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cDNA cloning and sequence analysis of genome segments S8 from rice black-streaked dwarf virus

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Abstract: Genome segments S8 of two Chinese isolates of rice black-streaked dwarf virus (RBSDV), one from Zhejiang Province and another from Hebei Province, were amplified by RT-PCR and sequenced. Both segments consisted of 1936 nts in full length (EMBL accession numbers were AJ297431 and AJ297432, respectively) and contained only one big open reading frame which encoded a polypeptide with molecular weight of 68kD. The two Chinese isolates shared 94.0% and 96.5% identity at nucleotide and amino acid level, respectively. They shared 94.5 - 94.9% and 92.5 - 92.9% homology with S8 of RBSDV Japanese isolate at nucleotide and amino acid level, respectively; shared 85.1 - 87.6% and 91.7 - 91.9% homology with S7 of Italian MRDV (maize rough dwarf virus).

Key word: Rice black-streaked dwarf virus (RBSDV); Genome segment S8, Sequence analysis, Biotechnology

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INTRODUCTION

Rice black-streaked dwarf virus (RBSDV), a member of the genus Fijivirus within the family Reoviridae, is propagatively transmitted to rice, maize, barley and wheat in a persistent manner by planthopper Laodelphax striatellus (Shikata et al., 1977). The infected plant usually shows darkening of leaves and pronounced stunting. The typical symptoms in rice are characteristically raised, smooth and whitish swellings along the veins on the undersurface of the leaf blades, sheaths and clums; later they become dark brown, forming black-streaked tumors of various lengths. Our previous studies showed dwarf diseases on rice, maize and wheat in different regions of China are caused by RBSDV (Zhang et al., 2000), which resulted in severe yield loses in recent years because no resistant variety was available. Electron microscopy of ultrathin sections revealed that both plant cells and insect cells infected with RBSDV contain viroplasms, virus crystals, and tubular structures; and that the virus particles were restricted to the area of the leaf veins, particularly the tumor cells (Shikata et al., 1977; Luisoni et al., 1973).

RBSDV has a multipartite genome consisting of 10 linear segments of double-stranded RNA (dsRNA), termed S1 to S10 based on their electrophoretic mobility in polyacrylamide gels, ranging in size from approximately 1.8 to 4.5 kb with a total genome size of approximately 30 kb. In this paper, we first report the complete nucleotide sequences of the genome segments S8 of two Chinese isolates, one from Zhejiang and another from Hebei.

MATERIALS AND METHODS

Source of virus isolate and extraction of viral dsRNA

Infected rice plants with typical dwarf

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symptoms were collected from Zhejiang Province in July 1998, and infected wheat plants with similar symptoms were collected from Hebei Province in May 1999. They were subsequently propagated by planthopper transmission on maize plant in the greenhouse of Virology Laboratory, Zhejiang Academy of Agricultural Sciences, respectively. Infected maize leaves were collected and frozen and stored at -80℃. The viral genomic dsRNA of both isolates were extracted directly from the infected maize plants using the method described by Uyeda et al. (1998). In brief, total nucleic acids were extracted with phenol-chloroform and then ethanol-precipitated. The dsRNA was further purified with CC41 (Whatman). About 2 μg viral dsRNA was obtained from 1 g freeze-dried maize plants by this method.

Denaturation of RBSDV dsRNA

The extracted dsRNA was suspended in 30 μl of H_2O and then denatured by adding 20 μl DMSO (dimethyl sulfoxide), incubated at 95 ℃ for 5 min and quickly transferred onto ice for 10 min. The denatured dsRNA was precipitated with 3 volumes of ethanol and 1/10 volume of 3 mol/L sodium acetate, pH 5.2.

Amplification of internal region by RT-PCR

Two primers, S8p-1 and S8p-2 (Table 1), were designed according to the published sequences of RBSDV Japanese isolate S8 and used to amplify the internal portion of two Chinese isolates S8. First strand cDNA synthesis was carried out 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 amplification with the two primers. The PCR procedures were carried out on a Perkin-Elmer PE480 DNA thermal cycler: one cycle of 3 min at 94 ℃; 30 cycles of 1 min at 94 ℃, 1 min at 56 ℃ and 1 min at 72 ℃; and a final extension at 72 ℃ for 10 min.

Table 1 Primers used for sequencing genome segments S8 from two Chinese isolates

Primer name	Position	Sequence (5' to 3')	T_m(℃)	Direction
S8p-1	493 – 514	GTG AAA ACT GAG ACT AAT GAT C	60	Forward
S8p-2	1545 – 1525	ACA ATA CGA ATG GAC GAA ATG	58	Reverse
S8p-3	571 – 551	CAA CGC TTG TTC TTT CAC ATC	60	Reverse
S8p-4	1428 – 1447	TCA CAG CAT CCA AGG TTC TA	58	Forward
Zhm-1		PO_4 – CTC TTC CCC TCC CTC CTC-NH_2	60	
Zhm-2		GAG GAG GGA GGG GAA GAG	60	

Note: Primers 1 and 2 were designed according to the Japanese RBSDV sequences and primers 3 and 4 to the Chinese sequences determined in this study

Amplification of the 5'-and 3'-terminal region by anchor-ligated PCR

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. Ligation reaction, in a total volume of 20 μl,, contained about 2 μg viral dsRNA, 200 pmol primer Zhm-1, 50 mmol/L Tris-HCl, pH 7.8, 10 mmol/L MgCl2 10 mmol/L dithiothreitol, 1 mmol/L ATP, 10 μg/ml BSA, 25% PEG (MW 8000), 10 U T4 RNA ligase (Biolab) and 10 U RNase inhibitor (Gibco), and incubated at 37 ℃ 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 ℃ for 60 min, the reaction was stopped by heating at 70 ℃ for 15 min, and the mixture was placed onto ice for 3 min. Thereafter, 1 μl of RNase H (2 U/μl) was added, and the mixture was incubated at 37 ℃ for 20 min. The resulting cDNA

was purified and used as a template for PCR with primer Zhm-2 and one of the two segment-specific internal primers, S8-p3 and S8p-4 (Table 1), designed based on our determined sequences. PCR reactions were initiated as previously described.

Cloning and screening of recombinant clones

The fragments amplified by RT-PCR were purified using PCR Gel Extraction Kit (QIAGEN), ligated into pGEM T-vector (Promega), and transformed into competent *E. coli* TG1 cells for cloning. The transformants were screened using the ampicillin resistance and α-complementation method. Recombinant plasmid DNA was isolated using alkaline lysis method according to Sambrook et al. (1989), analyzed by agarose gel electrophoresis, and confirmed by PCR.

Sequencing

Recombinant plasmid DNA used for sequencing were prepared using the QIAprep Spin Mini Prop Kit (Qiagen Ltd), and the inserts were sequenced entirely on both strands using BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PERKIN ELMER) on ABI PRISM™ 377 DNA Sequencer with universal primers of T7 and SP6.

Sequence analysis.

Sequence data were assembled and analyzed using the GCG programs (Program Manual for the Wisconsin Package, version 10.0, 1998, Genetics Computer Group, Madison, WI, USA).

RESULTS AND DISCUSSION

Amplification of internal regions by RT-PCR

The denatured genome of each RBSDV isolate was transcribed in the presence of the two primers, S8p-1 and S8p-2; the purified cDNA fragments were used as templates for PCR with the two primers, respectively, and specific fragments of expected size (1.05 kbp) were amplified from both isolates (Fig.1).

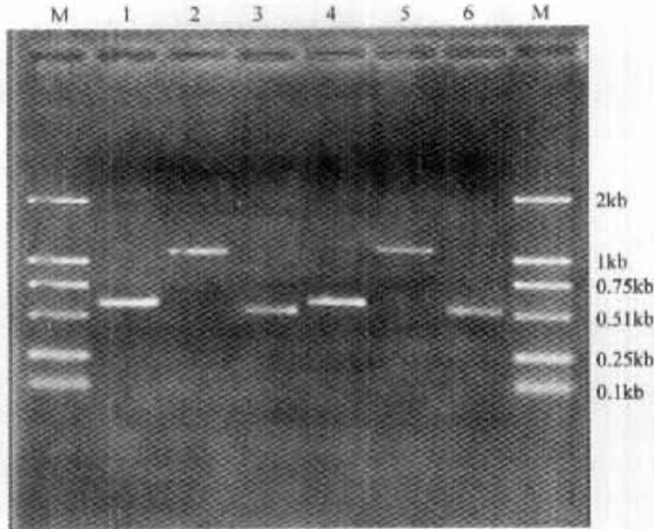

Fig.1 RT-PCR products for S8 of two Chinese isolates examined on a 1.2% agarose gel stained with ethidium bromide (M. Marker DNA, from up to down: 2 kb, 1 kb, 0.75 kb, 0.5 kb, 0.25 kb, 0.1kb, respectively)

1. RT-PCR product for 5' terminal region of RBSDV-Zhejiang S8;
2. RT-PCR product for internal region of RBSDV-Zhejiang S8;
3. RT-PCR product for 3' terminal region of RBSDV-Zhejiang S8;
4. RT-PCR product for 5' terminal region of RBSDV-Hebei S8;
5. RT-PCR product for internal region of RBSDV-Hebei S8;
6. RT-PCR product for 3' terminal region of RBSDV-Hebei S8.

Amplification of terminal region by anchor-ligated RT-PCR

To obtain the terminal sequences, the 3'-amino blocked primer (primer Zhm-1) was ligated to the 3'-ends of the two Chinese isolates genomic RNA. Total cDNA was then synthesized using a second primer (primer Zhm-2) complementary to primer Zhm-1, and subsequently the 5' terminal sequences were amplified by PCR with primer Zhm-2 and S8p-3, and the 3' - terminal sequences were amplified by PCR with primer Zhm-2 and S8p-4. Fragments of the expected size (about 0.59 kbp or 0.53 kbp) were obtained from both Zhejiang and Hebei isolates (Fig. 1).

Analysis of complete sequences of genome segments S8 from two Chinese isolates

The complete nucleotide sequences of the segments of the two Chinese isolates were assembled and deposited in the GenBank/EMBL/DDBJ databases (accession numbers AJ297431 and AJ297432). Both consisted of 1936 nts, which were 9 nts longer than S8 of Japanese RBSDV, but identical to S7 of Italian MRDV (Marzachi et al., 1995; Azuhata et al., 1993). Sequencing results showed that the sequence, 5' – AAGTTTTTTT......, was located immediately after the primer Zhm-2 and the sequence, ...GUC-3', was located immediately proceding the primer Zhm-1 in S8 of both RBSDV-Zhejiang and RBSDV-Hebei. The terminal sequences were identical to those of Japanese RBSDV and Italian MRDV. A perfect 9 bp inverted repeat, nt 7-15(5'-TTTTCGCAC-3') and nt 1915-1923 (5'-GTGCGAAAA-3'), were identified immediately adjacent to the conserved terminal sequences in full length of RBSDV-Hebei isolate S8, while the inverted repeat became imperfect because of base transition at position nt 1921 (Fig. 2a.) in S8 of Zhejiang RBSDV, Japanese RBSDV and in S7 of Italian MRDV. Conserved 5' and 3' terminal sequences and inverted repeats were also been found in all reoviruses sequenced to date. The conserved terminal sequences were thought to be genus-specific and to act as a packaging signal for viral and not host DNA; while the inverted repeats were thought to be genome segment-specific and may act as a signal to specify a particular segment (Anzola et al., 1987). At present, we do not know why the base transition occurred in such functional region, which suggested the base transition would not affect its function.

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RBSDV-Jap S8 (accession number: S63914)
MRDV S7 (accession number: L76562)
RBSDV-Heb S8 (accession number: AJ297432)

(a)

RBSDV-Heb S8p
RBSDV-ZJ S8p
RBSDV-Jap S8p
MRDV S7p

(b)

Fig.2 Multiple sequence alignments of the corresponding genome segments of Zhejiang RBSDV, Hebei RBSDV, Japanese RBSDV and Italian MRDV

(a) comparison of complete nucleotide sequences of the corresponding genome segments from four virus isolates; (b) comparison of polypeptides encoded by the corresponding genome segments from four virus isolates

Computer analyses revealed that S8 of both Chinese isolates contained one open reading frame (ORF). The ORF was 1773 nts from nt 25 to 1798 and potentially encoded a 591-amino-acid protein with calculated molecular mass of about 64 kD. Both isolates S8 contained similar organization (Table 2.) and shared 94.0% homology at nucleotide level and 96.5% homology at amino acid level with each other (Table 3). Comparison of the two sequences with the corresponding sequences of Japanese RBSDV and Italian MRDV revealed high identity at the nucleotide and amino acid level (Table 3). Their sizes and features were very similar to those of corresponding segments of Japanese RBSDV and Italian MRDV (Table 2).

Table 2 Sizes and its predicted open reading frames (ORFs) of segments S8 of isolates from Zhejiang, Hebei, and Japanese RBSDV and S7 of Italian MRDV

	Total size nts	GC content %	5'-UTR	ORF1		3'-UTR nts
				nts	kDa	
RBSDV-Zj S8	1936	34.8	24	1776	68.1	136
RBSDV-Heb S8	1936	35.0	24	1776	68.1	136
RBSDV-Jap S8	1927	34.6	24	1776	68.1	127
MRDV S7	1936	34.7	24	1776	68.1	136

Multiple sequence alignments (Fig. 2a) showed that most site mutations were base transitions (A/G or C/T substitution) (Fig. 2a). It was thought that viral RdRp lack proofreading ability (Li et al., 1998). It was noticeable that a 9 bp deletion was only found in 3'-UTR of Japanese RBSDV S8 and immediately adjacent to the termination codon, while the 9 bp insertion found at the position nt 1807-1815 in two Chinese isolates was just only homology to Italian MRDV (Fig. 2a).

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

S8	RBSDV-Hebei	Japanese	MRDV-S7
RBSDV-Zj	94.0(96.5)	94.9(92.9)	85.1(91.7)
RBSDV-Heb		94.5(92.5)	87.6(91.9)
RBSDV-Jap			85.1(87.8)

The amino acid sequence GNKFVGKS (aa356-362) (Fig. 2b.), regarded as purine NTP-binding motif GXXXXGKS, in S8s of both Chinese isolates were identical to that found in Japanese RBSDV and Italian MRDV, and were very similar to that found in all other known Fijiviruses. Moreover, Japanese RBSDV S8 encodes one of the core capsids of the virus particle (Isogai et al., 1995; 1998), but functional analysis is required to determine whether it can bind NTP in virus multiplication. Homology at level of amino acids was lower than that at level of nucleotides between S8s of Japanese RBSDV and two Chinese isolates because of some mutations, while homology at level of amino acids was higher than that at level of nucleotide between S7 of Italian MRDV and S8s of two Chinese isolates (Fig. 2b). which was not found in other known segments of the virus to date. At position aa100 – 163 were many amino acid mutations that occurred in S8 of Japanese RBSDV, but the regions shared high homology at amino acid sequences between Zhejiang RBSDV, Hebei RBSDV and Italian MRDV (Fig. 2b). The phylogenetic tree constructed from homologous proteins of OSDV (oat sterile dwarf virus), NLRV (nilaparvata lugens reovirus), MRDV, and three RBSDV isolates, also showed that the MRDV was clustered with three RBSDV isolates (data not shown). All those results clearly suggested that RBSDV and MRDV, although known as separate viruses, should

be considered as geographical races of the same virus and supported the previous proposal that the two viruses should be considered as geographical variants (Boccardo and Milne, 1984).

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