

Isolation, Purification, and Serology of Rice Tungro Bacilliform and Rice Tungro Spherical Viruses

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ABSTRACT

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Rice seedlings were inoculated by rice green leafhoppers (*Nephotettix virescens*) that had fed on rice plants infected with both rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV). Rice tungro spherical virus-infected plants were identified and selected using antiserum to rice waika virus which is very closely related, if not identical to, RTSV. Rice tungro spherical virus was propagated by inoculating rice seedlings using leafhoppers. To multiply RTBV, seedlings were inoculated by leafhoppers that had fed first on plants infected with both RTBV and RTSV, second on anti-RTSV immunoglobulin through membrane, and then on RTBV-infected plants. Rice tungro bacilliform virus and RTSV were purified separately from their respectively infected plants by heating sap 1 hr at 40 C, by driselase treatment, and by polyethylene glycol precipitation, differential centrifugations, and sucrose density gradient centrifugation. Purified RTBV fractions contained bacilliform particles 30–35 nm in width and 160–220 nm in length. Purified RTSV fractions contained isometric particles 30 nm in diameter. Both fractions had UV absorption spectra typical of nucleoprotein. Rabbit antisera obtained had titers of 1/2,560 for RTBV and 1/640 for RTSV by the ring-interface precipitin test. The latex test and ELISA specifically detected RTBV and RTSV in leaf extracts. The antisera were virus-specific.

Of all the known virus diseases of tropical Asian rice (*Oryza sativa* L.), tungro is the most important because of its wide distribution and destructiveness (12). It is a composite disease caused by rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV) (2,6,7,11,14,15). Both viruses are transmitted by the green leafhopper (GLH) (*Nephotettix virescens* Distant), and other leafhopper species in a semi-persistent manner (6,7,11). Rice tungro spherical virus is transmitted independently, while RTBV requires RTSV or a RTSV-related “helper” factor for transmission by GLH (2,7,11). Hence, RTBV is transmitted only when virus source is infected with both RTBV and RTSV, or when GLH acquires first RTSV then RTBV. Green leafhoppers that fed on plants infected with RTBV and RTSV generally transmit RTBV and

RTSV together or RTBV alone, and occasionally RTSV alone (7,11). Generally, plants infected with both viruses show severe tungro symptoms, including yellowing and stunting of plants, while RTBV-infected plants show mild stunting and yellowing. Rice tungro spherical virus-infected plants have indistinct symptoms.

Formulation of effective control measures and studies on epidemiology require rapid and accurate diagnosis. Diagnosis of tungro by symptomatology or the iodine test are unreliable, and the transmission test is time-consuming (12). Tungro-like symptoms can be caused by infection with a strain of grassy stunt virus (3,10), rice transitory yellowing virus, the orange leaf agent (12), by insect feeding, and by physiological disorders. Serology is more rapid and reliable for tungro diagnosis (1,13). However, serological identification of tungro is limited by the availability of antisera to RTBV and RTSV.

Concentration of RTBV and RTSV in plants is low, and purification, especially of RTBV, is difficult. For the first time, Omura et al (14) purified RTBV and RTSV separately from plants infected

with both viruses. They isolated RTSV by three cycles of sucrose density gradient (SDG) centrifugation followed by equilibrium centrifugation in cesium chloride. Also, after three cycles of SDG centrifugation, they isolated RTBV from the virus mixture by incubating virus fractions three times with antiserum to rice waika virus (RWV), which precipitates RTSV (8,15). Their purification procedure is complicated and time-consuming. In this paper, we describe separate propagation of RTBV and RTSV, and simplified purification and serology. A preliminary report was published (8).

MATERIALS AND METHODS

Virus, insect, and test plant. The tungro isolate was maintained in a greenhouse, and a virus-free GLH colony was reared on rice cultivar Taichung Native 1 (TN1). Only adult GLH were selected for virus transmission. Green leafhoppers were fed for 3 days on tungro-infected TN1 plants. For inoculation, GLH and 7-day-old TN1 seedlings were confined in test tubes at one leafhopper per seedling for 1 day. Inoculated seedlings were transplanted in clay pots and grown in screened cages.

Isolation and propagation of RTSV. One month after inoculation with tungro, all “healthy”-looking seedlings were tested for RTSV by the latex test using antiserum to RWV (courtesy of T. Omura, Tsukuba, Japan). Rice tungro spherical virus was transmitted from RWV-reactive plants. It was propagated in TN1 by serial transfer using GLH. At 50–60 days after inoculation, all inoculated plants were tested by the latex test and virus was purified from infected plants.

Isolation and propagation of RTBV. Seedlings showing tungro symptoms were also indexed for RTSV by the latex test (RTSV antiserum was produced first). Plants free from RTSV served as RTBV sources. Since GLH inefficiently inoculate RTBV alone from plants with both RTBV and RTSV, GLH that would

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transmit only or mostly RTBV were obtained by infectivity neutralization technique, as described by Hibino and Cabauatan (9). Green leafhoppers that fed on plants infected with both viruses were then fed for 16 hr through parafilm membranes on anti-RTSV immunoglobulin (IgG) diluted 25 times with 2% sucrose solution. Green leafhoppers were transferred to RTBV-infected plants for an acquisition access feeding of 0–24 hr, then were transferred to healthy seedlings. One month after inoculation, all plants with tungro symptoms were indexed for the presence of RTSV in ELISA. Plants free from RTSV were transplanted singly in clay pots and grown in screened cages. All selected plants were indexed once more in ELISA to confirm absence of RTSV 2 days before purification.

Purification of viruses. The same purification procedure was adapted for both RTBV and RTSV. About 500 g of plants (without roots) either infected with RTBV or RTSV were harvested about 50 days after inoculation and homogenized in 1 L of 0.01-M sodium ethylenediaminetetraacetate (EDTA) (pH 8.0). After incubation with 3 g of driselase (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) for 1 hr at room temperature, the extract was kept for 1 hr in a waterbath at 40 C then centrifuged for 10 min at 15,000 g. The supernatant was made 7% with respect to polyethylene glycol (mol wt 8,000), 0.2-M NaCl, and 1% Triton X-100, and then stirred for 1 hr at room temperature. The mixture was centrifuged for 30 min at 30,000 g and the pellet was suspended in 20 ml of cold 0.01-M EDTA (pH 8.0). The suspension was centrifuged at 11,000 g for 10 min and the supernatant at 100,000 g for 60 min. The pellet was resuspended in 2 ml of cold 0.01-M phosphate buffer (PB) (pH 7.4) and centrifuged at low speed. The supernatant was layered on 10–50% linear SDG in PB and centrifuged in a Beckman SW27 rotor at 25,000 rpm for 2.5 hr at 4 C. Tube contents were scanned at A_{254nm} and fractionated using Model 640 fractionator (ISCO, Lincoln, NE). The virus-containing fraction was recovered, diluted with 0.01-M PB, and centrifuged for 1 hr at 130,000 g. The pellet was suspended in 1-ml PB and centrifuged for 10 min at 11,000 g. This suspension was used for electron microscopy and immunization.

Electron microscopy. The purified virus suspension was diluted with distilled water and applied on grids coated with a collodion membrane. After removing excess solution with a filter paper, a drop of 2% uranyl acetate or neutralized 2% phosphotungstic acid was added to the grids. The excess stain was removed with a filter paper and grids were air-dried. The grids were examined in an EM 410 electron microscope (Philips, The Netherlands) at 80 kV.

Lumunization and serology. Rabbits

were separately immunized with RTBV and RTSV by four intramuscular injections followed by one intraveinal injection at weekly and biweekly intervals for RTBV and RTSV, respectively. Purified RTBV and RTSV suspensions, for which A_{260} (1-cm light path) were adjusted to 1.0 and 0.7, respectively, were injected directly into the vein or into the leg muscles after they were emulsified 1:1 with Freund's complete adjuvant. Rabbits were sacrificed 1 wk after the intraveinal injection.

Antiserum titer was determined by the ring-interface precipitin test (17). Immunoglobulin was purified by ammonium sulfate precipitation and diethylaminoethyl (DEAE)-cellulose column chromatography; IgG-alkaline phosphatase conjugate was prepared as described by Clark and Adams (4). Appropriate concentrations of coating IgG and conjugate were determined following standard ELISA procedures (4). Latex suspensions were sensitized with either anti-RTBV or anti-RTSV IgG as described by Omura et al (13). Leaf samples about 10 cm long were using a combined leaf and bud press (Erich Pollahne, Wennigsen, West Germany) and extracts were directly tested by ELISA or latex test, as described previously (1,13). Absorbance at 405 nm was determined with Microelisa Minireader II (Dynatech Corp., Chantilly, VA).

RESULTS

Isolation and propagation of viruses.

Of more than 200 plants inoculated with tungro, only two symptomless plants reacted to RWV antiserum. About 70% of adult GLH fed for 3 days on these two plants transmitted RTSV. All plants became infected when inoculated with three GLH per seedling. Thus, GLH rarely transmitted RTSV alone from doubly infected plants, but easily transmitted RTSV from single infections.

Transmission efficiency increased when the feeding period on RTBV-infected plants increased from 0 to 24 hr after sequential access feedings to RTBV-RTSV complex and then to anti-RTSV IgG (Table 1). Rice tungro bacilliform

virus transmission efficiency also increased when the feeding period was increased. Based on these results, GLH were fed on RTBV-infected plants for 8 hr before inoculation feeding. As a result, 95–100% of infected plants contained RTBV alone.

Purification and serology. RTSV.

After the SDG, a distinct band was observed about 3 cm below the meniscus. The purified RTSV fraction contained isometric particles about 30 nm in diameter (Fig. 1A). The purified fraction had a maximum UV absorption at 259–260 nm and minimum at 239–240 nm. The absorbance ratio at 260 and 280 nm ($A_{260/280}$) ranged from 1.48 to 1.72 (corrected for light scattering). The method yielded 1 ml of purified virus suspensions with A_{260} ranging from 1.0 to 2.8.

An antiserum of a titer by the ring-interface test of 1:640 was obtained. In the latex test, latex sensitized with 500 times diluted anti-RTSV IgG detected RTSV in 80 times diluted leaf extracts. In ELISA, optimum IgG concentration was 2 μ g/ml for coating, and optimum conjugate dilution was 500 times. ELISA detected RTSV antigen in 320 times dilution leaf extracts. Rice tungro spherical virus IgG did not react with either purified RTBV or healthy plant sap in either the latex test or ELISA. Absorbance at 405 nm of infected samples (1/10 dilution) in ELISA ranged from 0.25 to 2.24 compared with 0.00 to 0.05 for healthy controls. Both tests could detect RTSV in naturally infected plants.

RTBV. A distinct band was visible at almost the same depth as the band of RTSV after the SDG. The band was a little broader than RTSV. The purified fraction contained bacilliform particles about 30–35 nm in width with length (200 particles counted) ranging from 110 to 310 nm and a modal contour length of 180–190 nm (Fig. 1B; Fig. 2). Maximum UV absorption for purified RTBV was at 258–259 nm and minimum at 243–244 nm. $A_{260/280}$ ranged from 1.10 to 1.17 (corrected). About 1 ml of purified virus suspensions with A_{260} ranging from 0.8 to 1.5 were obtained.

The antiserum had a ring-interface

Table 1. Rice tungro bacilliform virus (RTBV) transmission by green leafhoppers (GLH) *Nephotettix virescens* given sequential access feeding on plants infected with RTBV and rice tungro spherical virus (RTSV) for 3 or 4 days; on anti-RTSV serum for 16 hr; and on RTBV-infected plants for 0, 8, or 24 hr

Access to RTBV (hr)	GLH tested (no.)	GLH that transmitted			Infective GLH ^a (%)	GLH that transmitted RTBV ^b (%)
		RTBV + RTSV	RTBV	RTSV		
0	245	7	53	0	24.4	88.3
8	193	6	117	0	63.7	95.1
24	150	3	96	0	66.0	96.9

^aTotal number of transmitters/total number of insects tested. Average of 4 trials.

^bNumber of RTBV transmitters/total number of transmitters. Average of 4 trials.

titer of 1:2,560. In the latex test, latex sensitized with 1,000 times dilution anti-RTBV IgG detected RTBV in 160 times dilution leaf extracts. In ELISA, optimum IgG concentration was 1 μ g/ml for coating, and optimum conjugate dilution was 1,000 times. ELISA detected RTBV in 320 times dilution leaf extracts. Rice tungro bacilliform virus IgG did not react with purified RTSV in either the latex test or ELISA. Absorbances at 405

nm of infected samples in ELISA ranged from 0.61 to 2.24 compared with 0.00 to 0.06 for healthy controls. Both tests detected RTBV in naturally infected plants.

DISCUSSION

Rice viruses are generally difficult to purify because rice tissues contain little virus and are difficult to homogenize. Both tungro-associated viruses are phloem restricted (5) and the two viruses are difficult to separate biologically. Susceptible cultivars are often infected with both RTBV and RTSV. Although propagation from doubly infected plants is much easier, purification of the viruses from these plants is laborious (14). Besides, purity of virus suspensions thus obtained is difficult to guarantee, especially without specific antisera. In these studies, RTSV was separated from RTBV and individually propagated before purification. Rice tungro bacilliform virus-infected plants were maintained by inoculating seedlings with GLH that had fed on anti-RTSV IgG (9). Using plants infected with either RTBV or RTSV, the viruses were easily purified although the yield was still low.

The purification procedure adapted in these studies was simple and applicable to both RTBV and RTSV. Heat treatment of extracts at 40 C for 1 hr was sufficient to clarify the preparation, thereby eliminating the need for organic solvents such as CCl_4 . Addition of driselase (16) eliminated most of the sticky precipitate that often accompanied polyethylene glycol treatment. As a result, virus fractions with high $A_{260/280}$ values were obtained even after only one cycle of SDG centrifugation. Two cycles of SDG centrifugation gave even higher $A_{260/280}$ values, but with a concomitant decrease in virus yield. ELISA and the latex test showed that the purification procedure gave serologically pure viruses. The antisera are currently being used in tungro disease diagnosis, epidemiological studies, and in evaluating cultivars for resistance to RTBV and RTSV.

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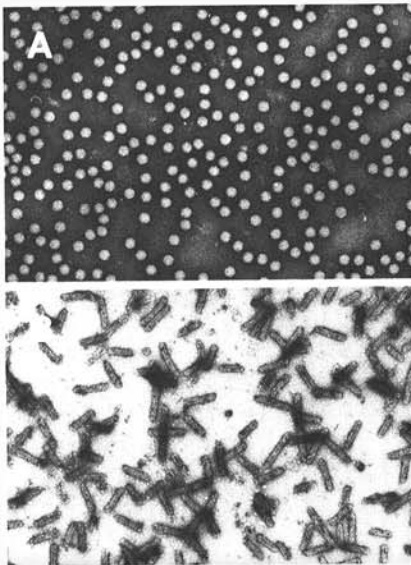


Fig. 1. Electron micrograph of (A) purified rice tungro spherical virus in neutralized 2% phosphotungstic acid and (B) rice tungro bacilliform virus in 2% uranyl acetate. ($\times 50,000$)

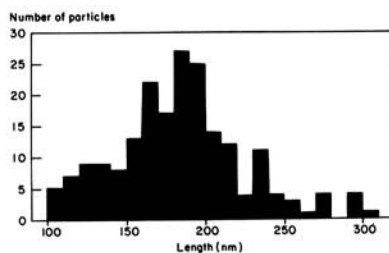


Fig. 2. Particle length distribution of purified rice tungro bacilliform virus.