

## Rice hoja blanca virus genome characterization and expression *in vitro*

Bertha-Cecilia Ramirez,<sup>1\*</sup> Gabriel Macaya,<sup>1†</sup> Lee A. Calvert<sup>2</sup> and Anne-Lise Haenni<sup>1</sup>

<sup>1</sup>Institut Jacques Monod, 2 Place Jussieu-Tour 43, 75251 Paris Cedex 05, France and <sup>2</sup>Centro Internacional de Agricultura Tropical (CIAT), Apartado Aereo 67-13, Cali, Colombia

No information exists on the organization and mechanisms of expression of the genome of rice hoja blanca virus (RHBV), a member of the tenuivirus group, but here we describe the first steps in its characterization. RHBV contains four ssRNA and three dsRNA species, the sizes of which were estimated by native and denaturing gel electrophoresis. Hybridization analyses using <sup>32</sup>P-labelled riboprobes of viral and viral complementary polarities showed that unequal amounts of the two polarities of at least the smallest RNA are present in the virion, and indicated

that the dsRNA species contain the same information as the ssRNA species of corresponding size. Total RHBV RNA directs the synthesis of two major proteins of 23K and 21K *in vitro*. RNA3 directs the synthesis of a 23K protein designated NS3, and RNA4 of a 21K protein designated NS4. The NS4 protein corresponds to the non-structural protein that accumulates in RHBV-infected rice tissue. The nucleocapsid protein is not translated from either total RHBV RNA or any individual RHBV RNA *in vitro*.

### Introduction

Rice hoja blanca (white leaf) virus (RHBV) has already caused large decreases in yield in several regions of tropical America where rice (*Oryza sativa* L.) is produced (Jennings, 1963; Vargas, 1985). It is transmitted by the planthopper *Sogatodes oryzaicola* Muir, and belongs to the tenuivirus group, which also includes (Gingery, 1987) rice stripe virus (RSV, the type member), maize stripe virus (MStV), European wheat striate mosaic virus and rice grassy stunt virus (RGSV).

Tenuiviruses exhibit properties that are quite different from those of other plant RNA viruses (Gingery, 1987). Some of these properties are: (i) virus particles appear as fine filaments 3 to 8 nm wide and of variable length that may adopt a circular configuration (Gingery *et al.*, 1981; Toriyama, 1982; Morales & Niessen, 1983; Hibino *et al.*, 1985); (ii) they are persistently transmitted by a delphacid planthopper in which they are also transovarially passed (Zeigler & Morales, 1990); (iii) a single species of nucleocapsid (NC) protein of 31K to 34K is part of the infectious nucleoprotein (Gingery *et al.*, 1981; Toriyama, 1982; Morales & Niessen, 1983; Hibino *et al.*, 1985); (iv) they induce the accumulation in infected plants of large amounts of a 16K to 21K non-structural (NS) protein, also designated non-capsid protein (Gin-

gery *et al.*, 1981; Falk & Tsai, 1983; Gingery *et al.*, 1983; Morales & Niessen, 1983); (v) RSV and MStV contain a multipartite RNA genome that uses an ambisense coding strategy (two genes are encoded by complementary RNAs) for the expression of the two smallest RNAs (Hayano *et al.*, 1990; Kakutani *et al.*, 1990, 1991; Huiet *et al.*, 1991); (vi) an RNA-dependent RNA polymerase is associated with the virion, a property common to negative strand RNA viruses (Toriyama, 1986). Not all of the properties described have been verified for each of the five known tenuiviruses.

RHBV has particle morphology and vector relations similar to those of other tenuiviruses, but no serological relationship between RHBV and either RSV (Toriyama, 1983), MStV (Falk *et al.*, 1987) or RGSV (Morales & Niessen, 1985) has been observed.

The genomes of RSV and MStV, the best studied members of the tenuivirus group, are composed of four and five RNA species, respectively (Toriyama, 1982; Falk & Tsai, 1984), designated RNA1 to RNA4 (or RNA5 in the case of MStV) in order of decreasing size. When analysed by non-denaturing gel electrophoresis, the RNA is found to be a mixture of double-stranded and single-stranded molecules. Each dsRNA is related to a corresponding ssRNA, and hence each dsRNA species is approximately the same size (but double the *M<sub>r</sub>*) as the corresponding ssRNA. Molecules of complementary polarities are present for each RNA, but ssRNA of one polarity occurs in excess over the other (Falk *et al.*, 1989; Toriyama & Watanabe, 1989).

† Permanent address: Universidad de Costa Rica, Ciudad Universitaria Rodrigo Facio, San José, Costa Rica.

*In vitro* translation experiments indicate that at least some of the viral RNA can serve as mRNA. Total RSV RNA stimulates the production of protein incorporating [<sup>3</sup>H]leucine, of which only a minor protein band corresponds in size to that of the NC protein (Toriyama, 1985). In contrast the NC and NS proteins have been identified among the translation products produced by total MStV RNA (Falk *et al.*, 1987), or from *in vitro* transcripts corresponding to specific fragments of RNA3 for the NC protein (Huiet *et al.*, 1991) or RNA4 for the NS protein (Huiet *et al.*, 1990).

The complete nucleotide sequences of RSV RNA3 (Katutani *et al.*, 1991) and RNA4 (Kakutani *et al.*, 1990), as well as that of MStV RNA3 (Huiet *et al.*, 1991) have been established. Each of these RNAs contains two long open reading frames (ORFs), one in the 5' half of the viral sense (v) RNA, and the other in the 5' half of the viral complementary sense (vc) RNA. This novel coding strategy is designated ambisense. The RSV NS protein is encoded by vRNA4 (Hayano *et al.*, 1990; Kakutani *et al.*, 1990) and the NC protein by vcRNA3 (Kakutani *et al.*, 1991). Similarly, the MStV NS protein is encoded by vRNA4 (Huiet *et al.*, 1990) and the NC protein by vcRNA3 (Huiet *et al.*, 1991).

No information exists on the organization and mechanisms of expression of the RHBV genome. In this paper we describe the first steps in the characterization of the viral genome, namely the number, size and nature of the RNA components, results of Northern blot analyses using cDNA clones, and analyses of *in vitro* translation products produced by the viral RNAs.

## Methods

**Maintenance of vectors, plants and virus.** Insect vectors (*S. oryzae*) were taken from a colony maintained at the Centro Internacional de Agricultura Tropical (Cali, Colombia) and used to inoculate the RHBV-susceptible rice cultivar Bluebonnet 50. Viruliferous vectors were fed on 10-day-old seedlings for 3 days; vectors and plants were contained in butyl acetate tubes covered with fine nylon mesh (Galvez, 1968). After the inoculation period, the vectors were removed and the plants were kept in the greenhouse at 24 °C with a 16 h light period. From 1 to 2 months after inoculation, the virus was purified from rice leaves (Morales & Niessen, 1983). RHBV NS protein was purified from rice leaves using the differential pH method (Falk & Tsai, 1983).

**RNA purification.** Total RNA was extracted from RHBV by incubating 3 mg [estimated using the extinction coefficient indicated by Morales & Niessen (1983)] of purified virus preparation in a solution (1.9 ml) containing 0.1 mg/ml of proteinase K, 14 mM-sodium phosphate pH 7.6, 1% SDS and 5% vanadyl-ribonucleoside complex (BRL) for 1 h at 37 °C, and then centrifuging the incubation mixture through a 5.7 M-CsCl cushion for 20 h at 120000 g in a swinging rotor to pellet the RNA (Chirgwin *et al.*, 1979). The yield was 150 µg of RNA.

**Isolation of individual RNAs.** The RNAs were separated by electrophoresis on native 1.2% low melting point agarose gels (Toriyama & Watanabe, 1989). The eluted RNAs were finally precipitated with ethanol using beef liver tRNA as the carrier.

**Purification of dsRNAs from infected tissue.** The dsRNA was extracted and purified from 20 g of freshly harvested RHBV-infected rice leaf tissue as described (Morris & Dodds, 1979), except that two cycles of chromatography on CF 11 cellulose (Whatman) columns were performed; the ethanol pellet was resuspended in 50 µl H<sub>2</sub>O. Control dsRNA was prepared from the killer virus of *Saccharomyces cerevisiae* (strain A8209B) as described by Fried & Fink (1978).

**Gel electrophoresis.** Analysis of the RNA under native conditions was performed by electrophoresis in 1% agarose gels containing TAE (10 mM-Tris-HCl, 10 mM-sodium acetate and 1 mM-EDTA, pH 7). Electrophoresis was for 1 h at 80 V. The gels were stained in ethidium bromide for 20 min and photographed under u.v. light.

Analysis of the RNA under denaturing conditions was performed using formaldehyde or glyoxal. Denaturing formaldehyde gel electrophoresis was according to Maniatis *et al.* (1982). After electrophoresis, the gels were washed four times for 30 min in H<sub>2</sub>O, incubated twice for 30 min in 0.1 M-ammonium acetate and stained in the dark for 90 min in 1 µg/ml ethidium bromide in 0.1 M-ammonium acetate. For glyoxal denaturation (Gerard & Miller, 1986), 3 to 4 µg of RNA was treated in 1.5 M-glyoxal, 50% DMSO and 10 mM-sodium phosphate pH 7.0 for 1 h at 50 °C prior to loading onto 1% agarose gels containing 10 mM-sodium phosphate pH 7.0 and 2 mg/ml sodium iodoacetate. Electrophoresis was for 3 h at 40 V in 10 mM-sodium phosphate pH 7.0 with rapid and continuous recycling of the buffer. The gels were stained in the dark after removal of glyoxal (Falk & Tsai, 1984).

**Terbium (Tb<sup>3+</sup>) quenching of dsRNAs.** After electrophoresis of the RNA under native conditions, the gels were soaked at 25 °C for 30 min in H<sub>2</sub>O, 30 min in 0.5 µg/ml ethidium bromide in H<sub>2</sub>O and 5 min in H<sub>2</sub>O, and photographed; quenching of the dsRNA was observed after immersing the gel in 0.25 mM-TbCl<sub>3</sub> (Aldrich Chemical), 10 mM-cacodylate-HCl pH 6.8 and 0.5 µg/ml ethidium bromide for 4 h (Al-Hakeem & Sommer, 1987).

**Synthesis and cloning of cDNA.** RHBV RNA (5 µg) was polyadenylated using 2 units (U) of poly(A) polymerase (BRL; Devos *et al.*, 1976). cDNA was prepared using the ZAP-cDNA synthesis kit (Stratagene). The cDNA library was packaged using the Gigapack II λ packaging extract (Stratagene) and plated onto the *Escherichia coli* strain PLK-F'.

**Screening and characterization of cDNA clones.** Individual RNA1 to RNA4 species (see above) and total RHBV RNA were partially fragmented (Toriyama & Watanabe, 1989) and 5' end-labelled (Maniatis *et al.*, 1982) with T4 polynucleotide kinase (Stratagene) and [<sup>32</sup>P]ATP (Amersham). The labelled fragments were phenol-extracted, ethanol-precipitated and fractionated on Sephacryl G-50 (Pharmacia) columns. The library was screened using these labelled probes essentially as indicated by the manufacturer (Stratagene). Plates containing up to 1000 plaques were screened with each probe and positive λ zap II plaques were converted to plasmid clones (pBlue-script SK<sup>-</sup>) using helper phage R408, and introduced into XL1-Blue *E. coli* cells. The second screening was performed on colonies using the same probes, and plasmid DNA was isolated from positive clones by the alkaline lysis procedure (Maniatis *et al.*, 1982). The cDNA inserts were mapped using restriction enzymes, labelled by nick translation (Maniatis *et al.*, 1982) and hybridized to total RHBV RNA to identify the origin of the cDNA.

**Preparation of riboprobes.** Riboprobes were synthesized using an RNA transcription kit (Stratagene). Clone RHB4C529 in pBlue-script SK<sup>-</sup> contains an insert of 700 bp corresponding to a region located near the 5' end of RHBV RNA4. Plasmid DNA prepared from this clone was digested with *Xho*I or *Xba*I, and 1 µg of linear DNA was transcribed with T3 or T7 RNA polymerase respectively, yielding riboprobes that allow the detection of vc- or vRNA. Transcription reactions were performed essentially as indicated by the manufacturer

with the addition of 10 U RNasin (Promega), 50  $\mu$ Ci [ $\alpha$ - $^{32}$ P]CTP (>400 Ci/mmol; Amersham) and 40 mM-CTP.

**Northern blotting and hybridization.** Native gel electrophoresis (see above) was in 1% agarose gels at 60 V for 90 min. One part of the gel was stained and photographed, and the other part was first treated with a solution containing 50 mM-NaOH and 150 mM-NaCl for 30 min, then with a solution containing 100 mM-Tris-HCl pH 7.6 and 150 mM-NaCl for 30 min, and transferred by capillary transfer overnight with  $10 \times$  SSC (SSC is 1.5 M-NaCl and 0.15 M-sodium citrate) to GeneScreen Plus (NEN) membranes (Maniatis *et al.*, 1982). Glyoxal gel electrophoresis was as described above. After removal of the glyoxal, one part of the gel was stained and photographed. The other part was transferred as described above.

Membranes were prehybridized at 65 °C for 2 h in a solution containing 50% deionized formamide, 50 mM-Tris-HCl pH 7.5, 5 mM-EDTA,  $5 \times$  SSC,  $5 \times$  Denhardt's solution [ $1 \times$  is 0.1% Ficoll 400 (Pharmacia), 0.1% polyvinylpyrrolidone (Sigma), 0.1% BSA fraction V (Sigma)], 1% SDS and 50  $\mu$ g/ml denatured salmon sperm DNA. Hybridization was carried out in the same solution and at the same temperature with  $10^6$  c.p.m./ml of  $^{32}$ P-labelled riboprobe. Washes under stringent conditions were performed at 65 °C in  $2 \times$  SSC, 0.1% SDS twice for 15 min each, and in  $0.1 \times$  SSC, 0.1% SDS twice for 15 min each. The membranes were autoradiographed for 25 min.

**Translation in vitro.** Translation experiments *in vitro* were performed using a rabbit reticulocyte lysate (Morch *et al.*, 1989). The incubation mixtures (10  $\mu$ l) contained 700 ng RHBV RNA and were incubated at 30 °C for 60 min. Following translation, the products were electrophoresed on 14.9% acrylamide-0.09% bis-acrylamide, 0.1% SDS (SDS-PAGE) slab gels (Laemmli, 1970) at 70 V for 16 h. The gels were fixed in a methanol:acetic acid:water solution (30:7.5:62.5), dried and autoradiographed for 1 to 7 days.

**Immunoprecipitation.** Rabbit polyclonal anti-RHBV antiserum was produced against pooled virus preparations obtained from RHBV-infected rice tissue (Morales & Niessen, 1983); rabbit polyclonal anti-NS protein antiserum was produced against the major RHBV NS protein purified by SDS-PAGE (Falk *et al.*, 1987). Both antisera were kindly provided by Dr F. Morales. Immunoprecipitation was carried out using a procedure derived from that described by Dulieu & Bar-Joseph (1990). After *in vitro* translation, 8  $\mu$ l of the reaction mixture was adjusted to 100  $\mu$ l with NP40 buffer [10 mM-sodium phosphate pH 7.2, 150 mM-NaCl, 1% NP40 (Sigma)], 1  $\mu$ l rabbit preimmune antiserum was added and the solution was incubated for 1 h at 4 °C. After the addition of 60  $\mu$ l of 10% Protein A-Sepharose CL-4B (Pharmacia) in NP40 buffer, the mixture was incubated overnight at 4 °C and the Sepharose recovered by centrifugation. The resulting pellet was stored at -70 °C for protein analysis by gel electrophoresis as indicated below. The supernatant was incubated overnight at 4 °C with 3  $\mu$ l of anti-RHBV or anti-NS antiserum. Protein A-Sepharose (60  $\mu$ l, 10%) was then added and incubated for 3 h at 4 °C and the Sepharose pellet was recovered by centrifugation. Both pellets were washed three times in 100  $\mu$ l NP40 buffer and finally resuspended in 40  $\mu$ l SDS protein sample buffer (Laemmli, 1970) for SDS-PAGE. In certain cases (as indicated), treatment with the rabbit preimmune antiserum was omitted.

## Results

RHBV RNA extracted from purified virus preparations was analysed by gel electrophoresis under native conditions together with the following controls: an

ssRNA ladder, turnip yellow mosaic virus (TYMV) ssRNA and yeast killer virus dsRNA. Seven clearly separated bands were observed (Fig. 1*a*). To distinguish dsRNA from ssRNA, quenching of the fluorescence of the dsRNA by  $Tb^{3+}$  was used.  $Tb^{3+}$  quenches the fluorescence of ethidium bromide bound to dsRNA producing a 40-fold reduction of emission, whereas quenching of ssRNA is less than fivefold (Al-Hakeem & Sommer, 1987). The gel shown in Fig. 1(*a*) was stained with ethidium bromide; when treated with a solution containing 0.25 mM- $TbCl_3$  only bands corresponding to ssRNA were visible under u.v. light (Fig. 1*b*). Using this procedure, it appeared that total RHBV RNA consistently contains four ssRNA species (designated ssRNA1, ssRNA2, ssRNA3 and ssRNA4 in order of decreasing size) and three dsRNA species (Fig. 1*a* and *b*). However, some or all of the dsRNAs were not visible in certain RHBV RNA preparations (see below).

The nature of the dsRNA bands was further assessed by examining the resistance of these RNAs to RNase A digestion under high ionic strength conditions (Lejour & Kummert, 1986) prior to analysis on native gels. Under these conditions, the bands corresponding to ssRNA1 to ssRNA4 were no longer visible (not shown). Their nature was also assessed by comparing the migration profile of the three presumed dsRNA species contained in the virions with that of the dsRNAs extracted from RHBV-infected rice tissue and purified by CF 11 cellulose chromatography. When analysed by native gel electrophoresis, three of the RNAs isolated from the nucleoprotein preparation migrated in the same positions as the three dsRNAs extracted from infected rice tissue (Fig. 2). Furthermore,  $Tb^{3+}$  quenching experiments confirmed that the RNA isolated from infected tissue is indeed dsRNA (not shown). No dsRNA was detected in non-infected tissue (Fig. 2).

When the individual ssRNA bands were eluted from a native gel, phenol-extracted, ethanol-precipitated and analysed on a second identical gel, a major ssRNA band appeared and a minor dsRNA band was clearly visible on the original photograph. Conversely, a dsRNA band, isolated and purified similarly, yielded a major dsRNA band and a minor ssRNA band. Fig. 3 presents the results of such an experiment performed with RNA4.

The size of the ssRNA species was estimated under denaturing conditions using glyoxal (Fig. 4) or formaldehyde (not shown), and the appropriate ssRNA markers. RNA2, -3 and -4 were clearly observed (Fig. 4); their average size, estimated from four gels, was 3800 nucleotides (nt), 2300 nt and 1900 nt ( $\pm 100$  nt), respectively. Scanning of the film negatives of the gels using a Joyce-Loebl microdensitometer consistently revealed two weak RNA bands, the average sizes of which were 1300 nt and 850 nt ( $\pm 40$  nt). The sensitivity of detection

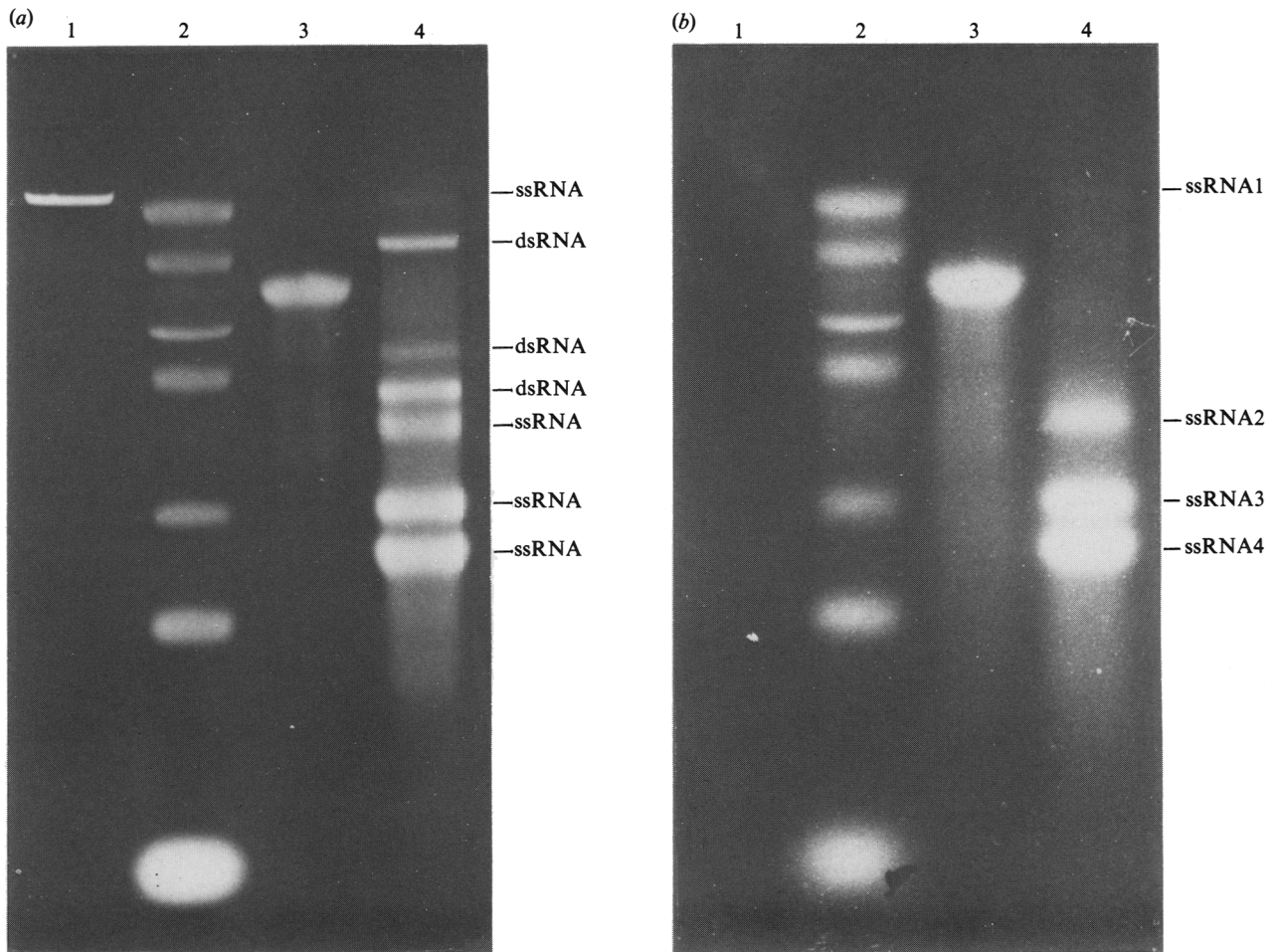


Fig. 1. Identification of ssRNA and dsRNA species by native gel electrophoresis and  $Tb^{3+}$  fluorescence quenching. Ethidium bromide-stained gel before (a) and after (b)  $Tb^{3+}$  treatment. The positions of the various RHBV RNAs (lanes 4) are indicated to the right of each panel. Lanes 1, yeast killer virus dsRNA (only one species is clearly visible); lanes 2, high  $M_r$  ssRNA ladder (BRL); lanes 3, TYMV ssRNA as controls.

of RNA using ethidium bromide is much lower under denaturing than under native conditions. Although the amount of RHBV RNA analysed under native conditions was as much as fourfold that under denaturing conditions, RNA1 was not present in sufficient amounts to be visualized on denaturing gels; however it could be detected using radiolabelled riboprobes (not shown).

The average size of RNA1, -2, -3 and -4 estimated from four native gels was 9800 nt, 3600 nt, 2500 nt and 1900 nt ( $\pm 300$  nt for RNA1 and  $\pm 100$  nt for RNA2 to RNA4), respectively. Using the dsRNA from the yeast killer virus as size standards (estimated by denaturing gel electrophoresis, Fig. 4), the average size of the three RHBV dsRNA species and of the three dsRNA species extracted from infected rice tissue was estimated from four native gels at 4200 bp, 2800 bp and 2300 bp ( $\pm 100$  bp); these are designated dsRNA2, dsRNA3 and

dsRNA4, respectively. The differences observed between the estimated sizes of the RHBV ss- and dsRNA may result from the fact that the sizes of the killer virus dsRNA markers were estimated from ssRNA under denaturing conditions.

RHBV RNA was analysed by Northern blotting under native and denaturing conditions using  $^{32}P$ -labelled riboprobes of v or vc polarity (Fig. 5). When RNA was electrophoresed under native conditions and denatured before transfer to the membrane, the T7 RNA polymerase-derived transcript that detects vRNA4 hybridized to ssRNA4 and dsRNA4 (Fig. 5a, lane 2); it also hybridized to a small RNA of about 800 nt which appeared to be derived from vRNA4 and has been designated subgenomic (sg) RNA4 for the sake of convenience (Fig. 5a, lane 2). The T3 RNA polymerase-derived transcript that detects vcRNA4 hybridized only

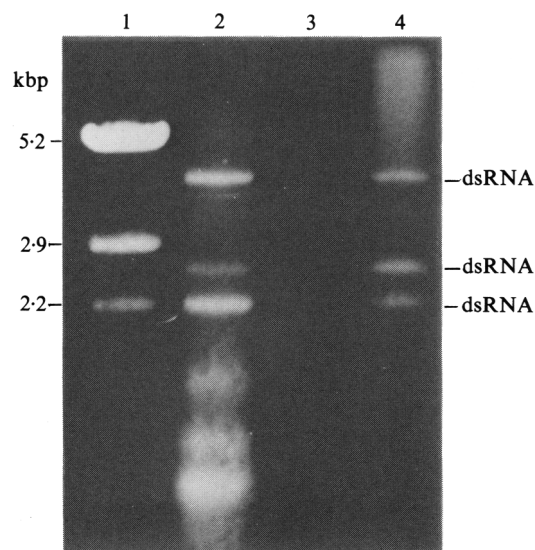


Fig. 2. Native gel electrophoresis of RHBV RNA (lane 2) and dsRNA from RHBV-infected rice tissue (lane 4). Lane 3, sample from non-infected tissue prepared under conditions identical to those used to prepare the sample from RHBV-infected tissue. The positions of the dsRNAs are indicated to the right. Yeast killer virus dsRNAs (lane 1, positions and sizes indicated to the left) serve as size markers. The gel was stained with ethidium bromide.

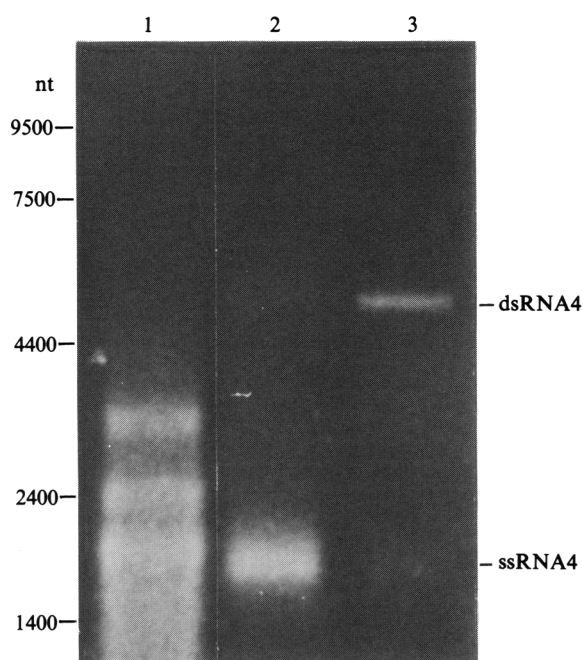


Fig. 3. Appearance of both dsRNA and ssRNA from isolated ssRNA or dsRNA, as detected by native gel electrophoresis. The ssRNA4 (lane 2) and dsRNA4 (lane 3) species were eluted from a native gel comparable to the one shown in Fig. 1, and phenol-extracted, ethanol-precipitated and analysed on the second native gel shown here. Lane 1, RHBV RNA. The positions of dsRNA4 and ssRNA4 are indicated to the right. The molecular sizes shown to the left are taken from a high  $M_r$  ssRNA ladder in a lane of the same gel (not shown). All lanes are from the same gel. The gel was stained with ethidium bromide.

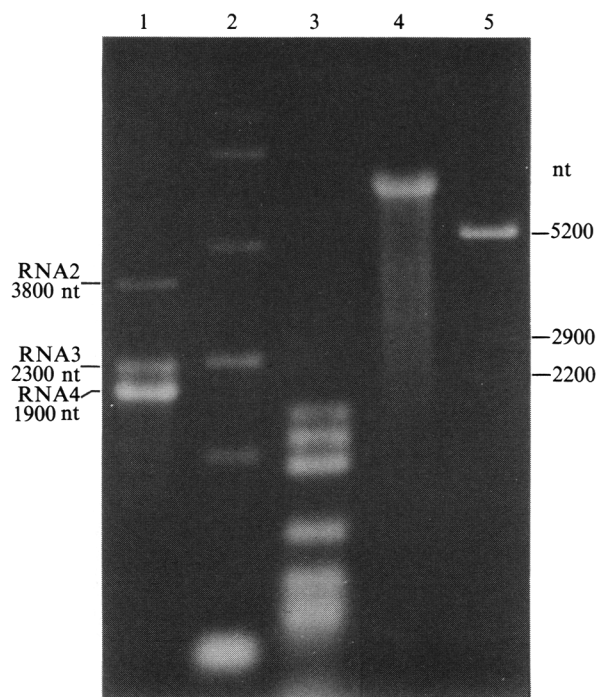


Fig. 4. Analysis by denaturing glyoxal gel electrophoresis of the size of the RHBV RNAs (lane 1). Lane 2, high  $M_r$  ssRNA ladder (9500 nt, 7500 nt, 4400 nt, 2400 nt, 1400 nt and 240 nt); lane 3, low  $M_r$  ssRNA ladder (1770 nt, 1520 nt, 1280 nt, 780 nt, 530 nt and 400 nt; BRL); lane 4, TYMV ssRNA (6318 nt) as size markers. The estimated sizes of the RHBV RNAs are indicated to the left, and those of the yeast killer virus RNAs (lane 5) to the right. All lanes are from the same gel. The gel was stained with ethidium bromide.

to dsRNA4 (Fig. 5a, lane 3). Hybridization signals were visible after 5 min exposure, but the riboprobe detecting vcRNA gave no signal with ssRNA4 or sgRNA4 after 24 h exposure (not shown). When the RHBV RNA was electrophoresed under denaturing conditions and analysed using the same riboprobes, the T7 RNA polymerase-derived transcript hybridized to ssRNA4 and sgRNA4 of about 900 nt (Fig. 5b, lane 2), whereas the T3 RNA polymerase-derived transcript hybridized to ssRNA4 only (Fig. 5b, lane 3). The intensity of the hybridization signal was stronger with the T7 RNA polymerase-derived transcript than with the T3 RNA polymerase-derived transcript. Identical results were obtained when different RHBV RNA preparations were analysed (not shown).

Total RHBV RNA was used as the template for *in vitro* translation experiments and the products were analysed by SDS-PAGE (Fig. 6). Two major proteins migrating at 23K and 21K were observed in a reticulocyte lysate (Fig. 6, lane 3) and in a wheatgerm extract (not shown); several minor proteins were also produced in one or the other system. To assign the translation products to

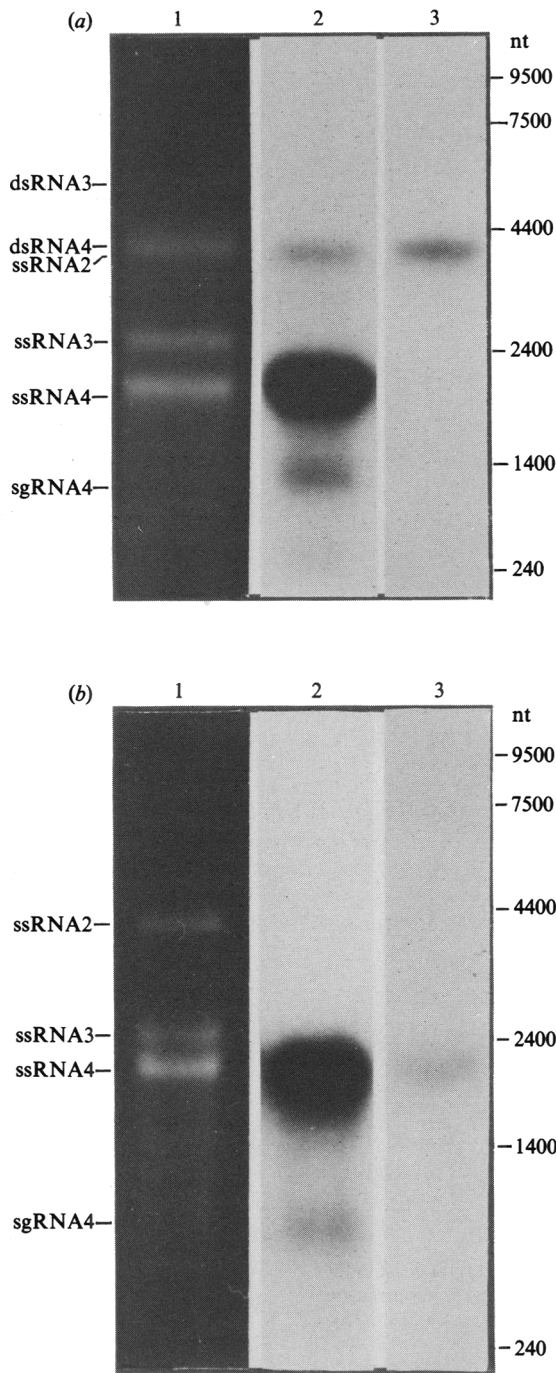


Fig. 5. Hybridization analysis of RHBV RNA using  $^{32}\text{P}$ -labelled riboprobes. (a) RHBV RNA (0.7  $\mu\text{g}/\text{lane}$ ) electrophoresed under native conditions. (b) RHBV RNA (1.5  $\mu\text{g}/\text{lane}$ ) electrophoresed under denaturing glyoxal conditions. Ethidium bromide-stained gel (lanes 1); Northern blots hybridized with a riboprobe detecting vRNA4 (lanes 2), or a riboprobe detecting vcRNA4 (lanes 3). The same amount of riboprobe of the same specific radioactivity was used throughout. Exposure time was 25 min. The molecular sizes shown to the right are from an ssRNA ladder (see legend of Fig. 4) electrophoresed on an adjacent lane of the same gel. The positions of the RHBV RNAs are indicated to the left of each panel.

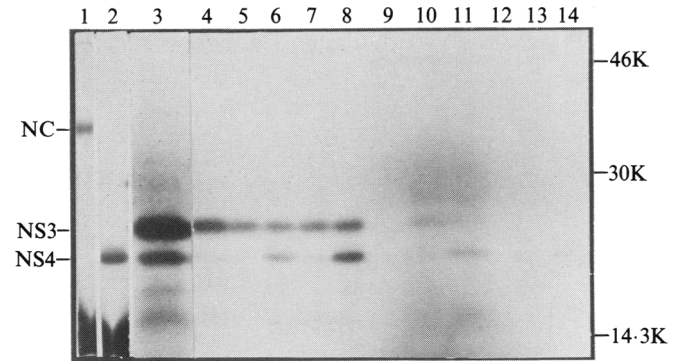


Fig. 6. SDS-PAGE and immunoprecipitation of RHBV RNA translation products synthesized *in vitro*. Purified RHBV NC (lane 1) and NS proteins (lane 2) were stained with Coomassie blue. [ $^{35}\text{S}$ ]Methionine-labelled translation products were electrophoresed either directly (lanes 3 and 9 to 11) or after immunoprecipitation (lanes 4 to 8 and 12 to 14), and detected by autoradiography. Translation was directed by total RHBV RNA (lanes 3 to 8), isolated RNA2 (lanes 9 and 12), isolated RNA3 (lanes 10 and 13) or isolated RNA4 (lanes 11 and 14). Immunoprecipitation was performed using preimmune rabbit serum (lane 4), preimmune serum followed by rabbit anti-RHBV antiserum (lane 5), preimmune serum followed by rabbit anti-NS protein antiserum (lane 6), anti-RHBV antiserum (lane 7) or anti-NS protein antiserum (lanes 8 and 12 to 14). The positions of NC (34K), NS3 (23K) and NS4 (21K) proteins are indicated to the left, and those of the  $^{14}\text{C}$ -methylated proteins (Amersham) used as size markers to the right. Lanes 1 and 2 are from lanes of the same gel in which total RHBV RNA translation products were also analysed. Lanes 3 to 14 are from another gel. The gels were electrophoresed under identical conditions.

specific viral RNAs, RHBV ssRNA2, ssRNA3 and ssRNA4 recovered from non-denaturing gels were used for *in vitro* translation studies. RNA3 produced a 23K protein designated NS3 (Fig. 6, lane 10) and RNA4 produced a 21K protein designated NS4 (Fig. 6, lane 11). Translation of RNA2 *in vitro* occasionally produced a 23K protein (not visible in lane 9 of the experiment presented in Fig. 6). The dsRNA species did not serve as a template for translation under the conditions used here either directly or after heat denaturation at 80 °C for 5 min followed by rapid cooling (not shown).

The translation products obtained with total RNA were immunoprecipitated with anti-NS protein or anti-RHBV antiserum. NS4 protein was specifically precipitated by anti-NS protein antiserum (Fig. 6, lanes 6 and 8); it migrates in the same position as NS protein purified from infected rice tissue (Fig. 6, lane 2). NS3 protein immunoprecipitated with all the antisera tested, i.e. preimmune antiserum (Fig. 6, lane 4), anti-RHBV antiserum (Fig. 6, lanes 5 and 7) and anti-NS protein antiserum (Fig. 6, lanes 6 and 8). None of the major translation products migrated in the same position as RHBV NC protein (Fig. 6, lane 1), nor were any of them

specifically recognized by the anti-RHBV antiserum. When the translation products obtained from purified RNA2, RNA3 and RNA4 were treated with anti-NS protein antiserum (Fig. 6, lanes 12 to 14, respectively), only the NS4 protein synthesized by RNA4 was immunoprecipitated (Fig. 6, lane 14).

## Discussion

Purified nucleoproteins of RHBV, as well as those of RSV (Toriyama, 1986) and MStV (Falk *et al.*, 1989), contain a mixture of dsRNA and ssRNA molecules. It is believed that the dsRNA species constitute extraction artefacts resulting from annealing of separately encapsidated complementary RNAs (Falk & Tsai, 1984; Ishikawa *et al.*, 1989). In total RHBV RNA the amount of dsRNA varies in different preparations, and the relative amount of an individual dsRNA also varies independently of the amount of the respective ssRNA; dsRNA species are always present in lower amounts than their ssRNA counterparts. These observations, together with the appearance of both dsRNA and ssRNA forms from an isolated ssRNA form and *vice versa* (Fig. 3) suggest an interconversion process, whereby extensive experimental manipulation of mixtures of vRNA and vcRNA strands produces varying amounts of dsRNA forms.

To establish whether RHBV encapsidates unequal amounts of vRNA and vcRNA strands, and whether the dsRNAs are related to the ssRNAs of corresponding size, hybridization analyses using cDNA clones to RNA4 were performed (Fig. 5). In native RHBV RNA denatured after electrophoresis, the riboprobe detecting vRNA4 (T7 RNA polymerase-derived transcript) recognizes both ssRNA4 and dsRNA4. Since hybridization was carried out under high stringency conditions, these results indicate that ssRNA4 and dsRNA4 have a high degree of identity, supporting the hypothesis that dsRNA4 contains the same information as ssRNA4. The riboprobe detecting vRNA4 gives a strong hybridization signal with both dsRNA4 and ssRNA4, whereas the one detecting vcRNA4 (T3 RNA polymerase-derived transcript) gives a signal with only dsRNA4, suggesting that the ssRNA4 in the native samples is primarily of viral polarity. In denatured RHBV RNA both vRNA4 and vcRNA4 are detected with the same riboprobes used to analyse native RNAs; however, the hybridization signal is stronger for the probe detecting vRNA4 than for the one detecting vcRNA4. Since riboprobes of opposite polarity were prepared under identical conditions and were of the same specific radioactivity, the differences in hybridization signal reflect quantitative differences between vRNA4 and vcRNA4. Consequently, unequal

amounts of vRNA4 and vcRNA4 are present. It can be postulated that RHBV, as well as RSV and MStV, encapsidate unequal amounts of vRNA and vcRNA strands, and that dsRNAs are probably formed during the extraction procedure. Assuming that the dsRNA species do not contain information absent from the ssRNA species, the RHBV genome is approximately 18000 nt in length.

In contrast to what is observed with MStV (Falk *et al.*, 1987; Huiet *et al.*, 1991), NC protein is not translated *in vitro* from either total RHBV RNA or individual RHBV RNA species; this situation is similar to that encountered with RSV RNA (Toriyama, 1985). If the ORF encoding NC protein is present in vcRNA, as it is in MStV and RSV, then this protein would not be expected to be produced *in vitro*, under the experimental conditions used here. This would hold true unless vcRNA remained as ssRNA in amounts sufficient to direct protein synthesis *in vitro*. As for MStV NS3 protein (Huiet *et al.*, 1991), RHBV NS3 protein is non-specifically immunoprecipitated by several antisera obtained from rabbits. It is conceivable that the RHBV and MStV NS3 proteins share epitopes with proteins already present in the rabbits and are recognized as non-self by these rabbits.

The fact that RHBV RNA cannot serve as a template for *in vitro* translation of all of its proteins implies that RHBV cannot be considered as a typical positive or negative strand RNA virus. It is probable that it uses the ambisense coding strategy for expression of RNA3 and RNA4, as do RSV and MStV. To confirm this hypothesis, complete nucleotide sequencing of the cDNA corresponding to RNA3 and RNA4 will be required.

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