Etiology

# Purification and Some Properties of Rice Gall Dwarf Virus, a New Phytoreovirus

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## ABSTRACT

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Double-shelled polyhedral particles about 65 nm in diameter were purified from rice plants infected with rice gall dwarf disease by carbon tetrachloride clarification, polyethylene glycol precipitation, differential centrifugation, and sucrose density-gradient centrifugation. The particles were not disrupted nor were their inner cores exposed when they were treated with chloroform, heat, freezing and thawing, or phosphotungstic acid staining. The purified particles were highly infectious to rice seedlings inoculated via injected insect nymphs. In gel diffusion tests, antiserum against the particle had a titer of 1:2,048 and 1:2 against the homologous antigen and double-stranded ribonucleic acid, respectively. No specific reaction was seen between the new virus and antisera against the rice dwarf and wound tumor viruses that belong to the Phytoreovirus group. These results suggest that the virus is a new member of Phytoreovirus in the plant reovirus group; thus, it was named rice gall dwarf virus.

Rice gall dwarf, recently found in Thailand, is a new virus disease of rice plants. Its characteristic symptoms are dark-green discoloration of the leaves, severe stunting of the plants, and galls that form along the leaf blades and leaf sheaths. Polyhedral particles about 65 nm in diameter were observed in diseased plants (13). The virus is transmitted in a persistent manner by five species of leafhoppers, *Nephotettix nigropictus*, *N. cincticeps*, *N. malayanus*, *N. virescens* (7), and *Recilia dorsalis* (12). Furthermore, the virus is transmitted transovarially to a high proportion of the progenies of *N. nigropictus* (7). The virus has been assumed to belong to the plant reovirus group based on particle morphology.

This paper describes the purification and some properties of the virus particles.

## MATERIALS AND METHODS

**Insects, plants, and viruses.** A colony of *N. nigropictus*, collected in Kagoshima Prefecture, Japan, was maintained on rice plants (cultivar Koshihikari) in an insect chamber at $26 \pm 2$ C with 12 hr fluorescent lighting per day. Rice plants (cultivar Taichung Native 1) at the three- to five-leaf stage were used for transmission tests and virus propagation. Rice gall dwarf (RGD) disease pathogen collected in Thailand (13) was maintained on the developmental stage of rice plants by successive insect vector transfers. Second- to third-instar nymphs were fed on diseased rice plants for 2 days and transferred to healthy rice plants for not less than 12 days with several changes of plants. For transmission tests and detection of virus particles in insect vectors, viruliferous insects were individually transferred to test seedlings for 3 days and then subsequently used for electron microscopic examination. The insects that transmitted the pathogen in the assay tests were counted as active transmitters. For virus propagation, rice seedlings were inoculated by allowing the viruliferous insects to feed for longer than 1 day. The inoculated plants were grown in an air-conditioned greenhouse ($27 \pm 3$ C).

**Purification.** Principally the method of purification for rice dwarf virus (RDV) (8) was employed for purifying rice gall dwarf virus (RGDV) because its particles were quite similar in morphology and its propagation host plant was the same as that of RDV. All the operations were done either in an ice bath or at 4 C. Fresh plants about 40 days after inoculation were ground with a meat chopper in two volumes (w/v) of 0.1 M potassium phosphate buffer (PB), pH 7.2. The homogenate was passed through a layer of finely woven cotton cloth, carbon tetrachloride ($CCl_4$) was added to a final concentration of 20%, and the homogenate was blended in a Waring Blendor for 2 min. After centrifugation for 15 min at 3,000 $g$, polyethylene glycol 6,000 (PEG) and NaCl were added to the supernatant fluid to final concentration of 6% (w/v) and 0.3 M, respectively. This mixture was stirred for 40 min and then centrifuged for 15 min at 6,000 $g$. The resulting pellets were homogenized in a Waring Blendor for 1 min with PB, pH 7.0 to which $CCl_4$ was added to a final concentration of 10%. After centrifugation for 15 min at 3,000 $g$, the supernatants were centrifuged for 30 min at 96,000 $g$ in a Hitachi RP-40 rotor. The pellets were resuspended in 0.01 M PB (pH 7.0) containing 0.01 M $MgCl_2$ (PB-Mg) (the $MgCl_2$ was added just before use), incubated for 30 min, and centrifuged for 15 min at 3,000 $g$. The supernatant fluid was layered on 10–40% (w/v) linear sucrose gradients in PB-Mg and centrifuged for 50 min at 60,000 $g$ in a Hitachi RPS-25 rotor. The zone containing virus particles was recovered with an ISCO model UA2 Scanner, layered on 40–60% (w/v) linear sucrose gradients in PB-Mg, and centrifuged for 15 hr at 36,000 $g$ in a Hitachi RPS-25 rotor. Gradients were fractionated by the scanner and appropriate fractions were concentrated by centrifugation for 90 min at 96,000 $g$ in a Hitachi RP-40 rotor. The final pellets were resuspended in PB-Mg. Each step in the purification procedure was monitored by electron microscopy.

**Artificial injection.** Purified preparations of RGDV ($A_{260nm}$ = 1.0–1.5) were injected into the abdomens of third- to fifth-instar nymphs of *N. nigropictus* by using fine glass capillaries. The amount of inoculum per insect was about 0.01 $\mu l$ and the injection was performed at room temperature. These insects were placed for 12 days on healthy rice seedlings with occasional transfers to new seedlings. They were individually transferred to test seedlings in test tubes ($2 \times 10$ cm) and were fed for 3 days. The injected insects and inoculated plants were kept in a controlled-environment greenhouse ($27 \pm 3$ C).

**Electron microscopy.** Insects were crushed in a drop (~0.01 ml) of neutralized 2% phosphotungstic acid (PTA), put on carbon-coated collodion grids, and excess liquid was removed. Purified preparations were stained in either PTA, 2% aqueous uranyl acetate (UA), or 2% ammonium molybdate (AM), pH 6.5, and then were examined with a Hitachi H-500 electron microscope.

**Physical and chemical treatments.** For heat treatment, the purified preparations in small test tubes were maintained in a water bath at 50 C for 10 min and then cooled to room temperature.

Purified preparations were frozen overnight at $-80$ C and later thawed at room temperature. The freeze-thaw procedure was repeated twice. Chloroform (20% v/v) was added to purified preparations, the mixture was vigorously shaken for 10 min at room temperature, and centrifuged for 15 min at 3,000 $g$. After these treatments, specimens were negatively stained with 2% PTA and were examined by electron microscopy.

**Serology.** A rabbit was immunized against purified RGDV by an initial intramuscular injection with the antigen emulsified with an equal volume of Freund's complete adjuvant followed by similar intravenous injections. Serum was stored at $-20$ C or at 4 C with 0.05% sodium azide. Dilutions of serum were made in 0.01 M PB, pH 7.0 containing 0.85% NaCl (PBS).

Serological tests were performed either by double gel diffusion or by immunoelectron microscopy. The gel medium consisted of 0.8% agar in PB containing 0.85% NaCl, 0.001 M ethylene-diaminetetraacetic acid (EDTA), and 0.05% sodium azide, pH 7.6. Hexagonal arrangements of wells 4 mm in diameter around a central well of the same size were used. The distance from the edge of the central well to that of any surrounding well was 3 mm. Purified virus preparation ($A_{260nm}$ = 2) was used as antigen. The plates were incubated for 2 days at room temperature.

TABLE 1. Transmission of particles by colony of green rice leafhopper *Nephotettix nigropictus* viruliferous for rice gall dwarf virus

| Insect colonies | Sex | Number of insects tested | No. of insect carriers of: | | Active transmitters |
| --- | --- | --- | --- | --- | --- |
| | | | Small particles[a] | Large particles[b] | |
| Viruliferous | female | 19 | 19 | 12 | 9 |
| | male | 21 | 21 | 12 | 11 |
| Acquisition + 2[c] | nymph | 10 | 10 | 0 | 0 |
| | female | 5 | 5 | 0 | 0 |
| | male | 5 | 5 | 0 | 0 |
| Virus-free | female | 10 | 10 | 0 | 0 |
| | male | 10 | 10 | 0 | 0 |

[a] Polyhedral particle about 35 nm in diameter.
[b] Polyhedral particle about 65 nm in diameter.
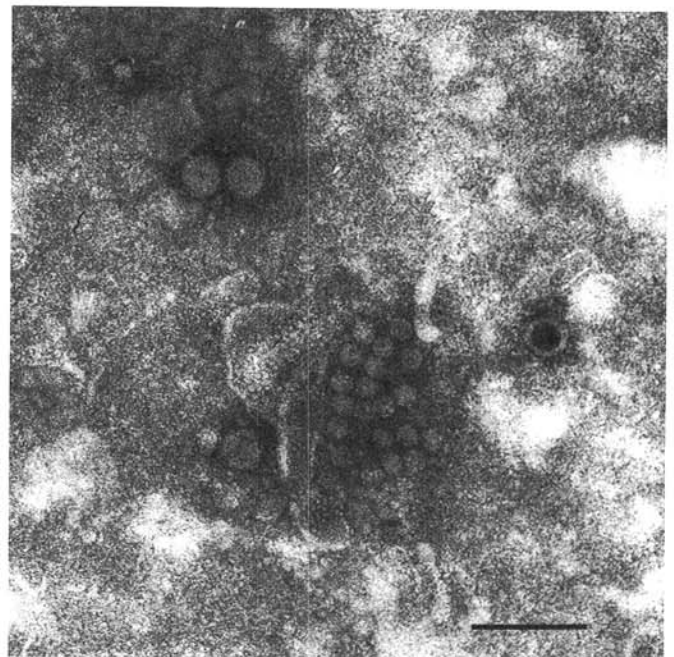[c] Acquisition access plus 2 days.



**Fig. 1.** Electron micrograph showing large and small particles in a crushed viruliferous insect preparation stained with 2% sodium phosphotungstic acid, pH 7.0. Bar represents 200 nm.

The clumping technique (11) was employed for immunoelectron microscopy. Individual insects were crushed in RGDV antiserum (1/100 dilution) and incubated for 15 min at room temperature. A carbon-coated collodion grid was then touched to the mixture. After 1 min, the grid was rinsed with 0.1 M PB (pH 7.0), then with water, and finally it was negatively stained with 2% PTA.

RDV antiserum provided by I. Kimura had a titer of 1:2,000 in precipitin ring interface test. A lyophilized antiserum against wound tumor virus (WTV) whose titer was 1:320 in precipitin ring interface test, prepared by L. M. Black, was obtained from American Type Culture Collection. Rice black-streaked dwarf

TABLE 2. Infectivity test of purified rice gall dwarf virus

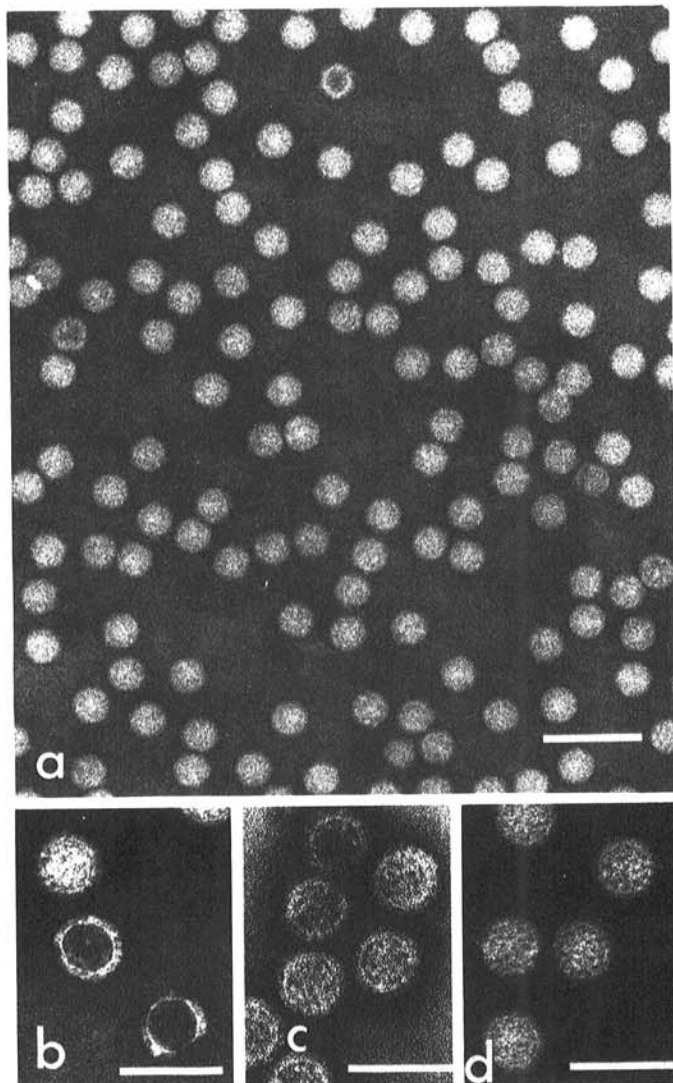| Exp. no. | Number of insects that: | | | Percentage of transmission |
|---|---|---|---|---|
| | were injected | survived 12 days | transmitted virus | |
| I | 50 | 7 | 4 | 57 |
| II | 50 | 32 | 26 | 81 |
| III | 50 | 13 | 11 | 85 |
| Control[a] | 120 | 21 | 0 | 0 |

[a] Buffer injected control.



**Fig. 2.** Purified rice gall dwarf virus particles. Preparations **a** and **d** were stained with ammonium molybdate, that in **b** with phosphotungstic acid, and that in **c** with uranyl acetate. Bars represent 200 nm in **a** and 100 nm in **b**, **c**, and **d**.

virus (RBSDV) antiserum (titer 1:1,024–2,048 by the precipitin ring interface test) was supplied by E. Shikata. Rice ragged stunt virus (RRSV) antiserum (titer 1:1,280 by the precipitin ring interface test) was provided by I. Kimura and H. Hibino. The fiji disease virus (FDV) antiserum (titer of 1:16 to viral protein and 1:2 to double-stranded RNA by the immunodiffusion test) was provided by R. Francki. E. Luisoni supplied maize rough dwarf virus (MRDV) antiserum with a homologous titer of 1:1,024, oat sterile dwarf virus (OSDV) antiserum with a homologous titer of 1:1,024, and pangola stunt virus (PSV) antiserum with a homologous titer of 1:256 all measured by using the agar gel diffusion test. Ribonucleic acid (RNA) of RGDV and RDV was prepared by the phenol-sodium dodecyl sulfate method (1). Poly(I):poly(C) $ds$-RNA was obtained from P-L Biochemicals Inc., Milwaukee, WI 53205 USA.

## RESULTS

**Detection of viruslike particles in plants and insect vectors.** In preliminary experiments (13) reoviruslike particles about 65 nm in diameter were detected in preparations from infected plants by electron microscopy. Systematic searches for the causal agent of the disease were also done on negatively stained specimens of viruliferous insects and virus-free insects in addition to those used for transmission tests or healthy plants. As shown in Table 1, many large polyhedral particles, about 65 nm in diameter (Fig. 1), were detected only in diseased plants and in viruliferous insects, most of which had transmitted the disease. The finding that large particle carriers do not always transmit the disease agrees with the results of Inoue and Omura (7) in that the transmission pattern of the disease is of an intermittent type.

Small polyhedral particles, about 35 nm in diameter (Fig. 1), were always detected in both viruliferous and nonviruliferous insects. These particles were not observed in healthy or diseased plants.

**Virus purification.** The highest yields of virus were obtained from fresh roots of plants ~40 days after inoculation. Contaminating host materials were lower in homogenates from roots than in those from leaves and leaf sheaths. Used as a clarifying agent, $CCl_4$ effectively removed host materials without significant effect on particle appearance. However, when the $CCl_4$ treatment was performed in a solution containing a weak buffer, significant particle loss occurred in the low-speed pellet. Among the buffers examined, 0.1 M PB (pH 7.0) and 0.1 M tris-HCl (pH 7.0) gave satisfactory results, but the use of 0.1 M glycine with or without $Mg^{++}$ (8) and STE buffer (0.1 M NaCl, 0.05 M tris-HCl, and 0.005 M EDTA, pH 7.5) (6) resulted in a significant loss of particles. Addition of $Mg^{++}$ very effectively removed host materials without causing significant loss of virus particles by low-speed centrifugation.

To prove that purified particles were the causal agent of the disease, purified virus preparations were injected into insect nymphs and these were placed on rice seedlings. As shown in Table 2, 57–85% of the insects given inoculation access feedings on test rice seedlings caused typical symptoms of RGD. The 65-nm particles were observed in negatively stained specimens from these diseased plants.

**Virus particles.** Electron micrographs of purified RGDV particles, negatively stained with PTA, UA, or AM (Fig. 2), showed that they are similar to the polyhedral particles of RDV (3) and WTV (14). Although there was a slight difference in penetration of the stains, the appearance and dimensions (diameter ~65 nm) of the RGDV particles were similar irrespective of the stains used (Fig. 2b–d). Preparations mixed a long time with PTA contained particles that were collapsed and penetrated by the stain (Fig. 2b). The diameter of the cavity penetrated by the stain was about 45 nm. Substructures about 50 nm in diameter surrounded with a thin layer of membraneous material, from which part of the shells were stripped, were observed rarely. The thin layer may be an inner membrane which is generally found in reovirus particles. Particles composed of only an inner core were not found. Surface projections observed with Fijivirus particles (10) were not observed

**Fig. 3.** Immunoelectron microscopy of crushed insect tissue mixed with rice gall dwarf virus antiserum. Bar represents 200 nm.

with RGDV in any stain.

Purified particles remained undamaged by repeated freezing and thawing, heat, and chloroform treatments; an inner core with B spikes as found in Fijivirus (10) was not observed after those treatments.

**Serological investigations.** Purified RGDV reacted to the antisera against RGDV, RDV, and WTV at a dilution of 2,048, 2, and 1, respectively. The antiserum against RGDV reacted at a dilution of 2 to RGDV-RNA, RDV-RNA, and poly(I):poly(C), respectively. The precipitin lines formed between RGDV and each antiserum against RDV and WTV, and that formed between RDV and RGDV antiserum up to 1:2 dilution, did not occur with antiserum that was cross absorbed with poly(I):poly(C). No serological cross reactions were observed with purified RGDV and antisera against RBSDV, FDV, MRDV, PSV, or OSDV tested at a 1/5 dilution.

The serological relation between the particles in insect vectors and those in diseased plants were examined by using the clumping technique. Aggregations of polyhedral particles about 65 nm in diameter were frequently observed in a mixture of viruliferous insects and RGDV antiserum (Fig. 3). However, no such aggregation was observed in mixtures of virus-free insects and RGDV antiserum. Thus, 65-nm particles in plants and insect vectors were shown to be serologically related.

## DISCUSSION

Transmission of the RGD disease by insects injected with the purified virus particles (Table 2) made it apparent that the polyhedral particles about 65 nm in diameter were the causal agent of RGD disease. The virus has been assumed to belong to the plant reovirus group from the standpoint of particle morphology. The plant reoviruses are divided into two groups (2,9): Phytoreovirus (plant reovirus subgroup 1) and Fijivirus (plant reovirus subgroup 2). The present investigations show that RGDV particles were stable after chloroform and heat treatment, repeated freezing and thawing, and PTA staining which degrade the outer capsid into spiked and smooth cores in Fijivirus, but not in Phytoreovirus (10). The genome of RGDV, like those of Phytoreovirus, has a similar amount of ds-RNA divided into 12 segments (4). Phytoreoviruses have cicadellid (leafhopper) vectors with a high percentage of transovarial transmission, whereas Fijiviruses have deliphacid (planthopper) vectors with either no, or a very low percentage of,

transovarial transmission (10). Inoue and Omura (7) reported RGDV was transmitted by several species of leafhoppers, but not by the planthoppers, *Laodelphax striatellus* and *Nilaparvata lugens* and RGDV was transmitted through leafhopper eggs with a high percentage. These data clearly show that RGDV is a Phytoreovirus. RDV was not suspected of being homologous with RGDV, even though RDV is a Phytoreovirus and infects rice plants, but the symptoms on rice are different (13). RGDV particles (65 nm) are slightly smaller than RDV particles (70 nm). No serological relationship was observed between RGDV and RDV when both viruses and antisera were examined reciprocally. These data demonstrate that RGDV is a new member of the Phytoreovirus, subgroup 1 in the plant reovirus group.

*Ds*-RNA is antigenic, but the antibody reacts with any *ds*-RNA. This is the reason for the positive serological reaction among viruses classified in the plant reovirus group (10). The reaction of RGDV antiserum against RGDV-RNA, RDV-RNA, and poly(I):poly(C) suggest that the serum contains antibody against *ds*-RNA as well as viral coat protein. The reaction of RGDV to each antiserum against RDV and WTV is supposed to be caused by nonspecific reaction of *ds*-RNA of RGDV and the antibody against the *ds*-RNA of RDV and WTV, which had been used as antigens.

Viruliferous *N. nigropictus* contained two kinds of polyhedral particles, RGDV and smaller ones (Fig. 1), whereas virus-free insects contained only smaller particles (Table 1). The smaller particles were not detected in diseased rice plants. Therefore, the smaller particles cannot be the causal agent of the rice disease but are probably insect virus particles which are similar to those reported in tissues of the planthopper *L. striatellus* in the presence or absence of MRDV and RBSDV (10) and in tissues of *N. lugens* in the presence or absence of RRSV (5).

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