

Rapid Induction and Severity of Symptoms in Zucchini Squash (*Cucurbita pepo*) Map to RNA 1 of Cucumber Mosaic Virus

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Received 11 December 1989. Accepted 10 January 1990.

Two strains of cucumber mosaic virus (cucumovirus group), Fny-CMV and Sny-CMV, were used to genetically map phenotypic differences in severity and timing of symptoms in zucchini squash. Pseudorecombinants were constructed between the two strains, and the genetic purity of the pseudorecombinant strains was verified by molecular heterogeneity analysis of the

viral RNAs using an RNase protection assay. Assessment of symptoms induced by the pseudorecombinant strains indicated that RNA 1 is responsible for both the severity and the rapid induction of symptoms elicited by Fny-CMV in zucchini squash. The potential role of RNA 1 in the induction of pathogenicity is discussed.

Additional keywords: pathogenicity domains, pseudorecombinant viruses.

Cucumber mosaic virus (CMV) of the cucumovirus group is a tripartite, plus-stranded RNA virus that infects a wide range of plant hosts encompassing nearly 800 species (Douine *et al.* 1979; Kaper and Waterworth 1981). CMV isolates fall into two major subgroups, based on hybridization analysis (Gonda and Symons 1978; Piazzolla *et al.* 1979; Owen and Palukaitis 1988) and serology (Devergne and Cardin 1975). The nucleotide sequence is known for strains from both major subgroups of CMV (Rezaian *et al.* 1984, 1985; Davies and Symons 1988; Nitta *et al.* 1988a; Rizzo and Palukaitis 1988, 1989; Hayakawa *et al.* 1989).

The CMV genome contains four open-reading frames (ORFs); RNAs 1 and 2 each contain one ORF encoding proteins of 111 and 97 kDa, respectively. RNAs 1 and 2 are necessary and sufficient for replication of the viral RNAs (Nitta *et al.* 1988b). These RNAs show significant homology with RNAs 1 and 2 of alfalfa mosaic virus and brome mosaic virus (BMV), which is a member of the bromovirus group (Rezaian *et al.* 1984, 1985). RNA 2 also shows homology with known polymerase genes (Argos 1988). RNA 3 contains two ORFs. The 5' half of RNA 3 encodes a 30-kDa protein thought to be involved in viral movement (Stussi-Garaud *et al.* 1987), and the 3' half of RNA 3 generates a subgenomic RNA, RNA 4, that acts as a messenger for the viral coat protein (Schwinghamer and Symons 1975; Gould and Symons 1978). Although translation products of all four ORFs can be detected using *in vitro* translation systems (Schwinghamer and Symons 1975, 1977), the gene products of the ORFs of RNAs 1 and 2 have not been observed *in vivo*.

Numerous strains of CMV have been identified that induce a variety of symptoms which range from mild mosaic to severe stunting and necrosis (Kaper and Waterworth 1981). In this study, two strains of CMV were used; they were originally isolated in eastern New York state from

muskmelon fields 15 miles apart (Banik *et al.* 1983). One strain, CMV-243, showed very severe symptoms in the field and spread rapidly. The other strain, CMV-241, showed much milder symptoms and spread more slowly throughout the field. In subsequent greenhouse studies using zucchini squash, plants inoculated with CMV-243 developed severe symptoms in 3 to 4 days. Plants inoculated with CMV-241 developed mild mosaic symptoms appearing in 6 to 7 days (Banik *et al.* 1983). We biologically purified strains CMV-243 and CMV-241, and the resulting local lesion-selected isolates were named Fny-CMV (Fast, New York) and Sny-CMV (Slow, New York), respectively.

To determine which RNA(s) was responsible for the different phenotypes of the two strains, viral RNAs from each strain were purified and reassorted to generate pseudorecombinant virus strains. These strains were then used to genetically map the phenotypic differences between Fny-CMV and Sny-CMV.

MATERIALS AND METHODS

Virus strains and plants. Plants used in this study were zucchini squash, *Cucurbita pepo* cv. Black Beauty; tobacco, *Nicotiana tabacum* L. cv. Xanthi-nc; and sugar beets, *Beta vulgaris* L. All plants were grown under greenhouse conditions before inoculation. Squash plants were inoculated at the cotyledon stage with viral RNA at a concentration of 100 µg/ml in 50 mM sodium phosphate, pH 9.2. Tobacco plants were inoculated at the three- to four-leaf stage, and sugar beets were inoculated 6 wk after planting as described below. Plants were maintained either under greenhouse conditions or in an environmentally controlled chamber with daytime temperatures of 23° C, nighttime temperatures of 19° C, and a day length of 16 hr.

Viral strains CMV-243 and CMV-241 were obtained from T. A. Zitter, Cornell University, and passaged through a local lesion host (sugar beet) twice to obtain biologically purified isolates. The resultant isolates were named Fny-

CMV and Sny-CMV, respectively. Virus purification was by the method of Lot *et al.* (1972). Viral RNA was purified as described by Palukaitis and Zaitlin (1984).

Construction of pseudorecombinants. Virus was purified from either squash (Sny-CMV) or tobacco (Fny-CMV), and viral RNA was extracted as described above. Viral RNAs were separated using 10 cm × 1 cm diameter tube gels containing 1.5% agarose in Tris-borate EDTA buffer (TBE) (Peacock and Dingman 1968). Gels were stained for 15 to 30 sec with 0.05% (w/v) toluidine blue, 20 mM sodium acetate, pH 7.8, and destained in sterile water. Bands were excised and minced with a sterile scalpel, and incubated overnight in 10 volumes of 0.2 M NaCl in TBE buffer at 4° C on a rocking platform. The eluate was then extracted with a 50:50 mixture of phenol:chloroform, followed by ethanol precipitation.

The RNA was collected by centrifugation, resuspended in water, and reprecipitated with ethanol. The final concentration of purified viral RNAs was estimated by coelectrophoresis with known quantities of viral RNA on slab gels containing 1.5% agarose in TBE. Purified viral RNAs were then reassorted and inoculated onto sugar beet leaves at a final concentration of 75 µg/ml of total RNA in 50 mM Tris-phosphate, pH 8.0. Individual local lesions were excised and either lyophilized and stored at -20° C or ground in 2 ml of 50 mM sodium phosphate, pH 7.0, and reinoculated onto sugar beet leaves. Local lesions from the second passage in sugar beets were then inoculated onto tobacco for amplification.

Construction of probes and heterogeneity mapping. The cDNA cloning of Sny-CMV was done essentially as described for Fny-CMV (Owen and Palukaitis 1988) except that cDNA fragments were initially cloned into the transcription vector pBS+ (Stratagene, La Jolla, CA). Clones were identified by colony hybridization (Maniatis *et al.* 1982) and Southern blot analysis (Southern 1975) using the appropriate probes generated from the Fny-CMV

cDNA clone bank (Owen and Palukaitis 1988; Rizzo and Palukaitis 1988, 1989).

Heterogeneity mapping was done as described by Owen and Palukaitis (1988) except that the annealing reaction time was shortened to 3 hr, and the RNase digestion was done at 15° C (from an article by K. Kirkegaard. Mapping sites of recombination and deletion in large RNA molecules, published in *Promega Notes* 12:3-4, 1988, Promega, Madison, WI). The following probes were used for heterogeneity mapping: for RNA 1, an 844 base pair *Hind*III restriction fragment from pFny100 (Rizzo and Palukaitis 1989), nucleotides 851 to 1694, was subcloned into transcription vector pBS+ and, after linearization with *Bam*HI, used to generate a radiolabeled antisense transcript with T3 RNA polymerase (Stratagene). For RNA 2, plasmid pFny206 (Rizzo 1989), which contains a full-length cDNA clone of Fny-CMV RNA 2, was linearized with *Sph*I and used to generate full-length antisense transcripts with SP6 RNA polymerase (U.S. Biochemical Corporation, Cleveland, OH). For RNA 3, an approximately 630-base pair cDNA fragment corresponding to the 3' end of Sny-CMV RNA 3 was cloned into pBS+, linearized with *Kpn*I, and used to generate antisense RNA 3 transcripts with T3 RNA polymerase. After linearization with *Hha*I (RNA 1 cDNA clone) or *Pst*I (RNAs 2 and 3 cDNA clones), unlabeled plus-sense RNA transcripts were generated from the above plasmids using T7 RNA polymerase (U.S. Biochemical Corporation). Restriction enzymes were purchased from U.S. Biochemical Corporation or New England Biolabs, Beverly, MA.

RESULTS AND DISCUSSION

Molecular analysis of pseudorecombinants. After viral purification and RNA extraction, 30 pseudorecombinant isolates were analyzed by heterogeneity assays to determine

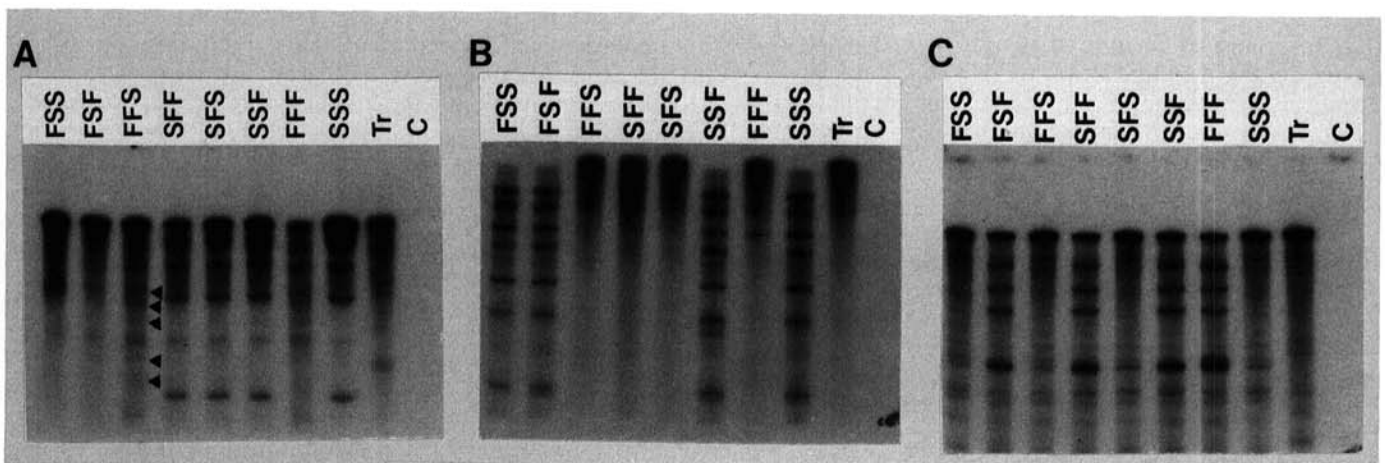


Fig. 1. Polyacrylamide gel electrophoretic analysis of heterogeneity in pseudorecombinant cucumber mosaic virus (CMV) strains. Each lane represents the protected fragments for the indicated reconstructed virus; Tr, *in vitro*-generated (+) sense transcript; and C, control, no RNA. **A**, RNA 1, using an 844-nucleotide probe generated from a cDNA clone of Fny-CMV RNA 1. **B**, RNA 2, using a 3.0-kilobase probe generated from a cDNA clone of Sny-CMV RNA 2. **C**, RNA 3, using a 630-nucleotide probe generated from a cDNA clone of Sny-CMV RNA 3. Pseudorecombinant strains are indicated as follows: FSS indicates RNA 1 from Fny-CMV, RNA 2 from Sny-CMV, and RNA 3 from Sny-CMV. Other pseudorecombinants follow similar designations. Arrows adjacent to lane FFS in **A** indicate the locations of minor bands diagnostic for Fny-CMV RNA 1, and these bands are present only in samples containing this RNA.

the purity of each strain. Antisense RNA was generated *in vitro*, in the presence of [³²P]UTP, from cloned cDNA fragments of each of the three genomic RNAs. Viral RNA was annealed separately with each radiolabeled antisense probe and subsequently incubated with ribonuclease to digest any single-stranded regions. Each RNA of the two strains could be clearly distinguished by analysis of the profiles of the protected fragments, thus providing a simple molecular assessment of the purity of the viral strains (Fig. 1).

RNAs 1 and 3 showed some fully protected fragments for both Fny-CMV and Sny-CMV (Fig. 1, A and C). This was probably due to the incomplete nuclease digestion of some of the mismatched sequences in the heterologous strain, because RNase A and T1 will not completely cleave all mismatches (Winter *et al.* 1985). In addition, some cleavage was also observed in the homologous strains with all three RNAs. This may have been due to either weak hydrogen bonding in AU-rich regions or heterogeneity within the population of a strain. These minor bands of lower molecular weight, found in the homologous strains, were sufficient to distinguish incomplete digestion from contamination in the heterologous strains; however, in the case of RNA 1 this required a longer exposure of the autoradiogram (data not shown) to determine either the presence or absence of the minor bands indicated in Figure 1A (arrows). These five bands were observed only in Fny-CMV RNA 1 and in pseudorecombinants containing Fny-CMV RNA 1. The lack of contamination of Sny-CMV RNA 1 by Fny-CMV RNA 1 was also confirmed by bioassay (see below). Isolates that contained no detectable contamination with the other strain were selected for further study. Only one isolate of each combination of Fny-CMV and Sny-CMV RNAs was used for further analysis.

Symptom production by pseudorecombinants. Pseudorecombinant strains were inoculated onto squash cotyledons and assessed for time of appearance and severity of symptoms. The results of these experiments are summarized in Table 1. All of the pseudorecombinant strains that contained RNA 1 from Fny-CMV (that is, FSS, FSF, and FFS) showed symptoms in 4 days, as did the regenerated parental strain FFF (Table 1 and Fig. 2A). Moreover, the symptoms were severe, consisting of mosaic, leaf curl, and severe stunting. All of the strains containing RNA 1 from Sny-CMV (SFF, SFS, SSF, and SSS) showed

symptoms in 6 to 7 days, and the symptoms were mild, consisting of chlorotic spots on the leaves and slight stunting (Table 1 and Fig. 2B). Hence, the determinant(s) for the severe pathogenic phenotype and the rapid induction of symptoms in squash resides in RNA 1 of the Fny-CMV strain. RNAs 2 and 3 do not appear to play any role in these phenotypic differences observed in Fny-CMV and Sny-CMV.

A second passage through squash of the pseudorecombinants containing Sny-CMV RNA 1 gave results identical to those described above, further reinforcing the purity of the pseudorecombinants. If the pseudorecombinants containing Sny-CMV RNA 1 had been contaminated by Fny-CMV RNA 1, then the pseudorecombinants SSF, SFS, and SFF would have shown a rapid and severe symptom induction, either initially or upon subsequent passage. This was not observed.

Role of RNA 1 in pathogenicity. Although the RNA 1 molecule is clearly required for replication, its specific function is not known. The conserved sequences for RNA polymerases, including the GDD motif, are encoded by RNA 2 (Argos 1988). The carboxy-terminal third of the putative 111-kDa protein encoded by RNA 1 contains the six conserved motifs found in both DNA and RNA helicases (Hodgman 1988), suggesting at least one function for this protein. However, given the limited genetic information contained in CMV, it seems likely that its protein products are multifunctional.

Most previous genetic mapping studies of CMV have implicated RNA 2 and/or RNA 3 in the induction of pathogenicity (Marchoux *et al.* 1974; Hanada and Tochiwara 1980; Rao and Francki 1982), although at least two previous studies (Lakshman and Gonsalves 1985; T. A. Zitter and D. Gonsalves. Analysis of pseudorecombinants of two strains of cucumber mosaic virus differing in symptom expression and aphid transmissibility in squash from pages 19-21 of the Proceedings of the Workshop on Epidemiology of Plant Virus Diseases, Orlando, FL, August 6-8, 1986) have suggested a role for RNA 1. In the latter study, both RNAs 1 and 3 were implicated in elevated virus titers in zucchini squash. In the former study, RNA 1 of a mutant strain was shown to be responsible for the loss of systemic infection in both zucchini squash and tobacco, whereas lesion size in a cultivar of cowpea was determined by RNA 2 of the same strain.

An earlier study, involving another related tricornavirus, compared the type strain of cowpea chlorotic mottle virus (T-CCMV, bromovirus group), which does not cause a systemic infection in some cultivars of cowpea, with a resistance breaking mutant of CCMV (R-CCMV) (Wyatt and Kuhn 1980). Plants infected with T-CCMV had a low virus titer in inoculated leaves, and progeny virus from the resistant host contained low levels of encapsidated RNA 3. R-CCMV caused a systemic infection in the resistant host, and the progeny had normal levels of RNA 3. Genetic mapping, however, indicated that RNA 1 of R-CCMV was responsible for the ability to overcome resistance in cowpea (Wyatt and Kuhn 1980).

Both of these studies suggest a role for RNA 1 in the ability of the virus to infect certain plants systemically.

Table 1. Timing and severity of symptoms in squash after inoculation with reconstituted viruses

Strain	Time ^a	Severity ^b
FSS ^c	4	S
FSF	4	S
FFS	4	S
SFF	6	M
SFS	6	M
SSF	6	M
FFF	4	S
SSS	6	M

^aTime, in days for first symptoms to appear after inoculation.

^bS, severe symptoms, consisting of leaf curl, mosaic, and severe stunting.

^cM, mild symptoms, consisting of mild mosaic and minimal stunting.

^dDesignation of strains is as described in Figure 1.

The reduction of encapsidated RNA 3 in the CCMV study suggests that this role is most likely an indirect one, involving timing and control of viral RNA synthesis in the replication cycle.

RNA 1 probably encodes functions that are required for interaction both with RNA 2 (and/or its gene product) and with the host. A specific interaction between RNA 1 and RNA 2 gene products is suggested by the observation that pseudorecombinants made between CMV and closely

related viruses such as tomato aspermy virus of the cucumovirus group are only viable if RNAs 1 and 2 are from the same virus, although RNA 3 can be from either virus (Rao and Francki 1981). Furthermore, pseudorecombinants made between RNAs 1 and 2 of BMV and CCMV replicate either poorly or not at all in protoplasts, whereas RNA 3 can again be freely exchanged between these two viruses (Bujarski *et al.* 1987; Allison *et al.* 1988). The pathogenic response described in this study for zucchini

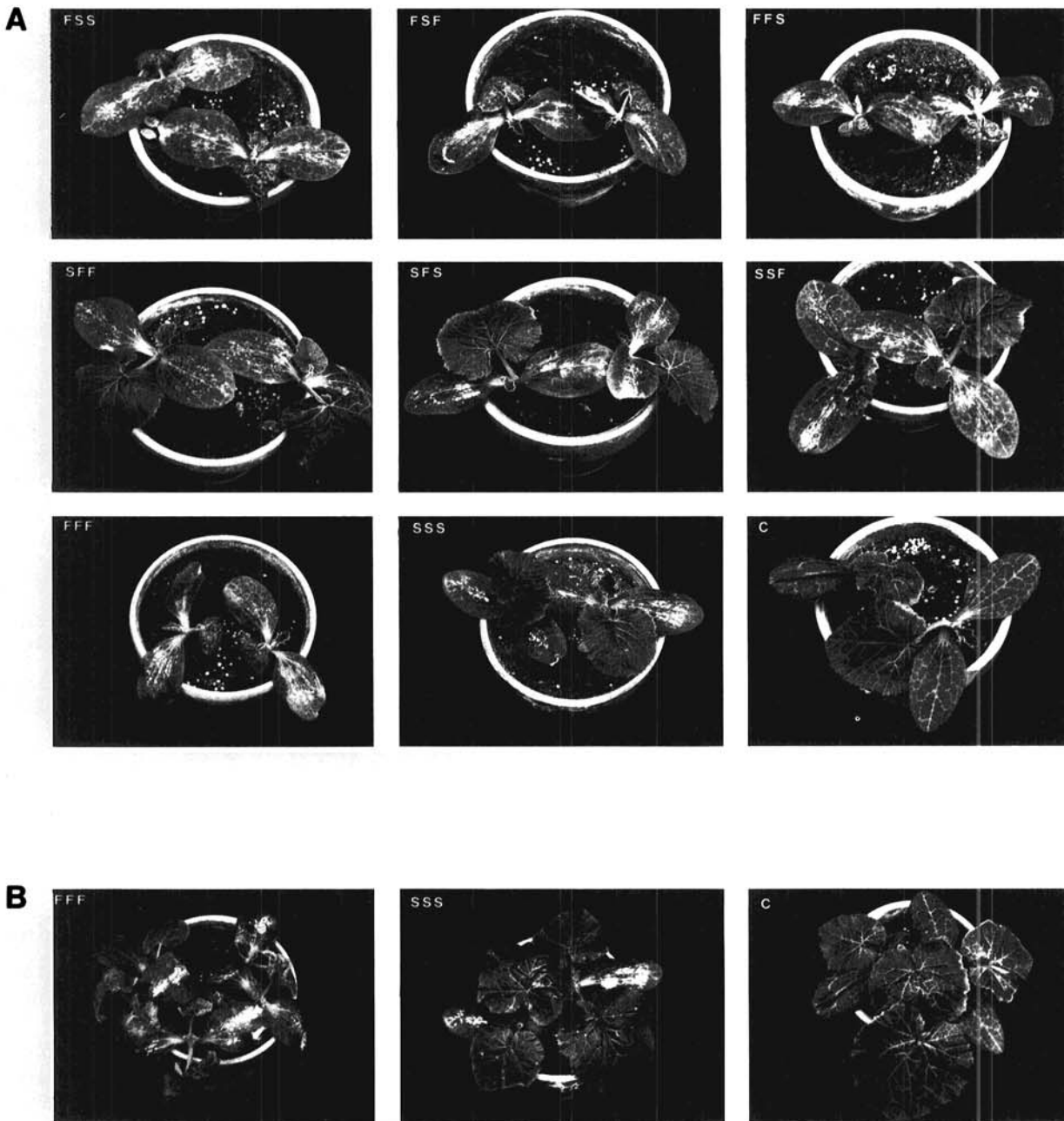


Fig. 2. Symptoms on zucchini squash inoculated with pseudorecombinant viruses. Virus strains are indicated in the upper left-hand corner of each segment of **A** and **B**; **C** indicates the uninoculated control plant in each panel. **A**, Plants 4 days after inoculation. **B**, The reconstituted parental strains 7 days after inoculation. Symptoms seen on the FFF strain were typical of all strains containing RNA 1 from Fny-CMV (FSS, FSF, and FFS). Symptoms appearing on the SSS strain were typical of all strains containing RNA 1 from Sny-CMV (SFF, SFS, and SSF). The apparent chlorosis on the cotyledons is due to inoculation damage. The designation of strains is as described in Figure 1.

squash does not occur in other cucurbits, such as cucumber, other squash cultivars, and some muskmelon cultivars (data not shown), suggesting that either RNA 1 or its encoded polypeptide plays a direct role in specific host-virus interactions.

It should be possible to map the differences between these two strains more precisely by making recombinants in the RNA 1 molecule using cDNA clones to both Fny-CMV and Sny-CMV. It will be interesting to determine if these differences in pathogenicity reside in the helicase portion of the RNA 1 ORF or in some other part of the molecule.

ACKNOWLEDGMENTS

We wish to thank Richard Francki for his advice and suggestions and Thomas Zitter for providing the initial CMV strains.

This work was supported by grant 88-37263-3806 from the USDA.

LITERATURE CITED

- Allison, R. F., Janda, M., and Ahlquist, P. 1988. Infectious *in vitro* transcripts from cowpea chlorotic mottle virus cDNA clones and exchange of individual RNA components with brome mosaic virus. *J. Virol.* 62:3581-3588.
- Argos, P. 1988. A sequence motif in many polymerases. *Nucleic Acids Res.* 16:9909-9916.
- Banik, M. T., Zitter, T. A., and Lyons, M. E. 1983. A difference in virus titer of two cucumber mosaic virus isolates as measured by ELISA and aphid transmission. (Abstr.) *Phytopathology* 73:362.
- Bujarski, J. J., Dzianott, A., and Kaesberg, P. 1987. Genomic RNAs of brome mosaic virus and cowpea chlorotic mottle virus: Molecular cloning, sequence homologies and activity of chimeric RNAs. (Abstr.) *Int. Cong. Virol.*, 7th. 9-14 Aug. 1987:288.
- Davies, C., and Symons, R. H. 1988. Further implications for the evolutionary relationships between tripartite plant viruses based on cucumber mosaic virus RNA 3. *Virology* 165:216-224.
- Devergne, J. C., and Cardin, L. 1975. Relations sérologiques entre cucumovirus (CMV, TAV, PSV). *Ann. Phytopathol.* 7:255-276.
- Douine, L., Auiot, J. B., Marchoux, G., and Archange, P. 1979. Recensement d'espèces végétales sensibles au virus de la mosaïque de concombre (CMV) étude bibliographique. *Ann. Phytopathol.* 11:439-475.
- Gonda, T. J., and Symons, R. H. 1978. The use of hybridization analysis with complementary DNA to determine the RNA sequence homology between strains of plant viruses: Its application to several strains of cucumoviruses. *Virology* 88:361-370.
- Gould, A. R., and Symons, R. H. 1978. Alfalfa mosaic virus RNA. Determination of the sequence homology between the four RNA species and a comparison with the four RNA species of cucumber mosaic virus. *Eur. J. Biochem.* 91:269-278.
- Hanada, K., and Tochihara, H. 1980. Genetic analysis of cucumber mosaic, peanut stunt, and chrysanthemum mild mottle viruses. *Ann. Phytopathol. Soc. Jpn.* 46:159-168.
- Hayakawa, T., Mizukami, M., Nakajima, M., and Suzuki, M. 1989. Complete nucleotide sequence of RNA 3 from cucumber mosaic virus (CMV) strain O: Comparative study of nucleotide sequences and amino acid sequences among CMV strains O, Q, D and Y. *J. Gen. Virol.* 70:499-504.
- Hodgman, T. C. 1988. A new superfamily of replicative proteins. *Nature* 333:22-23.
- Kaper, J. M., and Waterworth, H. E. 1981. Cucumoviruses. Pages 257-332 in: *Handbook of Plant Virus Infections and Comparative Diagnosis*. E. Kurstak, ed. Elsevier/North-Holland Biomedical Press, Amsterdam.
- Lakshman, D. K., and Gonsalves, D. 1985. Genetic analyses of two large-lesion isolates of cucumber mosaic virus. *Phytopathology* 75:758-762.
- Lot, H., Marrou, J., Quiot, J. B., and Esvan, C. 1972. Contribution à l'étude de virus de la mosaïque du concombre (CMV). II. Méthode de purification rapide du virus. *Ann. Phytopathol.* 4:25-38.
- Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Marchoux, G., Marrou, J., and Quiot, J. B. 1974. Complémentation entre ARN de différentes souches du virus de la mosaïque du concombre. Mise en évidence d'une interaction entre deux ARN pour déterminer un type de symptôme. *C. R. Acad. Sci. Paris, Ser. D.* 279:1943-1946.
- Nitta, N., Masuta, C., Kuwata, S., and Takanami, Y. 1988a. Comparative studies on the nucleotide sequence of cucumber mosaic virus RNA 3 between Y strain and Q strain. *Ann. Phytopathol. Soc. Jpn.* 54:516-522.
- Nitta, N., Takanami, Y., Kuwata, S., and Kubo, S. 1988b. Inoculation with RNAs 1 and 2 of cucumber mosaic virus induces viral RNA replicase activity in tobacco mesophyll protoplasts. *J. Gen. Virol.* 69:2695-2700.
- Owen, J., and Palukaitis, P. 1988. Characterization of cucumber mosaic virus. I. Molecular heterogeneity mapping of RNA 3 in eight CMV strains. *Virology* 166:495-502.
- Palukaitis, P., and Zaitlin, M. 1984. Satellite RNAs of cucumber mosaic virus: Characterization of two new satellites. *Virology* 132:426-435.
- Peacock, A. C., and Dingman, C. W. 1968. Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels. *Biochemistry* 7:668-674.
- Piazzolla, P., Diaz-Ruiz, J. R., and Kaper, J. M. 1979. Nucleic acid homologies of eighteen cucumber mosaic virus isolates determined by competition hybridization. *J. Gen. Virol.* 45:361-369.
- Rao, A. L. N., and Francki, R. I. B. 1981. Comparative studies on tomato aspermy and cucumber mosaic viruses. VI. Partial compatibility of genome segments from the two viruses. *Virology* 114:573-575.
- Rao, A. L. N., and Francki, R. I. B. 1982. Distribution of determinants for symptom production and host range on the three RNA components of cucumber mosaic virus. *J. Gen. Virol.* 61:197-205.
- Rezaian, M. A., Williams, R. H. V., Gould, A. R., and Symons, R. H. 1984. Nucleotide sequence of cucumber-mosaic-virus RNA 2 reveals a translation product significantly homologous to corresponding proteins of other viruses. *Eur. J. Biochem.* 143:277-284.
- Rezaian, M. A., Williams, R. H. V., and Symons, R. H. 1985. Nucleotide sequence of cucumber mosaic virus RNA 1. Presence of a sequence complementary to part of the viral satellite RNA and homologies with other viral RNAs. *Eur. J. Biochem.* 150:331-339.
- Rizzo, T. M. 1989. Molecular analysis of RNAs 1 and 2 from cucumber mosaic virus and *in vitro* generation of infectious transcripts. Ph.D. thesis, Cornell University, Ithaca, NY. 103 pp.
- Rizzo, T. M., and Palukaitis, P. 1988. Nucleotide sequence and evolutionary relationships of cucumber mosaic virus (CMV) strains: CMV RNA 2. *J. Gen. Virol.* 69:1777-1787.
- Rizzo, T. M., and Palukaitis, P. 1989. Nucleotide sequence and evolutionary relationships of cucumber mosaic virus (CMV) strains: CMV RNA 1. *J. Gen. Virol.* 70:1-11.
- Schwinghamer, M. W., and Symons, R. H. 1975. Fractionation of cucumber mosaic virus RNA and its translation in a wheat embryo cell-free system. *Virology* 63:252-262.
- Schwinghamer, M. W., and Symons, R. H. 1977. Translation of the four major RNA species of cucumber mosaic virus in plant and animal cell-free systems and in toad oocytes. *Virology* 79:88-108.
- Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.
- Stussi-Garud, C., Garud, J. C., Berna, A., and Godefroy-Colburn, T. 1987. *In situ* location of an alfalfa mosaic virus non-structural protein on plant cell walls: Correlation with virus transport. *J. Gen. Virol.* 68:1779-1784.
- Winter, E., Yamamoto, F., Almoguera, C., and Perucho, M. 1985. A method to detect and characterize point mutation in transcribed genes: Amplification and overexpression of the mutant c-Ki-ras allele in human tumor cells. *Proc. Natl. Acad. Sci. USA* 82:7575-7579.
- Wyatt, S. D., and Kuhn, C. W. 1980. Derivation of a new strain of cowpea chlorotic mottle virus from resistant cowpeas. *J. Gen. Virol.* 49:289-296.