Rice Grassy Stunt Virus: A Planthopper-Borne Circular Filament

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ABSTRACT

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Two viruslike particles, threadlike filaments 6–8 nm in diameter and small isometric particles 18–20 nm in diameter, were observed in extracts of rice plants affected by rice grassy stunt disease. The filaments were abundant and the isometric particles were scarce. The filaments were purified and antiserum to the filaments was obtained. The antiserum specifically reacted with extracts of infected plants and the vector *Nilaparvata lugens* that had fed on rice grassy stunt-affected plants. The purified filaments were often circular and the circular filaments were 950–1,350 nm in length. The filament was nucleoprotein containing RNA and a single protein species with a molecular mass of 31,000 daltons. Purified filaments were not infective. Serologically, the filament was distantly related to rice stripe virus but not to maize stripe and rice hoja blanca viruses. Purified rice stripe virus has a circular filament 11–13 nm in diameter, with a helical configuration and with varied length. The loosened helix appeared to be constructed of a filament 6–8 nm in diameter. The serological relationship and morphological similarity between the filaments and rice stripe virus indicate that the filament is the causal virus of grassy stunt. The name rice grassy stunt virus (RGSV) is proposed for the filament.

Additonal key words: brown planthopper.

Rice grassy stunt (RGS) (23) occurs widely in rice-growing areas in South, Southeast (18), and East Asia (2,15). From time to time it has severely damaged rice (*Oryza sativa* L.) production in the last 15 yr (21). The RGS causal agent is transmitted by the brown planthopper *Nilaparvata lugens* Stål in a persistent manner (23). RGS has been suspected of being caused by a virus, but the causal organism remains to be identified. Recent studies clearly indicated that the RGS infectious agent had characteristics of a virus (9,24). The occurrence of 70-nm viruslike particles (13) or mycoplasmalike bodies (14) in planthoppers exposed to RGS-affected rice tissues was reported but has not been confirmed. Viruslike particles, 20–25 nm in diameter, were reported in RGS-affected rice and RGS-exposed planthopper tissues (22,24).

In this report, we describe the association of filamentous virus particles with RGS-affected plants and the vector insects fed on RGS-affected plants. Preliminary reports have been published (11,12).

MATERIALS AND METHODS

Pathogens, plants, and insects. A rice plant infected with the RGS agent was collected on Okinawa, Japan, and the pathogen was maintained on rice seedlings by serial transmission with *N. lugens*. A rice stripe virus (RSV)-infected plant was collected at Tochigi, Japan, and similarly maintained by serial transmission with a planthopper *Laodelphax striatellus* Fallén. Virus-free *N. lugens* was reared on rice seedlings in a plastic cage. First- or second-instar nymphs of *N. lugens* were fed on RGS-affected rice plants for about 10 days and then allowed an inoculation access period of 2 days on 3-wk-old rice seedlings of cultivar Taichung Native 1 at two to five insects per seedling. The inoculated seedlings were planted in pots, grown in a greenhouse for 1 mo, and used for the test. Maize (*Zea mays* L.) seedlings similarly infected with RSV by means of *L. striatellus* were also grown in the greenhouse.

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Electron microscopy. Leaf extracts from RGS-affected plants were clarified by low-speed centrifugation and emulsification with CCl_4. Pellets obtained by high-speed centrifugation of the clarified extract were diluted with distilled water, mounted on grids coated with a collodion film, and stained with 1% uranyl acetate (UA). Purified fractions were similarly mounted and stained with UA or neutralized 1% phosphotungstic acid (PTA). The grids were examined in an H-500 electron microscope (Hitachi, Ltd., Japan). Filamentous particles were measured on prints at ×120,000 magnification with a graphic calculator.

Purification. About 300 g of RGS-affected rice plants with roots were ground in a mincer with 1.2 L of ice-cold 0.1 M borate buffer (pH 8.0) containing 0.01 M Na_2SO_3. The extract was squeezed through cheesecloth and centrifuged for 20 min at 13,000 *g*. The supernatant was mixed with 1.8–2.4 g of Mg-bentonite (6) and centrifuged again. The supernatant was mixed with one-tenth volume of CCl_4 and emulsified for 1 min with a blender. The emulsion was centrifuged for 20 min at 13,000 *g*. The aqueous solution was made 5% polyethylene glycol (PEG 6000) and 0.2 M NaCl, and then stirred for 30 min at 6 C. The mixture was centrifuged for 20 min at 13,000 *g* and the pellet obtained was resuspended in 60 ml of cold 0.1 M borate buffer (pH 8.0). The suspension was made up to 0.3% Triton X-100 and then treated once more with PEG and NaCl. The pellet obtained was resuspended in 2 ml of 0.01 M borate buffer (pH 8.0) and centrifuged for 15 min at 4,000 *g* to remove particulates. This partially purified preparation was layered on a 5–30% sucrose density gradient in 0.01 M borate buffer and centrifuged for 3 hr in the SW 27 rotor (Hitachi, Ltd., Japan) at 26,000 rpm. Centrifuged gradients were fractionated by a model 640 density gradient fractionator (ISCO, Lincoln, NE). Peak zones containing filamentous particles were pooled and the filaments were sedimented by centrifuging for 90 min at 130,000 *g*. The pellet was considered purified, suspended in 1 ml of 0.01 M borate buffer (pH 8.0), and used for further tests. RSV was purified from infected maize leaves by following the procedure of Koganezawa et al (17). For comparison, filamentous particles were also purified from RGS-affected rice plants following the same procedures. The extinction coefficient, $E_{260\ nm}$ (1-cm light path) of 2.3 mg/ml, estimated for a similar filamentous virus, maize stripe virus

(MStpV), was used to estimate the concentration of the purified filaments (7).

Antiserum. Five 1-ml aliquots of the purified filaments, of which $A_{260\ nm}$ (1-cm light path) was adjusted to 1.0, were injected into each rabbit. One week after the first intravenous injection, the aliquots were emulsified with an equal volume of Freund's complete adjuvant and given intramuscularly in 2- to 3-wk intervals, and the last injection was made intravenously. Antiserum was recovered 1 wk after the last injection. Antiserum to RSV (K. Shohara, *unpublished*) had the titer of the 1/2,048 by the ring interface precipitin test. Antisera to MStpV and rice hoja blanca virus (RHBV) were kindly supplied by R. E. Gingery, USDA-ARS, Ohio Agricultural Research and Development Center, and F. J. Morales, Centro Internacional de Agricultura Tropical (CIAT), Colombia, respectively.

Serological test. For the homologous and heterologous titration, the dilution end point of the antisera were determined by using the ring interface precipitin test with purified antigens adjusted to $A_{260\ nm}$ of 0.2. Immunodiffusion tests were conducted in 0.8% agar containing 0.1% sodium dodecyl sulfate (SDS) and 1% NaN_3 using undiluted sera and the purified antigens to $A_{260\ nm}$ of 0.5 or 1.0. Healthy antigen for serological tests was prepared from healthy rice or maize leaves by two cycles of differential centrifugation of a CCl_4-clarified extract. For the latex test, the procedures described by Bercks (1) were followed. The latex suspension (Bacto-Latex 0.81, DIFCO Laboratories, Detroit, MI) was sensitized with 0.05 M tris-HCl buffer (pH 7.2) containing antiserum at 1/1,000 dilution and the sensitized suspension was mixed with plant or insect extracts clarified with low-speed centrifugation. For ELISA, the procedure described by Clark and Adams (3) was followed. ELISA plates were coated with γ-globulin at 1 μg/ml; γ-globulin-alkaline phosphatase conjugate was diluted 500 times. Plant or insect samples were homogenized with 0.02 M phosphate buffer (pH 6.5) containing 0.15 M NaCl, 0.05% Tween-20, 0.02% sodium azide, and 2% polyvinylpyrolidone (10) and tested directly.

Nucleic acid and protein. Purified filamentous particles were dialyzed against 0.01 M borate buffer (pH 8.0) and then tested for RNA by orcinol, and for DNA by diphenylamine and cysteine-concentrated H_2SO_4 (4–6,19). Protein was released from the purified filamentous particles by boiling them for 1 min in 0.01 M borate buffer (pH 8.0) containing 1% SDS and 1% mercaptoethanol. Tobacco mosaic virus coat protein polymerized with glutaraldehyde was treated similarly as a reference. Electrophoresis was carried out by the procedure of Weber and Osborn (26) on 7.5% polyacrylamide gels under 60 mA per gel for 10 hr. The gels were stained with amido black 10B and scanned at 595 nm in a DU-8 spectrophotometer (Beckman, Palo Alto, CA).

Infectivity assay. Samples were injected into the abdomens of second-instar nymphs of *N. lugens* with fine glass capillaries. Injected nymphs were allowed to feed on rice seedlings for 6 days. Then, individual nymphs were given three sequential 2-day inoculation access periods on 5-day-old TN1 seedlings in a test tube. Inoculated seedlings were grown in a greenhouse. Symptoms appeared 1–2 wk after inoculation. The insects that transmitted grassy stunt in one incubation access were referred to as transmitters.

RESULTS

Viruslike particles in leaf extract. Numerous filamentous particles 6–8 nm in diameter and a few isometric particles 18–20 nm in diameter were observed in the clarified extracts (Fig. 1A and B). Isometric particles were often clustered (Fig. 1A) or aligned in tubules 22–30 nm in diameter (Fig. 1B). In some preparations, however, isometric particles were not observed and only tubules without the particles were observed in extracts. The filamentous and isometric particles were not observed in the preparations similarly obtained from healthy rice plants.

Purification of filamentous particles. Ultraviolet scanning patterns of centrifuged sucrose gradients layered with a partially purified preparation from RGS-affected rice plants and a similar preparation from virus-free rice plants showed that infected plants had a broad peak with one or two shoulders (Fig. 2). The peak fraction contained filamentous particles and few cellular components (Fig. 3). No isometric particles were observed in this fraction. The pattern obtained from virus-free plants did not have these peaks and no filamentous particles were found in fractions corresponding to the peak fraction from infected plants.

Yields of the filamentous particles from 300 g of fresh tissue ranged 0.5–1.5 mg. The yield was lower when filamentous particles were purified from rice plants infected longer than 2 mo.

Electron microscopy. Purified filamentous particles were long threads up to 2 μm long and 6–8 nm in diameter in UA (Fig. 4). They were often circular and of various lengths from 200 to 2,400 nm (Figs. 5 and 6). The length distribution of 332 circular filaments is shown in Fig. 3. Sixty-six percent of the filaments were in the 950–1,350 nm range. Filamentous particles were often shorter and wider and had branches. The filaments seemed unstable in the UA stain, and some branched filaments degenerated further and appeared to be clumping. One grid mounted with purified particles showed branched filaments at some sites, threadlike structures at other sites, and mixtures of both forms at still other sites. Threadlike filaments were observed less frequently in the freeze-stored fractions. In PTA, the purified filamentous particles appeared to be narrower, about 4 nm in diameter. The filaments also appeared to be threadlike structures and branched forms in PTA.

Properties of filamentous particles. The ultraviolet absorption spectrum of purified filamentous particles had a maximum absorbance at 259–260 nm and minimum of 246–247 nm. The $A_{260\ nm}/A_{280\ nm}$ ratio was 1.28 ± 0.03. Purified filamentous particles gave

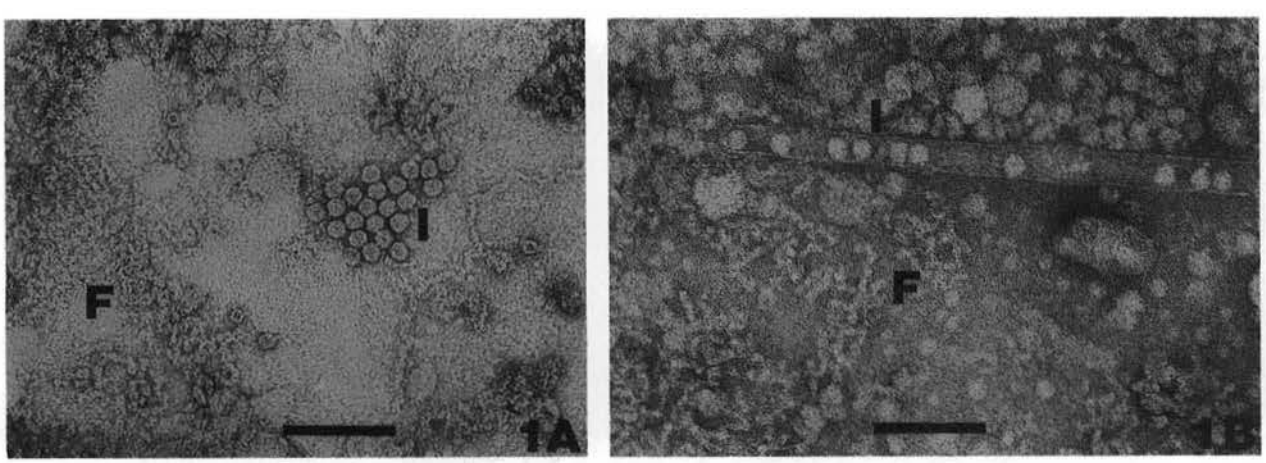


Fig. 1. Electron micrographs of viruslike isometric (I) and filamentous particles (F) in clarified extracts from grassy stunt-affected rice plants. Stained with 1% uranyl acetate. Scale bar represents 100 nm. **A,** A clump of isometric particles; **B,** isometric particles in a tubule.

a positive orcinol reaction, and negative reactions with diphenylamine or cysteine-concentrated H_2SO_4. Protein released from purified filamentous particles migrated on SDS-polyacrylamide gel as a single species of molecular mass of 31,000 daltons. These results indicated that the filamentous particles were ribonucleoprotein.

Pathogenicity of nucleoprotein. Fractions obtained at each step of purification were tested for infectivity. Infectivity was recovered from the fractions before sucrose density-gradient centrifugation. When the partially purified preparations were injected, 4–33% of

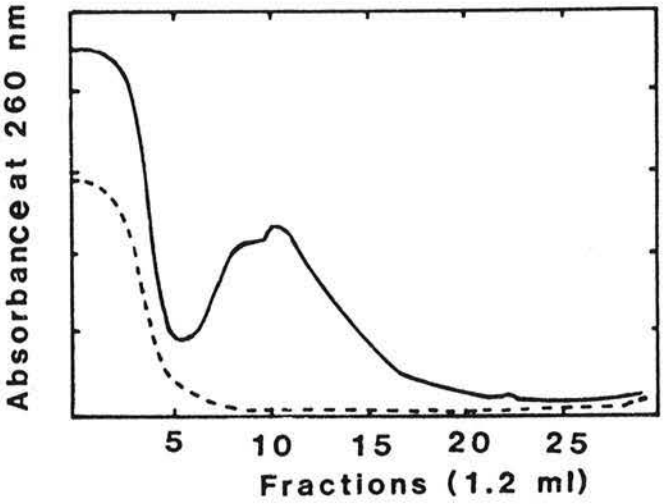


Fig. 2. Absorbance $A_{254\ nm}$ profile of partially purified filamentous particles from rice grassy stunt-affected rice plants centrifuged on 5–30% sucrose density gradients. Dotted line indicates absorbance profile of a similarly prepared sample from healthy rice plants.

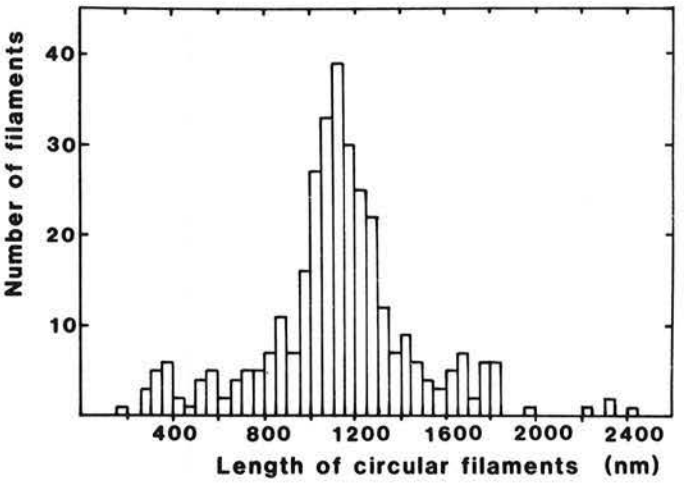
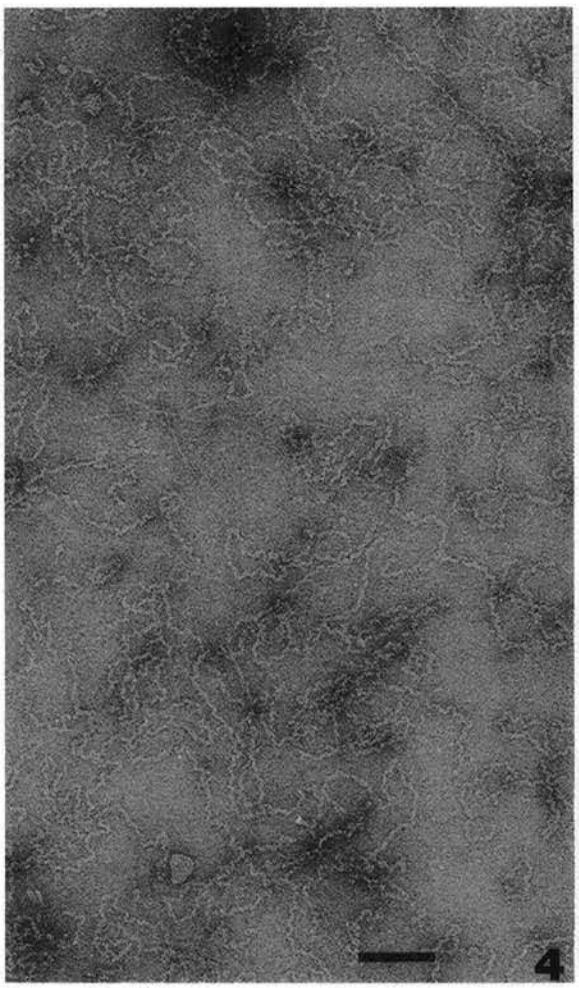
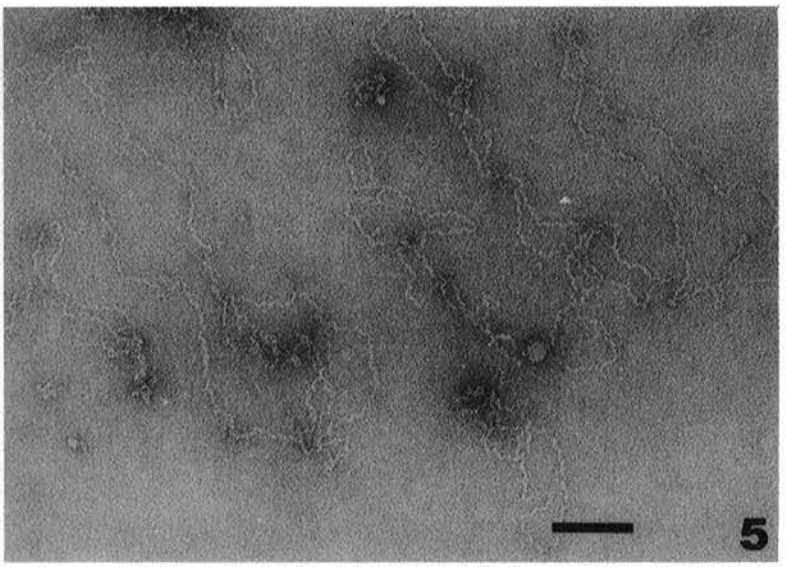


Fig. 3. Length distribution of circular filamentous particles observed in a nucleoprotein fraction purified from rice grassy stunt-affected rice plants. Stained with 1% uranyl acetate.





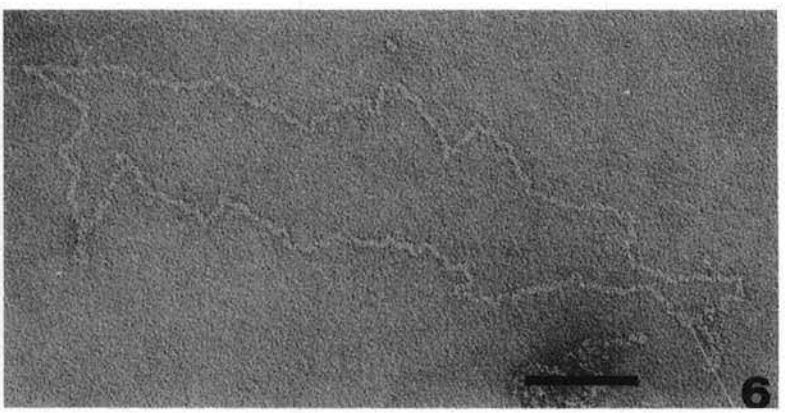


Fig. 4–6. 4, Purified preparation of filamentous particles from rice grassy stunt-affected rice plants. Stained in 1% uranyl acetate. Bar represents 100 nm. **5,** Circular filamentous particles of various lengths from rice grassy stunt-affected rice plants. Stained with 1% uranyl acetate. Bar represents 100 nm. **6,** A circular filament longer than 2 μm from rice grassy stunt-affected plants. Stained in 1% uranyl acetate. Bar represents 100 nm.

the planthoppers became infective. Infectivity of the purified filamentous particles in suspensions with $A_{260\ nm}$ values adjusted to 1.0 were tested four times with about 500 total *N. lugens* and none of the planthoppers became infective. Some rice plants that had been inoculated by using injected planthoppers were tested for the presence of the nucleoprotein antigen by the latex test and none reacted positively.

Serology. The antiserum recovered from the rabbits had a titer of 1/1,280 against purified filamentous nucleoprotein by the ring interface precipitin test. The titer against the healthy antigen was less than 1/10.

Nucleoprotein was detected in extracts of RGS-affected leaves and in planthoppers that had fed on RGS-affected plants diluted up to 1/4,096 and 1/1,024, respectively, by the latex test with the antiserum. Extracts of virus-free rice leaves and planthoppers were negative down to 1/4 dilutions. In ELISA, the nucleoprotein was detected from both leaf and planthopper extracts diluted up to 1/5,120, while extracts of virus-free leaves and planthoppers were negative down to 1/5 dilutions. These results suggested that the filamentous nucleoprotein was consistently associated with RGS.

In the ring test, RSV antiserum reacted with purified nucleoprotein but not with the healthy antigens (Table 1). The antiserum to RGS-associated nucleoprotein reacted weakly with purified RSV and also with healthy antigens. MStpV antiserum reacted with RSV but not with the RGS-associated nucleoprotein. The RHBV antiserum did not react with the nucleoprotein or RSV.

In SDS-immunodiffusion tests a single band was formed between RSV antiserum and RSV, and between the antiserum and the nucleoprotein. A spur formed in the reaction band between the RSV antiserum and RSV (Fig. 7A). A band formed between the antiserum to the nuceloprotein and the nucleoprotein, but not between the antiserum and RSV (Fig. 7B). An additional faint band was often formed adjacent to wells with antigens in homologous reactions. Faint single bands were also formed between the antiserum to the nucleoprotein and the healthy antigens but not between the RSV antiserum and the antigens. A band formed between MStpV antiserum and RSV but not the nucleoprotein. No band was formed between the RHBV antiserum and RSV or the nucleoprotein.

Morphology of RSV. Purified RSV in UA was a threadlike filament, 11–13 nm in diameter (Fig. 8A), with a helical configuration. The filaments were often circular, of various length from 100 to 1,250 nm (Fig. 8B). The length distribution of circular filaments showed a peak in 250–650 nm range (Fig. 9). The loosened helix appeared to be constructed of a filament, 6–8 nm in diameter. Filaments were often shortened and branched in UA as did filaments from RGS-infected plants (Fig. 8A). These degenerated filaments generally lacked the helical configuration. Particles purified from RGS-affected rice by following the procedures for RSV were also filamentous, 6–8 nm in diameter, and did not show a helical configuration.

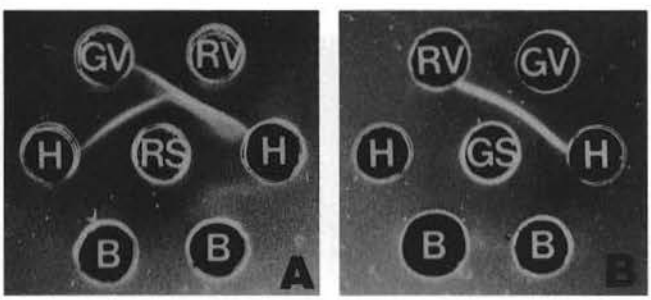


Fig. 7. Serological cross-reactivity between antiserum to rice grassy stunt-associated nucleoprotein (GS) to GV, and rice stripe virus (RV) from infected maize plants. **A,** Reactions between rice stripe virus antiserum (RS) to RV and GV, and an extract of virus-free rice plants (H) and buffer (B). **B,** Reactions between rice grassy stunt antiserum (GS) to GV, RV, H, and B.

TABLE 1. Serological reactions of rice grassy stunt-associated nucleoprotein (RGSN), rice stripe virus (RSV), and healthy antigens with antisera to RGSN, RSV, maize stripe virus (MStpV), rice hoja blanca virus (RHBV), and normal serum in the precipitin ring test

	Reaction[b] to antiserum				
Antigen[a]	RGSN	RSV	MStpV	RHBV	Normal
RGSN	1,280	80	0	0	0
RSV	20	1,280	160	0	0
Healthy rice	<10	0	0	0	0
Healthy maize	<10	0	0	0	0

[a] RGSN was purified from rice plants infected with grassy stunt. RSV was purified from RSV-infected maize plants. Healthy antigens were obtained from extracts of healthy rice or maize plants.

[b] Reciprocal of dilution end point of the antiserum. Zero indicates no reaction.

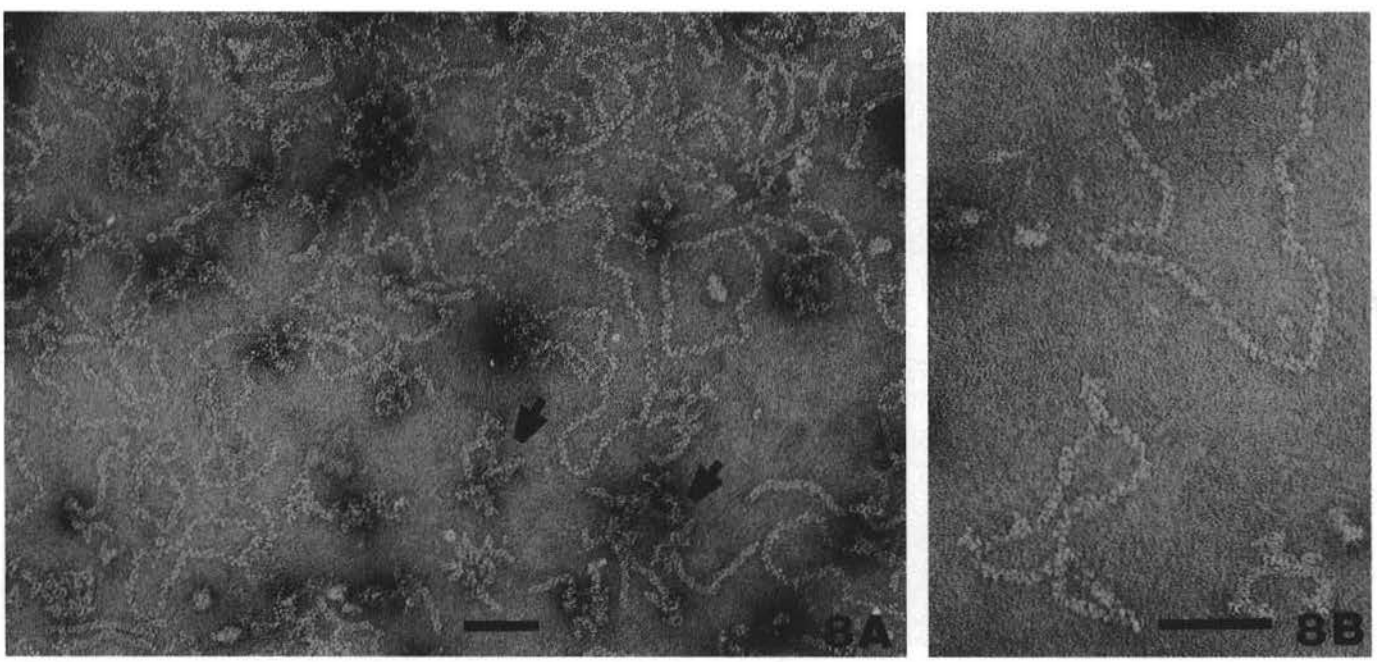


Fig. 8. Electron micrographs of a purified fraction of rice stripe virus in 1% uranyl acetate stain. Scale bar represents 100 nm. **A,** Filamentous particles with a helical configuration and degenerated filaments with branch (arrows). **B,** Two circular filaments.

DISCUSSION

Two types of particles, threadlike filamentous and small isometric, were observed in extracts of RGS-affected rice plants. The filamentous particles were abundant and the isometric particles were scarce. Association of similar isometric particles with RGS-affected plants and brown planthoppers fed on RGS-affected plants has been reported (22,24) but the filamentous particles had not been described. The filamentous particles may have been overlooked; they are difficult to see in the electron microscope because they stain with low contrast and are easily disintegrated in the stain.

Shikata et al (24) reported that infectivity of a fraction containing numerous isometric particles was high and that those particles were most likely the causal virus of RGS. Their conclusion conflicts with our data which indicate close association of filamentous viruslike particles with RGS. This discrepancy may indicate a possible association of two kinds of viruses with RGS. Further studies are needed to clarify the possible relationships between the filamentous and isometric particles.

Threadlike filamentous virus particles have been reported in plants infected with rice stripe (16,17,25), maize stripe (7) and rice hoja blanca (20) diseases. RSV and MStpV are similar in their relationships to vector planthoppers, and are serologically related (7,8,25) as confirmed in these experiments. RHBV is also similar to these two viruses in its relationships to a vector planthopper and in morphology, and may be another member of the newly recognized plant virus class (20). RSV and MStpV are RNA viruses (7,16) and RHBV contains RNA (F. J. Morales, *personal communication*). The molecular masses of the coat proteins of the three viruses ranged from 32,000 to 34,000 daltons (7,8,16,20,25). The morphology of the RGS-associated nucleoprotein is threadlike filaments, similar to MStpV (7), RHBV (20), and the unfolded form of RSV (5). RSV isolated in these experiments was a filament in a helical configuration and the unfolded helix appeared constructed of a filament similar to the RGS-associated nucleoprotein. The RGS nucleoprotein contained RNA and a single protein species with a molecular mass of 31,000 daltons. The similarities between the RGS nucleoprotein, and RSV, MStpV and RHBV in morphology and vector relationship suggest that the RGS nucleoprotein may be a virus, another member of the same class. Unlike the three viruses, however, the RGS agent is not transmitted through planthopper eggs (18). Further characterization of the nucleoprotein is needed to confirm its grouping with the three viruses.

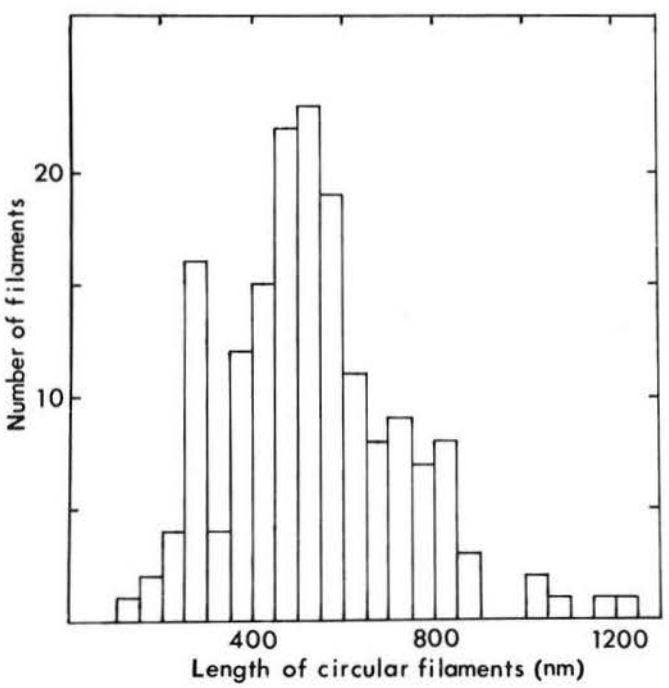


Fig. 9. Length distribution of circular filamentous particles observed in purified fraction of rice stripe virus in 1% uranyl acetate stain.

As yield of the RGS nucleoprotein was low, isopicnic gradient centrifugation was not applied to further purify the nucleoprotein after sucrose density gradient centrifugation. The purified RGS-associated nucleoprotein preparation contained few cellular components but no isometric particles. The antiserum to RGS-associated nucleoprotein was satisfactory for detecting the nucleoprotein by the latex test and in ELISA. The antiserum was highly specific to RGS and did not react with extracts of plants infected with other rice viruses such as rice tungro spherical and bacilliform viruses and rice ragged stunt virus (*unpublished*). The antiserum can be used for RGS diagnosis and epidemiology.

In the serological tests, RSV antiserum reacted with the RGS-associated nucleoprotein but, in the reciprocal test with the antiserum to the nucleoprotein, cross reactivity was not confirmed. This result suggested that the nucleoprotein and RSV were only distantly related and that the titer of the antiserum to the nucleoprotein might not be high enough to give cross reactivity. In immunodiffusion and microprecipitin tests conducted by R. E. Gingery (*personal communication*), the antiserum to the nucleoprotein failed to react with MStpV. In the immunodiffusion test conducted by F. J. Morales (*personal communication*), the antiserum to the nucleoprotein failed to react with RHBV.

The RGS nucleoprotein was circular filament, and RSV was also circular filament with a helical configuration. A circular form of particles has not been clearly demonstrated for RSV as well as for MStpV and RHBV (7,8,16,17,20,25). The circular form of RSV might be cut easily during preparation.

The purified RGS nucleoprotein was not infective. Toriyama (25) reported that the RSV component contained infectious rigid rods that appeared to be in a coiled configuration. In these experiments, RSV had a helical configuration but the RGS-associated nucleoprotein did not. A coiled rigid form may also be essential for the infectivity of the RGS-associated nucleoprotein. Infectivity of the purified RSV was not tested in these experiments.

It is also known that the infectivity of MStpV and RHBV was lost during the purification process and purified MStpV and RHBV particles were not in a coiled rigid form (7,20). The intact form of the RGS-associated nucleoprotein and the three filamentous viruses may be unstable and easily disintegrated during the purification and/or the staining process as indicated by Toriyama (25). It may also be possible that the small isometric particle is needed for infectivity of the RGS-associated nucleoprotein. The report of Shikata et al (24) may support this idea.

We propose the name rice grassy stunt virus (RGSV) for the filamentous nucleoprotein.

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