Etiology

Purification and Protein Characterization of Sorghum Stunt Mosaic Rhabdovirus

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ABSTRACT

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Sorghum stunt mosaic rhabdovirus (SSMV) was purified from infected sweet corn by Celite filtration and sucrose density centrifugation. Bacilliform particles measuring 71×218 nm were observed in preparations by electron microscopy. Transmission of membrane-acquired purified virus to sweet corn by *Graminella sonora* indicated that the purified virus was infectious. Polyacrylamide gel electrophoresis of dissociated virions revealed four major proteins with calculated molecular mass of 91, 59, 36, and 30 kDa. After treating the virus with 2% Triton X-100 and 0.4 M NaCl, centrifugation resulted in a pellet containing the 59-kDa protein (and traces of the 36-kDa protein), indicating the presence in the viral core particle of the 59-kDa protein. The 91-kDa protein stained positive with Schiff's reagent, indicative of a glycoprotein. The SSMV viral RNA was estimated to be 4.2×10^6 Da mol. wt., migrating identically to ssRNA of beet yellows virus in a 0.8% agarose gel. Antisera to a variety of other plant rhabdoviruses did not react with SSMV virions by indirect ELISA, and polyclonal rabbit antisera produced against SSMV did not react with any of the other plant rhabdovirus proteins assayed by western blotting.

Leafhopper- and planthopper-transmitted rhabdoviruses that infect monocotyledons are found throughout the world (2). Although these enveloped viruses cause serious losses to their cereal hosts, they have not been well studied. Four cereal-infecting rhabdoviruses have been reported in the United States: maize mosaic virus (MMV), wheat striate mosaic virus (WSMV), oat striate mosaic virus (OSMV), and sorghum stunt mosaic virus (SSMV). MMV, for many years a problem in the tropics, has been found in Florida (1), while WSMV has been found in South Dakota (16). Initial reports of OSMV were from Illinois (8), but all laboratory isolates of this virus are thought to have been lost.

SSMV is the only rhabdovirus infecting monocotyledons reported in California (11). Diseased plants show chlorotic streaking and mottling of the leaves and are stunted (11). Using electron microscopy, Mayhew and Flock associated the presence of rhabdoviruslike particles with the disease. They noted that while SSMV was not mechanically transmissible, the leafhopper *Graminella sonora* Ball could transmit the virus to corn, sorghum, and wheat. It was not demonstrated if the disease was caused by the rhabdovirus alone or in combination with another agent, or whether the virus was related to any other reported virus. This paper describes the purification, transmission, and preliminary characterization of SSMV RNA and its capsid proteins and compares SSMV serologically with other plant rhabdoviruses.

MATERIALS AND METHODS

SSMV isolate and leafhopper maintenance. SSMV-infected sweet corn was collected from Imperial County, California, by R. Flock. The virus was transmitted to maize (*Zea mays* L. Golden Bantam) with *Graminella sonora* and maintained in that host. The leafhopper vector, *G. sonora*, was obtained from W. Styer (The Ohio State University, Wooster) and reared in cages on oats (*Avena sativa* L. 'Cal Red Oats') and maize.

Transmission procedures. Nonviruliferous adult leafhoppers were given a 48-h acquisition access period (AAP) on detached leaves at 25 C. Ten leafhoppers per plant were then caged on healthy corn for an inoculation access period (IAP) of 15 days at 30 C/27 C diurnal temperature and light. Leafhoppers were removed, and the plants were sprayed with resmethrin (Whitmire Research Laboratories, St. Louis, MO). The plants were maintained in a leafhopper-free greenhouse.

Alternatively, leafhoppers were allowed to acquire purified virus through membrane feeding. Leafhoppers were placed into Parafilm membrane-covered vials, and starved for 30 min. Then 200 μl of purified SSMV (in 50 mM Tris-HCl, pH 7.5; 5 mM Mg Cl$_2$; 20 mM Na$_2$SO3; 10% sucrose) was placed on the membrane and another membrane was stretched over the virus suspension. Vials were illuminated from above. After a 4-h AAP, leafhoppers were transferred to corn as described above to test their ability to transmit virus.

Virus purification. Symptomatic leaves were harvested approximately 30 days after inoculation, and the midribs were removed. Virus was purified using a modification of the procedure described by Falk and Tsai (1). Tissue (60–80 g) was ground in a blender in 4 vol of extraction buffer (0.1 M Tris-HCl, pH 8.4, 0.01 M MgCl$_2$, 0.04 M Na$_2$SO$_3$), filtered through four layers of cheesecloth, and centrifuged for 15 min at 8,000 rpm in a Sorvall GSA rotor (Dupont Instruments, Wilmington, DE). The supernatant was filtered through cheesecloth, and layered onto a step gradient consisting of 5 ml of 30% sucrose, 6 ml of 60% sucrose in maintenance buffer (0.1 M Tris-HCl, pH 7.5, 0.01 M Mg Cl$_2$, 0.04 M Na$_2$SO$_3$). After centrifugation for 1 h at 25,000 rpm in a SW 28 rotor (Beckman Instruments, Fullerton, CA), the green band at the 30/60% sucrose interface was removed and diluted with 0.5 vol of maintenance buffer. One gram of Celite 503 (or 545) was added to the virus preparation, and the mixture was filtered through a 14-g Celite pad in an 11-mm-diameter Buchner funnel through Whatman No. 1 paper. The pad was rinsed with 100 ml of maintenance buffer, and the filtrates were combined. Virus was pelleted by centrifugation for 1 h at 25,000 rpm in a 50.2 Ti rotor (Beckman). The pellets were resuspended in maintenance buffer and layered onto sucrose gradients composed of 5, 10, 10, and 10 ml of 5, 10, 20, and 30% sucrose in the maintenance buffer, respectively. Gradients were centrifuged for 15 min at 26,000 rpm in an SW 28 rotor and fractionated. Virus from the fractions was concentrated by centrifugation for 30 min at 30,000 rpm in a 50.2 Ti rotor and the pellets resuspended in 200 μl maintenance buffer.

Analysis of SSMV RNA and virion proteins. Nucleic acids were extracted from purified virions as described by Reed and Falk (14), denatured with glyoxal (15), and analyzed by electrophoresis using an 0.8% agarose mini-gel in 10 mM sodium phosphate, pH 7.0 buffer, for 90 min at 100 V.

The molecular weights of the virion proteins were estimated with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on a 5% stacking gel, 12% resolving gel (9). After electrophoresis for 50 min at 200 V at room temperature in a Bio-Rad mini-gel apparatus (Bio-Rad Laboratories, Richmond, CA), proteins were stained with Coomassie Brilliant Blue (17). Protein molecular weights were estimated by comparing their relative mobility with molecular weight standards (Bio-Rad Laboratories, Richmond, CA). For glycoprotein analysis, polyacrylamide gels were stained with Schiff's reagent (19) instead of Coomassie Brilliant Blue.

To determine which proteins were associated with the "core" of the virion, a modification of Jackson's procedure (6) was used. Virions were treated with 0.4 M NaCl, with either 1 or 2% (v/v) Triton X-100 for 1 h at 4 C. The samples were centrifuged through a 3-ml cushion of 20% sucrose in maintenance buffer for 1 h at 35,000 rpm in a 70.1 Ti rotor. The resulting pellets were resuspended in water and analyzed by SDS-PAGE (9).

Electron microscopy. Preparations of SSMV were placed on a Formvar-coated heavy carbon-coated grid, stained with 2% uranyl acetate, and examined with a Hitachi H-600 electron microscope. Both leaf dip homogenates, made by grinding infected tissue in 0.1 M Tris-HCl, pH 8.0, and purified virion preparations were analyzed. The mean size of the virions was determined by averaging the measurements from 40 intact particles from purified preparations and nine intact particles from leaf dip homogenates.

Serological analysis. Antiserum to SSMV was produced by mixing equal volumes of purified virions with Freund's adjuvant, and injecting a rabbit subcutaneously with emulsion containing 800 μl of approximately 625 μg/ml of antigen. Four injections were made at 14-day intervals, with Fruend's complete adjuvant in the first injection, and incomplete adjuvant in subsequent injections. Bleedings began 14 days after the final injection and continued at 14-day intervals for 4 wk. Immunoglobulin (IgG) purified by Protein A affinity chromatography (4) from the first bleeding was used for this study.

Total protein extracts were prepared by grinding tissue in liquid N, adding 1 vol of Laemmli sample buffer (9), and boiling the mixture for 10 min.

Indirect enzyme linked immunosorbent assay (ELISA) was conducted using a modification of Lommel et al (10). Corn samples were ground with a sap expressor (Piedmont Tool and Die, Seneca, SC) and diluted 1:3 (w:v) in coating buffer. Samples (200 μl/well) were added to Immulon 2 microtiter plates (Dynatech, Chantilly, VA) and incubated overnight at 4 C. Antisera and IgG's were used at 1.6 μg/ml, alkaline phosphatase-conjugated goat anti-rabbit IgG was used at 1:600 dilution, and incubations were done for 2 h at 37 C. P-Nitrophenyl phosphate was added at 0.6 mg/ml for color development. Results were assessed spectrophotmetrically at 405 nm with a E Max microplate reader (Molecular Devices, Palo Alto, CA).

Western analysis was initiated by transferring SDS-PAGE gels to Immobilon-P membrane (Millipore, Bedford, MA), and carried out as described by Gingery and Nault (3), except that the membranes were blocked with 0.5% gelatin and the IgGs were used at 1.6 μg/ml. Alkaline phosphatase-conjugated goat anti-rabbit IgG (H&L) was used at 1:2,000 dilution, and nitrobluetetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (BCIP) were added for color development.

RESULTS

Virus purification and transmission. Virions were obtained from all purification attempts; however, the amount of contaminating host material present after Celite filtration depended on the source of the Celite. Filtration through Celite 503 yielded a nearly colorless filtrate, while the filtrate obtained using Celite 545 was green. This green color and accompanying host materials could not be removed in later steps. Two UV-absorbing peaks were observed following sucrose density gradient centrifugation of extracts from infected plants (Fig. 1). Electron microscopy of each of the fractions collected from these two peaks visualized virions that were similar in size (71 × 218 nm, data not shown) to those observed from leaf dip preparations (69 × 200 nm, Fig. 2). When *G. sonora* was allowed to acquire purified virus from peak 1 by membrane feeding, it transmitted the virus to healthy maize (six out of six plants). Symptoms in these plants were identical
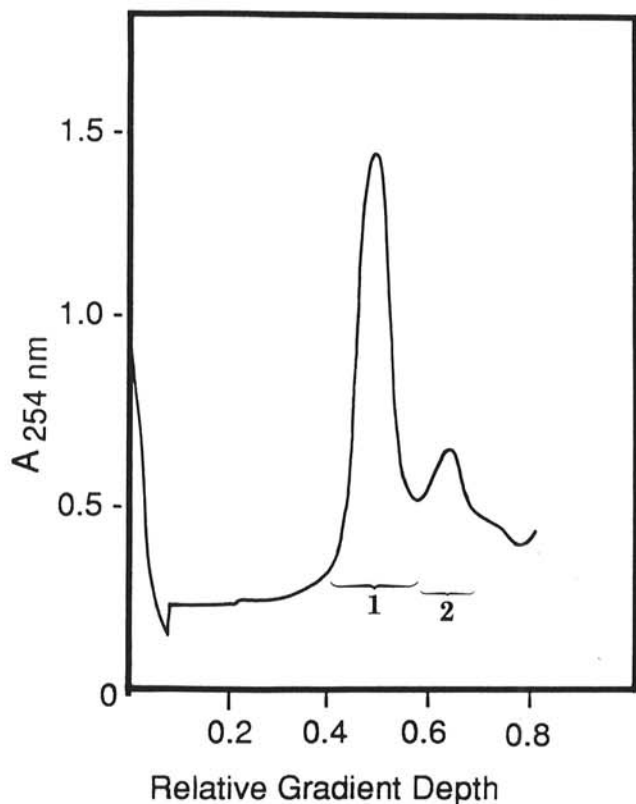

Fig. 1. Absorbance profile of sucrose density gradients of sorghum stunt mosaic virus after centrifugation for 15 min at 26 K in an SW 28 rotor at 4 C.
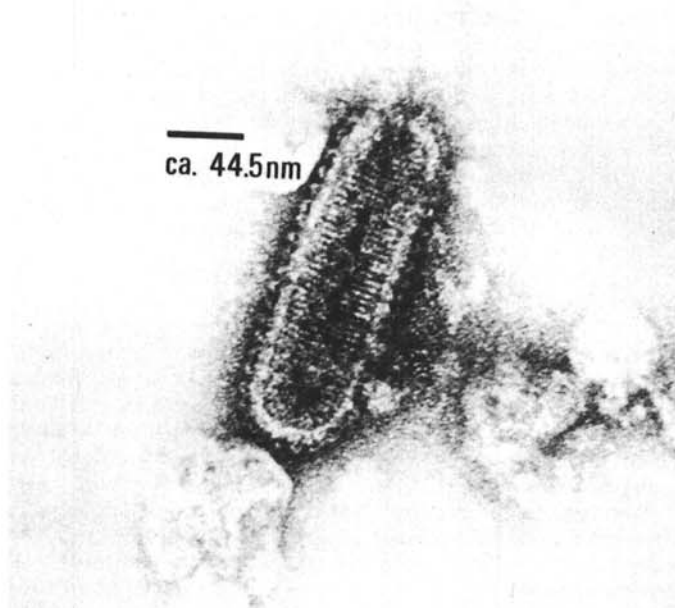

Fig. 2. Electron micrograph of a uranyl acetate stained leaf dip of sorghum stunt mosaic virus from infected corn.

to those of the plants used as a source for purification. Furthermore, the RNA and proteins isolated from virus purified from these plants were identical to those of the virus used for the transmission experiments. If purified SSMV was frozen and thawed prior to membrane feeding by *G. sonora*, no transmission of the virus was observed.

Analysis of SSMV RNA and virion proteins. RNA extracted from purified SSMV virions (peak 1) migrated similarly to that of beet yellows virus and migrated more slowly than that of tobacco mosaic virus when denatured with glyoxal and run on agarose gels (Fig. 3). The size of the RNA was estimated to be

14.5 kb. RNA extracted from each gradient peak migrated identically (data not shown).

Three prominent (91, 59, and 36 kDa) and two minor (197 and 30 kDa) protein bands were observed on 12% SDS-PAGE gels (Fig. 4). Protein banding patterns obtained from each gradient peaks were identical. The 91-kDa protein band reacted with Schiff's reagent (Fig. 5), suggesting that the protein was glycosylated. After detergent solubilization and centrifugation, the pellet contained only the 59-kDa protein, suggesting that this was contained in the nucleocapsid (Fig. 6).

Serological analysis. Neither purified SSMV nor total protein extracts from SSMV-infected corn reacted with antisera or IgG to SYNV, RTYV, AWSMV, NCMV, CA-SYDV, NY-SYDV,
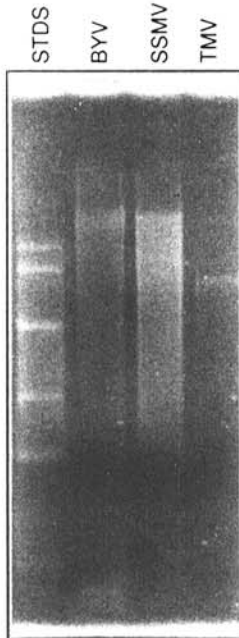

Fig. 3. An 0.8% agarose gel showing virion RNAs after denaturation with glyoxal and DMSO, electrophoresis, and staining with ethidium bromide. Lane 1, RNA molecular weight standards in decreasing size 9.49, 7.46, 4.4, 2.37, and 1.35 kb; lane 2, beet yellows virus; Lane 3, sorghum stunt mosaic virus; lane 4, tobacco mosaic virus, U1 strain.

Fig. 5. Electrophoresis of denatured sorghum stunt mosaic virus (SSMV) proteins through a 12% acrylamide gel. Lane 1, SSMV stained with Coomassie Brilliant Blue, Lane 2, SSMV stained with Schiff's reagent.
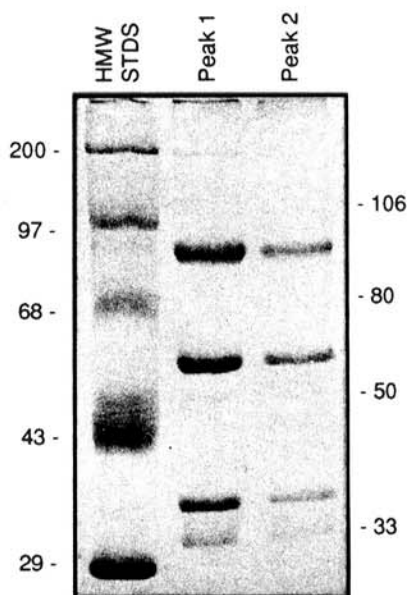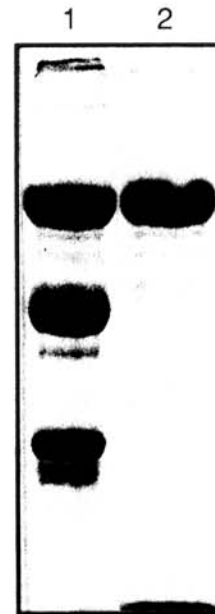

Fig. 4. Analysis of sorghum stunt mosaic virus proteins from sucrose gradients. Denatured samples were electrophoresed through a 12% acrylamide gel and stained with Coomassie Brilliant Blue. Molecular weight size standards are indicated in kDa.
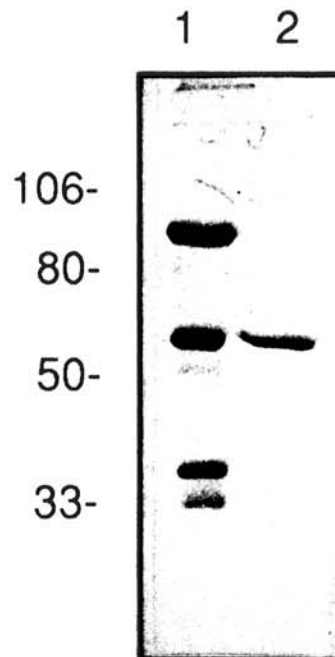

Fig. 6. Electrophoresis of sorghum stunt mosaic virus (SSMV) proteins following solubilization with 2% Triton X-100, 0.4 M NaCl and pelleted by centrifugation. Lane 1, SSMV, before solubilization, lane 2, SSMV, pellet fraction after solubilization and centrifugation.

CYDV, MMV-Ohio, or MMV-Florida in ELISA tests (data not shown). Because of reactions of some antisera with clarified extracts from healthy plants, only a subset of these IgGs were used for Western blotting. Using this technique, IgG to SYNV, CA-SYDV, CYDV, and NJ-SYDV did not react with purified SSMV nor total protein extracts from SSMV-infected corn. In a reciprocal Western blotting test, IgG to SSMV did not react with SYNV, RTYV, CA-SYDV, NY-SYDV, or CYDV infected tissue (data not shown). Using ELISA and Western blotting, IgG to SSMV reacted positively with SSMV-infected tissue and purified SSMV.

DISCUSSION

This study indicates that sorghum stunt mosaic is caused by the plant rhabdovirus, SSMV. The molecular weight of the RNA (4.2×10^6 Da) and capsid proteins (91, 59, 36, 30 kDa) differentiate it from any other characterized plant rhabdovirus. WSMV is composed of 145-, 92-, 59-, and 25-kDa proteins (17); MMV is composed of 75-, 54-, and 30-kDa proteins (1). The published molecular weights of WSMV and MMV RNAs are 2.2×10^6 (18) and 4.2×10^6 (1), respectively. The molecular weights of the proteins and RNA of OSMV are not known. However, the particle dimensions of OSMV (100×400 nm) (8) differ substantially from that of SSMV (71×218 nm). Additionally, the vector of OSMV (*Graminella nigrifrons* Forbes) (8) did not transmit SSMV (W. Styer, *personal communication*). The lack of serological reaction with any of the rhabdoviruses tested further confirms that SSMV is distinct.

Other purified rhabdoviruses are separated into multiple bands by sucrose gradient centrifugation (5). Some of these additional peaks have been attributed to the presence of defective interfering particles and others to virion aggregates. Because both fractions of SSMV contained virions with similar nucleic acid and protein profiles and similar-sized virions, the denser fraction may have contained virion multimers.

While other workers have injected purified rhabdoviruses into leafhoppers and planthoppers to show infectivity (12), this is the one of very few reports (13) of successful membrane feeding and subsequent transmission of purified rhabdovirus. This method has advantages of being able to use dilute virus preparations and of causing low insect mortality. In the future, use of this technique with antisera we have produced against the SSMV G protein could help us to determine the viral protein(s) responsible for virus transmission.

Rhabdovirus structural proteins, G, N, NS, M1, M2, and L, were named on the basis of the virion proteins of animal-infecting rhabdoviruses (7). The SSMV proteins appear to be analogous to several of these well-defined proteins. The presence of a glycoprotein moiety on the 91-kDa protein of SSMV suggests that this is the G protein. Recovery of the 59-kDa protein by centrifugation following treatment of puried virions with Triton X-100 provides evidence that it may be the N protein. The 36- and 30-kDa proteins are hypothesized to be M proteins, because they were removed by the detergent treatment.

In addition to the four prominent bands identified by PAGE, two other minor proteins with molecular masses of 50 kDa and 197 kDa were sometimes detected in virus preparations. The 197-kDa protein can be hypothesized to be analogous to the L protein (polymerase) due to its large size and low abundance. High molecular weight proteins found during rhabdovirus purification by other researchers (7) are thought to be L proteins or contaminating host proteins. The protein banding pattern, presence of probable G, N, and two M proteins, combined with evidence that SSMV replicates in the nucleus (11), suggests that SSMV should be placed in the plant rhabdovirus subgroup including SYNV and potato yellow dwarf virus.

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