Biological Variants of Rice Tungro Viruses in the Philippines

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ABSTRACT

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Tungro is a composite disease of rice induced by dual infection with rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV). Four strains of RTBV, designated L, G1, G2, and Ic, were isolated on the basis of their characteristic symptoms on rice cultivars FK135 and Taichung Native 1 (TN1). On FK135, strain Ic caused severe stunting, reduced tillering, narrow leaves, and distinct interveinal chlorosis (striping and/or mottling) on the leaf blades, while G1 and G2 caused only mild stunting and foliage was a normal green. On TN1, G1 and Ic caused mild stunting but no discoloration of the foliage; G2 caused severe stunting and yellow to orange discoloration similar to that caused by the type strain L. Thus, G1 and Ic can be readily differentiated by the reaction of cultivar FK135, while G1 and G2 can be differentiated by that of TN1. Cross-inoculation experiments showed that strains G1 and Ic were cross protective. In other studies, a virulent strain of RTSV (Vt6) was isolated from tungro-infected plants collected from Mindanao, Philippines. The cultivar TKM6, like many other cultivars resistant to type strain A, was susceptible to strain Vt6. TKM6 plants infected with both RTBV and strain Vt6 showed severe stunting and discoloration.

Additional keyword: resistance.

Tungro is a composite disease of rice (*Oryza sativa* L.) induced by dual infection with rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV). RTBV causes the tungro symptoms, including yellow orange discoloration and plant stunting. RTSV alone causes no clear symptoms except very mild stunting, but it enhances the symptoms caused by RTBV (11). The causal viruses are transmitted in a semipersistent or transitory manner by leafhoppers; the green leafhopper, *Nephotettix virescens* (Distant), is the most significant vector. RTSV is transmitted independently by leafhoppers, while RTBV is transmitted by leafhoppers only in association with RTSV (8).

The use of resistant cultivars is the most important component in managing the disease. Incorporation of virus resistance to tungro has become an important breeding objective for rice improvement. However, virus resistance may break down as a result of the appearance of new tungro virus strains. Several tungro virus strains have been reported previously (1,13,15). Rivera and Ou (15) isolated two strains, S and M, on the basis of symptoms on the rice cultivar FK135. A third strain, T, was later reported from the Philippines (2). In India, four strains (RTV1, RTV2a, RTV2b, and RTV3) were isolated on the basis of symptomatology and cultivar reaction (1). A fifth strain, RTV4, was also reported in India by Mishra et al (13). All of these reports were published before the discovery of the fact that two distinct viruses cause the tungro disease (11). Recent studies have demonstrated that rice cultivars react differentially to RTBV and/or RTSV and express variable symptoms depending on whether single or dual infections are involved (5,10). Thus, the reported strains of tungro could be the results of infection with either or both of the viruses. One way to determine the occurrence of tungro virus strains is to separate the two viruses and compare their symptoms on and/or infectivity to differential cultivars. By doing so, we differentiated four RTBV strains from the isolate maintained in the greenhouse and two RTSV strains, one of which originated in Mindanao, Philippines.

MATERIALS AND METHODS

Virus and plants. The tungro isolate used in this study was originally collected from Laguna, Philippines, and maintained for many years on rice cultivar Taichung Native 1 (TN1) by successive transfers via *N. virescens*. The original isolate contained RTBV and strain A of RTSV. A virulent strain (designated Vt6) was isolated from tungro-infected plants from Mindanao, Philippines, and was used for comparative transmission tests. Plants infected with either RTBV and RTSV or RTSV alone were used as virus sources. Seeds were obtained from the International Rice Germplasm Center at the International Rice Research Institute (IRRI).

Transmission. All transmission tests were done with the *N. virescens* colony that has been reared on TN1 for several generations. Occasionally, leafhoppers from the colony were tested on assay plants to ensure they were virus free. Six-day-old seedlings were used in all test tube inoculations. Adult *N. virescens* were allowed access to a virus source for 3–4 days. For RTBV transmission, two viruliferous insects were confined with a seedling in a test tube for a 24-h inoculation access period; for RTSV, three insects were used per seedling. Inoculated seedlings were transplanted into pots and grown in insect-proof cages for about 1 mo and then individually tested serologically for virus infection.

Cross-protection test. Cross inoculations of 2-wk-old FK135 plants with RTBV strains G1 and Ic were conducted to determine whether the two strains were cross protective. Plants dually infected with RTSV strain A and RTBV strain G1 or Ic were used as virus sources. The healthy plants were inoculated first with mild strain G1 and challenge inoculated with severe strain Ic at 1, 7, and 14 days after the first inoculation and vice versa. One month after inoculation with the second virus, symptoms were observed, and virus recovery was conducted for each cross-inoculated plant. Virus-free *N. virescens* were allowed access to cross-inoculated plants and used to inoculate FK135 seedlings. Inoculated seedlings were scored 1 mo after inoculation, and the strain recovered was determined on the basis of the presence or absence of interveinal chlorosis (mottling and/or striping).

To determine which of the two strains is dominant, FK135 plants were simultaneously inoculated with G1 and Ic. *N. virescens* were allowed acquisition access to each strain separately. Ten insects from each virus source were simultaneously confined with

a 2-wk-old FK135 plant for 24 h. Scoring and virus recovery were done as above.

Serological assay. The double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was used in all experiments to detect RTBV and RTSV as previously described (3,10). A portion approximately 10 cm long was taken from the second or third youngest leaf of each plant. The leaf was extracted with 1 ml of 0.1 M phosphate buffer (pH 7.0) containing 0.15 M NaCl, 0.05% Tween 20, and 1% NaN_3 in a leaf and bud press (Ehrich Pollahne, Wennigsen, Germany). An Immulon II microtiter plate (Dynatech Corp., Chantilly, VA) was coated with immunoglobulin at 0.5 μg/ml for RTBV and 1 μg/ml for RTSV, and an immunoglobulin-alkaline phosphate conjugate was diluted 1,000 times for RTBV and 500 times for RTSV. One well per sample was used for the detection of RTBV or RTSV. On each plate, four wells with extracts of healthy TN1 leaves were added along with two wells with extracts of TN1 leaves infected with both RTBV and RTSV. Presence or absence of the viruses in extracts was determined by measuring absorbance at 405 nm in an ELISA reader (Bio-Tek Instruments, Mt. Laurel, NJ). Absorbances over twice the mean of two healthy control readings were considered positive. In this assay, RTBV and RTSV were detected in dilutions (w/v) up to 1/1,280 and 1/640, respectively.

RESULTS

Isolation of RTBV strains. During routine tungro transmission tests in the greenhouse, symptom variation was repeatedly observed on TN1 plants. Although ELISA showed that plants were infected with RTBV alone, some showed very mild stunting and yellowing symptoms compared with the typically severe symptoms. Similar variations in symptoms were observed on FK135. Some plants were mildly stunted and did not show interveinal chlorosis (mottling and/or striping) characteristic of this cultivar when infected with tungro, as reported by Rivera and Ou (15). In our preliminary transmission tests on FK135, in which the original tungro isolate was used, five out of about 500 infected plants showed mild symptoms. Variants with distinct symptoms in the greenhouse were selected and transferred successively on FK135 and TN1. Because RTBV alone can not be transmitted, the leafhopper vectors were given 3–4 days of access to an RTSV-infected TN1 plant, transferred to an RTBV-infected plant of each variant for 24 h, and then used for inoculation of rice cultivars FK135 and TN1. With this method, the leafhoppers transmitted either RTBV, RTSV, or both to test plants. After confirmation by ELISA, plants infected with RTBV alone were selected and used as virus sources for the succeeding transmission experiments. This procedure was done repeatedly for each RTBV variant until all the RTBV-infected plants showed only the symptoms characteristic of that variant on both FK135 and TN1. At this stage, the variant was considered stable. After 10 successive transfers on TN1 and FK135, four RTBV strains (designated G1, G2, Ic, and L) were isolated.

The strains were differentiated mainly on the basis of the severity of symptoms on TN1 and the presence or absence of interveinal chlorosis (striping and/or mottling) on FK135. Among the four strains, L showed the typical severe symptoms on both TN1 and FK135 and was therefore considered the type strain. On FK135,

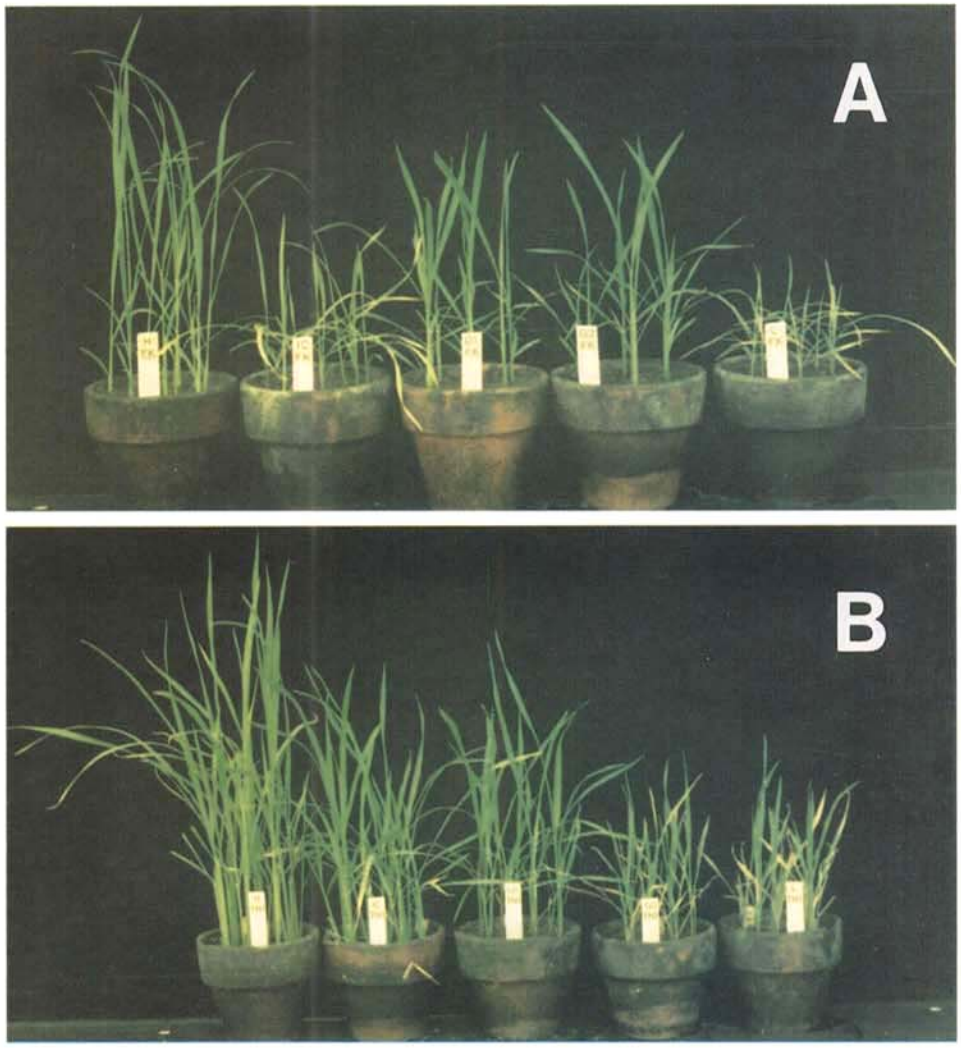

Fig. 1. Symptoms induced by rice tungro bacilliform virus strains on rice cultivars **A**, FK135 and **B**, TN1. Left to right: healthy plants (check) and plants infected with strains Ic, G1, G2, and L.

strain Ic induced distinct interveinal chlorosis, stunting, reduced tillering, and narrow leaves similar to symptoms caused by strain L, whereas G1 and G2 caused only mild stunting and no discoloration of the foliage. The symptoms caused by these three strains were milder than those caused by strain L, which causes very severe stunting and interveinal chlorosis (Fig. 1A). On TN1, Ic and G1 caused mild stunting and no discoloration of foliage, whereas G2 caused severe stunting and discoloration similar to that caused by strain L (Fig. 1B). Thus, G1 and Ic could be readily differentiated on FK135 (Fig. 2A), while G1 and G2 could be differentiated on TN1 (Fig. 2B). When the four RTBV strains were coinfected with RTSV, their characteristic symptoms were maintained on both FK135 and TN1, although the plants with dual infection were more stunted.

Tungro-infected plants were collected form Isabela, Iloilo, and North Cotabato (representative of isolates from the northern, central, and southern Philippines, respectively). When FK135 and TN1 were inoculated, RTBV symptom variations similar to those in our preliminary transmission tests were observed on collections from all three regions. A few RTBV-infected plants of both cultivars showed very mild stunting and discoloration symptoms.

Cross-protection test. Cross-inoculation experiments showed that strains G1 and Ic were cross protective. FK135 plants infected with G1 did not show changes in symptom pattern when challenge inoculated with Ic 1 or 2 wk after inoculation with G1 and vice versa. When virus-free leafhoppers were allowed access to cross-inoculated plants for 24 h and used to inoculate FK135 seedlings, only the strain inoculated first was recovered. Cross protection was incomplete when challenge inoculation was done 1 day after initial inoculation. However, most of the inoculated plants showed the symptoms of the strain inoculated first. Both strains were recovered from these plants, but more of the strain inoculated first was recovered (Table 1).

Results of simultaneous inoculations showed that symptoms typical of strain Ic were more dominant than those of G1. All plants but one showed symptoms of Ic but with varying degrees

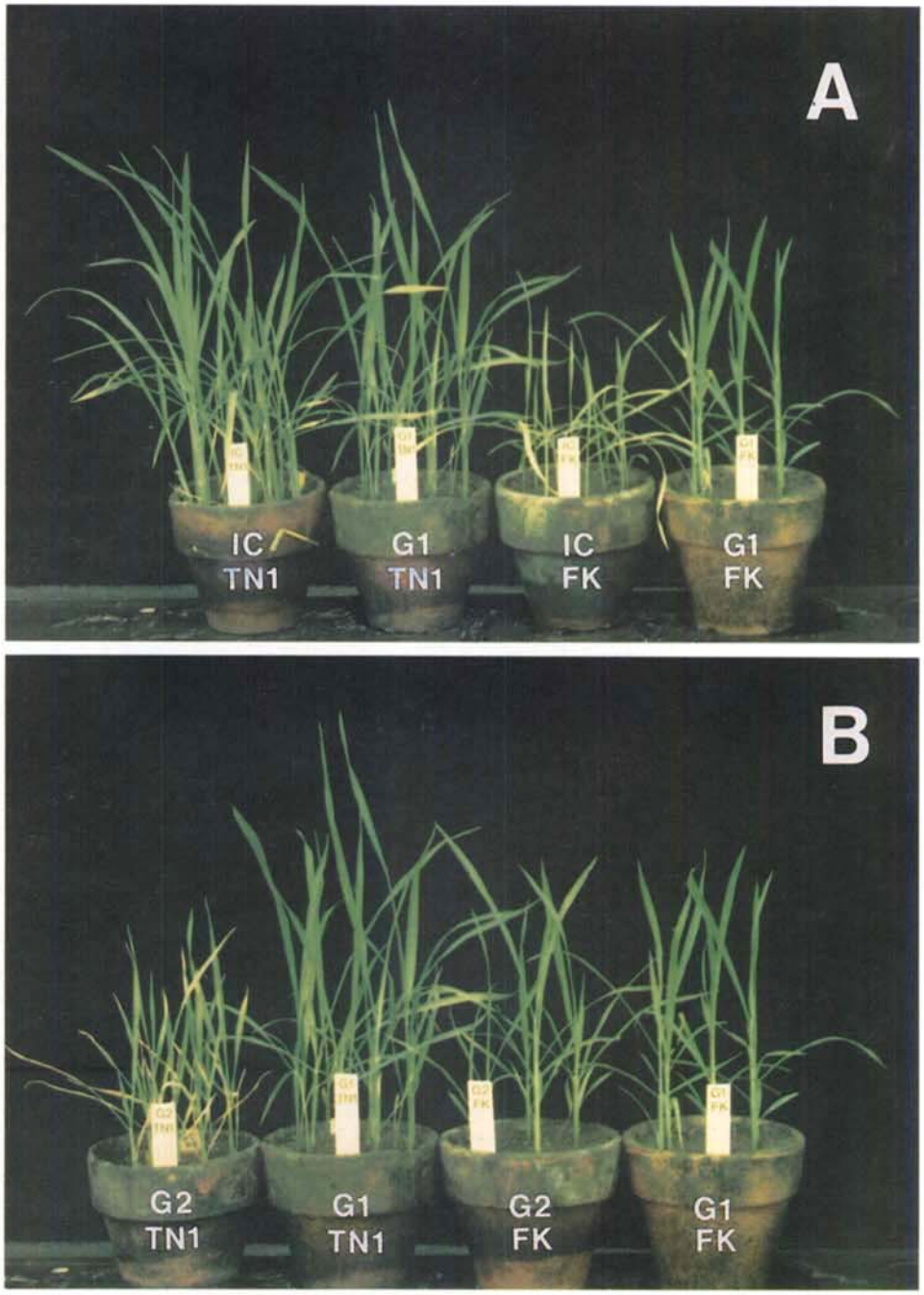

Fig. 2. Differential reactions of rice cultivars FK135 and TN1 to rice tungro bacilliform virus strains **A,** G1 and Ic and **B,** G1 and G2.

TABLE 1. Cross inoculation of the rice cultivar FK135 with rice tungro bacilliform virus strains GI and Ic at different time intervals and virus recovery from cross-inoculated plants 1 mo after the second inoculation

Interval between inoculations	Inoculation sequence[a]		Plants with symptoms[b] (no.)		Strains recovered[c] (no.)	
	1	2	1	2	1	2
2 wk	GI	Ic	19	0	94	0
	Ic	GI	17	0	69	0
1 wk	GI	Ic	33	0	129	0
	Ic	GI	17	0	88	0
1 day	GI	Ic	46	5	120	38
	Ic	GI	39	4	111	14
Control	GI	None	17	0	30	0
	Ic	None	17	0	61	0

[a] Five viruliferous leafhoppers were confined with 2-wk-old FK135 plants for a 24-h inoculation access period.
[b] Based on presence or absence of interveinal chlorosis 1 mo after the second inoculation.
[c] Virus-free leafhoppers were allowed a 24-h access to cross-inoculated plants showing symptoms of the first virus and used to inoculate 6-day-old FK135 seedlings. Plants were scored 1 mo after inoculation on the basis of the presence or absence of interveinal chlorosis.

TABLE 2. Symptom expression and virus recovery from rice cultivar FK135 plants simultaneously inoculated with rice tungro bacilliform virus strains GI and Ic[a]

Plant number	Symptoms	Strains recovered (no.)[b]	
		GI	Ic
1	Mild stunting, mottling	13	2
2	Severe stunting, striping	2	11
3	Mild stunting, mottling	13	1
4	Mild stunting, mottling	9	6
5	Severe stunting, striping	4	10
6	Mild stunting, mottling	5	11
7	Severe stunting, striping	3	11
8	Mild stunting, mottling	8	7
9	Mild stunting only	18	0
10	Mild stunting, mottling	7	3

[a] Leafhoppers were given access to each virus source, and 10 leafhoppers from each source were simultaneously confined on 2-wk-old FK135 plants.
[b] Virus-free leafhoppers were allowed access to a diseased plant and used to inoculate 6-day-old FK135 seedlings. Plants were scored 1 mo after inoculation on the basis of the presence or absence of interveinal chlorosis.

of stunting and interveinal chlorosis. *N. virescens* acquired both strains from these plants (Table 2).

Isolation of RTSV strains. Rice cultivar TKM6, which is highly resistant to strain A, was transplanted as a trap plant in Mindanao, Philippines, during the wet season of 1992. The plants showing tungro symptoms were collected and examined by ELISA for virus infection. A plant dually infected with RTBV and RTSV was selected. A few insects that were given access to this plant transmitted RTSV alone to TN1. RTSV-infected plants were selected, and the infecting RTSV isolate was designated Vt6.

RTSV strain Vt6 was not different from type strain A in terms of symptom expression (no visible symptoms) and its virus concentration in TN1. ELISA values of different rice cultivars infected with strain Vt6 were not significantly different from those of plants infected with strain A (data not shown). Transmission of the two strains was compared on rice accessions that were susceptible to the vector *N. virescens* but that had been previously identified as resistant to RTSV (4). Comparative transmission results showed that except for Utri Rajapan, which had an intermediate reaction, all the accessions tested were resistant to strain A. However, their reactions to strain Vt6 varied from highly resistant (0–10% infection ratio) to very susceptible (more than 60% infection ratio), comparable to the susceptible check, TN1 (Table 3). Eleven accessions, Adday Sel., Adday Local Sel., Utri Merah, ARC 11920,

TABLE 3. Comparative transmission of two strains of rice tungro spherical virus (RTSV)[a]

IRGC[b] accession no.	Cultivar name	RTSV infection[c]	
		Strain A	Strain Vt6
177	Adday Sel.	0/40	0/40
180	Adday Local Sel.	0/40	0/40
237	TKM 6	0/38	22/37
10262	G 378	0/39	37/37
11751	Habiganj DW8	0/36	10/38
12274	ARC 6561	3/40	33/33
12428	ARC 10312	0/40	37/40
12437	ARC 10343	0/38	26/33
12765	Kataribhog	2/37	25/34
14504	IR 580 420-1-1-2	1/28	23/40
14527	Barah	0/34	15/37
16680	Utri Merah	0/37	1/36
16684	Utri Rajapan	12/32	22/28
19680	ARC 10963	0/37	23/34
20600	ARC 7321	0/37	7/38
21310	ARC 11315	0/35	32/40
21474	ARC 11555	0/35	5/37
21745	ARC 11920	0/37	3/37
21958	ARC 12170	1/37	34/36
22199	ARC 12620	0/34	18/35
22215	ARC 12636	0/39	6/40
22307	ARC 12746	0/38	17/33
22331	ARC 12778	0/23	11/39
26253	Nep Bap	0/36	3/38
26410	Pala Bhir	1/35	1/36
26495	Konek Chul	1/38	3/36
26527	Shuli 2	0/40	35/40
26582	Buchi 2	0/38	38/38
26791	Sham Rosh	0/39	1/38
26813	Gogoj	0/39	1/36
27779	Bara Pashawari 390	0/38	38/39
27787	Basmati Nahan 381	5/40	20/38
27798	Basmati 1	4/40	37/37
27818	Basmati 242	0/40	11/38
27829	Basmati 377	1/38	31/38
27830	Basmati 388	4/38	29/31
27832	Basmati 405	0/38	38/39
27833	Basmati 406	0/40	5/40
27836	Basmati 433	0/40	39/40
27856	Begumi 302	4/40	7/40
27869	Chahora 144	1/40	40/40
27870	Chahora 148	0/36	30/36
27872	Chahora 292	0/38	37/38
27873	Chahora 382	0/40	39/39
27946	Hansraj 62	0/39	37/37
27947	Hansraj 189	0/37	34/34
27951	Hansraj 365A	0/34	35/38
28102	P 590	0/35	36/39
28522	Gundrikbhog	0/15	16/32
28867	Aus 4	0/35	39/39
36731	Firro E(1)	0/40	6/38
37215	Matichakma	0/37	24/36
37337	Urman Sardar	0/39	19/37
37482	Konekchul	0/38	34/37
37761	Maliabhangor 1096	0/34	1/38
49996	Ovarkondoh	0/40	1/34
11355	IR 20[d]	0/30	5/32
24153	IR 26[d]	0/40	3/38
30413	IR 30[d]	0/36	2/38
21473	ARC 11554[d]	0/36	0/36
105	Taichung Native 1[e]	39/40	38/40

[a] Leafhoppers were given 3 days' access to Taichung Native 1 plants infected with both rice tungro bacilliform virus and RTSV. Six-day-old seedlings were inoculated in test tubes using three insects per seedling for a 24-h inoculation access period.
[b] International Rice Germplasm Center.
[c] Numerator is the number of plants infected, and dominator is the number of plants tested.
[d] Vector-resistant variety. Other varieties are susceptible to vector.
[e] Susceptible check.

Nep Bap, Pala Bhir, Konek Chul, Sham Rosh, Gogoj, Maliabhangor 1096, and Ovarkondoh, showed low infection rates to both strains. Three cultivars, IR20, IR26, and IR30, which have the parentage of TKM6 and are resistant to *N. virescens,* showed low infection of two strains, while IR580-420-1-1-2, which has the parentage of TKM6 and is susceptible to *N. virescens,* had an infection rate similar to that of TKM6. TKM6 and other cultivars dually infected with strains Vt6 and RTBV showed severe stunting and discoloration.

DISCUSSION

The isolation of four distinct strains of RTBV indicates that tungro in the Philippines is heterogeneous. Around 1970, IRRI researchers reported the isolation of three tungro strains (2,15). The S strain showed striping symptoms, while the M strain showed mottling symptoms on FK135. The T strain caused narrow leaves and mild stunting on TN1 and striping symptoms on FK135. Although the tungro causal viruses were not yet known then, their report indicated the variability of the tungro agents in the Philippines. In our study, we also used FK135 and TN1 to differentiate RTBV strains, and our results show that RTBV symptoms are variable, even when both differential cultivars are infected with RTBV alone. If we consider only the degree of stunting on two differential hosts, strains S, M, and T resemble our strains L, G2, and Ic, respectively. Similar isolates were found in other areas of the Philippines, indicating that these variations are common in nature.

Cross-protection tests have been widely used to demonstrate strain relationships of some plant viruses (12). Our results show that RTBV strains G1 and Ic (strains that show very distinct contrasting symptoms on FK135) are cross protective. The failure of the challenge virus to induce characteristic symptoms and its failure to be recovered by the vector suggest that cross protection was complete, at least at 7 days after the first inoculation. Cross protection was also observed in later experiments involving the other strains, such as L and G1 (data not shown). Cross protection was also reported between tungro strains S and M in the Philippines (15). On the other hand, simultaneous inoculation with strains G1 and Ic resulted in mixed infection, as shown by the recovery tests, even though the inoculated plants predominantly showed symptoms typical of strain Ic. These results suggest that mixed infections with virus strains may occur in nature.

Recent molecular studies of RTBV indicate that the isolates from the Philippines are heterogeneous. RTBV DNA sequences reported by Hay et al (7) and Qu et al (14) showed that in addition to a difference in length of 2 nt, more than a hundred single nucleotide substitutions were found between the two RTBV DNA sequences; both RTBV DNAs that they sequenced came from the same source (IRRI) but were obtained at different times. Whether or not these molecular differences are related to the variation in RTBV symptom expression in the Philippines needs to be investigated. The RTBV and RTSV strains could be used for studying the molecular basis of virulence and/or other biological functions of viral genes.

Isolates of RTSV in the Philippines varied in their virulence to specific cultivars. Several rice cultivars highly resistant to strain A were very susceptible to strain Vt6. A difference in RTSV virulence on rice cultivar TKM6 of isolates from India and Philippines was also reported by Dahal et al (5). In our experiments, cultivars IR20, IR26, and IR30, which have resistance to RTSV derived from TKM6 (9), showed low infection rates. This may be the result of their resistance to the *N. virescens* colony that we used. Had we used a "virulent" leafhopper colony, infection rates could have been higher (6). Recent screening of rice cultivars showed that accessions with resistance to RTSV infection (strain A) are abundant in rice germ plasm (4,9,10). Use of resistant cultivars is anticipated to slow the spread of the disease (9,16). The results presented here, however, indicate that the number of cultivars resistant to strain Vt6 is limited. This contradicts our previous view that virus resistance is more durable than vector resistance. If rice cultivar IR26 is planted in an area where strain Vt6 exists, the cultivar may succumb to strain Vt6. It is interesting to note, however, that two accessions, Adday Sel. and Adday Local Sel., which are both susceptible to the vector, were not infected by either strain in repeated tests. These accessions may be used as sources of resistance genes to both strains.

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