Genetic Analysis of Cucumber Mosaic Virus in Relation to Host Resistance: Location of Determinants for Pathogenicity to Certain Legumes and *Lactuca saligna*

Michael C. Edwards, Dennis Gonsalves, and Rosario Provvidenti

Former graduate research assistant, associate professor, and senior research associate, respectively, Department of Plant Pathology, Cornell University, New York State Agricultural Experiment Station, Geneva 14456.
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ABSTRACT

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Pseudorecombinants of two naturally occurring strains of cucumber mosaic virus (CMV-B and CMV-LsS) were used to analyze the genetics of CMV in relation to pathogenicity to *Lactuca saligna, Phaseolus vulgaris, Pisum sativum*, and *Vigna unguiculata*. RNA 2 of CMV-B determined systemic infection of bean, pea, and cowpea. Ability to systemically infect *L. saligna* depended upon both RNAs 2 and 3 of CMV-LsS. Chlorotic and necrotic local lesion formation on *V. unguiculata* depended upon RNA 2 from the B and LsS strains, respectively. A simple and effective procedure using bentonite for the recovery of infectious RNA from polyacrylamide gels is also described.

Additional key words: bentonite, *Phaseolus vulgaris, Pisum sativum*, pseudorecombinant, reassortant, RNA, *Vigna unguiculata*.

Multipartite plant viruses are valuable for genetic studies of host-virus interactions. Many such studies have already demonstrated the usefulness of pseudorecombinants (8) for the determination of the genetic functions of individual RNA species (1,3,5,13,25).

Cucumber mosaic virus (CMV) possesses a functionally divided genome (16,20) and hence is suitable for genetic studies. It is well characterized, both biologically and biochemically (15). It also varies considerably in nature, reducing the need to induce mutations to obtain genetic markers (7,21,22). Its three genomic RNAs are designated 1, 2, and 3, in order of decreasing molecular weights of about 1.27, 1.13, and 0.82×10^6 (7). A subgenomic RNA of 0.35×10^6 mol wt (7) is encapsidated but is not required for infectivity. Still other satellite RNAs of approximately 1×10^5 mol wt are associated with certain CMV strains (9,14).

Some CMV pseudorecombinants have been studied previously. Habili and Francki (11) demonstrated that RNA 3 contains the coat protein gene. Other characters that probably depend upon properties of the coat protein, such as aphid transmissibility, electrophoretic mobility, and serological specificity (12,18,19), have also been assigned to RNA 3.

Whereas these studies have dealt with coat protein markers or symptomatology, we were interested in analyzing the genetics of CMV in relation to host resistance. CMV is an important pathogen of many vegetable crops and is the target of intense breeding programs for resistance. Two of the strains used in the New York program are CMV-B and CMV-LsS. CMV-B was originally isolated from *Phaseolus vulgaris* (bean) (21), and CMV-LsS was originally isolated from *Lactuca sativa* L. (lettuce) (22). CMV-B infects many legumes and is capable of causing economic damage to bean. CMV-LsS has been isolated from lettuce breeding lines resistant to other strains of the virus. The CMV resistance in these lettuce plants was derived from a related species, *L. saligna* L. (P.I. 261653). Before infection by the LsS strain, this plant line was considered resistant to CMV.

Identification of the RNA species allowing infection of otherwise resistant plants will provide a foundation for future studies of CMV-host interactions. This study was designed to examine the genetics of CMV in relation to its pathogenicity to *L. saligna* and to various legume species.

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MATERIALS AND METHODS

Propagation and purification of strains. The LsS and B strains of CMV were propagated in *Cucurbita pepo* L. 'President' (zucchini-type squash) and purified essentially by the procedures described by Lot et al (17). Virus preparations were resuspended in PEN buffer (0.01 M NaH_2PO_4, 0.001 M ethylenediamine tetraacetic acid [EDTA], and 0.001 M NaN_3, pH 7.0).

Isolation, fractionation, and recovery of RNA species. RNA was isolated from purified virus using a modification of the method of Brakke and Van Pelt (2), as previously described (10). After ethanol precipitation, RNA was resuspended in an appropriate volume of sterile PEN buffer.

RNA species were separated by a combination of sucrose density gradient centrifugation and polyacrylamide gel electrophoresis. One milliliter of unfractionated RNA (1 mg/ml) was heated at 60 C for 5 min and then rapidly cooled by immersion in an ice bath and addition of 2 ml of cold, sterile PEN. Each of six linear (7.5–30%, w/v) sucrose (dissolved in PEN) gradients was loaded with 0.5 ml of RNA and centrifuged at 30,000 rpm in a Beckman (Palo Alto, CA) SW40Ti rotor for 18–21 hr at 6 C. RNA fractions were collected from gradients, using the ISCO (Lincoln, NE) UA5 analyzer and 640 density gradient fractionator. Fractions containing the same RNA species were pooled, precipitated with ethanol, and resuspended in PEN. RNA 3 was further purified by at least two more cycles of density gradient centrifugation.

RNAs 1 and 2 sedimented as one component in sucrose gradients. To separate RNAs 1 and 2, the fraction containing both of them was collected after the first density gradient cycle and electrophoresed on 2.4% polyacrylamide slab gels (24) for 9 hr at 40 mA (constant current). Bands were visualized by staining with ethidium bromide (100 μg/ml) for 3–4 min. RNA was extracted from gels as follows: Approximately 3 ml of gel was combined with 6 ml of buffer (0.04 M Tris-HCl, 0.4 M NaCl, 0.004 M EDTA, pH 8.5) and homogenized for 30 sec, using a Tissumizer (Tekmar Co., Cincinnati, OH) at 20–25% of full speed. This suspension was centrifuged at 8,000 rpm for 10 min, using an SS34 rotor in a Sorvall RC2-B centrifuge. The supernatant was carefully drawn off and this procedure repeated twice more. The pooled supernatants

were placed in two 30-ml Corex tubes, and 0.2 ml of fractionated 2% bentonite suspension (6) was added to each during vortexing. The aggregated acrylamide was then removed from solution by centrifugation at 8,000 rpm for 10 min. The supernatant was given a second bentonite treatment followed by another low speed centrifugation. RNA was then precipitated with two volumes of ethanol.

After extraction from gels, RNAs 1 and 2 were further purified by density gradient centrifugation. Each RNA peak was collected as two samples, a light and a heavy fraction (23). All RNA samples were stored at −80 C until use.

Infectivity of RNA preparations. Infectivity tests were done using various combinations of homologous RNAs. To construct pseudorecombinants, all possible combinations of RNAs from each of the two strains were inoculated at various dilutions to *Chenopodium quinoa* plants (Table 1). All RNAs were in sterile PEN buffer containing about 800 μg of bentonite per milliliter. *C. quinoa* leaves were dusted with corundum and inoculated, using ground glass spatulas. Single-lesion isolates (pseudorecombinants) were increased by transferring them first to *C. quinoa* and then to squash. Test plants (*L. saligna*, beans, cowpeas, peas) were inoculated with infected squash.

Serological comparisons of strains. Antisera to CMV strains B, C, LsS, and WL (prepared in our laboratory) were used in Ouchterlony gel diffusion tests. Two types of gels, consisting of either 0.75% Ionagar, 0.85% NaCl, and 0.1% NaN_3 or 0.75% Ionagar and 0.1% NaN_3 were used. All tests were conducted using purified virus preparations at concentrations ranging from 0.1 to 1 mg/ml.

TABLE 1. Combinations of RNA species used to construct pseudorecombinants of cucumber mosaic virus strains B and LsS[a]

$1^L + 2^L + 3^B$	$1^B + 2^L + 3^B$
$1^L + 2^B + 3^L$	$1^L + 2^B + 3^B$
$1^B + 2^L + 3^L$	$1^L + 2^L + 3^L$
$1^B + 2^B + 3^L$	$1^B + 2^B + 3^B$

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

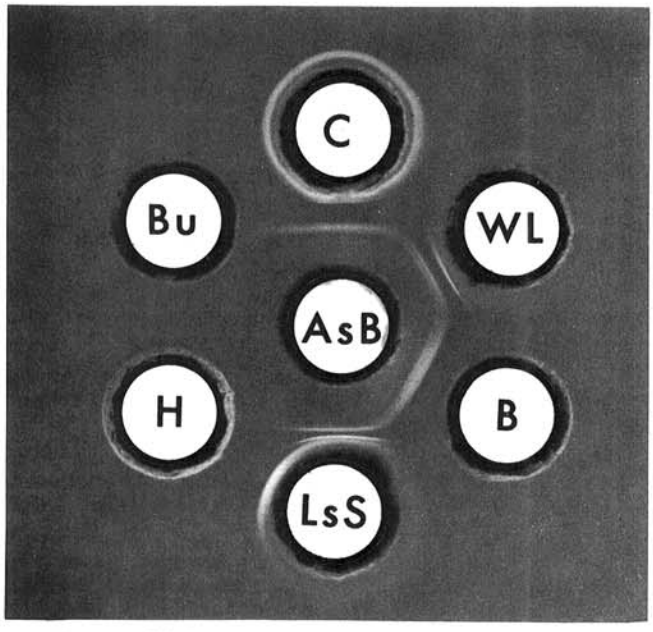

Fig. 1. Immunodiffusion patterns, showing reactions of identity between cucumber mosaic virus strains B, C, WL, and LsS. Center well was charged with antiserum to CMV-B. (H = healthy squash extract, Bu = buffer). Identical results were obtained with antisera to strains C, LsS, and WL.

RESULTS

Serological relationships. No serological differences were revealed after comparison of strains B, C, LsS, and WL through Ouchterlony gel diffusion tests with antisera to all four strains. Because CMV readily dissociates in the presence of sodium chloride, gels containing sodium chloride were used to compare the reactions of soluble proteins. Reactions of identity were observed regardless of the antiserum used (Fig. 1). Reactions of identity were also observed for intact virions when tests were conducted in the absence of sodium chloride.

Separation, isolation, and infectivity of RNA species. Although both strains were apparently serologically identical in double diffusion tests (Fig. 1), initial separation of RNA species using sucrose gradients revealed that both the number and relative amount of each RNA species varied with the strain (Fig. 2). RNA profiles of the B and LsS strains contained peaks representing low molecular weight RNAs, in addition to the typical three peaks found for RNAs 1+2, 3, and 4. A single, small RNA 5 was associated with the B strain, whereas two additional low molecular weight RNAs (RNAs 4a and 5) were associated with the LsS strain. Their functions have not yet been determined, but they may be similar to the satellite RNAs previously described for other CMV strains (9,14).

Although RNAs 1 and 2 could not be separated by sucrose density gradient centrifugation, we separated them by electrophoresis in polyacrylamide gels (Fig. 3). Initially, we compared several methods for extracting RNA from gels, including homogenization/hydroxyapatite chromatography (4), phenol

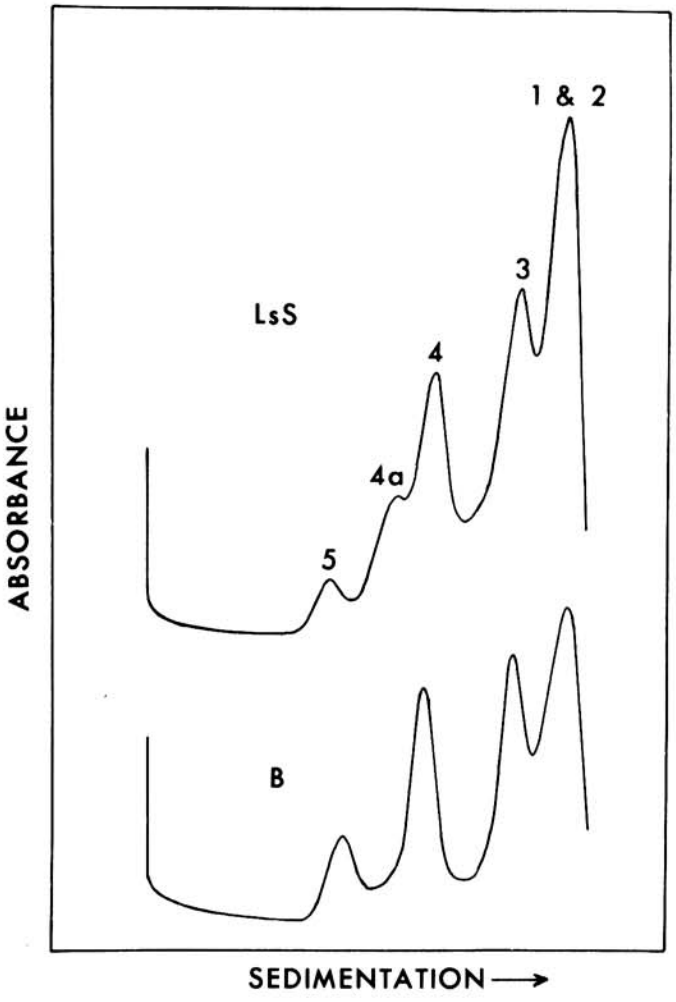

Fig. 2. Separation of RNA species 1+2, 3, 4, 4a, and 5 from the B and LsS strains of cucumber mosaic virus through sucrose density gradient centrifugation.

extraction (23), and electrophoretic elution (24). The most satisfactory in our hands was the phenol extraction technique of Schwinghamer and Symons (23). RNAs obtained in this manner were quite infectious, and mixing components significantly enhanced infectivity (data not shown). However, the extraction technique subsequently developed in our laboratory and described above was simpler, faster, and also yielded very infectious RNA preparations (Table 2). We found that bentonite precipitated polyacrylamide from RNA solutions (Fig. 4). Two successive bentonite treatments yielded a clear solution. RNAs extracted using our technique sedimented as sharper zones in sucrose gradients than did RNAs extracted using the phenol method.

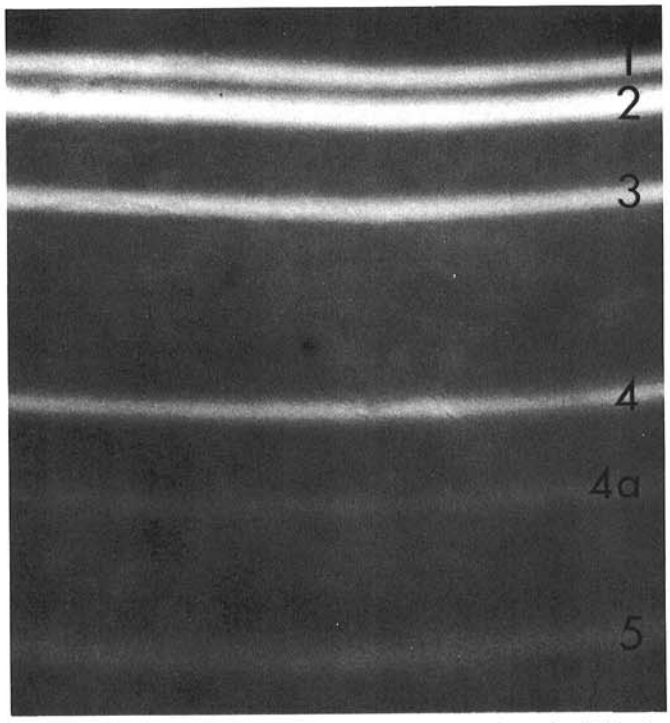

Fig. 3. Separation of cucumber mosaic virus (strain LsS) RNAs by electrophoresis in a 2.4% polyacrylamide slab gel (numbers designate RNA species).

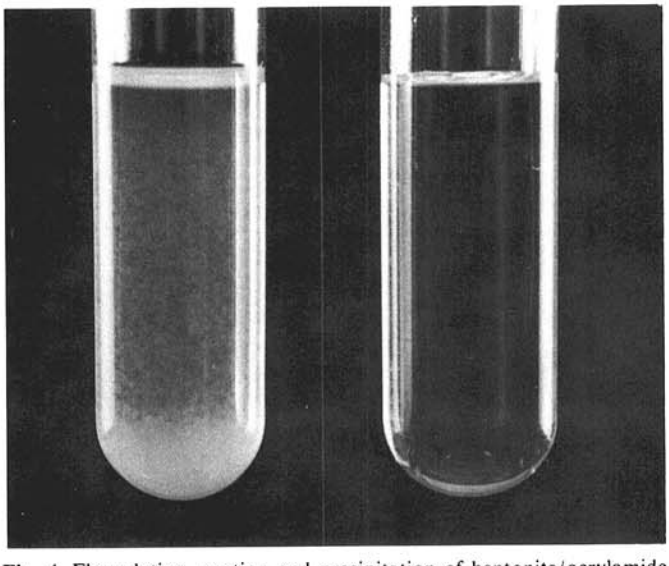

Fig. 4. Flocculation reaction and precipitation of bentonite/acrylamide complexes after the addition of a 2% bentonite suspension to an acrylamide-contaminated solution of RNA (left). On the right is a similar acrylamide-contaminated solution of RNA without bentonite.

Even after gel electrophoresis, RNAs 1 and 2 were detectably cross-contaminated. A second cycle of sucrose density gradient centrifugation significantly improved purity (Fig. 5).

Results of infectivity tests with the purified RNA preparations are shown in Table 2. RNAs were highly infectious and sufficiently pure to enhance infectivity when appropriately mixed. Most importantly, the data indicated that cross-contamination of RNA preparations could be minimized through dilution.

Effects of host passage on pathogenicity. LsS-infected *C. quinoa* could not be used to infect *L. saligna*. Only one out of 10 *L. saligna* plants inoculated with LsS from *C. quinoa* became infected, whereas all 10 plants inoculated with LsS from squash became infected. Consequently, infected squash tissue was used as inoculum.

Although the exact nature of the host passage effect was not determined, the low molecular weight RNAs associated with these

TABLE 2. Infectivity of combinations of RNAs 1, 2, and 3 from cucumber mosaic virus strains B and LsS

Strain	Inoculum dilution[a]	RNA combination				
		1,2,3	1+2	1+3	2+3	1+2+3
B	1/125	0[b]	1	12	68	414
	1/500	0	0	0	2	56
LsS	1/125	0	3	0	1	22
	1/500	0	0	0	0	0

[a] Actual RNA concentrations: 1/125 ≈ 0.65 μg/ml, 1/500 ≈ 0.16 μg/ml.
[b] Each number represents the average number of local lesions produced per leaf (five leaves).

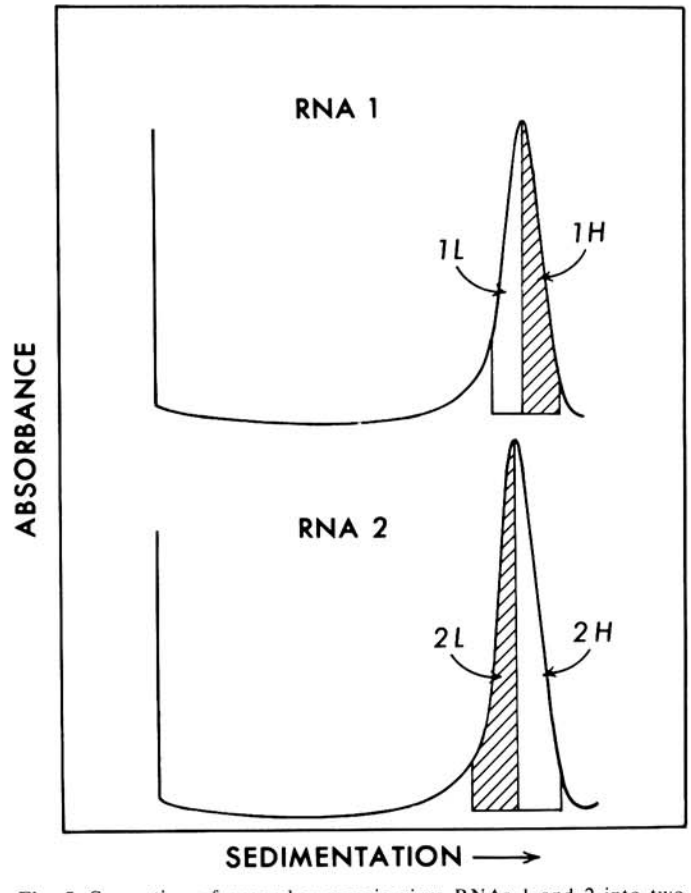

Fig. 5. Separation of cucumber mosaic virus RNAs 1 and 2 into two fractions after sucrose density gradient centrifugation. Only RNA fractions 1H and 2L were used for the construction of pseudorecombinants. H = heavy fraction, L = light fraction.

strains were apparently not involved. Direct inoculation of *L. saligna* plants with LsS-RNAs (L-RNAs) 1+2+3 produced typical LsS symptoms, indistinguishable from those produced by extracts from LsS-infected squash.

Reaction of test plants to CMV-B and -LsS. Responses of test hosts to infection by CMV strains B and LsS provided good markers for this study. *Ph. vulgaris* 'Red Kidney' (bean) was not infected by the LsS strain but was susceptible to the B strain. Inoculated primary and trifoliolate leaves developed severe epinasty. Trifoliolates were also mottled, and the plants were somewhat stunted. *Vigna unguiculata* 'Calif. Blackeye' (cowpea) and *Pisum sativum* 'Bonneville' (pea) were both systemically infected by the B strain but only locally infected by the LsS strain. Systemic symptoms included mottling, mosaic, and stunting. Primary leaves of cowpea inoculated with the LsS strain developed necrotic local lesions, whereas pea leaves completely collapsed. Occasional stem necrosis developed in pea inoculated with the LsS strain. Conversely, *L. saligna* was resistant to the B strain but susceptible to the LsS strain. Systemically infected leaves typically exhibited a mosaic.

During the course of this work, symptomatology was used only as a preliminary indication of a host infection by B or LsS. All plants not showing symptoms were routinely indexed on *C. quinoa*.

Pseudorecombinants constructed from strains B and LsS. Pseudorecombinants were constructed with all possible combinations of RNAs (Table 1). However, in initial experiments only the combinations B-RNA 1+2 plus L-RNA 3 and L-RNA 1+2 plus B-RNA 3 were used. In this case, all RNAs were purified by sucrose density gradient centrifugation so that RNAs 1+2 were isolated together. Single-lesion isolates (SLIs) having B-RNAs 1+2 infected beans, whereas the reciprocal cross was noninfectious to differential hosts (Table 3). Results indicated that either or both B-RNAs 1 and 2 were required for infection of bean and that L-RNA 3 was required in combination with either or both L-RNAs 1 and 2 for infection of *L. saligna*.

TABLE 3. Differential host reactions to pseudorecombinants constructed by exchanging RNAs (1+2) and 3 between cucumber mosaic virus strains B and LsS

Combination	Bean	*Lactuca saligna*
$(1+2)^B + 3^L$	18/20[a]	0/20
$(1+2)^L + 3^B$	0/20	0/20
$(1+2)^B + 3^B$	10/10	0/10
$(1+2)^L + 3^L$	0/10	10/10

[a] Numerator represents the number of plants infected. Denominator represents the number of plants inoculated.

To examine the role of individual components, RNAs 1 and 2 were separated using polyacrylamide gel electrophoresis as previously described, and the RNA combinations BLB, BLL, LBL, and LBB were tested. SLIs having the expected genotypes LBL and LBB infected most (17/20) bean plants in the first experiment (Table 4). SLIs possessing L-RNA 2 did not infect bean. SLIs containing B-RNA 2 systemically infected peas and cowpeas, whereas SLIs with L-RNA 2 induced necrotic local reactions with these hosts. Conversely, only those SLIs possessing both L-RNAs 2 and 3 infected *L. saligna*. Results for all hosts and pseudorecombinants were confirmed in a second experiment (Table 4).

As is evident in Table 4, a few exceptions to these results were found. Cross-contamination of the original RNA preparations is the most likely explanation for these few discrepancies. For example, in experiment 2, one out of 10 SLIs with the expected genotype BLB infected bean, pea, and cowpea plants without inducing necrotic lesion formation on pea or cowpea. Thus, the true genotype for this SLI was probably BBB, not BLB. Mixed infections were probably responsible for those exceptions that could not be accounted for in this manner.

DISCUSSION

Bentonite greatly facilitated removal of polyacrylamide from RNA preparations. Our extraction technique was simpler and faster than the other methods and yielded highly infectious RNA. Additional work indicates that using two consecutive runs of polyacrylamide gel electrophoresis produces still purer, yet highly infectious RNA preparations.

Although the B and LsS strains differ significantly in pathogenicity to certain hosts, their RNA components are sufficiently compatible for the formation of pseudorecombinants. All pseudorecombinants were able to infect squash and *C. quinoa*, despite the inability of certain pseudorecombinants to infect some of the differential hosts.

In agreement with those of Hanada and Tochihara (12), our data show B-RNA 2 conditions for pathogenicity to pea, cowpea, and bean (Table 5). Both the E and L strains of Hanada and Tochihara were originally obtained from pea. All three strains produce chlorotic local lesions on inoculated cowpea leaves followed by systemic infection, and in all three cases, RNA 2 determines systemic symptoms on cowpea. Apparently, RNA 2 conditions for necrotic local lesion production in cowpeas inoculated with strains incapable of systemic infection (Y strain of Hanada and Tochihara, LsS strain of ours). An alternative explanation may be that RNA 2 of the B strain is capable of suppressing the necrotic response. If so, the RNA that mediates the necrotic response cannot be determined

TABLE 4. Differential host reactions to pseudorecombinants constructed by exchanging RNAs 1, 2, and 3 between cucumber mosaic virus strains B and LsS

Host	Reaction	Experiment No.	RNA Combination					
			BLB[a]	BLL	LBL	LBB	BBB	LLL
Bean		1	0/10[b]	0/10	8/10	9/10	5/5	0/5
		2	1/10	0/10	8/10	10/10	5/5	0/5
Pea	LR[c]	1	10/10	10/10	2/10	1/10	0/5	5/5
		2	9/10	10/10	2/10	0/10	0/5	5/5
	SR	1	0/10[d]	0/10	8/10	9/10	5/5	0/5
		2	1/10	0/10	8/10	10/10	5/5	0/5
Cowpea	LR	1	10/10	10/10	2/10	1/10	0/5	5/5
		2	9/10	10/10	2/10	0/10	0/5	5/5
	SR	1	0/10	1/10	8/10	9/10	5/5	0/5
		2	1/10	1/10	8/10	10/10	5/5	0/5
L. saligna		1	2/10	8/10	2/10	0/10	0/5	5/5
		2	0/10	10/10	2/10	0/10	0/5	5/5

[a] BLB = B–RNA 1 + LsS–RNA 2 + B–RNA 3, etc.
[b] Numerator represents the number of plants infected; denominator represents the number of plants inoculated.
[c] LR = necrotic local reaction, SR = systemic reaction.
[d] In experiment 1, systemic reaction on pea was judged visually only, without bioassay.

TABLE 5. Assignment of genetic determinants of cucumber mosaic virus for pathogenicity to bean, pea, cowpea, and *Lactuca saligna*

Host	RNA species
Phaseolus vulgaris cv. Red Kidney (bean)	B–RNA 2
Pisum sativum cv. Bonneville (pea)	B–RNA 2
Vigna unguiculata cv. Calif. Blackeye (cowpea)	B–RNA 2
Lactuca saligna P. I. 261653	L–RNAs 2 + 3

by these experiments.

In general, the results of Marchoux et al (18) are consistent with our results. They found that RNA 2 determined systemic infection of cowpea by strain DS. RNA 2 also determined whether chlorotic or necrotic lesions were produced on inoculated leaves, although RNA 3 influenced lesion size. No effect of this latter type was observed with our strains.

The importance of RNA 2 for infecting legumes, then, is not limited to our strains but apparently holds for several strains widely distributed around the world. This indicates an interesting interrelationship between RNA 2 and legumes. Some factor(s) common to many legumes probably mediates susceptibility to certain CMV strains.

Both L-RNAs 2 and 3 are essential for infection of *L. saligna*. Previously, RNA 3 alone was reported to govern pathogenicity to *Beta vulgaris* and *Zea mays*, as well as systemic symptom development in *Nicotiana tabacum* var. Xanthi nc (18). However, the requirement of both RNAs 2 and 3 for pathogenicity to a particular host has not been previously reported.

Any differences related to pathogenicity between B-RNA 3 and LsS-RNA 3 may not be associated with differences in coat protein, because these strains are serologically indistinguishable (Fig. 1). These results are inconclusive, however, because serological tests can detect only a fraction of the potential differences between the LsS and B coat proteins.

Our results are epidemiologically significant. Because infection of *L. saligna* requires both RNAs 2 and 3 of CMV-LsS, CMV-B is unlikely to infect lettuce cultivars having the resistance gene from this source. CMV-B would require at least two mutations to infect *L. saligna*. Alternatively, commercial legume cultivars should not be susceptible to strains such as CMV-LsS. This strain would require at least one mutation to infect bean. Strains that can infect both legumes and lettuce possessing the CMV resistance from *L. saligna* cannot be generated by genetic reassortment of the B and LsS strains. However, some other natural strains may conceivably exist that can reassort with CMV-LsS or CMV-B to produce a strain capable of infecting both hosts.

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