Genetic Analyses of Two Large-Lesion Isolates of Cucumber Mosaic Virus

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ABSTRACT

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Four strains of cucumber mosaic virus (CMV) induced small (≤0.1 cm diameter) necrotic lesions upon inoculation to cowpea, *Vigna unguiculata* ssp. *cylindrica* 'Catjang.' In addition, a few large (0.4–0.6 cm) necrotic lesions appear at the rate of 0.11–5.26% (0.53% average) of total lesions. It was shown that these large lesion-producing isolates were mutants derived from their parent strains (17). In this work, pseudorecombination analysis showed that RNA 2 carries the genetic determinant for the large-lesion phenotype in two large-lesion isolates derived from CMV-C and CMV-N strains. In addition, RNA 1 of a large-lesion isolate of CMV-N was found to be responsible for induction of necrotic local lesions on cotyledons of *Cucurbita pepo* cultivar President and the inability to move systemically in *Nicotiana tabacum* cultivar H-423.

Cucumber mosaic virus (CMV) is a multicomponent virus with worldwide distribution and numerous strains (15). Its high variability is exemplified by the presence of several strains affecting vegetable crops within New York state (6,24,25). During this investigation, we observed that four CMV strains (CMV-C [25], CMV-N [30], CMV-WL [6], and CMV-L-2 [25]) which were known to produce small necrotic lesions on cowpea (*Vigna unguiculata* ssp. *unguiculata* 'Blackeye') also occasionally produced large necrotic lesions on *V. unguiculata* ssp. *cylindrica* 'Catjang.' In our previous communication (17), we reported the isolation and characterization of those large-lesion-producing isolates of CMV which were found to be both biologically and biochemically identical with the parental strains. On that basis we have postulated that the large-lesion isolates are mutants of the parent CMV strains. Viral mutants are useful in investigations of gene structure, function, and regulatory mechanisms. To obtain more information on the CMV-host interaction at the molecular level, it was considered important to investigate the distribution of gene(s) that determine lesion size on Catjang.

The three genomic RNAs of CMV (18,23) are single-positive strands (coding strands) designated as RNA 1, RNA 2, and RNA 3 in order of decreasing molecular mass (i.e., 3.97, 3.53, and 2.56 kilobases) (5). A subgenomic RNA (1.9 Kb and designated as RNA 4) has a nucleotide sequence identical to that of the 3′-terminus of RNA 3 (9) and codes for CMV coat protein (27). Small, single-stranded RNAs, variously known as RNA 5, satellite RNA, or CARNA 5 (8,12), are associated with some strains of CMV. These RNAs, in association with CMV, may induce new disease symptoms (6,14,29) and/or regulate symptom expression and CMV replication in particular host plants (11,13,31). The tripartite genome of CMV facilitates genomic analysis of CMV strains. Many such studies have already demonstrated the usefulness of pseudorecombinants for genetic functions of RNA species in naturally occurring CMV strains (3,10,20,21,26).

In this paper we report the results of our experiments designed to locate the distribution of gene(s) in two CMV mutants (CMV CL #5 and CMV NL #1) determining large-lesion production on Catjang by in vitro pseudorecombination.

MATERIALS AND METHODS

Virus isolates. CMV-C (25) and CMV-N (30) were maintained in zucchini squash (*Cucurbita pepo* 'President') and periwinkle (*Vinca rosea*), respectively. Selection of large-lesion mutants following inoculation of CMV-C and CMV-N strains to Catjang was described previously (17). It has been shown that large-lesion mutants reverted to small-lesion types after four serial mass passages each through *Chenopodium quinoa*, cucumber (*Cucumis sativus* 'Marketer'), squash, or tobacco (17). However, they remained stable if passed through Catjang. Considering this instability, four serial single-lesion transfers of the large-lesion mutants CMV-CL #5 and CMV-NL #1 were made through Catjang and then further multiplied in Catjang. The infected Catjang leaves were used for inoculation to squash and cucumber with CMV-CL #5 and CMV-NL #1, respectively. Inoculum from systemically infected squash and cucumber leaves produced only large lesions on Catjang. Purification of the isolates was carried out with those infected squash and cucumber leaves. The CMV-C and CMV-N were propagated in squash and cucumber, respectively.

Purification of virus and separation of RNAs. Virus was purified from systemically infected squash or cucumber leaves by the procedure of Lot et al (19). After the final high-speed centrifugation, the virus pellet was resuspended in PEN buffer (0.01 M NaH_2PO_4, 0.001 M EDTA, 0.001 M NaN_3, pH 7.0). RNA was extracted from purified virus by the phenol-SDS method (2, 7), and suspended in sterile PEN buffer. RNA was separated into genomic components by two successive cycles of electrophoresis in 2.4% polyacrylamide-*bis* acrylamide (20:1) gels (28), and the RNA species were recovered from the gel by the bentonite extraction procedure of Edwards et al (3).

Tests of purity and infectivity of RNA preparations. Purity of the separated RNA components was determined by enhancement tests on *C. quinoa* with various combinations of homologous RNA (Table 1). To construct in vitro pseudorecombinants, RNA 1, RNA 2, and RNA 3 of CMV-C, which produced small lesions on Catjang, were exchanged with those of either CMV-CL #5 or CMV-NL #1. About 800 μg of bentonite per milliliter was added to each RNA preparation before corundum-dusted *C. quinoa* leaves were inoculated with the aid of sterile ground-glass spatulas. Following inoculation, leaves were rinsed with sterile, distilled water. The pathogen in about 20 discrete single lesions was individually isolated and inoculated to leaves of *C. quinoa* which were subsequently used as inoculum sources. The pseudorecombinants were designated on the basis of exchanged RNAs of the donor CMV. Thus, CCL denotes C-RNA 1 + C-RNA 2 + CL #5 RNA 3.

Test of pseudorecombinants. Single-lesion isolates of each pseudorecombinant were inoculated to young primary leaves of four-to-six Catjang seedlings. Pseudorecombinants constructed from CMV-C and CMV-NL #1 were passed through cucumber

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seedlings before being inoculated to tobacco and squash. Parent strains were inoculated as controls. All seedlings were grown and maintained in the greenhouse at 25–28 C and supplemented with fluorescent light to ensure 18 hr of light per day.

RESULTS

Characteristics of CMV isolates used for pseudorecombination. The CMV-C and CMV-N strains could be distinguished on a set of

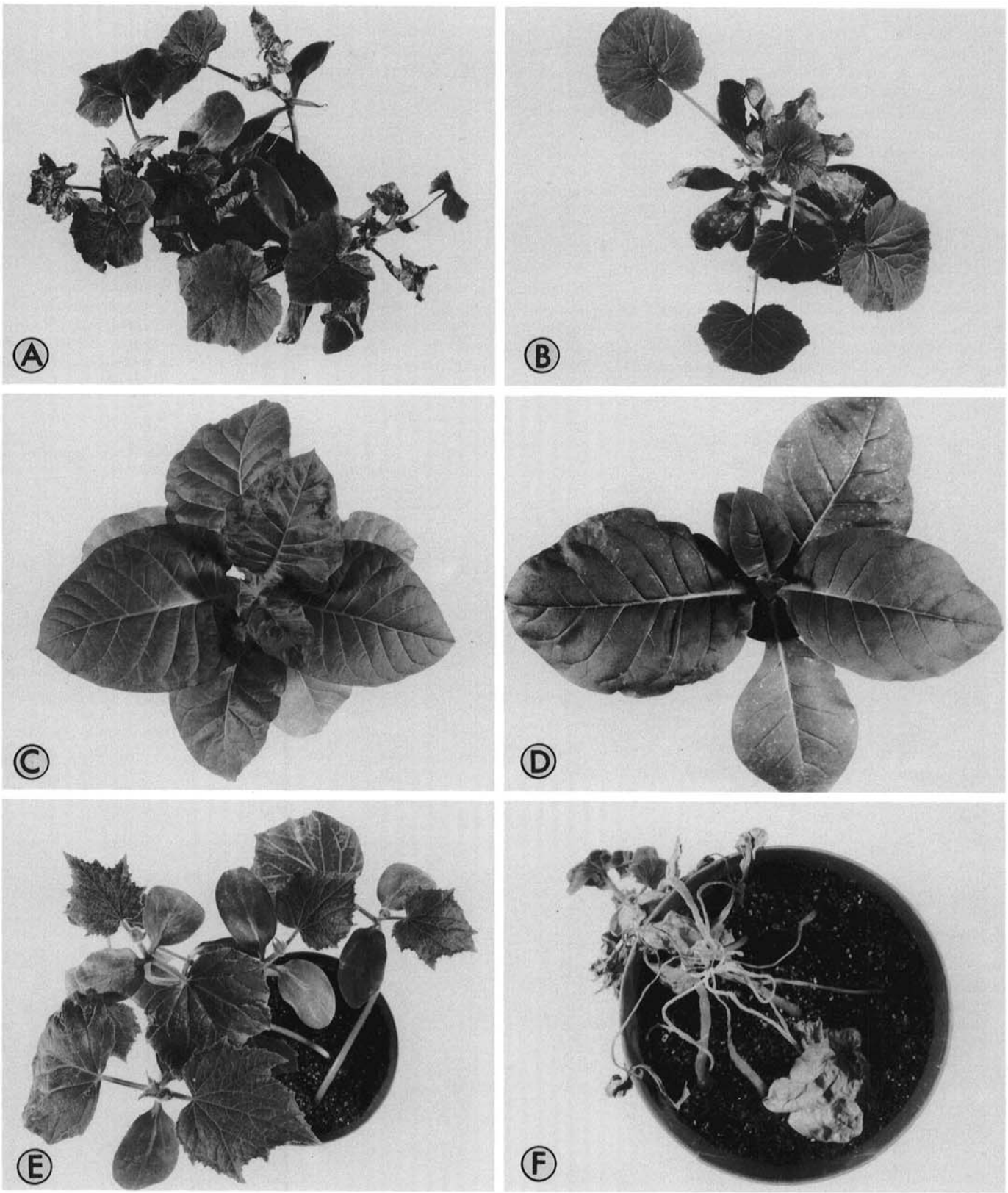

Fig. 1. Characteristic symptoms induced by cucumber mosaic virus (CMV) strain CMV-C and mutant CMV-NL #1 derived from CMV-N on different hosts. **A,** Chlorotic spots on the cotyledons and systemic mosaic on *Cucurbita pepo* 'President' inoculated with CMV-C. **B,** Local necrotic spots on the cotyledons of *C. pepo* 'President' inoculated with CMV-NL #1. **C,** Systemic mosaic on *Nicotiana tabacum* 'H-423' inoculated with CMV-C. **D,** Local chlorotic and necrotic spots on *N. tabacum* 'H-423' inoculated with CMV-NL #1. **E,** Systemic mosaic on *Cucumis sativus* 'Marketer' inoculated with CMV-C. **F.** Systemic mosaic and wilting of *C. sativus* 'Marketer' inoculated with CMV-NL #1.

diagnostic hosts like *C. pepo* cultivar President, *Nicotiana tabacum* cultivar H-423, and *Cucumis sativus* cultivar Marketer (Table 2). The characteristic symptoms produced on these hosts are shown in Fig. 1. The large-lesion isolates, CMV-CL #5 and CMV-NL #1, produced symptoms similar to CMV-C and CMV-N, respectively. On the primary leaves of Catjang, however, CMV-C and CMV-N produced small (≤0.1 cm) necrotic lesions, whereas large-lesion isolates produced necrotic lesions 0.4–0.6 cm in diameter (Fig. 2). Polyacrylamide gel electrophoresis of RNA showed that CMV-C contained detectable RNA 5 and that CMV-CL #5 contained a trace of RNA 5. Inoculation of this virus preparation to tomato did not induce necrosis (*unpublished*). The CMV-N and CMV-NL #1 did not contain a detectable amount of RNA 5 (Fig. 3).

Pseudorecombination analyses of the large-lesion phenotype. The homologous mixtures of fractionated RNAs from CMV-C, CMV-CL #5, and CMV-NL #1 produced phenotypic responses similar to their intact virus from infective plant sap (Table 3). Among the six pseudorecombinants constructed by exchange of RNA 1, RNA 2, and RNA 3 between CMV-C and CMV-CL #5, as well as between CMV-C and CMV-NL #1, those with RNA 2 from CMV-CL #5 and CMV-NL #1 induced large lesions on Catjang. In such cases, the number of large lesions ranged from 30 to 50 per leaf. Pseudorecombinants with RNA 2 from CMV-C produced 15–30 small lesions per leaf. This indicated that the factor(s) responsible for large-lesion production reside in the RNA 2 of both CMV-CL #5 and CMV-NL #1. Some anomalies were observed. For example, one of 20 single-lesion isolates (SLI) with the expected LCL genotype (RNA 1 and RNA 3 of CMV-CL #5 + RNA 2 of CMV-C) caused large lesions on Catjang. Similarly, two of 12 SLIs expected to have genotype CNC produced small lesions, and one of 11 SLIs expected to have genotype NCN (RNA 1 and RNA 3 of CMV-NL #1 + RNA 2 of CMV-C) produced large lesions. Cross contamination of the original RNA preparations was the most likely explanation for these exceptions.

Pseudorecombination analyses of some pathological properties diagnostic for CMV-N strain. Since CMV-C and CMV-NL #1 could be distinguished by their symptoms on squash, tobacco, and cucumber (Table 2), pseudorecombinants constructed from CMV-C and CMV-NL #1 (cf., Table 3) were inoculated to squash, tobacco, and cucumber to determine which RNA(s) code for specific symptoms. A homologous mixture of RNA 1, RNA 2, and RNA 3 from CMV-C and CMV-NL #1 produced phenotypic responses similar to the respective virus from infective plant sap (Table 4). Among the heterologous combinations, most of the single-lesion isolates with the expected RNA 1 of CMV-NL #1 RNA 1 conditioned: production of necrotic local lesions on the cotyledons of squash, poor systemic movement in squash, and lack of systemic infection on tobacco. Limited observations (*unpublished*) also suggest that RNA 1 enables this isolate to cause necrotic local lesions on cotyledons and necrotization of stems of cucumber. Similarly, CMV-C RNA 1 determined systemic movement in tobacco. However, some discrepancies were noticed

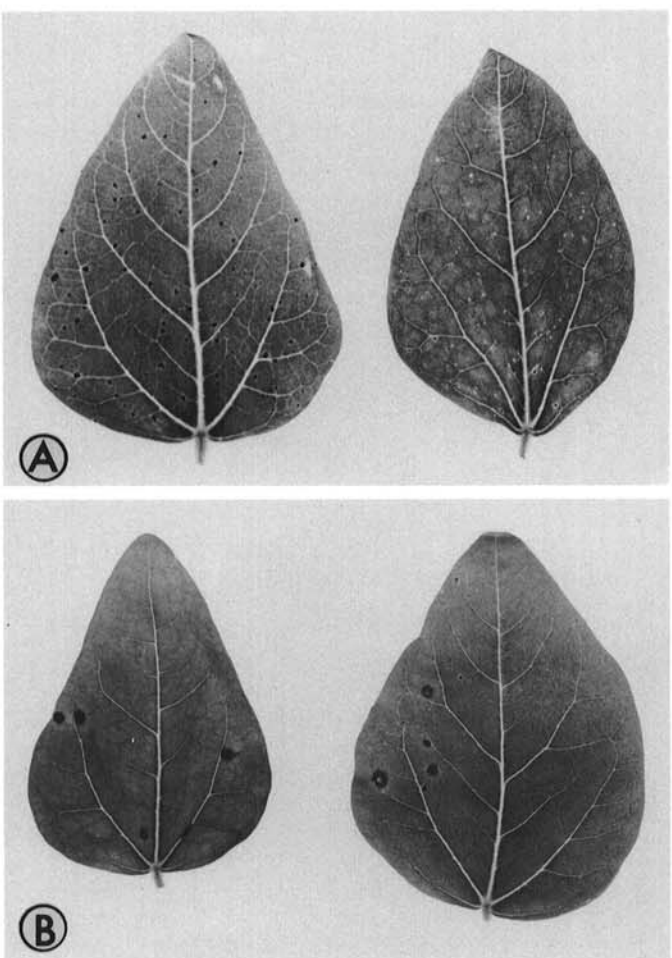

Fig. 2. Symptoms induced by cucumber mosaic virus (CMV)-C and a large-lesion isolate derived from CMV-C on *Vigna unguiculata* ssp. *unguiculata* 'Blackeye' and *V. unguiculata* ssp. *cylindrica* 'Catjang.' **A,** Small necrotic spots on Blackeye (right) and Catjang (left) primary leaves inoculated with CMV-C (white spots on Catjang were not due to infection). **B,** Large necrotic spots on Blackeye (left) and Catjang (right) primary leaves inoculated with large-lesion isolate CMV-CL #5.

TABLE 1. Test of purity and infectivity of RNA 1, RNA 2, and RNA 3 of cucumber mosaic virus (CMV)-C, CMV-NL#1, and CMV-CL#5 separated by two cycles of gel electrophoresis

Isolate	Inoculum dilution	Combination of RNAs				
		1, 2, 3	(1+2)	(1+3)	(2+3)	(1+2+3)
C	1/25	0.0[z]	0.0	0.0	0.40	3.10
	1/50	0.0	0.0	0.0	0.30	1.40
CL#5	1/50	0.0	0.8	2.4	0.60	34.60
	1/100	0.0	0.6	2.0	0.20	6.20
	1/150	0.0	0.0	0.0	0.00	5.00
NL#1	1/25	0.0	0.0	0.0	0.18	1.33
	1/50	0.0	0.0	0.0	0.09	0.36
NL#1	1/25	0.0	0.0	1.2	0.50	8.70
	1/50	0.0	0.0	0.4	0.10	7.50

[z] Number represents the average number of local lesions on 10–12 leaves of *Chenopodium quinoa*.

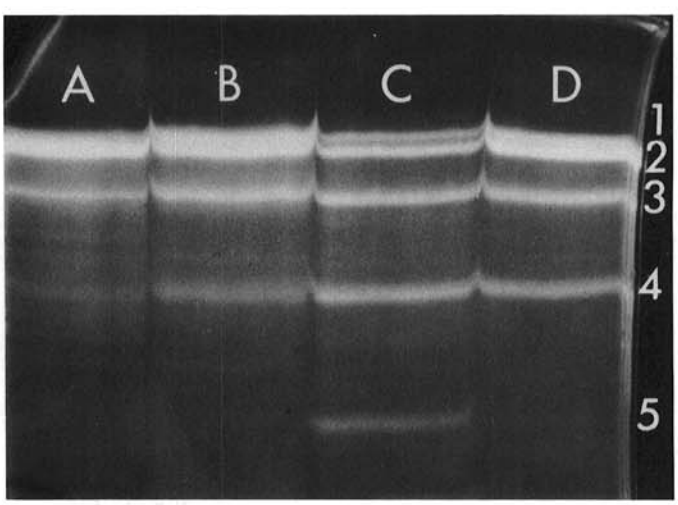

Fig. 3. Electrophoretic patterns of total RNA from cucumber mosaic virus (CMV) strains C and N, as well as the large-lesion isolates CMV-CL #5 and CMV-NL #1 in 2.4% polyacrylamide slab gel (numbers designate RNA species). **A,** CMV-N; **B,** CMV-NL #1; **C,** CMV-C; and **D,** CMV-CL #5.

TABLE 2. Symptoms induced by cucumber mosaic virus (CMV)-C, CMV-N, and large-lesion mutant CMV-CL#5 and CMV-NL#1 on some diagnostic hosts

CMV isolates	Host reactions[x]			
	Cucurbita pepo 'President'	*Nicotiana tabacum* 'H-423'	*Cucumis sativus* 'Marketer'	*Vigna unguiculata* ssp. *cylindrica* 'Catjang'
C	Cys (cotyledon) M	M	M	Snl
CL#5	Cys (cotyledon) M	M	M	Lnl
N	Nls (cotyledon) Ns[y]	Nls Ns[z]	Nls (cotyledon) Mm, Ssn	Snl
Nl#1	Nls (cotyledon) Ns[z]	Cnl Ns[z]	Nls (cotyledon) Mm, Ssn	Lnl

[x]Cnl = Chlorotic and necrotic lesions, Cys = chlorotic yellow spots, Lnl = large necrotic lesions (0.4–0.6 cm in diameter), M = mosaic, Mm = mild mosaic, Nls = necrotic local lesions, Ns = nonsystemic, Snl = small necrotic lesions (≤0.1 cm in diameter), and Ssn = severe stem necrosis.
[y]Occasionally mild mottle and necrosis appear on uninoculated leaves.
[z]Rarely systemic yellow spots appear on uninoculated leaves.

TABLE 3. Symptoms on *Vigna unguiculata* ssp. *cylindrica* 'Catjang' inoculated with pseudorecombinants constructed by exchanging RNA 1, RNA 2, and RNA 3 between cucumber mosaic virus (CMV)-CL #5 and CMV-C as well as between CMV-NL #1 and CMV-C

Inoculum	SLI (no.)	Reaction of SLI[w] on Catjang	
		Large lesions[x]	Small lesions[x]
Exp. #1			
C virus	10	0[y]	10
CL #5 virus	10	10	0
CCC[z]	10	0	10
LLL	10	10	0
CCL	21	0	21
LLC	19	19	0
CLC	22	22	0
LCL	20	1	19
CLL	20	20	0
LLC	19	19	0
Exp. #2			
C virus	10	0	10
NL #1 virus	10	10	0
CCC	7	0	7
NNN	5	5	0
CCN	15	0	15
NNC	10	10	0
CNC	12	10	2
NCN	11	1	10
NCC	10	1	9
CNN	10	8	2

[w]SLI = single lesion isolates.
[x]About 30–50 large lesions and 15–30 small lesions appeared per cultivar Catjang leaf inoculated with different SLI.
[y]Number of SLI of each pseudorecombinants producing large or small lesions.
[z]Letters refer to RNA 1, RNA 2, and RNA 3 of exchanging strains; thus, CCL = C-RNA 1 + C-RNA 2 + CL #5-RNA 3, and CCN = C-RNA 1 + C-RNA 2 + NL #1-RNA 3.

among SLIs involving the exchange of RNA 1 and RNA 2. Again, cross contamination is the most likely explanation for such minor exceptions.

DISCUSSION

In a separate communication (17) we demonstrated that the large-lesion-producing CMV isolates originated from their parental strains by mutation. Hence, we were interested in locating the site of mutation on the tripartite genome of CMV. Although genetic analyses of RNAs of CMV have been made with several

TABLE 4. Expression of symptoms on *Cucurbita pepo* 'President' and *Nicotiana tabacum* 'H-423' after inoculation with pseudorecombinants constructed by exchanging RNA 1, RNA 2, and RNA 3 of cucumber mosaic virus (CMV)-NL #1 and CMV-C

Inoculum	SLI tested (no.)	Reaction[w] of SLI[x]		
		President		H-423
		Cotyledons inoculated	Leaves uninoculated	
		Chl/Nec	Sm/Mm/Ns	Sm/Ns[y]/Ys
Exp. #1				
C virus	10	10/0	10/0/0	10/0/0
NL #1 virus	10	0/10	0/5/5	0/9/1
CCC[z]	12	12/0	12/0/0	12/0/0
NNN	10	0/10	0/2/8	0/10/0
CCN	15	14/1	14/1/0	14/1/0
NNC	16	0/16	0/2/14	0/13/3
CNC	15	14/1	14/1/0	14/1/0
NCN	15	0/15	0/8/7	0/11/4
NCC	13	1/12	1/4/8	1/12/0
CNN	15	15/0	15/0/0	15/0/0
Exp. #2				
NCC		0/5	0/5/0	3/6/0
CNC		5/0	5/0/0	7/1/0
CCN		5/0	5/0/0	10/0/0

[w]Chl = chlorotic yellow spots, Mm = mild mottling with necrosis, Nec = necrotic spots, Ns = nonsystemic, Sm = mosaic, Ys = rare systemic yellow spots on uninoculated top leaves.
[x]SLI = single lesion isolates.
[y]Top leaves of symptomless plants bioassayed on *Chenopodium quinoa* produced no local lesions.
[z]Letters refer to RNA 1, RNA 2, and RNA 3 of exchanging strains; thus, CCN = C-RNA 1 + C-RNA 2 + NL #1-RNA 3.

naturally occurring strains of CMV (3,10,20,21,26), ours is the first attempt to utilize this technique to locate host-selected pathogenic mutations in CMV strains. Thorough examination of the pseudorecombinants constructed between CMV isolates that produce small and large lesions on Catjang, indicated that the genetic determinant for large-lesion type resides on RNA 2. In addition, we also observed that some of the large-lesion-producing mutants from CMV-C, CMV-N, CMV-WL, and CMV-L-2 could systemically infect Blackeye and produce a severe mosaic with mild necrosis on uninoculated leaves (17). Interestingly, several workers have established that the systemic infection in cowpea of naturally occurring CMV strains E and L (10), DS (20), and B (3) is due to RNA 2. It is reasonable to assume that spontaneous mutation in the RNA 2 of local-lesion-producing CMV strains followed by selection through suitable host(s) could lead to the appearance of new CMV strains, which would overcome the hypersensitive resistance of cowpea. However, present observations do not exclude other possibilities. Appearance of new CMV strains by host selection mechanism was also reported by Yarwood (34).

Pseudorecombination analysis with CMV-C and CMV-NL #1 also revealed that CMV-NL #1 RNA 1 determined some of its diagnostic pathogenic properties. To our knowledge, this is the first report of pathogenic properties solely determined by CMV RNA 1. Several artificially-induced temperature-sensitive mutations have been located in RNA 1 of alfalfa mosaic virus (AMV) (4) and cowpea chlorotic mosaic virus (CCMV) (1). RNA 1 of CCMV is also responsible for systemically infecting a selection (PI 186465) of cowpea (32). Interestingly, each of these tripartite genomic viruses in which RNA 1 was found to determine pathogenic properties was either a spontaneous or artificially induced mutant of the parent strain (1,4,30,32).

The molecular mechanism of lesion enlargement or systemic spread of CMV is unknown. However, for two other "tricorna viruses" (brome mosaic virus [BMV] [16] and AMV [22]) RNA 1 and RNA 2 appear to affect RNA synthesis while RNA 3 is necessary for cell-to-cell spread and coat protein synthesis. Since CMV is a "tricorna virus," host protein(s) in Catjang may specifically interact with CMV RNA 2 protein(s) and enhance the

rate or extent of RNA synthesis. The rate of CCMV accumulation in protoplasts of resistant and susceptible cowpea have been investigated by Wyatt and Wilkinson (33). They observed that the rate of CCMV accumulation in the individual protoplast is strongly correlated with the susceptibility of cowpea. Similar work with CMV large-lesion isolates and cowpea protoplasts should be helpful in understanding CMV-host interaction at the molecular level.

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