Rice Grassy Stunt Virus Strain Causing Tungrolike Symptoms in the Philippines

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ABSTRACT

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A virus disease of rice causing tungrolike symptoms (yellowing and stunting) was observed in the Philippines. The virus is transmitted by the brown plant hopper (*Nilaparvata lugens*) in a persistent manner and has an incubation period in the insect ranging from 5 to 21 days. Based on symptomatology, virus-vector interaction, serological reaction, and particle morphology, the causal virus was identified as a strain of rice grassy stunt virus. The new strain was designated rice grassy stunt virus 2 (RGSV-2), and the type strain was designated rice grassy stunt virus 1 (RGSV-1). Aside from causing tungrolike symptoms in the field, RGSV-2 differs from RGSV-1 in pathogenicity to rice varieties. In inoculation tests, rice varieties resistant to RGSV-1 were susceptible to RGSV-2.

An unknown virus disease of rice (*Oryza sativa*) characterized by stunting, yellowing of leaves, spreading growth habit, and premature death of infected plants was observed in Laguna and South Cotabato, Philippines, during 1982–1983. Symptoms were similar to those caused by rice tungro disease (15) (Fig. 1). In our preliminary studies (1), however, the causal virus could not be transmitted by the tungro vectors *Nephotettix virescens, N. nigropictus,* and *Recilia dorsalis*. Instead, the virus was transmitted by the brown plant hopper (BPH) (*Nilaparvata lugens* (Stål)). We are reporting results of further studies conducted on the "new" virus disease, which we identified as a distinct strain of rice grassy stunt virus (RGSV) and designated it RGSV-2.

MATERIALS AND METHODS

Source of infected plants. RGSV-2 was isolated from a plant collected from the field at the International Rice Research Institute (IRRI), Laguna, Philippines, using the BPH. An RGSV isolate (RGSV-1) that has been maintained for many years at IRRI was used for comparing symptoms, pathogenicity to different rice cultivars, and serological reactions.

Transmission. Second-instar BPH nymphs were given access to RGSV-2-diseased plants for 1, 2, or 4 days, then transferred singly to 7-day-old rice seedlings, Taichung Native 1 (TN1), in test tubes and kept for 24 hr. The test insects were transferred to fresh TN1 seedlings daily until they died. All inoculated plants were transplanted to pots and kept in the greenhouse until symptoms appeared. In symptom-comparison experiments, test plants were inoculated simultaneously with RGSV-1 and RGSV-2 when either 7 days old (seedling stage) or 30 days old.

To determine the shortest acquisition access time, second-instar BPH nymphs were given access to RGSV-2-diseased plants for 15, 30, 60, and 120 min. The insects were kept in healthy TN1 seedlings until the presumed incubation period of the virus in the insect (9–10 days) was over. The insects were then used to inoculate TN1 seedlings in test tubes serially for 20 consecutive days. The percentage of infective insects for each acquisition access time was recorded.

The shortest inoculation access time was determined by exposing healthy TN1 seedlings planted in pots to BPH nymphs, previously given 10 days of access to RGSV-2, for 5, 10, 15, 30, and 60 min. The insects were removed, and inoculated seedlings were placed in screened cages until symptoms appeared. The percentage of infected plants was noted for each inoculation access time.

Tests were also conducted to transmit the virus by sap, seed, or soil (soil-inhabiting organisms). TN1 plants at the three-leaf stage were inoculated manually by rubbing the leaf blades with the sap of RGSV-2-infected plants, using Carborundum (600-mesh) as an abrasive. Mature seeds from infected plants were germinated and grown until maturity to test transmission by seed. Also, diseased plants together with the surrounding soil were collected from the field and placed in plastic trays. Healthy TN1 seedlings were transplanted around the diseased plants to test transmission by soil-inhabiting organisms. All test plants were kept in screened cages and observed for 2 mo for appearance of symptoms.

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Fig. 1. Discoloration and stunting of rice plants (foreground) caused by rice grassy stunt virus 2.

Results of the transmission studies were compared with those of RGSV-1 transmission studies conducted at IRRI (12). No transmission study was conducted for the Japanese isolate of RGSV (RGSV-J).

Virus purification and electron microscopy. Three hundred grams of RGSV-2-infected plants with roots were homogenized in 900 ml of ice-cold extraction buffer (0.1 M phosphate buffer, pH 7.2, containing 0.01 M Na_2SO_3 and 0.01 M sodium diethyldithiocarbamate) using a commercially available meat grinder. The homogenate was filtered through cheesecloth and centrifuged at 13,000 *g* for 20 min. Magnesium bentonite (1.25 mg/ml) (5) was then added to the supernatant, stirred for about 5 min, and centrifuged for 20 min at 13,000 *g*. Carbon tetrachloride (20%, v/v) was added to the supernatant, stirred for about 3 min, and centrifuged at 13,000 *g* for 20 min. The aqueous layer was collected. Polyethylene glycol 6000 (5%), Triton X-100 (1%), and NaCl (0.2 M) were added, stirred for about 40 min, and centrifuged at 13,000 *g* for 20 min. The pellet was resuspended in 30 ml of 1:10 extraction buffer (pH 7.2) and centrifuged at 10,000 *g* for 15 min. The resulting supernatant was again centrifuged at 130,000 *g* for 90 min. The pellet was suspended in 1 ml of 0.01 M phosphate buffer (pH 7.2) and centrifuged at 10,000 *g* for 15 min. Then the supernatant was centrifuged at 54,000 *g* for 180 min in 10–45% linear sucrose density gradient columns prepared in 0.01 M phosphate buffer (pH 7.2). The gradient columns were scanned at 254 nm and fractionated with an ISCO (model 640) density gradient fractionator. The virus-containing fraction (ultraviolet absorbing zone) was collected and centrifuged at 130,000 *g* for 90 min. The virus pellet was resuspended in 1 ml of 0.01 M phosphate buffer (pH 7.2) and centrifuged at 10,000 *g* for 15 min. The resulting supernatant was collected and stored at 4 C. RGSV-1 and RGSV-J were purified similarly. RGSV-1 and RGSV-2 were multiplied in IRRI and purified in Japan; RGSV-J was multiplied and also purified in Japan.

For electron microscopic examination, purified virus in 0.01 M phosphate buffer was mounted on a grid covered with carbon-coated collodium membrane and negatively stained with uranyl acetate (1%). The prepared grids were examined under a Hitachi H-500 electron microscope.

Serology. A rabbit was immunized against the purified virus fraction by two intramuscular injections, with the antigen emulsified with an equal volume of Freund's complete adjuvant followed by one intravenous injection. Injections were made at 2-wk intervals; 1 ml of virus fraction with A_{260} adjusted to 1.0 was used in each injection. The antiserum was collected about 1 wk after the third injection. The ring-interphase precipitin test was performed using purified virus fraction with an A_{260} of 0.2. An immunodiffusion test was conducted in 0.8% agar gel containing 1% sodium dodecyl sulfate and 0.005 M NaN_3. Antiserum prepared in Japan against RGSV-J (11) was used in reciprocal tests.

Varietal reaction. Different rice varieties were inoculated with RGSV-2 using the mass screening method for RGSV (12) at IRRI. The reactions of these varieties to RGSV-2 were compared with their reactions to RGSV-1.

RESULTS

Transmission. RGSV-2 was acquired and transmitted by *N. lugens* in a persistent manner. The incubation period of the virus ranged from 5 to 21 days (av. 8.4 days). Five to 20% of the insects used in the test were infective. The shortest acquisition and inoculation access times were 1 hr and 15 min, respectively. About 2% of the insects transmitted RGSV-2 after 1 hr of acquisition access, whereas 10% of the plants were infected after 10 min of inoculation access. The percentage of infective insects and infected seedlings increased as the acquisition and inoculation access times were increased to 4 days and 24 hr, respectively. The virus was not transmitted by the nymphs that hatched from eggs of virus-exposed BPH females, which indicates that transovarial passage of the virus did not occur. Attempts to transmit the virus by sap, soil, and seed gave negative results. Transmission characteristics of RGSV-2 did not differ significantly from those reported for RGSV (12,16).

Symptoms. Symptoms appeared 7–14 days after inoculation of 7-day-old TN1 seedlings. Early symptoms consisted of stunting, yellow to orange discoloration and rusty spotting of the lower leaves, and narrowing of the leaf blades. Young emerging leaves were pale green or sometimes almost entirely chlorotic. Striping or mottling symptoms on emerging leaves were observed also. Severely infected seedlings produced very few small tillers and died 3–4 wk after inoculation. TN1 seedlings that were mildly affected by RGSV-2 tillered profusely (similar to RGSV-1) about 6 wk after inoculation, but they were more stunted and leaves remained yellow or pale yellow even when adequately fertilized (Fig. 2). In contrast, RGSV-1 symptoms appeared 17–21 days after inoculation. The first discernible symptoms were stunting and paling of the newly unfolded leaves. Later, numerous diminutive tillers were produced and

Fig. 2. TN1 plants healthy or infected with rice grassy stunt virus 1 and 2 (RGSV-1 and RGSV-2) 6 wk after inoculation.

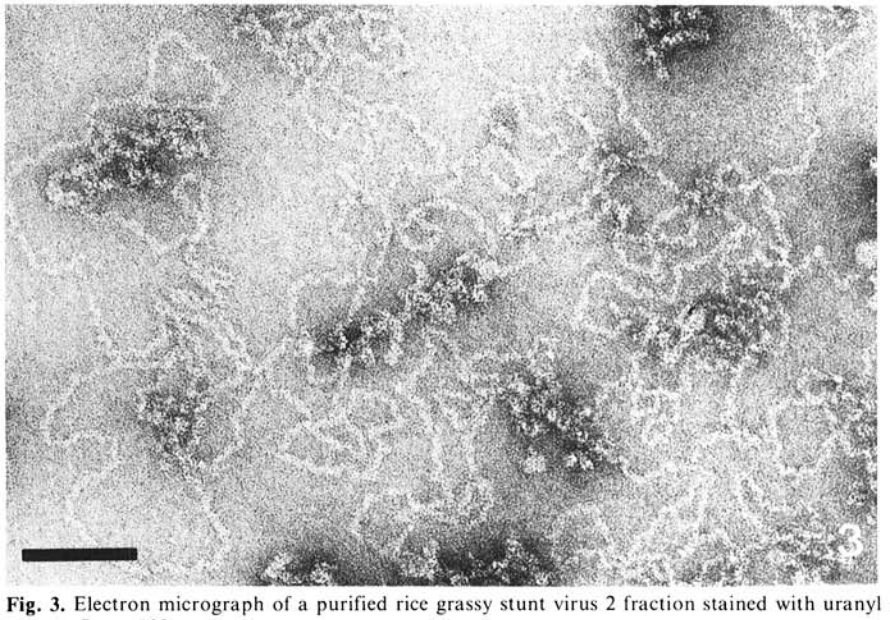

Fig. 3. Electron micrograph of a purified rice grassy stunt virus 2 fraction stained with uranyl acetate. Bar = 100 nm.

occasional yellowing and rusty spotting of the basal leaves were observed. Mature leaves turned dark green when nitrogen fertilizer was applied. Except for these differences, the plants infected with either RGSV-1 or RGSV-2 at seedling stage were generally similar; however, RGSV-2 caused tungrolike symptoms (stunting and yellow to orange discoloration of leaves) on different rice varieties infected under field conditions. This type of symptom was reproduced in the greenhouse when TN1 and other IRRI varieties were inoculated with RGSV-2 when 30 days old or more. On the other hand, RGSV-1 did not show similar symptoms when plants of the same age were inoculated. TN1 plants infected with either RGSV-1 or RGSV-2 at a later stage of growth produced small and incompletely emerged panicles bearing mostly empty dark brown to black grains.

Virus purification and electron microscopy. After sucrose density gradient centrifugation, a single light-scattering zone was observed 1.5–2 cm below the meniscus. The absorption spectrum of the purified virus fraction had a maximum absorbance at 260 nm and minimum at 246–247 nm, which is typical for a nucleoprotein. The average ratio of ultraviolet absorption of 260 and 280 nm ($A_{260/280}$) for the purified virus fraction was 1.29.

Electron microscopic examination of the purified virus fractions revealed filamentous particles (6–8 nm in diameter) of various lengths and some cellular components (Fig. 3). Some of the filaments were circular.

Serology. In ring-interphase precipitin tests, the antiserum to RGSV-2 had a titer of 1/640 against both purified homologous virus and RGSV-J. Antiserum to RGSV-J reacted with RGSV-2 and homologous virus up to dilutions of 1/640 and 1/1,280, respectively. In immunodiffusion tests, single reaction bands were formed between antiserum to RGSV-J and either RGSV-2 or RGSV-J, and the reaction bands fused (Fig. 4A). Similarly, reaction bands between the antiserum to RGSV-2 and the antigen of RGSV-1 and RGSV-2 also fused (Fig. 4B).

Varietal reaction. *O. nivara* (wild rice species), the only known source of genes for resistance against RGSV-1, was susceptible to RGSV-2. Two and 93% of *O. nivara* plants inoculated with RGSV-1 and RGSV-2, respectively, were infected. Consequently, IRRI varieties that possess *O. nivara* genes for resistance to RGSV-1 are also susceptible to RGSV-2 (Table 1).

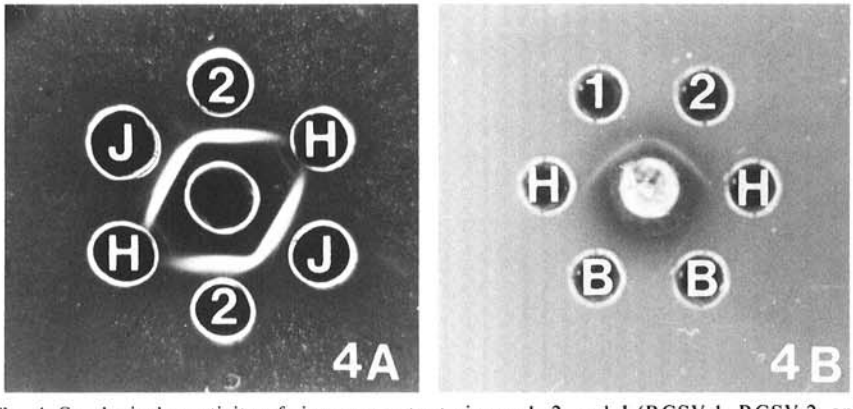

Fig. 4. Serological reactivity of rice grassy stunt viruses 1, 2, and J (RGSV-1, RGSV-2, and RGSV-J). **(A)** Center well contains antiserum to RGSV-J and peripheral wells contain RGSV-J (J), RGSV-2 (2), and clarified healthy sap (H). **(B)** Center well contains antiserum to RGSV-2 and peripheral wells contain RGSV-1 (1), RGSV-2 (2), clarified healthy sap (H), and buffer (B).

Table 1. Reactions of International Rice Research Institute varieties to rice grassy stunt virus 1 (RGSV-1) and virus 2 (RGSV-2)[a]

	RGSV-1		RGSV-2	
Variety	No. infected/ no. inoculated	Percent infection	No. infected/ no. inoculated	Percent infection
IR29	48/183	26.2	66/73	90.4
IR30	81/309	26.2	64/77	83.1
IR32	156/548	28.5	78/87	89.7
IR34	101/489	20.7	59/65	90.8
IR38	73/367	19.9	65/75	86.7
IR40	57/308	18.5	49/58	84.5
IR45	16/175	9.1	82/85	96.5
IR48	35/342	10.2	67/77	87.0
IR50	91/560	16.3	81/92	88.0
IR52	27/264	10.2	69/75	92.0
IR54	3/41	7.3	69/72	95.8

[a] Varieties with 0–30% infected plants are considered resistant; 31–60%, moderately resistant; and 61–100%, susceptible.

DISCUSSION

Three rice virus diseases have been reported to be transmitted by *N. lugens*: grassy stunt (RGSV) (16), ragged stunt (RRSV) (9,13), and wilted stunt (RWSV) (4,5). RRSV is easily separable from the other two diseases by its distinct symptoms and particle morphology (9,13). RGSV-2, on the other hand, resembles RGSV-J, RGSV-1, and RWSV in symptomatology. In this study, however, RGSV-2 was compared only with RGSV-1 because of quarantine regulations.

In all varieties tested, RGSV-2 caused more severe symptoms than RGSV-1 under both greenhouse and field conditions. RGSV-2 infection often resulted in premature death, particularly when plants were infected at the seedling stage. Those that did not die, however, showed profuse tillering very similar to that caused by RGSV-1 but remained pale yellow and were more stunted than the RGSV-infected plants. This type of symptom is very similar to that of RWSV (2,3). In addition, RGSV-2 caused tungrolike symptoms in both greenhouse and field conditions. This symptom was produced only when the plants were infected at a later stage of growth. In our field observations, it became very difficult to distinguish plants infected with tungro disease from those infected with RGSV-2. In most cases, RGSV-2 infection was mistaken for tungro disease. This kind of symptom was not observed for RGSV-1 and has not been reported before for this type strain (16).

A more significant difference between RGSV-1 and RGSV-2 was the ability of the latter to attack RGSV-1-resistant varieties. Rice varieties with *O. nivara* genes for resistance to RGSV-1 were susceptible to RGSV-2 under both greenhouse and field conditions. It is not uncommon, however, for plant virus strains to differ in symptom severity and/or virulence. Some rice varieties resistant to RGSV at IRRI were susceptible in India (6). It is possible that a similar strain occurs there.

In transmission characteristics, RGSV-2 did not markedly differ from RGSV-1 (15). The minor differences could be due to the difference in transmissive ability of insect colonies used. Variation in proportion of active transmitters among insect colonies has been observed before (12).

The filamentous nucleoproteins isolated from RGSV-2-infected plants were morphologically and serologically similar to those of RGSV-J, which confirmed our previous finding on the serological relation between the two (8). Such filamentous particles were not observed from healthy plants. Moreover, antisera to RGSV-2 and RGSV-J did not react to clarified extract from healthy plants. Similar filamentous particles were reported to be associated with plants

infected with rice *hoja blanca* (14), rice stripe (17), and maize stripe disease (6). However, we were not able to test the infectivity of the RGSV-2-associated filamentous nucleoprotein.

A serological relationship between RSV and RGSV-J has been demonstrated (10). The morphological similarity and serological relationship between RSV and RGSV-J, together with homologous serological reactions shown in this study, indicated that the isolated filamentous nucleoprotein is the grassy stunt virus. Our results showed that RGSV-J, RGSV-1, and RGSV-2 were morphologically and serologically indistinguishable. The relationship between RGSV-2 and RWSV remains uncertain.

In our recent field survey, RGSV-2 was predominant at Laguna, Philippines. A disease similar to RGSV-2 occurred also in Thailand (7), and rice plants showing symptoms similar to those of RGSV-2 were observed in Indonesia in 1981 and 1982 (H. Hibino, *unpublished*).

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