RICE GALL DWARF VIRUS

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Abstract

A previously undescribed virus disease was found on rice plants in central Thailand in 1979 and named rice gall dwarf. Symptoms consisted of gall formation along leaf blades and sheaths, dark green discoloration, twisted leaf tips, and reduced number of tillers.

Of the plants inoculated the followeing were infected and back-inoculated to rice; wild rice *Oryza rufipogon,* barley *Hordeum distichum,* wheat *Triticum aestivum,* rye *Secale cereale,* oat *Avena sativa* and Italian ryegrass *Lolium multiflorum* and suzumenoteppo *Alopecurus aequalis.*

The disease was transmitted by *Nephotettix nigropictus, N. cincticeps, N. malayanus, N. virescens* and *Recilia dorsalis* in a persistent manner and was also transmitted through the eggs of *N. nigropictus.* Transmission efficiency showed marked differences among vector species and colonies. Incubation period of the virus in every vector species was about 2 weeks at 25°C. Rate of congenital infective progeny born from infective females showed wide variations from 0 to 100% among test broods of *N. nigropictus* and, in addition, there was a steep decrease of infective rate of progeny with the advance of leafhopper generations.

Double-shelled polyhedral particles about 65 nm in diameter were purified from infected rice plants by carbon tetrachloride clarification, polyethylene glycol precipitation, differential centrifugation, and sucrose density-gradient centrifugation.

The purified particles were highly infectious to rice seedlings inoculated via injected insect nymphs. In gel diffusion tests, antiserum against the particles 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.

Nucleic acid of purified virus was composed of double-stranded RNA divided into 12 segments.

Virus antigens both in infected plants and individual insect vectors were detected by latex flocculation test.

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1. Introduction

In August 1979, dark green, stunted rice plants with small galls on the leaf blades and sheaths were found in paddy fields at Uthaithani, central Thailand. Tips of some leaves of diseased plants were slightly twisted. None of the known virus diseases of rice produces these symptoms. Preliminary experiments showed that the green rice leafhopper *Nephotettix nigropictus* transmitted the causal agent of the disease and that polyhedral particles about 65 nm in diameter were always associated with the disease.

This paper describes the host range and symptomatology of the disease, transmission of the disease, purification procedure, the properties of the virus particles, and serological detection of the virus both in infected plants and insect vectors.

2. Symptomatology and host range

1) Symptomatology

Early-instar nymphs of *N. nigropictus,* collected at Kagoshima, Japan, and maintained at Kyushu National Agricultural Experiment Station, were used for transmission of the disease. After a 1-day acquisition access on diseased source plants, the insects were transferred to healthy rice seedlings for one day (cv. Taichung Native 1) for an inoculation access in a controlled-environment greenhouse (23-27°C). Inoculated seedlings were grown in the same greenhouse. About 1 month after inoculation of rice seedlings at the one- to three-leaf stage, marked dwarfing, small galls along the leaf blades and sheaths, and dark green discoloration and twisting of some leaf tips appeared. Symptoms were milder when plants were inoculated at the 8- to 10-leaf stage. Dwarfing and discoloration were the characteristic symptoms of diseased plants in the field.

Galls or vein swellings appeared on the undersurfaces of leaf blades as well as the outer sides of leaf sheaths. Galls were more abundant in plants infected early, and some leaves bore more than 10 galls. Galls varied from 0.4 to 8 mm in length, with most of them being less than 2 mm long, and from 0.4 to 0.5 mm in width.

2) Host range

Second- and third-instar nymphs of the rice green leafhopper *N. nigropictus* that had fed on infected rice plants for 2 to 3 days were reared on healthy TN1 seedlings for 10 to 14 days, then *N. nigropictus* was used to inoculate 11 plant species grown in pots: maize *Zea mays,* sorghum *Sorghum nervosum,* timothy grass *Phleum pratense,* orchard grass *Dactylis glomerata,* Italian ryegrass *Lolium multiflorum,* suzumenoteppo *Alopecureus aequalis* var. *amurensis,* wild rice *Oryza rufipogon,* barley *Hordeum distichum,* wheat *Triticum aestivum,* rye *Secale cereale,* and oat *Avena sativa.* At the second leaf stage, seedlings were inoculated by placing 2 viruliferous insects on each plant for 2 to 3 days.

Virus symptoms developed 15 to 30 days after inoculation. Inoculated plants were examined by electron microscopy using a negative stain preparation. They were then back-inoculated to healthy rice seedlings. Barley, wheat, rye, oat, Italian ryegrass, suzumenoteppo, and wild rice showed typical symptoms. Polyhedral particles 65 nm in diameter were observed in the negatively stained preparations. Rice plants

exhibited symptoms after back-inoculation.

3. Transmission of rice gall dwarf virus by zig-zag and green leafhoppers in Thailand

1) Serial transmission tests by vectors

The third instar nymphs of *R. dorsalis* and the fourth to fifth instar nymphs of *N. nigropictus* were allowed to feed on diseased rice for 1 day. Each species was tested for retention period by serial daily transfers on healthy plants. Part of the results is shown in Table 1. Both species of insects transmitted the virus in a persistent manner, and most individuals transmitted it intermittently. Eleven out of 94 *N. dorsalis* and 12 out of 130 *N. nigropictus* transmitted the virus. The incubation period ranged from 5 to 11 days (7.9 days average) in *R. dorsalis* and 4 to 12 days (7.6 days average) in *N. nigropictus*. No effect on transmission was observed by molting. Young nymphs of *N. virescens* were fed on the diseased plant for 1, 3, and 6 days. After 10 to 13 days of rearing on healthy feeding material for incubation, each insect was tested for its transmission ability by 5 to 8 transfers of 2-day intervals on test plant. Three out of 100 *N. virescens* transmitted the virus when they were allowed to feed on the diseased rice for 6 days.

2) Acquisition feeding period of the virus

The second instar nymphs of *R. dorsalis* were allowed feeding access on the diseased rice plants for 1, 2, 4, 8, 16, 24, and 48 hr. After acquisition feeding, the insects were reared on healthy rice seedlings for 10 days as incubation period. Each insect was tested for transmission ability by serial inoculation for 10 days on each healthy seedling by 5 transfers of 2-day intervals. The insects were unable to transmit the virus within 4 hr or shorter acquisition feeding period, but 3 individuals out of 50 were capable of transmitting the virus within 8 hr feeding. Transmission of the virus was more efficient with a longer acquisition period.

3) Inoculation feeding period for the transmission

The second instar nymphs of *R. dorsalis* were allowed an acquisition access period of 5 days on the diseased rice plants. The insects were reared on healthy rice seedlings for 10 days. Each insect was then allowed an inoculation feeding period of 1, 2, 4, 8, 16, 24 and 48 hr on individual test plants. Three individuals out of 38 were able to transmit the virus within an 1 hr inoculation access period.

4) Effect of vector development stage on virus acquisition

Nymphs of *R. dorsalis* at different instar stages and adults were allowed acquisition feeding access on the diseased rice plants for 1 day and were tested on test plants. Transmission percentages of the second, the third to fourth, the fifth instar nymphs and adults were 38, 29, 12, and 13, respectively. Young nymphs, especially the second instar nymphs, acquired the virus more efficiently than the older ones.

Table 1. Serial daily transmission of rice gall dwarf virus by leafhoppers

Insect no.	Days after virus acquisition started																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Recilia dorsalis																								
1	–	–	–	–	–	+	+	+	+	+	–	–	–	–	–	+	+	+	+	–				
2	–	–	–	–	–	*	–	+	+	+	+	+	–	+	+	+	+	+	+	+				
3	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+				
Nephotettix nigropictus																								
1	–	–	–	–	–	–	–	*	–	+	+	–	+	–	–	+	+	+	+	–	+	+	+	–
2	–	–	–	–	+	–	+	+	+	+	+	+	–	–	+	+	+	+	+	*	–	+		
3	–	–	–	–	–	–	–	+	+	+	–	+	+	+	+	+	–	–	–	+	+	+	+	+

* test plant died

5) Nontransovarian transmission of virus in R. dorsalis

Viruliferous females were obtained by a 1-day acquisition feeding access from diseased rice plants and a 2-week incubation period. They were allowed oviposition access on healthy rice seedlings for 1 day. Immediately after hatching, the young nymphs were transferred to healthy rice seedlings to avoid virus acqusition from the oviposited rice plants. The infectivity of individual nymphs was tested for transmission. None of the 89 nymphs from 10 viruliferous females was found to be infective.

6) Seed transmission of the virus

Mature seeds of the rice variety TN1 were collected from diseased plants and sown in seedling boxes kept in a screenhouse. None of the 1,461 seedlings showed symptoms of the disease.

The results obtained in the present study are summarized in Table 2. In Thailand, *R. dorsalis, N. nigropictus,* and *N. virescens* were shown to be the insect vectors of RGDV. According to Inoue and Omura (1982), *N. cincticeps, N. malayanus,* and *N. virescens,* as well as *N. nigropictus* transmit RGDV. *R. dorsalis* is one of the vectors of rice dwarf virus (RDV) (Fukushi, 1937) and the vector of rice orange leaf disease (Rivera *et al.,* 1963). *N. nigropictus* (Fukushi, 1937; Nasu, 1963) and *N. cincticeps* (Fukushi, 1937) also transmit RDV. RGDV has a wider vector range than RDV.

Incubation periods of *R. dorsalis* and *N. nigropictus* were 7.9 and 7.6 days on the average respectively and that of *N. virescens* was 13 days in Thailand. Transmission percentages of RGDV in higher instar nymphs of leafhoppers were relatively low in Thailand, whereas, incubation periods in *Nephotettix* leafhoppers in Japan were about 2 weeks with a higher transmission percentage (Inoue and Omura, 1982). *N. nigropictus* females produce both infective and noninfective progenies in Japan (Inoue and Omura, 1982), but nontransovarian transmission of *R. dorsalis* was detected in

Table 2. Interaction between rice gall dwarf virus and insect vectors in Thailand

	R. dorsalis	*N. nigropictus*	*N. virescens*
Transmission mode	persistent	persistent	persistent
Transmission efficiency	11.2%	9.2%	3.0%
Incubation period in insect	5—11 (7.9) days	4—12 (7.6) days	13 days
Shortest acquisition feeding period	8 hr		
Shortest inoculation feeding period	1 hr		
Acquisition efficiency in nymphs	2nd instar (38%)		
Transstadial passage	yes	yes	
Transovarian passage	no (0/89)	*	
Seed transmission of rice	no (0/1461)		

* yes: *N. nigropictus* in Japan (Inoue and Omura, 1982)

Thailand. The difference cannot be clearly determined because of the difference in insect species and origin. The insect vectors from Thailand and Japan should be compared in their transmission ability in relation to affinity of RGDV. No seed transmission of rice on RGDV was detected as in the case of the other leafhopper-borne rice viruses.

4. Characteristics of rice gall dwarf virus transmission by Cicadellid leafhoppers

1) Transmission mode

Groups of *N. nigropictus* nymphs of the Kagoshima colony at the 1st instar were given an acquisition access period of 1 day on the diseased source rice, and the test leafhoppers were then allowed to feed individually on test seedlings in test tubes at 25°C. The test leafhoppers were transferred serially to new test seedlings at 3- or 4-day intervals during their life. In the next test, 9 infective females were paired with healthy males and the eggs laid by the females were removed from the seedlings. The eggs were, then, kept on moistened filter paper for nymphal hatching. The nymphs were tested individually for their infectivity twice with a 3-day inoculation access.

Of the 128 individuals of *N. nigropictus* tested, 62 transmitted the RGDV. The transmission pattern suggested a persistent virus-vector relationship since the infective insects, after the virus latent period in the insects, retained the infectivity during their entire life and, moreover, the virus passed from the infective females to their progeny through eggs.

2) Vector species range and transmission efficiency

In the test of vector species range, 7 leaf- and planthopper species, as shown in Table 3, were fed on the diseased source plants for an acquisition access of 2 to 15 days and test insects were allowed to feed individually for an inoculation access on test seedlings in test tubes. The test insects were transferred serially to healthy test seedlings at 2-day intervals during their entire life. In the test on transmission

Table 3. Transmission efficiency and latent period of rice gall dwarf virus in leaf- and planthoppers

Species (localities of collection)	Number of insects tested	Percent infectivity	Latent period in day (avg. ± SD)
N. nigropictus (Kagoshima)	49[1)]	51.0	14.5 ± 0.8
N. cincticeps (Chikugo)	134[1)]	15.7	14.8 ± 1.7
N. malayanus (Ishigaki)	86[1)]	9.4	14.5 ± 2.5
N. virescens (Kagoshima)	272[1)]	0.7	18.0 ± 4.3
R. dorsalis (Fukuoka)	49[2)]	4.1	16, 18
Nilaparvata lugens (Chikugo)	40[3)]	0	
Laodelphax striatellus (Chikugo)	40[3)]	0	

N.: the genus *Nephotettix*
Test virus acquisition periods were 2 days for 1), 15 days for 2) and 3 days for 3)

efficiency, a total of 10 colonies in 3 *Nephotettix* species were tested for a virus acquisition access of 3 to 12 days and for inoculation access during their life. The test leafhoppers were transferred serially to new seedlings at 2-day intervals.

As shown in Table 3, *N. nigropictus, N. cincticeps, N. virescens, N. malayanus* and *Recilia dorsalis* could transmit the virus. No transmission was obtained with males and females of *Nilaparvata lugens* and *Laodelphax striatellus,* even though all the test insects survived at least 2-day after the onset of the virus acquisition period.

The average virus latent period in vector insects taken as the start of virus acquisition and first effective inoculation was about 2 weeks in *N. nigropictus, N. cincticeps* and *N. malayanus,* but *N. virescens* and *R. dorsalis* required a 2 to 4 day longer period of virus latency.

There were considerable variation in the rate of infective individuals among the colonies within the species of *N. nigropictus* and *N. cincticeps,* as shown in Table 4. The highest rate of transmission was found in the Amami colony in *N. nigropictus* (95%) or Chikugo colony in *N. cincticeps* (42%). Also both species included inefficient vector colonies, i.e. the Thai colony in *N. nigropictus* (1.8%) or Amami colony in *N. cincticeps* (1.4%). There was no essential difference in the infective efficiency between the 2 *N. virescens* colonies. Based on the maximum rate of transmission in each species, *N. nigropictus* showed the highest efficiency followed by *N. cincticeps., N. malayanus. R dorsalis* exhibited a slightly lower rate of transmission than *N. cincticeps,* and *N. virescens* was a very inefficient vector.

Table 4. Variation of transmission efficiency of rice gall dwarf virus in different colonies in 3 *Nephotettix* spp.

Species	Site of collection	Number of insects tested	Virus acquisition access (days)	Percent infectivity
N. nigropictus	Kagoshima	40	9	47.5
	Amami	40	9	95.0
	Thailand (1)	38	3	7.9
	Thailand (2)	57	4	1.8
	Thailand (2)	20	12	10.0
N. cincticeps	Joetsu	51	4	33.3
	Chikugo	42	4	42.7
	Amami	69	4	1.4
N. virescens	Kagoshima	70	4	0.1
	Thailand	231	4	0.1

3) Transovarian transmission in N. nigropictus

Infective adult females, 2 - 4 days after emergence, which were given previously a virus acquisition feeding on diseased rice plant for 3 days at the 2nd instar nymph stage were paired with healthy male adults of the same colony and cv. Reiho seedlings were supplied in test tubes for oviposition site and food. The eggs were removed from the rice plant tissues before being hatched and hatched nymphs were obtained by keeping the eggs on moistened filter paper to prevent reacquisition of the virus. Ten

individuals in each of the 29 broods of the Amami colony and 9 to 32 individuals in each of the 12 broods of the Kagoshima colony at the 5th instar nymph or young adult stages were collected randomly and were tested for virus transmission with an inoculation access of 4 days.

Thereafter, transovarian transmission of the virus was tested over 3 successive generations, V_1, V_2 and V_3, for 8 broods of the Amami colony. Infective females which acquired the virus at the nymphal stage with a 3-day acquisition access and in which the infectivity was confirmed were paired with healthy male adults, and the progenies (V_1) from the respective females were grown in separate groups. V_2 and V_3 generations were obtained by inbreeding within groups. Nine to forty individuals in each brood in each generation were tested individually for their virus infectivity twice with a 3-day inoculation access to test seedlings.

Table 5. Percent distribution of RGDV-congenitally infective individuals born from infective females of Amami and Kagoshima colonies of *Nephotettix nigropictus*

Percent	Amami colony	Kagoshima colony
	(%)	(%)
0	0	17
~ 10	14	17
~ 20	3	0
~ 30	7	25
~ 40	10	8
~ 50	17	17
~ 60	7	0
~ 70	7	8
~ 80	10	0
~ 90	7	8
~100	17	0
Total	100	100
Number of test broods	29	12

As shown in Table 5, the rate of infective progenies produced by the infective females varied considerably in the broods, from 10 to 100% in the Amami colony and from 0 to 83% in the Kagoshima colony. The average infectivity rate was 57.5% for the Amami colony and 31.5% for the Kagoshima colony, reflecting the order of transmission efficiency between the colonies.

With regard to the start of the virus transmission by the progeny, there was no demonstrable virus latent period.

In the next test, as shown in Table 6, the average rate of infective progenies decreased steeply with the advance of the insect generation when the progenies did not feed on diseased rice plant: the average infectivity rate of the V2 individuals was only 4.95, about 1/3 against the expected rate of infectivity (= 2.5%) calculated from the rate of infective V2 individuals (4.9% × 4.9%). These findings indicate the deleterious effects of the virus on the fecundity and life span of infective leafhoppers, as observed in *N. cincticeps* for the rice dwarf virus (Nasu, 1963; Nakasuji and Kiritani, 1970) and *Sogatodes orizicola* for the Hoja blanca virus (Maramorosch, 1969).

Table 6. Percent transovarian transmission of RGDV over 3 successive generations in *Nephotettix nigropictus* (Amami colony)

	First generation (V_1)		2nd generation (V_2)		3rd generation (V_3)	
No. brood	Number of test progenies	Percent infectivity	Number of test progenies	Percent infectivity	Number of test progenies	Percent infectivity
1	31	87	39	13	42	2
2	9	67	36	11	40	0
3	16	44	40	3	40	0
4	29	38	37	8	40	0
5	14	29	37	3	—	
6	26	27	38	3	40	0
7	12	25	40	0	40	0
8	32	6	40	0	40	0
Average	—	39.6	—	4.9	—	0.4

RGDV can be distinguished from the 15 known virus and viruslike diseases of rice on the basis of symptomatology, morphology and serological relationships of the causal agent. The present study provides further basis for distinguishing RGDV. RGDV belongs to the plant reovirus group that also includes rice ragged stunt virus, rice black streaked dwarf virus and rice dwarf virus. However, the first 2 viruses are transmitted by planthoppers. Furthermore, while RGDV was transmitted by 4 *Nephotettix* spp. (Table 3), only *N. nigropictus* and *N. cincticeps* are vectors of the rice dwarf virus, whereas *R. dorsalis* is the vector of both RGDV and rice dwarf virus.

Transmission efficiency showed considerable variations among the vector colonies within the species, suggesting differences in the affinity of virus-vector relationships. It was interesting to note that the Thai colony of *N. nigropictus* exhibited the lowest efficiency of transmission among the test colonies within the species though the disease occurs in Thailand.

Previously, transovarian transmission has not been shown for any of the rice viruses in the tropics and the subtropics in Asia. The only known viruses which are endemic to the temperate region, namely, rice dwarf virus transmitted by *N. cincticeps, N. nigropictus* and *R. dorsalis* and rice stripe virus by *L. striatellus, Unkanodes albifascia* and *U. sapporonus*. RGDV is thus the first virus disease of rice in the tropical region of Asia for which transovarian transmission has been demonstrated. Such transmission is probably of great importance in the epidemiology and perpetuation of the virus together with the year-round occurrence of vector

leafhoppers.

5. Purification and preparation of antiserum

1) Relation with cells and tissues

Ultrathin sections of virus infected plants were observed under an electron microscope and compared with healthy materials to identify the causal agent of the disease.

Leaf samples were fixed with 2.5% glutaraldehyde in 0.1 M. phosphate buffer (pH 7.0) for 2 hr. They were washed in cold buffer and then fixed with 2% osmium tetroxide in the same buffer for 3 hr, dehydrated in an acetone series, and embedded in Epon 812. Thin sections were cut transversely from samples by using a diamond knife mounted on a Sorvall MT-2 ultramicrotome. After staining with uranyl acetate and lead citrate, the sections were observed under a Hitachi H-500 electron microscope.

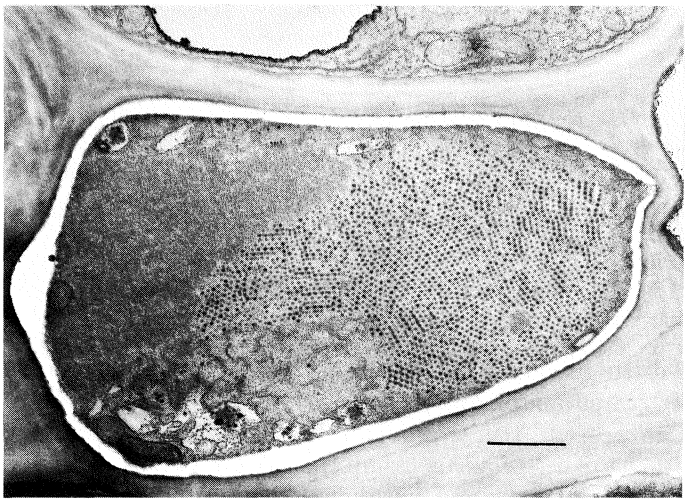


Fig. 1. Electron micrograph showing virus particles in a phloem cell of a rice plant infected with rice gall dwarf virus. Bar represents 1,000 nm. (data from Omura *et al.*, 1980)

Polyhedral particles about 65 nm in diameter were frequently observed in diseased plants (Fig. 1). No such particles were found in healthy controls.

2) Detection of virus-like particles in plants and insect vectors

Systematic searches for the causal agent of the disease were also conducted on negatively stained specimens of viruliferous insects and virus-free insects in addition to those used for transmission tests of healthy plants. As shown in Table 7, many large polyhedral particles, about 65 nm in diameter (Fig. 2) were detected only in diseased plants and in viruliferous insects, most of which had transmitted the disease.

Table 7. Transmission of particles by a colony of green rice leafhopper *Nephotettix nigropictus* viruliferous for rice gall dwarf virus

Insect colonies	Sex	Number of insects	Small[a] particle carriers	Large[b] particle carriers	Active transmitters
Viruliferous	female	19	19	12	9
	male	21	21	12	11
Virus-free	female	10	10	0	0
	male	10	10	0	0

a) Polyhedral particles about 35 nm in diameter.
b) Polyhedral particles about 65 nm in diameter.
(data from Omura *et al.*, 1982)

The finding that large particle carriers do not always transmit the disease agrees with the results of Inoue and Omura (1982) who showed that the transmission pattern of the disease is of the intermittent type.

Small polyhedral particles, about 35 nm in diameter (Fig. 2) were always detected in both viruliferous and nonviruliferous insects. These particles were not observed in healthy or diseased plants.

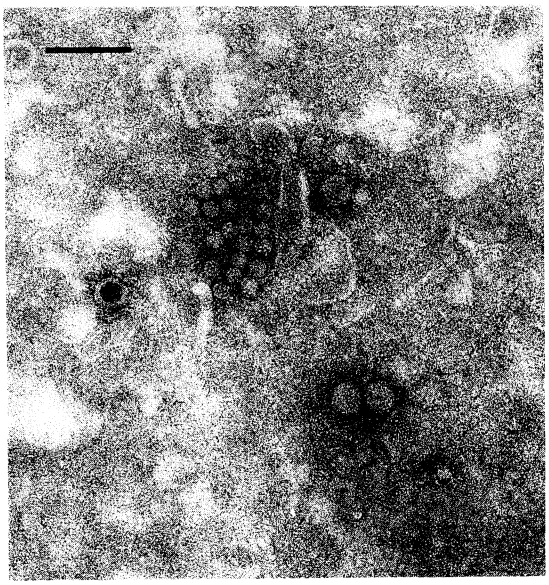


Fig. 2. Electron micrograph showing large and small particles in a crushed viruliferous insect preparation stained with 2% sodium phosphotungstic acid, pH 7.0. Bar represents 200 nm. (data from Omura *et al.*, 1982)

3) Purification

According to the studies described above, polyhedral particles about 65 nm in diameter observed only in diseased rice and viruliferous insects were purified to identify the causal agent of the disease.

Principally the method of purification for rice dwarf virus (Kimura, 1976) was employed for purifying rice gall dwarf virus because the particles were similar in morphology and the propagation host plant was the same as that for RDV. All the procedures were performed either in an ice bath or at 4°C. Fresh plants about 40 days after inoculation were ground with a meat chopper in 2 vol (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 and carbon tetrachloride (CCl_4) was added to a final concentration of 20% and then the homogenate was blended in a Waring Blender for 2 min. After centrifugation for 15 min at 3,000 g, polyethylene glycol 6,000 (PEG) and sodium chloride (NaCl) were added to the supernatant fluid to a 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 Blender 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 magnesium chloride ($MgCl_2$) ($MgCl_2$ was added just before use) (PB-Mg), incubated 30 min and later were 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 then centrifuged for 50 min at 60,000 g in a Hitachi PRS-25 rotor. The zone containing virus particles was recovered with an ISCO Model UA2 Scanner, and layered on 40–60% (W/V) linear sucrose gradients in PB-Mg and then 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.

The highest yield of virus was achieved with fresh roots of plants about 40 days after inoculation. The contamination of host materials was lower in homogenates from roots than in homogenates from leaves and sheaths. Carbon tetrachloride (CCl_4) used as clarifying agent 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-HC1, pH 7.0 gave satisfactory results, whereas the use of 0.1 M glycine with or without Mg^{++} and STE buffer (0.1 M NaCl, 0.05 M Tris-HCl, 0.005 M EDTA, pH 7.5) resulted in a significant loss of particles. Addition of Mg^{++} was very effective in removing host materials without significant loss of virus particles by low speed centrifugation.

4) Particle morphology

Electron micrographs of purified RGDV particles, negatively stained with PTA, UA or AM (Fig. 3) showed that they are similar to the polyhedral particles of RDV (Fukushi and Shikata, 1963) and WTV (Streissle and Granados, 1968). Although there was a slight difference in the penetration of the stains, the appearance and dimensions of the RGDV particles were similar irrespective of the stains used (Fig. 3b,

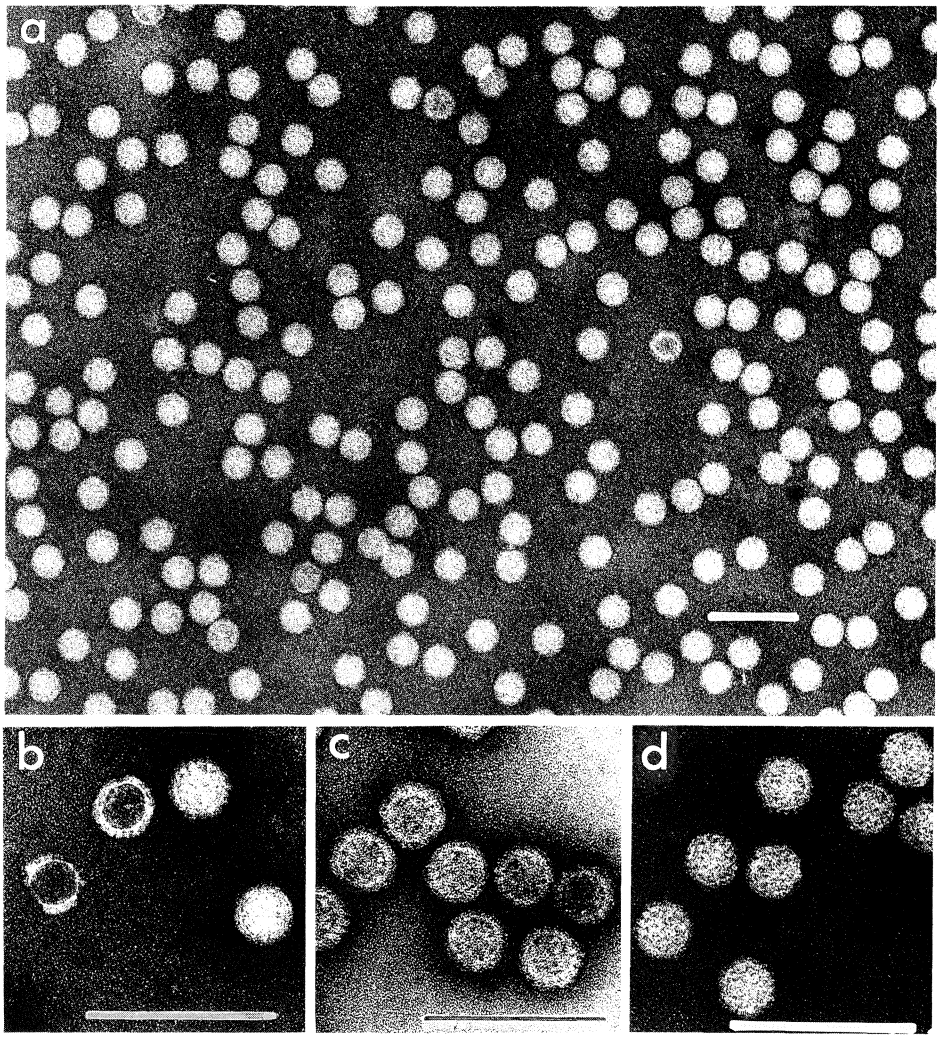


Fig. 3. Purified rice gall dwarf virus particles. Preparations (a) and (d) were stained with ammonium molybdate; (b) with phosphotungstic acid; and (c) with uranyl acetate. Bars represent 200 nm in (a) and 100 nm in (b), (c) and (d). (data from Omura *et al.*, 1982)

c, d). The diameter of the particles was about 65 nm. Preparations mixed a long time with PTA contained particles that had collapsed and were 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 membranous material, from which part of the shells was stripped, were rarely observed. The thin layer may be the inner membrane which is generally found in Reovirus particles.

5) Artificial injection

To confirm that the purified particles were the causal agent of the disease, purified

virus preparations were injected into insect nymphs and these were placed onto rice seedlings. Purified preparations of RGDV (A_{260} = 1-1.5) were injected into the abdomens of third to fifth instar nymphs of *N. nigropictus* using fine glass capillaries. The amount of inoculum per insect was about 0.01 μl and the injection was performed at room temperature. These insects were placed for 12 days on healthy rice sedlings with occasional transfers to new seedlings. They were individually transferred to test seedlings in test tubes (2 × 10 cm) and fed for 3 days. The injected insects and inoculated plants were kept in a controlled-environment greenhouse (27 ± 3C).

As shown in Table 8, 57-85% of the insects given inoculation access feedings on test rice seedlings exhibited typical symptoms of rice gall dwarf. The 65 nm particles were observed in negatively stained specimens from these diseased plants. These results demonstrate that the polyhedral particles about 65 nm in diameter are the causal agent of rice gall dwarf disease.

Table 8. Infectivity test of purified rice gall dwarf virus

Exp. no.	Injected	Number of insects which survived for 12 days	Transmitted	Percentage of transmission
I	50	7	4	57
II	50	32	26	81
III	50	13	11	85
Cont [1)]	120	21	0	0

a) Buffer-injected control.
(data from Omura *et al.*, 1982)

6) Preparation of antiserum

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 three 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 MPB, pH 7.0 containing 0.85% NaCl (PBS).

Serological tests were performed using the double gel diffusion method. The gel medium consisted of 0.8% agar in PB containing 0.85% NaCl, 0.001 M ethylenediaminetetraacetic 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_{260} = 2) was used as antigen. The plates were incubated for 2 days at room temperature.

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 appear when the antiserum had been previously absorbed with poly (I):poly (C).

6. Nucleic acid

The properties of the nucleic acid extracted from purified rice gall dwarf virus (RGDV) were characterized as follows.

1) Extraction of nucleic acid

Nucleic acid from purified RGDV was extracted by the phenol-cresol-SDS method (Reddy and Black, 1973). The nucleic acid preparation showed ultraviolet absorbance spectra with a peak at 260 nm, a trough at 232 nm and an A260/A280 ratio of 1.89.

2) Effects of nucleases on nucleic acid

Native and denatured (100°C, 10 min) nucleic acids were digested with ribonuclease A or nuclease S1 and the kinetics of digestion of the nucleic acids was estimated by

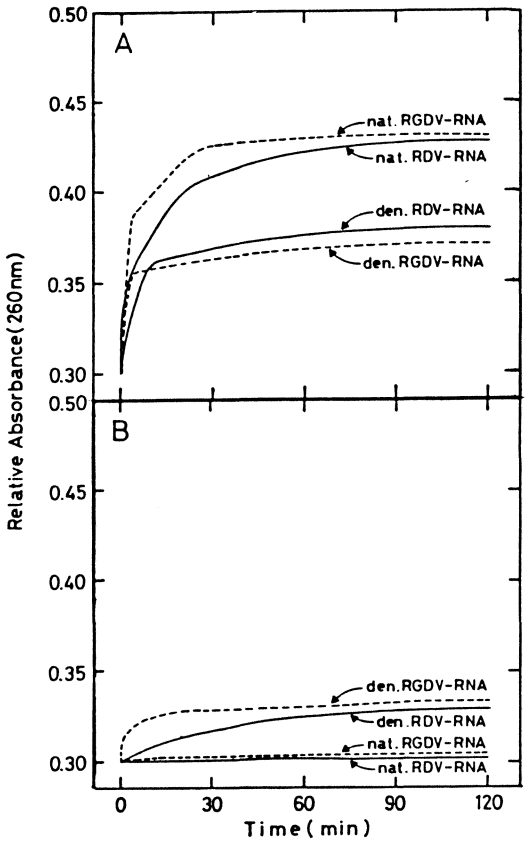


Fig. 4. Comparison of the kinetics of digestion of RGDV RNA and RDV RNA by RNase A as measured by the increase in absorbance at 260 nm. About 15 μg/ml of native (nat.) or heat-denatured (den.) nucleic acids were incubated at 25°C in either 0.1 × SSC (a) or 1 × SSC (b) with 1 μg/ml (a) or 10 μg/ml (b) of RNase. (data from Hibi *et al.*, 1984)

the rate of increase in absorbance at 260 nm. When incubated with RNase A in 0.1 × SSC, the rate of increase in absorbance at 260 nm of both native and denatured nucleic acids of RGDV, and RDV used as a control was rapid. When incubated in 1 × SSC, native nucleic acid was highly resistant to RNase digestion, whereas denatured nucleic acid was digested at a slower rate than in 0.1 × SSC (Fig. 4). Nuclease S1 also digested the denatured but not the native nucleic acid (Fig. 5). These results indicate that RGDV nucleic acid consists of double-stranded RNA.

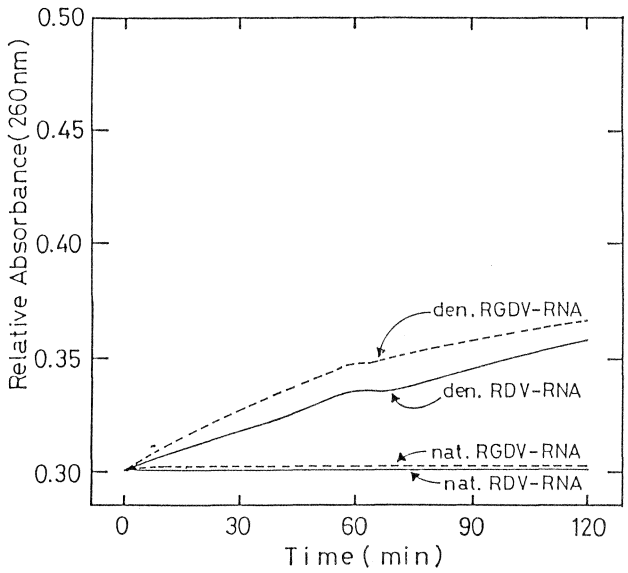


Fig. 5. Comparison of the kinetics of digestion of RGDV RNA and RDV RNA by nuclease S1 as measured by the increase in absorbance at 260 nm. About 15 μg/ml of native (nat.) or heat-denatured (den.) nucleic acids were incubated at 45°C in 30 mM sodium acetate containing 0.2 M NaCl and 1 mM $ZnSO_4$, pH 4.6, with 200 units/ml nuclease S1. (data from Hibi *et al.*, 1984)

3) Reactivity with formaldehyde

Nucleic acid was treated with formaldehyde as reported by Miura *et al.* (1966). The ultraviolet absorbance spectrum of native RGDV RNA was little affected by incubation with 1.8% formaldehyde at 37°C for 22 hours. On the other hand, similar treatment of denatured RNA caused a hyperchromic shift of 24.3% at the peak absorbance and a 3 to 4 nm shift of maximum absorbance wavelength (Fig. 6).

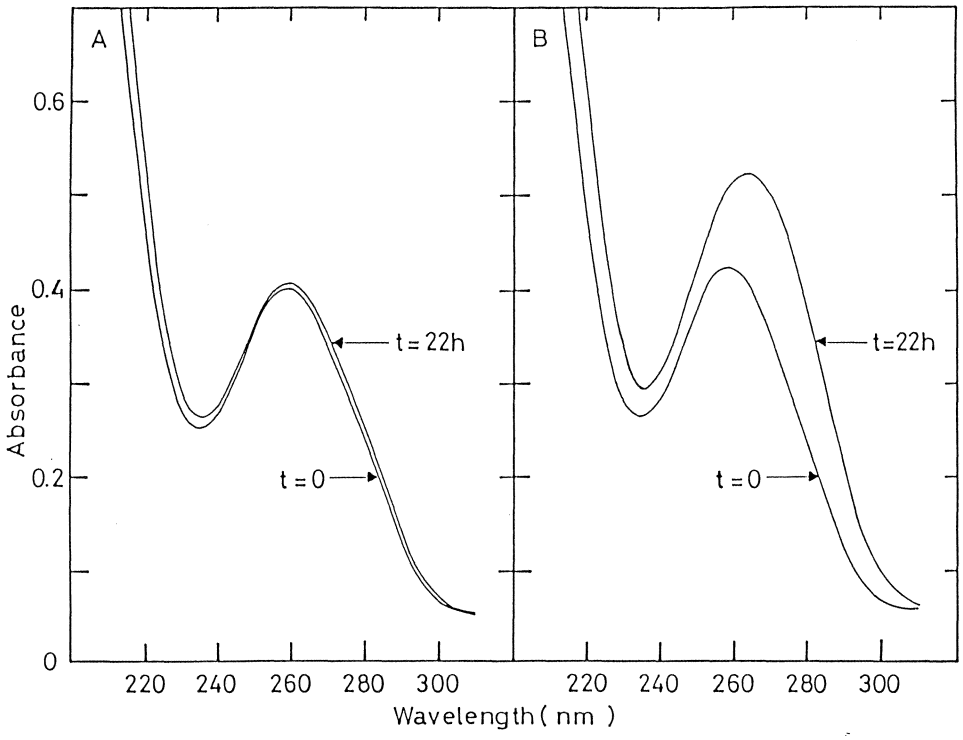


Fig. 6. Reaction of native (a) and heat-denatured (b) RGDV RNA with formaldehyde. The ultraviolet absorbance spectra of the RNA (about 20 μg/ml in 0.1 M NaCl) were recorded before (t = 0) and 22 hr after (t = 22h) incubation at 37°C in 1.85 formaldehyde. (data from Hibi *et al.*, 1984)

4) Thermal denaturation

Thermal denaturation kinetics of nucleic acid in 0.01 × SSC was estimated from the rate of increase in absorbance at 260 nm. RGDV RNA, unlike TMV RNA used as a control, showed a sharp thermal transition with a melting temperature of 76.2°C and a hyperchromicity of 30.6% (Fig. 7), which also indicates its double-stranded nature. Under the same conditions, the Tm of RDV RNA was 78.6°C.

5) Buoyant density

Isopycnic ultracentrifugation was performed as described by Szybalski (1968). The buoyant density of native RGDV RNA in Cs_2SO_4 was 1.596 g/ml (Fig. 8) and that of native RDV RNA was 1.599 g/ml.

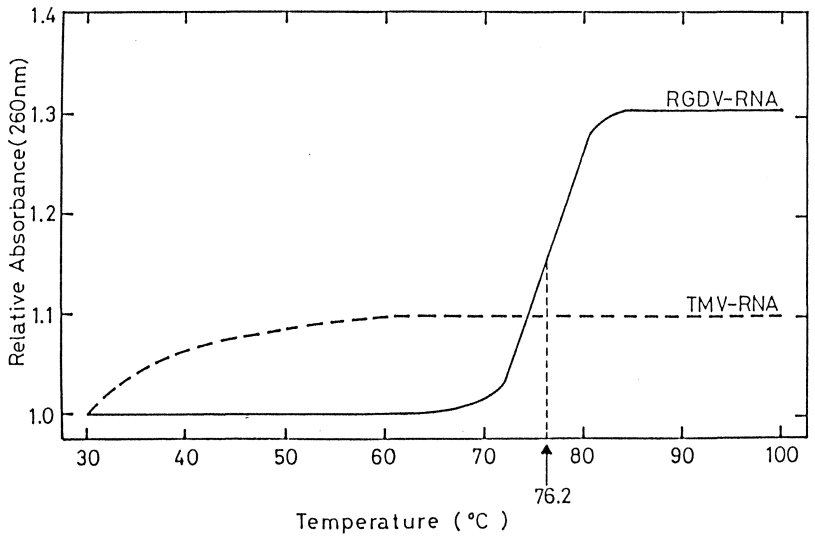


Fig. 7. Comparison of the thermal denaturation kinetics of native RGDV RNA and TMV RNA. RNA (about 20 μg/ml) was suspended in 0.01 × SSC and its absorbance at 260 nm was measured when the temperature was increased at 0.50°C/min. (data from Hibi *et al.*, 1984)

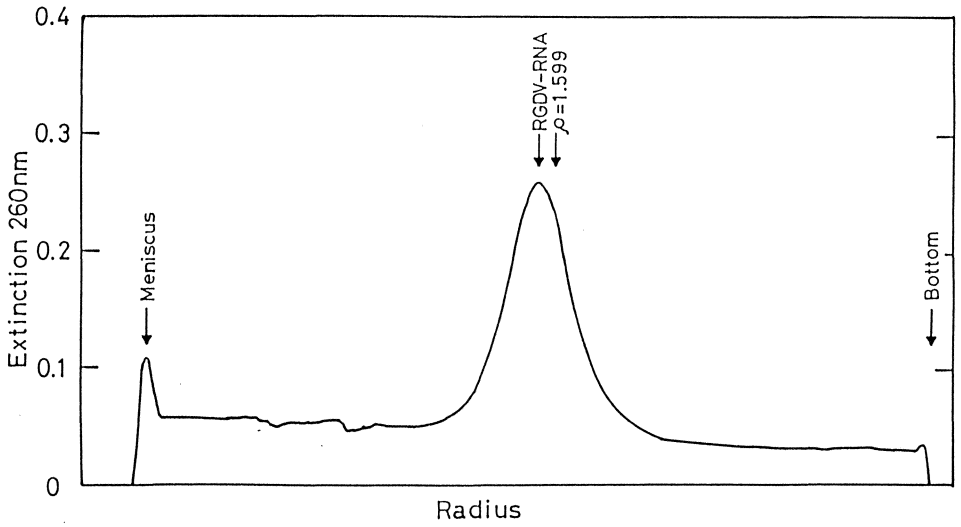
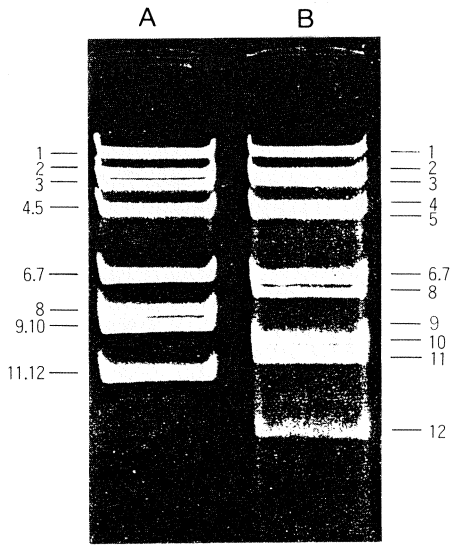


Fig. 8. Absorbance profile of native RGDV RNA after isopycnic ultracentrifugation in Cs_2SO_4. (data from Hibi *et al.*, 1984)

6) Molecular weight and segmentation of RGDV RNA

Electrophoresis of the RNA was performed in 2.5% polyacrylamide slab gels using 36 mM Tris, 30 mM NaH_2PO_4, 1 mM EDTA, pH 7.8 as the electrophoresis buffer. It resolved 11 distinct bands (Fig. 9b). Electropherograms also suggested that the 6th band from the origin contained two different co-migrating RNA segments, because the peak area of the band was larger than that expected from the assumption that all virus particles contain a set of 12 distinct RNA segments. Thus, it was concluded that the RNA genome of RGDV consists of 12 segments. The molecular weights of these were calculated by comparing their electrophoretic mobilities to those of RDV RNA segments separated in the same slab gel. The approximate molecular weights of the RGDV RNA segments ranged from 0.32×10^6 to 3.10×10^6 and the sum of molecular weights for the total RGDV genome was about 16.92×10^6 (Table 9). The approximate molecular weights of the genome segments of RGDV, RDV and WTV (Table 9) suggest that the molecular weights of segments 1 to 7 of RGDV are similar to those of the corresponding segments of RDV, but not to those of WTV, and that the molecular weights of segments 8 to 12 of RGDV are different from those of the corresponding segments of RDV.

The properties of the nucleic acid of RGDV described show that its particles contain double-stranded RNA in 12 segments, with a total molecular weight of approximately 16.92×10^6. These results, in addition to the biological and morphological properties, confirm that RGDV is a new virus and a third member of the genus Phytoreovirus.



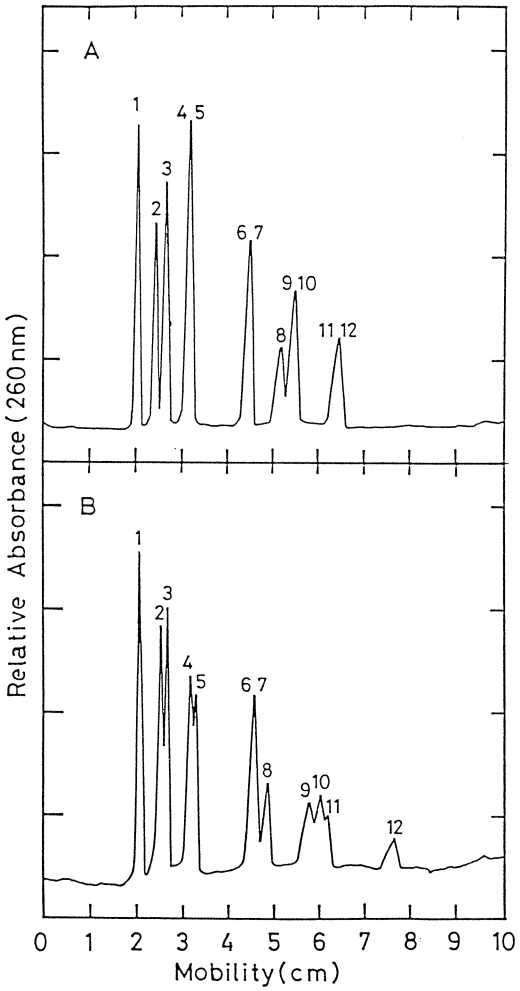


Fig. 9. Electropherograms of double-stranded RNA segments from RDV (A) and RGDV (B) in 2.5% polyacrylamide gel. About 20 μg/slot of each RNA preparation was electrophoresed for 10 hr. Each lane of the gel was scanned at 260 nm. (data from Hibi *et al.*, 1984)

Table 9. Approximate molecular weights of double-stranded RNAs of RGDV

Segment No.	Molecular weight ($\times 10^{-6}$)		
	RGDV[a)]	RDV[b)]	WTV[b)]
1	3.10	3.10	2.90
2	2.48	2.50	2.48
3	2.25	2.25	2.25
4	1.90	1.90	1.80
5	1.82	1.80	1.80
6	1.11	1.15	1.20
7	1.11	1.13	1.15
8	0.96	0.86	0.90
9	0.68	0.78	0.63
10	0.61	0.75	0.61
11	0.58	0.52	0.60
12	0.32	0.52	0.35
Total	16.92	17.26	17.67

a) Calculated by using double-stranded RNA segments of RDV as standards.
b) Data from Reddy *et al.* (1974)
(data from Hibi *et al.*, 1984)

7. Serological detection

1) Detection in plants

Latex flocculation test (LF) was employed to detect virus antigen both in infected rice and insect vectors. Antiserum obtained above was used in this experiment.

The procedure of Bercks and Querfurth (1971) was principally employed for latex sensitization with serum. The fraction which precipitates when the serum is 50% saturated with ammonium sulphate was used. Phosphate buffer (0.01 M), pH 7.0 containing 0.01 M $MgCl_2$ and 0.1% Tween 20 (PB-Mg-Tween) was used to homogenize and to dilute virus infected samples.

The homogenate was centrifuged at 3,000 g for 10 min. Serial twofold dilutions of the supernatant were made with buffer and 0.1 ml of each dilution was placed in a small test tube (11 × 75 mm). Two drops (ca. 0.1 ml) of sensitized latex suspension were then added to each tube and the tubes were shaken 20 min on a shaker (Taiyo Incubator M-100, Taiyo Sci. K. K. Chiyoda, Tokyo, Japan) at 150 oscillations per min at 30°C. As controls, sensitized latex was mixed with (i) buffer, (ii) sap from healthy plants and (iii) sap containing viruses unrelated to antiserum used to sensitize the latex. The latex particles formed aggregates in a positive reaction, or remained as a milky suspension in a negative one. Doubtful readings were checked under a microscope (× 200). All the tests were duplicated.

The transition from a positive to a negative reaction indicating the dilution end point usually was abrupt. Very weak agglutination was regarded as negative. The most distinct flocculation occurred at globulin dilutions of 1:1,200 when the protein concentration in the globulin suspension was calculated to be 1%. Intensity of flocculation did not increase when shaking was extended beyond 50 min.

As shown in Table 10, all the virus antigens were detected by LF. Maximum titers of the antigens were obtained 30—50 days after inoculation and little change in titer occurred during the period.

Table 10. Detection of RGDV antigen by latex flocculation test[a] in rice plants at various times after inoculation

Infected material	Exp. no.	Days after inoculation					
		20	30	40	50	60	70
Leaf	I	20[b]	20	20	40	40	40
	II		40	40			
Root	I	80	160	160	160	160	160
	I		160	160			

a) No flocculation was observed in healthy controls tested.
b) Figures in the Table indicate reciprocal of highest dilution with positive reaction.
(data from Omura *et al.*, 1984)

2) Detection in insects

Individual insects were crushed in 0.4 ml buffer and the crude sap was used either directly or after centrifugation at 8,000 g for 10 min. Crude sap (0.1 ml) or supernatant after centrifugation was used for the detection of virus after twofold serial dilutions. Ten insects with a positive reaction were used for each experiment. Results were the same for all the experiments both using crude sap or supernatant after centrifugation. As shown in Table 11, antigen titers did not vary significantly after the 15th day after

Table 11. Detection of virus antigen by latex flocculation test[a] in viruliferous *N. nigropictus* at various times after acquisition access

Sex	Days after acquisition access started				
	5	10	15	25	25
Female			32	32	32
Nymph	0[b]	8[c]			
Male			16	16	16

a) No flocculation was observed in healthy controls tested.
b) Negative reaction.
c) Figures in the Table indicate reciprocal highest dilutions of crushed insects as mentioned in Materials and Methods.
(data from Omura *et al.*, 1984)

the acquisition access. The result was negative when Tris-PVP was used as extraction buffer for RGDV. Use of PB-Mg improved flocculation, however, non-specific flocculation occurred in healthy control. The addition of Tween-20 to PB-Mg dispersed non-specific aggregate without affecting the specific reaction.

8. Conclusion

The present study conducted by researchers in various fields in many countries disclosed the characteristics of rice gall dwarf disease. It was important that information on the disease was accumulated before epidemics occurred (Faam *et al.*, 1983). The information on the characteristics of the disease together with the establishment of serological detection methods of the virus antigen in both infected plants and individual insect vectors will undoubtedly contribute to the control of the disease in future.

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