Resistance

Sunn-Hemp Mosaic Virus Facilitates Cell-to-Cell Spread of Southern Bean Mosaic Virus in a Nonpermissive Host

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ABSTRACT

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Accumulation of the cowpea strain of southern bean mosaic sobemovirus (SBMV-C) in bean, a nonpermissive host, was facilitated by coinfection with sunn-hemp mosaic (SHMV), a tobamovirus. The rate of spread in bean of SBMV-C in the presence of SHMV was compared with the rate of spread of the bean strain of SBMV (SBMV-B) coinoculated with SHMV. Virus accumulation in the leaf blade, lateral veins, midrib, petiole, stem, and roots revealed similar amounts of both strains in the nonvascular tissue of the inoculated leaf; however, a sharp decline in

SBMV-C accumulation was observed starting from the lateral veins towards the mid- and distal parts of the petiole, where virtually no virus could be found. This contrasted with the uniform presence of SBMV-B throughout these plant parts. Bean protoplasts sustained replication of SBMV-C in single infection upon in vitro inoculation. Antigen accumulation of the bean and the cowpea strains of SBMV in bean protoplasts was similar, and coinoculation of bean protoplasts with SHMV and SBMV-C showed no effect on the accumulation of the latter.

Additional keyword: viral movement.

The cowpea and bean strains of southern bean mosaic virus (SBMV-C and SBMV-B) share similar chemical and physical properties (7) and are serologically related, but they differ in their host ranges. SBMV-B infects most common bean cultivars but does not infect cowpea, whereas SBMV-C is restricted to *Vigna* cultivars but cannot infect bean (18).

Molefe et al (13) showed that bean (*Phaseolus vulgaris* L. 'Pinto') singly inoculated with SBMV-C sustained only subliminal viral replication in inoculated primary leaves (no symptoms developed and very low amounts of virus were recovered). However, SBMV-C does not establish a systemic infection in this host for reasons that are presently unknown. On the other hand, plants coinfected with sunn-hemp mosaic virus (SHMV), which infects bean systemically, and SBMV-C showed local lesions in the inoculated primary leaves due to the replication of SBMV-C. Furthermore, SBMV-C replicated to levels detectable by ELISA and bioassay in the inoculated primary leaves, but not in the trifoliate leaves, of three other bean cultivars coinfected with SHMV (6).

In this study the "helper effect" of SHMV on SBMV-C in mixed infections of bean was shown to be limited to short distance (cell-to-cell) movement in the primary inoculated leaves, with no virus (SBMV-C) moving into the vascular system. Inoculation of isolated bean protoplasts with SBMV-C, SBMV-B, and SBMV-C plus SHMV showed that SBMV-C is capable of replicating in isolated bean cells to the same extent as SBMV-B, and that its replication is not affected by the presence of SHMV.

MATERIALS AND METHODS

Virus propagation and purification. SBMV-B and SBMV-C were maintained in *P. vulgaris* 'Bountiful' and *Vigna unguiculata* L., respectively. Both strains were purified from infected leaf tissue 12–18 days after inoculation according to established protocols (19). SHMV was propagated in Bountiful and purified by the method of Kassanis and Varma (9).

Virus inoculation. Four cultivars of *P. vulgaris* (Bountiful, Pinto, Top Crop, and Tendergreen) were inoculated by applying 0.1 mg/ml of purified SBMV-C (this concentration of virus was used for practical purposes because, when applied to the local lesion host *V. unguiculata* 399419, it gave an average of 40 lesions per leaf), SHMV, or a 1:1 mixture of both virus preparations to the surface of Carborundum-dusted primary leaves.

Protoplast isolation. All manipulations were performed under sterile conditions in a laminar flow hood. Protoplasts were isolated from fully expanded leaves of Bountiful bean by the one-step method (14). Tissue was surface-sterilized with 70% ethanol and rinsed three times in distilled water. Leaves then were cut into 1-mm sections and incubated overnight in 0.6 M mannitol, pH 5.4, 0.2% cellulase (Onozuka R-10), and 0.025% Macerozyme (Yakult Honsha Co., Minato-ku, Tokyo, Japan). The suspension was filtered through Miracloth (Calbiochem Corp., La Jolla, CA), centrifuged at 1,000 g for 3 min, and washed twice with 0.6 M mannitol. Viability was assessed in a 10% solution of Evans blue (10). Preparations with 80% or higher viability were used for inoculation with viruses.

Protoplast inoculation and incubation. Freshly prepared proto-

plasts were inoculated with SBMV-C, SBMV-B, or SHMV in a 30% polyethylene glycol (PEG) solution (12). Briefly, 10 or 20 μg of virus was added to approximately 2×10^6 protoplasts that then were resuspended in a minimal volume of 30% PEG in 0.6 M mannitol and 3 mM CaCl. After 10–20 s of incubation, the suspension was diluted 10 times with a solution of 0.6 M mannitol and 1 mM CaCl$_2$ to reduce the concentration of PEG. Protoplasts were allowed to stand for 5 min at room temperature, centrifuged, washed three times, and finally resuspended in a modified minimal salt medium (16) (mannitol concentration of 0.6 M instead of 0.7 M and no 2-4-dichlorophenoxyacetic acid). Incubation of protoplast suspension cultures was at 25 C under constant illumination of about 10,000 lx. At selected intervals, protoplast samples were collected, centrifuged, and stored at −70 C.

Virus assays. Presence of SBMV-C in plants and protoplasts was assayed by double antibody sandwich enzyme-linked immunosorbent assay (ELISA) (4) and by bioassay (infectivity test) on cowpea (*V. unguiculata* 399419). Antiserum for ELISA was raised in rabbits against purified SBMV-C.

Frozen, inoculated protoplasts were thawed, resuspended in 1 ml of 1$\times$ PBS (20 mM phosphate buffer, pH 7.4, and 150 mM NaCl), and sonicated.

To test for subliminal infection, primary bean leaves were harvested 7 and 15 days after inoculation with SBMV-C. To remove any viral inoculum from the leaf surface, leaves were washed under running tap water for 2 h. They then were ground in 0.01 M potassium phosphate buffer, pH 7.0, and the homogenate was rubbed onto primary leaves of a systemic host, *V. unguiculata*.

Spread of virus through the plant. Various tissues from Bountiful inoculated with SHMV and SBMV-C or SHMV and SBMV-B were harvested from 4 to 6 different plants 5, 7, and 11 days postinoculation. Inoculated primary leaves were washed under tap water for 2 h, and interveinal tissue was separated from the lateral veins, main vein, and petiole with the aid of a scalpel. Stems, roots, and trifoliate leaves also were separated. Equal weights of tissue were ground in 1$\times$ PBS (for ELISA) or 0.01 M potassium phosphate buffer, pH 7 (for bioassay), and tested accordingly for the presence of SBMV-C or SBMV-B. Total leaf RNA from different leaf sections was extracted (15) and presence of SBMV-C RNA was assessed by dot blotting using a nick-translated ^{32}P-labeled probe (10^7–10^8 cpm/μg) of SBMV cloned DNA (kindly provided by Dr. Claire A. Rinehart, University of Wisconsin, Madison).

RESULTS

Virus accumulation and symptoms in singly and doubly inoculated plants. Upon inoculation with SBMV-C, four bean cultivars (Bountiful, Pinto, Tendergreen, and Top Crop) proved to be nonpermissive hosts, allowing only subliminal replication in inoculated primary leaves (data not shown). Moreover, very few infected protoplasts were detected by SBMV-C antibody staining of protoplasts isolated from SBMV-C-inoculated primary leaves of bean (cv. Bountiful) compared with those isolated from primary leaves of the same cultivar inoculated with both SBMV-C and
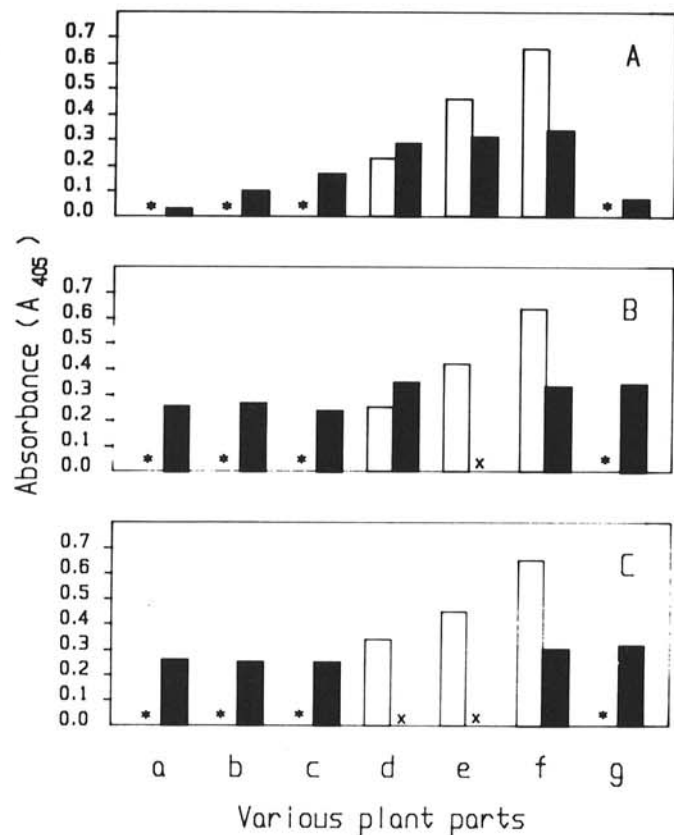

Fig. 1. Accumulation of capsid proteins, detected by enzyme-linked immunosorbent assay in bean (*Phaseolus vulgaris* 'Bountiful'), of cowpea and bean strains of southern bean mosaic virus (□ and ■ for cowpea and bean strains, respectively) in mixed infections with sunn-hemp mosaic virus in a, roots; b, stem; c, petiole from inoculated primary leaf; d, main vein from inoculated primary leaf; e, lateral vein from inoculated primary leaf; f, mesophyll from inoculated primary leaf; and g, trifoliate leaves. **A, B,** and **C,** Accumulation profiles 5, 7, and 11 days postinoculation, respectively. X indicates that the tissue was necrotic at sampling time; * indicates that values for these samples were < 0.025.
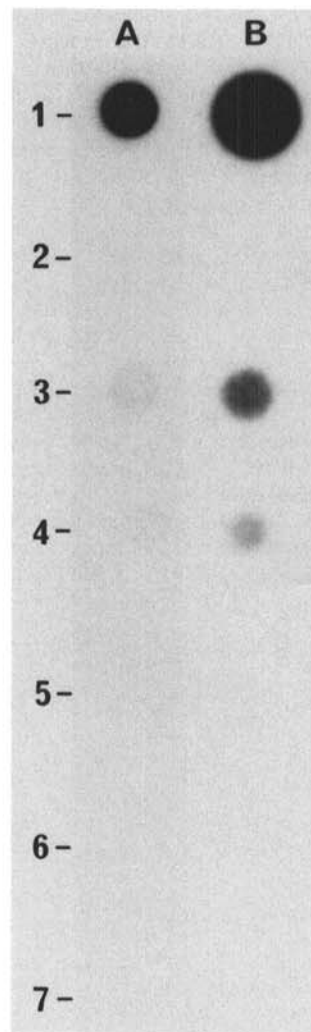

Fig. 2. Detection of the cowpea strain of southern bean mosaic virus (SBMV-C) RNA in different parts of bean plants (*Phaseolus vulgaris* 'Bountiful') by dot-blot hybridization using a nick-translated ^{32}P c-DNA probe of SBMV-C. Total RNA was extracted from each plant part and 100 ng added for each sample. 1, mesophyll; 2, extraction buffer; 3, lateral veins; 4, main vein; 5, healthy tissue; 6, petiole; 7, stem. A and B, 4 and 12 h exposure, respectively.

SHMV (data not shown). When these same bean cultivars were inoculated with a mixture of SHMV and SBMV-C, SBMV-C was readily detected in the inoculated primary leaves by bioassay on local lesion hosts and by ELISA. This observation indicates that SHMV acts as a helper for SBMV-C, facilitating its accumulation in inoculated bean plants. This "helper effect" occurred after simultaneous inoculation with both viruses but also could be observed when SBMV-C was inoculated up to 72 h after SHMV inoculation.

Symptoms of SBMV-C/SHMV-mixed infections varied between the seasons in three of the four cultivars (Bountiful, Tendergreen, and Top Crop), yet were qualitatively similar to the SHMV single infection. However, in Pinto, mixed infections resulted in pinpoint local lesions in the inoculated primary leaves that were not produced upon single infection with either virus but were similar, although smaller, to those produced by single infection with SBMV-B. Severity of symptoms increased when plants were grown at 32 C, although they remained qualitatively similar, with vein clearing and ringlike lesions being particularly noticeable in the case of Bountiful.

Trifoliate leaves of the four doubly inoculated cultivars were harvested 14, 20, and 60 days after inoculation and tested for the presence of SBMV-C by bioassay and ELISA. No virus was detected in the trifoliate leaves of any of the cultivars at any time. Neither was any SBMV-C detected in the trifoliate leaves after inoculation of these leaves with SHMV and SBMV-C.

Spread of SBMV-C within inoculated leaves and throughout the plant. Accumulation of SBMV-C capsid protein in mixed infections with SHMV, as compared with accumulation of SBMV-B capsid protein in mixed infections with SHMV, is shown in Figure 1. Estimates of virus concentration in the interveinal tissue were similar for both, although SBMV-C was usually higher. These results are likely accounted for by the fact that primary leaves infected with SBMV-B and SHMV suffered severe wilting and yellowing as early as 4 days postinoculation. Further dissection of the leaf and petiole revealed that the concentration of SBMV-C decreased from the lateral veins towards the mid- and distal parts of the petiole, where virtually no virus could be found. In later stages of infection, the bean strain of SBMV was present in the roots and trifoliate leaves, whereas the cowpea strain remained confined to the inoculated primary leaves, with no antigen detectable in stem, roots, or trifoliates even at 2 mo postinoculation.

The absence of infective SBMV-C outside the inoculated leaves was confirmed by bioassay of plant extracts on a local lesion host. A similar distribution of SBMV-C RNA in different plant parts was found by dot-blot hybridization analysis (Fig. 2), although the presence of very small amounts of infectious SBMV-C RNA cannot be totally excluded by these detection methods.
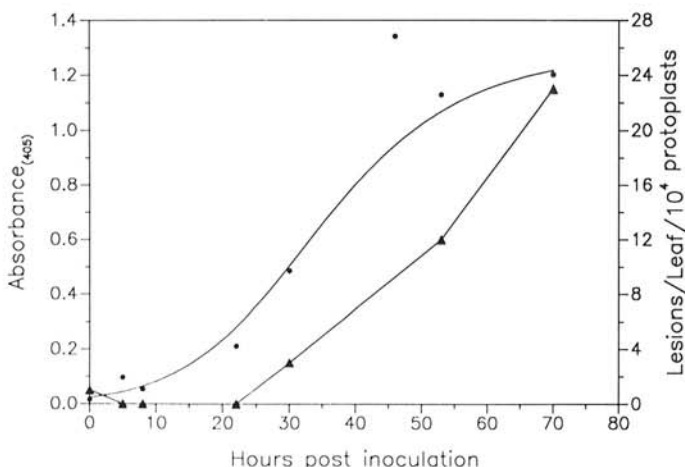
Thus, in the presence of SHMV, the cowpea strain of SBMV appeared to move through the epidermal and mesophyll cells of inoculated primary leaves at the same rate as the bean strain. However, movement of SBMV-C into the vascular tissue was limited or nonexistent. The antigen detected in lateral veins and midrib could be due to the presence of SBMV-C-infected mesophyll tissue that could not be totally eliminated during dissection.

Replication of SBMV-C in bean protoplasts. To confirm that bean cells can support viral replication, Bountiful protoplasts were inoculated with SBMV-C. Viral antigen in infected protoplasts could be detected 22 h after inoculation, increased until 42 h, and remained constant thereafter. Bioassays of virus infectivity were positive about 30 h after inoculation, and lesion numbers increased steadily until 68 h (Fig. 3).

Parallel inoculation of bean protoplasts with either SBMV strain resulted in similar infection courses and antigen accumulation. The levels of SBMV-C antigen accumulation after double infection of protoplasts with SHMV and SBMV-C also were similar to the levels attained when the same lot of protoplasts was singly inoculated with SBMV-C (Table 1). Thus, individual bean protoplasts sustain replication of SBMV-C and SBMV-B with the same efficiency, and the level of replication of SBMV-C in the isolated cells is unaffected by coinoculation with SHMV.

DISCUSSION

The results of these experiments provide an example of a system in which one virus (SHMV) enables another virus (SBMV-C) to move from cell to cell within the inoculated leaf, but not systemically, in a plant that would not normally be a host of the latter.

The cowpea strain of SBMV can replicate only subliminally in bean plants, yet it can replicate and produce infective particles in bean protoplasts to the same degree as the bean strain of SBMV. This suggests that the limited recovery of SBMV-C from inoculated bean leaves is not due to the inability of the cells to support virus replication, but to other factors that influence viral movement. This could be a plant and/or a viral factor because spread of a virus within the plant depends on the genome of the plant (20) and also on the translation product(s) of the virus genome (1). One such factor is the "movement protein," which is important in determining the host range of plant viruses (17).

Mixed infection of bean with SBMV-C and SHMV resulted in high levels of SBMV-C antigen and infectious material present in the inoculated primary leaves. SHMV seems to supply a transport function for SBMV-C, because coinoculation of bean protoplasts with SHMV and SBMV-C did not affect the accumulation attained by the latter. In this system, the helper effect provided by SHMV was limited to short distance (cell-to-cell) transport. Malyshenko et al (11) obtained similar results from

Fig. 3. Time course of the replication of the cowpea strain of southern bean mosaic virus in *Phaseolus vulgaris* 'Bountiful' protoplasts detected by enzyme-linked immunosorbent assay (●) and by bioassay (▲) on *Vigna unguiculata* 399414.

TABLE 1. Replication of the cowpea and bean strains of southern bean mosaic virus (SBMV-C and SBMV-B) and replication of SBMV-C in single and mixed infections with sunn-hemp mosaic virus (SHMV) in cultivar Bountiful protoplasts detected by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA)

	Absorbance ($A_{405nm}/10^4$ protoplasts)			
	Experiment A		Experiment B	
Time postinoculation	SBMV-C[a]	SBMV-B	SBMV-C	SBMV-C + SHMV
0	0.089	0.031	0.314	0.438
5	0.044	0.008	0.272	0.371
12	0.022	0.003	ND	ND
24	0.377	0.414	0.275	0.345
46	ND[b]	ND	0.749	0.867

[a] Experiment in first two columns was done simultaneously with the same preparation of Bountiful bean protoplasts that was divided into two equal parts, one for each treatment; the last two columns represented another lot of Bountiful bean protoplasts also divided into two equal parts, one for each treatment.

[b] Not determined.

complementation experiments between different viruses within the tobamovirus group and between unrelated viruses from different groups.

Comparison of the accumulation of SBMV-C and SBMV-B viral antigen in different parts of the plant showed that, in mixed infections with SHMV, SBMV-C moved at the same rate as SBMV-B between epidermal and parenchyma cells in the leaf blade. On the other hand, the comparatively low quantities of SBMV-C associated with the veinal tissue of inoculated leaves and its absence in petioles, stems, roots, and trifoliate leaves suggest that the virus was not being transferred from the parenchyma into the conducting tissue and from there back into the mesophyll. The absence of infectious SBMV-C material outside the inoculated leaves also was confirmed by bioassay and dot-blot hybridization analysis. The fact that another strain of the virus, SBMV-B, in single infection, does move systemically in the host implies that a specific virus-plant interaction, probably distinct from the mechanism involved in cell-to-cell movement, regulates viral movement from the parenchyma cells into the vascular tissue.

Recent reviews have suggested that viral movement is probably regulated at several levels by different host and viral genes (2,8). If viruses move through plasmodesmata and if plasmodesmatal connections function differently in different symplastic domains (5), then different genes should regulate cell-to-cell and systemic movement of viruses. This could explain why SHMV complements SBMV-C in cell-to-cell movement but does not effect systemic infection of bean. Specific viral proteins have been implied in cell-to-cell movement for several viruses (for reviews, see 2,8).

Complementation of the movement function can occur among viruses belonging to different taxonomic groups. This relative lack of specificity could be explained, in some cases, by the recent finding that the transport protein of TMV binds single-stranded RNAs nonspecifically in vitro (3). Citovsky et al propose that the transport protein sequesters viral RNA and transports it into adjacent host cells. Since this binding by the transport protein is nonspecific, an analogous transport protein of SHMV may bind SBMV-C single-stranded RNA and transport it from cell to cell. Systemic movement, on the other hand, might require a different mechanism, either to overcome the defense response of the plant and/or to modify the plasmodesmata and allow the virus to enter the vascular bundles. Given the complexity of the vascular system itself, it would be useful to investigate the ultrastructural localization of SBMV-C and determine whether it is entering the companion cells but not the sieve tubes, or whether the block to systemic spread occurs before it enters the companion cells. This would contribute to a better understanding of the differences between short and long distance movement and the mechanisms involved in these two stages of virus movement.

LITERATURE CITED

1. Atabekov, J. G., and Dorokhov, Y. L. 1984. Plant virus-specific transport function and resistance of plants to viruses. Adv. Virus Res. 29:313-364.
2. Atabekov, J. G., and Taliansky, M. E. 1990. Expression of a plant virus-coded transport function by different viral genomes. Adv. Virus Res. 38:201-248.
3. Citovsky, V., Knorr, D., Schuster, G., and Zambryski, P. 1990. The P30 movement protein of tobacco mosaic virus is a single-strand nucleic acid binding protein. Cell 60:637-647.
4. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
5. Erwee, M. G., and Goodwin, P. B. 1985. Symplastic domains in extrastellar tissues of *Egeria densa* Planch. Planta 163:9-19.
6. Fuentes, A. L., and Hamilton, R. I. 1988. Spread of the cowpea strain of southern bean mosaic virus in a non permissive host is facilitated by infection with sunn-hemp mosaic virus. Abstr. Int. Congr. Plant Pathol., 5th, Kyoto, Japan. 1-2-114.
7. Ghabrial, S. A., Shepherd, R. J., and Grogan, R. G. 1967. Chemical properties of three strains of southern bean mosaic virus. Virology 33:17-27.
8. Hull, R. 1989. The movement of viruses in plants. Annu. Rev. Phytopathol. 27:213-240.
9. Kassanis, B., and Varma, A. 1975. Sunn hemp mosaic virus. No. 153 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, England.
10. Larkin, P. J. 1976. Purification and viability determination of plant protoplasts. Planta 128:213-216.
11. Malyshenko, S. I., Kondakova, O. A., Taliansky, M. E., and Atabekov, J. G. 1989. Plant virus transport function: Complementation by helper viruses is non-specific. J. Gen. Virol. 70:2751-2757.
12. Maule, A. J. 1983. Infection of protoplasts from several *Brassica* species with cauliflower mosaic virus following inoculation using polyethylene glycol. J. Gen. Virol. 64:2655-2660.
13. Molefe, T. L., Wakarchuk, D. A., Kalmar, G., and Hamilton, R. I. 1983. Interaction of cowpea strains of southern bean mosaic and tobacco mosaic viruses in cowpea and bean. Page 120 in: Abstr. Int. Congr. Plant Pathol., 4th, Melbourne, Australia.
14. Power, J. B., and Cocking, E. C. 1969. A simple method for the isolation of very large numbers of leaf protoplasts by using mixtures of cellulase and pectinase. Proc. Biochem. Soc. Biochem. J. 111:33.
15. Siegel, A., Hari, V., Montgomery, I., and Koalacz, K. 1976. A messenger RNA for capsid protein isolated from tobacco mosaic virus-infected tissue. Virology 73:363-371.
16. Takebe, I. 1977. Protoplasts in the study of plant virus replication. Pages 237-283 in: Comprehensive Virology. Vol. II. H. Fraenkel-Conrat and R. R. Wagner, eds. Plenum Publishing, New York.
17. Taliansky, M. E., Malyshenko, S. I., Pshennikova, E. S., and Atabekov, J. G. 1982. Plant virus-specific transport function. II. A factor controlling virus host range. Virology 122:327-331.
18. Tremaine, J. H., and Hamilton, R. I. 1983. Southern bean mosaic virus. No. 274 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, England.
19. Tremaine, J. H., Ronald, W. P., and Kelly, E. M. 1981. Comparison of highly basic cyanogen bromide peptides from strains of southern bean mosaic virus. Can. J. Microbiol. 27:654-663.
20. Zaitlin, M., and Hull, R. 1987. Plant virus-host interactions. Annu. Rev. Plant Physiol. 38:291-315.