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SHORT NOTES

Sequence analysis of RNA3 of *Maize stripe virus* associated with stripe disease of sorghum (*Sorghum bicolor*) in India

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Summary. *Maize stripe virus* (MSpV), one of the distinct species of the genus *Tenuivirus,* has been associated with stripe disease of sorghum in India. In this study, we report the complete sequence analysis of ambisense RNA3 of four MSpV isolates associated with this disease, to confirm its correct identity. The RNA3 of four MSpV-Sorg isolates is 2357 nucleotides in length with two ORFs, one in virion sense (594 nucleotides, non-structural protein 3, NS3) and the other in complementary sense (951 nucleotides, coat protein, CP). The intergenic region between these two ORFs is 653 nucleotides in length, which is rich in U and A residues. The deduced molecular weights of NS3 and CP are ≈22 and ≈34 kDa, respectively. RNA3 has ≈82% sequence identity at nucleotide level with RNA3 of MSpV infecting maize in Florida, USA and Reunion. NS3 and CP ORFs shared ≈94% and ≈95% identities at amino acid levels, respectively with MSpV isolates of maize from Florida and Reunion. The internal non-coding region between two ORFs has 67–68% identity at nucleotide level with the reported MSpV isolates from Florida and Reunion. The sequence identity was more than ≈98% among the four isolates of MSpV-Sorg. Compared to maize-infecting MSpV isolates in USA and Reunion, the sorghum-infecting MSpV isolates in India had more amino acid substitutions in both NS3 and CP. This is the first report of complete sequence analysis of MSpV RNA3 from Asia.

Key words: Sorghum stripe disease, MSpV-Sorg, India, RNA3 and Genetic relationships.

Introduction

Maize stripe virus (MSpV) belongs to the unusual plant virus genus *Tenuivirus*. These viruses have characteristic thin (3–6 nm in width) highly flexuous thread-like particles of undefined lengths. MSpV infecting maize has been reported from tropical regions of Asia, Africa, Australia and Americas (Ramirez *et* al, 1994; Falk and Tsai, 1998; Ramirez, 2008). The virus is transmitted by the plant hopper *Peregrinus maidis* in a persistent manner. Most of the MSpV isolates from maize were found to be serologically related (Huiet *et al.,* 1992; Falk and Tsai, 1998; Mahmoud *et al.,* 2007). The MSpV genome consists of five segments of either negative or ambisense ssRNA with a total estimated size of ≈ 18 kb (RNA1- 8.8 kb, RNA2- 3.4 kb, RNA3- 2.3 kb, RNA4- 2.2 kb and RNA5- 1.3 kb) (Ramirez, 2008). The MSpV RNAs have 5´ and 3´ terminal base complementarity, a characteristic feature of all the tenuiviruses (Ramirez, 2008). The RNA3 of all the tenuiviruses including MSpV have ambisense gene organization. MSpV RNA3 contains coat protein (CP) and non-structural protein 3 (NS3) ORFs in an ambisense gene organization, both ORFs separated by large intergenic regions (IR). The reported Mr of structural protein of virus particles ranges from 32-35 kDa. So far, the complete sequence of the four smaller ssRNAs (RNAs 2,3,4 and 5) of MSpV–Florida, USA (MSpV-Flo) and complete sequence of RNA3 and partial sequence of RNA1 of MSpV-Reunion (MSpV-RE) isolate infecting maize have been reported (Huiet *et al.,* 1992; Huiet *et al,* 1993; Falk and Tsai, 1998; Mahmoud *et al.,* 2007).

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Sorghum, the fifth most important world cereal crop, is grown in several tropical and sub-tropical countries, mostly cultivated for food in semi-arid zones and for animal feed in developed countries. India is the third largest country cultivating sorghum for food (Food and Agricultural Organization, 2010). Nearly 20 viruses have been reported to naturally infect sorghum worldwide (Frederiksen and Odvody, 2010; Srinivas *et al.*, 2010). *Maize mosaic virus, Sorghum mosaic virus, Sugarcane mosaic virus, Sugarcane streak mosaic virus* and an isolate of *Maize stripe virus* have been reported to naturally infect sorghum in India (Naidu *et al.*, 1989; Peterschmitt *et al.*, 1991; Srinivas *et al.*, 2010). In Andhra Pradesh state of India, a virus isolate associated with stripe disease of sorghum was identified as MSpV-Sorg based on typical symptoms, particle morphology, transmission by delphacid vector (*Peregrinus maidis*), antigenic relationships and genome nature, composition and size (Peterschmitt *et al.*, 1991). Subsequently, similar disease was also reported based on symptomatology, vector transmission and serology from Karnataka and Maharashtra states in India (Narayana and Muniyappa, 1995; Garud *et al.*, 2000). The affected plants were dwarfed and had poor or no panicle formation. MSpV-Sorghum isolate in India failed to infect maize under experimental inoculations using *P. maidis* (Peterschmitt *et al.*, 1991). In earlier studies, the isolate infecting sorghum in India was designated as MSpV-Sorg and the same designation is used in the present paper. Although serological relationships between various MSpV isolates were established from different countries, reports on genetic relationships among these isolates infecting maize / sorghum around the world are limited. Hence, RNA3 of four MSpV-Sorg isolates from Andhra Pradesh, India have been completely sequenced to understand its genetic relationships with isolates of the same virus from other geographic regions and also with other tenuiviruses.

Materials and methods

Collection and screening of stripe disease affected sorghum samples

Sorghum bicolor (local cultivar) leaf samples exhibiting either chlorotic and/or whitish interveinal streaks and/or bands were collected during the rainy season of 2009–2011 from Cuddapah (Kadapa), Kurnool and Chittoor districts of Andhra Pradesh state, India. The samples were initially screened with polyclonal antisera of MSpV-Flo and –RE isolates by direct antigen coating- enzyme-linked immunosorbant assay (DAC-ELISA) (Clark and Bar-Joseph, 1984). The ELISA positive samples were also confirmed by western blotting using the same antisera (Koenig and Burgermeister, 1986).

RT-PCR, cloning and sequence analysis of RNA3

The leaf samples from sorghum plants, already confirmed for MSpV by DAC-ELISA and western blot assays, were used for the isolation of total RNA using Tri reagent (Sigma-Aldrich) according to the manufacturer's protocol. Total RNA isolated from healthy sorghum leaf samples served as healthy controls. The extracted RNA was suspended in 20 μL of RNase-free water and stored at -20°C. The primers for the amplification of MSpV-RNA3 were designed based on the RNA3 sequences of MSpV-Flo and -RE isolates deposited in the GenBank (A/C numbers M57426 and AJ969410) using the Primer3 programme (http://frodo.wi.mit.edu/primer3/). The designed primers (1st set of primers) MSpV 3-F: 5′-TATATATTACTTCTGTCCATCGAAC-3′ and MSpV 3-R: 5′-ACACAAAGTCTGGGTAATCGTT-3′ (Table 1) were expected to amplify ≈2.2 kb fragment from 3′ end of MSpV-RNA3. Eight μL of the isolated total RNA (from MSpV associated samples collected from Kadapa district-MSpV-Sorg-Kad1) was denatured at 55–60°C for 10 min and then subjected to first strand cDNA synthesis by adding 20 pmol of the MSpV 3-R primer involving M-MuLV-reverse transcriptase (Fermentas) according to the manufacturer's protocol. The reverse transcribed cDNA was then subjected to PCR in a 25 μL reaction volume involving 2.5 μL of 10 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix (Fermentas), 20 pmol of each of MSpV 3-forward and reverse primers and 1 U of *Taq* DNA polymerase (Fermentas). The PCR mix was subjected to thermal cycling conditions of 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 52°C for 45 sec and 72°C for 2 min, with a final extension of 72°C for 7 min. The PCR products were electrophoresed in agarose gel (Sambrook and Russell, 2001), and the amplified products (≈2.2 Kb) were gel extracted using Qiaquick mini gel extraction kit (Qiagen) according to the manufacturer's protocol. The gel eluted PCR products were cloned into pTZ57R/T vector (Fermentas) and transformed

into *E.coli* $DH_5\alpha$ cells (Sambrook and Russell, 2001). The recombinant clones were confirmed by restriction enzyme (*Eco R*1 and *Hind* III) analysis. The confirmed positive recombinant clones were then sequenced at a commercial sequencing facility (Eurofins Biotech, India). Second set of primers (5´ end-F: 5´-ACACAAAGTCCTGGGT-3´ and 5´ end –R-5'-CCGGACCCCTTAGTAGGC-3') (Table 1) were designed for the amplification of 5´ end of the RNA3 of MSpV-Sorg isolate from Kadapa district (MSpV-Sorg-Kad1) to cover its full length sequence. The second reverse primer is an internal primer designed from the sequence obtained by using the first set of primers. The synthesized cDNA was again subjected to PCR thermal cycling conditions of 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 52°C for 45 sec and 72°C for 2 min with a final extension of 72°C for 7 min involving 5´ end primers. The amplified products (≈250 bp) were cloned and sequence analyzed as above. The clones were sequenced three times to avoid any sequencing errors.

The RNA3 of MSpV-Sorg isolates from Chittoor (MspV-Sorg-Chi), Kadapa (MSpV-Sorg-Kad2) and Kurnool (MSpV-Sorg-Kur) were later amplified by RT-PCR using the third set of primers, MSpV 5´end-F: 5´-ACACAAAGTCCTGGGT-3´ and MSpV RNA3-R: 5´-ACACAAAGTCTGGGTAATCGTT-3´ (Table 1), employing the RT-PCR conditions as described above. This facilitated the complete amplification of RNA3 (2357 nt) and the amplified products were gel extracted, cloned, transformed and screened as described above. The respective clones were sequenced using a commercial sequencing facility. The sequences obtained were then initially analyzed using the BLAST programme of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and further analyzed at the nucleotide and amino acid levels using MEGA version 4.0 (Tamura *et al.*, 2007) by comparing with the MSpV maize isolates, RSV and other tenuiviruses.

Results and discussion

The virus isolates associated with stripe disease of sorghum in Andhra Pradesh, India were found to be serologically related to MSpV-Flo and -RE isolates in DAC-ELISA (A_{405} values range: for the buffer control, 0.09–0.12; for the healthy sorghum control, 0.11–0.16; and for infected sorghum, 0.41–1.12). In western blot analyses, the present MSpV isolate's structural protein was resolved as one major polypeptide with Mr of ≈34 kDa (Figure 1). The first set of primers (Table 1) amplified ≈2.2 kb fragment from 3´ end of RNA3 (Figure 2a). The initial BLAST analysis of the sequence of recombinant clones having the ≈2.2 kb insert clearly indicated that the virus associated with the stripe disease of sorghum in Andhra Pradesh, India is indeed MSpV. The 5´ end of the RNA3 was also successfully amplified using the second set of designed primers (Table 1) (Figure 2b). The sequences of two fragments were aligned to form complete MSpV RNA3 (2357 nucleotides in length). The later designed third set of primers, based on the generated RNA3 sequence of MSpV-Sorg-Kad1 isolate (RNA3 sequence of MspV-Sorg isolate1 from Kadapa district), were used successfully for the amplification of complete RNA3 of other MSpV-Sorg isolates collected from Chittoor, Kadapa and Kurnool districts of Andhra Pradesh state, India. The RNA3 of the four respective MSpV isolates (two from Kadapa, one from Chittoor and one from Kurnool districts) contained two open reading frames (ORFs) that were separated by a large IR. The first ORF, non-structural protein 3 (NS3) was 594 nucleotides in length, while the second ORF, structural coat protein (CP) was 951 nucleotides in length. These two ORFs were separated by a large IR which is 653 nucleotides in length. The 5´- untranslated region (UTR) and 3´-UTR are, respectively, 66 and 93 nucleotides in length. These

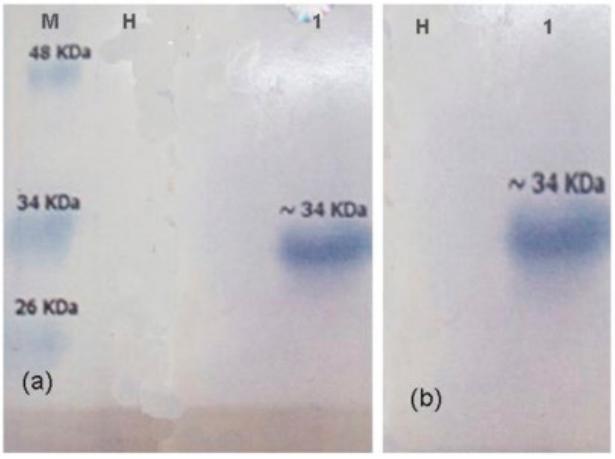

Figure 1. Western blot analysis of sorghum leaf samples suspected of MSpV infection, probed with MSpV-Flo (a) and MSpV-RU (b) antisera. Lane M, prestained protein markers (Fermentas); lane H, healthy sorghum; lane 1, sorghum leaf with stripe symptoms.

Table 1. Primers used for the amplification of RNA3 sequences of MSpV-Sorg isolates.

Primer	Set of primers	Sequence (5'to 3')	Size
MSpV-RNA3-F	1st set	TATATATTACTTCTGTCCATCGAAC	≈2.2 Kbp (partial RNA3)
MSpV-RNA3-R		ACACAAAGTCTGGGTAATCGTT	
MSpV5´-F	2nd set	ACACAAAGTCCTGGGT	≈250 bp (5´ end of RNA3)
MSpV5´-R		CCGGACCCCTTAGTAGGC	
MSpV5´-F	3rd set	ACACAAAGTCCTGGGT	≈2.3 Kbp (complete RNA3)
MSpV-RNA3-R		ACACAAAGTCTGGGTAATCGTT	

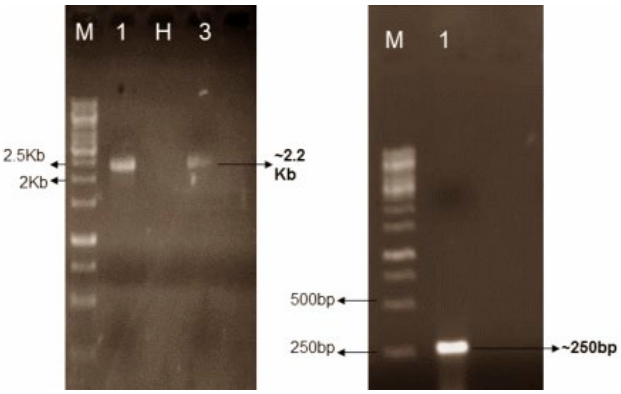

Figure 2. PCR amplicons resolved by 1% agarose gel electrophoresis. (a) The PCR amplification of ≈2.2 kb with first set of primers. Lane M, 1 kb Fermentas DNA ladder; lane H, healthy control; lane 1 and 3, samples with 2.2 kb amplicons. (b) PCR amplification with second set of primers. Lane M, 1 kb Fermentas DNA ladder; lane 1, amplicons of ≈250 bp covering the 5´end.

sizes are similar among the analyzed MSpV-Sorg isolates. The complete sequence (2357 nucleotides, GenBank A/C- MSpV-Sorg-Kad1: JN591726; MSpV-Sorg-Kad2: JN591724; MSpV-Sorg-Chi: JN579656; MSpV-Sorg-Kur: JN591725) of RNA3 of MSpV-Sorg isolates shared maximum identity of 82.4 and 82.6% at the nucleotide level with the RNA3 of MSpV-Flo and -RE isolates infecting maize, respectively. NS3 shared 88.2% identity at the nucleotide level with both MSpV-Flo and -RE isolates, while at the amino acid levels it has 93.9 and 94.4% identities with the respective isolates. The CP shared 87.3 and 86.6% identities with MSpV-Flo and -RE isolates at the nucleotide level, while it had 94.9 and 95.8% identities at the amino acid level with the respective isolates (Table 2). The ORF encoding NS3 protein was present in the viral sense strand while the ORF encoding CP was in complementary strand. Similar ambisense organization of genes was also reported in RNA3 and RNA4 of other tenuiviruses (Kakutani *et al.*, 1990; Kakutani *et al.*, 1991; Zhu *et al.*, 1991; Ramirez, 2008). The 5´ and 3´ termini of MSpV isolates were reported to be complementary (Mahmoud *et al.*, 2007; Ramirez, 2008). This complementarity of 5´ and 3´ ends facilitated the successful amplification of complete RNA3 of MSpV-Sorg isolates.

The internal large non-coding spacer had 67.1 and 68.4% identity with the maize infecting MSpV-Flo and -RE isolates, respectively. The 5´-UTR of the MSpV-Sorg isolate shared 100% identity with the maize infecting MSpV isolates, while the 3´-UTR of the isolate had 93.5 and 92.4% identities with the MSpV-Flo and -RE isolates, respectively. The percent identities of RNA3 of MSpV-Sorg isolates from the present study with RNA3 of other tenuiviruses that have more than 50% identity at complete nucleotide level, NS3 and CP at nucleotide and amino acid levels, IR at nucleotide level are given in Table 2. RNA3 percent identities among the four MSpV-Sorg isolates from the present study were found to be more than 97%, except the MSpV-Sorg-Kunool isolate which had 89.6% identity with the other three isolates especially at IR (Table 2). In the phylogenetic analyses based on the complete RNA3 nucleotide sequence, the MSpV-Sorg isolates from India clustered along with the MSpV isolates infecting maize in Florida and Reunion (Figure 3). The same clustering pattern was also observed with NS3 and CP at

Table 2. Comparison of sequence identities of RNA3 of MSpV-Sorg isolates with MSpV-maize isolates.

Virus	RNA3 Nucleotide level	NS 3 Nucleotide level	NS 3 Amino acid level	IR Nucleotide level	CP Nucleotide level	CP Amino acid level
MSpV-Sorg-Kad1	100	100	100	100	100	100
MSpV-Sorg-Kad2	97.7	100	100	99.0	100	100
MSpV-Sorg-Chi	98.7	99.3	99.4	97.7	99.4	100
MSpV-Sorg-Kur	98.4	99.3	99.4	89.6	100	100
MSpV-Flo	82.4	88.2	93.9	67.1	87.3	94.9
MSpV-RU	82.6	88.2	94.4	68.4	86.6	95.8
RSV	55.2	60.6	62.9	30.4	63.9	66.7

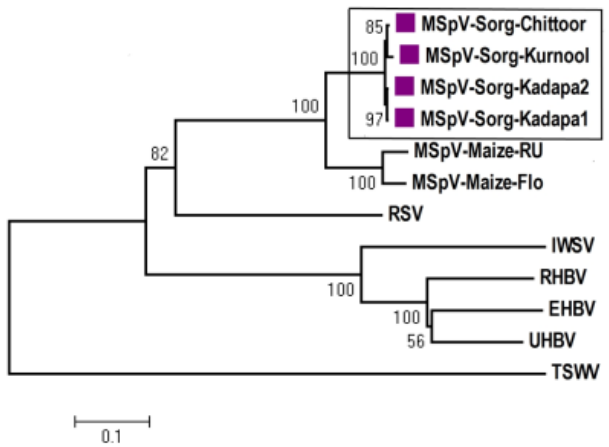

Figure 3. Phylogenetic tree based on the alignment of MSpV-Sorg sequences at complete RNA3 nucleotide level, constructed using MEGA version 4.0. The values at the forks indicate the number of trees that this grouping occurred after bootstrapping the data. The scale bar shows the number of substitutions per base. Virus acronyms: RSV, *Rice stripe virus*; IWSV, *Iranian wheat stripe virus*; RHBV, *Rice hoja blanca virus*; EHBV, *Echinochloa hoja blanca virus*; UHBV, *Urochloa hoja blanca virus*; TSWV, *Tomato spotted wilt virus*. TSWV was included as an out group.

the amino acid levels (data not shown).

Both the NS3 and CP of the MSpV-Sorg isolates showed variations in their amino acid sequences when compared to maize infecting isolates of MSpV (Table 2). All four MSpV-Sorg isolates had 12 and nine amino acid changes in the CP and NS3 proteins, respectively when compared to their maize infecting counterparts (data not shown). Although maize is also extensively grown in India, MSpV was reported to infect only sorghum in this country. Although the plant hopper vector, *P. maidis* transmitting MSpV, infests both sorghum and maize, only sorghum was observed to be affected by the stripe disease. The observed genetic variation within the MSpV-Sorg isolates could be responsible for its adaptation to sorghum only.

The complete RNA3 sequences of the four virus isolates provided the genetic basis for confirmation of the association of MSpV with stripe disease of sorghum. This is the first complete RNA3 sequence-based study on MSpV-Sorg isolates associated with stripe disease of sorghum from Asia.

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Note: The RNA3 of MSpV-Sorg isolates were deposited in the GenBank with accession No. JN591726; JN591724; JN579656; JN591725.

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