Detection of Rice Viruses in Plants and Individual Insect Vectors by Latex Flocculation Test

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ABSTRACT

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The latex flocculation test (LF) was employed to detect virus antigens in infected rice plants and viruliferous insects. Reciprocal of dilution end points for infected plants tested 40 days after inoculation were 160, 40, 5,120, 5,120, 10,240, 40, and 80 for rice gall dwarf (RGDV), rice ragged stunt (RRSV), rice dwarf (RDV), rice grassy stunt (RGSV), rice stripe (RSV), rice tungro bacilliform (RTBV), and rice tungro spherical (RTSV) viruses, respectively. Virus antigens were detected in the following individual insects: *Nephotettix nigropictus* viruliferous for RGDV or RDV, *Nilaparvata lugens* viruliferous for RGSV, and *Laodelphax striatellus* viruliferous for RSV. The inability to detect RRSV in viruliferous *N. lugens* was caused by interference by an insect-host component. Sensitivity of LF for detecting RGDV was compared with the agar gel diffusion test and enzyme-linked immunosorbent assay.

Virus-infected rice plants have been identified mainly by visual symptoms or by insect transmission of the virus to assay plants. Visual symptoms are not always specific in the field because more than one virus may cause similar symptoms in rice plants, and many nonpathogenic disorders such as nutritional deficiencies, excess water after draught, or insect injury may cause viruslike symptoms. Insect transmission assays are laborious as well as time-consuming; some persistent viruses require 2 wk of incubation in the vector and another 2–3 wk for symptom development in test plants. Moreover, virus-free vector populations must be available at various stages for transmission assays. On the other hand, serological assays are usually specific, and in some cases, sensitive enough to detect viruses, even in individual insects (4,10,19); however, serological techniques are often complex and may require special facilities.

For rapid identification of rice viruses and for epidemiological studies of rice virus diseases, serological tests with the following attributes are needed: 1) a simple method that can be used in the laboratory without sophisticated equipment, 2) able to detect virus antigens in crude sap, 3) sensitive enough to detect virus antigens in individual insects, and 4) applicable for many samples in a short time. In this report, the latex flocculation test (LF), which seems to satisfy the abovementioned conditions, was employed and compared with other serological techniques to detect rice gall dwarf (RGDV) (11), rice dwarf (RDV) (16), rice ragged stunt (RRSV) (5), filamentous nucleoprotein associated with rice grassy stunt disease (RGSV) (14), rice stripe (RSV) (6,9), rice tungro bacilliform (RTBV), and rice tungro spherical (RTSV) viruses (15). Virus antigens were detected in infected plants as well as in viruliferous individual insects. Most of the important rice viruses found in Asia were included in these tests.

MATERIALS AND METHODS

Viruses, insects, and plants. All of the viruses used were maintained in rice plants at the Institute for Plant Virus Research, Tsukuba Science City, Japan. RGDV (11) and RDV (16) were maintained by successive transfers using the green rice leafhopper (*Nephotettix nigropictus*), and RRSV (5) and RGSV (14) were maintained by successive transfers using the brown plant hopper (*Nilaparvata lugens*). For transmission of RSV (16), the small brown plant hopper (*Laodelphax striatellus*) was used. For each of these viruses, second to third instar nymphs were fed on diseased plants for 2 days and transferred to healthy plants (cultivar Taichung Native 1) at the three- to five-leaf stage. The two semipersistent viruses associated with rice tungro disease (RTBV and RTSV) (15) were transferred using the green rice leafhopper (*Nephotettix virescens*). Adult insects were allowed to feed for 2 days on infected plants, then transferred to healthy rice seedling for 2 days or more. All inoculated plants were grown in an air-conditioned greenhouse (27 ± 3 C).

Adsorption of antibody onto latex. Antisera of RGDV (12), RDV (12), RRSV (4), RGSV (6), RTBV, and RTSV (13) had titers of 1:2,560, 1:2,000, 1:1,024, 1:1,600, 1:2,560, and 1:320, respectively, in ring-interface microprecipitin tests. Antiserum against RSV (titer 1:2,048 in precipitin ring-interface tests) was

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obtained from the Plant Virus Laboratory, Institute of the Japan Plant Protection Association.

The procedure of Bercks and Querfurth (1) was employed for latex sensitization with serum. The fraction that precipitated when serum was 50% saturated with ammonium sulfate was used. The protein concentration of globulin was measured with a Hand Protein Refractometer PRP-B (Hitachi, Ibaraki, Japan). Fractionated globulins were serially diluted (twofold to fivefold) in 0.05 M Tris-HCl, pH 7.2. One volume of the diluted globulin was mixed with 1 volume of Bacto-Latex (0.81 μm, Difco, Detroit, MI 48232) diluted 1:14 (v/v) in 0.85% NaCl and incubated for 30 min at 30 C. The mixture was centrifuged for 15 min at 8,000 *g*. Pellets were washed twice by suspending in 0.05 M Tris-HCl, pH 7.2, which contained 0.02% (w/v) polyvinylpyrrolidone (PVP, mol wt 40,000) (Tris-PVP), and centrifuging for 15 min at 8,000 *g*. The pellet was finally resuspended in one volume of Tris-PVP containing 0.05% (w/v) sodium azide and stored at 4 C.

Reactions. Based on preliminary experiments, the following buffers were used both for plant and insect tissues. Tris-PVP was used to homogenize and dilute samples infected with RGSV or RSV; this buffer caused little or no aggregation in controls. However, 0.01 M phosphate buffer, pH 7.0, containing 0.01 M $MgCl_2$ (PB-Mg) and 0.1% Tween-20 (PB-Mg-Tween) was used for RGDV, RRSV, RDV, RTBV, and RTSV because the aggregation was generally more pronounced in this buffer. Virus-infected plants were homogenized in nine times the amount of buffer (w/v). The homogenate was centrifuged 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 (about 0.1 ml) of sensitized latex suspension were then added to each tube and tubes were shaken for 20 min when using RDV, RGSV, and RSV or 50 min when using RGDV, RRSV, RTBV, and RTSV (Taiyo Incubator M-100^N, Taiyo Sci. K. K. Chiyoda, Tokyo, Japan) at 150 oscillations per minute at 30 C. For controls, sensitized latex was mixed with buffer, sap from healthy plants, and sap containing viruses unrelated to the antiserum used to sensitize the latex. 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 tests were duplicated.

Direct detection of virus antigens from infected plants was performed using glass rods to crush small pieces of the specimen in test tubes containing 0.1 ml buffer; two drops of sensitized latex were then added and treated as described before.

Individual insects were crushed in 0.4 ml buffer and used either directly or after centrifugation at 8,000 *g* for 10 min. Crude insect macerates (0.1 ml) or supernatants after centrifugation were used for detection of viruses after twofold serial dilutions. Ten viruliferous insects were used in each experiment.

Infectivity assay. For transmission tests and detection of virus antigens in insect vectors, viruliferous insects were individually transferred onto test seedlings for 2 days, then used for serological examination as mentioned before. The insects that transmitted virus in the assays were counted as active transmitters.

Gel diffusion test (GD). Concentrated samples for the gel diffusion test were prepared by high-speed centrifugation. Infected material was homogenized in nine times the amount of buffer and centrifuged at 3,000 *g* for 10 min; the supernatant was then centrifuged at 96,000 *g* for 30 min. The pellet was resuspended in one-fourth the amount of original material (v/w) with buffer and twofold dilutions were made up to four times the dilution of the original material. The method for the GD test was the same as described previously (12).

Enzyme-linked immunosorbent assay (ELISA). The procedure described by Clark and Adams (2) was followed for purification of gamma-globulin, conjugation of alkaline phosphatase with gamma-globulin, and coating and washing of polystyrene plates (Linbro Sci. Co. Inc.). Coating gamma-globulin was used at 1 μg/ml; conjugation was used at a 1:400 dilution. The method described by Hibino and Kimura (4) was followed for the process of enzyme-substrate reaction and assessment of the reaction.

RESULTS

Detection of virus antigens in rice plants. The transition from positive to negative reaction, indicating the dilution end point, was usually abrupt. Very weak agglutination was regarded as negative. The most distinct flocculation occurred at globulin dilutions of 1:1,200, 1:270, 1:800, 1:1,200, 1:1,000, 1:150, and 1:120 for RGDV, RRSV, RDV, RGSV, RSV, RTBV, and RTSV, respectively, when the protein concentration in the globulin

Table 1. Detection of virus antigens in rice plants at various times after inoculation by latex flocculation test[a]

Virus	Infected material	Days after inoculation					
		20	30	40	50	60	70
RGDV	Leaf	20[b]	20	20	40	40	40
	Root	80	160	160	160	160	160
RRSV	Leaf	10	10	10	10	0	0
	Root	20	40	40	20	20	10
RDV	Leaf	1,280	5,120	5,120	5,120	5,120	2,560
RGSV	Leaf	5,120	5,120	5,120	5,120	5,120	5,120
RSV	Leaf	10,240	10,240	10,240	10,240	10,240	10,240
RTBV	Leaf	20	80	40	40	40	40
RTSV	Leaf	40	80	80	80	80	80

[a] No flocculation was observed in any of the healthy controls for all viruses tested.
[b] Reciprocal of the highest dilution with positive reaction.

Table 2. Detection of virus antigens in viruliferous insects at various times after acquisition access by latex flocculation test[a]

Virus	Insect	Sex	Days after acquisition access started					
			5	10	15	25	30	35
RGDV	*Nephotettix nigropictus*	Female	···	···	32	32	···	32
		Nymph	0[b]	8[c]	···	···	···	···
		Male	···	···	16	16	···	16
RRSV	*Nilaparvata lugens*	Female	···	···	0	0	···	0
		Nymph	0	0	···	···	···	···
		Male	···	···	0	0	···	0
RDV	*N. nigropictus*	Female	···	···	32	64	···	32
		Nymph	0	8	···	···	···	···
		Male	···	···	16	32	···	16
RGSV	*N. lugens*	Female	···	···	64	64	64	···
		Nymph	0	16	···	···	···	···
		Male	···	···	32	16	16	···
RSV	*Laodelphax strialellus*	Female	···	···	16	16	16	···
		Nymph	0	4	···	···	···	···
		Male	···	···	8	8	8	···

[a] No flocculation was observed in any of the healthy controls for all the viruses tested.
[b] Negative reaction.
[c] Reciprocal of the highest dilutions of crushed insects as mentioned in Materials and Methods.

Table 3. Relationship between infectivity of vectors and the ability to detect viral antigens in their extracts by latex flocculation test (LF)[a]

Virus	Insect	Sex	Number of vectors				
			Tested	Positive[b]	Negative[c]	Transmitters	Nontransmitters
RGDV	*Nephotettix nigropictus*	Female	60	58		55	3
					2	0	2
		Male	60	58		56	2
					2	0	2
RDV	*N. nigropictus*	Female	60	25		11	14
					35	0	35
		Male	60	35		12	23
					25	0	25
RGSV	*Nilaparvata lugens*	Female	60	11		6	5
					49	0	49
		Male	60	15		8	7
					45	0	45
RSV	*Laodelhax striatellus*	Female	60	21		3	18
					39	0	39
		Male	60	27		4	23
					33	0	33

[a] Thirty healthy insects (female or male) of each species were used as controls. All produced negative reactions in LF and transmission tests.
[b] Positive reaction by LF test.
[c] Negative reaction by LF test.

suspension was calculated to be 1%. Intensity of flocculation did not increase when shaking was extended beyond 20 min for RDV, RGSV, and RSV and 50 min for RGDV, RRSV, RTBV, and RTSV.

As shown in Table 1, 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 this period with all the viruses tested. Antigen concentrations were higher in roots than in leaves of plants infected with RGDV or RRSV. Antigen titer was the same using the leaf with galls or that without galls for RGDV or RRSV. Antigen concentrations were higher in leaves than in roots with the rest of the viruses. Antigen concentrations decreased with time after inoculation in RRSV-infected plants, which recovered from the disease with increasing time. Antigen titers of plants infected with RDV, RGSV, or RSV were much higher than those of plants infected with RGDV, RRSV, RTBV, or RTSV.

In direct detection of virus antigens, reactions were easily detected with the naked eye using crude sap from 1–32 mm^2 of leaf tissue infected with RDV, RGSV, or RSV. To detect RTBV and RTSV, leaf pieces larger than 8 mm^2 were needed and the reactions had to be checked under a microscope. When leaf pieces larger than 32 mm^2 were homogenized in 0.1 ml buffer, the homogenate become viscid and the reaction was not observed visually or under a microscope. Virus antigens of RGDV and RRSV were never detected from leaves or roots with this method. No flocculation was observed in any of the controls used in these experiments.

Detection of virus antigens in insect vectors. Results were the same for all experiments, using both crude insect macerates and supernatants after centrifugation. As shown in Table 2, antigen titers did not vary greatly after the 15th day postacquisition access for all the viruses with positive reactions. The result was negative when Tris-PVP was used as the extraction buffer for RGDV and RDV. Use of PB-Mg improved flocculation; however, nonspecific flocculation occurred in healthy controls. Addition of Tween 20 to PB-Mg dispersed nonspecific aggregates without affecting the specific reaction as described previously with potato (3). Flocculation was clearly observed for RGSV and RSV when individual insects were homogenized with either Tris-PVP or PB-Mg-Tween. Reactions were clear when individual insects were homogenized in 0.1 ml buffer for RGSV and RSV; however, no reaction occurred for RGDV and RDV when individual insects were homogenized in the same amount of buffer. Flocculation occurred when more than 0.4 ml buffer was used to homogenize single insects for RGDV and RDV. For RRSV, no flocculation occurred under a variety of test conditions, including variable incubation temperatures (20–40 C), shaking times (0–3 hr), oscillations (0–180 per min), amounts of the buffers used (0.1–3.2 ml), ratios of sap and sensitized latex solution (3:1–1:3), use of unfractionated serum or globulin fraction that precipitated when the serum was one-third saturated with ammonium sulfate, use of 0.1 M glycine containing 0.01 M $MgCl_2$, pH 7.0, PB-Mg-Tween, for homogenizing insects, or use of different buffers to suspend latex. Virus antigens were not detected from the insects viruliferous for RTBV and RTSV.

Average weights of insects used for assay were 3.5 and 2.4 mg for females and males of *Nephotettix nigropictus*, respectively, 2.7 and 1.2 mg for females and males of *Nilaparvata lugens*, respectively, and 1.2 and 0.8 mg for females and males of *L. striatellus*, respectively. The dilution end point of virus in each insect at the 25th day in Table 2 was 900 and 700 (v/w) for females and males viruliferous for RGDV, respectively, 1,800 and 1,300 for females and males viruliferous for RDV, respectively, 2,400 and 1,300 for females and males viruliferous for RGSV, respectively, and 1,300 and 1,000 for females and males viruliferous for RSV, respectively.

To confirm the reliability of LF for detecting virus antigens in insect vectors, infectivity of viruliferous insects was tested and each insect was then homogenized with the appropriate buffer. As shown in Table 3, all insects that transmitted the virus gave positive reactions in LF. Results were negative in all healthy controls.

Interference of LF by insect-host component. Although more virus particles were usually observed under the electron microscope in insect vector extracts than in plant tissue extracts (*unpublished*), virus antigens of RRSV were not detected in viruliferous insect extracts by LF. Because we suspected that materials in the insect-host component may have interfered with the reaction, we conducted the following experiments: Purified RRSV was obtained from I. Kimura. Purification of RGSV was done according to the method reported by Hibino et al (6). As shown in Table 4,

flocculation of RRSV and RRSV antibody-sensitized latex were affected by the insect homogenate from *N. lugens*. On the other hand, presence of the insect homogenate had no effect on the reaction between RGSV and homologous latex antiserum.

Comparison of sensitivity of LF, GD, and ELISA. The RGDV antigen was detected only in samples concentrated 2× or more using GD (Table 5), but in ELISA, the antigen was detected in dilutions up to 1:5,120. The LF (Table 5) was over 256 times more sensitive than GD, and ELISA was 32 times more sensitive than LF in detecting the RGDV antigen in plants. GD and ELISA required 2 days to obtain results; in contrast, the LF could be completed within 30–90 min if the sensitized latex was prepared in advance.

Table 4. Effect of insect homogenates on flocculation of latex sensitized to rice ragged stunt virus and rice grassy stunt virus

Virus	Material homogenized in virus suspension	Reciprocal of dilution[a]							
		1[b]	2	4	8	16	32	64	128
RRSV	Female[c]	+[d]	+	+	+	−	−	−	−
	Male[e]	+	+	+	+	+	−	−	−
	Buffer	++	++	++	++	++	+	+	−
RGSV	Female	++	++	++	++	++	++	+	−
	Male	++	++	++	++	++	++	+	−
	Buffer	++	++	++	++	++	++	+	−

[a] Homogenate of one insect homogenized in 0.1 ml purified virus (A_{260} = 0.1) suspension was presented as 1, ie, original homogenate.
[b] Purified virus concentration was adjusted to become end point 64 times of dilution.
[c] Female *Nilaparvata lugens*.
[d] + = Positive reaction, with flocculation not so large; ++ = large flocculation, making mixture translucent; and − = negative reaction.
[e] Male *N. lugens*.

Table 5. Comparison of agar gel diffusion test, latex flocculation test, and enzyme-linked immunosorbent assay (ELISA) for detection of rice gall dwarf virus[a,b]

Test	Dilutions														
	4×[c]	2×	1	2	4	10	20	40	80	160	320	640	1,280	2,560	5,120	10,240
Agar gel	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Latex	···	···	···	···	···	+	+	+	+	+	−	−	−	−	−	−
ELISA	···	···	···	···	···	+	+	+	+	+	+	+	+	+	+	−

[a] Root of RGDV infected plant was used as material.
[b] All the healthy controls of each method resulted in negative reaction.
[c] Four times of concentration as mentioned in Materials and Methods.

DISCUSSION

Several serological methods were compared for their sensitivity in detecting virus antigens. Usugi (18) compared ELISA, hemagglutination (HA), LF, complement fixation, precipitin ring interface, and GD to detect satsuma dwarf virus and stated that sensitivity decreased in the same order as mentioned before. Serum-specific electron microscopy (SSEM), ELISA, and HA are generally considered more sensitive than LF; however, each method has its characteristic disadvantages. SSEM is inadequate for large numbers of samples and requires the availability of an electron microscope. Two days and several steps are needed to detect virus antigen in one sample using ELISA. In HA, the life of red cells is short and high concentrations of antiserum globulin preparation are needed to sensitize the red cells optimally. Only 20 min was required to detect virus antigens of RDV, RGSV, and RSV in both plant and insect extracts and RTBV and RTSV in plant extracts when LF was employed. Working time was less than 1 min for one sample with this method. Furthermore, LF was sensitive enough to detect RGDV, RDV, RGSV, and RSV antigens from individual viruliferous insects. From infected plants, LF easily detected RGDV, RRSV, RTBV, and RTSV, all of which are considered to be restricted to phloem tissue. For RDV, RGSV, or RSV, antigens were detected from a 1-mm^2 area of leaf tissue. The LF method proved useful for detecting virus antigens in rice plants naturally infected with each of the viruses used in this report (*unpublished*). As reported earlier (17), all sensitized latex particles used in this experiment could be stored for many months (at least 14) at 4 C.

Our study is the first report of the detection of virus antigens in individual leafhopper or plant hopper vectors by LF. In these experiments, LF proved to be an excellent and convenient serodiagnostic method for detecting all the rice viruses tested except RRSV in the insect vector. So far, ELISA (4,10) is recommended for detecting RRSV in the insect vector.

Use of roots is recommended for detecting RGDV and RRSV because antigen concentrations appear to be higher in roots than in leaves. Antigen titers in plants infected with RDV, RGSV, and RSV were more than 32 times greater than those infected with the other viruses (Table 1). This result may be due to the fact that RGDV, RRSV, RTBV, and RTSV are restricted to the phloem tissue of infected plants.

LF was more sensitive than the infectivity test for detecting RGDV, RDV, RGSV, and RSV antigens in insect vectors. It is interesting that some insect vectors contained large amounts of virus antigen but did not transmit RGDV, RDV, RGSV, and especially RSV (Table 3). This may be related to the intermittent transmission pattern reported for all viruses (7,8,14,16) in which transmission efficiency decreases gradually with time. On the contrary, antigen titers in the individual insect did not decrease with time for RGDV, RDV, RGSV, and RSV (Table 2).

Data in Table 4 show that the insect homogenate could interfere with the flocculation of latex sensitized to RRSV antibody. This is probably one reason for our inability to detect the RRSV antigen in viruliferous insects (Table 2). It is not known why flocculation of latex sensitized to RGSV was not affected similarly.

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