Characterization of a Cucumber Mosaic Virus Isolate and Satellite RNA from the Ornamental Host *Ajuga reptans* ‘Royalty’

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ADDITIONAL INDEX WORDS. virus purification, DNA sequencing, double-stranded RNA

ABSTRACT. Cucumber mosaic virus (CMV) was isolated from the perennial ornamental mint, *Ajuga reptans* L. ‘Royalty’, using melon aphids (*Aphis gossypii* Glover). The isolate and its associated satellite RNA (satRNA) were biologically and chemically characterized. The satRNA was cloned and sequenced and is 338 nucleotides long and does not induce lethal necrosis on ‘Rutgers’ tomato (*Lycopersicon esculentum* Mill.) or severe chlorosis on tobacco (*Nicotiana* L. spp.). The virus is ≈28 to 30 nm in diameter and reacts to CMV serological subgroup I antibodies. The virus is able to infect ‘Black Beauty’ squash (*Cucurbita pepo* L.), cucumber (*Cucumis sativus* L.), and ‘Howden’ pumpkin (*Cucurbita pepo*) but is not able to infect green bean (*Phaseolus vulgaris* L.) or cowpea [*Vigna unguiculata* (L.) Walp. ssp. *unguiculata*]. The virus is able to efficiently replicate its satRNA in tobacco and ‘Black Beauty’ squash but replication is less efficient in cucumber, based on accumulation of double-stranded satRNA.

Ajuga reptans is a perennial ornamental mint grown commonly for its foliage in borders or as a groundcover. Cucumber mosaic virus (CMV, genus *Cucumovirus*), alfalfa mosaic virus (AMV), tobacco streak virus (TSV, genus *Ilarvirus*), and broad bean wilt virus (BBWV, genus *Fabavirus*) were reported in this host in Australia (Shukla and Gough, 1983), CMV in Denmark (Kristensen, 1956), and AMV in the United States (Schroeder and Provvidenti, 1972).

CMV is the type member of the cucumovirus group of plant viruses, which includes tomato aspermy (TAV) and peanut stunt viruses (Francki et al., 1979). It is distributed worldwide, has a very wide host range, and is transmitted mechanically, through seed, and by >60 aphid species in a nonpersistent manner (Francki et al., 1979). CMV particles are spherical, 28 to 30 nm in diameter, and encapsidate four functional segments of single-stranded RNA (Francki, 1985; Francki et al., 1979).

Satellite RNAs (satRNAs) are nucleic acids that are dependent on a helper virus for replication and encapsidation, and CMV satRNAs lack significant nucleotide sequence identity to the helper virus genome (Collmer and Howell, 1992; Diaz-Ruiz and Kaper, 1977). SatRNAs may influence the symptoms induced by the helper virus alone. This influence can range from symptom amelioration to symptom exacerbation, and even induction of new symptoms not associated with the helper virus alone (Collmer and Howell, 1992).

Recently CMV, AMV, and TSV were reported in *A. reptans* cultivars in the United States, and a satRNA was found to be associated with CMV-infected *A. reptans* ‘Royalty’ (Fisher and Nameth, 2000). In this paper we report characterization of a CMV isolate and its associated satRNA, including the satRNA nucleotide sequence, from *A. reptans* ‘Royalty’ (CMV-‘Royalty’).

Materials and Methods

APHID TRANSMISSION. Melon aphids (*Aphis gossypii* L.) were used to isolate CMV from *A. reptans* ‘Royalty’ and transmit the

virus to tobacco (*Nicotiana* spp.). A colony of melon aphids was maintained on ‘Howden’ pumpkin (*Cucurbita pepo*) leaves. Three groups of 25 aphids each were placed in petri plates containing moist filter paper. The aphids were starved for 3 h before a 1 min acquisition feeding period on an *A. reptans* ‘Royalty’ leaf and then transferred to 3-week-old *Nicotiana tabacum* L. ‘Glurk’, *N. benthamiana* L., *N. rustica* L., and *N. clevelandii* L. seedlings. Three pots of each species containing three seedlings each were inoculated with at least two to three aphids per seedling. One pot of each species was mock-inoculated with aphids that were starved but not acquisition fed. About 30 d after inoculation, seedlings were transplanted into individual pots and observed over the next several months under greenhouse conditions. Direct antibody-sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was used to test the plants for CMV, AMV, TSV, and TAV (see below). The aphid-transmission experiment was conducted twice and the CMV-‘Royalty’ isolate was maintained in *N. tabacum* ‘Glurk’ and *N. rustica*.

DAS-ELISA. Samples were tested using a variation of the method described previously (Clark and Adams, 1977). Commercially produced antibodies (Agdia, Inc., Elkhart, Ind.) against CMV, AMV, TSV, and TAV were used. Healthy tissue and extraction buffer alone were used as negative controls. Tissue from an established source known to be infected with the above viruses was used as positive control. Each 100-μL sample was dispensed into two test wells (Nunc Maxisorp 96 well polystyrene microtitre plates, Thomas Scientific, Swedesboro, N.J.), and the average absorbance of the two wells at 405 nm was determined using a microtitre plate reader (EAR 400SF Plus EIA plate reader; SLT Lab Instruments, Hillsboro, N.C.). A sample was considered to be positive if its absorbance was twice the mean of two negative control wells, and a plate was considered valid only if the positive control reacted.

HOST RANGE. *Nicotiana rustica*, *N. tabacum* ‘Glurk’ and ‘Samsun’, cowpea (*Vigna unguiculata* ssp. *unguiculata*), green bean (*Phaseolus vulgaris*), ‘Black Beauty’ squash (*Cucurbita pepo*), ‘Howden’ pumpkin (*Cucurbita pepo*), cucumber (*Cucumis sativus* L.) and ‘Peto 696’, ‘Nema 1401’, and ‘Rutgers’ tomato (*Lycopersicon esculentum*) were used in host range studies. Individual seeds were sown directly into 492-cm³ pots containing sterile soilless growing medium (Metro Mix 250; W.R. Grace & Co., Cambridge, Mass.) and germinated under mist. A set of

Received for publication 22 Jan. 2002. Accepted for publication 1 Nov. 2002. Melon aphids were provided by Karen Magnuson and Celeste Welty, Dept. of Entomology, The Ohio State Univ., Columbus. ‘Peto 696’ tomato seedlings were provided by R.M. Riedel, Dept. of Plant Pathology, The Ohio State Univ., Columbus. Lyophilized tobacco tissue containing CMV-fny was provided by Keith Perry, Purdue Univ., West Lafayette, Ind. We also thank Doris Majerczak for technical advice.
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seven pots of each species was planted for each host range study.

Inoculum was prepared by grinding symptomatic *N. tabacum* 'Glurk' tissue infected with the CMV-'Royalty' isolate at a 1:10 ratio in 0.02 M sodium phosphate buffer containing 0.1% (w/v) thioglycolic acid (pH 7.0), or in 0.03 M sodium phosphate buffer without thioglycolic acid, and mixed with a small amount of celite ($\approx 1\%$ w/v). Squash, pumpkin, cucumber, cowpea, bean, and tomato were inoculated at the cotyledon stage and tobacco was mock-inoculated with buffer as a negative control. Following inoculation, seedlings were placed in a growth chamber set at 18 °C with a 12-h photoperiod, and observed for symptoms over 4 weeks. The host range study was conducted three times and tissue samples from each pot were collected and tested for the presence of CMV by DAS-ELISA. Symptomatic or ELISA-positive samples were also tested for the presence of viral-associated double-stranded RNA (dsRNA).

LETHAL NECROSIS BIOASSAY. 'Rutgers' tomato seeds were sown directly into 50-cm³ cell pack containing Metro Mix 250 and germinated under mist in a greenhouse. Inoculum was prepared as above, and the cotyledons inoculated (White and Kaper, 1987). There were three replications each with 10 plants inoculated with CMV-'Royalty' and one buffer mock-inoculation as a negative control. A fourth replication consisted of 55 'Rutgers' seedlings inoculated with CMV-'Royalty' and six buffer mock inoculations. Seedlings were grown in a growth chamber under the same conditions as the host range plants, and observed over 4 to 6 weeks. All seedlings were tested for the presence of CMV by DAS-ELISA as described previously.

VIRAL dsRNA ANALYSIS. Viral-associated double-stranded ribonucleic acid (ds-RNA) was extracted and purified as described previously (Jordan et al., 1983; Morris and Dodds, 1979). DsRNA was

loaded onto 5% or 10% polyacrylamide gels and analyzed by electrophoresis. Ethidium bromide-stained dsRNA bands were visualized on a transilluminator (302 nm) and photographed with Polaroid type 667 black and white film (Polaroid Corp., Waltham, Mass.).

VIRUS PURIFICATION. CMV-'Royalty' and subgroup I strain CMV-Fny were purified from *N. tabacum* 'Glurk' or *N. rustica* using a modification of the protocol of Lot et al. (1972). Following emulsification and clarification, the preparation was subjected twice to differential centrifugation and purified further by ultracentrifugation in 10% to 40% sucrose density gradients. The virus was repelleted by ultracentrifugation, resuspended in borate buffer (5.0 mM sodium tetraborate, 0.5 M EDTA, pH 9.0), and the preparations used for electron microscopy (EM), sodium dodecyl sulfate poly-

roysat
dsat
gsat
esat
b1sat
b2sat
b3sat
w11sat
w12sat
ysat
oy2sat

roysat
dsat
gsat
esat
b1sat
b2sat
b3sat
w11sat
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b3sat
w11sat
w12sat
ysat
oy2sat

roysat
dsat
gsat
esat
b1sat
b2sat
b3sat
w11sat
w12sat
ysat
oy2sat

GTTTTGTTTGTAGAGAACTGCGTAGAGGGGTTGTATCTACGTGAGGATCTGTCACTC--
GTTTTGTTTGTAGAGAACTGCGTAGAGGGGTTATATCTGCGTGAGGATCTGTCACTC--
GTTTTGTTTGTAGAGAACTGCGTAGAGGGGTTGTATCTACGTGAGGATCTATCACTC--
GTTTTGTTTGTAGAGAACTGCGTAGAGGGGTTGTATCTGCGTGAGGATCTGTCACTC--
GTTTTGTTTGTAGAGAACTGCGTAGAGGGGTTATATCTACGTGAGGATCTATCACTC--
GTTTTGTTTGTAGAGAACTGCGTAGAGGGGTTATATCTATGTGAGGATCTATCACTC--
GTTTTGTTTGTAGAGAACTGCGTAGAGGGGTTATATCTACGTGAGGATCCGTCACTC--
GTTTTGTTTGTAGAGAACTGCGTAGAGGGGTTATATCTACGTGAGGATCTATCACTC--
GTTTTGTTTGTAGAGAACTGCGTAGAGGGGTTATATCTGCGTGAGGATCCATCACTC--
GTTTTGTTTGTAGAGAACTGCGTAGAGGGGTTATATCTGCGTGAGGATCTATCACTC--
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GGCGGTGTGGGTACCTCCCTGCTACGGCGGGTTGAGT-----TGACG-CACCTCGGA
GGCGGTGTGGGTACCTCCCTGCTAAGGCGGGTTGAG-----TGATGTTCCCTCGGA
GGCGGTGTGGGTACCTCCCTGCTACGGCGGGTTGAG-----TGACG-CACCTCGGA
GGCGGTGTGGGTACCTCCCTGCTAAGGCGGGTTGAG-----TGACG-TATCTCGGA
GGCGGTGTGGGTACCTCCCTGCTAAGGCGGGTTGAG-----TGACG-CACCTCGGA
GGCGGTGTGGGTACCTCCCTGCTACGGCGGGATGAG-----TGACG-CACCTCGGA
GGCGGTGTGGGTACCTCCCTGCTACGGCGGGATGAG-----TGACG-CACCTCGGA
GGCGGTGTGGGTACCTCCCTGCTAAGGCGGGTTGAGT-----TGACG-CACCTCGGA
GGCGGTGTGGGTACCTCCCTGCTAAGGCGGGTTGAGT-----TGACG-CACCTCGGA
GGCGGTGTGGGTACCTCCCTGCTAAGGCGGGTTGAG-----AGTG-TATCTCGGA
GGCGGTGTGGGTAACTCCCTGCTAAGGCGGGTTGAGACTGCGCCGAGACAGGCCGGA
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127|

148|149

CT---GGGGACCGCT-GGCTTGCGAGCTATGTCCGCTACTC-----
CT---GGGGACCGCT-GGCTTGCGAGCTATGTCCGCTACTC-----
CT---GGGGACCGCT-GGC---CGAGCTATGTC-GCTACTC-----
CT---GGGGACCGCT-GGCTTGCGAGCTATGTCCGCTACTC-----
CT---GGGGACCGCT-GACTTGCGAGCTATGTCCGCTGCTC-----
CT---GGGGACCGCT-GACTTGCGAGCTATGTCCGCTGCTC-----
CTC---GGGGACCGCTGTTTGCGAGTATCGTCCGCT-ATTC-----
CT---GGGGACCGCT-GACTTGCGAGTATCGTCCGCTGCTC-----
CTG---GAGGCGGGATGTCTGCGGGTGTTCGCTGCTGCCACGATGGTGGGAGCCACCC
CCTTGGGGGAGCCACGAGCCGCGTGGGAACGTAGCGGTTTCCGGTTGAAGTGGCGCCG
* * * * * * * * * * * * * * * * *

153|

-----T-CAGCACTACGCA-----CTCATTTGAGCCCCCGC-TCAGTTTGCTA
-----T-CAGTACTACACT-----CTCATTTGAGCCCCCGC-TCAGTTTGCTA
-----T-CAGCACTACGCA-----CTCAATTGAGCCCCCGC-TCAGTTTGCTA
-----T-CAGCACTGCGCT-----CTCATTTGAGCCCCCGC-TCAGTTTGCTA
-----T-CAGCACTACGCA-----CTCAATTGAGCCCCCGC-TCAGTTTGCTA
-----TTTACGACACGCA-----CTCATTTGAGCCCCCGC-TCAGTTTGCTA
-----TTTACGACACGCA-----CTCATTTGAGCCCCCGC-TTAGTTTGCCA
-----TTTAGCACTACGCG-----CCAATTGAGCCCCCGC-CTAGTTTGCCA
-----TTTACGCACTACGCA-----TCAATTGAGCCCCCGCCTCAGTTTGCTA
CAGGGGCGACTTTTTTTCAGCTCTGCATTT---CTCATTTGAGCCCCCGC-TCAGTTTGCTA
AGGCCCTCAGCGGCTGTTTTCGCGCTTCCCTCCATTTGAGCCCCCGC-CCAGTTTGCTA
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WESTERN TRANSFER AND IMMUNOLOGICAL DETECTION. Protein samples were denatured and electrophoresed on discontinuous SDS-polyacrylamide gels as above. The proteins were electroblotted onto a piece of 0.2 μ m nitrocellulose membrane using standard procedures (Sambrook et al., 1989). The membranes were blocked, incubated for 2 h at 25 °C with mouse anti-CMV subgroup I or subgroup II monoclonal antibodies (Agdia, Inc.) and then 1 h at 25 °C in rabbit anti-mouse alkaline phosphatase-conjugated antibodies. Protein bands were detected in alkaline phosphatase substrate

Insert DNA was mixed with plasmid DNA at a molar ratio of 1:1. Ligations, including controls using only plasmid DNA digested

roysat CGCGTATGTACAT-CATACCTTACCAGGACCC
dsat CGTGAATGTCTAT-CATTCCCTCTGCAGGACCC
gsat CTTGGATGTTTAT-CATTCCCTACCAGGACCC
esat CGCGTATGTTTAA-CATACCTTAACAGGACCC
b1sat CGTGAATGTCTAC-CATTCCCTACAGGACCC
b2sat CGTGAATGTCTAC-CATTCCCTACAGGACCC
b3sat CGTGGATGTTTAT-CATTCCT-CTACCGGACCC
w11sat CGTGAATGTCTA--CATTCCCTACAGGACCC
w12sat CGTGAATGTCTA--CATTCCCTACAGGACCC
ysat CGTGAATGTCTATACATTCTCTACAGGACCC
oy2sat CATGGATGTCTACACATTCTCTACAGGACCC
* * * * *

with SmaI, were carried out using standard procedures (Sambrook et al., 1989). The T4 ligase was not heat-inactivated following the reaction incubation, and the ligation reaction products were used directly to transform competent *E. coli* DH5 cells (Sambrook et al., 1989). Putative transformant colonies were evaluated using a standard small scale plasmid isolation procedure (Sambrook et al., 1989). Plasmid DNA was digested with HindIII and XbaI restriction endonucleases and the minipreps evaluated on 1% agarose gels. Clones with inserts similar in size to an (S)CARNAS (ATCC 45124) cDNA were prepared for sequencing using the Promega WizardPlus Mini Prep DNA Purification kit (Promega Corp., Madison, Wis.),

and the DNA purity and quantity assessed spectrophotometrically at A_{260/280}. Clones were sequenced by the DNA Sequencing Facility, The Ohio State University, Columbus, and CLUSTAL-W (version 1.4), and DNA Strider version 1.3f3 used to do multiple sequence alignments and pairwise alignments, respectively.

SOUTHERN HYBRIDIZATION. A digoxigenin (DIG) labeled (S)CARNA-5 (ATCC 45124) cDNA probe was prepared (Genius II DIG-DNA Labeling Kit, Boehringer-Mannheim Biochemical, Indianapolis, Ind.). Putative satRNA cDNA clones were screened by hybridization to the DIG-labeled cDNA. Following electrophoresis in 1% or 1.5% agarose gels, cDNA inserts that had been excised from plasmids were chemically denatured, blotted onto nylon membrane, and hybridized to the probe. Southern blot hybridization was performed at 68 °C and high stringency washes performed at 68 °C in 0.1× SSC buffer (Sambrook et al., 1989).

Results and Discussion

CMV-‘ROYALTY’ SATELLITE RNA. Seventy-four colonies were screened for inserts similar in size to (S)CARNA-5 and 43 of those sequenced. Both strands of the clones were sequenced, CLUSTAL-W (version 1.4) was used to align the clone sequences, and a 338 base sequence was obtained (Fig. 1). CLUSTAL-W was also used to align the ‘Royalty’ satRNA sequence with the 10 satRNA sequences used to design the cloning primers (Fig. 1), and DNA Strider was used to make pairwise alignments and comparisons of the Royalty satRNA sequence with those 10 satRNA sequences. The percentage

identity of the CMV-‘Royalty’ satRNA sequence to those 10 satRNAs is presented in Table 1. A BLASTN (gapped BLASTN with Repeat Masker; Baylor College of Medicine website) search of the DNA database was performed to locate other CMV satRNA sequences with higher percentage identity to ‘Royalty’ satRNA. These results are shown in Table 1.

Disease phenotype sequence domains and key nucleotide positions have been identified in CMV-satRNAs. A nucleotide change at position 149 from U to C alters host specificity of the chlorosis phenotype from tomato to tobacco (Sleat and Palukaitis, 1992). Other nucleotide positions important in the chlorosis phenotype are 127: G in nonchlorosis inducing satRNAs and A in chlorosis-inducing satRNAs; 148: G in chlorosis-inducing satRNAs and A in nonchlorosis-inducing satRNAs; and 153: U in all chlorosis-inducing satRNAs and deleted in all others (Sleat and Palukaitis, 1992). The nucleotides at these positions in the CMV-‘Royalty’ satRNA clones are G (127), A (148), C (149), and a deletion at position 153 (Fig. 1), and are conserved among all the clones, suggesting the nonchlorosis-inducing phenotype. The 5' 223 bases of necrogenic satRNA variants are identical but nonnecrogenic satRNA variants have at least nine variable regions, five of which are in the 5' two-thirds of the molecule (Kaper et al., 1988). The CMV-‘Royalty’ satRNA differs from the necrogenic D-satRNA in 14 nucleotide positions in the 5' 220 bases of the molecule (Fig. 1). Necrogenic satRNAs also have a three-base deletion relative to their non-necrogenic counterparts. In D-satRNA this deletion is between nucleotides 300 to 302 (Sleat and Palukaitis, 1990), and is not present in the CMV-‘Royalty’ satRNA (Fig. 1). The greatest number of nucleotide differences between necrogenic and ameliorative satRNAs is

Table 1. Comparison of percentage identity between the CMV-‘Royalty’ satRNA (roysat) nucleotide sequence, 10 previously published satRNA sequences, and the 11 satRNA sequences obtained from a BLASTN database search.

Satellite RNA/ (accession no.)	No. of nucleotides	No. of conserved nucleotide positions ^z	Identity with CMV ‘Royalty’ satRNA ^y	Necrogenic phenotype ^x
Royalty satRNA	338	NA ^w	100	–
g satRNA (m16585)	333	314/338	92.9	–
e satRNA (m20844)	341	312/341	91.5	–
b1 satRNA (m16586)	336	308/338	91.1	–
b2 satRNA (m16587)	339	309/340	90.9	–
b3 satRNA (m16588)	337	310/339	90.5	–
w12 satRNA (m16590)	340	303/341	88.9	–
w11 satRNA	336	294/341	86.2	–
d satRNA	335	292/338	86.4	+
y satRNA	368	289/373	77.5	+
oy satRNA	386	280/389	72.0	–
(z75876)	339	321/339	94.7	
(z75871)	339	320/339	94.4	
(z75870)	340	321/340	94.4	
(z75882)	339	319/339	94.1	
(j02060)	336	318/338	94.1	–
(z75872)	339	319/340	93.8	
(z75873)	339	319/339	93.8	
(x86408)	340	317/341	93.0	
(x53534)	339	315/339	92.9	–
(x69136)	339	313/339	92.3	–
(m14934)	339	312/339	92.0	–

^zVariation between the number of nucleotides in column 2 and the total number of positions in column 3 due to spaces added to make the alignment.

^yPercentage of conserved nucleotide positions with ‘Royalty’ satRNA.

^xSatellite RNAs expressing the necrogenic (+) or nonnecrogenic (–) disease phenotype, where known.

^wNA = not applicable.

Table 2. Summary of symptoms and ELISA results for host range study.

Host	Symptoms	Days postinoculation	ELISA ^z
'Black Beauty' squash	Chlorotic spots/veinal chlorosis	7	+
Cucumber	Chlorotic spots/veinal chlorosis	10–12	+
'Howden' pumpkin	Chlorotic spots/veinal chlorosis	13–14	+
Cowpea	None	---	—
Green bean	None	---	—
'Peto 696' tomato	None	---	—
'Nema 1401' tomato	None	---	—
'Rutgers' tomato	None or mild mosaic	45	+

^zResults of DAS-ELISA when using CMV antibodies.

located in the 3' one-third of the molecule between nucleotides 286 to 310 (Sleat and Palukaitis, 1990). There are four regions of variability between nucleotides 280 to 320 in D-satRNA relative to 'Royalty' satRNA (Fig. 1), suggesting 'Royalty' satRNA is a nonnecrogenic satRNA.

Of the 10 published sequences used for primer design, 'Royalty' satRNA has the highest percentage identity to E-satRNA (92.9%), G-satRNA (91.5%), B1-satRNA (91.1%) and B2-satRNA (90.9%) (Table 1), all ameliorative satRNAs (Garcia-Arenal et al., 1987; Hidaka et al., 1988), and only 86.4% identity with the necrogenic D-satRNA (Table 1).

LETHAL NECROSIS BIOASSAY. Six of thirty 'Rutgers' tomato plants tested were positive for CMV by DAS-ELISA. All six of the plants appeared initially asymptomatic. After ≈45 d one plant developed a very mild mosaic symptom, and was slightly paler in color than the controls. After ≈50 to 52 d the new leaflets on this plant began to show slight distortion. The other five plants remained symptomless. Forty-eight of the fifty-five plants in the fourth replication tested positive for CMV by DAS-ELISA. One plant showed a slight leaf distortion similar to that seen on the plant from the first replication. All others were asymptomatic. It has been reported that contamination of a nonnecrogenic satRNA isolate with as little as 0.5% of a necrogenic satRNA can cause tomato necrosis death of 100% of the test plants (Kaper et al., 1988), and there was no evidence of lethal necrosis in any of the 'Rutgers' tomato plants after >45 d. These results support the nucleotide sequence data, and demonstrate that 'Royalty' satRNA is nonnecrogenic and ameliorative in nature.

APHID TRANSMISSION AND HOST RANGE. Mild mosaic symptoms appeared on *N. rustica* and *N. tabacum* 'Glurk' ≈5 weeks after aphid inoculation. The symptoms developed into severe mosaic and leaf puckering on younger *N. rustica* leaves, and vein clearing and mild mosaic on younger *N. tabacum* 'Glurk' leaves. *Nicotiana benthamiana* seedlings were stunted compared to the mock-inoculated controls, but showed no other symptoms, and no symptoms developed on *N. clelandii* seedlings. CMV was detected in all three *N. rustica* samples, and two of three *N. tabacum* 'Glurk' samples, but was not detected in any of the *N. clelandii* samples by DAS-ELISA. The *N. benthamiana* plants were too small to test. Once CMV was transmitted to *N. tabacum* by *A. gossypii*, the virus was then easily mechanically transmissible to tobacco, squash, cucumber, and pumpkin using symptomatic *N. tabacum* 'Glurk' as inoculum. CMV-'Royalty' induced mild mosaic and mild vein clearing on *N. tabacum* 'Glurk' and 'Samsun', and a very mild mosaic symptom on *N. clelandii* which disappeared after 28 d. The presence of CMV in mechanically inoculated *N. clelandii* was confirmed by ELISA and demonstrates that *N. clelandii* is a host for CMV-'Royalty' even though *A. gossypii* failed to transmit it to this host. These

results also support the nucleotide sequence data, and demonstrate that the 'Royalty' satRNA is nonchlorosis-inducing on tobacco. None of the test plants developed the brilliant chlorosis that has been described for chlorosis-inducing satRNAs (Sleat and Palukaitis, 1992).

Results of the host range study are shown in Table 2. and were similar for all three replications. Symptoms on 'Black Beauty' squash developed the fastest, became progressively more severe over 28 d, but did not kill the plants. Symptoms on cucumber were milder and took longer to develop, and symptoms on 'Howden' pumpkin were even milder. DAS-ELISA results confirmed the presence of CMV.

No local lesions developed on cowpea cotyledons in any of the three host range replications. Some CMV strains such as CMV-Y infect cowpea systemically (Francki et al., 1979), but ELISA confirmed that there was no systemic infection. Thus, cowpea is neither a local lesion nor systemic host for CMV-'Royalty'. Likewise, no symptoms were observed on green bean or 'Peto 696' or 'Nema 1401' tomato, and ELISA confirmed lack of infection. Certain CMV strains, such as CMV-B, infect *Phaseolus* L. spp. systemically while others, such as CMV-C, do not (Provvidenti, 1976). CMV-'Royalty' is a strain unable to infect *P. vulgaris*.

VIRAL dsRNA ANALYSIS. DsRNA analysis of selected symptomatic *N. tabacum* 'Glurk', squash, and cucumber samples from the host range studies revealed a dsRNA banding pattern consistent with that of CMV and an associated satellite RNA in 'Glurk' tobacco, squash, and cucumber (Dodds et al., 1984; Fisher and Nameth, 2000; Valverde et al., 1990). The dsatRNA appeared to accumulate to a higher level in 'Black Beauty' squash than in cucumber (Fig. 2). These results are consistent with reports that satRNA replication in cucurbits is generally poor, while replication in solanaceous hosts, such as tobacco, is more efficient

Fig. 2. Ten percent polyacrylamide gel electrophoresis of dsRNA extracted from host range samples. Lane 1 = asymptomatic 'Peto 696' tomato; lane 2 = symptomatic cucumber; lane 3 = symptomatic 'Black Beauty' squash; and lane 4 = CMV-'Royalty' isolate in *N. tabacum* 'Glurk'. Arrow indicates double-stranded satRNA band.

Fig. 3. Five percent polyacrylamide gel electrophoresis of dsRNA extracted from 'Rutgers' tomato positive for CMV- 'Royalty' by DAS-ELISA. Lane 1 = CMV- 'Royalty' isolate in *N. tabacum* 'Glurk', lanes 2 and 3 = asymptomatic 'Rutgers' tomato samples, lane 4 = 'Rutgers' tomato sample showing slight leaf distortion, and lane 5 = healthy 'Rutgers' tissue. Arrow indicates dssatRNA.

(Roossinck et al., 1992). It also has been reported that efficient replication of the WL1 satRNA in 'Black Beauty' squash is helper strain-dependent (Roossinck and Palukaitis, 1991). CMV-Fny replicates the WL1 satRNA efficiently in all hosts, including 'Black Beauty' squash. CMV-Sny, however, is incapable of efficiently replicating the WL1 satRNA in this squash cultivar (Roossinck and Palukaitis, 1991). The dsRNA data indicate that CMV- 'Royalty' is capable of efficiently replicating its satRNA in *N. tabacum* 'Glurk' and 'Black Beauty' squash, but replication is not as efficient in cucumber based on the accumulation of dssatRNA (Fig. 2) when equal gram equivalents of tissue were analyzed. The six 'Rutgers' tomato plants from the lethal necrosis assay were analyzed for the presence of dsRNA and five of the six plants produced dsRNA banding patterns consistent with that reported for CMV and satRNA (Dodds et al., 1984; Valverde et al., 1990). The sixth plant produced a banding pattern that lacked the dssatRNA (Fig. 3). The five plants which produced the dsRNA banding pattern including the dssatRNA were all asymptomatic while the plant that produced the dsRNA banding pattern lacking the dssatRNA showed mild mosaic and slight leaf distortion of the young leaflets. The 48 CMV-positive 'Rutgers' plants from the fourth replication were not tested for dsRNA. It has been proposed that dssatRNA plays a role in the disease modu-

lating effects of satRNAs (Habili and Kaper, 1981). Clearly the dssatRNA was accumulating to high levels in the five 'Rutgers' plants (Fig. 3), and this accumulation correlated with the absence of symptoms. The lack of dssatRNA correlated with mild symptoms.

THE VIRUS. Purification of the CMV- 'Royalty' isolate from aphid-inoculated *N. rustica* produced a single light-scattering band in 10% to 40% sucrose density gradients. Purified CMV-Fny also produced a single light-scattering band in sucrose gradients. When the two purified preparations were mixed, the particles were indistinguishable under the TEM. CMV- 'Royalty' particles were estimated to be ≈ 27.43 nm (± 1.53 nm) in diameter, which falls within the 28 to 30 nm range reported for CMV (Francki et al., 1979).

Purified CMV- 'Royalty' and CMV-Fny proteins produced similar banding patterns when subjected to SDS-PAGE (Fig. 4A). A major band corresponding to the coat protein was located between the 23 to 29 ku markers (u = unified atomic mass unit) (Fig. 4A). Several higher molecular weight bands were visible in both preparations. CMV- 'Royalty' and CMV-Fny proteins both reacted positively with the CMV subgroup I antibodies, but not the subgroup II antibodies (Fig. 4B). Since we lacked a positive control to demonstrate reactivity of the CMV-II antibodies in our western blot, we had tissue samples tested commercially (Agdia, Inc.) against CMV-I and CMV-II. Results confirmed that CMV- 'Royalty' reacted to CMV-subgroup I antibodies, but not CMV-II antibodies. These results indicate that CMV- 'Royalty', like CMV-Fny, belongs to the CMV serological subgroup I (Owen and Palukaitis, 1988; Sleat and Palukaitis, 1990a), and is not a mixed population of subgroup I and II strains.

It is important to be aware of *A. reptans* as a potential reservoir for CMV and satRNA since it is a perennial host. Furthermore, even though the 'Royalty'-satRNA appears to have an ameliorative phenotype it has been shown that a single nucleotide change

Fig. 4. (A) 10% SDS-PAGE gel stained with Coomassie brilliant blue. Lanes 1 and 5 = purified CMV- 'Royalty'; lanes 2 and 4 = purified CMV-Fny; and lane 3 = benchmark protein ladder. Apparent molecular weights of markers are indicated at right in ku (u = unified atomic mass unit). Arrow next to lane 1 indicates coat protein. (B) Western blot of SDS-PAGE gel in Fig. 4A. Lanes 1 and 5 = purified CMV- 'Royalty'; lanes 2 and 4 = purified CMV-Fny; lane 3 = benchmark protein ladder (69 ku marker indicated at right). The left half of the membrane (lanes 1 and 2, and half of 3) was treated with CMV subgroup I to lane 1 indicates the coa antibodies, and the right half (half of lane 3, and lanes 4 and 5) was treated with CMV subgroup II antibodies. Arrow next to protein.

can alter the phenotype of an ameliorative satRNA to necrogenic (Sleat and Palukaitis, 1990). Given the potential that RNA viruses have for rapid mutation (Holland et al., 1982; Steinhauer and Holland, 1987) it is important to be aware of the potential threat that a satRNA in a perennial host may pose.

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