11. PURIFICATION AND PROPERTIES OF RICE STRIPE VIRUS

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Rice stripe disease has become a widespread and destructive virus disease coinciding with the extension of early planting of the rice crop in Japan. The disease has been reported in every rice growing area except the northern parts of Tohoku district in Japan. Rice stripe virus (RSV), the causal agent, is transmitted by leafhoppers such as *Laodelphax striatellus, Unkanodes sapporonus, Ribautodelphax albifascia,* and *Terthron albovittatus.*

A high rate of transovarial passage of the virus and serial passage of the virus by micro-injection method showed virus multiplication in vector insects. Leafhopper transmission or varietal resistance has been studied by many workers.

Prior to the perfect purification of RSV, Yasuo and Yanagita (1963) succeeded in preparing the antiserum against the virus by injecting the crude sap from RSV-infected rice leaves into rabbits. Okuyama (1959) first reported the purification of RSV from RSV-infetced rice leaves. They found spherical particles of 30–50 nm in diameter in the purified preparation. Saito et al. (1964) also purified RSV and demonstrated that RSV was a spherical virus of about 30 nm in diameter.

Kitani and Kiso (1968) isolated RSV as spherical particles of the same size which showed infectivity in micro-injection tests into *Laodelphax striatellus.* In a preliminary experiment, the author could not find any spherical virus particle in preparations obtained by the method described by Kitani and Kiso (1968). So the author attempted to isolate and find RSV particles.

Detection of branched filamentous particles

Doi et al. (1969) devised a rapid method for detecting virus particles in living plant materials and designated it the direct negative staining method. According to this method, RSV-infected leaves were crushed in a drop of 2% phosphotungstate solution on a carbon-coated grid. After air-drying, the preparations were examined under electron microscope. As a result, the preparations revealed the presence of branched filamentous particles of about 400 nm in total length and about 8 nm in width. These particles were quite different in morphology from any other virus particle so far reported, and never found in control preparations from healthy rice leaves.

Purification

The branched filamentous particles were purified from RSV-infected rice leaves according to the following procedure. The infected leaves were chopped and macerated in 0.2 M phosphate buffer containing 0.01 M diethyl dithiocarbamate and 0.01 M ascorbic acid by the use of a Waring blendor. The expressed sap was centrifuged at 8,000 rpm for 20 min. The supernatant was centrifuged on 10% sucrose cushion at 30,000 rpm for 3 hrs. The pellet was resuspended in 0.01 M phosphate buffer. After adding 20%

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chloroform, the suspension was stirred for 10 min. and centrifuged at 8,000 rpm for 10 min. The supernatant was again centrifuged at 30,000 rpm for 3 hrs. The resultant pellet was suspended in 0.01 M phosphate buffer to which was added Triton X-100 in concentration of 5%, and then differential centrifugations were repeated. Finally 10–40% sucrose density gradient centrifugations were repeated three times. Instead of sucrose density gradient centrifugation, D_2O-sucrose equilibrium centrifugation or CsCl equilibrium centrifugation was also employed.

Ultraviolet absorption

The purified preparation showed an ultraviolet absorption spectrum characteristic of a nucleoprotein with a maximum at 260 nm and a minimum at 246 nm. The A 260/280 ratio was 1.49, indicating that the nucleic acid content of the particles were about 12%.

Electron microscopy

Examination by electron microscope showed that the purified preparation contained a large number of the same branched filamentous particles as mentioned above (Fig. 1). When observed at high magnifications, each particle appeared to be a slender filament of 3 nm wide which usually took the form of a super-coiled helix (Fig. 2). The pitch

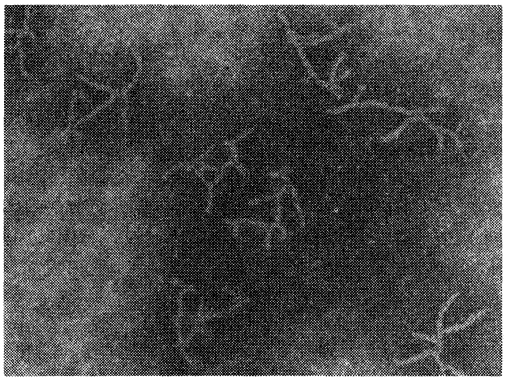

Fig. 1. Electron micrograph of branched filamentous particles in purified preparation.

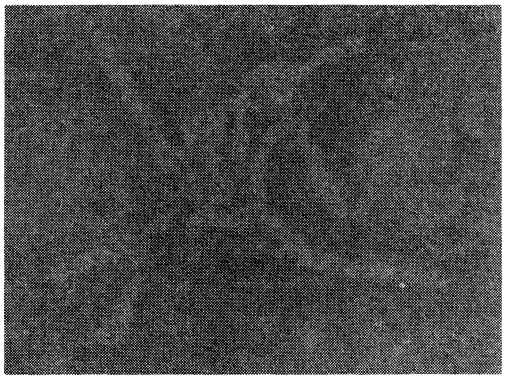

Fig. 2. Electron micrograph of a branched filamentous particle at high magnification.

of the helix was 6 nm. The number of branches differed with particles. Sometimes some parts of the particle showed loop-like structures due to partial loosening of the super-coiled helix. Some circular particles, 800 nm in contour length, were also observed among the branched filamentous particles. It is noteworthy that the branched filamentous particles closely resemble the complex of gene 5 protein from fd phage and single-stranded circular DNA from the same phage in morphology (Alberts et al. 1972).

Infectivity test

Infectivity of the purified preparation was tested by injecting the preparation into nonviruliferous insects of *Laodelphax striatellus*. The injected insects were fed on healthy rice seedlings for ten days, and then transferred individually to a healthy rice seedling in a test tube. After 4 days the insects were removed, and rice seedlings were transplanted in pots to observe the development of rice stripe symptoms. More than 50% of the injected insects died, but about 40% of survived insects transmitted RSV to the rice

seedlings. Leaves of these RSV-infected seedlings were examined under electron microscope by the direct negative staining mthod. A large number of branched filamentous particles were again observed.

From these results it is concluded that rice stripe virus is a branched filamentous particle.

Polymorphism

The purified RSV preparation was shown to contain three components by sucrose density gradient centrifugation. Top component in a faint broad band, about 1.4 cm below the meniscus, contained both single circular filaments of various, but short, length and single linear filaments of 800 nm long. The linear filaments were twice as long as the branched filamentous particles. Middle component was obtained in the largest quantity. It contained branched filamentous particles and a small number of circular particles of 800 nm in contour length. Bottom component contained the same particles in morphology as middle component. Sedimentation coefficient of top component and middle component ($OD_{260}=10$) in 0.01 M phosphate buffer was about 40 S and 72 S respectively.

Nucleic acid

Nucleic acid was isolated from the purified RSV preparation according to the SDS-bentonite-phenol method. It gave a positive result in orcinol tests for RNA, but a negative result in diphenylamine tests for DNA. It showed a maximum ultraviolet absorption at 257 nm and a minimum one at 240 nm. The A 260/280 ratio was 2.1–2.2, indicating minimal contamination of protein. Nucleic acid of RSV gave a positive reaction with formaldehyde. Twenty-four hours after the reaction with formaldehyde, the nucleic acid exhibited both an increase and a shift to a longer wavelength in ultraviolet absorption. In thermal denaturation tests the nucleic acid increased ultraviolet absorption gradually with temperature. These results suggest that RSV contains a single stranded RNA.

Specific soluble protein

Kitani and Kiso (1968) reported that a large quantity of specific soluble protein was produced in RSV-infected leaves. By disc electrophoresis the protein could be detected in RSV-infected rice leaves, but not in healthy ones. It was soluble at higher than pH 7.0, but precipitated as needle crystals at pH 5.4, the isoelectric point. Based on this property, the specific soluble protein was precipitated in sap from RSV-infected rice leaves by addition of 1/10 N HCl. The precipitate was resolved in alkaline phosphate solution. The procedure was repeated several times, and finally purified preparation of the protein was obtained. Usually 50–110 mg of the purified protein was isolated from 1 kg of RSV-infected rice leaves. The purified protein showed a maximum absorption at 278 nm and a minimum one at 250 nm. The sedimentation coefficient of the protein measured by analytical centrifugation was 3 S. SDS-acrylamide gel electrophoresis showed that the protein is a single component with the molecular weight of 21,000 daltons. The specific protein was concluded to be different from RSV coast protein because the molecular weight of the latter protein was 32,000 daltons. Fine parallel lattice of 4 nm wide was observed in needle crystals of the specific protein under electron microscope by negative staining with uranylacetate. The antigen of winter wheat mosaic virus reported by Atabecov et al. (1968) is very similar to the specific protein in its properties excepting that it takes a helical form. The function of the specific protein is still unknown.

Electron microscopy of thin sections

Ultrathin sections from RSV-infected rice leaves were observed under electron microscope. It was very difficult to discern RSV particles leaf cells because of their unique morphology, but they could be found here and there in the cytoplasm and in vacuoles, and sometimes as virus aggregates enclosed by membrances, of different kinds of leaf cells. Crystalline inclusions of the specific protein were observed in vacuoles or in xylem vessels probably due to their acid pH condition.

In conclusion, rice stripe virus is very unique in shape and fine structure as reported here. Its morphology is quite different from that of any other virus so far reported in plants, animals, and bacteria. However, European wheat striate mosaic virus, oat zakuklivanie virus, and winter wheat mosaic virus have been known to be very similar to rice stripe virus in virus-vector relationships and appearance of specific soluble protein in host cells. These three viruses may be very similar to rice stripe virus in particle morphology although their particles are still unknown.

Discussion

F. Nakasuji, Japan: (1) What is the particle which have been discovered by Okuyama and Asuyama (1959) etc.?

(2) Does the discovery of the new particle of RSV influence on the serological studies, e.g., measurement of percentage of viruliferous infected insect which was conducted by some entomologists?

(3) Is the particle of RDV which have been discovered the true particle?

Answer: (1) I think that spherical particles are artifacts, may be normal cell component or cell debris.

(2) A large amount of RSV particles are found in infected rice leaves. Antibody obtained by injecting the crude sap of contaminated plant into rabbits has a high titer. So, I think that discovery of new particles does not influence on the past serological studies.

(3) Yes. RDV is a large spherical particle.

K. C. Ling, IRRI: (1) How many insects were used for the infectivity test?

(2) How do you know the insects used for your infectivity test are free from virus?

Answer: (1) Fifty insects were used.

(2) The insects were maintained on rice seedlings for three years, and none of the seedlings have infected.

T. Soelaeman, Indonesia: Could it be possible that the branched filamentous particle is actually an aggregate of smaller particles?

Answer: I don't think so.

E. W. Kitajima, Brazil: (1) Have you tried to detect these branched particles in vector's tissues?

(2) Could you give further detail on gradient centrifugation studies, concerning the infectivity or the different zones obtained?

(3) You mentioned that you extracted viral RNA. Was it infective? What is its MW?

Answer: (1) No. I have not undertaken such experiment. But, Dr. Kiso told me that he detected branched filamentous particles in vector insects.

(2) and (3) I should like to test the infectivity of three components and extracted RNA. But I haven't made the experiment yet.