

RICE TRANSITORY YELLOWING VIRUS

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Abstract

Rice transitory yellowing virus was first identified in the tropics, in northern Thailand, and designated as the Thailand isolate. The bullet-shaped virus particles about 90 nm wide and about 120 nm long were observed in the inner part of the double nuclear membrane and in the cytoplasm of the phloem cells of diseased rice plants. The leafhopper vectors, *Nephotettix nigropictus*, *N. cincticeps* and *N. virescens* were capable of transmitting the Thailand isolate as well as the Okinawa isolate, collected in Okinawa, in a persistent manner, but the latent period of the Thailand isolate in the vectors was slightly shorter than that of the Okinawa isolate. The symptoms induced by the Thailand isolate were somewhat more severe and appeared earlier than those induced by the Okinawa isolate. The Thailand isolate did not appear to be identical with the Okinawa isolate in virulence, suggesting the existence of different strains. The virus was first purified by the Percoll density gradient centrifugation method and the size of the particles, 95 to 120 nm wide and 120 to 180 nm long was similar to that observed in both ultrathin sections and dip preparations from diseased rice plants. However, no virus-transmitting individuals were recovered by injection of the purified virus into leafhoppers.

1. Introduction

Rice transitory yellowing virus (RTYV), a leafhopper-borne virus transmitted by green rice leafhoppers in a persistent manner, was detected by Chiu *et al.* (1965) in Taiwan. The disease was also observed in Okinawa (Saito *et al.*, 1979) and in the south-eastern part of mainland China (Doi, personal communication). Therefore it had been considered that the disease was endemic in the east coast of Asia. In August 1979, however, characteristic symptoms of yellowing were observed on wet-season rice in Chiengrai and Chiengmai, in northern Thailand, and the causal agent was identified as RTYV based on its transmission mode by the leafhopper and the association of bullet-shaped virus particles with the diseased rice plants. This is the first record of a RTYV outbreak in the tropical region of Asia.

Surveys of the affected fields revealed that there was a sporadic occurrence of the disease in the area, and the rate of diseased hills in these affected fields ranged between 18 and 32%; the disease occurred in early-transplanted rice in the area. In the following year, however, the disease was no longer observed.

The present investigation aimed at comparing characteristics of the

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symptomatology, virus transmission, morphology and intracellular localization between the Thailand and Okinawa isolates of the virus, along with purifying the virus.

2. Comparison of RTYV symptoms between Thailand and Okinawa isolates in various rice varieties

The characteristic symptoms of RTYV on rice consist of a yellow discoloration of leaf blades which develops 2 or 3 weeks after virus infection. Also reduction of plant height and number of tillers is common when plants are infected with the virus in early growth stages. According to Su (1976), RTYV symptoms vary depending on the rice varieties and, in general, japonica varieties are more susceptible to the virus than indica varieties. For instance, a reddish-yellowing discoloration of affected leaf blades without rusty flecks is observed on Taichung Native 1, whereas the virus has an almost lethal effect on Tainan 3 (japonica).

In the present chapter, the symptoms on young rice plants were compared using japonica and indica varieties.

1) Materials and methods

During the field survey, we observed symptoms on rice at the tillering stage in the affected fields in Chiengrai and Chiengmai, northern Thailand, in late August 1979. The rice varieties were Niaw Sanpatong, a leading variety in the area, and some local varieties including glutinous and nonglutinous types.

For virus inoculation tests, diseased plants were originally collected in Chiengrai in 1979. Thereafter the disease was maintained on cv. Reiho (japonica) using an efficient vector, *Nephotettix nigropictus*. Also diseased plants were collected in Nago, Okinawa, in 1977 and the diseased rice plants (cv. Reiho) were maintained consecutively by the inoculation via vectors in a greenhouse. Tests of virus inoculation to rice seedlings were carried out by group using viruliferous adults of *N. nigropictus*. Nymphs collected from stocks in our laboratory were allowed a virus acquisition feeding of 3 days on diseased rice plants with an inoculation period of 10 days. Fifteen adult insects or 5th instar nymphs and 5 test seedlings were confined together in a plastic cage (10 × 10 cm) for 2 days for inoculation feeding with three replications. The inoculated seedlings were transplanted in a greenhouse and symptoms were observed every other day for 2 months. Two series of tests were conducted; the 1st test for japonica varieties and the 2nd test for indica varieties. The symptom development in the 1st test was slightly delayed due to the low temperature in the greenhouse.

2) Results

In affected paddy fields in Chiengrai and Chiengmai, diseased rice plants were scattered in the fields. In typical diseased plants, 2 or 3 middle and upper leaves became distinctly yellow or orange-buff and several lower leaves remained healthy. The leaves with a yellow discoloration exhibited interveinal discoloration. There were brown necrotic flecks on drooped leaves at an advanced stage. The drooping of leaves was particularly apparent from the joint portion between the leaf sheath and leaf blade. Plant height was not significantly affected but tillering was inhibited to a certain extent.

In the inoculation tests for both the Thailand and Okinawa isolates, symptom development on japonica varieties was earlier than that on indica varieties as shown in Table 1, and, in addition, the symptoms on japonica varieties were slightly more severe than those on indica varieties. Symptoms on japonica varieties inoculated with the Thailand isolate consisted of distinct yellowing on leaf blades which turned gradually orange-buff in the following acute stage, and plant growth was arrested for a long time. Rusty flecks were often observed on yellow leaf blades and the diseased plants occasionally withered up. On the other hand, symptoms on indica varieties showed a greater variation than those on japonica varieties, ranging from a typical yellow discoloration and rusty flecks on leaf blades on cv. Te-tep and Tadukan to mild symptoms as yellowing and no plant height reduction on IR 20 or IR 24. In contrast symptoms on japonica varieties inoculated with the Okinawa isolate included a yellow discoloration of the leaf blades which then often became bright yellow and plant growth was slightly arrested for about 1 month after inoculation followed by recovery of plant growth thereafter. On the other hand, all the tested indica varieties showed

Table 1. Differences in the latent period and severity of symptoms between Thailand and Okinawa isolates of RTYV on some japonica and indica varieties in seedling tests.

Variety	RTYV isolate	Number of diseased seedlings	Latent period of symptoms in days	Characteristics of symptoms
			Average (Min. — Max.)	
Test I				
Akanemochi	T	9	25 (25 — 25)	Necrosis
	O	9	29 (26 — 31)	
Miyazakimochi	T	13	25 (25 — 25)	Necrosis
	O	15	29 (25 — 32)	
Todorokiwase	T	10	28 (28 — 28)	Necrosis
	O	9	32 (27 — 34)	
Yamajiwase	T	11	26 (26 — 33)	Necrosis
	O	9	31 (28 — 33)	
Koganenishiki	T	9	26 (24 — 27)	Necrosis
	O	9	28 (27 — 30)	
Test II				
IR 8	T	11	15 (13 — 20)	Necrosis
	O	12	22 (22 — 22)	
IR 20	T	10	15 (13 — 16)	
	O	10	19 (16 — 22)	
IR 24	T	12	18 (16 — 20)	
	O	8	26 (24 — 27)	
IR 26	T	15	13 (13 — 13)	Stunt
	O	13	18 (16 — 20)	
Te-tep	T	11	20 (18 — 20)	Necrosis
	O	10	21 (18 — 27)	
TN 1	T	10	23 (21 — 23)	
	O	8	25 (25 — 28)	
Tadukan	T	9	25 (24 — 28)	Recovered
	O	8	28 (25 — 33)	

Yellowing and slight stunting were symptoms common to every test variety.

only 2 or 3 yellow leaves and green leaves were produced at a later stage. These experimental evidences indicate that the Thailand isolate has a greater virulence than the Okinawa one.

3. Transmission study

RTYV is transmitted by *Nephotettix* species in Taiwan (Chiu *et al.*, 1965; Chiu *et al.*, 1968; Hsieh *et al.*, 1970) and in Japan (Inoue, 1970); *N. nigropictus*, *N. cincticeps* and *N. virescens*. The virus persists in the leafhopper vectors but there is no transovarian transmission (Su, 1967). Also, transmission by mechanical means or seed transmission has not been demonstrated (Chiu *et al.*, 1965; Chiu *et al.*, 1967).

1) Materials and methods

The Thailand isolate that had originally been collected in Chiengrai, northern Thailand, in 1979 and the Okinawa isolate collected originally in Nago, Okinawa, in 1977 were transmitted successively to the cv. Reiho using *N. nigropictus* and maintained in a greenhouse. The diseased rice plants within one month after virus inoculation served as virus source plants for the investigations. The test insect colonies originated from our stocks at the Kyushu National Agricultural Experiment Station and were maintained on young rice seedlings in a growth chamber at 25°; *N. nigropictus* and *N. virescens* were originally collected in Kagoshima, southern Japan, *N. cincticeps* in Chikugo, western Japan, and *N. malayanus* on Ishigaki Is., subtropical Japan. Second or third instar nymphs were allowed to feed on the diseased source plants for a virus acquisition access of 2 days, then were tested for virus transmission with an inoculation feeding on cv. Reiho seedlings. For the inoculation test, the leafhoppers were confined individually in test tubes with rice seedlings and the seedlings were exchanged serially at 1-day intervals during their entire life. Virus transmission was evaluated by the appearance of symptoms on seedlings transplanted in a greenhouse. All series of tests were conducted at 25°C.

2) Results

(1) Transmission mode by vectors

Transmission of the Thailand isolate was recognized in 21 *N. nigropictus* out of 41 individuals tested and 9 *N. cincticeps* out of 43 individuals tested, the former species showing a higher rate of transmission than the latter one. In addition, the proportion of infective males was higher than that of females in both species. The period that elapsed between the onset of the virus acquisition feeding and the first effective inoculation feeding was considered to correspond to the virus latent period in the insects. The period ranged from 8 - 16 days, 11.5 days on the average, in *N. nigropictus* and 8 - 20 days, 14.4 days on the average, in *N. cincticeps*. The virus latent period of males was shorter than that of females by 1 or 2 days on the average, reflecting the higher rate of transmission in males than in females.

The daily transmission pattern of the virus by individual insects of the two species is shown in Table 2. The virus persisted in vectors; the vectors remained infective for their entire life. The longest retention period of the virus was 27 days for *N. nigropictus* and 18 days for *N. cincticeps*, and the females remained infective for a longer period than the males due to the longer life span of the females than that of the

males. The transmission pattern was intermittent rather than consecutive and there were individual variations in the rate of daily effective transmission (number infected seedlings/number test seedlings). The infectivity of the vector often decreased at an old stage.

Table 2. Serial daily transmission of RTYV by two leafhopper species

Test insect	Days after onset of virus acquisition feeding																
	10	15	20	25	30	35											
<i>Nephotettix nigropictus</i>																	
1	-	-	-	-	+	+	+	+	-	+	+	+	+	-	+	+	+
2	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
3	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+
4	-	-	-	-	-	-	+	+	+	+	-	+	-	+	-	-	-
5	-	-	-	-	+	+	+	+	+	-	+	+	-	+	-	+	-
1	-	-	-	-	-	+	+	+	+	-	+	+	-	+	+	-	+
2	-	-	-	+	+	+	+	+	-	+	-	+	+	+	+	+	+
3	-	-	-	-	-	+	+	+	+	x	-	+	+	-	-	-	-
4	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
5	-	-	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+
<i>N. cincticeps</i>																	
1	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-
2	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-
3	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
4	-	-	-	-	-	-	+	+	+	+	-	+	-	+	+	+	-
5	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-
1	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
2	-	-	-	-	-	-	+	+	+	+	-	+	-	+	-	+	-
3	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
4	-	-	-	-	-	-	+	-	-	+	+	+	+	+	+	+	-

+ : transmission - : no transmission x : test seedling died

(2) Efficiency of *Nephotettix* leafhoppers in the transmission of the Thailand and Okinawa isolates

As shown in Table 3, *N. nigropictus*, *N. cincticeps* and *N. virescens* transmitted the Thailand isolate as well as the Okinawa isolate. No transmission of the virus was demonstrated in *N. malayanus* even though tests for the inoculation access were performed 26 days after the onset of the 2-day virus acquisition access for a total of 40 insects. For both isolates, the transmission efficiency among the 3 vector species was in the following descending order; *N. nigropictus* showed the highest efficiency followed by *N. cincticeps* and *N. virescens* was the least efficient vector. The average latent period of the virus in *N. nigropictus* was significantly shorter than that of *N. cincticeps*, and *N. virescens* required the longest retention period among the 3 species reflecting the order of transmission efficiency of the 3 species.

As for the difference in transmission efficiency of vectors between the two isolates, every vector species showed a slightly shorter latent period for the Thailand isolate

Table 3. Efficiency and latent period of 3 vector species in transmission of Thai and Okinawa isolates of RTYV

Species	Test	Thai isolate			Okinawa isolate		
		Number of test insects	% transmission	Average latent period in days (\pm SD)	Number of test insects	% transmission	Average latent period in days (\pm SD)
<i>Nephotettix nigropictus</i>	1	35	57.2	10.9 \pm 1.9	35	57.6	11.7 \pm 2.1
	2	39	14.1	—	44	43.2	—
<i>N. cincticeps</i>	1	38	31.6	15.0 \pm 3.2	34	23.5	16.3 \pm 4.7
<i>N. virescens</i>	1	39	2.6	21	40	2.5	19
<i>N. malayanus</i>	1	40	0				

than for the Okinawa isolate, though there was no difference in the rate of infective individuals between the two isolates. As in the case of symptom development the incubation period of the Thailand isolate was shorter than that of the Okinawa isolate as described before. Furthermore, no difference in the daily transmission pattern was detected between the two isolates.

4. Purification

As RTYV is a member of plant rhabdoviridae, the procedures of virus purification which had been used for other viruses belonging to this group were employed (Toriyama, 1972). However, it was difficult to obtain purified virus in the course of the procedures mainly due to the labile nature of RTYV which underwent degradation during its homogenization with the buffer solution. This particularity may be attributable to the vacuolar structure of the virus particles surrounded by a membrane at the truncated end (Saito *et al.*, 1978), and to the fact that RTYV particles aggregate easily with each other or with the cell organelles of the host plant. Once the virus particles aggregate by either low or high speed centrifugation, it is difficult to resuspend them without destroying them with the detergents employed. Furthermore, since some fragments of plant cell organelles and virions are similar in size, it is difficult to separate RTYV particles from cell organelles by centrifugation due to co-sedimentation.

1) Materials and methods

The Percoll® (Pharmacia Japan) density gradient centrifugation method was employed for the purification of RTYV. RTYV was collected originally in Okinawa, southern Japan. The disease was maintained by inoculation to cv. Taichung Native 1 or Koshihikari using *N. cincticeps*. Rice plants, cv. Koshihikari, infected with RTYV were used as materials for virus purification. To minimize the contamination of the cell organelles of rice plants, especially fragments of plant chloroplasts, and to obtain a highly purified virus preparation, diseased rice plants which showed conspicuous symptoms were used. Young seedlings of rice inoculated with RTYV were harvested

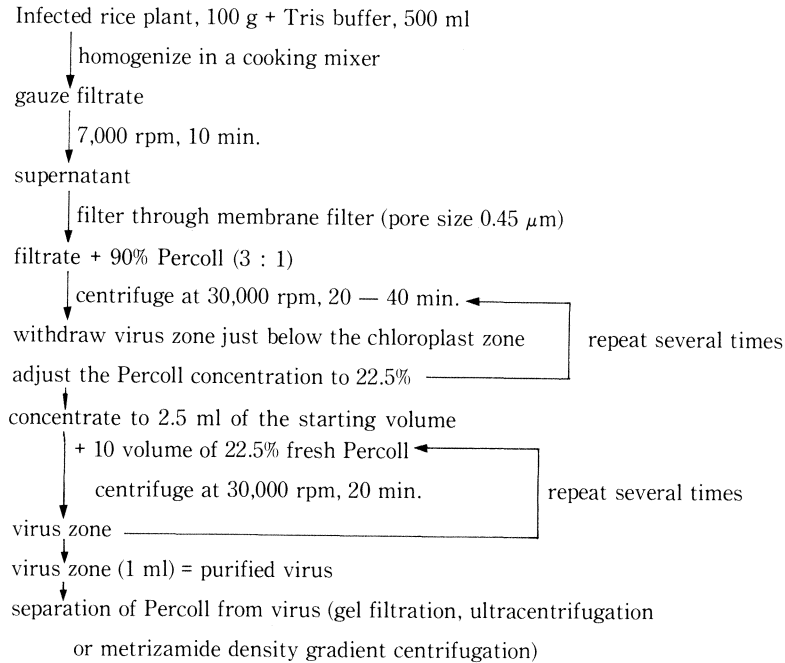


Fig. 1. Purification procedure of rice transitory yellowing virus.

when they showed severe symptoms before withering-up, one to one and a half months after virus inoculation. In other cases, affected rice plants inoculated with RTYV at a later stage of growth were harvested after the ear-emergence stage, then the stalks were used for virus purification as starting materials.

Procedure of virus purification is shown in Fig. 1. One hundred gram of infected rice plant was mixed with 500 ml of Tris-HCl buffer (0.01 M Tris-HCl, 0.005 M MgCl₂, pH 8.0) and homogenized in a cooking mixer. The homogenate was filtered through a gauze and the filtrate was centrifugated at 7,000 rpm (\times 5,000 g) for 10 min. The supernatant was again filtered through a membrane filter (Millipore®, pore size 0.45 μ m). Three volumes of the filtrate were mixed with 1 volume of 90% Percoll (in 0.01 M Tris-HCl, 0.05 M MgCl₂, pH 8.0) and centrifuged at 30,000 rpm for 40 min in a RP 42 rotor (Hitachi). After the centrifugation, the opalescent zone of the virus could be seen just below the green chloroplast zone, as shown in Fig. 2. This virus solution was collected by a glass capillary and then the Percoll concentration was adjusted to 22.5% by a hand refractometer. This adjusted virus solution was again centrifuged at 30,000 rpm for 40 min in the same manner. After several repeated centrifugations at 30,000 rpm and adjustment of Percoll concentration in the virus solution, the virus zone was concentrated and the volume decreased to approximately 1/200 of the starting volume. In the course of the concentration, the centrifugation conditions were adjusted depending on the decrease of the volume of the virus zone to 30 min for a RP 50 rotor or 20 min for a RP 65 rotor at 30,000 rpm. This concentrated virus zone was further mixed with 10 volumes of 22.5% fresh Percoll and again centrifuged at 30,000 rpm for 20 min in a RP 65 rotor. After the centrifugation, the virus zone was withdrawn and this procedure was repeated several times. Finally the volume of the virus zone was

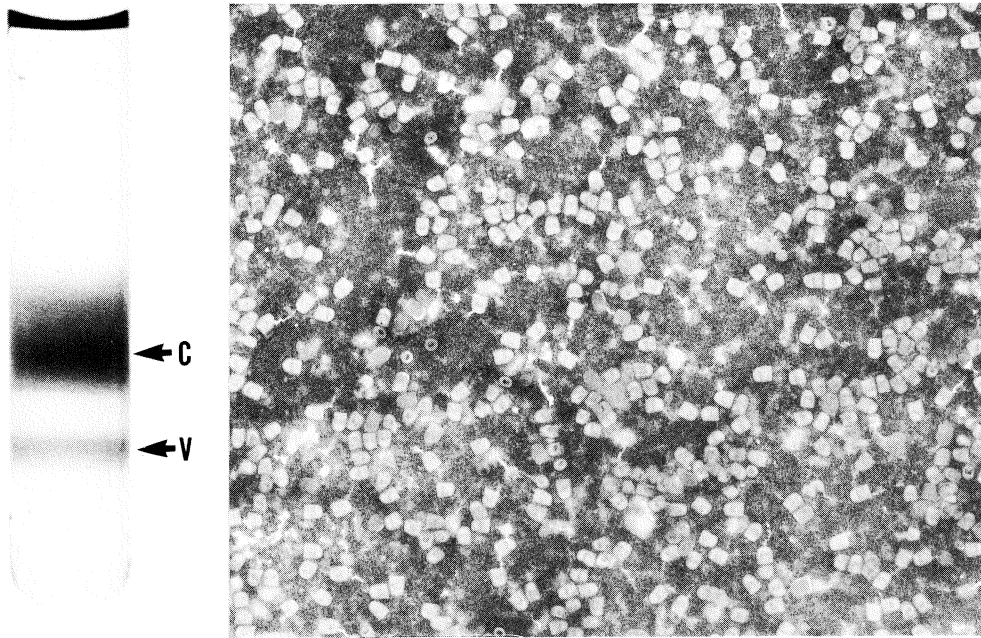


Fig. 2. Homogenate of rice plant infected with RTYV after Percoll density gradient centrifugation at 30,000 rpm for 20 min in RP 65 rotor. C: chloroplast zone, V: RTYV zone (left).

Fig. 3. Purified RTYV in the Percoll solution. Fine grains in the background are Percoll particles (right).

concentrated to 1/500 of the starting volume. This virus suspension could be used for experiments for various purposes except for electron microscopy, as Percoll adversely affected the quality of electron micrographs, as shown in Fig. 3.

To separate the virus from Percoll, the following three methods were used.

(1) Ultracentrifugation - As mentioned earlier in this chapter, RTYV was easily coagulated by the precipitation during centrifugation. Therefore, we centrifuged the virus suspension in a Percoll solution at 50,000 rpm for 1 hr in a RP 65 rotor. This procedure enabled the virus to precipitate in the form of a sheet on the transparent pellet of Percoll. This virus sheet was scraped off and resuspended in Tris buffer solution. The suspension was centrifuged at a low speed of less than 5,000 rpm to precipitate virus aggregates, leaving Percoll in the supernatant as Percoll did not precipitate under such conditions.

(2) Gel filtration - Virus and Percoll could be separated by gel filtration in using Sephacryl S 1,000 superfine® (Pharmacia Japan).

(3) Density gradient centrifugation in metrizamide - The virus suspension in Percoll was layered on 15 - 50% of linear metrizamide gradient (Pharmacia Japan) and was centrifuged for 2 hr at 65,000 rpm in a RPS 65T rotor (Hitachi).

In the next experiment, infectivity of the purified virus was examined. The fluid containing virus particles was injected into virus-free *N. cincticeps* nymphs at the 3rd instar using a glass capillary needle. The injected insects fed on rice seedlings for 16 days to complete the virus latent period in the insect. The infectivity of the surviving

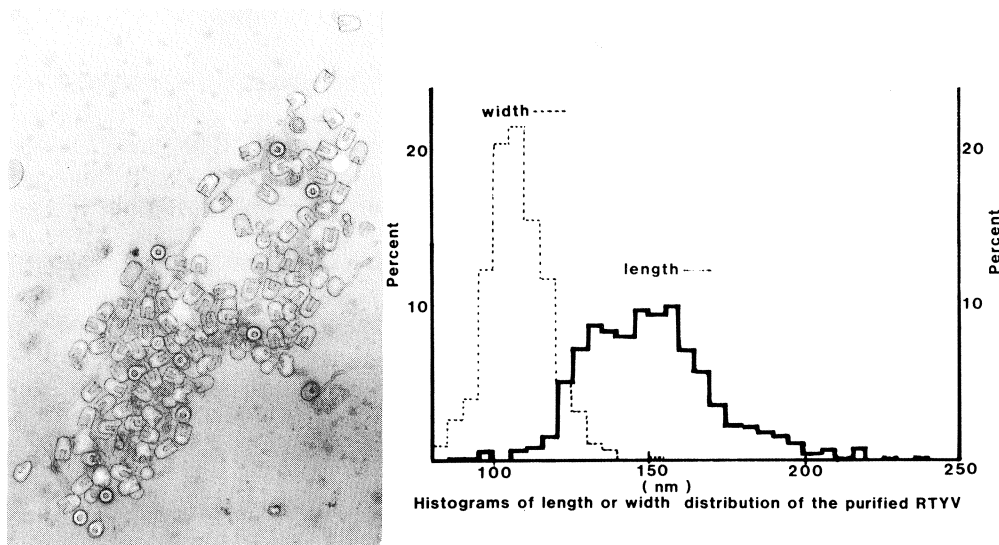


Fig. 4. Purified RTYV particles after metrizamide density gradient centrifugation (left).

Fig. 5. Histograms of length of width distribution of purified RTYV (right).

insects was tested in a group with an inoculation feeding on test rice seedlings.

2) Results

The virus precipitated at the zone around the buoyant density of $\rho = 1.2$ and, as shown in Fig. 4, numerous bullet-shaped particles were detected by electron microscopy. No pellet formation was observed during centrifugation. As shown in Fig. 5, the size of the virus particles ranging from 95 to 120 nm in width and from 120 to 180 nm in length was similar to the size described previously (Shikata and Chen, 1969).

In the test for the injection of purified virus into the leafhoppers, no transmitter was obtained even when the test insects were allowed to feed on healthy rice seedlings after the completion of the virus latent period in the insect.

5. Relation with cells and tissues of rice plant

1) Materials and methods

Leaf samples of RTYV-infected rice plants were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 2 hr. After being washed with cold buffer, they were post-fixed with 2% osmium tetroxide in the same buffer for 3 hr. After dehydration in an acetone series, they were embedded in Epon 812. Thin sections were cut transversely from samples using a diamond knife mounted on a Sorvall MT2-B ultramicrotome. The sections were stained with uranyl acetate and lead citrate. They were observed under a Hitachi H-500 electron microscope.

2) Results

Bullet-shaped particles were observed in ultrathin sections of the infected rice leaves. The particles were found in the inner part of the double nuclear membrane (Fig. 6B) as well as in the cytoplasm of the phloem cells (Fig. 6A). The particles were usually scattered in the cytoplasm, but they were frequently aligned with their axis perpendicular to the nuclear membrane in the inner part of the double nuclear membrane. No such particles were found in healthy leaves.

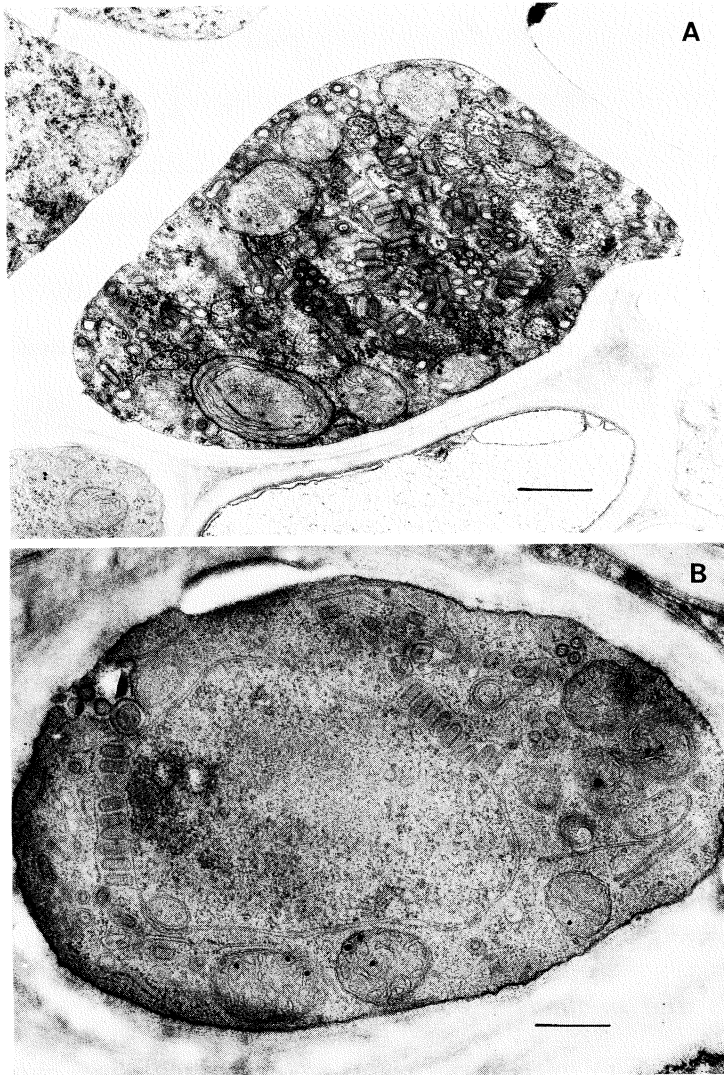


Fig. 6. Electron micrograph showing virus particles in phloem cell of a rice plant infected with RTYV. Bar represents 500 nm.

- A. Virus particles scattered in cytoplasm.
- B. Virus particles aligned with their axis perpendicular to the nuclear membrane in the inner part of the double nuclear membrane.

6. Morphology of virus particles

1) *Materials and methods*

Leaf samples of diseased rice plants were dipped in 2% neutral phosphotungstic acid and examined under a Hitachi H-500 electron microscope.

2) *Results*

Bullet-shaped particles (Fig. 7) were consistently observed with various patterns, i.e. uniformly dense particles without visible inner structure, particles with deep central cores or with cross striations, presumably due to the difference in the penetration of phosphotungstic acid. Numerous upright fine surface projections were aligned over the surface of each virus particle. The particles, excluding the surface projections, were about 90 nm wide and about 120 nm long. In the different patterns, sheath-like membranes surrounding the virus particles were also frequently observed.

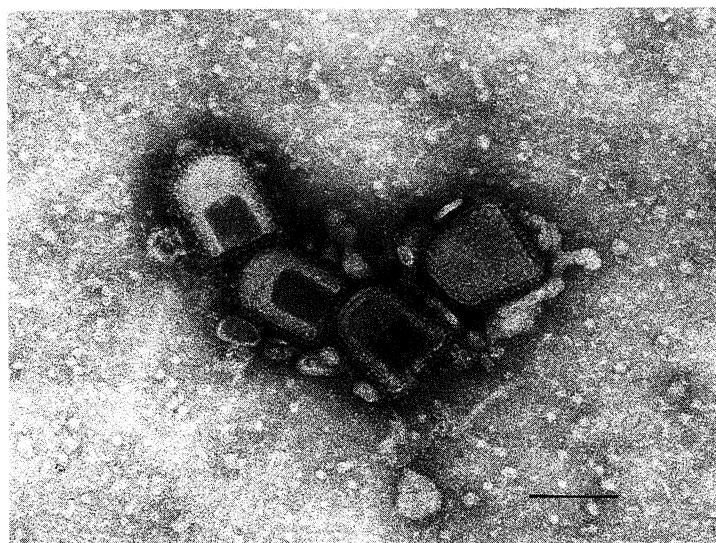


Fig. 7. Electron micrograph showing virus particles in dip preparation from the sample infected with RTYV. Bar represents 100 nm.

7. Discussion

Based on the field observations of disease incidence, the symptoms of RTYV such as yellow discoloration and drooping of leaf blades closely resembled those of rice tungro virus disease. However, the distribution pattern of diseased hills in affected fields was different: RTYV-affected hills exhibited individually a scattered pattern whereas in rice tungro disease the area involved was circular or irregular in shape over a surface of approximately 100 m² or more. These facts are attributable to the difference in the mode of transmission of the respective viruses by the vectors, i.e. RTYV is

transmitted in a persistent manner with a latent period after virus acquisition feeding whereas rice tungro virus is transmitted in a semi- or non-persistent manner without a virus latent period in the vector. These results together with the electron microscopic observations (Fig. 6A, 6B and 7) confirm that the disease is caused by RTYV.

Tests on virus inoculation to seedlings from several varieties showed that the Thailand isolate caused slightly more severe symptoms than the Okinawa isolate on every Japonica variety. Also, tests on virus transmission revealed that the latent period of the virus in *N. nigropictus* and *N. cincticeps* was slightly shorter for the Thailand isolate than for the Okinawa isolate. These results suggest that there are 2 strains of RTYV in Thailand and in Okinawa with different degrees of virulence to rice plants and vectors. Furthermore, RTYV occurrence in Thailand may not result from recent propagation of the virus from Okinawa to the south, although RTYV strains in Taiwan and in Okinawa have not been identified yet.

RTYV has previously been known to occur endemically in the southeastern coastal area of subtropical Asia such as Taiwan, Okinawa and a part of mainland China. However, the detection of the virus from northern Thailand in 1979 has led to the belief that the disease had been present in these areas for some time before it was identified; it may have been identified wrongly as tungro previously. Furthermore, there is a possibility that the disease may be widespread in the northern part of tropical Asia in view of the wide distribution of *N. nigropictus* which is an efficient vector species.

There are several problems associated with RTYV purification. First, the yield of purified RTYV as protein equivalent was, at most, 1 mg from 100 g of diseased rice plant materials. This value is rather low compared with that of other plant viruses. Therefore, diseased plant materials with adequate RTYV propagation are necessary to obtain a higher yield of virus in the purification procedure. In general, the efficiency of virus acquisition feeding by vector insects varies depending on the growth stage of the diseased rice plants as a virus source. To obtain a greater yield of purified RTYV, the plant age for virus inoculation and for test materials should be reexamined precisely. Second, there was a failure in the transmission by *N. cincticeps* which was injected with purified virus. It appears that the method of purification must be improved further. At any rate, prolonged storage of the purified virus at 4°C is impossible due to the intrinsic instability of the virus. Furthermore, thawing of the virus frozen at -70°C may result in alterations of the structure of the virus.

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