
N1-05 (340 nt)	NN, NC	ACTCT-CAGCACTACGCACTCA-	AAAACCT-ATAAGGTCATGC
0100 (340 nt)	not tested	ACTCT-CAGCACTACGCGCTCA-	AAAACCT-ATAAGGTCATGC
N1-04 (390 nt)	NN, NC, A	GAACTGGCGCCGGAGGCCCTCAG	AAAACCT-ATAAGGTCATGC
N1-02 (389nt)	NN, NC	GAACTGGCGCCGGAGGCCCTCAG	AAAACCT-ATAAGGTCATGC
0100 (389 nt)	not tested	GAACTGGCGCCGGAGGCCCTCAG	AAAACCT-ATAAGGTCATGC
WL2sat	NN, C	GTTCTTCAGCACTACGCACTCAA	AAAACCT-ATAAGGTCATGC
B1sat	NN, C	GTTCTTCAGCACTACGCACTCA-	AAAACCT-ATAAGGTCATGC

[s] Necrogenic on tomato (N), non-necrogenic on tomato (NN), chlorosis-inducing on tomato or tobacco (C), non chlorosis-inducing on tomato or tobacco (NC), ameliorative (A).

[t] G in chlorosis satRNAs, A in non-chlorosis.

[u] Chlorosis on tomato (T) or tobacco (C).

[v] T in chlorosis satRNAs, deleted in all others.

[w] A determines helper strain specificity of chlorosis on tomato (white chlorosis with CMV-WL and yellow chlorosis with CMV-LS), deleted in other satRNAs.

[x] G in necrogenic satRNAs, A in non-necrogenic.

[y] T in necrogenic satRNAs, G in non-necrogenic.

[z] C in necrogenic satRNAs, T in non-necrogenic.

Blue font indicates key nucleotides involved in the necrosis and chlorosis disease states. Red font indicates variations in the CMV-Vinca satRNA isolates.

Two regions of variability in the 3' half of N1-05 (340nt) and 0100 (340nt) satRNAs distinguished them from N1-03 (339nt), N1-02 (339nt), and 0100 (339nt) satRNAs. The nt188-262 region consistently differed at 16 nt positions between N1-05 (340nt) and 0100 (340nt) satRNAs, and N1-03 (339nt), N1-02 (339nt), and 0100 (339nt) satRNAs. The domain inducing the tomato lethal necrosis phenotype spans nt 286-310 which are conserved in necrogenic satRNAs and a substitution at any one of three sites (nt 293, 299, 304) abolishes necrogenicity on tomato (7,14). The N1-03 (339nt), N1-02 (339nt), and 0100 (339nt) satRNA necrosis domains are identical to the necrogenic DsatRNA domain except for an additional A residue at nt 299 (Table 5). These results demonstrate that the 339 and 340 nt CMV-Vinca satRNAs, although largely conserved in the 5' half of the molecule, diverge and are distinct from one another in the 3' half. The lack of necrosis on Rutgers tomato inoculated with CMV-Vinca N1-02 and N1-03 after 8 weeks suggests an insertion at nt 299 may abolish the necrogenic phenotype. The N1-05 (340nt), N1-04 (390nt), N1-02 (389nt), 0100 (340 nt), and 0100 (389 nt) satRNAs have necrosis domains identical to the non-necrogenic WL2 and B1 satRNAs (Table 5). The 389 and 390 nt satRNAs are highly conserved, although N1-04 (390nt) satRNA has an additional A residue at nt 274 that is absent in the others. None of the 389 or 390 nt CMV-Vinca satRNAs have necrosis domains resembling other necrogenic satRNAs (Table 5).

Phylogenetic analysis of the CMV-Vinca satRNAs grouped the molecules in three clades. The 389 and 390 nt satRNAs of CMV-Vinca N1-04, N1-02, and 0100 have the greatest nt differences and grouped with other large satRNAs such as OY2 and T43 satRNAs. The 339 and 340 nt satRNAs grouped in two separate clades. N1-03 (339nt), N1-02 (339nt), and 0100 (339nt) satRNAs grouped in the same clade as necrogenic DsatRNA and ameliorative WL1satRNA, and N1-05 (340nt) and 0100 (340 nt) satRNAs grouped in the same clade as chlorosis-inducing B1, B2, B3, and WL2 satRNAs and ameliorative S satRNA (Fig. 5).

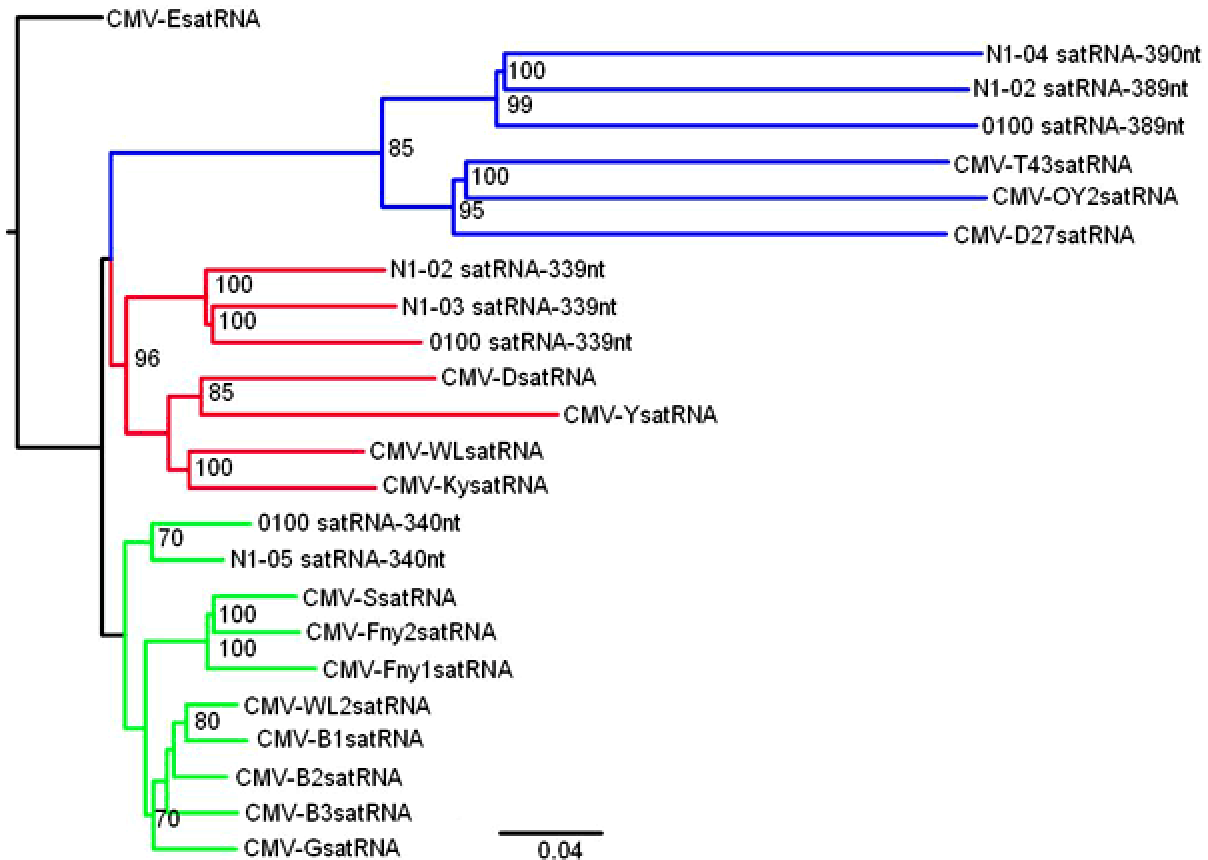

Fig. 5. Unrooted phylogram of CMV-Vinca satRNA nucleotide sequences. 1000 bootstrap replicates were performed and bootstrap values ≥ 70% are shown.

Eight individual satRNAs of four size classes were isolated and sequenced from CMV-Vinca. The 389 and 390 nt satRNAs are highly conserved and represent a non-necrosis, non-chlorosis inducing variant based on symptoms on tobacco and tomato and the chlorosis and necrosis domains. The 339 and 340 nt satRNAs are conserved in the 5' half of the molecules, including the chlorosis domain, but diverge in the 3' half and can be differentiated by their necrosis domains--the 339nt class having a necrogenic-like domain and the 340nt class having a non necrogenic-like domain. Both the 339nt and 340nt satRNAs represent non-necrosis, non-chlorosis inducing variants based on symptoms on tobacco and tomato, but it appears that a single nucleotide deletion at nt 299 in the 339nt satRNAs may potentially be sufficient to change the phenotype from non-necrogenic to necrogenic on tomato. Work by others has shown that a single nucleotide change within the necrosis domain changed the phenotype from ameliorative to necrogenic (14), and a benign satRNA can spontaneously mutate to a pathogenic variant (10).

These findings have implications for ornamental and vegetable growers with operations in proximity to *V. minor* plantings or nursery stock that may be serving as reservoirs for CMV and satRNAs. The CMV symptoms observed on *V. minor* were extremely subtle so a grower scouting for diseased plants would

need a sharp eye when roguing plant material. Likewise, infected source material used for divisions or cuttings may go unnoticed by the unwary eye. The effects of satRNA on tomato and tobacco are well studied but less so on the more than 1000 other CMV hosts (10), and there may be unstudied regions of the molecule that play unknown roles in disease on other hosts. Likewise, the effect of the helper CMV strain cannot be ignored. Others have shown that various CMV strains and satRNA combinations produce varying symptoms on tobacco and tomato (9,20). The uncertainty of the threat these satRNAs may pose to high value nursery stock should serve as the impetus for growers to exclude CMV and its satRNAs from their operations by roguing out diseased plants, controlling aphid vectors, and employing good sanitation practices. The research presented here helps to increase awareness of the potential threat that CMV and its satRNAs may pose to ornamental and vegetable crops. The three classes of satRNA variants identified in *V. minor* currently appear to be benign when associated with the subgroup 1A CMV strain studied here, but at least one has the potential to become pathogenic on tomato, and their effect on other hosts remains to be determined.

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