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Present status of rice tungro disease in West Bengal: occurrence and characterization of viruses

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ABSTRACT

Rice tungro disease is one of the most severe virus diseases of rice (Oryza sativa L.) and significant threat to rice production in Southeast Asia. In India, it can cause an estimated loss of 2% at the national level. Rice tungro is a composite disease caused by joint infection of two unrelated viruses, rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV), appears in the form of severe yellowing and stunting in susceptible rice cultivars. Exhaustive surveys have been made to identify the locations of tungro incidence in West Bengal during 2007-2008. The samples showing tungro like symptoms were collected and checked for the presence of tungro through polymerase chain reaction (PCR) using primers specific for the tungro viruses. But all the collected samples were virus free except that collected from Rice Research Station, Chinsura, Hoogly, West Bengal. Previous studies reported all the RTBV isolates from the India as the "South Asian type". Recently considerable variations at the genomic level have been detected within the above group. In this present experiment, attempts were taken to sequence a part of the genome of both RTBV and RTSV from West Bengal to reveal any changes that might have been occurred in these segments. These sequences have been compared with the previous reports available from NCBI. Similarity analysis of the sequenced genome of RTBV exhibited about 95% similarity with the previously reported isolate from West Bengal. Where as sequenced segment of RTSV exhibited near about 96% similarity.

Key words: Rice tungro; detection; PCR, RTBV, RTSN; sequence.

Rice tungro disease (RTD) is one of the most severe virus diseases of rice (*Oryza sativa* L.) and significant threat to rice production in Southeast Asia, where outbreaks of the disease are sporadic and, therefore, difficult to control (Ou, 1985). Rice tungro is a composite disease caused by a bacilliform DNA pararetro virus (rice tungro bacilliform virus, RTBV) and a spherical RNA plant picorna virus (rice tungro spherical virus, RTSV) leading to yellow-orange discoloration and stunting of the infected plants (Muralidharan *et al*., 2003). RTBV has a circular double-stranded DNA molecule of about 8 kbp, containing two site-specific discontinuities, one on each strand (Hay *et al*., 1991). The genome has four large open reading frames (ORFs), potentially capable of encoding proteins of 24 (P1), 12 (P2), 194 (P3) and 46 (P4) kDa (Hay *et al.*, 1991; Laco *et al*., 1994). P3 (146kDa) is a large polyprotein and contains four functional domains: the viral movement protein, coat protein, aspertate protease, reverse transcriptase and ribonuclease H (Hay *et al*., 1991 and Laco *et al*., 1994). On the other hand, RTSV has a polyadenylated RNA genome which is more than 12kb in length. RTSV has one large open reading frame (ORF), encoding a polyprotein that is thought to be cleaved by virus- and/or cell-encoded proteases. The genome also contains two other small ORFs, which are thought to be expressed from sub genomic mRNAs (Shen *et. al.*, 1993). The RTSV capsid comprises of three coat proteins, CP1, CP2 ad CP3, with predicted molecular masses of 22.5, 22.0 and 33 kDa, respectively.

In nature the tungro virus complex are transmitted exclusively by green leafhopper (*Nephotettix virescens* Dist.) and by other leafhopper species (Hibino *et al*, 1978) in a non-persistent manner. A major outbreak of this disease occurred in Tamil Nadu in 1984 and 1992. Whereas, an epidemic outbreak of tungro during 2001 in three districts of West Bengal caused an un-milled rice production loss of 0.5 million tones valued at Rs. 2911 millions at current prices. So far reported, the sporadic appearance of the disease cause significant grain yield losses (Muralidharan *et al*., 2003). Considering the overall importance of RTD in India, it is essential to check the status of RTD in the fields as well as at the genome level of the viruses. It will help us to monitor the disease properly. the present study is undertaken to check the availability of the disease in the field of South Bengal and simultaneously polymerase chain reaction techniques for virus detection has been used to detect the disease besides visual detection.

MATERIALS AND METHODS

Collection of samples

The surveys were made during kharif season, 2007 and 2008 in the three rice growing districts of West Bengal *viz*. Burdwan, Hooghly and Nadia were targeted. The samples were collected from the farmers' field and from Rice Research Station,(RRS) Chinsura, Hooghly. Total DNA was extracted from rice leaves by crushing the 4-5 cm length leaf in a prechilled mortar pastel in 600 µl extraction buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0 and 1% SDS). Extracted DNA was dissolved in 20 µl of water and used in PCR. Total RNA from the leaves was extracted by using plant RNA extraction kit

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(Qiagen; Catalogue No. 74903). Total RNA extracted from leaves was used for synthesizing the cDNA and it was done by using the cDNA synthesis kit (Fermentus; Catalogue No. K621). Oligo dT was used as the primer during CDNA synthesis.

The primers for RTBV and RTSV were designed from the previously available sequences of both the viruses. The sequences of the West Bengal isolate of both the viruses were collected from NCBI database. For RTBV forward 5'AGATGAATCAGAAGAAGGATGG 3' and reverse 5'AGAATCCCCTGAGGAATTCCATATCC 3' primers were designed to cover a1.1 kb region between 5444 to 6553 nucleotides (i.e. covering the parts of RT/RNAse H and ORF IV) of the RTBV genome. Similarly for RTSV forward 5' TTGGAAGAAGCCTATCGTGTTCGC 3' and a reverse 5' TCTGGACCCACTTGCTTCTTCGAC 3' primers were designed to amplify an 848 bp region of RTSV coat protein III. For both the viruses polymerase chain reaction PCR were same. The PCR amplifications were performed in a Mastercycler (Eppendorf AG, Germany), and carried out in a final volume of 25 µl in a reaction mixture containing 1µl (10–20 ng) template DNA, 0.5 µM of each forward and reverse primer, 0.4 mM dNTPs, 2.5 µl of 6X PCR buffer [containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 0.1 mM gelatin, 0.45% NP40, and 0.45% Tween 20], 2 mM $MgCl_2$, 1.0 units of DNA *Taq* polymerase (Fermentus, India). The PCR program consisted of 1 cycle of 5 min at 94 ˚C; 30 cycles of 30s at 94 ˚C, 30s at 55 ˚C, and 1 min at 72 ˚C; a final extension of 7 min at 72 ˚C and ultimately kept at 4 ˚C. The amplified PCR products were subjected to electrophoresis on 1% agarose gels at 120 V for 1 h. The electrophoresis results were visualized through ethidium bromide staining and photographed.

The PCR products of were used directly for cloning by using PCR cloning kit (CloneJET, Fermentus; Catalogue No. k1231). At first the PCR products were ligated to the pJET1.2/blunt cloning vector and then transformed into *Escherichia coli* strain DBH10 with the help of bacterial transformation kit (Fermentus, TransformAid, catalogue No. k2710). The cloned fragments ware sequenced form GeNei, Bangalore, India. The derived DNA sequences have been deposited in the NCBI Sequence Database and have been assigned the accession numbers: EU684541 (RTBV) and EU684542 (RTSV). The sequences were compared with the published sequences in the database of NCBI using BLAST programme (Blastn: www.ncbi.nlm.nih.gov/blast/Blast.cgi).

RESULTS AND DISCUSSION

Occurrence of rice tungro and its detection through PCR

From our field level it was observed that tungro is not available in these three districts (Table 1). Presence of RTBV and RTSV was detected only in the rice plants collected from Rice Research Station, Chinsura and a field of Bhatar, Burdwan. Although the samples from the other places of these districts showing tungro like symptoms but neither RTBV nor RTSV were detected. Polymerase chain reaction of the samples collected from RRS, Chinsura and Bhatar, Burdwan, had yielded characteristic bands of 1.1 kb and 848 bp for RTBV and RTSV respectively (Fig. 1). No such amplifications were found in other samples of the three districts. Our survey. The rice tungro disease is very scarce and also it is not present in considerable amount in the areas like Bhatar, Chakdah etc., though these areas were reported as tungro prone areas. The reason behind such a non availability of tungro disease may be that the viral inoculum is not present at the field level sufficiently. So, the disease is not spreading by the vector, green leaf hopper at the field level.

Sequence analysis of RTBV genome segment

The length of the sequenced fragment of RTBV represented by EU684541 was 1111bp. The sequence was compared with previously reported two RTBV isolates from India i.e., WB (West Bengal) and AP (Andhra Pradesh) isolates. The amplified segment showed 95% similarity with WB isolate (EMBL accession No. AJ314596) at the nucleotide position 5444 – 6554, encoding the terminal part of ORF III and initial part of the ORF IV along with small intergenic region between ORF III and ORF IV. On the other hand only 91% similarity was observed with AP isolate (EMBL accession No. AJ292232) from 5442 – 6522 nucleotide (nt) position encoding the same region. The sequence alignment (Fig. 2) showed that there was approximately 41 nt substitution between recent and old WB isolates, but there was no addition or deletion of nt in the sequenced region. Where as the sequenced part exhibited marked difference with AP isolate having 48 nt substitution along with nt addition and/or deletion. After nt position 377 in our sequence, a stretch of 30 nts was unique in both our present and previous WB isolate. But in AP isolate those 30 nts were deleted or missing. These resulted in an addition of 10 amino acid residues close to the C-terminus of ORF III in WB isolate. Previously all the RTBV isolates from within the Indian subcontinent were designated as "South Asian" type (Fan *et al*., 1996). But Nath *et al.* (2002) reported variations among the Indian isolates (WB and AP isolates) due to presence of

hypervariable region in ORF III. The present findings points out marked similarity with previously reported WB isolate and important difference with AP isolate.

Sequence Analysis of RTSV genome segment

The sequenced fragment of RTSV having the length of 848 bp was represented by EU684542. Comparing the sequence with previously reported West Bengal (WB, EMBL accession No. AM234049) and Orissa (Ori, EMBL accession No. AM234048) isolate of RTSV (Fig. 3), no marked difference was observed. The sequence exhibited 96% similarity with both the isolate at the neucleotide position 3675 – 4522, a partial gene for polyprotein. From the sequence alignment 18 nt substitution between recent and old WB isolate was noticed, where as only 21 nt substitution in case of comparison with Ori isolate. No addition or deletion of nucleotide has been found in our derived sequence. Thus the sequenced fragment of RTSV of prevalent WB isolate exhibits no marked difference with the previously reported isolates of West Bengal and Orissa. Previously the nucleotide sequence of the full length RNA of a Phillipine isolate of RTSV (Shen *et al*., 1993) and the partial nucleotide sequence of isolates from Malayasia, Thiland, India and Bangladesh (Zhang *et al*., 1993) have been determined, revealed some variation in these sequences. Also the nucleotide sequence of one (CP3) of the three coat protein (CP) genes, the partial sequence of CP1 gene and the 3' terminal part of the genome of and India RTSV isolate have been reported (Druka *et al*., 1996; Zhang *et al*., 1997). Recently the full genome of RTSV from India has been done (Verma and Dasgupta, 2007), which also indicated the conserved nature of the RTSV genome. Our finding also corroborates with these reports.

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Table 1: Occurrence of rice tungro disease

Collection Place	Presence of RTBV/RTSV
Bhatar	+/+
Memari	-/-
Galshi	-/-
Shaktigarh	-/-
Rasulpur	-/-
Panduah	-/-
Magra	-/-
Adisaptagram	-/-
Singur	-/-
Chinsura	+/+
Chakdah	-/-
Ghoragachha	-/-
Ranaghat	-/-
GAngnapur	-/-

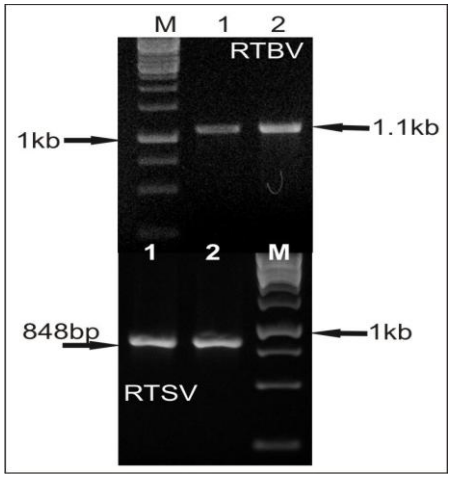

Fig. 1. PCR amplification for RTBV and RTSV genome

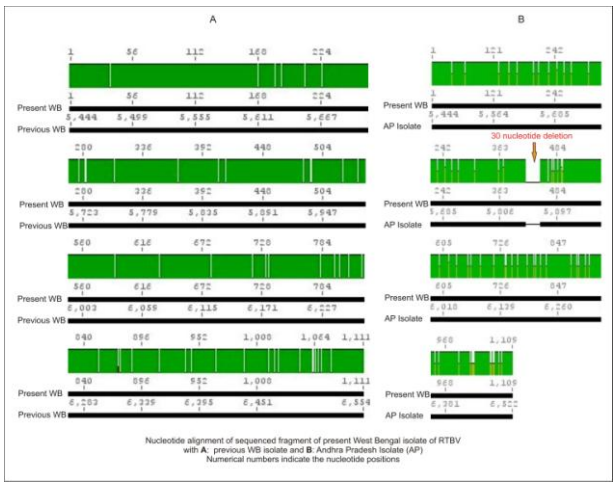

Fig. 2. Nucleotide alignment of sequenced fragment of RTBV

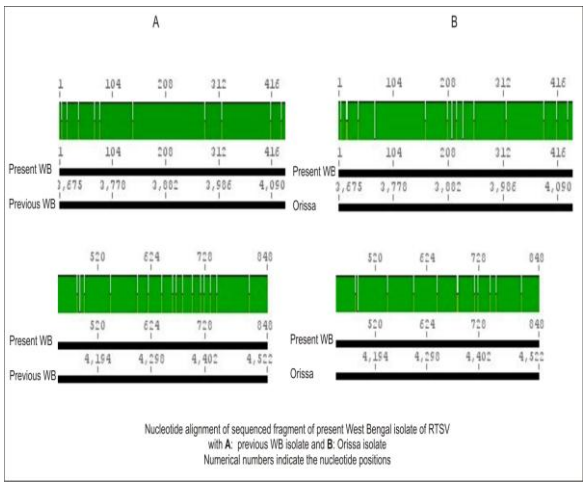

Fig. 3. Nucleotide alignment of sequenced fragment of