Molecular Diagnosis of *Rice Black-Streaked Dwarf Virus* in Japan and Korea

Masamichi Isogai*, Ichiro Uyeda[1] and Jang Kyung Choi[2]

Plant Pathology Laboratory, Faculty of Agriculture, Iwate University, Ueda 3-chome, Morioka 020-8550, Japan

[1]*Department of Agrobiology and Bioresources, Faculty of Agriculture, Hokkaido University, Kita 9, Nishi 9, Kita-ku, Sapporo 060-8589, Japan*

[2]*Division of Biological Environment, College of Agriculture and Life Sciences, Kangwon National University, Chunchon 200-701, Korea*

Rice black-streaked dwarf virus (RBSDV) and *Maize rough dwarf virus* (MRDV) are closely related viruses. Since the two viruses produce identical symptoms on maize, barley, and wheat, diagnosis of infected plants is difficult. Previously, we reported that partial cDNA clones of RBSDV S5 and S6 from the Japanese isolate (RBSDV-H) have lower sequence homology to MRDV than do cDNA clones from other genomic segments. In order to test whether cDNA clones of RBSDV-H S5 and S6 can be used for molecular diagnosis, RBSDV field isolates from Korea and from Hokkaido, Japan were tested in dot blot hybridizations probed with RBSDV-H S5 and S6 cDNA clones. Hybridization with these probes was more intense against the RBSDV genome than against the MRDV genome. Therefore, RBSDV-H S5 and S6 cDNA clones can be used to differentiate between the two viruses. Furthermore, RBSDV-H S5 and S6 clones reacted more strongly against the viruses from stunted maize plants from Korean fields than to MRDV, indicating that RBSDV may be the causal disease agent in maize plants in Korea.

Keywords : *Fijivirus, Maize rough dwarf virus*, plant reovirus, *Rice black-streaked dwarf virus*.

Plant reoviruses have been classified into three genera: *Phytoreovirus, Fijivirus*, and *Oryzavirus* (Van Regenmortel et al., 2000). The genus *Fijivirus* includes the largest number of related virus species (Boccardo and Milne, 1984). In particular, *Rice black-streaked dwarf virus* (RBSDV) and *Maize rough dwarf virus* (MRDV) are considered very closely related (Azuhata et al., 1993; Isogai et al., 1995a; MacMahon et al., 1999). They are serologically related and have common plant hosts (maize, wheat, and barley), in which both produce similar symptoms (Luisoni et al., 1973). Both RBSDV and MRDV are transmitted by a com-

mon plant hopper vector, *Laodelphax striatellus* (Milne and Lovisolo, 1977; Shinkai, 1962). However, some differences do exist; the major differences are the symptoms produced on rice. RBSDV naturally infects rice and induces tumors as well as a distinctive stunting effect, while MRDV cannot naturally infect rice. If MRDV is forced to infect rice experimentally, only mild symptoms are induced (Milne and Lovisolo, 1977). Additionally, MRDV is transmitted through the eggs of the insect vector, unlike RBSDV. Nucleotide sequence comparison reveals that the viruses are closely related, but distinct (Azuhata et al., 1992; Azuhata et al., 1993; Isogai et al., 1998; Marzachí et al., 1991; Marzachí et al., 1995; Marzachí et al., 1996; Uyeda et al., 1990). Therefore, correct identification of RBSDV and MRDV is difficult, and previous identification methods based on biological properties, such as host range and symptom analysis, should be reexamined.

Previously, we showed that all MRDV genomic segments have some sequence homology to their counterparts in the RBSDV-H genome (Isogai et al., 1995a). However, partial cDNA clones of RBSDV-H genomic segments S1, S4, S5, and S6 showed low signal intensity against the MRDV genome in dot blot analysis. In particular, the signal intensities suggested that RBSDV-H S5 and S6 cDNAs have lower homology to MRDV than do RBSDV-H cDNAs from other genomic segments. In this respect, partial cDNA clones from RBSDV-H S5 and S6 are promising candidates for distinguishing between RBSDV and MRDV.

In this study, we tested the use of molecular hybridization using RBSDV-H S5 and S6 cDNA to distinguish RBSDV from MRDV. We also used these cDNAs to analyze whether field virus isolates collected from diseased maize samples in Korea were RBSDV or MRDV.

Materials and Methods

Viral strains. RBSDV-H has been maintained at Hokkaido University for over 20 years. RBSDV-AC, -AG, and -AS were isolated from stunted rice plants growing in fields in the city of

---
*Corresponding author.
 Phone) +81-19-621-6176, FAX) +81-19-621-6177
 E-mail) isogai@iwate-u.ac.jp

Asahikawa, Hokkaido, Japan, in 1992. RBSDV-KR1 and -KR2 were isolated from diseased rice plants, Korea, in 1994. RBSDV-K1 to -K5 isolates were from maize plants collected in Gyeong-sangnam-Do, Korea, by Dr. M. U. Chang, of the Dept. of Biology, Yeugnam University.

Purification of genomic dsRNA and polyacrylamide gel electrophoresis. Double-stranded RNA (dsRNA) of RBSDV was extracted directly from approximately 0.5 g of leaf tissue as described by Murao et al. (1994). Briefly, total nucleic acid was extracted by phenol-chloroform and ethanol precipitated. The ssRNA was excluded with 2M LiCl precipitation, and dsRNA was further purified by CC41 (Whatman). MRDV dsRNA genome was kindly supplied by Dr. G. Boccardo (Institute of Applied Plant Virology, National Research Council, Italy).

Polyacrylamide gel electrophoresis (PAGE) for the separation of genomic dsRNA was performed with a Tris-acetate (TAE) (40 mM Tris, 20 mM sodium acetate, 20 mM EDTA, pH 7.8) buffer system (Sambrook et al., 1989).

Dot blot hybridization. Heat-denatured dsRNA genomes of RBSDV and MRDV were spotted on nylon Hybond-N^+ membrane (Amersham). The spotted membrane was baked for 2 hr at 80°C. Prehybridization was performed at 42°C for at least 2 hr in 50% formamide, 5 × SSC, 50 mM sodium phosphate (pH 6.7), 500 μg/ml denatured salmon sperm DNA, 500 μg/ml tRNA, 0.1% SDS and 5 × Denhardt's solution. Hybridization was done overnight at 52°C. Probes were used at greater than 10^6 cpm/ml. After hybridization, the membrane was washed for 10 min four times at room temperature in 2 × SSC, 0.1% SDS, followed by two 20-min washes in 0.1 × SSC, 0.1% SDS at 60°C. The membrane was dried and developed by autoradiography.

Amplification of RBSDV-KR1 S10 using the polymerase chain reaction. RBSDV-KR1 S10 was amplified by the adapter PCR method described by Isogai et al. (1998). The 3' termini of both strands of the total dsRNA genome were polyadenylated. The polyadenylated RNA was used as a template for reverse transcription using the following oligo(dT)-containing adapter primer (AP): 5'-CGATGGTACCTGCAGGCGCGCC(T)$_{17}$-3'. The AP initiated cDNA synthesis at polyadenylated regions. Products of the first strand cDNA synthesis reaction were amplified by the polymerase chain reaction (PCR), using a primer containing the adapter region sequences identical to that of the AP (AUPA): 5'-CGATGGTACCTGCAGGCGCGCC-3'.

Results

Comparative electrophoresis of RBSDV and MRDV genomes. RBSDV-H, -AC, -AG, -AS, -KR1, and -KR2 were identified as RBSDV. However, we could not determine whether RBSDV-K1 to -K5 were RBSDV or MRDV isolates, because they were isolated from diseased maize plants, which can be infected by either virus. Additionally, maize plants contain unknown components detrimental to L. striatellus (Shikata, 1974), making it difficult to inoculate the viruses from diseased maize plants to healthy rice plants through L. striatellus, although insect transmission is the

only way to inoculate RBSDV and MRDV into plants (Shikata, 1974).

Electrophoretic profiles of the viral genomes were first compared by polyacrylamide gel electrophoresis (PAGE) in a 7.5% gel. Genomic segments were numbered S1 to S10, from the slowest migrating band to the fastest. The migration of the corresponding viral genomic segments was similar, but distinct (Fig. 1). In particular, the electrophoretic migration of MRDV S10 (S10a and S10b) was faster than that of the S10 segments from other virus isolates. However, the difference in mobility was not sufficiently significant to determine whether RBSDV-K1 to -K5 were RBSDV or MRDV isolates. Furthermore, the mobility differences of other genomic segments were as great within RBSDV-K1 to -K5 as they were between RBSDV and MRDV (Fig. 1). The MRDV genome contained many minor bands (Fig. 1), probably because the MRDV was isolated from naturally infected maize plants from Italy, and may have contained genomes from many MRDV isolates (Isogai et al., 1995b). RBSDV-AS and RBSDV-KR1 had two forms of S9 and S1, respectively. The two forms of RBSDV-AS S9, and RBSDV-KR1 S1 appeared to be present in about half the amount of the other genomic segments. This suggests that genomic variants of RBSDV exist even within one RBSDV isolate.

Dot blot hybridization. Previously, two partial cDNA probes to pRB796 and pRB596 (RBSDV-H S5 and S6, respectively) clearly differentiated between the RBSDV genome and the MRDV genome on dot blot hybridization analyses (Isogai et al., 1995a). To test whether the cDNA probes to RBSDV-H S5 and S6 are able to differentiate between RBSDV and MRDV from infected plants, the hybridization signals of RBSDV-H S5, and S6 cDNA probes to the genomes of MRDV and RBSDV isolates (RBSDV- AC, -AG, -AS, -KR1, and -KR2) from Korea and from Hokkaido, Japan were examined quantitatively by dot blot hybridization. The results showed that all the RBSDV isolates produced a stronger signal intensity than did MRDV isolates. Furthermore, the hybridization signals of the unidentified virus isolates from the maize plants (RBSDV-K1 to -K5) were also stronger than those of MRDV isolates (Fig. 2). Thus, it is likely that RBSDV-K1 to -K5 are RBSDV isolates, not MRDV.

Cloning and sequencing of RBSDV-KR1 S10. The RBSDV-KR1 S10 was cloned by adapter PCR (Isogai et al., 1998). The full-length PCR products of RBSDV-KR1 S10 were digested with PstI and inserted into the PstI site of the plasmid vector pBluscript II^{SK-} (Stratagene). RBSDV-KR1 S10 is 1,801 nucleotides (nt) long and contains a single long open reading frame (ORF). RBSDV-KR1 S10 has the same conserved terminal sequence and inverted repeat as RBSDV-H S10 and MRDV S10 (Azuhata et al.,
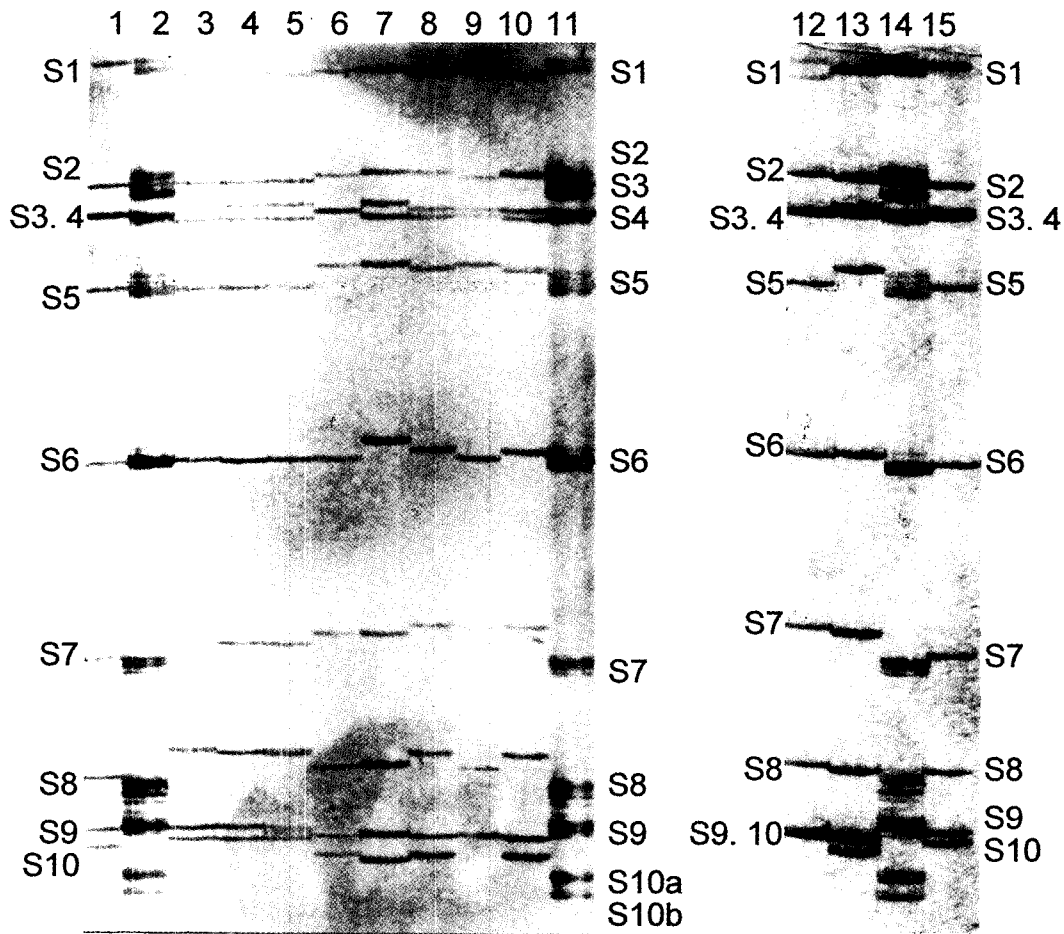
Fig. 1. Comparison of the genomic dsRNA migration profiles of the virus isolates in a 7.5% polyacrylamide gel stained with silver. Lane 1, RBSDV-H; lane 2, MRDV; lane 3, RBSDV-AC; lane 4, RBSDV-AG; lane 5, RBSDV-AS; lane 6, RBSDV-K1; lane 7, RBSDV-K2; lane 8, RBSDV-K3; lane 9, RBSDV-K4; lane 10, RBSDV-K5; lane 11, MRDV; lane 12, RBSDV-KR2; lane 13, RBSDV-KR1; lane 14, MRDV; lane 15, RBSDV-H.

1992; Marzachí et al., 1991; Marzachí et al., 1995; Uyeda et al., 1990). The ORF of RBSDV-KR1 initiates around nt 22 to 24 (from the 5' end) and terminates around nt 1,696 to 1,698. RBSDV-KR1 S10 has a predicted molecular weight of 62,942 Da. Compared to the sequences of RBSDV-H S10 and RBSDV-KR1, MRDV S10 has a T inserted at nt 11 (Isogai et al., 1998). However, some RBSDV isolates from Japan also have a T inserted at nt 11. The insertion of a T at nt 11 is therefore not a defining characteristic of MRDV S10. The nucleotide sequences of the coding strands of RBSDV-H S10, RBSDV-KR1 S10, and MRDV S10 were aligned. The overall homology between RBSDV-H S10 and RBSDV-KR1 S10 is 94% and between RBSDV-KR1 S10 and MRDV S10 is 88%. At the deduced amino acid sequence level, the overall homology between RBSDV-H S10 and RBSDV-KR1 S10 is 98% and between RBSDV-KR1 S10 and MRDV S10 is 92%. Based on these results, we conclude that RBSDV-KR1 is

more closely related to RBSDV-H than to MRDV at the nucleotide and amino acid sequence level of these S10s as well as the segments corresponding to RBSDV-H S5 and S6.

Discussion

Although all MRDV genomic segments showed some level of sequence similarity in hybridization analyses to their counterparts in RBSDV-H, the signal intensities differed depending on the genomic segments used (Isogai et al., 1995a). RBSDV-H S5 and S6 cDNAs showed lower signal intensities against the MRDV genome than RBSDV-H cDNAs from other segments, suggesting that S5 and S6 could be used in distinguishing the two viruses. Every RBSDV isolate (RBSDV- AC, -AG, -AS, -KR1, and -KR2) from Hokkaido and Korea that hybridized with the probes had a stronger signal than MRDV. Furthermore,
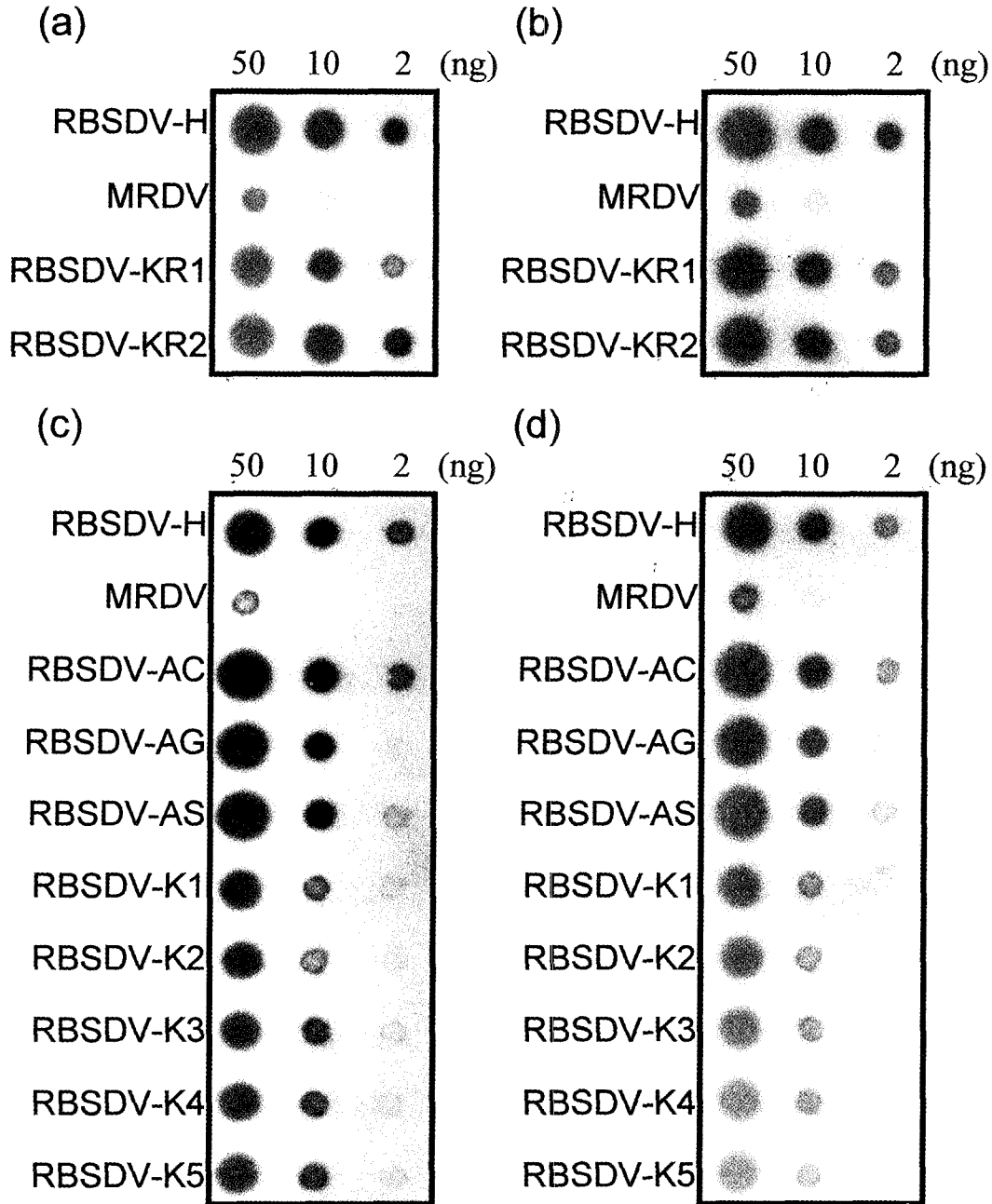
Fig. 2. Dot blot hybridization using cDNA probes of RBSDV-H S5 (a and c), and S6 (b and d). The genomes of virus isolates (RBSDV-H, -AC, -AG, -AS, -KR1, -KR2, -K1, -K2, -K3, -K4, -K5) were dotted at various dilutions (50 ng, 10 ng, and 2 ng).

comparison of the nucleotide and amino acid sequences of RBSDV-KR1 S10, RBSDV-H S10, and MRDV S10 clearly showed that RBSDV-KR1 S10 also has higher similarity to RBSDV-H than to MRDV. Therefore, we have demonstrated that RBSDV-H S5 and S6 cDNAs are powerful tools for distinguishing between RBSDV and MRDV.

MRDV has been reported in Scandinavia and in areas bordering the northern and eastern Mediterranean, while

RBSDV has been reported in Japan, Korea, and China (Boccardo and Milne, 1984). However, a molecular diagnosis of RBSDV has not been conducted previously, making it difficult to interpret the presence of maize, barley, and wheat plants with MRDV-like symptoms in Korea and China. This study demonstrates that RBSDV-K1 to -K5 are genetically closer to RBSDV than to MRDV, and that RBSDV, not MRDV, may be the agent causing disease in maize plants in Korea.

Acknowledgment

References

Azuhata, F., Uyeda, I. and Shikata, E. 1992. Conserved terminal nucleotide sequences in the genome of rice black-streaked dwarf virus. *J. Gen. Virol.* 73:1593-1595.

Azuhata, F., Uyeda, I., Kimura, I. and Shikata, E. 1993. Close similarity between genome structures of rice black-streaked dwarf and maize rough dwarf viruses. *J. Gen. Virol.* 74:1227-1232.

Boccardo, G. and Milne, R. G. 1984. *Plant reovirus group.* CMI/AAB Description of Plant Viruses No. 294.

Isogai, M., Azuhata, F., Uyeda, I., Shikata, E. and Kimura, I. 1995a. Genomic relationship between rice black-streaked dwarf and maize rough dwarf fijiviruses detected by nucleic acid hybridization. *Ann. Phytopath. Soc. Japan* 61:513-518.

Isogai, M., Uyeda, I. and Kimura, I. 1995b. The diversity of field isolates of rice black streaked dwarf fijivirus. *Ann. Phytopath. Soc. Japan* 61:575-577.

Isogai, M., Uyeda, I., Lee, B. C. and Shikata, E. 1998. Detection and assignment of proteins encoded by rice black streaked dwarf fijivirus S7, S8, S9, and S10. *J. Gen. Virol.* 79:1487-1494.

Isogai, M., Uyeda, I. and Hataya, T. 1998. An efficient cloning strategy for a viral double-stranded RNA with an unknown sequence. *Ann. Phytopathol. Soc. Japan* 64:244-248.

Luisoni, E., Lovisoro, O., Kitagawa, Y. and Shikata, E. 1973. Serological relationship between maize rough dwarf virus and rice black-streaked dwarf virus. *Virology* 52:281-283.

MacMahon, J. A., Dale, J. L. and Harding, R. M. 1999. Taxonomic implication for fijiviruses based on the terminal sequences of Fiji disease fijivirus. *Arch. Virol.* 144:2259-2263.

Marzachí, C., Boccardo, G. and Nuss, D. L. 1991. Cloning of the maize rough dwarf virus genome ñ Molecular confirmation of the plant reovirus classification scheme and identification of two large nonoverlapping coding domains within a single genomic segment. *Virology* 180:518-526.

Marzachí, C., Boccardo, G., Milne, R., Isogai, M. and Uyeda, I. 1995. Genome structure and variability of Fijiviruses. *Semin. Virol.* 6:103-108.

Marzachí, C., Antoniazzi, S., D'Aquilio, M. and Boccardo, G. 1996. The double-stranded RNA genome of maize rough dwarf Fijivirus contains both mono and dicistronic segments. *Eur. J. Plant Pathol.* 102:601-605.

Milne, R. G. and Lovisolo, O. 1977. Maize rough dwarf and related viruses. *Adv. Virus Res.* 21:267-341.

Murao, K., Suda, N., Uyeda, I., Isogai, M., Suga, H., Yamada, N., Kimura, I. and Shikata, E. 1994. Genomic heterogeneity of rice dwarf phytoreovirus field isolates and nucleotide sequences of variants of genome segment 12. *J. Gen. Virol.* 75:1843-1848.

Sambrook, J., Fritsch, F. and Maniatis, T. 1989. *Molecular Cloning, A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York.

Shikata, E. 1974. *Rice black-streaked dwarf virus.* CMI/AAB Description of Plant Viruses No. 135.

Shinkai, A. 1962. Studies on insect transmission of rice virus diseases in Japan. *Rep. Nat. Agric. Sci. (Japan)* C14:1-112.

Uyeda, I., Azuhata, F. and Shikata, E. 1990. Nucleotide sequence of rice black-streaked dwarf virus genome segment 10. *Proc. Japan Acad.* 66:37-40.

Van Regenmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L., Carstens, E. B., Estes, M. K., Lemon, S. M., McGeoch, D. J., Maniloff, J., Mayo, M. A., Pringle, C. R. and Wickner, R. B. (eds) 2000. *Virus Taxonomy. Classification and Nomenclature of Viruses.* Seventh ICTV Report, Academic Press.