Serological Studies on Rice Transitory Yellowing Virus (RTYV) in Rice Plants and Green Leafhoppers

By TAKAHARU HAYASHI and YUZO MINOBE

Department of Molecular Biology, National Institute of Agrobiological Resources
(Yatabe, Ibaraki, 305 Japan)

Introduction

Rice transitory yellowing virus (RTYV) is one of the most important viruses infecting rice plants in China and Southeast Asia[6,13]. In Japan this virus is also sporadically observed at the restricted area (Okinawa)[9,11]

In some experiments[4,5,6,8], it was demonstrated that RTYV was transmitted by several species of green leafhoppers (*Nephotettix apicalis* Motsch., *N. cincticeps* Uhler, etc.).

Thus far, epidemiological, etiological or ecological studies[4-6,8,9,13] and electron microscopic studies[2,3,12] have been done on RTYV, but there are few biochemical or serological ones. This study revealed the characteristics of virions and their protein constituents by the immunoblotting method[10] which utilized the antibodies derived from the purified virus proteins. The feature of virus multiplication in rice plants and their vector insect green leafhoppers was revealed by the same method as well.

Purification of RTYV and preparation of antibodies

The difficulties of purifying this virus were its fragility and the tendency of virions to coaggulate each other after they were precipitated. These difficulties could be successfully overcome by the use of Percoll[9]. The analysis of the purified virus by polyacrylamidegel electrophoresis revealed that RTYV has 4 species of protein components nominated G, N, NS and M[7]. Antisera against the whole virions or each protein were obtained by immunizing rabbits or mice with the whole virions or each protein separated by polyacrylamide gel electrophoresis (Plate 1A). As shown in Plate 1B, antisera against 4 species of constituent proteins did not cross-react each other except for M and NS. Antiserum prepared from the whole virions had high precipitation titer of G and N, but much lower titer of NS and M.

In this investigation anti M reacted with NS as well as M. Therefore, there is a possibility that NS protein contains some antigenic regions common to M protein. But the amount of NS protein was so small that antibody against NS could not be obtained. Consequently, it was impossible to demonstrate that they have common antigenic region.

Antigen distribution of RTYV in rice plants and vector insects green leafhoppers

Another experiment was performed to investigate the antigen distribution of RTYV in infected rice plants and its insect vector green leafhoppers.

As previously described, RTYV can be persistently transmitted by several species of green leafhoppers[4-6,8,9]. Namely, RTYV multiplies in the insects after aquisition feeding and these insects get an ability to transmit the virus to rice plants, though transovarial transmission has not been proved. Besides, it has been pointed out that there are some differences of *in situ* feature of RTYV between rice cells and insects[3]. For instance, it was expressly pointed out that there were extraordinarily larger particles in leafhoppers

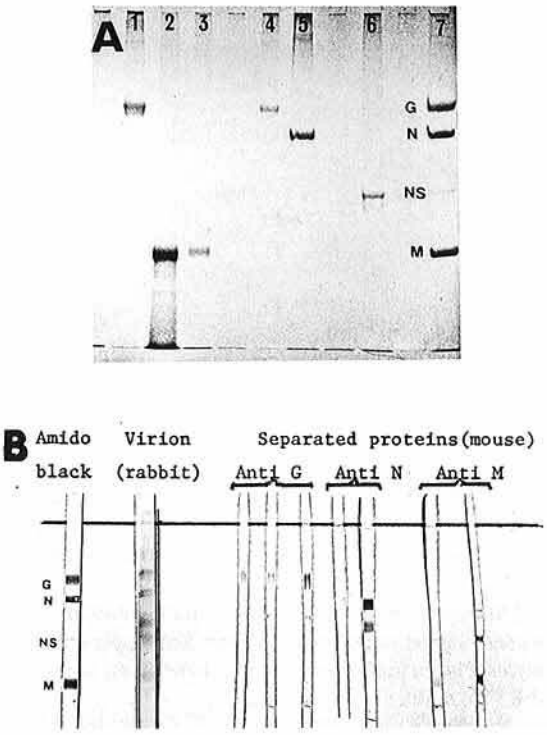

Plate 1. RTYV constituent proteins and immunoblotting of them with their antisera

A. RTYV constituent proteins separated by polyacrylamide gel electrophoresis. Lane 1–6 are the separated protein from purified whole virions (lane 7). Lane 1,4: G protein, lane 2,3: M protein, lane 5: N protein and lane 6: NS protein. Each protein was injected into mice and each of their antisera was obtained. Antiserum against whole virions was also prepared by injecting whole virions into rabbit. **B**. Immunoblotting of RTYV protein with their antisera. Purified RTYV protein was transferred from electrophoresed polyacrylamide gel to nitrocelulose filter papers and stained with amidoblack or immunoblotted with antisera against whole virion, anti G, anti N and anti M.

(1,100–2,000 nm, 5–10 times larger than those of typical virus particles). On the other hand, ability of virus transmission varies in every insect which has equally aquired the virus by feeding or in the different species of insects[8]. To elucidate the mechanisms of these phenomena, several experiments were performed by investigating the antigen distribution of RTYV in infected rice plants and its insect vector green leafhoppers. In the infected rice plants (*Oryza sativa* L. cv. Koshihikari) the 4 species of virus protein could be detected (Plate 2A).

In addition to these 4 species of main viral protein, some minor band could often be seen, especially in the plant infected for a long term (more than 2 months). These minor components seem to be the degradation products of main constituent proteins G, N and M as shown in Plate 2A (G′, N′ or M′).

Meanwhile, in green leafhoppers (*N. cincticeps* Uhler) kept on the infected rice plants for more than a month, that is the sufficient time to get transmission ability for the insects, antigen distribution in the insects mashed in collective showed different feature from rice plants. In green leafhoppers characteristic features were that they had a very small amount of G protein which is embedded in the lipid bilayer of virions borne in rice plants, and that an extraordinarily large amount of N protein was often seen in them (Plate 2B and 2C). When individual insect was examined, they had generally some irregular antigen distribution from each insect to insect (Plate 3). Among them only the insects which had a large amount of N protein had the ability to transmit virus to rice plants (Table 1).

In the next experiment the time of the appearance of viral antigens after aquisition feeding was investigated. After three days of acquisition feeding each insect was kept on a healthy seedling for certain periods, then the insects were stored at −70°C. The seedlings were planted in pots to see if the infection was achieved 2 months later. The freezed insects were mashed and immunoblotted to see the viral antigen distribution in them. According to these experiments, the earliest appearance of viral antigen could be seen on the 3rd day after the end of acquisition feeding. With the lapse of time the number of insects which contained viral antigen gradually increased. Finally, not every insect showed viral antigen, and the distribution of viral antigen in them was also irregular.

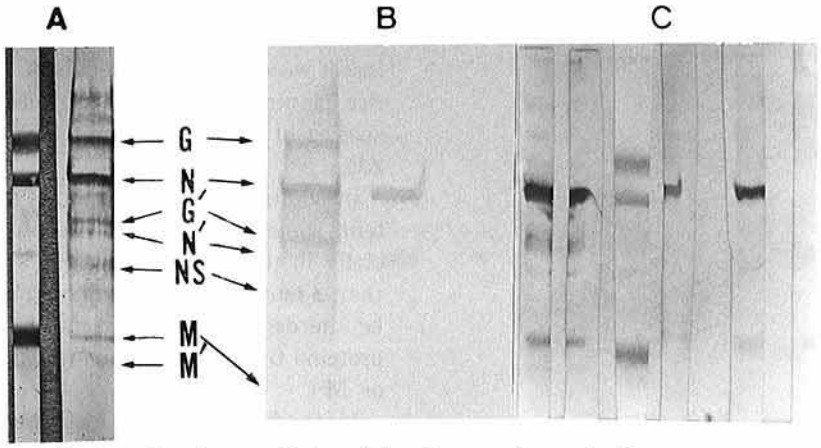

Plate 2. Immunoblotting of crude sap of infected rice plants and green leafhoppers

The infected rice plants or five collectives of green leafhoppers were mashed with 0.01 M Tris-HCl and 0.005 M $MgCl_2$ pH 7.5 and denatured by 1% SDS and 2% mercaptoethanol. Then these were centrifuged for 10 min at 7,000 rpm. After centrifugation the supernatants were electrophoresed and immunoblotted with antiserum against whole virion. **A.** Left lane: Purified RTYV protein stained with amidoblack. Right lane: Crude sap of infected rice plants. **B.** Parallel immunoblotting of infected rice plants (left lane) and green leafhoppers (center lane). Healthy green leafhopper was also immunoblotted (right lane). **C.** Different collectives of green leafhoppers immunoblotted in the same way. The center lane is the reference (purified RTYV stained with amidoblack).

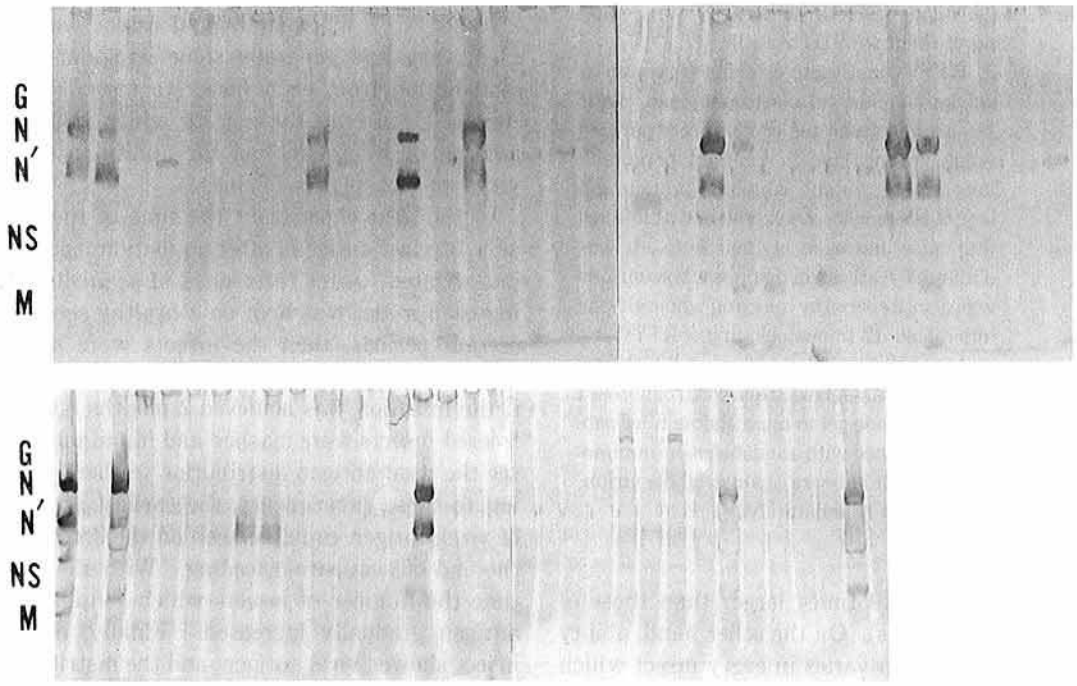

Plate 3. Immunoblotting of the individual insects (*N. cincticeps*) kept on the infected rice plants for more than a month

Each insect was mashed with buffer, electrophoresed and immunoblotted described in Plate 2.

Table 1. Relationships between the amounts of N antigen and transmission ability of green leafhoppers

Amounts of N antigen detected	Number of insects	Number of insects transmitter	% transmission
N. apicalis			
Little	47	0	0
A little	28	0	0
In large amounts	10	6	60
Total number of insects	85	6	7
N. cincticeps			
Little	72	0	0
A little	12	0	0
In large amounts	16	2	13
Total number of insects	100	2	2

The amount of N antigen was estimated from another series of experiments in Plate 3.

Antigen distribution of virus in the infected rice plants and green leaf hoppers analysed by Ficoll density gradient centrifugation

To examine the morphological differences between rice-borne and insect-borne RTYV, Ficoll density gradient centrifugation was performed. The infected plants or insects were homogenized with buffer in a motar. The homogenate was centrifuged at 7,000 rpm for 10 min. The supernatant was loaded on 5–20% Ficoll density gradient and centrifuged at 16,000 rpm for 90 min in Hitachi RPS 40 T rotar. In this Ficoll density gradient centrifugation analysis, virus antigen from rice plants sedimented in the middle of the centrifuge tube (Plate 4A). On the other hand, virus antigen from leafhoppers did not sediment in the same position as ones from rice plants, but it sedimented far slowly (Plate 4B). These results suggest that the structure of RTYV in insects differs from ones in rice plants in accordance with the results of antigen distribution in insects seen in the previous experiments.

Supposed defective interfering particles (DI) of RTYV

The irregular antigen distribution of RTYV in the leafhoppers suggests a possibility that they have so-called deffective interfering particles (DI). DI particles can often be seen in the group of rhabdoviruses and interfere the multiplication of normal viruses[1].

According to the histograms of the purified RTYV by Percoll density gradient centrifugation, RTYV has a wide size distribution in particle length (90–200 nm) being consisted of mainly two peaks (120–140 nm and 145–160 nm) (Fig.1). The size of normal or standard RTYV is thought to be 145–160 nm and that of DI particles is 120–140 nm. The width of RTYV is invariably constant (90–100 nm).

Ficoll density gradient pattern also suggested two peaks as shown in Fig. 2. The main peak seems to be normal virus particles and upper peak sedimenting slowly is supposed DI particles.

Discussion

In this study RTYV structural protein was characterized serologically. Each of RTYV constituent proteins (G, N, NS and M) which had been identified by polyacrylamide gel electrophoresis[7] had independent antigenicity except for NS. NS had antigenicity common to M. As anti NS antibody can not be obtained so far and the contamination of NS to M, with which rabbits or mice were immunized, can not be denied, it is impossible to come to a clear conclusion that they have common antigenic region.

Difference of the antigen distribution between rice plants and leafhoppers suggests that there are

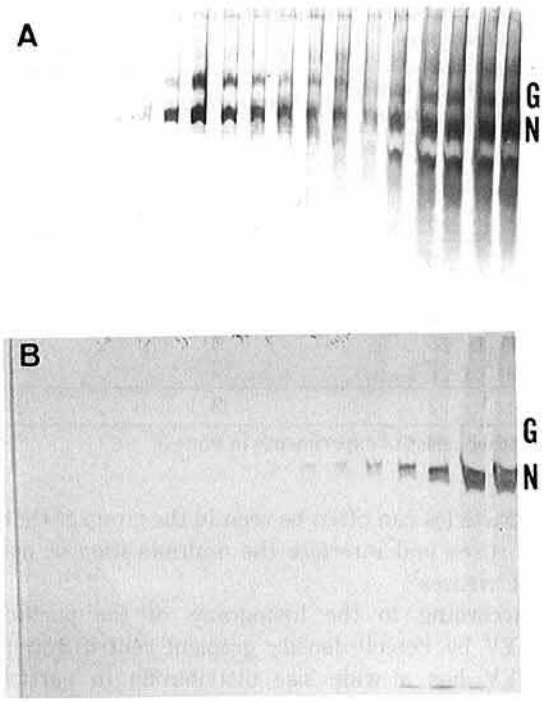

Plate 4. Immunoblotting of RTYV after Ficoll density gradient centrifugation

Infected rice plants or green leafhoppers (*N. cincticeps*) were mashed in 0.01 M Tris-HCl, 0.005 M $MgCl_2$ and centrifuged for 10 min at 7,000 rpm. The supernatants were loaded on 5–20% linear Ficoll density gradients and centrifuged for 90 min at 16,000 rpm in Hitachi RPS 40T rotar. After the fractionation, aliquots of each fraction were denatured with 1% SDS and 2% mercaptoethanol and electrophoresed in 10% acrylamide gel. Then electrophoresed protein was transferred on nitrocellulose filter paper and immunoblotted with antiserum against whole virions. **A**. RTYV antigen in infected rice plants. **B**. RTYV antigen in infected green leafhoppers. Right sides are the tops of the gradients.

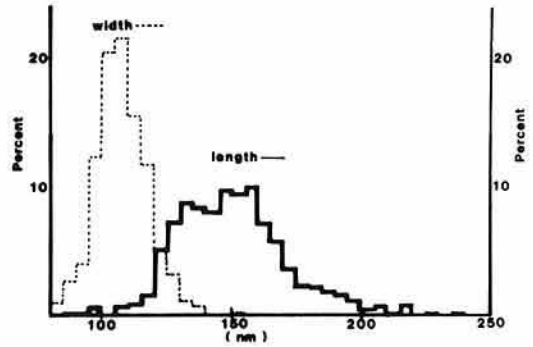

Fig. 1. Histograms of length or width distribution of purified RTYV

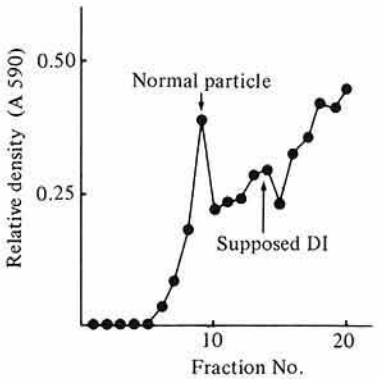

Fig. 2. Ficoll density gradient pattern of RTYV in the infected rice plants

The density of G protein bands in Plate 4A was traced by a densitometer at 590 nm and shown by a diagram.

some diversity of virus multiplication and virion structure between rice plants and leafhoppers.

In addition to the mode of virus multiplication, the irregular antigen distribution suggests the presence of DI particles, which is suggested by the short particles found in electron microscopy of purified virus and in Ficoll density gradient. DI particles are usually found in the group of rhabdoviridae. Its primary function is to inhibit normal virus multiplication, which is modulated by the ratio of DI to normal virus at the infection. Taking the presence of DI particles of RTYV into consideration, irregular antigen distribution, difference of transmission rate in the same species of leafhoppers or between species of leafhopper as well as the symptom of "transitory" yellowing can be understood reasonably. The evidences of DI participation in those phenomena should be demonstrated in the further studies.

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(Received for publication, October 2, 1986)