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Instructions for use

Detection and assignment of proteins encoded by rice black streaked dwarf fijivirus S7, S8, S9 and S10

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The proteins encoded by rice black streaked dwarf fijivirus (RBSDV) genomic segments 7–10 (S7–S10) were characterized. Open reading frames (ORFs) from these segments were expressed as fusion proteins in *Escherichia coli*. Antibodies raised against the expressed products were used as probes to determine whether the viral ORFs encode structural proteins. In Western blots, antibodies to the expressed S8 and S10 products reacted with a core capsid (65 kDa) and a major outer capsid (56 kDa) protein, respectively, while none of the antibodies to S7 and S9 products reacted with structural proteins. Antisera to RBSDV S7 ORF1 and S9 ORF1 each detected a single protein of the predicted size in total protein extracts from infected rice plants and viruliferous *Laodelphax striatellus*. Immuno-electron microscopy revealed that antibodies to RBSDV S7 ORF1 and RBSDV S9 ORF1 reacted with tubular structures and viroplasm, respectively, in sections of both infected maize plants and viruliferous *L. striatellus*. Antisera to ORF2 of S7 and S9 failed to detect any proteins in the infected tissue using either Western blotting or immuno-electron microscopic techniques.

Introduction

Rice black streaked dwarf fijivirus (RBSDV) is a member of the genus *Fijivirus* within the family *Reoviridae*. At present, little is known of the proteins encoded by the 10 fijivirus genomic double-stranded RNA (dsRNA) segments (S1–S10), including those of RBSDV, and the nucleotide sequences of only some of them have been determined (Uyeda *et al.*, 1990; Azuhata *et al.*, 1993; Marzachí *et al.*, 1991, 1996). The size and number of structural polypeptides has only been reported for maize rough dwarf fijivirus (MRDV) (Milne *et al.*, 1973).

Nucleotide sequence analyses of MRDV S6 have revealed the presence of two nonoverlapping open reading frames (ORFs) (Marzachí *et al.*, 1991); a similar dicistronic structure has been found in RBSDV S7 (Azuhata *et al.*, 1993) and MRDV S8 (Marzachí *et al.*, 1996). It is not known how the downstream ORFs in these segments are expressed, if they are expressed at all, as *in vitro* translation of the full-length mRNA from MRDV S6 failed to express the second ORF (Marzachí *et al.*, 1991).

RBSDV is propagatively transmitted to rice, maize, barley and wheat by the planthopper *Laodelphax striatellus* (Shikata & Kitagawa, 1977). Plant hosts infected with RBSDV produce white tumours along the vein on the back of the leaves and on leaf sheaths that originate from phloem-derived neoplastic cells (Kuribayashi & Shinkai, 1952; Shinkai, 1962). Electron microscopy of ultrathin sections from leaves of diseased rice, maize, wheat and barley plants shows that RBSDV is restricted to the area of the leaf veins, particularly the tumour cells (Shikata & Kitagawa, 1977). Microscopy of ultrathin sections of both plant cells and insect cells infected with RBSDV revealed viroplasm, virus crystals and tubular structures (Shikata, 1969; Shikata & Kitagawa, 1977). The same structures are observed in MRDV-infected plants and insects (Gerola & Bassi, 1966; Vidano, 1970) and in oat sterile dwarf fijivirus-infected plants (Milne, 1980).

In this study, RBSDV S9 was found to have two nonoverlapping ORFs (ORF1 and ORF2) transcribed as a single piece of mRNA, as is the case in RBSDV S7. Moreover, the translation products of S7–S10 were studied using immunoblotting and immuno-electron microscopy.

Methods

■ **cDNA cloning and sequencing.** cDNA clones were generated from either viral transcripts (Azuhata *et al.*, 1993) or genomic dsRNA (Uyeda *et al.*, 1990) and those from S9 were selected by hybridization using S9 as a probe. One clone that covered about 80% of S9 from the 3′ end and four clones that covered the 5′-terminal region were used for sequencing. Sequences of the cDNA clones were read in both directions and 14 nucleotides from the 5′ end, which was not covered by the cDNA,

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Table 1. Primers used in cDNA synthesis and PCR amplification

RBSDV cDNA clone	Sense primer*	Antisense primer†	Cloning site of pMAL2c	Fused amino acid sequence of ORF
pMAL7-1	5′ ATGGATAGACCTGCACGGGA	5′ CCCTGCAGTTAAGCGGAAGGAG	XmnI and PstI	Full-length
pMAL7-2	5′ ATGAATTACACTTTAAGCGA	5′ GGCTGCAGACATCAGCTGCATT	XmnI and PstI	Full-length
pMAL8	5′ GGGGGATCCATGACTGGCACCCATGAC	5′ GGGCTGCAGTTACACAATAATA	BamHI and PstI	Full-length
pMAL9-1	5′ ATGGCAGACCAAGAACGG	5′ GGGCTGCAGTCAAACGTCCAGTTTCAA	XmnI and PstI	Full-length
pMALΔ9-2	5′ GGGGAATTCAAGCTTACTCTTA	5′ GGGCTGCAGTTAATTAAAAAGTGTGTA	EcoRI and PstI	Truncated
pMAL10	5′ GGGGAATTCATGGCTGACATAAGACTC	5′ GGGCTGCAGTCATCTTGTTACTTTA	EcoRI and PstI	Full length

* BamHI site underlined; EcoRI site in bold.

† PstI site underlined.

were read directly from viral transcripts by a primer extension method. Sequencing was performed by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) using a Sequenase version 2.0 DNA sequencing kit (US Biochemical). The deduced nucleotide sequences were assembled and analysed with the computer program DNASIS (Hitachi Software Engineering).

■ **Northern blot hybridization.** RBSDV S9 ORF1 and ORF2 DNA segments were amplified separately from cloned cDNA by PCR. The amplified DNAs were labelled with [α-^{32}P]dCTP using a random primer DNA labelling kit (Takara) and used as probes.

RNA samples were separated on 1·5% agarose gels with 1 × MOPS (0·4 M MOPS, 0·1 M sodium acetate, 0·02 M EDTA) containing 0·66 M formaldehyde using the method described by Sambrook *et al.* (1989).

The RNA was transferred to a nylon membrane, Hybond-N+ (Amersham), in capillary blotting apparatus in 2 × SSC (300 mM NaCl, 30 mM trisodium citrate dihydrate), for more than 12 h. The membrane was then washed in 2 × SSC, with shaking, for 10 min and dried at room temperature. The dried membranes were fixed by UV cross-linking.

Prehybridization and hybridization were performed using the method described by Isogai *et al.* (1995).

■ **Purification of RBSDV particles from infected maize plants.** All procedures involved in the preparation of intact particles were performed as rapidly as possible in a cold room or on ice because of the instability of the particles. About 100 g fresh, infected maize plants were ground with a meat grinder in 35 ml phosphate buffer (PB; 0·1 M Na_2HPO_4, 0·1 M KH_2PO_4, pH 5·8). The homogenate was further extracted with an ELISA juice press and squeezed through a double layer of medical gauze. After addition of Triton X-100 to give a 3% concentration, the mixture was stirred for 1 h and then centrifuged at 1500 **g** for 30 min at 4 °C. The supernatant was centrifuged at 8000 **g** for 1·5 h at 4 °C through 40% sucrose (w/v) in a volume of extraction buffer equal to one-third of the tube capacity. The pellet was gently suspended in 5 ml PB with a Pasteur pipette and centrifuged at 3500 **g** for 5 min at 4 °C. The supernatant was then layered onto a 10–40% sucrose density gradient tube and centrifuged at 8000 **g** for 1·5 h at 4 °C. The virus zone in the middle of the tube was collected and dialysed against PB for 2 h at 4 °C. The resulting sample was deemed to contain intact RBSDV particles.

For purification of B-spiked subviral particles of RBSDV, the intact particle was treated with CCl_4 before treatment with Triton X-100. The CCl_4 was added to the product of the first extraction to give a concentration of 30%, the mix was stirred for 1 h, and then centrifuged at 1500 **g** for 30 min at 4 °C.

Core particles were obtained by removing both the outer capsids and B-spikes from purified intact particles. The virus preparation was incubated in 1·9 M $MgCl_2$ for 5 min at room temperature and centrifuged at 100 000 **g** for 1 h at 4 °C. The pellet was used as a sample of the core particle.

■ **Subcloning into pMAL2c.** The cDNA for RBSDV S7–S10 ORFs was synthesized by reverse transcription of the RBSDV genome using antisense primers coupled with PCR using sense and antisense primers, and then ligated into pMAL2c (New England Biolabs) as shown in Table 1. The plasmids containing sequences of RBSDV S7 ORF1 and ORF2, RBSDV S8, RBSDV S9 ORF1 and ORF2 and RBSDV S10 were designated pMAL7-1, pMAL7-2, pMAL8, pMAL9-1, pMALΔ9-2 and pMAL10, respectively. pMAL7-1, pMAL7-2, pMAL8, pMAL9-1 and pMAL10 contained full-length ORF sequences. pMALΔ9-2 contained the truncated RBSDV S9 ORF2 sequence nt 1436–1789. The fusion proteins from pMAL8, pMALΔ9-2 and pMAL10 had 6, 4 and 4 extra amino acids between the factor Xa cleavage site and the viral amino acid sequences, respectively.

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AAGUUUUUAAGCCUGGAACUGACACAUUCCUACAGGAACGAGCGUAAGAGAAUGGCAGACCAAGAGCGGAGAACGUUUGG    80
                                            M  A  D  Q  E  R  R  T  F  G
AUCAUAUAAAAUAGAAGGAAUUGACAAUUAGAAACGACCAACCAAAUAGAAACAUUCAUCGAAUUCUCAAUCCACUG   160
 S  Y  K  I  E  E  L  T  I  R  N  D  Q  P  N  R  N  T  N  S  S  N  S  Q  S  T  E
AAAAUCGUCUUUCAACCAAGAAAAUCCCCCUCCUUGAUGACGGCAUCUUCGAACUUUUGAACUACCUUAUCGAUGGAACU   240
 N  R  L  S  T  K  K  I  P  L  L  D  D  G  I  F  E  L  L  N  Y  L  I  D  G  T
AAUUUUUGACAAAACUUGUUAUUGCGGGUUUUAAUUAUUCUCAUCUUCCCAACCUUGAACGUGAUUUUAACGUUGCAUCAAU   320
 N  F  D  K  T  C  Y  C  G  F  N  Y  S  H  L  P  N  L  E  R  D  F  N  V  A  S  I
UUACGUCCGCGAAAAUUUUGAGUUGUGCACUGAACAUUUAAAUUUGAAAGAUUAUGAUAGACAGGCAAACAUAAGCGUGA   400
 Y  V  R  E  N  F  E  L  C  T  E  H  L  N  L  K  D  Y  D  R  Q  A  N  I  S  V  K
AAUCACCUGAUUUUACUUUGUCUUUAGAAUACACUCUCAAACCAUCUUCAGAAACCGAUUCUCCUAUUCCAGAAAAAGAA   480
 S  P  D  F  T  L  S  L  E  Y  T  L  K  P  S  S  E  T  D  S  P  I  P  E  K  E
AGCGAAGAGAAUGAGAAAAAAGCUACUCCUAAAGUCGUCACUCCCAAAGAAGAGAAAAUACGUUGAAAUGUCUUUAUU   560
 S  E  E  N  E  K  K  A  T  P  K  V  V  T  P  K  E  E  K  I  P  V  E  M  S  L  L
GCCCAUUUUAAAACCGUGAAUCUGAAGAAUCGUUAAGCUCUGAAAUUCUCGAAGGUGAAGCUGCUGUCGUCAAUGUAUUCA   640
 P  I  L  N  R  E  S  E  E  S  L  S  S  E  I  L  E  G  E  A  A  V  V  N  V  F  K
AAAUGUAUAUCAAAGGAUUUCUAAUGUAUUUAGGUGAAAAUCCAAAUCUUAUGAUCGCAAUUAAAUCGAAAAGUAU   720
 M  Y  I  K  G  F  L  M  Y  L  G  E  N  P  N  S  Y  D  R  Q  L  N  I  E  K  Y
CGGCCUUUGUUGAUUUCAAUUAUCGGAUACGAACAUCUCAUUGGAACAAGAGUUACUAGUAAAGAGAUUAAUCACAUUUU   800
 R  P  L  L  I  S  I  I  G  Y  E  H  L  I  G  T  R  V  T  S  K  E  I  N  I  H  F
UUACCAAUUGGCAACUUUCGACAACUAUCCUUGUAGAUUCCAACUUAGAAUUCCAACUUCUUCACUCAUCUCAUACCUGCUU   880
 Y  Q  L  A  T  F  D  N  Y  P  F  D  L  L  R  F  Q  L  S  S  L  I  S  T  P  A  L
UAAUUCGUGAGAAAAUAGCGAAAGAAGGACUGUUCAAAAUCAUCACAUCAAACGCCCUGCGUGGGUGCUCCCCGUCAAACU   960
 I  R  E  K  I  A  K  E  G  L  F  K  I  I  T  S  N  A  L  R  G  A  P  R  Q  T
GUCUUAUUUUAGAGGAAUUAAUGGAAGCGAAUCGUUUUUGACAUUAAAAGAUAUCGUAAAUUUAGAACACGAGUCGCUGG   1040
 V  L  F  R  G  I  N  G  S  E  S  F  L  N  I  K  R  Y  R  K  F  R  T  R  V  A  G
UAACGUUGACAGUGUGAUUAAGUCUGAUUUCUCCUCCUUGAAACUGGACGUUUGAUCUUUUACUUUUUGUGUUCUGAAAA   1120
 N  V  D  S  V  I  K  S  D  F  S  S  L  K  L  D  V  *
UUGAAUAUCACUGGUUGAUGAAUUAUUUCCUUUAAUUUUAUGAACCCCAGUCUUCAGUAAAUGUUGAUACCUAUACCUU   1200
                                          M  N  P  Q  S  S  V  N  V  D  T  Y  T  F
UAACUGUCCCUUCGAAUUAGCAAAAAUCCAAAUUGAAUCGAUGAAACCAAUUAUGCAAGAUUUUAGUAAUUUCGAUGAUA   1280
 N  C  P  F  E  L  A  K  I  Q  I  E  S  M  K  P  I  M  Q  D  F  S  N  F  D  D  I
UUUUUGAGCGCACAUAUCUGAUUCGAAAUUGAUGAUCGUGUAGAACAACUGUGAUGGUUGAAUCAAAAGUUGAU   1360
 F  E  R  T  L  S  D  S  E  I  D  D  R  V  E  Q  L  E  V  D  V  E  S  K  V  D
CCAAUCGUGAGAAGAAGAUACGGUAAAAUAGGACAUAUUAUUAUUAUGAUUAUCAGUUCGUUGUUUUUGGUAUUUUUGAA   1440
 P  I  V  R  R  R  Y  G  K  I  G  H  I  I  I  M  I  I  S  F  V  F  F  G  I  F  K
GCUUACUCUUAAAAAUGUUUUAUCAUUUAUUUCGUUGUGUGUGUUGUAAUCCUUUAAUUAGAGGUAUACUUAGCAUCAUUU   1520
 L  T  L  K  M  F  Y  H  L  F  R  C  V  C  C  N  P  L  I  R  G  I  L  S  I  I  F
UUACCAUACUAAAUUACUUCACGUUAUGGUGUUUGUAUUUAUUUGUUUACUUUUUUUGGAGAUCAAAAUUUCGUUAGCU   1600
 T  I  L  F  Y  F  T  L  C  V  C  I  Y  F  V  Y  Y  F  F  G  D  Q  I  S  L  A
UAUAAUACUUUAAAUCAAAUUGACAGCUCUAAUUUUAUUAAUUCCACUCAAGUUGAAGAGAAAGUUAAGAACAUAAUUCA   1680
 Y  N  T  L  N  Q  I  D  S  S  N  F  I  N  S  T  Q  V  E  E  K  V  K  N  I  I  H
CGAUGGCUCACUCUUUUUUUGUGAACUCGCGAUCAAGCUACAGGUCAGGAUUAAACGAAGUUGUAAAAUGGCG   1760
 D  G  S  L  F  F  G  T  R  D  Q  A  T  G  Q  I  N  E  V  E  T  Q  V  V  N  G  G
GCACUGUAAACUACACACUUUUUUAAUUAACGUCUAUUUUAUGUGACUCUGAAAAUAUUCACGCUCUCAAGUCGAUUCUCU   1840
 T  V  N  Y  T  L  F  N  *
UAUAGUUAUGUCUUUUGGAGGUAUGACAAACUCGUUCUCAAGCCGGCUUACAGCUAUCGUC.                       1900
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Fig. 1. Complete nucleotide and predicted amino acid sequences of RBSDV S9. * denotes the positions of the predicted termination codons.

■ **Expression and purification of pMAL2c fusion proteins.** Fusion proteins were expressed from pMAL2c constructs using the procedure described by Masuta *et al.* (1992). Expressed proteins were purified with amylose resins (New England Biolabs) and used to immunize Japanese white rabbits. Proteins encoded by RBSDV S7 ORF1 and ORF2, RBSDV S8, RBSDV S9 ORF1 and ORF2 and RBSDV S10 were designated P7-1, P7-2, P8, P9-1, P9-2 and P10, respectively.

■ **Western blot analysis.** Rice plants and insect vectors were homogenized in liquid nitrogen and proteins were extracted in grinding buffer (10 mM KCl, 5 mM MgCl$_2$, 400 mM sucrose, 10 mM mercapto-ethanol, 100 mM Tris–HCl, pH 8·0, 10% glycerol) containing protease inhibitors aprotinin, leupeptin and pepstatin A (all at 0·2 μg/ml) as well as 2 mM PMSF; approximately 2 ml/g tissue was used. The protein concentrations of the samples were determined by the Bradford method (Bradford, 1976) using a kit from Bio-Rad and IgG as a standard.

All samples were analysed by SDS–PAGE (Laemmli, 1970). Following electrophoresis, the fractionated proteins were transferred onto an Immobilon PVDF membrane (Millipore) using a Bio-Rad Miniprotean II. Blots were developed using phosphatase-conjugated goat anti-rabbit IgG as described by Blake *et al.* (1984).

■ **Immuno-electron microscopy.** White tumours of maize plants infected with RBSDV were fixed in a mixture of 4% paraformaldehyde, 1% glutaraldehyde and 0·06M sucrose in cacodylate buffer, pH 7·4. Viruliferous insect vectors of *L. striatellus* were fixed in a mixture of 4% paraformaldehyde and 0·2% glutaraldehyde in 0·1 M PB, pH 7·2 (Suzuki *et al.*, 1993). Each fixed sample was dehydrated in a graded series of dimethylformaldehyde and embedded in white resin (London Resin) at 55 °C for 5 h. Ultrathin sections were cut with a diamond or sapphire knife, incubated with antiserum and immunogold-labelled with gold (15 nm)-conjugated protein A. Samples were viewed by immuno-electron microscopy in a JEOL 1200EX at 80 kV.

Results

Complete nucleotide sequence of RBSDV S9

RBSDV S9 was 1900 nt long and contained the conserved terminal oligonucleotide sequences (＋) 5′ AAGUUUUU and AGCUXXXGUC 3′ followed by a stretch of the segment-specific inverted repeat, as in the case of RBSDV S7, S8 and S10 (Azuhata *et al.*, 1992). It contained two large nonoverlapping ORFs of different sizes (Fig. 1). ORF1 began at nt 45–47, ended at nt 1086–1088, and encoded a polypeptide of 40 kDa. ORF2, from nt 1152–1154 to 1780–1782, encoded a polypeptide of 24 kDa. No sequence similarity was found between ORF1- and ORF2-encoded polypeptides.
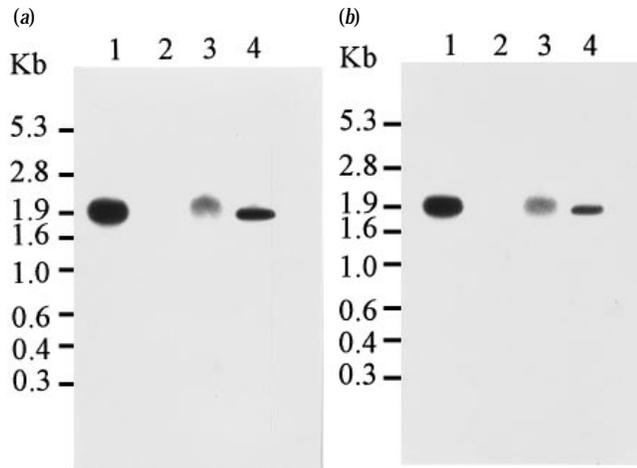
Fig. 2. Northern blot analysis of RBSDV S9 RNA in rice plants infected with RBSDV. Northern blot hybridization was carried out using probes for RBSDV S9 (*a*) ORF1 and (*b*) ORF2. Samples were denatured, fractionated on 1·5% agarose gels and transferred to nylon membranes. Lane 1, total RNA from RBSDV-infected rice plants (10 μg); lane 2, total RNA from healthy rice plants; lane 3, total mRNA from RBSDV-infected rice plants (10 μg); and lane 4, total dsRNA and low-molecular-mass single-stranded RNA from RBSDV-infected rice plants.

Fig. 3. Purification of RBSDV particles. RBSDV particles were negatively stained with 2% uranyl acetate: (*a*) and (*d*), intact particles; (*b*) and (*e*), B-spiked particles; (*c*) and (*f*), core particles. Bars, 200 nm. Single and double arrows indicate A- and B-spikes, respectively.

The sequence homology between RBSDV S9 and MRDV S8 (Marzachí *et al.*, 1996) was high (85%), and the proteins encoded by RBSDV S9 ORF1 and ORF2 had 87 and 93% amino acid sequence identity with those encoded by MRDV S8 ORF1 and ORF2, respectively (data not shown). Moreover, proteins encoded by RBSDV S9 ORF1 and ORF2 had local similarities with those of *Nilaparvata lugens* reovirus (NLRV; a putative member of *Fijivirus*) S9 ORF1 and ORF2 (Nakashima *et al.*, 1996) (data not shown).

Detection of RBSDV S9 mRNA

In order to test the possibility that mRNA from RBSDV S9 ORF2 was transcribed as subgenomic RNA, the size of RBSDV S9 mRNA *in vivo* was analysed by Northern blot hybridization. Total mRNA from RBSDV-infected rice plants was isolated by the guanidinium thiocyanate–phenol–chloroform extraction method of Chomczynski & Sacchi (1987) and precipitated with 2 M LiCl (Diaz-Ruiz & Kaper, 1978). In Northern blot hybridizations using probes for RBSDV S9 ORF1 and ORF2, only a full-length mRNA was detected (Fig. 2). No subgenomic RNA, or smaller RNA fragments that could be generated from the full-length mRNA, were found, suggesting that RBSDV S9 was transcribed only as a full-length mRNA.

Purification and structural protein analysis of RBSDV intact, B-spiked and core particles

To determine the number, size and location of structural proteins in the particles, methods of purifying intact, B-spiked and core particles were developed. A low pH buffer (0·1 M PB, pH 5·8) was used to stabilize the intact particles. Treatment of RBSDV particles with organic solvents removed the A-spikes
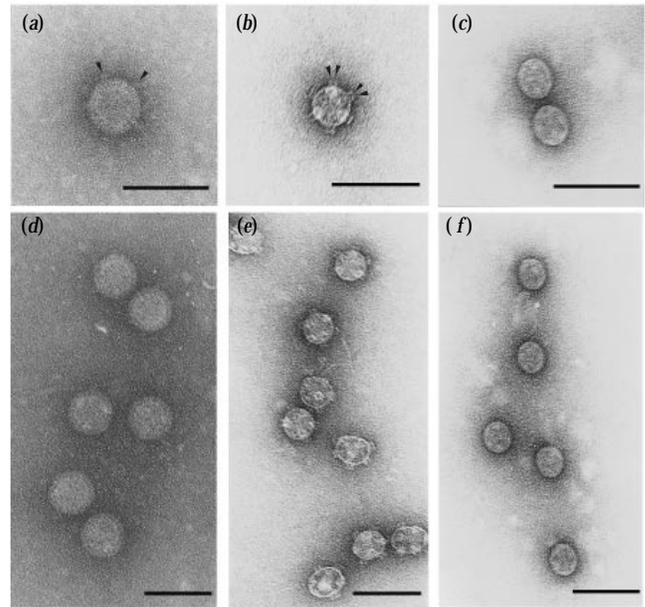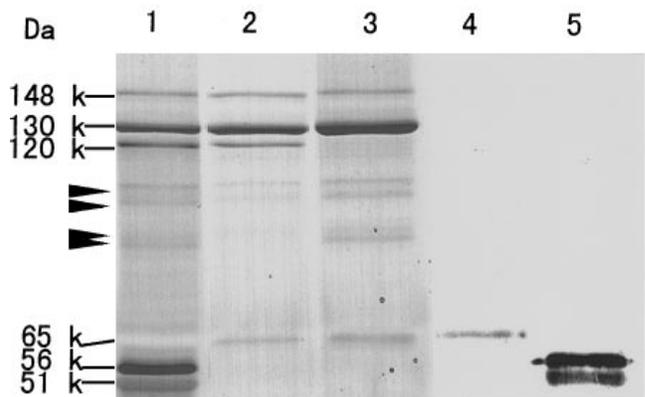

Fig. 4. Comparison of RBSDV structural proteins from intact, B-spiked and core particles, and immunodetection of the structural proteins encoded by RBSDV S8 and S10. Proteins were separated by 7·5% SDS–PAGE and stained with silver (lanes 1–3) or transferred to PVDF membranes for immunoblotting (lanes 4 and 5). Lane 1, intact particles; lane 2, B-spiked particles; lane 3, core particles; lane 4, immunodetection of B-spiked particles using antibody to P8; lane 5, immunodetection of intact particles using antibody to P10. Antibodies to P8 and P10 were used as a primary label at a dilution of 1:400.

and outer capsids, so this step was omitted in the purification of intact particles (Fig. 3 *a*, *d*). A-spikes were removed from most intact particles during the purification process. B-spiked particles could be purified by organic solvent treatment (Fig. 3 *b*, *e*). Treatment with 1·9 M MgCl$_2$ effectively removed the B-spikes, and core particles with a smooth appearance were thus obtained (Fig. 3 *c*, *f*).

Structural proteins of intact, B-spiked and core particle samples were resolved by SDS–PAGE (Fig. 4). Proteins from
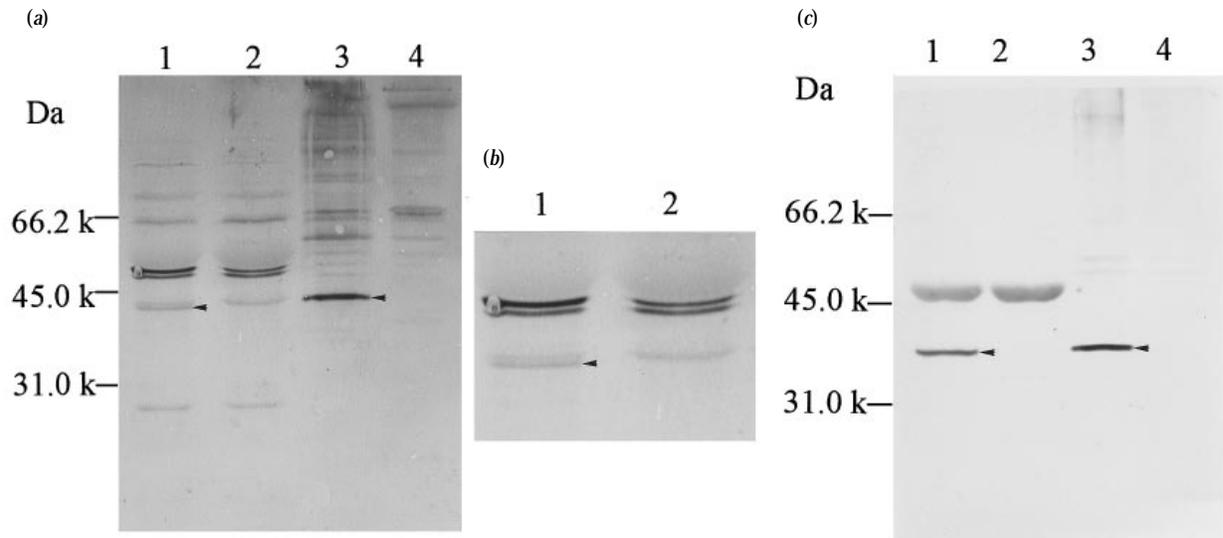
Fig. 5. Products of RBSDV S7 ORF1 (*a*) and (*b*) and S9 ORF1 (*c*) were visualized by Western blotting from 12·5 % polyacrylamide gels. Rice plant and *L. striatellus* samples were homogenized in extraction buffer (see text), boiled for 5 min, fractionated on polyacrylamide gels and blotted onto PVDF membranes probed with antisera to (*a*) P7-1 and (*c*) P9-1. Lanes 1 and 2 of (*a*) are magnified in (*b*). Lane 1, total proteins from RBSDV-infected rice plants (40 μg); lane 2, total proteins from healthy rice plants (40 μg); lane 3, total proteins from RBSDV-infected *L. striatellus* (20 μg); lane 4, total proteins from healthy *L. striatellus* (20 μg). Arrows indicate specifically detected proteins. Antibodies to P7-1 and P9-1 were used as a primary label at a dilution of 1:200.

intact particles were resolved into 148, 130, 120, 65, 56 and 51 kDa proteins (Fig. 4, lane 1). The 65 kDa protein was negatively stained by silver staining (Fig. 4, lane 1), whereas it stained positively with Coomassie brilliant blue (data not shown). The reason for this effect is unknown. The 148, 130, 120 and 65 kDa proteins were detected in the B-spiked particles (Fig. 4, lane 2), while the 148, 130 and 65 kDa proteins were detected in core particles (Fig. 4, lane 3). In addition, four minor bands, indicated by arrows, were commonly seen in all the particles. The morphology of NLRV is similar to that of RBSDV and the 148, 130 and 65 kDa proteins of the RBSDV core particle corresponded to the 160, 140 and 65 kDa structural proteins of the NLRV core particle, respectively. The origin of the four minor bands (indicated by the arrows) remained unknown and they were not characterized further. They may have been maize plant components that remained as contaminants during the purification process. The 56 and 51 kDa proteins, which were not detected in either the B-spiked or core particles, are thought to make up the outer shell, while the 120 kDa protein, which was not detected in the core particles, is thought to be a component of the B-spike.

Assignment of RBSDV structural proteins by Western blot analysis

The entire coding regions of S7 ORF1, S7 ORF2, S8, S9 ORF1 and S10 were successfully expressed as fusion proteins. A construct designed to express the full-length protein of S9 ORF2 failed to produce a product of the expected size. To raise antiserum to the S9 ORF2 protein, a truncated S9 ORF2 sequence was ligated into pMAL2c (Table 1; pMALΔ9-2). Antibodies to P8 reacted with the 65 kDa protein, suggesting that RBSDV S8 encodes one of the core capsid proteins (Fig. 4, lane 4). Antibodies to P10 reacted with the 56 and 51 kDa structural proteins (Fig. 4, lane 5). The 51 kDa protein is presumably formed by degradation of the 56 kDa protein, since it was not found in freshly prepared samples.

No proteins reacted in the Western blot probed with antisera raised against P7-1, P7-2, P9-1 and P9-2 from the purified virus particles (data not shown). These results suggest that ORFs 1 and 2 of RBSDV S7 and S9 encode non-structural proteins.

Immunological detection of non-structural proteins encoded by S7 and S9 *in vivo*

In vivo detection of non-structural proteins using antibodies to P7-1, P7-2, P9-1 and P9-2 was carried out. Total protein extracts were prepared from RBSDV-infected rice plants and the insect vector, separated in 12·5 % polyacrylamide gels and blotted onto PVDF membranes. The membranes were probed with antisera raised against P7-1 or P9-1 (Fig. 5). Using antiserum against P9-1, a 41 kDa protein was detected in infected rice plants and the insect vector (Fig. 5*c*). The size of this protein was similar to that predicted by the encoding ORF. However, the P7-1 antiserum detected proteins in the infected rice plants and insect vector which differed in migration (Fig. 5*a* and *b*); the protein from infected rice plants migrated more slowly than that from the insect vector. The apparent sizes of the proteins detected in the plants and insect vector were
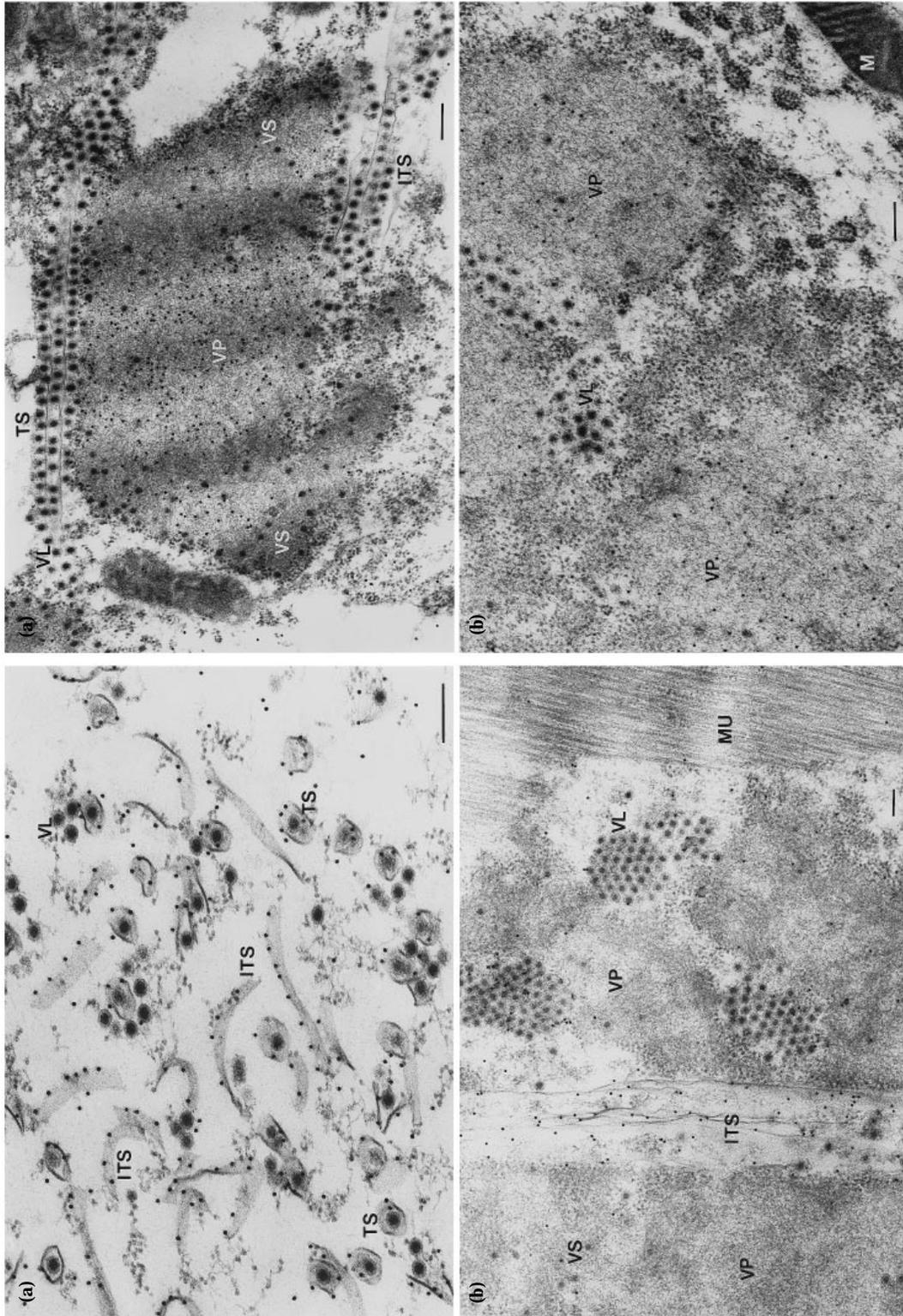
Fig. 7

Fig. 6. Immunogold labelling of protein encoded by RBSDV S7 ORF1 (P7-1) in cells of (*a*) white tumour of maize plants and (*b*) viruliferous planthopper *L. striatellus*. VP, Viroplasm; ITS, incomplete tubular structure; VS, small incomplete virions; VL, large complete virions; MU, muscle. Bars, 200 nm. Antibody to P7-1 was used at a dilution of 1:200.

Fig. 7. Immunogold labelling of protein encoded by RBSDV S9 ORF1 (P9-1) in cells of (*a*) white tumour of maize plants and (*b*) viruliferous planthopper *L. striatellus*. VP, Viroplasm; TS, tubular structure; ITS, incomplete tubular structure; VS, small incomplete virions; VL, large complete virions; M, mitochondrion. Bars, 200 nm. Antibody to P9-1 was used at a dilution of 1:200.

Fig. 6

about 41 and 44 kDa, respectively. This may indicate different post-translational modifications of P7-1 in these different backgrounds. Antibodies to P7-2 and P9-2 failed to react with any of the proteins (data not shown). No P7-2 or P9-2 was detected when a 1000 g pellet containing cell wall-associated material was solubilized by boiling in guanidine–HCl (Deom *et al.*, 1990) (data not shown).

Intracellular localization of P7-1 and P9-1

Previous studies have revealed that RBSDV is restricted to the veins of the diseased plant, while it invades infected insects systemically (Shikata & Kitagawa, 1977). Ultrathin sections of the white tumour of diseased maize plants and whole bodies of the viruliferous insect *L. striatellus* showed virus particles, viroplasm and a tubular structure (Figs 6 and 7). The virus crystals were composed of large particles of about 70 nm in diameter, while smaller particles of about 60 nm in diameter were scattered throughout the viroplasm. Tubular structures containing large particles were found in the cytoplasm of infected cells. The tubes were often incompletely closed, forming scrolls.

Immunogold labelling was used to examine the intracellular localization of P7-1 and P9-1 (Figs 6 and 7). Antibodies to P7-1 reacted with the tubular structure. Although the completely closed tubular structures reacted, these bound fewer gold particles than incompletely closed tubular structures (data not shown), suggesting that the incomplete structure has more exposed epitopes than the intact structure. Viroplasms in maize and insects were labelled with antibodies to P9-1. None of the structures in healthy plants and virus-free insects reacted with these antisera. These results show that P7-1 and P9-1 are components of the tubular structure and viroplasm, respectively.

Discussion

In this paper, we report the molecular masses of all the structural proteins of the RBSDV particle, except for the A-spike, and show that the core capsid (65 kDa) protein and major outer capsid (56 kDa) protein are encoded on RBSDV S8 and S10, respectively. This result is supported by the observation that comparable structural proteins are encoded in similar positions on NLRV S7 and S8, respectively. The sequences of the core capsid and major outer capsid proteins encoded by RBSDV S8 and S10 are homologous to the NLRV amino acid sequences [see the accompanying paper by Isogai *et al.* (1998)].

Immuno-electron microscopic examination demonstrated that P7-1 accumulated in tubule-like structures and that P9-1 accumulated in the viroplasm. Tubular structures are also formed in infection with cowpea mosaic virus and tomato spotted wilt virus (TSWV) and are thought to be involved in cell-to-cell movement (van Lent *et al.*, 1990; Storms *et al.*,

1995). The RBSDV tubular structure is induced in plant and insect cells, as with TSWV. However, the RBSDV tubular structure is not restricted to the plasmodesmata of plants and does not emerge from the surface of the insect cells. In both plants and insects, P9-1 accumulated in the intracellular viroplasm. In RBSDV infection, the viroplasm is thought to be the site of virus formation in the cell, because small incomplete virions approximately 60 nm in diameter appear mainly within the viroplasm. These correspond to the inner core of the large complete virions and are never found in the cytoplasm or tubular structures. This suggests that P9-1 plays an important role in viral morphogenesis.

P7-2 and P9-2 were not detected by Western blot analysis of plant and insect tissue, although the antiserum to the fusion protein of S9 ORF2 diluted by more than 1 in 10 000 can detect the purified protein expressed from pMALΔ9-2 (data not shown). It is likely that P7-2 and P9-2 are expressed *in vivo*, because both these ORF2s are conserved in RBSDV S9, MRDV S8 and NLRV S9, and between RBSDV S7 and MRDV S6. Given the time-span involved in the divergence of these viruses, this suggests that these translation products are indispensable for virus multiplication. In Northern blot hybridization using RBSDV S9 ORF1- and ORF2-specific probes, only full-length mRNAs of RBSDV S9 were observed. This is consistent with the *in vitro* transcription products being obtained from purified RBSDV virions (I. Uyeda, unpublished data). Therefore, a dicistronic mode of translation for the mRNA of S7 and S9 is favoured. An undetectable level of expression of P7-2 and P9-2 *in vivo* may result from a low level of translational efficiency of the dicistronic mRNA. In these mRNAs, initiation at the ORF2 AUG triplet might depend on the reinitiation mechanism (Kozak, 1987) or the internal ribosome entry model (Jang *et al.*, 1988; Pelletier & Sonenberg, 1988). In the internal ribosome entry model, these elements consist of large stretches of highly structured RNA, which either guide ribosomes directly to the initiating AUG codon in conjunction with associated proteins, or guide a scanning complex to an internal position upstream of the start codon. However, the upstream regions of RBSDV S7 and S9 ORF2s are not highly structured and these sequences are very rich in adenine and uridine. With the reinitiation mechanism, scanning may resume after translation of the first ORF, and a start codon located further downstream may be recognized. The translational efficiency of the downstream ORF in a dicistronic construct is only 0·3–2·5 % of that in a monocistronic construct (Kaufman *et al.*, 1987) when artificially engineered transcription units consisting of several normal-sized cistrons are introduced into mammalian cells. In plants, Angenon *et al.* (1989) reported that a value 500-fold lower was obtained with a dicistronic unit.

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