Differentiation of Pseudorecombinants of Two Cucumber Mosaic Virus Strains by Biological Properties and Aphid Transmission

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ABSTRACT

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Pseudorecombinants prepared from the RNAs of two distinct strains of cucumber mosaic virus (CMV) were studied to determine reasons for differences in symptom severity and transmission efficiency by *Aphis gossypii*. Strain CMV-FNY, readily transmitted to and from summer squash, and CMV-SNY, a moderately transmitted strain, were used. Genomic RNAs 1, 2, and 3 of CMV-FNY and CMV-SNY were used to construct pseudorecombinants in all combinations. Symptom severity ratings were higher when RNA 1 from CMV-FNY was present than when recombinants were constructed with RNA 1 from CMV-SNY. The average number of days required to reach 100% symptom expression was lower when RNA 1 from CMV-FNY was present. Maximum aphid transmission and symptom severity occurred for the combination of RNAs 1 and 3 from CMV-FNY (e.g., F-S-F), followed by the combinations of F-F-S, S-S-F, and S-F-S. Results suggest that RNA 1, associated with virus replication, is most important in achieving maximum transmission and symptom severity. RNA 3 appears to have an intermediate role in aphid transmission and symptom expression.

Cucumber mosaic virus (CMV) is an aphid-transmitted agent that causes a serious disease in many vegetable crops, especially cucurbits such as zucchini summer squash (*Cucurbita pepo* L.) and muskmelon (*Cucumis melo* L. var. *reticulatus* Naudin) which are grown in many countries (5,6,10,13). The virus can exist in nature as many strains that are differentially transmitted (3,9). Reasons for differences in aphid transmission efficiency have been investigated. Factors that affect aphid behavior are known to influence transmission efficiency (4). Similarly, differences in virus concentration in some instances account for variation in transmissibility (11). In more recent studies, pseudorecombinants or transcapsidations were used to show that the transmissibility of CMV strains was associated with coat protein (3,8).

While studying the occurrence of CMV in summer squash and muskmelon in New York State, Banik and Zitter found that two strains of CMV from muskmelon differed markedly in aphid acquisition and transmission (1). The first strain, designated CMV-FNY (F = fast; strain spreads rapidly) was recovered from a muskmelon farm with a long history of early-season infections with CMV; these infections resulted in annual losses that exceeded those of most other locations within the state. The farm had been in melon production for more than 60 yr. Early efforts to reduce the rate of spread of the virus by using the best available techniques such as oil sprays (12) were unsuccessful (T. A. Zitter, *unpublished data*). A second strain, designated CMV-SNY (S = slow; strain spreads slowly), was recovered from a mixed vegetable farm 24 km away, where the rapid spread of CMV was not a limiting factor for crop production. Studies conducted in growth chambers showed that CMV-FNY was more efficiently transmitted by aphids than was CMV-SNY, and this was correlated with higher virus titers in muskmelon (1). Symptoms caused by CMV-FNY in summer squash also tended to be more severe than those caused by CMV-SNY.

In this study, pseudorecombinants were prepared from the RNAs of these strains to determine what effect each RNA could exert on aphid transmission and symptom development and if enhanced aphid transmission could be separated from the increase in virus titer as an explanation for the differences observed in virus epidemiology.

MATERIALS AND METHODS

Virus propagation and assay. The two CMV strains (CMV-FNY and CMV-SNY) were maintained by either mechanical or aphid transmission in *C. melo* 'Saticoy' and occasionally in *C. pepo* 'Zucchini Elite.' For mechanical assays done at Geneva, NY, the zucchini cultivar President was used, while Zucchini Elite plants were used in all tests performed at Ithaca, NY. To maintain uniformity among the trials in Ithaca, all plants were grown in an environmental chamber (24 C during the day, 18 C at night, under 10,000 lx fluorescent light for a 14-hr photoperiod) during the test period.

Virus and RNA purification. CMV-FNY and CMV-SNY were propagated in zucchini squash cultivar President and purified

as described by Lot et al (7). The yield of CMV-FNY was approximately 120 mg/470 g of tissue, and for CMV-SNY it was approximately 136 mg/290 g of tissue. RNAs 1, 2, and 3 of CMV-FNY and CMV-SNY were extracted and separated by two cycles of polyacrylamide gel electrophoresis with the method developed by Edwards et al (2). The RNA samples were stored at −80 C.

Infectivity of RNA preparations and construction of pseudorecombinants. Infectivity tests of the RNA species were done with various combinations of homologous RNAs. RNA concentrations were not determined because of the low levels on the gels. Instead, RNA constructs from each of the two strains were inoculated at various dilutions into *Chenopodium quinoa* Willd. plants. All RNAs were kept in sterile PEN (0.01 M sodium phosphate, 0.001 M EDTA, 0.01% sodium azide, pH 7.0) buffer containing 800 μg of bentonite per milliliter. *C. quinoa* leaves were dusted with corundum and inoculated with glass spatulas. Subsequently, pseudorecombinants between CMV-FNY and CMV-SNY were constructed in all combinations (Table 1) (2). Twenty single-lesion isolates (pseudorecombinants) from each combination were transferred to a single *C. quinoa* leaf, harvested, stored in a refrigerator, and assayed to three President zucchini squash plants per 10-cm pot (60 plants total). Plants were held in a greenhouse at Geneva, and symptoms were rated once after 19 days. Each plant was assigned a symptom category with the following scale: 0 = no visible symptoms; 1 = isolated chlorotic spots on the mechanically inoculated cotyledons and a few chlorotic spots on the first true leaves systemically infected; 2 = systemic symptoms of more numerous chlorotic spots on the first and second true leaves but no symptoms on additional leaves; 3 = more extensive systemic symptoms with chlorotic spots and mosaic on two or more leaves; 4 = moderate to severe mosaic symptoms with leaf-cupping; 5 = severe symptoms resulting in plant death. Symptom ratings were calculated by multiplying the number of plants by the assigned value and an average value determined for each group. These ratings and subsequent selection of representative plant samples were made by the first author, who did not know the genomic composition of the groups. Six samples, consisting of pooled tissue from the three plants per pot and spanning the symptom rating for each group, were collected. The tissue was stored at −135 C for subsequent testing.

Symptom expression of CMV-FNY, CMV-SNY, and pseudorecombinants. Average symptom ratings for the two parent strains and the pseudorecombinants were based on six trials conducted over a 5-mo period with 20 Zucchini Elite plants per trial. Plants were inoculated mechanically in the cotyledonary stage. The number of days for symptoms to appear on the cotyledons and on the first and second leaves were recorded daily for 10 days. Each plant was assigned a symptom rating according to the following severity categories: 0 = no visible symptoms; 1 = isolated chlorotic spots appear on the cotyledon(s) or the first true leaf; 2 = isolated chlorotic spots on systemically infected leaves 1, 2, and 3; 3 = more extensive systemic symptoms with chlorotic spots and mosaic on three or more leaves; 4 = moderate virus symptoms without leaf-cupping; 5 = most severe symptoms resulting in leaf-cupping, distortion, and stunting. Average symptom ratings were calculated as previously described. Because these experiments were conducted in an environmentally controlled chamber and the symptoms were recorded daily for the first 10 days, the symptom severity readings were more precise than the Geneva readings. After the 10-day period, plants were held in a greenhouse (24 C) for an additional 2 wk, and were discarded after a final symptom reading to note any change in symptom severity. Twenty-one plants were inoculated with known CMV-FNY and CMV-SNY and were included with each test as controls (126 plants per strain).

Aphid transmission of strains and pseudorecombinants. Zucchini Elite plants were used as source plants (acquisition access) and test plants (inoculation access) in all aphid transmission trials. A colony of melon aphids (*Aphis gossypii* (Glover)) was obtained from G. G. Kennedy, North Carolina State University, and was reared on CMV-resistant cucumber plants in an insectary. Late instar apterous aphids were starved for 1–3 hr before being used in transmission tests. For transmission to and from squash plants, one aphid per 20 test plants was used after 1 min of acquisition-access feeding. Time-course studies were done in growth chambers to determine the efficiency of aphid transmission of the two parent strains and four of the eight pseudorecombinants (groups 2, 3, 6, and 7) from different leaf positions over a 5-wk period. Squash source plants were mechanically inoculated in the cotyledonary stage and the leaves selected for assay were the lowest ones that first showed systemic symptoms (experiment 1), or ones that subsequently developed and became fully expanded (progressive leaf samples, experiment 2). Both experiments were performed twice, and data on the percentage of transmission and the symptom ratings are averages. After a 1-hr exposure period, all test plants were fumigated with dichlorvos and then returned to the chambers. Symptoms were recorded daily for a 3-wk period.

TABLE 1. Combination of RNA species used to construct pseudorecombinants of cucumber mosaic virus strains FNY and SNY

Recombinant no.	Combination[a]
1 (original fast)	$1^F + 2^F + 3^F$
2	$1^F + 2^F + 3^S$
3	$1^F + 2^S + 3^F$
4	$1^F + 2^S + 3^S$
5 (original slow)	$1^S + 2^S + 3^S$
6	$1^S + 2^S + 3^F$
7	$1^S + 2^F + 3^S$
8	$1^S + 2^F + 3^F$

[a] Numbers represent RNA species. Superscripts indicate strain donating RNA species.

RESULTS

Separation and infectivity of RNA species. Data in Table 2 show that RNA species 1, 2, or 3 was not infectious alone. The highest infectivity was found with the combination of RNA preparations (1 + 2 + 3). Cross-contamination of RNA preparations could be minimized by dilution (2). Pseudorecombinants between CMV-FNY and CMV-SNY (Table 1) were then prepared with RNAs of CMV-SNY at 0.2 and CMV-FNY at 0.04 dilution.

Symptom severity ratings from initial inoculation. Of the 480 President squash plants inoculated with the local lesion inoculum, 42 plants failed to express symptoms (rated 0 for uninfected). For the most part, this was found for those recombinants when RNA 1 came from CMV-SNY (recombinant numbers 5–8; Table 1). An exception was noted with recombinant 4 (F-S-S) in which seven negative transmissions occurred. In contrast, 12 plants died as a result of infection; this occurred for recombinants when RNA 1 came from CMV-FNY (numbers 1

TABLE 2. Infectivity of separated and mixed genomic RNAs of cucumber mosaic virus (CMV) strain FNY or strain SNY

Strain	Inoculum dilution[a]	RNA combination				
		1,2,3[b]	1+2[c]	1+3	2+3	1+2+3
FNY	0.2	0[d]	4	34	75	...[e]
	0.04	0	1	5	11	86
SNY	0.2	0	0	1	1	14
	0.04	0	0	0	0	1

[a] Initial RNA concentrations not determined because of low levels.
[b] Inoculum consisted of either RNA 1, 2, or 3 of strain FNY or strain SNY.
[c] Inoculum consisted of equal concentrations of RNA 1 and 2 of strain FNY or strain SNY.
[d] Numbers refer to the average number of local lesions per leaf on *C. quinoa*.
[e] Average number of local lesions exceeded 120 per leaf.

[five dead], 2 [five dead], 3 [one dead], and 4 [one dead]). Although symptoms were read late (19 days after inoculation) and some plants showed signs of recovery, two main groups for symptom severity were observed; recombinants 1–4 averaged a 4.35 rating compared with 3.41 for recombinants 5–8.

Influence of genomic composition on time and severity of symptoms. The range of symptoms produced on CMV-infected Zucchini Elite squash by either mechanical or aphid inoculation was very consistent between trials (Fig. 1). No additional infected plants were found after the initial observation period, and symptom ratings remained unchanged during the 3-wk observation period. This symptom rating method was used in all tests. Several attempts to recover virus from plants rated as 0 (no symptoms) were all unsuccessful.

The reaction of squash plants to the eight RNA groupings is shown in Table 3. When the overall average symptom rating for each group was calculated, plants in groups 1–4 had the most severe symptom ratings (0.6–4.8); groups 2 and 4 were intermediate for symptom expression. Plants in groups 5–8 had less severe symptoms (0.4–3.2). These data indicated that RNA 1 from CMV-FNY produced severe symptoms and agreed with the initial inoculations. Analysis of the reaction showed that RNAs

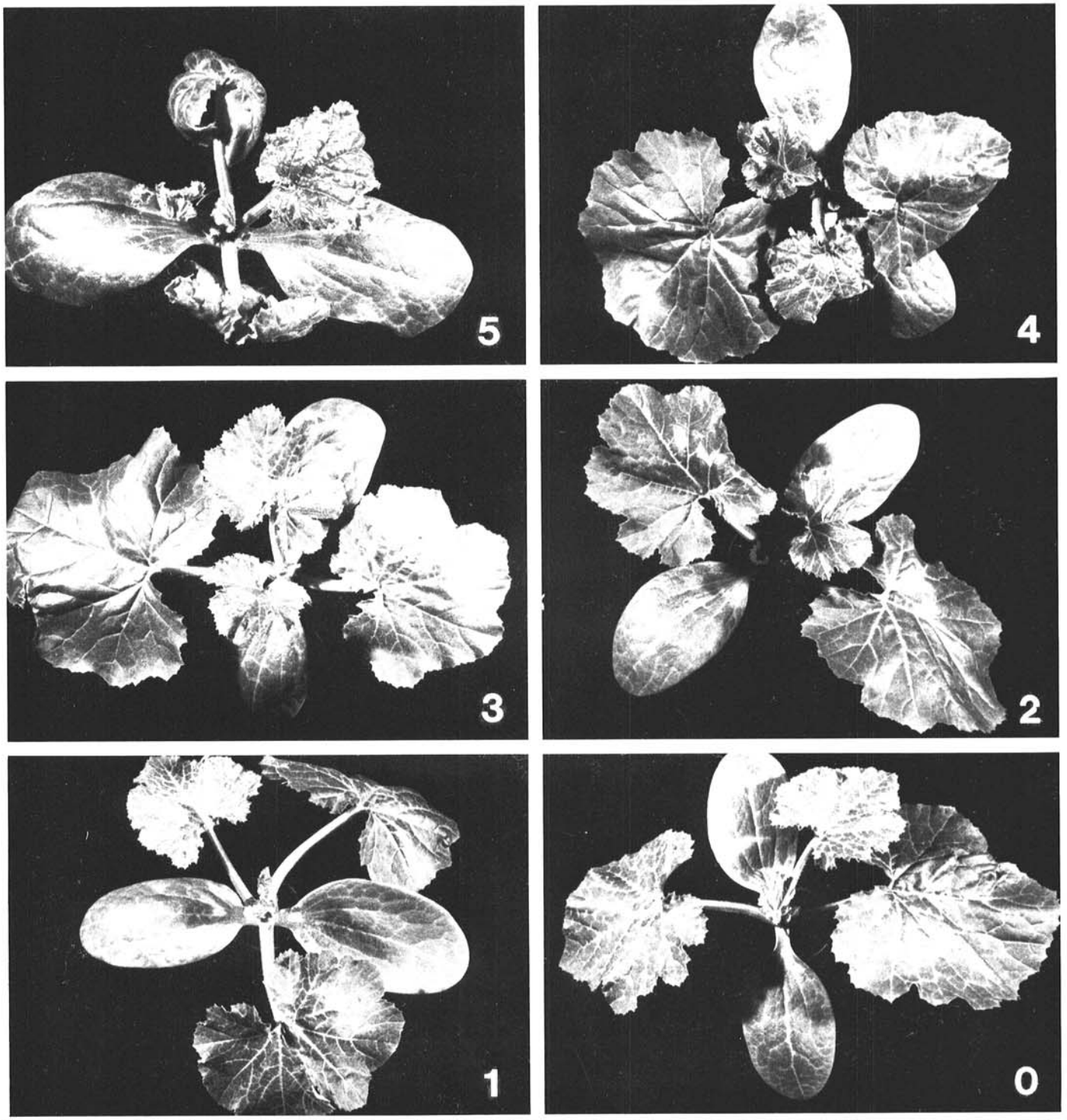

Fig. 1. Virus symptom rating method used in tests, done in Ithaca, NY, for evaluating Zucchini Elite squash infected with cucumber mosaic virus. 0 = no visible symptoms; 1 = an isolated chlorotic spot on cotyledon(s) or first true leaf; 2 = isolated chlorotic spots on systemically infected leaves 1 and 2 and occasionally leaf 3; 3 = more extensive systemic symptoms with chlorotic spots and mosaic on several leaves; 4 = moderate virus symptoms without leaf-cupping; 5 = most severe symptoms resulting in leaf-cupping, distortion, and stunting.

1 and 3 from CMV-FNY produced consistent severe symptoms, which is typical for CMV-FNY (groups 1 and 3 and the original CMV-FNY; average symptom rating 4.3–4.5). The average time to achieve 100% symptom expression was usually within 10 days. When RNA 3 from CMV-SNY was substituted in the third position (groups 2 and 4), average symptom ratings decreased to an intermediate level and infections reached a maximum of 74% after a 10-day incubation period. When RNA 1 from CMV-SNY appeared in the first position (groups 5–8), average symptom ratings were low (0.7–2.1), and a corresponding delay in achieving maximum infection occurred. The highest symptom ratings and shortest time to maximum symptom development occurred when RNA 3 from CMV-FNY appeared in the third position (groups 6 and 8). There was excellent agreement for highest symptom rating and the shortest time to maximum symptom development between group 1 (F-F-F) and the original strain of CMV-FNY maintained in Ithaca. The agreement between group 5 (S-S-S) and the parent strain of CMV-SNY was not as close; parent CMV-SNY had a more severe reaction.

Aphid transmission of pseudorecombinants. To test the effects of various RNA combinations on aphid transmission, a single representative sample was selected for groups 2, 3, 6, and 7 (Table 3). Data showing aphid transmission of these pseudorecombinants when the lower leaves were sampled over a 5-wk period are presented in Figure 2A. Aphid transmission of groups 3, 7, and 6 decreased from the initial levels during the first 3 wk. Subsequently, groups 2, 7, and 6 showed increased transmission in the fourth week with a corresponding decrease in the fifth week. However, aphid transmission of group 3 (F-S-F) increased in the fifth week. When average aphid transmission was calculated over the 5-wk period, the highest percent transmission occurred for group 3 (53%), followed by group 2 (F-F-S, 21%), group 7 (S-F-S, 16%), and group 6 (S-S-F, 12%). The overall symptom ratings assigned to the positive transmissions are shown in Table 4. There is a clear difference between F-S-F and F-F-S, but not between S-F-S and S-S-F.

Data on aphid transmission, with newly developed leaves with symptoms (progressive leaf source) as sources, are shown in Figure 2B. As with the previous data (Fig. 2A), group 3 had much higher transmission (~ 70%) than the other groups (~ 15–22.5%) during the first 2 wk. Aphid transmission of group 3 decreased markedly in the third through the fifth weeks. Aphid transmission of groups 2, 6, and 7 was generally highest during the first week (15–25%) but progressively decreased. By the fifth week, aphid transmission was near 0% for all groups except group 6 (~ 7.5%). When average aphid transmission was calculated over the 5-wk period, the highest percent transmissions again occurred for group 3 plants (39%), followed by group 2 (24%), group 6 (24%), and group 7 (19%). Symptom ratings were not as consistent with the first test (Table 5). Group 3 again had the highest symptom severity rating, but group 6 plants were more severely affected than either group 2 or 7. Results indicated that when RNA 3 from CMV-FNY was present (along with RNA 1 from CMV-FNY), maximum aphid transmission occurred and symptom severity was greatest. When RNA 3 from CMV-FNY was replaced with RNA 3 from CMV-SNY, aphid transmission diminished for groups 2 and 7, although overall transmission was greater for group 2, which reflects the importance of RNA 1 for virus replication.

TABLE 3. Influence of genomic composition on time and severity of symptom expression in Zucchini Elite squash after mechanical transmission of pseudorecombinants of cucumber mosaic virus (strains FNY and SNY)

		Average no. of days for 100% symptom development (maximum % achieved within 10 days)			
Group[a]	Genome	Cotyledon	First leaf	Second leaf	Av. symptom rating[b]
1	1F+2F+3F	5	10	10	4.5
2	1F+2F+3S	10	10+(65%)	10+(74%)	2.8
3	1F+2S+3F	4	10	10+(96%)	4.3
4	1F+2S+3S	10+(87%)	10+(51%)	10+(53%)	2.1
5	1S+2S+3S	10+(74%)	10+(28%)	10+(32%)	1.0
6	1S+2S+3F	10+(91%)	10+(66%)	10+(64%)	2.1
7	1S+2F+3S	10+(53%)	10+(21%)	10+(19%)	0.7
8	1S+2F+3F	6 (97%)	10+(67%)	10+(69%)	2.1
CMV-FNY[c]	1F+2F+3F	4	7	8	4.3
CMV-SNY[c]	1S+2S+3S	4	10+(84%)	10+(72%)	2.0

[a] For each group, six isolates per group were inoculated to 20 plants (120 plants total). Example: In group 3, all plants (120) showed symptoms on cotyledons within 4 days, and symptoms on first leaf within 10 days. In addition, 115 (96%) of those 120 plants showed symptoms on the second leaf within 10 days. A 10+ indicates that more than 10 days was required for additional plants to show symptoms, with the final percent noted in parentheses.

[b] Rating was calculated by multiplying the number of plants assigned to each symptom category (0–5) and then averaging the score for the six isolates tested.

[c] CMV-FNY and -SNY standards were included as controls during each test and totaled 126 for the six trials.

DISCUSSION

Previous studies with CMV-FNY and CMV-SNY suggest that virus titer exerted a major role in the early days of infection (up to 10 days after inoculation) in determining differences in aphid transmission and time for symptoms to appear with the two strains (1). This was shown by determining the relative virus titer for the two strains in squash as monitored by aphid transmission and enzyme-linked immunosorbent assay (ELISA) done on the same leaves. In the present study, use of pseudorecombinants with these two strains allowed us to determine that RNAs 1 and 3 from CMV-FNY exert a major

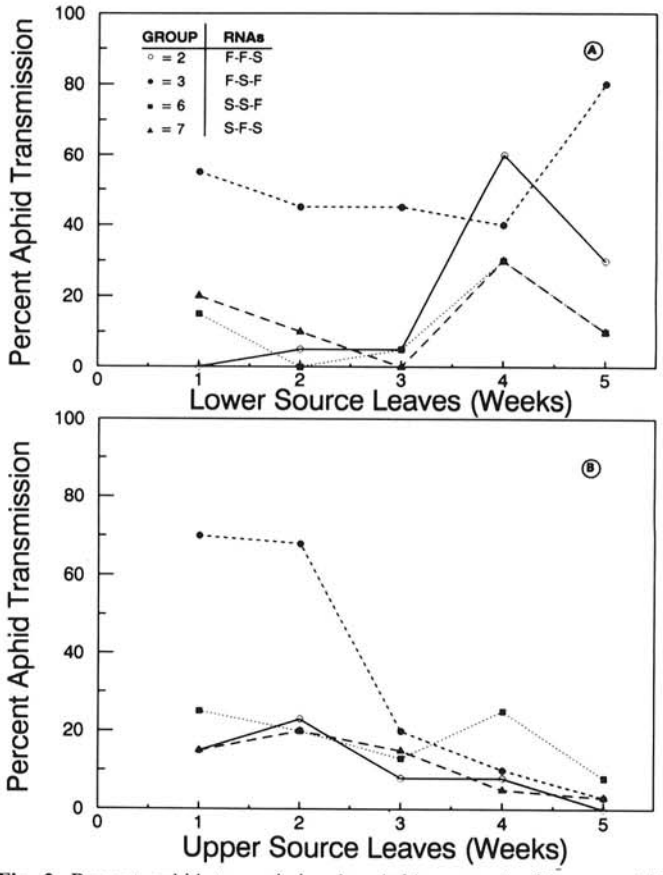

Fig. 2. Percent aphid transmission by *Aphis gossypii* with one aphid per test plant of four pseudorecombinants of cucumber mosaic virus from source plant leaves of Zucchini Elite squash assayed over a 5-wk period: **A,** lower squash leaves that show first systemic symptoms; **B,** upper squash leaves progressively infected and fully expanded. Both experiments were repeated.

TABLE 4. Foliar disease rating of 100 Zucchini Elite squash plants inoculated with four pseudorecombinants of cucumber mosaic virus using one aphid per test plant from lower leaves assayed over a 5-wk period (see Fig. 2A)

		Symptom rating and distribution[a]						
Pseudorecombinant	RNAs	0	1	2	3	4	5	Average
Group 2	F-F-S	79	1	7	2	5	6	3.4
Group 3	F-S-F	47	0	1	1	4	47	4.0
Group 6	S-S-F	88	1	6	3	2	0	2.5
Group 7	S-F-S	84	2	7	3	3	1	2.7

[a]Rating system: 0 = no symptoms (no transmission); 5 = severe symptoms. (See Fig. 1 for symptom appearance.)

TABLE 5. Foliar disease rating of 100 Zucchini Elite squash plants inoculated with four pseudorecombinants of cucumber mosaic virus using one aphid per test plant from progressively infected leaves assayed over a 5-wk period (see Fig. 2B)

		Symptom rating and distribution[a]						
Pseudorecombinant	RNAs	0	1	2	3	4	5	Average
Group 2	F-F-S	76	9	3	4	3	5	2.6
Group 3	F-S-F	61	5	2	4	4	24	4.0
Group 6	S-S-F	76	5	1	2	3	13	3.7
Group 7	S-F-S	81	7	3	2	1	6	2.7

[a]Rating system: 0 = no symptoms (no transmission); 5 = severe symptoms. (See Fig. 1 for symptom appearance.)

influence on symptom severity and aphid transmissibility. Separation of the original eight recombinants into two broad groups based on symptom severity and length of time for symptoms to appear (used here as length of incubation period) revealed that when RNA 1 was from CMV-FNY, groups 1–4 could be separated from groups 5–8 in which RNA 1 was from CMV-SNY. These results were subsequently confirmed (Table 3). The subtle influence of RNA 3 from CMV-FNY in these mechanical transmission studies was apparent from the intermediate symptom ratings (2.1) and incubation periods for the combinations S-S-F and S-F-F. Aphid transmission data for four selected RNA groupings revealed that maximum transmission (and symptom severity) was achieved when the RNA from CMV-FNY was present in positions 1 and 3 (average of 39% transmission over a 5-wk period), which most closely approximates the performance of the original strain (1). Presence of CMV-FNY RNA in position 1 (F-F-S) or 3 (S-S-F) resulted in equivalent low transmissions (average of 24%). These results indicate that RNA 1 from CMV exerts an important influence on initial (Fig. 2A and B) or subsequent (Fig. 2A) virus replication, which enhances aphid transmission. Substitution of RNA 3 from CMV-FNY failed to restore high aphid transmissibility. These results suggest that the differences observed in the spread of the two strains in New York can be explained on the basis of the higher virus titers achieved by CMV-FNY in the early stages of plant infection.

Two lines of evidence indicate that the intrinsic aphid transmissibility of CMV is controlled by the coat protein. Mossop et al (8) used pseudorecombinants to show that RNA 3, which codes for coat protein, was necessary to render CMV aphid transmissible. And, Gera et al (3) showed that virions reconstituted in vitro from RNAs of a poorly aphid transmitted strain and coat protein of a highly aphid transmitted strain were efficiently transmitted by aphids acquiring the virions through membranes. In our case, both CMV-SNY and CMV-FNY were aphid-transmissible. Thus, depending on the virus strain selected for study, maximum aphid transmissibility can be influenced by both RNA 3 (3,8) or by RNA 1, as shown in this work.

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