Association of Double- and Single-Stranded RNAs with Each Four Components of Rice Stripe Virus

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Abstract

Rice stripe virus (RSV) particles in purified preparations were circular filaments and found to be composed of four components (nB, B, M2 and M1 in the order of rapid sedimentation) after several cycles of sucrose density gradient centrifugations. When nucleic acids extracts from the purified RSV were electrophoresed, four double stranded (ds-) RNAs and four single stranded (ss-) RNAs were obtained. The size of the ss-RNA (designated as ss-1 to 4 in the order of decreasing molecular weights) under nondenaturing condition was 3.1, 1.5, 1.2 and 1.0×10^6 Mr, respectively, and that of ds-RNA (designated as ds-1 to 4 in order of decreasing molecular weights) under the same condition was 5.0, 2.8, 2.1 and 1.7×10^6 Mr, respectively. The nB, B, M2 and M1 components contained ss-1 and ds-1, ss-2 and ds-2, ss-3 and ds-3, and ss-4 and ds-4, respectively. Comparison of the size of ss-RNA and denatured ds-RNA in each component, and hybridization analysis suggest that ss-RNAs are counterparts of ds-RNAs and that the RNAs associated with the four components are not subgenomic. Thus all components are associated with RSV. These results are almost similar to those reported on maize stripe virus except that the number of components of the two viruses are different, which confirms that the both viruses are related each other.

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Key words: rice stripe virus, double-stranded RNA, viral components, hybridization.

INTRODUCTION

Rice stripe virus (RSV) causes severe damage to rice production in China, Japan, Korea and USSR. The virus is persistently transmitted by the smaller brown planthopper *Laodelphax striatellus* Fallén with a high percentage of transovarial transmission, which makes control of this disease very difficult. RSV particles were first reported to be branched filaments¹⁵⁾ and then to be circular filaments¹³⁾. RSV induces the accumulation of a specific protein¹⁴⁾ in infected plants, although the role of the protein has not yet been clarified. RNA-dependent RNA polymerase activity is associated with RSV²⁷⁾.

Nucleic acids of RSV have been reported to be composed of four species of single-stranded (ss-) RNAs^{24,26)}. RSV has three components, middle (M), bottom (B), and infective (nB) ones, and all the three components are in association with the four RNAs^{24,26)}. Maize stripe virus (MStpV), proposed to be placed in the same group with RSV¹⁰⁾, has been reported to contain five species of both ss- and double stranded (ds-) RNAs⁷⁾.

In the present investigation, we found that RSV had four components, each of which was associated with a pair of ss- and ds-RNAs.

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MATERIALS AND METHODS

Virus and plant materials. RSV collected from Konosu, Saitama Prefecture, Japan was maintained in rice seedlings by transmission via the viruliferous smaller brown planthopper, *L. striatellus*. Infected rice and wheat leaves with typical RSV symptoms were used as materials for virus purification.

Purification of RSV. RSV was purified following the method modified from that of Toriyama^{24,25} and Hibino¹². About 100 g of infected leaf tissues was ground in 1 liter of 0.1 M potassium phosphate buffer, pH 7.0 (PB), containing 10 mM sodium diethyldithiocarbamate and 10 mM ascorbic acid. The extracts were squeezed through two layers of gauze and emulsified with 20% CCl₄. After low-speed centrifugation for 10 min at 3,000×g, the supernatant was made 8% polyethyleneglycol (PEG 6000), 0.1 M NaCl and 1% Triton X-100, and then stirred for 1 hr at 4 C. The mixture was centrifuged for 15 min at 10,000×g. The pellet was resuspended in 100 ml of 0.1 M PB. After centrifugation for 10 min at 3,000×g, the supernatant was treated once more with PEG and NaCl. The pellet was resuspended in 20 ml of 0.1 M PB, and centrifuged for 10 min at 3,000×g. The supernatant was layered on a 10~40% sucrose density gradient in 0.1 M PB and centrifuged for 3.5 hr at 88,000×g (RPS27-2, Hitachi, Ltd., Japan). Virus bands and the zones between the bands were separately collected with syringes. Each fraction was diluted with 0.1 M PB and centrifuged for 1.5 hr at 150,000×g. The pellet was finally suspended in 0.01 M PB.

Purified RSV in each band was further centrifuged in a 10~40% sucrose density gradient in 0.1 M PB for 75 min at 165,000×g (RPS 50-2, Hitachi, Ltd., Japan) and fractionated through a model UA5 ultraviolet analyzer (ISCO, Lincoln, Nebr., USA).

Electron microscopy. Purified RSV containing all components was diluted with distilled water, mounted on grids coated with a collodion film, and stained with 4% uranyl acetate. The grids were examined using a JEM-1200 EX electron microscope (JEOL, Japan).

Preparation and electrophoretic analysis of RSV RNA. Mixture of purified RSV with 1/5 vol of 10% SDS and an equal amount of water-saturated phenol (pH 7.0), was shaken for 15 min at room temperature, and then centrifuged for 10 min at 15,000×g. The aqueous phase was reextracted with phenol and the nucleic acids were precipitated with 2.5 vol ethanol for 30 min at -70 C. The precipitate was recovered by centrifugation for 10 min at 15,000×g, dried *in vacuo*, and dissolved in sterile distilled water. Nucleic acids were also released from the purified RSV by heating for 5 min at 55 C in a solution containing 1% SDS, 0.5% 2-mercaptoethanol, 0.05 M sodium phosphate, 2 mM EDTA and 10% sucrose (pH 7.4) just prior to electrophoresis⁷.

Nondenaturing electrophoresis was carried out on composite slab gels of 0.5% agarose/2.0% acrylamide^{17,21} using the buffer system of Loening¹⁶. Nucleic acids were stained with acridine orange (30 µg/ml)¹⁷ or ethidium bromide (1 µg/ml). Denaturing electrophoresis of ds-RNAs was carried out on 1.2% agarose horizontal gels using the buffer system of Peacock and Dingman²⁰ to compare the molecular weights of ss-RNAs and denatured ds-RNAs. Ds-RNAs were denatured with 5 mM methylmercuric hydroxide for 10 min at room temperature¹. After electrophoresis, the gels were soaked in 0.5 M ammonium acetate for 30 min and then stained with ethidium bromide.

The following RNA species were used as molecular weight standards: tobacco mosaic virus (TMV) RNA, 2.05×10^6 Mr²²; turnip mosaic virus (TuMV) RNA, 3.06×10^6 Mr²³; 23S and 16S ribosomal RNAs of *Escherichia coli*, 1.009 and 0.534×10^6 Mr, respectively^{3,4}; replicative form of citrus tristeza virus (CTV RF), 13.3×10^6 Mr²⁴; TMV RF, 4.1×10^6 Mr; RDV RNA, 3.29, 2.65, 2.39, 2.01, 1.91, 1.22, 1.20, 0.912, 0.827, 0.795, 0.553 and 0.553×10^6 Mr, respectively²⁵.

RNase A (Sigma) treatment in high- and low-salt buffers was carried out¹¹. The follow-

ing RNAs or DNA were used as controls: TMV RNA; 23S and 16S ribosomal RNAs; RDV RNAs; lambda phage DNA digested with *Hind* III (Pharmacia). After electrophoresis, gels were stained with ethidium bromide, soaked in RNase A (10 $\mu\text{g/ml}$) in $2\times\text{SSC}$ (0.3 M NaCl, 0.03 M sodium citrate) for 1 hr at 37 C, washed with $2\times\text{SSC}$ and $1\times\text{SSC}$, and then restained with ethidium bromide. Thereafter, the gels were soaked in RNase A in $0.1\times\text{SSC}$ for 1 hr at 37 C and restained. After electrophoresis, gels were also treated with DNase I (5 $\mu\text{g/ml}$) (Sigma) in the presence of 5 mM MgCl_2 .

Northern analysis of RSV RNA. Nucleic acids were electrophoresed on composite gel. After electrophoresis, the nucleic acids were denatured by soaking the gels in 0.1 M NaOH for 20 min. Then the gels were soaked twice in $20\times\text{SSC}$ for 20 min²³⁾. The nucleic acids were transferred²³⁾ to Hybond N membranes (Amersham International plc). The membranes were baked for 2 hr at 80 C.

For preparing ds-RNA probes, each ds-RNA was recovered from the gel after electrophoresis by phenol extraction of the excised band located by staining with ethidium bromide and labeled with 100 μCi of [γ -³²P]ATP. The probes were denatured for 3 min at 95 C and quenched on ice prior to hybridization. Hybridization was carried out using Amersham's rapid hybridization system (Amersham International plc). The membranes were immersed in rapid hybridization buffer and shaken for 15 min at 65 C. Then the probes were added to the buffer. After incubation for 1 hr at 65 C, the membranes were washed once in $2\times\text{SSC}$ containing 0.1% SDS, once in $1\times\text{SSC}$ containing 0.1% SDS, and then two times in $0.1\times\text{SSC}$ containing 0.1% SDS for 15 min at 65 C, respectively.

RESULTS

Purification of RSV

Purified RSV preparations were generally separated into three bands by the first sucrose density gradient centrifugation as described already by Toriyama²⁴⁾ (Fig. 1). The pellet obtained after the density gradient centrifugation was found to contain a large amount of RSV by the latex flocculation test (LF)¹⁹⁾. When the pellet was suspended in 0.1 M PB and centrifuged again in the density gradient, the same three bands appeared. When M band was recovered, placed on the density gradient and centrifuged, the three bands appeared again. A band corresponding to M component was recovered again and centrifuged in the density gradient. By the several cycles of the centrifugation, M component was separated from others (Fig. 2A). Similarly, B and nB components were separated from others.

The upper and lower portions (Fig. 2A, "a" and "b") of the M component were separately collected and then subjected to sucrose density gradient centrifugation. After several cycles

Fig. 1. Sucrose density gradient profile of purified RSV. Each peak are designated as M, B and nB component, respectively.

of centrifugations, a fraction that appeared to be a definite shoulder on the left side of a peak (Fig. 2B, "c") was obtained from "a". Similarly, a fraction that appeared to be a single peak (Fig. 2C, "d") was obtained from "b".

Electron microscopy

Purified RSV appeared as particles like entangled yarn. Circular filamentous particles were sometimes observed in the preparations (Fig. 3). Circular filaments were approximately 8 nm in width and ranged in length from 200 to 2,300 nm.

Fig. 2. Sucrose density gradient profiles of the isolated M component (A), a fraction obtained from the upper portion "a" after several cycles of sucrose density gradient centrifugation (B), and a fraction obtained similarly from the lower portion "b" (C). The portion "c" was referred as shoulder fraction and the portion "d" as peak fraction in the text.

Fig. 3. Electron micrograph of circular filaments of purified RSV stained with 4% uranyl acetate. Scale bar represents 200 nm.

Electrophoretic analysis of RSV RNA

Four broad and four sharp bands were observed on the composite gels after electrophoresis of nucleic acids extracted using SDS and phenol (Fig. 4). When nucleic acids were stained with acridine orange, the broad bands fluoresced red and the sharp bands fluoresced green (data not shown). When nucleic acids were treated with RNase A in $2\times\text{SSC}$, the broad bands disappeared as did those of TMV RNA and ribosomal RNAs. Thereafter, all bands except those of lambda DNA disappeared by following treatment with RNase A in $0.1\times\text{SSC}$ (Fig. 5). Treatment of nucleic acids with DNase I resulted in digestion of only lambda DNA (data not shown). These results suggest that the broad bands were ss-RNAs and the sharp bands were ds-RNAs.

The electrophoretic patterns of RNAs extracted by SDS and phenol were compared with those released directly by heating the purified RSV in SDS prior to the electrophoresis. No significant difference was observed in the relative amount of ds- and ss-RNAs extracted by the two methods (data not shown).

Fig. 4. Electrophoresis of RSV RNAs on composite gel. ss-1 to 4 and ds-1 to 4 show single stranded RNA 1 to 4 and double stranded RNA 1 to 4, respectively.

Fig. 5. Effect of RNase A on RSV RNAs following electrophoresis. (A) Electrophoresis of nucleic acids on composite gels. Lane 1, RSV RNAs; lane 2, RDV RNAs; lane 3, TMV RNA, 23S and 16S ribosomal RNAs; lane 4, lambda DNAs. (B) Digested in $2\times\text{SSC}$. (C) Digested in $0.1\times\text{SSC}$ followed by treatment of "B".

The sizes of ss-RNAs (designated as ss-1 to 4 in the order of decreasing molecular weight) under nondenaturing condition were 3.1 , 1.5 , 1.2 and 1.0×10^6 Mr, respectively, and those of ds-RNAs (designated as ds-1 to 4 in the order of decreasing molecular weight) under the same conditions were 5.0 , 2.8 , 2.1 and 1.7×10^6 Mr, respectively.

The ds-RNAs were denatured by methylmercuric hydroxide and electrophoresed on 1.2% agarose horizontal gels (Fig. 6). The sizes of the denatured ds-1 to ds-4 were 3.3 , 1.5 , 1.1 and 1.0×10^6 Mr, respectively, and coincided with those of ss-1 to 4, respectively.

When RNAs extracted from nB, B and M components were separately electrophoresed, ss-1 and ds-1, ss-2 and ds-2, and ss-3,4 and ds-3,4 were obtained, respectively (Fig. 7A). The

Fig. 6. Electrophoresis of denatured ds-RNAs of RSV on composite gel. Lane 1, non-denatured ds-RNAs of RSV; lane 2 to 5, denatured ds-4, 3, 2 and 1, respectively; lane 6 to 8, denatured 23S and 16S ribosomal RNAs, TMV RNA and TuMV RNA, respectively.

Fig. 7. Electrophoresis of RNAs extracted from each component of RSV on composite gel. (A) Lane 1 to 3, M, B and nB components, respectively; lane 4, four components. (B) Lane 1 to 3, M1, M2 and M, components, respectively.

Fig. 8. Northern blot analysis of RSV RNAs. RNAs were fractionated on composite gel, transferred to membrane and hybridized with each ds-RNA as the probe. Lane 1, four ds-RNAs used as a probe. Lane 2 to 5, ds-1 to 4 used as probes, respectively.

shoulder fraction (Fig. 2B, "c") obtained after the density gradient centrifugation of the upper portion of M band contained ss-4, ds-4 and a small amount of ss-3. The peak fraction (Fig. 2C, "d") obtained after the density gradient centrifugation of the lower portion of the M band contained ss-3, ds-3 and a small amount of ss-4 (Fig. 7B). These results suggest presence of two components in M band. We named the shoulder and the peak fractions, M1 and M2, respectively.

Northern analysis of RSV RNA

Northern blot hybridization was carried out to examine the relationship among RNAs. The ds-1 used as the probe hybridized with ds-1 and ss-1. In the same manner, ds-2, ds-3 and ds-4 hybridized with ds-2 and ss-2, ds-3 and ss-3, and ds-4 and ss-4, respectively (Fig. 8).

DISCUSSION

Each band obtained after the first sucrose density gradient centrifugation contained all the components (M, B and nB) as reported already by Toriyama²⁴⁾. Furthermore, the M component was found to be composed of two components, M1 and M2, though they were not completely separated by additional sucrose density gradient centrifugations. The association of different RNAs with M1 and M2 components suggested that they were not T components reported previously by Toriyama²⁵⁾ nor other artifacts. After the first sucrose density gradient centrifugation, the parts between bands and the pellet also contained large amounts of all the components. These results suggest that RSV particles are easily become entangled and are difficult to separate completely into the individual components. This entanglement may result from the filamentous nature of the virus^{13,15,24)} (Fig. 3). In fact, we observed particles like entangled yarn in purified RSV under the electron microscope. Bands other than those described above were sometimes observed and found to contain all the four components (data not shown), suggesting that the entanglement may be affected by virus concentration, purity and some other factors. MStpV was reported to have three to six sedimenting components⁹⁾. The variation in the number of components may also be due to similar entanglement.

RSV was found to be composed of four components containing one pair each of ss- and ds-RNA (Fig. 7A, B). The ss-RNAs were thought to be the counterparts of the respective ds-RNAs from a comparison of the sizes of the ss-RNAs and denatured ds-RNAs. This idea was confirmed by the hybridization experiment (Fig. 8). Some possible explanations for the presence of both ss- and ds-RNAs in the RSV RNA preparations are: 1) ds-RNAs and ss-RNAs are separately encapsidated in particles; 2) only ds-RNAs are encapsidated in particles, and ss-RNAs are denatured products of the ds-RNAs; 3) only ss-RNAs are encapsidated in particles, and ds-RNAs are annealing products of the ss-RNAs. If the first hypothesis were the case, two types of particles should have been observed by electron microscopy. We found no particles of different widths. If the second were the case, ds-RNA should have been obtained after the annealing of each ss-RNA reextracted from the gels, but such annealing experiments showed negative results (data not shown). Thus, the ss-RNAs detected on the gels seem to be composed of one sense. Based on these considerations, both plus and minus sense RNAs seem to be encapsidated separately as suggested for MStpV⁷⁾, and the amounts of particles of plus and minus sense RNAs are not equal.

This study clarified the number of RSV components, the nature of RNAs, and their relation with these components. The results of the hybridization experiment suggest that RNAs associated with the four components of the RSV are not subgenomic and each component is independently associated with RSV.

MStpV and RSV have been compared serologically, chemically and physically¹⁰⁾. The present study revealed the association of ds-RNAs and ss-RNAs with RSV as is the case with MStpV⁷⁾. The major difference between RSV and MStpV is the number of the nucleic acids, *i.e.*, RSV has four ss- and four ds-RNAs, while MStpV has five ss- and five ds-RNAs. The association of both ss- and ds-RNAs, a novel feature common to both viruses, suggests that they are related each other and should be placed in the same group probably together with rice grassy stunt virus¹³⁾ and rice hoja blanca virus¹⁸⁾. This characteristic may become one of the criteria of this novel virus group.

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和 文 摘 要

石川浩一・大村敏博・土崎常男：イネ縞葉枯ウイルスの4成分から検出される一本鎖および二本鎖 RNA

イネ縞葉枯ウイルス (RSV) の粒子および核酸について検討した。RSV 粒子は電顕下で環状のひも状粒子として観察された。純化 RSV より抽出した核酸をコンボジットゲル (2% ポリアクリルアミド-0.5% アガロース) で電気泳動したところ4本の一本鎖 RNA (ss-1, 2, 3, 4) の他に同数の二本鎖 RNA (ds-1, 2, 3, 4) が検出された。未変性条件下における一本鎖 RNA の分子量は 3.1, 1.5, 1.2 および 1.0×10^6 , 二本鎖 RNA の分子量は 5.0, 2.8, 2.1 および 1.7×10^6 であった。ショ糖密度勾配遠心 (10~40%) を繰り返して粒子を分離したところ従来報告の M 成分は2成分から成り、合計4成分 (nB, B, M2, M1) であった。単離成分から核酸を抽出したところ nB 成分より ss-1 と ds-1, B 成分より ss-2 と ds-2, M2 成分よりおもに ss-3 と ds-3, および M1 成分よりおもに ss-4 と ds-4 が検出された。ds-RNA を変性させて得た ss-RNA の分子量が各成分中の ss-RNA に対応すること、および ds-RNA をプローブとしたハイブリダイゼーションの結果から二本鎖 RNA の片方は同一成分より検出される一本鎖 RNA と同一であることが示唆された。