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Short communication

Sequence analysis shows that a dwarfing disease on rice, wheat and maize in China is caused by *Rice black-streaked dwarf virus*

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Abstract

Isolates of plant reoviruses causing severe stunting and dark leaf symptoms on wheat in Hebei province, on maize in Hubei province and on rice in Zhejiang province, China have been characterized. Four of the ten genome segments corresponding to *Rice black-streaked dwarf virus* (RBSDV) S7, S8, S9, S10 were amplified by RT-PCR from the Hebei and Zhejiang isolates and sequenced. Sequences of S9 and S10 were also obtained from the Hubei isolate. Sequences of corresponding segments of the Chinese isolates were very similar to each other (94.0–99.0% identical nucleotides and 96.3–100% identical amino acids) and were closer to those of a previously reported Japanese RBSDV isolate (90.0–94.9% identical nucleotides and 91.1–98.6% identical amino acids) than to those of an Italian *Maize rough dwarf virus* isolate (85.1–88.1% identical nucleotides and 85.5–94.3% identical amino acids). The Chinese isolates should be classified as RBSDV.

Abbreviation: MRDV – *Maize rough dwarf virus*; RBSDV – *Rice black-streaked dwarf virus*.

Rice black-streaked dwarf virus (RBSDV) and *Maize rough dwarf virus* (MRDV) are closely related members of the genus *Fijivirus* (family *Reoviridae*). Both viruses are transmitted by the planthopper *Laodelphax striatellus* Fallen, and can be experimentally transmitted to rice, maize, wheat and other cereal and grass hosts, producing similar symptoms. However, MRDV does not naturally infect rice whereas RBSDV can be found in both rice and maize (Boccardo and Milne, 1984). Virions of the two viruses have the same particle morphology and are serologically related (Luisoni et al., 1973). Both virus genomes have ten segments of dsRNA. Serological comparisons and hybridization experiments using cDNA probes (Isogai et al., 1995) suggest that they are distinct viruses. Although this has been adopted in the latest ICTV report (van Regenmortel et al., 2000), there have been other suggestions that they should be considered as geographical races of the same virus (Marzachi et al., 1995). The segments S7, S8, S9 and S10 of RBSDV are homologous to S6, S7, S8 and S10 of MRDV respectively and share 85–87.5% identical nucleotides between them (Azuhata et al., 1993; Marzachi et al., 1995). RBSDV segments S8 and S10 are both monocistronic and encode the core capsid (68 kDa) and the major outer capsid (63 kDa) proteins respectively, while segments S7 and S9 each encode two nonstructural proteins (Isogai et al., 1998). The sequences of other segments have not yet been determined.

RBSDV was first reported causing an important disease on rice in Japan and when an isometric virus producing symptoms of severe stunting and leaf darkening on rice, and transmitted by the planthopper *L. striatellus,* caused severe damage in eastern China in the 1960s, it was thought to be RBSDV. In the following 30 years, the disease incidence declined greatly and

infected plants became very difficult to find. However, since 1991, the virus has occurred again in most parts of Zhejiang province and north Fujian province, causing severe damage on rice with a disease incidence exceeding 90% in some areas. This is the result of widespread release of susceptible rice cultivars, the abundance of the planthopper vector in rice fields and the introduction of cultivation patterns for late-season hybrid rice that favour infection (Dong et al., 1999).

MRDV has been reported from several European and Mediterranean countries. In China, stunting and leaf darkening symptoms on maize, caused by an isometric virus transmitted by *L. striatellus*, became severe in Hebei province in the 1970s. Since 1990 this disease, usually referred to as MRDV, has occurred each year in northern, northwestern and northeastern China, causing very severe damage (Li et al., 1999). The virus overwinters in wheat, in which it causes symptoms, but much less damage.

The purpose of this study was to clarify the status of the virus isolates in China by investigating isolates from rice in Zhejiang province, from wheat in Hebei and from maize in Hubei.

Plants showing typical RBSDV/MRDV dwarfing symptoms were collected from Zhejiang province in July 1998 (rice), from Hebei province in May 1999 (wheat) and Hubei province in August 2000 (maize). The leaves were frozen and stored at −80°C until used for virus purification. The dsRNA was extracted from purified virus particles as previously described (Uyeda et al., 1998) and suspended in 30 µl of H_2O. It was then denatured by adding 20 µl DMSO (dimethyl sulfoxide), incubating at 95°C for 5 min and quickly transferring to ice for 10 min. The denatured dsRNA was precipitated with 3 volumes of ethanol and 1/10 volume of 3M sodium acetate, pH5.2.

From each of four genome segments (two segments only for the maize isolate), an internal portion was first amplified using primers designed from the published sequences of a Japanese isolate of RBSDV (Table 1). First strand cDNA synthesis was done using denatured RNA as a template and Superscript Reverse Transcriptase (MBI) according to the manufacturer's instructions. The resulting cDNA was purified using the PCR Gel Extraction Kit (QIAGEN) and used as a template for PCR. After cloning, the products were sequenced (see below) and further primers were designed from these sequences to amplify the remaining regions of the genome segments (Table 1). To ensure that complete terminal sequences were obtained, primer zhm-1 (Table 1) was first ligated to both 3′ ends of the viral RNA. To prevent concatenation of primer zhm-1 in subsequent dsRNA/DNA ligation reactions, the 3′-terminus of the primer was blocked by amine. For the ligation reaction, 10 U T4 RNA ligase (Biolab) were included in a total volume of 20 µl, containing about 2 µg viral dsRNA, 200 pmol primer zhm-1, 50 mM Tris-HCl, pH7.8, 10 mM $MgCl_2$ 10 mM dithiothreitol,

Table 1. Primers used for sequencing genome segments S7–10 from the Chinese isolates

Primer name	Position	Sequence (5′ to 3′)	Tm (°C)	direction
S7p-1	922–941	GAG CTC TTC TAG TTA TTG CG	58	forward
S7p-2	1468–1450	TGT CAC ACC ACT CTT CTC C	58	reverse
S7p-3	1135–1116	CAA GAT TAA GCA GAA GGA GA	56	reverse
S7p-4	1183–1204	ATG AAT TAC ACT TTA GGT GAT C	58	forward
S8p-1	493–514	GTG AAA ACT GAG ACT AAT GAT C	60	forward
S8p-2	1640–1621	ACT TGT CCG TTT CTA GGA AC	58	reverse
S8p-3	571–551	CAA CGC TTG TTC TTT CAC ATC	60	reverse
S8p-4	1512–1533	GAG TCA AAC TTG TCA TTT CGT C	62	forward
S9p-1	432–451	CAC TCT CAA ACC ATC TTC AG	58	forward
S9p-2	1491–1472	GGA TTA CAA CAC ACA CAA CG	58	reverse
S9p-3	590–571	GAT TCT TCA GAT TCA CGG TT	56	reverse
S9p-4	1299–1320	CTG ATT CTG AAA TTG ATG ATC G	60	forward
S10p-1	167–185	TCA AAG CGC CCC ACG TTG C	62	forward
S10p-2	1278–1261	CGC GCT CAA CAC TTC GCC	60	reverse
S10p-3	505–486	CAT CAG CGG AAA AGG GCT TC	62	reverse
S10p-4	1208–1229	TTG TAG TTC AAA CTC ATA AAG G	58	forward
zhm-1		PO_4-CTC TTC CCC TCC CTC CTC-NH_2	60	
zhm-2		GAG GAG GGA GGG GAA GAG	60	

For each segment, primers 1 and 2 were designed from the Japanese RBSDV sequences and primers 3 and 4 from the Chinese sequences determined in this study.

1 mM ATP, 10 μg/ml BSA, 25% PEG (MW 8000), and 10 U RNase inhibitor (Gibco), which was incubated at 37 °C for 15 min. To remove the unligated primer zhm-1 molecules, the RNA/zhm-1 ligation product was purified by the QIAGEN RNeasy plant minikit following the manufacturer's protocol. Primer zhm-1-tailed genomic RNA was transcribed to cDNA in the presence of 90 ng of primer zhm-2 (Table 1, complementary to primer zhm-1) with Superscript Reverse Transcriptase (MBI). After incubation at 42 °C for 60 min, the reaction was stopped by heating at 70 °C for 15 min, and the mixture was placed on ice for 3 min. Thereafter, 1 μl of RNase H (2U/μl) was added, and the mixture was incubated at 37 °C for 20 min. The resulting cDNA was purified and used as a template for PCR with one of the segment-specific internal primers and primer zhm-2.

PCR reactions were done as described by Chen et al. (2000). Fragments amplified by PCR were purified using the PCR Gel Extraction Kit (QIAGEN), ligated into pGEM T-vector (Promega), and used to transform competent *E. coli* TG1 cells for cloning. Plasmids were isolated using the QIAprep spin mini prep kit (Qiagen Ltd), and the inserts were sequenced by a commercial company (TaKaRa Co. Ltd., Dalian City, China).

Sequence analysis was done with programs from the Wisconsin (GCG) package (Genetics Computer Group, 1998). Pairwise comparisons of sequences were made using GAP with a gap creation penalty of 50 and a gap extension penalty of 3 for nucleotide sequences or with a gap weight of 8 and a gap extension penalty of 2 for amino acid sequences.

The sizes and features of these genome segments (Table 2) were very similar to those of the corresponding Japanese RBSDV and Italian MRDV segments. The complete nucleotide sequences of the segments of the Chinese isolates were deposited in the DDBJ/GenBank/EMBL databases and assigned the accession numbers shown in Table 2. Comparisons of nucleotide and amino acid identities between the homologous segments showed that the Chinese isolates were the most similar to one another and that they shared higher percentage identities with the corresponding segments of the Japanese RBSDV than with those of the Italian MRDV (Table 3). This

Table 2. Sizes of genome segments and predicted open reading frames (ORFs) on the isolates from Zhejiang, Hebei and Hubei, with the corresponding sizes for the Japanese RBSDV and Italian MRDV sequences

	Total size	GC content	5′-UTR	ORF1		Intergenic region	ORF2		3′-UTR
	nts	%	nts	nts	kDa	nts	nts	kDa	nts
Zhejiang S7	2193	34.3	41	1089	41.0	52	930	36.3	81
Hebei S7	2190	34.4	41	1086	40.9	52	930	36.3	81
Japanese S7	2193	34.0	41	1089	41.1	52	930	36.4	81
MRDV S6	2193	34.7	41	1089	41.0	52	930	36.3	81
Zhejiang S8	1936	34.8	24	1776	68.1	—	—	—	136
Hebei S8	1936	35.0	24	1776	68.1	—	—	—	136
Japanese S8	1927	34.6	24	1776	68.1	—	—	—	127
MRDV S7	1936	34.7	24	1776	68.1	—	—	—	136
Zhejiang S9	1900	33.4	51	1044	39.9	64	630	24.2	111
Hebei S9	1898	33.8	51	1044	40.0	64	630	24.2	109
Hubei S9	1900	33.6	51	1044	39.9	64	630	24.3	111
Japanese S9	1900	34.1	51	1044	39.9	64	630	24.2	111
MRDV S8	1900	34.3	51	1044	39.9	64	630	24.2	111
Zhejiang S10	1801	36.6	21	1677	63.1	—	—	—	103
Hebei S10	1801	36.3	21	1677	63.1	—	—	—	103
Hubei S10	1801	36.6	21	1677	63.1	—	—	—	103
Japanese S10	1801	36.3	21	1677	63.2	—	—	—	103
MRDV S10	1802	36.6	22	1677	62.9	—	—	—	103

Sequence accession numbers are: Zhejiang S7 (AJ297427), Hebei S7 (AJ297428), Japanese S7 (S63917), MRDV S6 (X55701), Zhejiang S8 (AJ297431), Hebei S8 (AJ297432), Japanese S8 (S63914), MRDV S7 (L76562), Zhejiang S9 (AJ297430), Hebei S9 (AJ297429), Hubei S9 (AJ291706), Japanese S9 (AB011403), MRDV S8 (L76561), Zhejiang S10 (AJ297433), Hebei S10 (AJ297434) , Hubei S10 (AJ291707), Japanese S10 (D00606) and MRDV S10 (L76560).

Table 3. Homologies (% identical nucleotides, with % identical amino acids in the open reading frame(s) in brackets) between the respective RBSDV and MRDV segments

S7	Hebei	Japan	MRDV-S6
Zhejiang	99.0 (100.0, 98.4)	93.4 (98.1, 97.8)	85.1 (92.3, 85.5)
Hebei		93.8 (98.1, 96.5)	85.3 (92.3, 86.8)
Japan			85.1 (91.2, 86.1)

S8	Hebei	Japan	MRDV-S7
Zhejiang	94.0 (96.5)	94.9 (92.9)	85.1 (91.7)
Hebei		94.5 (92.5)	87.6 (91.9)
Japan			85.1 (87.8)

S9	Hebei	Hubei	Japan	MRDV-S8
Zhejiang	98.5 (96.3, 100.0)	98.8 (98.9, 99.0)	90.2 (93.1, 98.6)	86.3 (89.1, 94.3)
Hebei		98.5 (97.4, 99.0)	90.0 (91.1, 98.6)	85.9 (89.1, 94.3)
Hubei			89.9 (93.7, 97.6)	86.4 (89.9, 94.3)
Japan				85.3 (89.1, 93.3)

S10	Hebei	Hubei	Hubei-hb	Hubei-hbr	Henan	Shaanxi	Japan	MRDV
Zhejiang	97.1 (98.2)	97.3 (98.6)	96.3 (98.4)	97.2 (98.0)	97.0 (98.4)	97.1 (98.7)	93.8 (96.2)	87.7 (92.0)
Hebei		98.9 (99.3)	97.4 (99.1)	98.7 (98.7)	98.5 (99.1)	98.2 (99.5)	93.2 (96.0)	88.1 (92.5)
Hubei			97.5 (99.5)	98.7 (99.1)	98.8 (99.5)	98.3 (99.8)	93.5 (96.4)	87.9 (92.7)
Hubei-hb				97.3 (98.9)	97.6 (99.3)	97.1 (99.6)	93.4 (96.2)	88.0 (92.8)
Hubei-hbr					98.3 (98.9)	98.3 (99.3)	93.3 (95.9)	88.1 (92.3)
Henan						98.2 (99.6)	93.5 (96.2)	88.3 (92.4)
Shaanxi							93.4 (96.6)	88.0 (92.8)
Japan								87.7 (90.9)

Sequence accession numbers are as in Table 2, with the addition of the Chinese S10 sequences: Hubei-hb (AF227205), Hubei-hbr (AF227206), Henan (AF227207) and Shaanxi (AF227208).

was also apparent with S10, for which the sequences of four other Chinese isolates (from maize) have been recently deposited in the database and were included in this analysis. The primers used in these experiments were designed to RBSDV sequences but those for RBSDV S8 would also be a good match for MRDV S7. Primers were also designed from the published MRDV S6 (X55701)and S8 (L76561) sequences and used in RT-PCR. For S6, the primers were MRS6-1 (5′-GCCGTTCTCATCACTATA-3′; forward primer matching nt 924–941) and MRS6-2 (5′-GTCTGCAAAATTGTGAATC-3′; reverse primer complementing nt 1485–1467). For S8, zhm-2 was used in combination with MRS8-1 (5′-GGGTCTGTTTTGAAGCTTCG-3′; reverse primer complementing nt 505–486). These reactions did not amplify genome fragments from any of the three Chinese templates. It therefore seems unlikely that the experiments had selected only one component from a mixed infection.

The reoviruses isolated from wheat in north China (Hebei) and from rice in east China (Zhejiang) had similar biological properties. Both had isometric particles 65 nm in diameter and an antiserum prepared against the Zhejiang isolate reacted equally well with both isolates in ELISA tests (data not shown). Both virus isolates could be transmitted efficiently to maize and rice from their original hosts (data not shown) and shared high nucleotide and amino acid percent identities between their four genome segments S7–10. The isolate from maize in Hubei had very high nucleotide and amino acid percent identities to the other isolates in S9 and S10. Based on these criteria, we consider that the Chinese isolates from Hebei, Hubei and Zhejiang, are isolates of the same virus species. The four additional Chinese isolates from maize, represented by their S10 sequences, were also very similar. No biological or serological comparisons have been made between the Chinese isolates and known isolates of RBSDV and MRDV. However, because of the high nucleotide and amino acid similarity to the Japanese RBSDV (90.0–94.9% identical nucleotides and 91.1–98.6% identical amino acids) and the somewhat lower similarity to MRDV (85.1–88.1% identical nucleotides and 85.5–94.3% identical amino acids), we suggest that the Chinese isolates should be classified as RBSDV. However, it is clear that the magnitude of the difference between RBSDV and MRDV is not great

with many proteins sharing over 90% identical amino acids. The second ORF of RBSDV S9 (MRDV S8), which encodes a non-structural protein of 24.2 kDa, is particularly highly conserved. Further biological comparisons, and sequences from additional MRDV isolates are needed but it must be considered doubtful whether the two viruses should continue to be classified as different species.

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