

Rice tungro disease is caused by an RNA and a DNA virus

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We present evidence that rice tungro spherical virus (RTSV) has a genome of polyadenylated single-stranded RNA of about 10 kb whereas rice tungro bacilliform virus (RTBV) contains double-stranded circular DNA. RTBV DNA has been mapped and shown to have two discontinuities, one in each strand,

at specific sites; it thus resembles that of the caulimoviruses. Gel electrophoresis of RTSV preparations revealed two protein bands (M_r 35K and 26K). RTBV yielded two major protein bands of 37K and 33K together with several minor species of higher and lower M_r which react with antiviral serum.

Introduction

Tungro, the most important virus disease of rice in south-east Asia, effects annual losses in excess of $\$1.5 \times 10^9$ (Herdt, 1988). It is caused by a complex of two viruses (Saito *et al.*, 1976; Hibino *et al.*, 1978). Rice tungro spherical virus (RTSV) is transmitted in a semipersistent manner by the green leafhopper, *Nephotettix virescens*, but induces few symptoms; rice tungro bacilliform virus (RTBV) causes the severe symptoms of the disease but, on its own, is not leafhopper-transmitted. Both viruses are transmitted together by leafhoppers to cause the disease (Hibino *et al.*, 1978, 1979; Hibino, 1983*a, b*; Cabauatan & Hibino, 1985). Despite the importance of rice tungro disease little is known about the molecular characteristics of the two viruses mainly owing to the low concentration of virus particles in infected plants and consequent difficulties in virus purification (Omura *et al.*, 1983; Cabauatan & Hibino, 1988). In this report we describe some molecular properties of the two viruses which show that RTSV is an RNA virus and that RTBV has a double-stranded DNA genome with some properties similar to that of the retroid cauliflower mosaic virus (CaMV).

Methods

Viruses and virus purification. A sample of tungro disease virus complex collected near Los Banos, The Philippines in 1987 and purified RTSV and RTBV were kindly provided by Dr H. Hibino,

International Rice Research Institute. The tungro virus complex was propagated in rice cv. TN1 and was purified by the method described by Cabauatan & Hibino (1988), a procedure which separates the two viruses. Alternatively, a newly developed method was used which gives a rapid preparation of unseparated viruses. In this procedure infected leaves were frozen in liquid N_2 , ground and thawed into 4 ml of 0.1 M-sodium citrate pH 5.9 per g tissue (i.e. 4 v/w). Celluclast (Novo Enzymes) was added to 5% (v/v) and, after incubation for 2 h at 30 °C, the mixture was centrifuged at 10000 r.p.m. for 10 min in a Sorvall GSA rotor. The pellet was resuspended in 2 v/w citrate buffer and 5% Celluclast, reincubated for 1 h at 30 °C and centrifuged as above. The supernatant fractions were pooled and polyethylene glycol 6000, NaCl and Triton X-100 added to 7%, 0.2 M and 1%, respectively. After incubation for 2 h at room temperature the preparation was centrifuged at 12000 r.p.m. for 10 min in a Sorvall GSA rotor and, after resuspension in 0.2 v/w citrate buffer, the pelleted material was centrifuged through a 5% sucrose cushion at 36000 r.p.m. for 2.5 h in a Beckman Ti40 rotor at 4 °C. This pellet, resuspended in 0.04 v/w citrate buffer, contained both RTSV and RTBV and is referred to as our 'unfractionated virus preparation'.

Viral coat proteins. Virus particles were dissociated by heating at 90 °C in Laemmli sample buffer (62.5 mM-Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue) and the coat proteins were separated by electrophoresis in 15% polyacrylamide gels containing SDS (Laemmli, 1970). Proteins were detected either by staining with Coomassie blue or by immunoblotting (Towbin *et al.*, 1979) using antisera kindly provided by Dr Hibino which were specific to each virus.

Viral nucleic acids. Viral nucleic acids were extracted by digestion of the coat protein of virus preparations for 2 h at 37 °C with 0.5 µg/µl Pronase in 100 mM-Tris-HCl pH 7.4, 10 mM-EDTA, 1% SDS, followed by phenol extraction, and recovered by ethanol precipitation. Selection on oligo(dT) columns was by standard procedures (Maniatis *et al.*, 1982). Nucleic acids were treated with either RNase-free DNase in 10 mM-Tris-HCl pH 7.5, 1 mM-MgCl₂ or 0.5 units of DNase-free RNase in 10 mM-Tris-HCl pH 7.5 without (low salt) or with 0.3 M-NaCl (high salt) at 37 °C.

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RTSV nucleic acid was electrophoresed in a 1% agarose gel containing formaldehyde (Maniatis *et al.*, 1982). RTBV nucleic acid was also electrophoresed in a 1% agarose gel in TBE; denaturation was by heating to 100 °C for 2 min and rapid cooling on ice. Both RTSV and RTBV nucleic acids were transferred to nitrocellulose membranes (Schleicher & Schuell BA85) using standard techniques (Maniatis *et al.*, 1982).

First-strand cDNAs were made to RTSV nucleic acid by standard techniques (Maniatis *et al.*, 1982) using both oligo(dT) and random priming. Second-strand cDNAs were made by the method of Gubler & Hoffman (1983). The cDNAs were made blunt-ended with T4 DNA polymerase and cloned into *Sma*I-cut pUC18 (Norrander *et al.*, 1983) or pBluescript (Short *et al.*, 1988) plasmids. For making RTSV probes, [α - 32 P]dCTP was incorporated into the first-strand cDNA synthesis. RTBV probes were prepared by nick translation of viral double-stranded DNA (Rigby *et al.*, 1977) also using [α - 32 P]dCTP.

Results

Virus purification

The purification procedures of Omura *et al.* (1983) and Cabauatan & Hibino (1988) give individual preparations of highly purified RTBV and RTSV either by separating the two viruses during purification or by purifying them from separately infected plants. Both these procedures are relatively laborious and therefore a modified procedure as described in Methods was developed. In developing this procedure F(ab')₂-ELISA (Barbara & Clark, 1982) was used to monitor the losses of both viruses at various stages. The viruses were captured by F(ab')₂ fragments of antisera to RTSV and RTBV. The second antibody was to the individual viruses and its binding was detected using protein A-peroxidase conjugate with tetramethylbenzidine as the chromogenic substrate. Freezing the leaves in liquid N₂ made them much easier to grind and gave more reliable yields of both viruses. There was relatively little loss of the two viruses when extracts were digested with Celluclast; the use of Dreselase (Cabauatan & Hibino, 1988) resulted in significant yield losses. The yield was usually about 1 to 2 mg virus per kg leaves from plants that had been infected for 4 to 6 weeks; the ratio of RTSV to RTBV particles was approximately 15:1.

Viral coat proteins

The Coomassie blue staining of gels containing the coat protein of RTSV which had been prepared individually (Fig. 1*a*, lane 1) revealed two predominant bands (M_r 35K and 26K) and a minor band of 37K. Each of these reacted with RTSV antiserum in immunoblots of either individually prepared virus or unfractionated virus (Fig. 1*b*, lane 1). There was no cross-reaction between RTSV and RTBV and their respective antisera (see Fig. 1*b*,

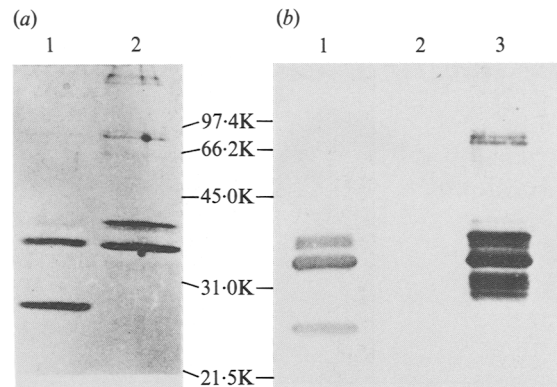


Fig. 1. Characterization of RTSV and RTBV coat proteins by gel electrophoresis. The viruses were purified individually (Cabauatan & Hibino, 1988), then dissociated and electrophoresed as described in Methods. (*a*) Gel stained in Coomassie blue: lane 1, RTSV; lane 2, RTBV. (*b*) Immunoblot: lane 1, RTSV probed with RTSV antiserum; lane 2, RTSV probed with RTBV antiserum; lane 3, RTBV probed with RTBV antiserum. The M_r s shown are from unstained protein markers from Bio-Rad.

lane 2) and no reaction between either antisera and healthy plant protein preparations.

Preparations of highly purified RTBV were also subjected to SDS-PAGE and major protein bands of 37K and 33K were detected by Coomassie blue staining (Fig. 1*a*, lane 2); there were also some minor bands of both higher and lower M_r . Immunoblotting showed reactions with all these protein bands (Fig. 1*b*, lane 3). In some preparations the 37K band predominated and in others the 33K band was most abundant, which suggests that the lower M_r band was derived from the larger.

RTSV nucleic acid

Nucleic acid isolated from purified RTSV was retained on oligo(dT) columns (data not shown). Clones of cDNA, made by priming with either oligo(dT) or random primers, had virus-specific inserts of up to 2.9 kbp which were recognized by probing against nucleic acid extracts from both virus-infected and healthy plants. When blots of nucleic acid from unfractionated virus preparations were probed with cloned DNA (Fig. 2*b*) a major nucleic acid species of about 10 kb was detected. This nucleic acid was sensitive to treatment with RNase under both high and low salt conditions but not to DNase treatment (Fig. 2*a*). This indicates that the RTSV genome is single-stranded RNA and its binding to oligo(dT), coupled with the priming of cDNA synthesis by oligo(dT), suggests that it is polyadenylated.

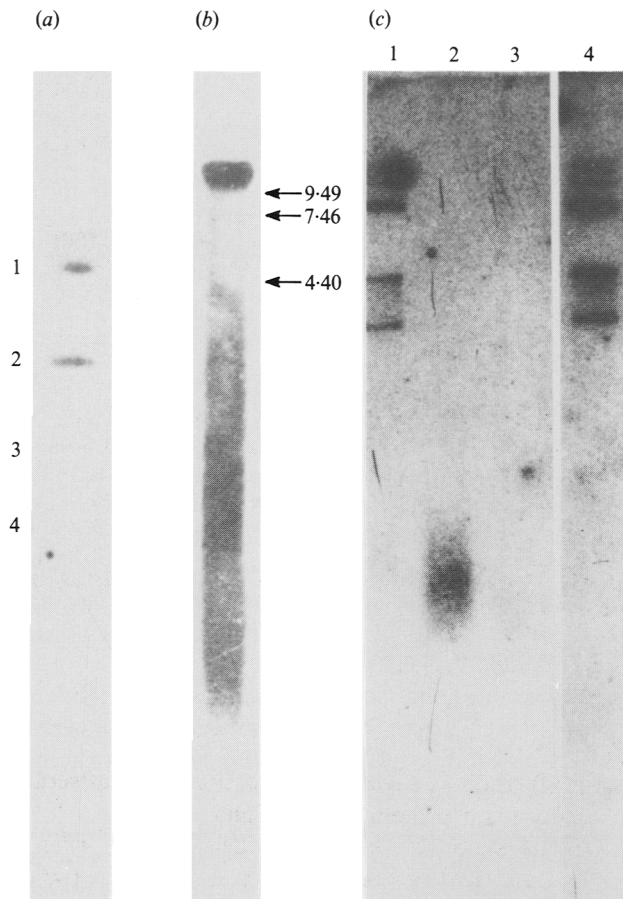


Fig. 2. Characterization of RTSV and RTBV genomes. (a) Slot blot analysis of RTSV nucleic acid treated with: 1, no enzyme; 2, 5 µg RNase-free DNase; 3, DNase-free RNase in the presence of high salt solution; 4, DNase-free RNase in low salt solution. The reactions were at 37 °C for 30 min and the blot was probed with ³²P-labelled RTSV cDNA. (b) RTSV nucleic acid, denatured in formaldehyde, electrophoresed in a 1% agarose gel, blotted onto nitrocellulose and probed with ³²P-labelled RTSV cDNA. The positions of molecular size markers (kb) are shown on the right-hand side. (c) RTBV nucleic acid treated with: lane 1, no enzyme; lane 2, RNase-free DNase (0.0027 units); lane 3, RNase-free DNase (0.027 units); lane 4, DNase-free RNase (0.5 units). The reactions were at 37 °C for 60 min. The samples were electrophoresed into a 1% agarose gel, transferred to a Hybond-N membrane and probed with ³²P-labelled cloned RTBV DNA.

RTBV nucleic acid

Nucleic acid extracted from preparations of RTBV migrated as several bands in agarose gels (Fig. 2c, lane 1; Fig. 3, lane 6), each of which was sensitive to DNase but not to RNase (Fig. 2c). These DNA molecules were cut by a variety of restriction endonucleases two of which, *Bam*HI and *Sal*I each gave a single major band of 8.3 kbp in gels and a minor band of 7.5 kbp (Fig. 3, lanes 2

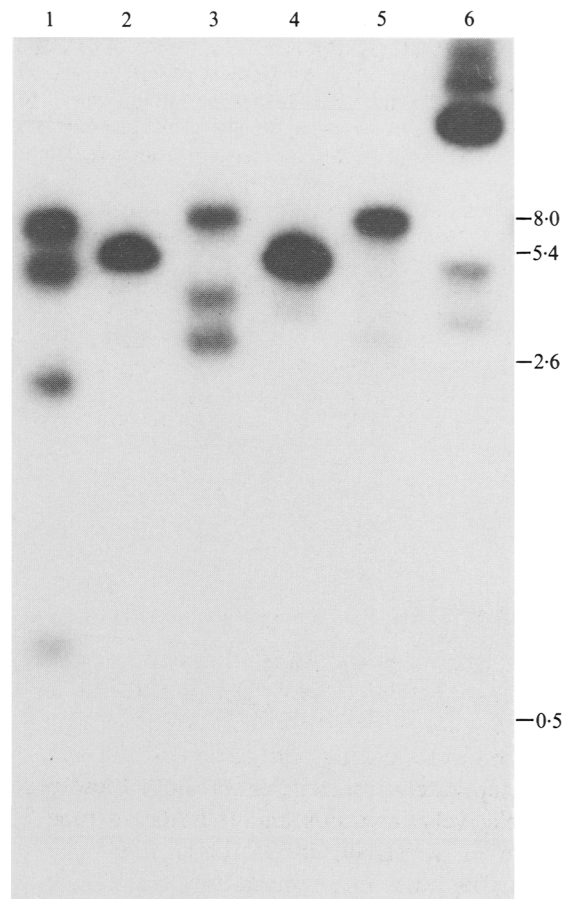


Fig. 3. Analysis of nucleic acid isolated from purified RTBV. The nucleic acid was digested with *Bam*HI (lanes 1 and 2), with *Sal*I (lanes 3 and 4) or was undigested (lanes 5 and 6). Nucleic acid in lanes 1, 3 and 5 was denatured by heating and quick-cooling; that in lanes 2, 4 and 6 was undenatured. The samples were electrophoresed in a 1% agarose gel, transferred to a Hybond-N membrane and probed with ³²P-labelled cloned RTBV DNA. Under these gel conditions denatured DNA migrates more slowly than undenatured DNA. The size markers (kb) on the right-hand side are denatured CaMV DNA.

and 4); digestion with other enzymes gave major bands which added up to approximately 8.3 kbp. This indicates that the viral genome is circular double-stranded DNA. RTBV DNA cut with either *Bam*HI or *Sal*I was cloned into pUC18 (cut with either *Bam*HI or *Sal*I) and a circular restriction endonuclease map was produced (Fig. 4) using a variety of restriction enzymes. Restriction endonuclease mapping indicated that the 7.5 kbp minor band lacked much of the region between the *Bam*HI and *Sal*I sites (data not shown). Gel electrophoresis of the viral DNA, which had been denatured by heating and rapidly cooling or by alkali treatment, produced a major band of 8.3 kb and minor bands of 7.5 and 4.0 kb (Fig. 3, lane 5). When the viral DNA was cut by *Bam*HI before denaturation, bands of 7.2, 5.2, 2.5 and

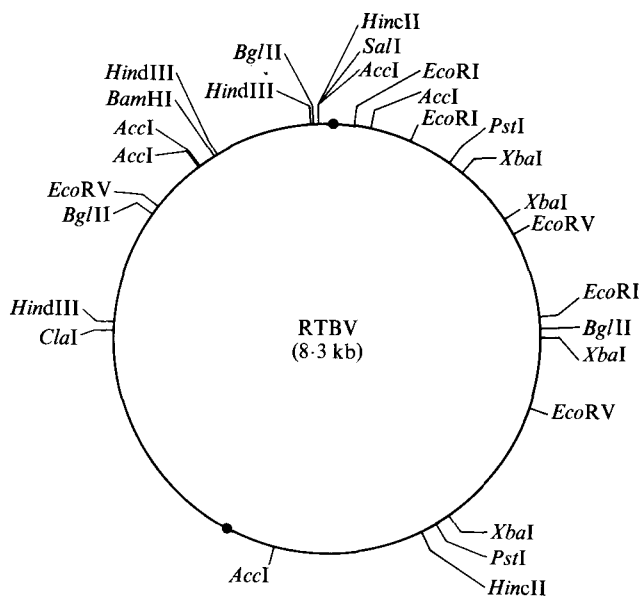


Fig. 4. Map of the restriction sites and positions of the discontinuities (●) in RTBV DNA.

0.7 kb were found upon gel electrophoresis; when cut with *SalI*, denatured DNA gave bands of 8.0, 4.5 and 3.5 kb (Fig. 3, lanes 1 and 3). These data suggest that RTBV DNA has two discontinuities, one in each strand at specific sites, whose approximate map positions based on the restriction enzyme analysis are shown in Fig. 4.

Discussion

The results presented demonstrate that the viruses which make up the rice tungro complex are very different from each other. RTSV has a single-stranded RNA genome which is polyadenylated. The size of RNA, the particle type and the transmission characteristics do not place RTSV in any currently recognized group of plant viruses. However, it does show similarities with maize chlorotic dwarf virus (MCDV) (Gingery, 1988; Ge *et al.*, 1989) in form and size of nucleic acid and in having a leafhopper vector with which it has a semipersistent relationship. Our data suggest that RTSV might have more than one coat protein species, also a feature of maize chlorotic mottle virus, but this has yet to be confirmed.

RTBV DNA has several features in common with the genomic DNAs of caulimoviruses. Caulimoviruses have circular genomes of 7.8 to 8.0 kbp (for reviews see Covey, 1985; Mason *et al.*, 1987) which always have a single discontinuity in one strand (the minus strand) and one to three in the other strand depending on the virus. Most strains of the type member of the group, CaMV, have

two discontinuities in the other strand but one strain (CM4-184) has a single discontinuity in each strand (Hull & Howell, 1978). These discontinuities in CaMV are sites for priming DNA synthesis. The native viral DNA of caulimoviruses has several conformations which produce multiple bands on gel electrophoresis as was observed for RTBV DNA (Fig. 2c, lanes 1, 3 and 6). There was no cross-hybridization between RTBV and CaMV DNAs (data not shown).

The caulimoviruses are the only group of plant viruses that is thus far known to replicate by reverse transcription. Unlike RTBV, the caulimoviruses have isometric particles. Recently Lockhart (1990) reported that several mealybug-transmitted viruses with bacilliform particles of similar size to RTBV contain double-stranded DNA genomes of about 7.5 kbp. Thus, RTBV could belong to a new group of plant viruses with DNA genomes. If the similarities between RTBV and caulimoviruses extend to the mode of replication, these bacilliform viruses could be a second group of plant pararetroviruses (Temin, 1989; Hull & Wills, 1989).

The interactions between the two very different viruses which cause rice tungro disease will be of considerable interest. Understanding the relationships of rice tungro viruses to other viruses and how they function together could assist in the prediction of other possible virus combinations which could result in new serious diseases. More immediately, approaches can be followed for new methods of conferring resistance in rice to the tungro disease.

The study of CaMV has had a considerable input into the genetic engineering of dicotyledonous plants due mainly to the use of the strong constitutive 35S promoter. If RTBV resembles CaMV, the finding of a pararetrovirus which infects a monocotyledon may have significant implications for the genetic engineering of rice and other cereal crops.

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