Rice dwarf disease in North Vietnam in 2009 is caused by southern rice black-streaked dwarf virus (SRBSDV)

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Abstract

During the summer of 2009, a serious rice dwarf disease occurred in several provinces in North Vietnam. The appearance of white to black waxy galls along the veins of the leaves and culms on the diseased plants suggested the infection of a fijivirus like rice black-streaked dwarf virus (RBSDV) or a related virus, namely southern rice black streaked dwarf virus (SRBSDV) = rice blacked streaked virus 2 (RBSDV2), which was recently identified in South China in 2008. The universal primers specific for these viruses were designed based on the conserved regions of the S10 segment. RT-PCR tests on typical samples collected from different provinces gave specific products using these primers. Four RT-PCR products amplified from diseased plants that are representative for the four North Vietnam provinces, Nghe An, Thanh Hoa, Nam Dinh, and Son La, were directly sequenced. Blast searches and sequence analyses revealed that they showed all of the isolates of SRBSDV.

Keywords: North Vietnam, S10, sequencing, southern rice black-streaked dwarf virus.

Introduction

Reoviridae is the family of RNA viruses that infect vertebrates, arthropods, fungi and plants. Particles of reoviruses have icosahedral symmetry but may appear spherical in shape. All reoviruses have a fragmented genome encompassing 10, 11 or 12 linear double stranded (ds) RNA molecules, depending on the genus (Mertens *et al*., 2005). The plant reoviruses are classified in one of three genera, *Oryzavirus*, *Phytoreovirus*, and *Fijivirus*, on the basis of the number of genome segments, capsid structure and sequence variation, particularly in the more conserved genome segments (Mertens *et al*., 2005).

All known fijiviruses, Fiji disease virus (FDV), maize rough dwarf virus (MRDV), mal de Rio Cuarto virus (MRCV), pangola stunt virus (PaSV), rice black-streaked dwarf virus (RBSDV), oat sterile dwarf virus (OSDV), garlic dwarf virus (GDV) and Nilaparvata lugens reovirus (NLRV), have double-shelled, 65-70 nm in diameter virions encapsidating 10 genomic dsRNA molecules assigned S1-S10

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(Milne *et al*., 2005).

Recently, on the bases of sequence analyses of the S9 and S10 segments and vector transmission, a putatively novel fijivirus, namely southern rice black-streaked dwarf virus (SRBSDV) or RBSDV2, has been identified in rice in South China (Zhang *et al*., 2008; Zhou *et al*., 2008). In this paper, we use the name SRBSDV to avoid confusion as the two names are synonymous (Zhang *et al*., 2008) .

In the summer of 2009, a serious viral rice disease occurred in many provinces of North Vietnam, expanding a total of 5506 ha. Of this area, approximately 3510 ha were totally lost. Infected plants showed severe stunting, darkening and deformation of leaves, waxy galling along veins of culms, and poor heading. Some of these symptoms were similar to those caused by rice ragged stunt virus (RRSV) in rice in South Vietnam.

This paper reports the identification of the causal agents of rice dwarf disease in North Vietnam based on symptomatic evaluation, electron microscope observation, and sequence analyses.

Materials and methods

Samples

Infected rice plants showing typical symptoms were collected in outbreak regions of Nghe An, Nam Dinh, Thanh Hoa, Hai Phong and Son La in 2009. Samples were used immediately after collection and dried under silica gel for long term storage.

RT-PCR and Sequencing

Two primers, RB-S10-F2 (5'-TCCATAATGGCTGACATAAGAC3-') and RB-S10-R2 (5'-CATTT-GAGCAGGAACTTCACG-3'), were selected in the conserved regions of 17 S10 sequences available in GenBank of three fijiviruses, RBSDV, SRBSDV and MRDV. These primers can generate products with an expected size of approximately 0.6 kb.

Total RNAs were extracted from the samples using an RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. RT-PCRs were directly performed from RNA extracts using a Super-script III One-Step RT-PCR System (Invitrogen). The reactions were performed on the initiation step at 45 ˚C for 30 min and at 94 ˚C for 2min, and then subjected to 35 cycles of denaturation at 94 ˚C for 30 sec, annealing at 52 ˚C for 40 sec and extension at 68 ˚C for 1min, finishing with 5 min at 68 ˚C.

RT-PCR products were purified from agarose gel using an AccuPrep Gel Purification Kit (Bioneer), estimated for the DNA concentration using agarose gel electrophoresis. DNAs were directly sequenced in both directions with RT-PCR primers using a BigDye Terminator v3.1 Cycle Sequencing Kit and sent to the Institute of Biotechnology at Ha Noi for reading.

Sequence analyses

After being assembled using the SeqMan program (DNASTAR,Madison,WI), the sequences were initially compared to known viral sequences using the BLAST program available at the National Centre for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were aligned with the ClustalX program (Thompson *et al*., 1997). Phylogenetic trees were constructed from the

ClustalX-aligned sequences using MEGA version 4 (Tamura *et al*., 2007) using a neighbor-joining (NJ) method with the Kimura 2-Parameter model for estimating the distances or the Maximum Parsimony (MP) method. All phylogenetic analyses were bootstrapped with 1000 replicates.

Electronic microscope

The presence of virions or inclusion bodies in ultrathin sections from stems of infected plants was examined using a JEOL 1010 electron microscope at the Institute of Hygiene and Epidemiology (Hanoi).

Results

Symptoms

Observation of typically diseased plants (Fig. 1) collected in the Northern provinces, Nghe An, Nam Dinh, Thanh Hoa, Hai Phong, Son La, Ha Noi, showed that the disease exhibited some symptoms similar to those of ragged stunt disease caused by RRSV, a phytoreovirus, in rice in South Vietnam. The confusable symptoms were dwarf plants, dark green and twisted leaves, and white waxy galls along the veins of the leaves. However, the infected plants from North Vietnam also expressed some distinct symptoms. Firstly, the twisted (Fig. 1 b and c) or recovering leaves did not show the yellow ragged appearance in one edge of leaves which are always observed in typical RRSV plants. Secondly, there were white to black waxy galls along the veins of the culms (Fig. 1 e and f).

These two symptoms, particularly the second one, suggested that the rice dwarf disease in North Vietnam was not caused by RRSV, but probably by a fijivirus like RBSDV, SRBSDV or MRDV.

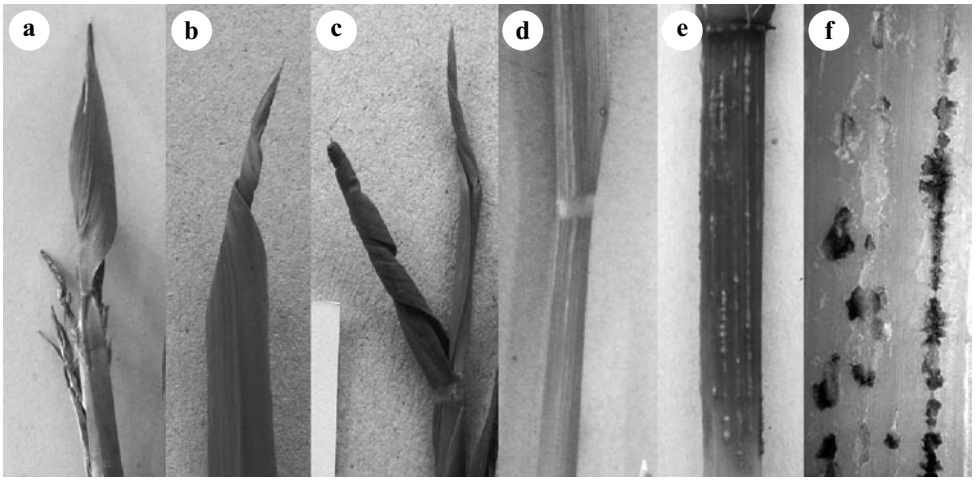

Fig 1. Appearances of diseased rice plants in North Vietnam in 2009. Twisted leaves (a, b, c). Poor heading with browning seeds (a). White waxy galls along major veins of leaf blade and sheath (d), and culm (e). Waxy galls turn black (f, closed up with a stereo microscope).

Electron microscope

Electron micrographs from ultrathin sections of typically diseased rice plants revealed the presence of numerous crystalline arrays of aggregated reovirus-like particles in the phloem cells. The individual particles were 60-70 nm in diameter, typical for a reovirus (Fig. 2).

RT-PCR and sequencing

From suggestions based on symptom and electron micrograph observations, two universal primers specific for RBSDV, SRBSDV, and MRDV were designed from highly conserved regions of 17 S10 sequences available in public databases. S10 was chosen for primer selection as a much larger number of sequences of this segment were available compared to other segments among fijiviruses.

RT-PCR tests using the universal primers on typical samples collected from different provinces

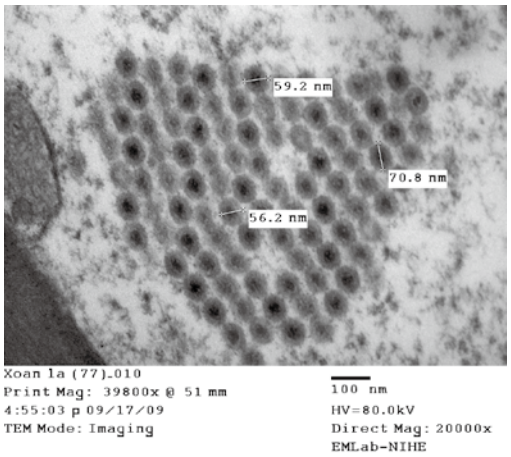

Fig 2. Electron micrograph from ultrathin section of a diseased rice plant collected from Nghe An province.

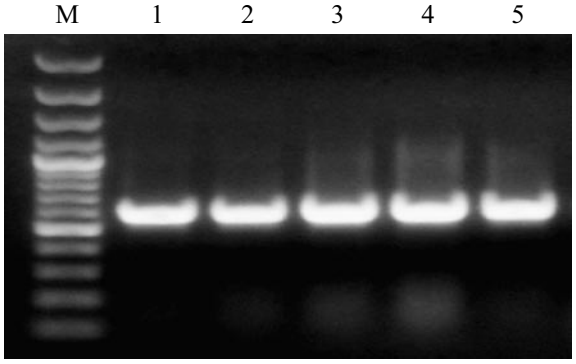

Fig 3. RT-PCR with RB-S10-F2 and RB-S10-R2 primers. Lane M is 100 bp DNA ladder (GeneRuler, Fermantas). Lanes 1-5 indicate sample locations: Nghe An (1 and 2), Son La (3), Thanh Hoa (4) and Nam Dinh (5). Size of RT-PCR products is 0.6 kb.

having outbreaks of dwarf disease in North Vietnam generated products with expected sizes of 0.6 kb (Fig. 3) but were negative for primers specific to RRSV (data not shown).

To identify the causative virus, four RT-PCR products that are representative of the four provinces, Nghe An, Thanh Hoa, Nam Dinh and Son La, were directly sequenced on both strands using RT-PCR primers. After removing the sequences with poor quality at two ends which normally occur in direct sequencing, a 570 bp sequence of each product was obtained. All four sequences were deposited on GenBank under accession numbers GU017739 (Thanh Hoa), GU017740 (Son La), GU017741 (Nam Dinh) and GU017742 (Nghe An).

Sequence analyses

The nucleotide sequences of the four isolates were initially searched for corresponding viral sequences in the database using the BLAST program. The Blast searches evidenced that all four isolates from Vietnam were most closely related to two SRBSDV isolates originating from South China (Hainan island, EU523360 and Guangdong province, EU784840).

To assess the level of genetic diversity, we compared the nucleotide and putative amino acid sequences of the four isolates from Vietnam with those of the two above database available SRBSDV isolates from China, and a representative isolate of RBSDV (AF459813), which is most closely related to SRBSDV. Analyses (Table 1) showed the four Vietnamese samples all shared very high nucleotide sequence identities with the two Chinese SRBSDV isolates (98.4 – 99.8 %). Interestingly, most of the nucleotide substitutions over the 570 bp fragment were non-synonymous. Consequently, these substitutions did not show any differences in the amino acid sequence among Vietnamese isolates compared with the Guangdong isolate, but only one compared with the Hainan isolate. However, when compared with the representative of RBSDV, all Vietnamese isolates showed much higher sequence variations (75.6 – 76.1 % nucleotide identities and 92.1 % amino acid similarity).

Table 1. Sequence comparison* of four Vietnamese isolates and database partners

GenBank isolate	Vietnamese isolate	Nucleotide		Amino acid	
		Number of difference	Identity (%)	Number of difference	Similarity (%)
SRBSDV- EU523360 (Hainan, China)	Thanh Hoa	9	98.4	1	99.5
	Son La	3	99.5	1	99.5
	Nam Dinh	8	98.6	1	99.5
	Nghe An	2	99.6	1	99.5
SRBSDV**-EU784840 (Guangdong, China)	Thanh Hoa	7	98.8	0	100.0
	Son La	2	99.6	0	100.0
	Nam Dinh	7	98.8	0	100.0
	Nghe An	1	99.8	0	100.0
RBSDV-AF459813 (China)	Thanh Hoa	139	75.6	15	92.1
	Son La	138	75.8	15	92.1
	Nam Dinh	136	76.1	15	92.1
	Nghe An	137	76.0	15	92.1

* Sequence differences were calculated over the 570 bp fragment after aligned with the ClustalX program

** Known under name RBSDV2 (Zhang *et al*., 2008)

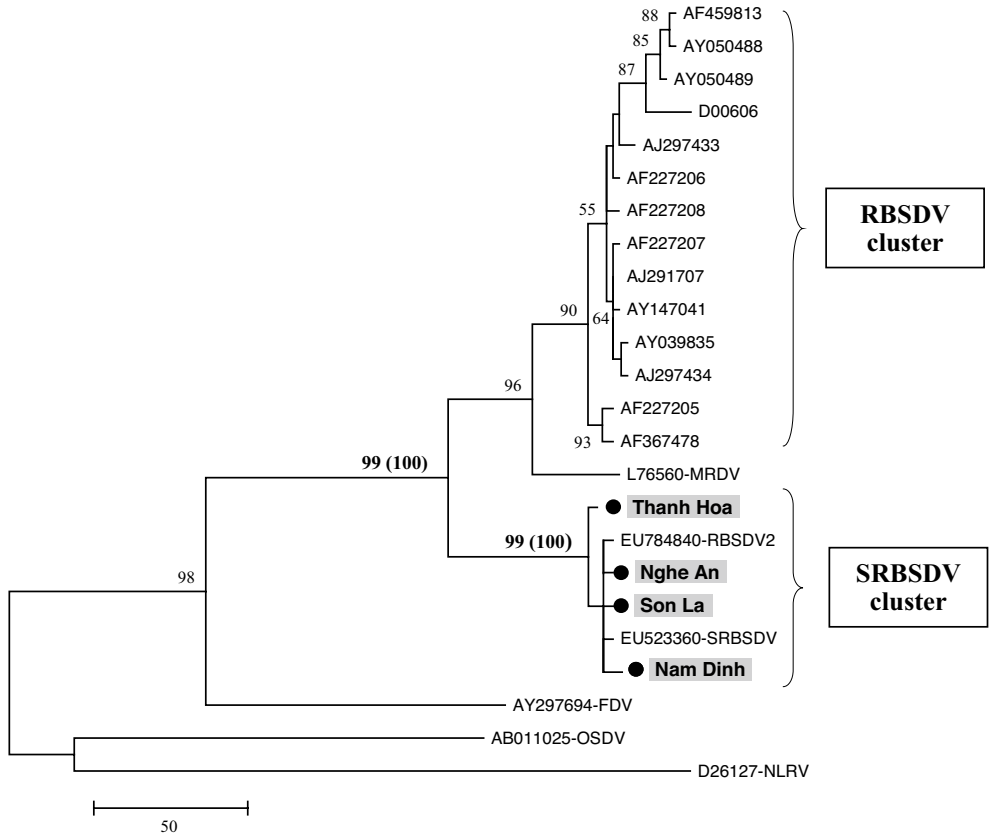

Fig 4. A maximum parsimony tree based on the 570 bp fragment of the S10 segment showing relationship of Vietnamese isolates with other fijiviruses. The bar presents the number of nucleotide substitutions. The four Vietnamese isolates from this study are dotted, underlined and in bold. Only bootstrap values greater than 50% (1000 replicates) are shown. Bootstrap values in parentheses are from neighbor-joining analysis.

Phylogenetic analysis

To determine the relationship of the four Vietnamese isolates with other fijiviruses, a phylogenetic tree was constructed (Fig. 4) based on the 570 bp fragments. As shown on the tree, all four Vietnamese and two Chinese SRBSDV isolates formed a distinct species cluster which was well supported through bootstrap analyses (99 % in MP and 100% in NJ).

Discussion

There were considerable debates among the plant pathologists of Vietnam about the causal agent of the rice dwarf disease in North Vietnam. Mostly based on the symptoms (dwarf plants and twisted leaves), the disease was initially though to be caused by the RRSV that has been infecting rice in South Vietnam since 2006 and has the natural vector of brown plant hoppers (PBH, *Nilaparavata lugens*).

Several lines of evidence indicated that the disease in North Vietnam was caused by a putatively

novel fijivirus, SRBSDV. Firstly, the infected plants exhibited several characteristic symptoms similar to those of rice black streaked dwarf disease caused by RBSDV (Shikata, 1974) or SRBSDV (Zhou *et al*., 2008) such as pronounced stunting, darkening and twisting of leaves, and particularly, the presence of white to black waxy galls (epinations) along the major veins of the leaves and culms.

Secondly, while the electron micrographs evidenced the presence of reovirus-like molecules in the infected plants, the sequence and phylogenetic analyses based on amplified fragments of the S10 segment demonstrated clearly that the viruses infecting rice in North Vietnam were different isolates of SRBSDV. Currently, the complete genome of SRBSDV has not yet been reported except for the S9 and S10 segments (Zhang *et al*., 2008; Zhou *et al*., 2008). Like other fijiviruses, S10 encodes the outer capsid proteins of virions, which are known to confer the vector specificity of many plant viruses including rice dwarf virus (RDV, a reovirus) (Hogenhout *et al*., 2008). In our study, the amino acid sequence of all Vietnamese isolates was 100 % similar with that of the Guangdong isolate or 95.5 % similar (different by only one residue) with that of the Hainan isolate. These very high levels of sequence conservation suggest that the Vietnamese and Chinese isolates all share a common vector specificity.

Finally, it is known that RBSDV is naturally transmitted through small brown plant hoppers (SBPH, *Laodelphax striatellus*) (Shikata, 1974). However, SRBSDV transmission experiments with three common rice hopper species, PBH, SBPH and white back plant hoppers (WBPH, *Sogatella furcifera*), conducted by Zhou *et al*. (2008) demonstrated that SRBSDV could be transmitted from rice to rice by both SBPH and WBPH and from rice to corn by only WBPH. They also confirmed that PBH and SBPH could not transmit SRBSDV and RBSDV, respectively. In Vietnam, Dinh *et al*. (2008) reported significant changes among rice plant hopper populations in recent years. The Northern PBH population dropped from approximately 70 % in 1981 to nearly 30 % in 2007, whereas, the figures of WBPH are 35 % and 70 %, respectively. In addition, SBPH has reappeared at a high rate in May and June of 2009 in many Northern provinces such as Hung Yen, Hai Duong, Thai Binh and Bac Ninh. The occurrence of the outbreaks of SRBSDV dwarf disease in North Vietnam, therefore, could be the consequence of the prevalence of WBPH and SBPH in this area.

In this experiment, the origin of SRBSDV in Vietnam is unknown. While RBSDV, which is closely related to SRBSDV and induces a similar disease, was firs recognized in 1952 in Japan (Shikata, 1974) and distributes only in the northern hemisphere such as Japan (Isogai *et al*., 2001), Korea (Lee *et al*., 2005) and the Northern provinces of China (Bai *et al*., 2002), SRBSDV was identified only recently in the south part of China (Zhou *et al*., 2008; Zhang *et al*., 2008). Otuka (2008) suggested that the main migration sources for immigrants of WBPH in the southern provinces of China like Guangdong and Guangxi were estimated to be in the Red River delta in North Vietnam. The occurrence of the disease outbreaks at almost the same time in geologically closed areas, the high level of sequence conservation, and the interchangeable vector populations imply that the SRBSDV populations in South China and North Vietnam may represent a unique population.

While in recent reports, we have provided strong evidence that Vietnam and South China is one of origins of diversity of many plant viruses (Ha *et al*., 2008a; Ha *et al*., 2008b), we still do not know if SRBSDV has evolved from a distinct lineage for a long period of time or newly separated from a common ancestor with RBSDV. Complete genome and more sequenced isolates are needed to shed light on the origin and evolution of SRBSDV.

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