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Taxonomic characteristics of fijiviruses based on nucleotide sequences of the oat sterile dwarf virus genome

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Sequence determination of full-length cDNA clones of genomic segments 7–10 (S7–S10) of oat sterile dwarf fijivirus (OSDV) revealed that the 5' and 3' ends of the plus strands of these segments had the same conserved terminal sequences, 5' AACGAAAA and UUUUUUAGUC 3'. These sequences are similar, but not identical, to the conserved terminal nucleotide sequences of the genomic segments of rice black streaked dwarf fijivirus (RBSDV) and maize rough dwarf fijivirus (MRDV). The coding strands of S7 and S10 each contained two large nonoverlapping open reading frames (ORFs), as do RBSDV S7 and S9, MRDV S6 and S8 and *Nilaparvata lugens* reovirus (NLRV; a putative member of *Fiji-*

virus) S9. These results strongly suggest that the dicistronic nature of certain genomic segments is characteristic of fijiviruses. Computer analyses revealed sequence homology between RBSDV S7 ORF2, MRDV S6 ORF2 and OSDV S7 ORF2, suggesting that this protein is conserved among plant fijiviruses. No counterparts were found in the genome of NLRV, which is a nonphytopathogenic insect reovirus. Furthermore, phylogenetic trees derived from multiple sequence alignments of each of the homologous proteins from OSDV, RBSDV, MRDV and NLRV suggest that NLRV did not evolve from either *Fijivirus* group 2 (RBSDV and MRDV) or group 3 (OSDV).

Introduction

The plant reoviruses are classified into three genera, *Phytoreovirus*, *Fijivirus* and *Oryzavirus*, on the basis of particle morphology, the number and size of their genomic segments, and vector specificity (Holmes *et al.*, 1995). Fijiviruses have 10 double-stranded RNA (dsRNA) segments and are propagatively transmitted by delphacid planthoppers (Milne *et al.*, 1973; Hatta & Francki, 1977). Three groups of fijiviruses have been recognized on the basis of antigens associated with core particles (Milne & Lovisolo, 1977): group 1 contains only the Fiji disease fijivirus (FDV); group 2 includes the rice black streaked dwarf fijivirus (RBSDV), maize rough dwarf fijivirus (MRDV) and Pangola stunt fijivirus; and group 3 has a single member, oat sterile dwarf fijivirus (OSDV).

Sequence analyses of RBSDV and MRDV have suggested that 5' AAGUUUUU and GUC 3' are conserved, genus-specific, terminal genomic sequences (Marzachi *et al.*, 1991;

Azuhata *et al.*, 1992). However, RBSDV and MRDV belong to the same *Fijivirus* group and are believed to be closely related (Azuhata *et al.*, 1993). To determine whether these terminal sequences represent a genus-specific characteristic, fijiviruses from groups other than group 2 should be analysed. *Nilaparvata lugens* reovirus (NLRV) was recently classified as a putative member of the genus *Fijivirus* (Nakashima *et al.*, 1996). NLRV found in a healthy colony of the brown planthopper, *N. lugens*, has the same morphology as fijiviruses and contains 10 dsRNA genomic segments (Noda & Nakashima, 1995; Nakashima *et al.*, 1996). However, the biological properties of NLRV differ from those of plant fijiviruses. NLRV does not reproduce in rice plants, although the virus is transmitted from a viruliferous to a nonviruliferous planthopper through the rice plant (Nakashima & Noda, 1995). Moreover, although the conserved terminal sequences of NLRV, 5' AGU and GUU-GUC 3', are similar to those of RBSDV and MRDV, they are distinct (Noda *et al.*, 1994).

The deduced amino acid sequence of open reading frame 2 (ORF2) of RBSDV S7, and of MRDV S6, its counterpart in the MRDV genome, shares no similarity with any part of the NLRV genome (Nakashima *et al.*, 1996). The other proteins that have been analysed in RBSDV (S7 ORF1, S8 and S10) and

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MRDV (S6 ORF1, S7 and S10) all have counterparts in NLRV. As already mentioned, NLRV is unable to propagate in rice plants. These findings suggest that RBSDV S7 ORF2 and MRDV S6 ORF2 may be involved in multiplication within the plant (Nakashima *et al.*, 1996). It would be interesting to know whether the proteins corresponding to RBSDV S7 ORF2 and MRDV S6 ORF2 are also conserved in OSDV.

In this study, we further investigated the genus-specific, terminal, conserved sequences of *Fijivirus* by determining the terminal sequences of the OSDV genome. We also determined the complete nucleotide sequences of OSDV S7–S10 to see whether the OSDV genome has a counterpart to RBSDV S7 ORF2 and to examine the evolutionary relationships of OSDV, RBSDV, MRDV and NLRV.

Methods

■ **Source of virus and direct extraction of genomic dsRNA from infected rice plants.** The OSDV isolates originated from infected *Javesella pellucida* collected in 1994 from an oat field in Dalarna in the central part of Sweden, and were subsequently propagated in oat plants.

The dsRNA genomes of OSDV were extracted directly from about 1 g freeze-dried oat plants infected with OSDV using the method described by Murao *et al.* (1994). In brief, total nucleic acids were extracted with phenol–chloroform and then ethanol-precipitated. Single-stranded RNA was removed using 2 M LiCl and the dsRNA was further purified by binding with CC41 (Whatman). About 2 µg OSDV dsRNA was obtained by this method.

■ **Cloning of the OSDV genome.** The OSDV dsRNA genomes were cloned using the adapter PCR method (Isogai *et al.*, 1998a). The 3' termini of both strands of the dsRNA were first polyadenylated. The polyadenylated dsRNA was used as a template for an initial reverse transcription using an oligo(dT)-containing adapter primer (AP), 5' GGCCACGCGTCTGACTAGTAC(T)₁₇ 3' (Gibco BRL). The AP initiated cDNA synthesis in the polyadenylated regions. Products of the cDNAs were amplified by PCR using a primer-containing adapter region sequence identical to that of the AP (AUAP), 5' GGCCACGCGTCTGACTAGTAC 3' (Gibco BRL). The total amplified products were cut with *Sall*, which has a restriction site in AUAP, and were inserted into the *Sall* site of pBluescriptII SK(–) (Stratagene). *Escherichia coli* strain MV1184 was used for transformations.

■ **5' RACE cloning.** To analyse both termini of the plus strand of the OSDV genomic segment, 5' RACE cloning was performed using the method described by Frohman *et al.* (1988). For the initial reverse transcription, 1 µg OSDV genome was used. To analyse the 5' termini, the OSDV S9-specific internal antisense primer, 5' ATACACTTTGCGA-CATATG 3' (nt 302–284), was used for reverse transcription. The OSDV S10-specific internal sense primer, 5' ATCTTGTGTGTTCTGACTCG 3' (nt 1247–1265), was used to analyse the 3' termini. The first-strand cDNA was tailed with dC, and then amplified with the anchor primer, 5' GGCCACGCGTCTGACTAGTACGGGIIGGGIIGGGIIG 3', and the primer used for the respective reverse transcription. The amplification products were inserted into the *SpeI* and *SmaI* sites of pBluescriptII SK(–) using the *SpeI* site of the anchor primer; transformations were carried out in *E. coli* strain MV1184.

■ **Sequencing.** The cDNA was sequenced by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) using a Thermo Sequenase

fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP in a Li-Cor Sequencer (Amersham). The nucleotide sequences were assembled and analysed using the computer program DNASIS (Hitachi Software Engineering).

Results

Comparative electrophoresis of OSDV and RBSDV genomic RNAs

OSDV genomic RNA segments were extracted separately from individual infected oat plants and compared with the RBSDV genome using PAGE (7.5% polyacrylamide gel in Tris–acetate buffer). Although both OSDV and RBSDV have 10 dsRNA segments, their electrophoretic profiles differed (Fig. 1). Reddy *et al.* (1975a, b) reported that the electrophoretic profiles of FDV (*Fijivirus* group 1) and RBSDV and MRDV (group 2) genomes are very similar. These results suggest that groups 1 and 2 are more similar to each other than either group is to group 3, which comprises OSDV. The OSDV genomic segments were numbered S1–S10, from the least mobile to the most mobile band, following the convention of Boccardo & Milne (1980) (Fig. 1, lane 5). Most OSDV isolates had two forms of OSDV S4 (Fig. 1, lanes 2–4). Since the two forms of OSDV S4 appeared to be present in about a half of the genomic segments, natural genomic variants may exist, as in the case of RBSDV and MRDV (Isogai *et al.*, 1995a, b).

The terminal nucleotide sequence of the plus strands of OSDV genomic segments

Since the nucleotide sequence of the OSDV genome was unknown and no specific PCR primers could be designed, the adapter primer PCR method for unknown dsRNA was used. To identify the terminal nucleotide sequence of OSDV, all the cDNA clones were sequenced across the plasmid–insert junctions. Two kinds of conserved terminal sequences were identified: 5' AACGAAAAA and 5' GACTAAAAAAA (data not shown). When 5' AACGAAAAA was designated as the plus strand, all the cDNA clones had long ORFs. From this result, we concluded that the conserved terminal nucleotide sequences of OSDV on the plus strands were 5' AACGAAAAA and UUUUUUUUAGUC 3'. When the adapter primer PCR method is used, however, the 3' ends of the plus and minus strands of the dsRNA genome are polyadenylated and used as the template for the initial reverse transcription with an oligo(dT)-containing adapter primer. Therefore, whenever the 5' terminal of the plus strand was T, or the 3' terminal of the plus strand was A, these nucleotides would be assimilated into the polyadenylated sequences. 5' RACE was used to analyse both terminal sequences of the plus strand. An oligo(dC) tail was added to the first-strand cDNAs of 5' RACE in order to distinguish between the terminal sequences and the polyadenylated sequences obtained with the adapter primer PCR method. Using a dG tail, the 5' end of the plus strand was shown to begin with the sequence 5' AACGAAAAA and the

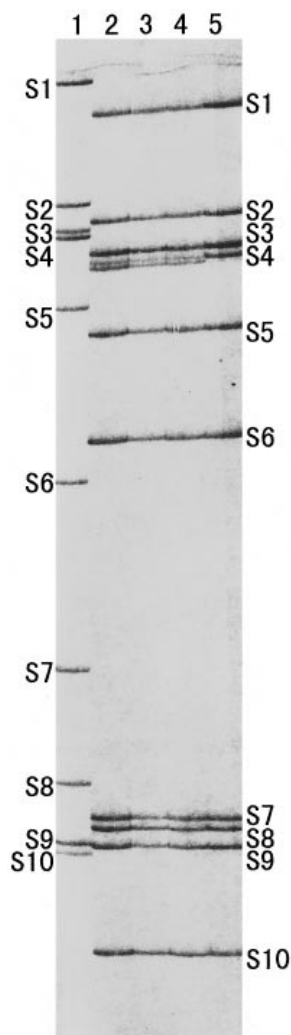


Fig. 1. Electrophoretic separation of the genomic dsRNA segments of OSDV and RBSDV in a 7.5% polyacrylamide gel stained with silver. Lane 1, RBSDV; lanes 2–5, OSDV genomes extracted separately from individual infected plants.

3' end of the plus strand to end with the sequence UUUUUUUUAGU 3' (data not shown). The 5' RACE and adapter primer PCR methods both gave the same 5' end

terminal sequence, while the 3' terminal sequence determined by the 5' RACE method was one base shorter than that determined by the adapter primer PCR method. Based on these results, the conserved terminal sequences of the OSDV genomic segments in the plus strand were determined to be 5' AACGAAAA and UUUUUUUUAGUC 3'. Analyses of RBSDV and MRDV have shown the *Fijivirus* genus-specific, conserved, terminal nucleotide sequences to be 5' AAG-UUUUUU and GUC 3' (Marzachi *et al.*, 1991; Azuhata *et al.*, 1992). Comparison of these sequences with those from the OSDV genome suggests that the genus-specific, terminal, conserved sequences are 5' AA and GUC 3'.

Complete nucleotide sequence of OSDV S7, S8, S9 and S10

Full-length cDNA clones of OSDV S7–S10 and partial cDNA clones representing portions of S1–S6 were obtained by the adapter primer PCR method. The full-length cDNA clones of OSDV S7–S10 were identified by dot-blot hybridization of each OSDV genomic segment on a nylon membrane with randomly labelled [³²P]cDNA inserts. The lengths and G + C content of OSDV S7–S10 are summarized in Table 1. OSDV S7, S8, S9 and S10 were completely sequenced; they comprised 1944, 1874, 1893 and 1761 bp, respectively. Although OSDV S9 was 19 bp longer than S8, S9 migrated faster than S8 on polyacrylamide gels. This phenomenon has been observed in many other plant reoviruses, including RBSDV, MRDV, NLRV, wound tumour phytoreovirus, rice dwarf phytoreovirus and rice ragged stunt oryzavirus (Uyeda *et al.*, 1995a).

The sequenced RBSDV and MRDV genomic segments have a G + C content of 33.9–36.5% and are AT-rich compared with other plant reoviruses, including all the rice dwarf phytoreovirus genomic segments (G + C, 41.4–48.4%; Uyeda *et al.*, 1995a) and rice ragged stunt oryzavirus S9 (44.9%; Uyeda *et al.*, 1995b) and S10 (45.4%; Yan *et al.*, 1995). OSDV S7–S10 have a similar nucleotide composition and are also AT-rich. OSDV S7 had the highest G + C content (35.6%) and OSDV S8 the lowest (33.8%). The genomic segments of NLRV, which is a putative member of the genus *Fijivirus*, have

Table 1. Properties associated with nucleotide and deduced amino acid sequences of OSDV S7, S8, S9 and S10

Segment	Size (bp)	G + C content (%)	Coding strategy	Size (aa)	Molecular mass (kDa)
S7	1944	35.6	Dicistronic	368 and 225	41.97 and 26.98
S8	1874	33.8	Monocistronic	587	66.23
S9	1893	34.6	Monocistronic	586	69.36
S10	1761	34.5	Dicistronic	314 and 194	35.73 and 22.72

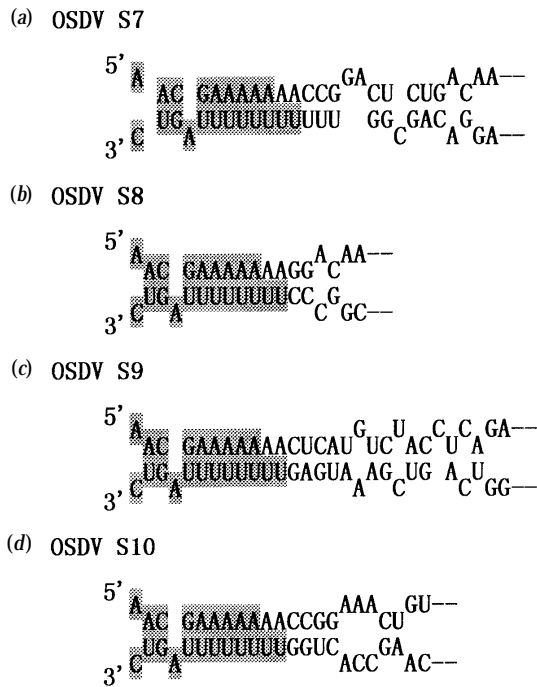


Fig. 2. Segment-specific inverted repeats in the terminal region of the plus strands of OSDV S7, S8, S9 and S10. Shaded nucleotides are the 5'- and 3'-terminal conserved sequences of OSDV.

a G + C content of 33.1–39.0% (Nakashima *et al.*, 1996). These results suggest that an AT-rich composition is one of the characteristics of fijiviruses and related viruses.

Analysis of the terminal sequences identified segment-specific inverted repeats in OSDV S7–S10 (Fig. 2). In all these segments, each inverted repeat had a long base pair between the oligo-A of the 5'-terminal region and the oligo-U of the 3'-terminal region. This is also a unique characteristic of the OSDV genome.

Genome organization and sequence homology in fijiviruses

Computer analyses revealed that the OSDV genome contained both monocistronic and dicistronic segments (Table 1). OSDV S8 and S9 both contained one long ORF, while S7 and S10 contained two long ORFs each. The two ORFs in S10 did not overlap, but there was an 11 bp overlap between ORF1 and ORF2 in S7. However, there were two other candidate initiation codons (nt 1186–1188 and 1198–1200), 37 and 49 bp downstream of S7 ORF1, respectively, within S7 ORF2 (data not shown). With the exception of OSDV S7, all other dicistronic segments have an intercistronic region of about 30–50 bp between ORF1 and ORF2. This includes OSDV S10, RBSDV S7 (Azuhata *et al.*, 1992) and S9, MRDV S6 (Marzachi *et al.*, 1991) and S8 (Marzachi *et al.*, 1996), and NLRV S9 (Nakashima *et al.*, 1996). Therefore, it is likely that one of the initiation codons 37 or 49 bp downstream of OSDV S7 ORF1

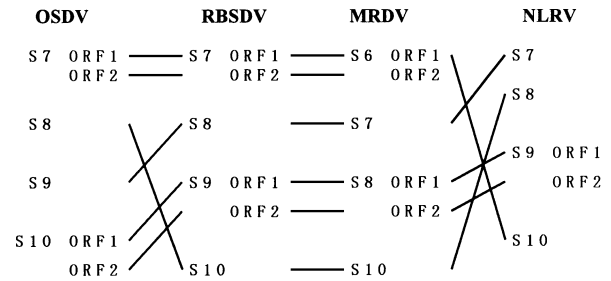


Fig. 3. Correspondence between homologous proteins encoded by OSDV S7–S10, RBSDV S7–S10, MRDV S6–S8 and S10, and NLRV S7–S10.

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OSDV S9 : GCSLQLI*GNKGC**STLIRGM373 aa
RBSDV S8 : GQDVQIIGNKGVGKSEIGVML368 aa
MRDV S7 : GQDVQIIGNKGVGKSEIGTML368 aa
NLRV S7 : RMPiWVVA*GNKGS***GKT*VL*RKEL414 aa
    
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Fig. 4. Comparison of the purine NTP-binding motif of the deduced amino acid sequences encoded by OSDV S9, RBSDV S8, MRDV S7 and NLRV S7. * indicates the purine NTP-binding motif. Black regions indicate identical amino acids.

is the actual initiation codon for OSDV S7 ORF2. In Table 1, we assume that OSDV S7 ORF2 begins 37 bp after ORF1.

The amino acid sequences encoded by OSDV S7–S10 were compared with the proteins encoded by RBSDV S7–S10, MRDV S6–S8 and S10, and all the genomic segments of NLRV, using the Macaw program version 2.0.5. The program was used to generate statistically significant alignments between the amino acid sequences encoded by OSDV, RBSDV, MRDV and NLRV (data not shown). All these viruses contained proteins corresponding to OSDV S7 (ORF1), S8, S9 and S10 (ORF1 and ORF2), as shown in Fig. 3. Only NLRV lacked the protein that corresponded to the ORF2 of OSDV S7, RBSDV S7 and MRDV S6. OSDV S9 had a purine NTP-binding motif GXXXXGKS (Gorbalenya & Koonin, 1989), which is identical to that found in RBSDV S8 and MRDV S7, and very similar to that (AXXXXGKT) of NLRV S7 (Fig. 4). The proteins encoded by RBSDV S8 and MRDV S7 were homologous; OSDV S9 and NLRV S7 encoded similar proteins (Fig. 3).

Fig. 5(a) shows multiple sequence alignments of homologous proteins encoded by OSDV S8, RBSDV S10, MRDV S10 and NLRV S8, constructed using the program Clustal W version 1.5. The phylogenetic tree obtained from these proteins is shown in Fig. 5(b). Phylogenetic trees were constructed by the UPGMA method using the formula of Zukerkandl & Pauling (1965). For each homologous protein, all the phylogenetic trees had the same shape, confirming the close evolutionary relationship between OSDV, RBSDV, MRDV and NLRV. Phylogenetic analysis also suggested that NLRV is not derived from either of *Fijivirus* groups 2 or 3.

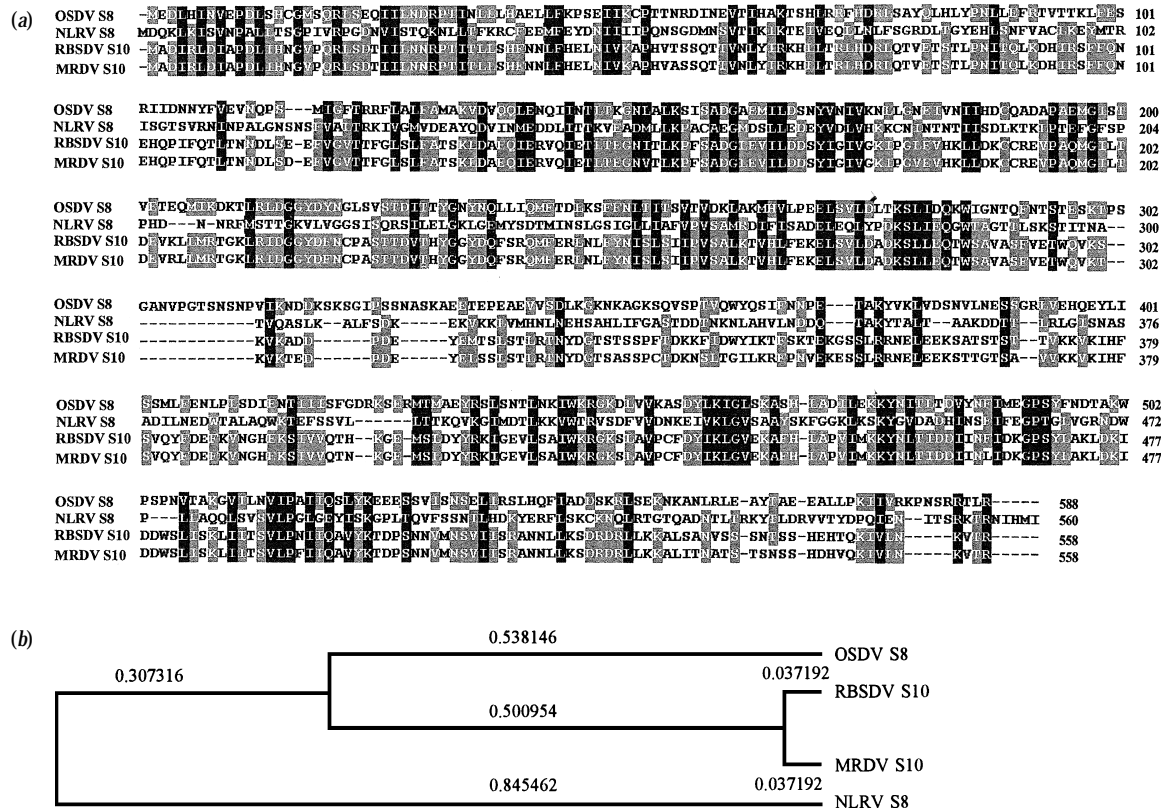


Fig. 5. (a) Multiple sequence alignment of homologous proteins from OSDV S8, NLRV S8, RBSDV S10 and MRDV S10, constructed using the program Clustal W version 1.5. Black and shaded regions represent conserved amino acids in four and three viruses, respectively. (b) Phylogenetic tree constructed from homologous proteins of OSDV S8, NLRV S8, RBSDV S10 and MRDV S10 using the UPGMA method (Zukerkandl & Pauling, 1965). The average numbers of substitutions per amino acid in the aligned sequence in (a) are shown.

Instead, the split from the ancestral virus of NLRV apparently occurred before the separation of *Fijivirus* groups 2 and 3.

Discussion

It was previously thought that the conserved terminal oligonucleotides of the group 2 fijiviruses RBSDV and MRDV were 5' AAGUUUUU and GUC 3' (Marzachi *et al.*, 1991; Azuhata *et al.*, 1992). In this study, analysis of the sequences of the group 3 fijivirus, OSDV, revealed that the genus-specific, terminal sequences are 5' AA and GUC 3'. The fijiviruses seem to have a relatively short genus-specific sequence when compared with phytoreoviruses and oryzaviruses (Uyeda *et al.*, 1995a). Furthermore, the conserved 5' and 3' sequences of 5' A and GUC 3', respectively, are identical to those of NLRV, an insect virus. The diversity of fijiviruses and the close affinity of NLRV to fijiviruses may necessitate a future revision of the taxonomy of plant reoviruses.

In this study, the complete nucleotide sequences of OSDV S7–S10 were determined. It was shown that OSDV S8 and S9 each contain a single large ORF, while S7 and S10 each contain two large nonoverlapping ORFs. These results, together with

previous reports of two nonoverlapping ORFs in RBSDV S7 (Azuhata *et al.*, 1992) and S9, MRDV S6 (Marzachi *et al.*, 1991) and S8, and NLRV S9 (Nakashima *et al.*, 1996), suggest that this dicistronic pattern is unique to fijiviruses in the family *Reoviridae*.

Computer analyses of proteins encoded by OSDV S7–S10 revealed that all the ORFs of OSDV, except for S7 ORF2, had counterpart ORFs in RBSDV, MRDV and NLRV; S7 ORF2 had a counterpart in RBSDV and MRDV, but not in NLRV. When each group of homologous proteins was analysed using the Macaw program, statistically significant regions of each protein were generated (data not shown). These results suggest that each protein has a similar function. Genomic segments OSDV S9, RBSDV S8, MRDV S7 and NLRV S7 all contain a similar purine NTP-binding motif. Moreover, RBSDV S8 encodes one of the core capsids of the virus particle. Based on the calculated molecular mass of the ORF product, NLRV S7 might also encode a core capsid protein (Nakashima *et al.*, 1996; see also the accompanying paper, Isogai *et al.*, 1998b). On the other hand, RBSDV S10 and NLRV S8 each encode the major outer capsid protein of the virus particle (Nakashima & Noda, 1994; Isogai *et al.*, 1998b).

Noda & Nakashima (1995) reported that RBSDV S7 ORF2 might be involved in the multiplication of RBSDV in rice plants. RBSDV has the ability to multiply in rice plants, while NLRV does not. The most distinct difference between RBSDV and NLRV is that the deduced amino acid sequence of ORF2 from RBSDV S7 is not similar to any of the NLRV sequences. Our study revealed that a protein corresponding to RBSDV S7 ORF2 is also conserved in OSDV. This suggests that all the phytopathogenic fijiviruses specify a protein corresponding to RBSDV S7 ORF2. This protein has not yet been detected *in vivo* and functional analysis is required to determine how it is involved in virus multiplication.

It has been hypothesized that plant reoviruses originated from insect viruses (Nault, 1994). This hypothesis is derived from the following observations: (1) plant reoviruses have a greater affinity for their insect vectors than their plant hosts (plant reoviruses induce severe symptoms in plants but not in insects); and (2) there are some nonphytopathogenic reoviruses in plant reovirus insect vectors. Noda & Nakashima (1995) hypothesized that NLRV is an ancestor of the fijiviruses because it does not multiply in rice plants, which are the only natural host of *N. lugens*, and is nonphytopathogenic. In this study, we constructed phylogenetic trees using each of the homologous proteins found in OSDV, RBSDV, MRDV and NLRV. The phylogenetic trees suggested that NLRV did not split from *Fijivirus* groups 2 and 3 and then lose its ability to amplify within plants. However, it is still not clear whether the ancestor of the fijiviruses is an insect virus and fijiviruses subsequently acquired the ability to amplify within plants, or whether the ancestor is a plant virus and NLRV subsequently lost the ability to amplify within plants. Further analyses of *Fijivirus* group 1 and other nonphytopathogenic viruses like NLRV are needed to clarify the origin of fijiviruses.

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