Serological and Biochemical Properties of the Capsid and Major Noncapsid Proteins of Maize Stripe, Rice Hoja Blanca, and Echinochloa Hoja Blanca Viruses

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ABSTRACT

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The two major virus-specific proteins found in plants infected by maize stripe virus (MStpV), rice hoja blanca virus (RHBV), and Echinochloa hoja blanca virus (EHBV) were compared by serological and one-dimensional peptide-mapping analysis. When total cellular proteins from healthy plants and plants singly infected with MStpV, RHBV, or EHBV were compared by SDS-PAGE, the samples from the virus-infected plants showed a prominent protein of about 32,000 M_r in the high-speed pellet fraction and a prominent protein of about 16,000 M_r in the high-speed supernatant fractions. These were identified as the virion capsid and virus-specific major noncapsid proteins, respectively. Antisera produced to these six proteins reacted specifically with virus-infected and not healthy plants by indirect ELISA. The two MStpV protein antisera reacted only with MStpV-infected plants, whereas the RHBV and EHBV protein antisera reacted with plants infected with either virus but not MStpV. Immunological analysis of Western blots showed that the antisera were specific in that none of the noncapsid protein antisera reacted with any of the capsid proteins and vice versa. One-dimensional peptide-mapping analysis of the individual proteins gave results that agreed with the serological data. The MStpV capsid and noncapsid proteins were distinct from the corresponding proteins of RHBV and EHBV, which were indistinguishable.

Recently, several planthopper-borne viruses affecting the Gramineae have been shown to have similar properties that probably place them as a distinct group of plant viruses that includes maize stripe virus (MStpV), rice hoja blanca virus (RHBV), Echinochloa hoja blanca virus (EHBV), rice stripe virus (RSV), European wheat striate mosaic virus (EWSMV), and possibly, rice grassy stunt virus (RGSV) (1,7,9,10,13,17,19). Some of the properties associated with these viruses include a capsid protein of about 32,000 M_r associated with infectious nucleoprotein (7,9,17,19); large amounts of a 16,000 M_r noncapsid protein produced in infected plants (6,9,10,17); and fine-stranded particles, sometimes with a spiral or helical structure, that are believed to be virions (9,17,19). Morphologically similar inclusion bodies also have been observed in RHBV, MStpV, and EWSMV-infected plants (1). The virion-associated RNAs of MStpV and RSV also show somewhat similar patterns after denaturing electrophoresis (7,19), but the significance of the array of RNAs for each is not yet known.

The relationships of the planthopper-borne viruses to each other have not yet been studied in detail. Difficulties such as strong host plant specificity of the vector have made comparisons of the viruses in common host plant species difficult (10,17), and whether some of these viruses are closely related strains of a given virus or different viruses has been recently questioned (1,10). We have compared three of these viruses that overlap in their geographical and plant host ranges. By examining the serological and biochemical relationships of the two major virus-specific proteins found in infected plants, similarities as well as distinct differences between these viruses are readily apparent.

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MATERIALS AND METHODS

Virus and vector maintenance. The Florida isolate of MStpV (MStpV-FL) and its vector *Peregrinus maidis* Ashmead are the same as used previously (6,7,20). MStpV-FL and *P. maidis* were maintained using maize (*Zea mays* L.) as described (20). Itchgrass (*Rottboellia exaltata* L.) was inoculated with MStpV-FL using inoculative *P. maidis* 15–20 days after acquisition on MStpV-FL-infected maize source plants. MStpV-Co was isolated from naturally infected maize collected near Cali, Colombia.

The maintenance host for RHBV and its vector, *Sogatodes orizicola* Muir., was rice (*Oryza sativa* L.), and the host for EHBV and its vector, *Sogatodes cubanus* Grawford, was *Echinochloa colona* L. These viruses and vectors were maintained in Colombia as described (17).

Viral protein purification. Cellular proteins from healthy and virus-infected plants were prepared as described (5,22). Extracts were centrifuged at 10,000 *g* for 10 min. The pellet was discarded, and the supernatant was subjected to high-speed centrifugation (145,000 *g*) for 2 hr. Both the supernatant and pellet fractions were analyzed for virus-specific proteins.

The major viral noncapsid proteins were gel-purified from MStpV-FL-infected maize and itchgrass, MStpV-Co-infected maize, RHBV-infected rice, and EHBV-infected *E. colona* by the methods described (6). After electroelution from SDS-gels, the purified noncapsid proteins were stored at −20 until used.

MStpV-FL virion capsid protein was prepared by purifying the MStpV nucleoprotein as described (7). The RHBV and EHBV nucleoproteins were purified as described (17) or in some cases by electroelution from SDS-gels from preparations enriched by differential pH precipitation for noncapsid protein. As was found for MStpV (6), differential pH precipitation concentrates significant amounts of RHBV and EHBV virion capsid protein as well as noncapsid protein.

SDS-polyacrylamide gel electrophoresis of virion proteins was done using the system of Laemmli (16). Samples were routinely labeled with MDPF (2-methoxy-2.4-diphenyl-3(2H)-furanone) before electrophoresis (6). Electrophoresis was done using either

5% stacking and 12% resolving acrylamide gels at 200V for 1 hr in Mini-slabs (Idea Scientific, Corvallis, OR) as described (5–7) or in 10–20% linear gradient gels using a Hoefer Sturdier apparatus at 100V for 18 hr. Proteins were visualized by exposing the gel to 302 nm illumination or by silver staining (18).

Serological and peptide-mapping analysis. Antisera to the MStpV, RHBV, and EHBV virion capsid proteins were produced in New Zealand white rabbits by intramuscular injection of purified nucleoprotein. Antisera to the MStpV, RHBV, and EHBV noncapsid proteins were produced by intramuscular injection of SDS-gel-purified noncapsid proteins. Noncapsid protein antigens were dialyzed against PBS (0.02 M phosphate, 0.15 M NaCl, pH 7.4) before injection. For the first intramuscular injection, antigens were emulsified with an equal part of Freund's complete adjuvant, and for second and third injections, antigens were emulsified with incomplete adjuvant. Blood was collected by marginal ear vein bleeding starting 10 days after the final immunization. Antisera were processed as described (6) and stored at −20.

Western blotting and immunological analysis of SDS-PAGE-analyzed proteins was done as described (3), using a Trans-blot cell (Bio-Rad Laboratories, Richmond, CA). After transfer, nitrocellulose membranes were washed in PBS and then "blocked" by soaking in 5% nonfat evaporated milk (Carnation) in PBS for 15–30 min at room temperature with shaking. Membranes were washed in PBST (PBS containing 0.3% polyoxyethylene sorbitan monolaureate), then probed by incubation in the desired antiserum for 2 hr at room temperature with shaking. Concentrations were 1/500 (in PBST) for the capsid protein antisera. The noncapsid protein antisera were used at 1/1,000 for MStpV, 1/2,000 for RHBV, and 1/500 for EHBV. Blots were then thoroughly washed three times, 10 min each in PBST, then incubated with a 1/2,000 dilution of horseradish peroxidase-conjugated protein A (Boehringer Mannheim, Indianapolis, IN) for 2 hr at room temperature with shaking. Membranes were thoroughly washed three times with PBST and incubated in the dark with *o*-phenylenediamine substrate for 30 min (5). Membranes were rinsed with PBST, allowed to dry, and photographed.

One-dimensional peptide mapping of MDPF-labeled purified viral proteins was done essentially as described by Cleveland et al (4). Purified viral proteins were treated with V-8 protease (Miles Scientific, Naperville, IL) or chymotrypsin (Sigma, St. Louis, MO) at 33 μg/ml for 100 min at room temperature. Samples were placed in boiling water for 2 min to stop proteolysis, then analyzed by SDS-PAGE. Virion capsid and the major noncapsid proteins were analyzed in 10–20% linear acrylamide gradient gels. The major noncapsid proteins also were analyzed in SDS-6 M urea gels of 15% acrylamide (2).

Indirect ELISA (enzyme-linked immunosorbent assay) was used to compare the serological reactions of MStpV-, RHBV-, and EHBV-infected plants by using antisera to the virion capsid and the major noncapsid proteins. Microtiter plates (Dynatech Immulon II, Alexandria, VA) were coated with plant extracts diluted 1/10 (w/v) in sodium carbonate buffer as described (6). Immunoglobulins from the polyclonal rabbit antisera were used at 2 μg/ml, and alkaline phosphatase-conjugated goat antirabbit immunoglobulins (Miles Scientific. Naperville. IL) were used at 1/1,000 in PBST. Plates were incubated for 60 min with 0.6 mg/ml *p*-nitrophenylphosphate in diethanolamine substrate buffer, and reactions were assessed by measuring the absorbance of each well (A_{405nm}) with a Bio-Tek (Burlington, VT) EIA reader.

RESULTS

Viral proteins. Protein preparations from MStpV-, RHBV-, and EHBV-infected plants all showed two prominent virus-specific proteins (Fig. 1). The virion capsid protein was detected primarily in the 145,000 *g* pellet fractions from infected plants, whereas the major noncapsid protein was detected in the 145,000 *g* supernatant preparation. The capsid proteins of MStpV, RHBV, and EHBV all had apparent molecular weights (M_r) of about 32,000. The major noncapsid proteins also had M_rs of 16–18,000. No proteins similar to the viral capsid and major noncapsid proteins were apparent in corresponding preparations from healthy control plants.

The purified major noncapsid proteins from MStpV-, RHBV-, and EHBV-infected plants were resolved by SDS-PAGE (Fig. 2). Only one protein was detected in each preparation, indicating the purity of the noncapsid proteins. No consistent differences in mobility were detected among the MStpV, RHBV, and EHBV noncapsid proteins. In some gels, the EHBV and RHBV noncapsid proteins migrated slightly ahead of the MStpV noncapsid proteins, but this was inconsistent. However, MStpV noncapsid protein never migrated ahead of the RHBV and EHBV noncapsid proteins. Also, no differences were detected for MStpV-FL noncapsid proteins purified from maize and itchgrass (*R. exaltata*) or for MStpV-Co noncapsid protein purified from maize. The purified capsid proteins of MStpV-FL, EHBV and RHBV also were indistinguishable by one-dimensional SDS-PAGE in linear or gradient slab gels.

Serological analysis. All of the purified proteins elicited specific antibodies when they were used as immunogens in rabbits. Indirect ELISA against plant extracts showed that all six antisera reacted

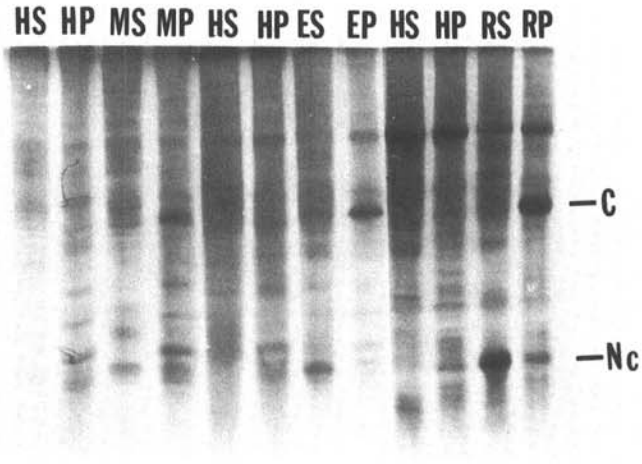

Fig. 1. Silver-stained SDS-12% polyacrylamide gel showing the 145,000 *g* supernatant and pellet proteins from healthy and virus-infected plants. The first letter for each lane shows whether the sample is from a healthy (H) or a virus-infected sample (M = MStpV, R = RHBV, and E = EHBV). The second letter shows whether the sample is the 145,000 *g* pellet (P) or supernatant (S) protein preparation. The C and Nc to the right of the gel show the locations of the capsid and major noncapsid proteins, respectively.

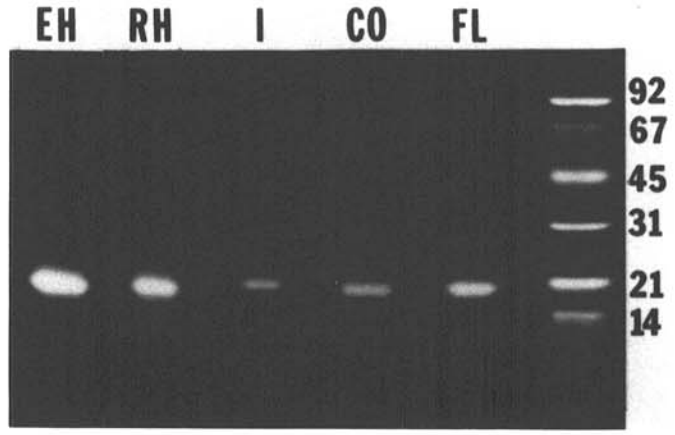

Fig. 2. Linear 10–20% gradient SDS-polyacrylamide gel showing purified noncapsid proteins of maize stripe virus (MStpV), rice hoja blanca virus (RH), and Echinochloa hoja blanca virus (EH). I, CO, and FL refer to MStpV Florida isolate noncapsid proteins purified from Rottboellia exaltata, MStpV Colombia isolate purified from *Zea mays*, and MStpV Florida isolate purified from *Z. mays*, respectively. Molecular mass markers are shown at right.

with their respective infected and not healthy plants (Table 1). Similar results were obtained when plant samples were tested by immunodiffusion using the six antisera in 0.6% agar plates in PBS. RHBV- and EHBV-infected plants both gave positive reactions with antisera to the RHBV and EHBV capsid and noncapsid proteins, indicating a serological relationship for the RHBV and EHBV capsid proteins and also for the RHBV and EHBV noncapsid proteins. Neither the MStpV capsid protein antiserum nor the noncapsid protein antiserum gave positive reactions with the RHBV- or EHBV-infected plants, and conversely, the RHBV and EHBV antisera did not react with MStpV-infected plants. In all indirect ELISAs, absorbance values were slightly higher for heterologous virus-infected samples than for healthy checks when using any of the noncapsid protein antisera (e.g., MStpV extract with antiserum to RHBV noncapsid protein or vice versa). Indirect ELISAs as done here are not quantitative, and it is difficult to interpret the significance of these slight cross-reactions using noncapsid protein antisera.

To demonstrate that the antisera reacted only with the desired proteins that were used as immunogens, immunological analyses of Western blots were performed using each of the antisera and both purified proteins and total protein extracts from healthy and infected plants. The MStpV noncapsid protein antisera reacted strongly with purified MStpV noncapsid protein from maize (both FL and CO isolates) and from *R. exaltata* but did not react with the RHBV and EHBV purified noncapsid proteins (Fig. 3). The antiserum are shown). None of the noncapsid protein antisera both RHBV and EHBV noncapsid proteins but not with the MStpV noncapsid protein or with any of the MStpV, RHBV, or EHBV capsid proteins (only data for EHBV noncapsid protein antisera are shown). None of the noncapsid protein antisera reacted with the capsid proteins, showing that the noncapsid protein antisera were specific. In most cases, when total protein and purified protein blots were probed using any of the noncapsid protein antisera, besides the strong reaction for the noncapsid protein, a somewhat less intense reaction at about 35,000 M_r also was prominent. This was interpreted to be noncapsid protein dimer, because the purified noncapsid proteins both used as immunogens and as checks on the western blots all had been electroeluted from SDS gels as part of their purification, and the reaction was at about twice the M_r of the noncapsid protein monomer.

In some of the Western blots, the RHBV and EHBV noncapsid protein antisera also appeared to give general nonspecific reactions with several proteins on the blots including the M_r standards. However, proteins were routinely labeled with MDPF so they could be visibly detected on blots before probing (5). When unlabeled samples were electrophoresed, blotted, and then probed with these same antisera, the nonspecific reactions were eliminated without affecting the homologous reactions.

Western blot analysis using the capsid protein antisera showed the specificity of the antisera for the virus capsid protein and that the RHBV and EHBV capsid proteins are serologically related to each other and not to MStpV (Fig. 4). There was some reaction for the MstpV, EHBV, and RHBV capsid proteins in the supernatant as well as in the pellet fractions in all cases. This may have been due to disrupted capsids that did not sediment by ultracentrifugation. Both the RHBV and EHBV capsid protein antisera reacted with fraction 1 protein in both healthy and virus-infected samples in initial experiments. These reactions were eliminated by cross-absorbing these two antisera with extracts from healthy rice.

Peptide-mapping analysis. The peptide-mapping analysis for the noncapsid proteins agreed with the serological data. The MStpV-FL and MStpV-Co noncapsid proteins purified from infected maize and the MStpV-FL noncapsid protein purified from *R. exaltata* were indistinguishable by one-dimensional peptide mapping after cleavage with either V-8 protease or chymotrypsin (Fig. 5). V-8 protease cleavage gave an identical series of polypeptides for the three MStpV noncapsid protein preparations, whereas chymotrypsin digestion gave three minor products in addition to some uncleaved noncapsid protein. When MStpV-FL noncapsid protein was compared with the RHBV and EHBV noncapsid proteins, the MStpV noncapsid protein was easily differentiated from the other two noncapsid proteins (Fig. 6). The MStpV noncapsid protein showed a pattern similar to previous experiments (see Fig. 5), but the V-8 protease-treated RHBV and EHBV noncapsid proteins both gave one prominent cleavage product immediately below the uncleaved noncapsid protein and some unresolved much fainter products farther down in the gel. Treatment with chymotrypsin gave three products plus undigested noncapsid protein for MStpV, whereas the RHBV and EHBV noncapsid proteins were not affected by chymotrypsin under the conditions used here.

When the MStpV, RHBV, or EHBV capsid proteins were compared after cleavage using V-8 protease, the EHBV and RHBV capsid protein peptide patterns were indistinguishable, yet they were obviously different from that for the MStpV capsid protein (Fig. 7). The MStpV capsid protein was cleaved into a series of distinct polypeptides smaller than the capsid protein, whereas both the RHBV and EHBV capsid proteins yielded two major cleavage products and a series of smaller unresolved polypeptides.

DISCUSSION

MStpV, RHBV, and EHBV share several properties that include planthopper vectors, production of similar symptoms in their respective host plants, and morphologically similar nucleoproteins. Plants infected by each of these viruses also contain two major virus-specific proteins. The respective major noncapsid protein from MStpV, RHBV, and EHBV are similar in M_r, as are the capsid proteins. However, the MStpV noncapsid protein and capsid proteins can be readily differentiated from those of EHBV and RHBV by serological and one-dimensional peptide-mapping analyses, showing that MStpV is a distinct virus. In contrast, RHBV and EHBV are not distinguishable by the biochemical and serological tests used here. Thus, we were unable to determine whether RHBV and EHBV are distinct or closely related viruses. However, based on the overall similar properties of the two virus-specific proteins for MStpV, RHBV, and EHBV, these three viruses are all most likely members of the same taxonomic group.

The other planthopper-borne viruses of the Gramineae that show overall similar properties to MStpV, RHBV, and EHBV

TABLE 1. Indirect ELISA reactions for healthy and MStpV-, RHBV-, and EHBV- infected plants using antisera to virion capsid and major noncapsid proteins

	Antiserum					
Test plant	MStpV-C[a]	MStpV-NCP	RHBV-C	RHBV-NCP	EHBV-C	EHBV-NCP
MStpV-maize	0.400[b]	0.600	0.006	0.070	0.020	0.10
Healthy maize	0.001	0.001	0.010	0.001	0.001	0.05
RHBV-rice	0.001	0.020	0.810	1.100	0.520	0.16
Healthy rice	0.001	0.001	0.050	0.001	0.040	0.01
EHBV-*E. colona*	0.001	0.040	0.300	1.000	0.400	0.18
Healthy *E. colona*	0.001	0.001	0.020	0.001	0.020	0.03

[a] MStpV = maize stripe virus, RHBV = rice hoja blanca virus, and EHBV = Echinochloa hoja blanca virus. C and NCP refer to capsid and major noncapsid protein, respectively, for the given virus.
[b] Numbers are mean absorbance values at 405 nm for two replicates measured after 60 min of incubation.

have not as yet been comparably characterized. MStpV and RSV are serologically related when tested using antisera to their capsid proteins, but MStpV noncapsid protein antiserum does not react with RSV-infected rice (10). This might imply that although the capsid proteins of RSV and MStpV are related, the major noncapsid proteins are not; however, reciprocal tests are needed to help resolve this question. Using the properties of both the noncapsid and the capsid proteins for determining relationships of the planthopper-borne viruses of the Gramineae therefore may be

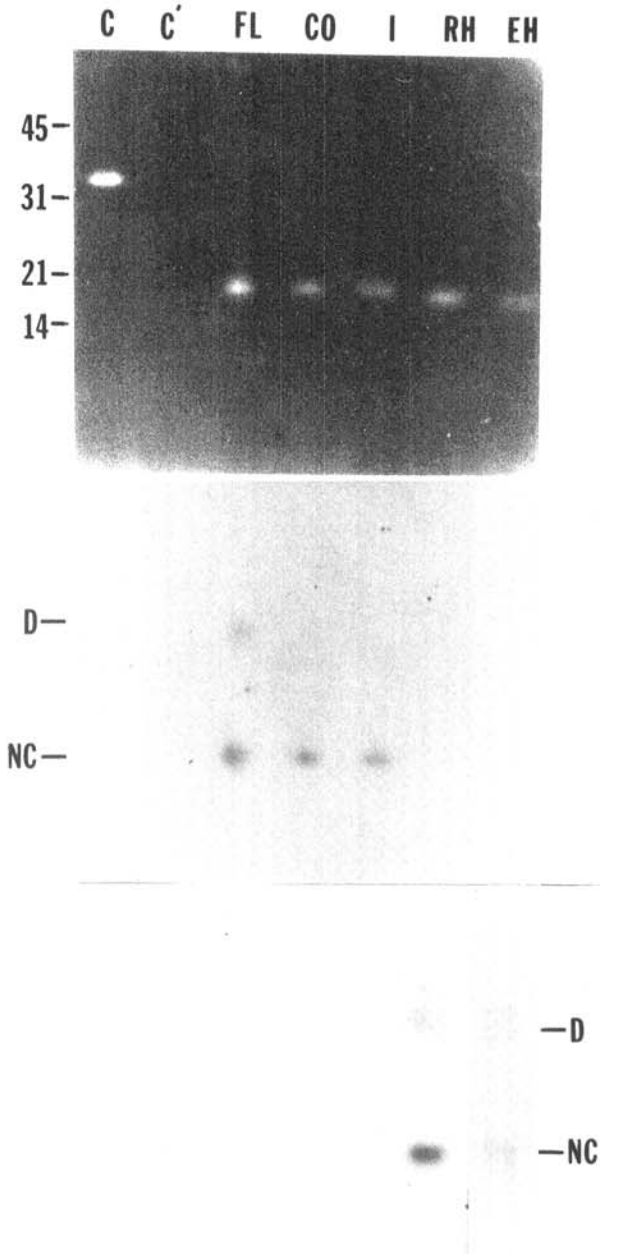

Fig. 3. Top, Linear 10–20% gradient SDS-polyacrylamide gel showing purified fluorescent-labeled maize stripe virus (MStpV) capsid protein (C), purified unlabeled MStpV-capsid protein (C'), and the purified major noncapsid proteins of MStpV Florida isolate from maize (FL), MStpV Colombia isolate from maize (CO), MStpV Florida isolate from *Rottboellia exaltata* (I), RHBV from rice (RH), and EHBV from *Echinochloa colona* (EH). Molecular weight markers are shown at left. **Middle,** Proteins from the above gel after transfer to nitrocellulose by electroblotting and subsequent serological analysis with antiserum to the MStpV noncapsid protein. NC and D indicate reactions for noncapsid protein monomer and dimer, respectively. **Bottom,** Proteins from a duplicate of the above gel after transfer to nitrocellulose by electroblotting and subsequent serological analysis with antiserum to the RHBV noncapsid protein.

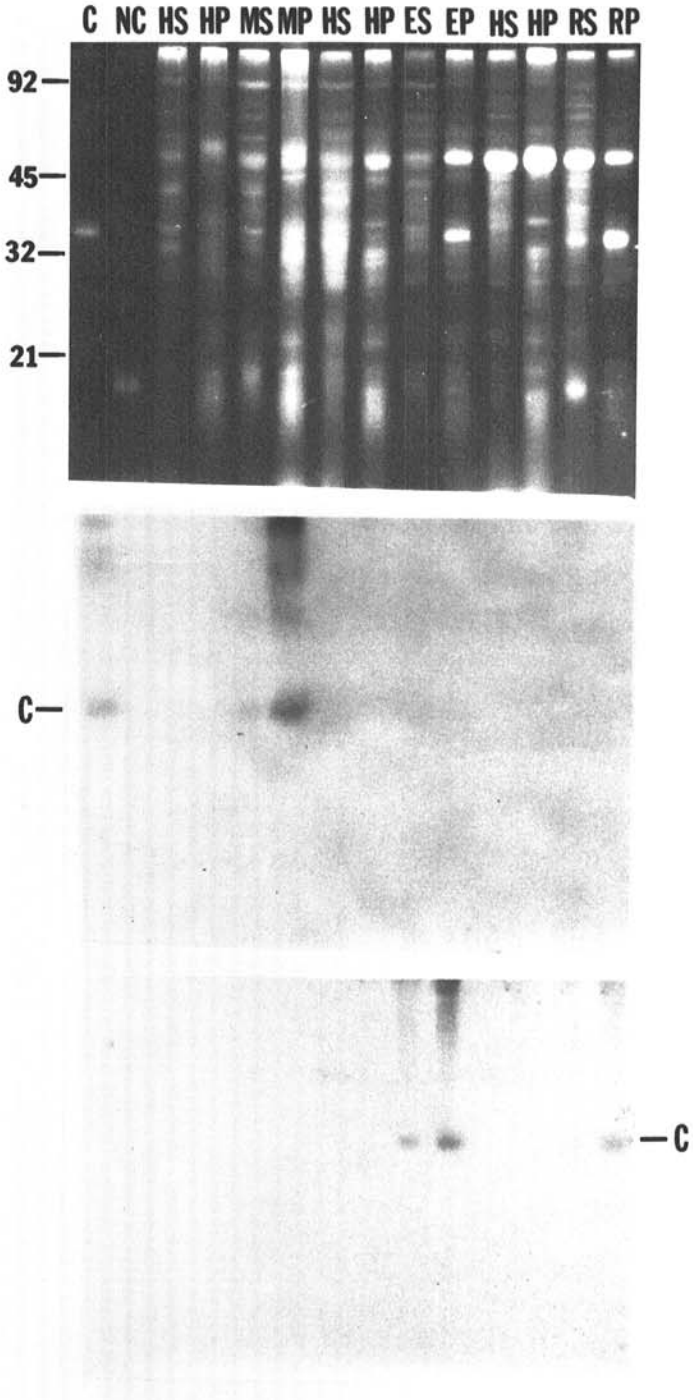

Fig. 4. Top, SDS-polyacrylamide (12%) gel showing cellular proteins extracted from healthy plants and plants infected with maize stripe virus (MStpV), rice hoja blanca virus (RHBV), and Echinochloa hoja blanca virus (EHBV). Proteins were separated by high-speed centrifugation (145,000 *g* for 2 hr), and the pellet (P) and supernatant (S) proteins were fluorescent-labeled and analyzed by gel electrophoresis. Locations of protein standards (phosphorylase B, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor from top to bottom, respectively), are shown at left. Lanes C and NC show purified MStpV capsid and noncapsid proteins, respectively. For the remaining lanes, the first letter shows whether the sample is from a healthy (H) or a virus-infected sample (M = MStpV, R = RHBV, and E = EHBV). The second letter shows whether the sample is the 145,000 *g* pellet (P) or supernatant (S) protein preparation. **Middle,** Proteins from the above gel after they were transferred to nitrocellulose by electroblotting, then serologically analyzed with antiserum to the MStpV capsid protein. **Bottom,** Proteins from a duplicate of the above gel after they were transferred to nitrocellulose by electroblotting, then serologically analyzed with cross-adsorbed antiserum to the EHBV capsid protein.

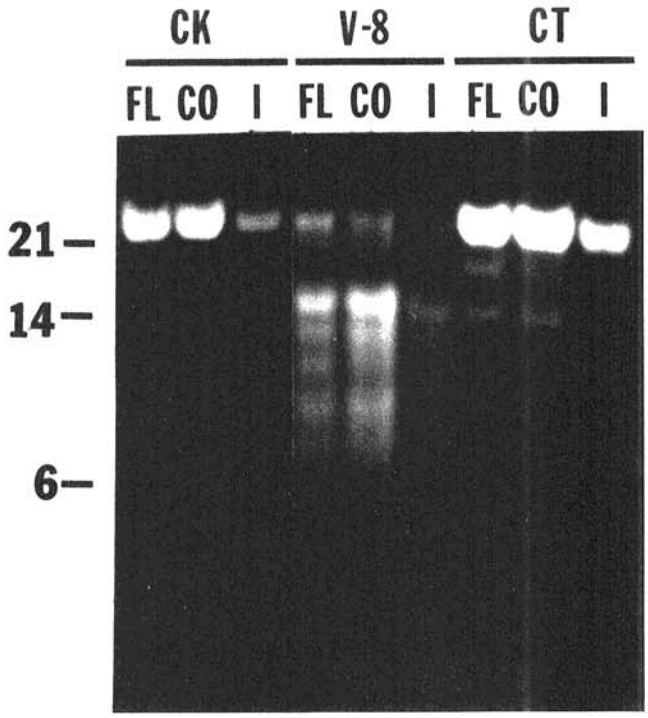

Fig. 5. A 6 M urea 15% acrylamide gel showing one-dimensional peptide-mapping analysis of the major noncapsid proteins of maize stripe virus (MStpV), Florida (FL) isolate and Colombia (CO) isolate, purified from maize and the MStpV Florida isolate purified from *Rottboellia exaltata* (I). Proteins were untreated (CK), treated with *Staphylococcus aureus* V-8 protease (V-8), or treated with chymotrypsin (CT) before electrophoresis.

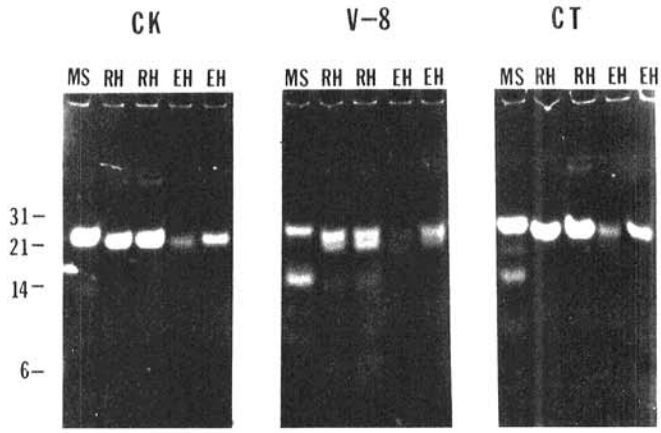

Fig. 6. Three 6 M urea 15% acrylamide gels showing one-dimensional peptide-mapping analysis of the major noncapsid proteins of maize stripe virus (MS) purified from maize, rice hoja blanca virus (RH) purified from rice, and Echinochloa hoja blanca virus (EH) purified from *Echinochloa colona*. Proteins were untreated (CK), treated with *Staphylococcus aureus* V-8 protease (V-8), or treated with chymotrypsin (CT) before electrophoresis.

useful and somewhat similar in certain respects to studies or potyvirus relationships by analysis of capsid and inclusion body proteins (14,23). The noncapsid and capsid proteins of MStpV, RHBV, and EHBV are distinct proteins. The noncapsid proteins do not cross-react serologically with the capsid proteins. Also, when the MStpV noncapsid and capsid proteins were directly compared by one-dimensional peptide mapping and immunological analysis of the peptides on Western blots, the peptide patterns were distinct and there were no serological cross-reactions (*unpublished*).

Whether the noncapsid proteins are viral-coded or host-coded proteins has been recently questioned, at least for MStpV (11,15). All of the available data suggest that for MStpV and most likely other similar viruses, the noncapsid protein is coded by the viral

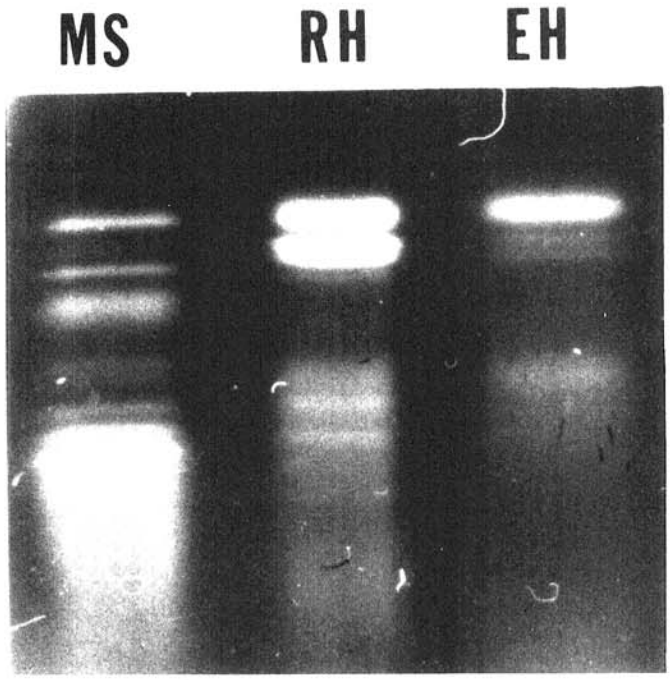

Fig. 7. A 10–20% linear gradient SDS-polyacrylamide gel showing the maize stripe virus (MS), rice hoja blanca virus (RH), and Echinochloa hoja blanca virus (EH) capsid proteins after digestion with V-8 protease and subsequent electrophoresis.

genome. The MStpV noncapsid protein is indistinguishable by peptide mapping and serological analyses when purified from such diverse hosts as maize and *R. exaltata*. We previously reported that antiserum to the MStpV noncapsid protein reacted only with MStpV-infected plants and not healthy plants or maize plants infected with other maize viruses (6). Recent experiments done by immunological analysis of Western blots on total proteins from healthy and various virus-infected plants also have shown that although there are proteins of similar M_r to the noncapsid protein in both healthy plants and those infected by other maize viruses, these proteins do not react with antiserum to the MStpV noncapsid protein. Similar results have been obtained by using antiserum to the RHBV and EHBV noncapsid proteins. Recently, a protein that coelectrophoreses with purified MStpV noncapsid protein was specifically immunoprecipitated with MStpV noncapsid protein antiserum from MStpV RNA in vitro translation products (8), providing further evidence that the MStpV noncapsid protein is coded by the viral genome.

Serological analyses of plant virus capsid proteins has been routinely used for identifying relationships of plant viruses (21), even though the viral capsid protein represents a small amount (10–20%) of the total coding capacity of the viral genome. Serological relationships are then based on a limited percentage of the viral genome products (12,14). Thus when more than one major viral protein can be used for serological comparisons, a larger proportion of the viral genome can be indirectly compared (14).

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