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Identification of a virus naturally infecting sorghum in India as *Sugarcane streak mosaic virus*

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Abstract The virus associated with mosaic disease of sorghum growing around the sugarcane fields in Andhra Pradesh state, India was found to be serologically related to the *Sugarcane streak mosaic virus* (SStMV) and Sorghum mosaic Parbhani virus (SMPV). The reverse transcription-polymerase chain reaction (RT-PCR) of the total RNA from the enzyme-linked immunosorbent assay positive sorghum samples with the potyvirus specific degenerate primers yielded an amplicon of ~500 bp. This amplicon sequence had a 95% identity to the SStMV-Andhra Pradesh (SStMV-AP) and SStMV-Coimbatore isolates reported to naturally infect sugarcane in India. Further confirmation was made by RT-PCR of these samples with the SStMV-AP sequence specific primers that yielded ~1,000 bp amplicon comprising the entire coat protein and 3′ UTR of the viral genome. This amplicon sequence also had a identity of 95% at nucleotide level with the SStMV-AP sugarcane isolate, but at the CP amino acid level it had 97.8% identity. This partial sequence data confirmed the association of SStMV with the mosaic disease of sorghum in Andhra Pradesh state, India. To our knowledge, this is the first report on association of SStMV with mosaic disease of sorghum and designated as SStMV-sorghum isolate.

Keywords Sorghum · Mosaic disease · *Sugarcane streak mosaic virus* · India

The sequences reported in this paper were submitted to GenBank (Accession numbers EU883390, EU883391).

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Sorghum (S*orghum* spp.) is the fifth major cereal crop of the world and India is the third largest producer (Food and Agricultural Organization 2008). Although sorghum is used as animal feed in the developing world, it is mostly used as a human food in the African and Asian countries, especially in the semi-arid zones. More than 18 viruses have been reported to naturally infect sorghum (Gordon and Thottappily 2003). Among these, the potyviruses like *Maize dwarf mosaic virus* (MDMV), *Sugarcane mosaic virus* (SCMV), *Johnson grass mosaic virus* (JGMV), *Sorghum mosaic virus* (SrMV), *Zea mosaic virus* (ZeMV) and *Pennisetum mosaic virus* (PenMV) have been reported to naturally infect the sorghum crop in different countries. MDMV and SCMV infect sorghum worldwide. These two viruses were also

reported to infect the sorghum in India (Gordon and Thottappily 2003). Sorghum red stripe virus (SRSV), reported to infect various grain sorghum cultivars in the Parbhani and Marthwada regions of Maharashtra state in India, induces mosaic, red stripes and general necrosis on sorghum (Mali 2001). Occurrence of SrMV is limited but this virus is thought to infect sorghum in India (Mali 1999). Many of these mosaic diseases on sorghum in India caused by viruses were reported earlier, but none of the causal viruses have been characterized at the molecular level.

In the present study, samples from mosaic disease affected sorghum plants grown near by or some distance away from sugarcane fields in Andhra Pradesh (A.P.) state were collected during the field surveys in 2006–2008. The virus from a mosaic diseased sorghum sample that reacted strongly in ELISA was partially characterized at molecular level and identified as *Sugarcane streak mosaic virus* (SStMV)-sorghum isolate. So far, SStMV was reported to naturally infect only sugarcane (Hema et al. 2008).

Sixty four (64) samples exhibiting mosaic symptoms were collected from the various sorghum (*Sorghum bicolor*) fields located around the sugarcane crops in Andhra Pradesh state, India. These samples were initially screened by direct antigen coating–enzyme linked immunosorbent assay (DAC-ELISA) using antisera to SStMV (Dr. P.Sreenivasulu, Sri Venkateswara University, Tiruapti, India), SCMV, MDMV, Sorghum mosaic Parbhani virus (SMPV) and *Maize mosaic virus* (MMV) (Dr. Lava Kumar, ICRISAT, Hyderabad, India). The virus isolates from ELISA strong positive samples were maintained by sap inoculation of carborundum (600 mesh) dusted sorghum (*Sorghum bicolor* cv Rio, 25–30 seedlings at 3–4 leaf stage) leaves. The leaf extracts were prepared (1/10, weight/volume or g/ml) by grinding the infected sorghum leaves in cold 0.01 M potassium phosphate buffer, pH 7.0 containing 0.2% 2-mercaptoethanol and sap inoculated to sorghum. Carborundum dusted sorghum seedlings inoculated only with inoculation buffer served as control plants.

The extracts from 64 symptomatic sorghum samples were tested against the antisera to the potyviruses MDMV, SCMV, SStMV, SMPV and with a *Nucleorhabdovirus* (MMV) by DAC-ELISA (Clark and Bar-Joseph 1984). The SStMV-AP sugarcane isolate maintained at the Department of Virology, S.V. University, Tirupati was used as a positive control (Hema et al. 1999).

For western blot analyses, the virus antigen was prepared from ELISA-positive samples by grinding the leaf sample in 0.1 M Tris-HCl (1/10, weight/volume or g/ml) followed by a brief centrifugation at 10,000X g for 5 min. The supernatant was taken as an antigen sample for electroblot immunoassay. The leaf extracts along with the marker proteins (Prestained protein molecular weight marker, Fermentas, USA) were electrophoresed in 12% SDS-PAGE system (Laemmli 1970) and the electrophoretic transfer of proteins to a nitrocellulose membrane (NCM, BIO-RAD, USA) was performed using Tris-glycine buffer containing 20% methanol (Koenig and Burgermeister 1986). The gel separated proteins were probed with the SStMV-AP antiserum at 1:3000 vol/vol dilutions. The alkaline phosphatase (ALP) conjugate and 5-Bromo-4-chloro-3-indolyl phosphate/ Nitro blue tetrazolium (BCIP/NBT) substrate (Fermentas, USA) were used as the detection system for western blotting. Appropriate healthy controls were also included.

For reverse transcription–polymerase chain reaction (RT-PCR), the total RNA was extracted from 100 mg of fresh virus infected sorghum leaf (*S. bicolor* cv Rio) as well as from the healthy sorghum leaf samples using trizol reagent (Gibco BRL, UK) according to the manufacturer's protocol and resuspended in 20 µl of RNase-free distilled water. Five µl of RNA was denatured at 55–60°C for 10 min and set for first strand cDNA synthesis involving oligo $d(T)_{18}$ (Promega, USA) and reverse transcriptase (M-MLuV-RT) (Fermentas, USA) according to the manufacturer's protocol. The PCR amplification was performed by taking 2 µl of 1:10 diluted cDNA in a 25 µl reaction involving 2.5 µl of 10X PCR buffer with $(NH_4)_2SO_4$, 2.0 mM $MgCl_2$, 0.8 µl of 10 mM dNTP mix, 1 U of Taq DNA polymerase (Fermentas, USA) involving 20 pmol of the degenerate primers viz; potyvirid primer 1 (5^1-CACGGATCCCGGG $(T)_{17}$(AGC)-3^1) and potyvirid primer 2 (5^1-ACCA CAGGATCCGG(TCG)AA(CT)AA(CT)AGCGG (GTA)CA(AG)CC-3^1) as reported by Gibbs and Mackenzie (1997). The above PCR mix was subjected to the following programme: initial denaturation of 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 51°C for 2 min and 72°C for 3 min

and a final extension step of 72°C for 10 min. After amplification, the PCR product along with the marker DNA (100 bp DNA ladder, Fermentas, USA) were electrophoresed on 1% agarose gel (Sambrook and Russell 2001) and the amplified product was extracted from the gel using the Qiaquick gel extraction kit (Qiagen, USA).The gel extracted PCR product was cloned into the pGEMT-Easy vector (Promega, USA) and transformed into *E. coli* $DH_5\alpha$ cells and the recombinant clones were selected by blue-white colony selection (Sambrook and Russell 2001). The positive clones were sequenced at commercial sequencing facility (MWG Biotech, Bangalore, India). The sequence analyses were performed using MEGA version 4.0 (Tamura et al. 2007). The sequence identity of the present virus isolate with the other viral data base sequences were primarily determined using the BLAST programme of the NCBI public data base.

The SStMV-AP isolate specific primers (Primer 1: 5^1-TTTTTTCCTCCTCACGGGGCAG GTTGATTG-3^1 and Primer 2: 5^1-GGACAAGGAACGCAGC CACCTCAG-3^1) were used for further confirmation of the sorghum virus isolate (Hema et al. 2003). After the first strand cDNA synthesis using oligo $d(T)_{18}$ (Promega, USA) as described above, the PCR amplification with the SStMV-AP specific primers was performed in a 25 µl reaction involving 2.5 µl 10X PCR buffer (Applied Biosystems, USA), 2.5 mM $MgCl_2$, 1 µl of 10 mM dNTP mix, 20 pmol of each primer, 1 U of ampliTaq Gold DNA polymerase (Applied Biosystems, USA) and 4 µl of 1:10 diluted cDNA. The PCR mix was subjected to the following programme: initial denaturation of 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 56°C for 45 s and 72°C for 1 min 15 s and a final extension step of 72°C for 10 min. After amplification, the PCR product along with the marker DNA (1 Kb DNA ladder, Fermentas, USA) were electrophoresed on 1% agarose gel (Sambrook and Russell 2001) and the amplified product was extracted from the gel using the Qiaquick gel extraction kit (Qiagen, USA), cloned into pGEMT-Easy vector and sequenced as described above. The sequence data was analyzed using MEGA version 4.0 and the phylogenetic trees were constructed for CP amino acid sequences using the SStMV sequences deposited in the GenBank, considering only those SStMV partial sequences whose length has been about 1 kb or more and CP amino acid sequences of viruses representing five genera of the family *Potyviridae* (Table 1).

The virus from a sorghum sample with mosaic symptoms and that reacted strongly with potyvirus antisera in ELISA was mechanically sap inoculated to sorghum plants which showed the development of interveinal chlorotic streaks and spots 14-days after inoculation. These streaks and spots gradually increased in size and adjacent spots coalesced resulting in alternate chlorotic and green areas (Fig. 1). The infected plants were stunted with obvious mosaic symptoms when compared to the buffer inoculated control plants.

In the DAC-ELISA tests performed with the crude extracts of 64 symptomatic sorghum samples, strong positive reactions were observed with 30 samples using antisera against SStMV-AP (A_{405} 0.62–1.65) and SMPV (0.44–1.66). These 30 samples that reacted with SStMV-AP and SMPV, failed to react with the antisera against SCMV, MDMV and *Nucleorhabdovirus* MMV (A_{405} 0.12–0.19). The remaining 34 samples failed to react with the tested antisera. The positive control, SStMV-AP-sugarcane isolate, being maintained on sorghum gave a strong reaction (A_{405} 0.66–1.85). Only samples with ELISA A_{405} values that were twice or greater than twice the value of the healthy sorghum (A_{405} 0.09–0.11) samples were considered positive. A 40 kDa protein from crude leaf extracts of virus infected sorghum strongly reacted with the antiserum of SStMV-AP. No band was obtained with healthy leaf extracts (Fig. 2).

The RT-PCR of the total RNA extracted from the virus infected sorghum sample with the degenerate primers (Gibbs and Mackenzie 1997) yielded an amplicon of ~500 bp. After sequencing, the length of this amplicon was 467 bp excluding the poly (A) tail. Surprisingly, the BLAST data of the virus isolate from sorghum revealed that the viral sequence was closely related to the SStMV. The sequence of 467 bp included the partial CP and 3′-UTR. This 467 bp viral sequence showed 95% sequence identity with the SStMV-AP and SStMV-Co isolates. The RT-PCR of SStMV positive sorghum samples with the SStMV-specific primers resulted in the amplification of ~1 Kbp fragment from the 3′end of the viral genome. The sequenced amplicons (1,045 bp) that comprised complete CP and 3′-UTR, was 95% identical with SStMV-AP isolate. The SStMV-sorghum isolate had a maximum identity of 95% and 97.8% at the CP gene

Table 1 Virus isolates selected for sequence comparisons and phylogenetic analysis.

Virus	Host	Location	GenBank Number	% CP amino acid identity	References
SStMV-sorghum	Sorghum	Andhra-Pradesh, India	EU883391	100 (Reference)	This study
SStMV-AP	Sugarcane	Andhra-Pradesh, India	Y17738	97.8	Hema et al. 1999
SStMV-TN	Sugarcane	Tamil Nadu, India	AY193784	94.3	Hema et al. 2003
SStMV-KA	Sugarcane	Karnataka, India	AY193783	94.3	Hema et al. 2003
SStMV-TA	Sugarcane	Andhra-Pradesh, India	AY189681	93.9	Hema et al. 2003
SStMV-PAK	Sugarcane	Pakistan	U75456	96.1	Hall et al. 1998
SStMV-CO 7219_30	Sugarcane	Tamil Nadu, India	AM749406	96.1	Prabu. Direct submission
SStMV-CO 86032_20	Sugarcane	Tamil Nadu, India	AM920686	96.1	Prabu. Direct submission
SStMV-CO 86032_19	Sugarcane	Tamil Nadu, India	AM920685	96.1	Prabu. Direct submission
Sugarcane mosaic virus (SCMV)	Sugarcane	Tamil Nadu, India	EU089686	17.9	Viswanathan. Direct submission
Wheat streak mosaic virus (WSMV)	Wheat	Iran	EU914918	25.2	Maumi. Direct submission
Cardamom mosaic virus (CdMV)	Cardamom	India	AY823986	12.5	Smitha. Direct submission
Cassava brown streak virus (CBSV)	Cassava	Uganda	EU916829	18.4	Mbauzibwa et al. 2009
Ryegrass mosaic virus (RGMV)	Ryegrass	Australia	AF035818	15.7	Gibbs. Direct submission

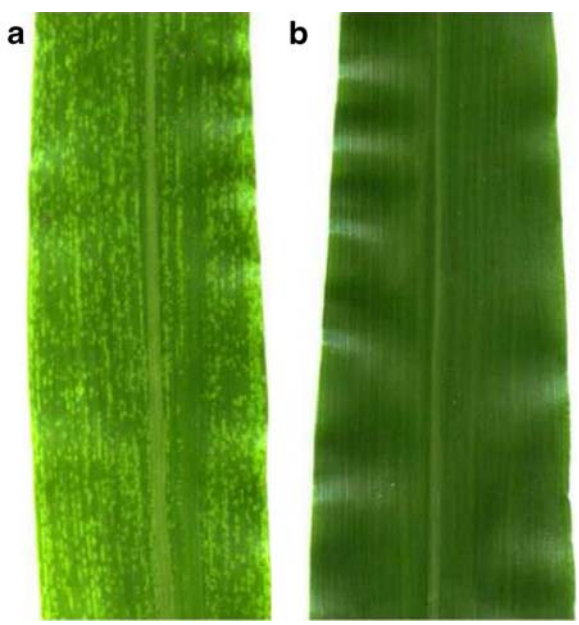

Fig. 1 *Sugarcane streak mosaic virus* (SStMV) infected sorghum leaf showing interveinal chlorotic streaks and stripes **a** and healthy sorghum leaf **b**

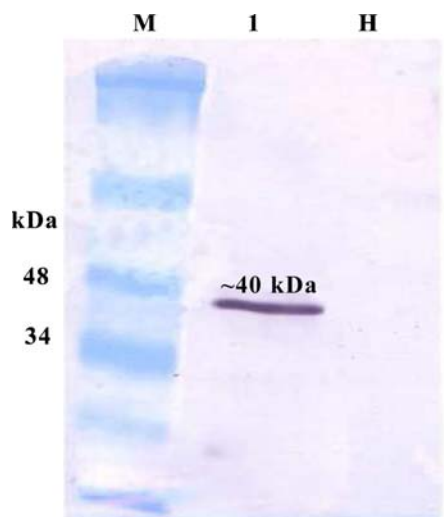

Fig. 2 Western blot analysis of healthy and SStMV infected sorghum leaf extracts with antiserum of *Sugarcane streak mosaic virus* (SStMV)-AP-sugarcane isolate. Lane M, pre-stained protein markers; Lane 1, SStMV sorghum isolate capsid protein band; Lane H, healthy sorghum

nucleotide and amino acid levels, respectively with the SStMV-AP-sugarcane isolate. The CP amino acid based phylogenetic tree revealed that SStMV-sorghum isolate is close to SStMV-AP-sugarcane isolate infecting sugarcane but not to five other distinct potyviruses representing different genera of the family *Potyviridae* (Fig. 3).

The occurrence of mosaic disease on sorghum growing in the vicinity of sugarcane fields was observed in field surveys conducted during 2006–2008. Initially, the causal virus was suspected to be SrMV reported previously by Mali (1999). The present sorghum virus isolate was readily sap transmissible to sorghum plants under experimental conditions. The virus associated with mosaic disease of sorghum samples reacted with the antisera of SStMV-AP and SMPV in DAC-ELISA, suggesting that it is antigenically related to these two potyviruses. Hema et al. (1999) have also reported that SStMV–AP-sugarcane isolate is serologically related to SMPV. As the molecular data on SMPV are not available, it is not possible to further compare SMPV and SStMV-sorghum. The western blot analysis of crude sorghum leaf extract with the antiserum of SStMV-AP clearly

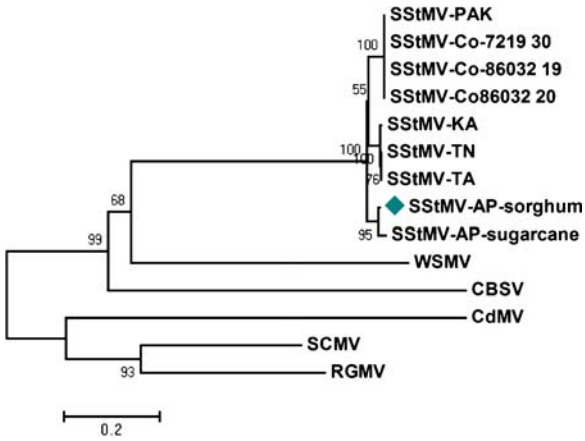

Fig. 3 Phylogenetic tree constructed from the alignment of CP amino acid sequences of *Sugarcane streak mosaic virus* (SStMV)-sorghum isolate with those of other reported SStMV sequences and other monopartite viruses representing *Tritimovirus* (*Wheat streak mosaic virus*, WSMV), *Potyvirus* (*Sugarcane mosaic virus*, SCMV), *Rymovirus* (*Ryegrass mosaic virus,* RGMV), *Ipomovirus* (*Cassava brown streak virus*, CBSV) and *Macluravirus* (*Cardamom mosaic virus,* CdMV) genera of the family *Potyviridae*. The phylogenetic tree was 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

indicated the presence of a 40 kDa viral CP, an unusual size for most of the potyviruses. Coat proteins of the size of 39–40 kDa have been previously reported for viruses such as *Narcissus latent virus*, *Maclura mosaic virus* and SStMV-AP, although the deduced amino acid sequences of these viruses indicate a range of only 32–34 kDa (Badge et al. 1997; Hema et al. 1999). The M_r of the virus studied herein, deduced from the CP (~32 kDa) sequence, was also lower than the M_r determined by western blot analysis (Fig. 2). This apparent increase in the molecular weight of the CP may be due to glycosylation as reported for SStMV-AP (Hema et al. 1999).

Degenerate primers designed by Gibbs and Mackenzie (1997) to detect potyviruses infecting cereals and millets results in the amplification of the complete CP gene and a part of the NIb gene in certain cases. The sequence of the RT-PCR amplicons obtained herein with the degenerate primers clearly matched with that of the SStMV sequence existing in the GenBank data base (Table 1). Based on this initial finding, further confirmation was made using the SStMV-AP specific primers that yielded 1,045 bp amplicons covering 3′-UTR and complete CP gene by RT-PCR of virus infected sorghum samples. Comparison of sequences of 1,045 bp amplicons further confirmed that the virus associated with mosaic disease of sorghum around Tirupati is genetically related to SStMV infecting sugarcane in India (Hema et al. 2008; Viswanathan et al. 2008). The phylogenetic analyses of the SStMV-sorghum isolate sequences (EU883390 and EU883391) along with the various SStMV sequences already existing in the GenBank showed that the virus isolate from sorghum had highest identity of 95% and 97.8% at CP gene nucleotide and amino acid levels, respectively with SStMV-AP isolate infecting sugarcane (Table 1). The phylogenetic analyses performed with the amino acid sequences of the CP gene suggests that the SStMV-sorghum isolate is closely related to SStMV-AP isolate from sugarcane and clustered as a distinct group nearer to *Tritimovirus* (WSMV) (Fig. 3). The phylogenetic relationships of SStMV-sorghum isolate with other SStMV isolates based on 3′-UTR sequences indicated that this sorghum isolate has greater identity with SStMV-TA sugarcane isolate (GenBank A/C AY189681) from Tamil Nadu state (data not shown). Full length genome sequence of SStMV-AP

sugarcane isolate confirmed that it may represent a distinct genus (Sacchamovirus proposed) in the family *Potyviridae* (GenBank A/C GQ246187, Subba Reddy 2009).

Based on antigenic relatedness and partial genome sequence identities, the virus associated with the mosaic disease of sorghum in India is identified as SStMV-sorghum isolate. To our knowledge, this is the first report on natural infection of sorghum by SStMV. Earlier reports indicated that the SStMV natural infection was restricted to sugarcane only (Hema et al. 2002, 2003, 2008), but the present study revealed that sorghum is also a natural host of SStMV. The transmission of SStMV by a specific insect vector could have a crucial role for host variation, but the vector responsible for the transmission of SStMV has so far not been identified. Although a recent work revealed the presence of SStMV in sugarcane aphids (*Melanophis indosacchari*) by RT-PCR (Viswanathan et al. 2008), the spread of SStMV under natural conditions by an aphid vector is doubtful because the SStMV CP amino acid sequence lacks DAD or DAG motifs that are necessary for aphid transmission (Lopez-Moya et al. 1999). The transmission of SStMV by mites can not be ruled out because its full length genome sequence analysis suggested that it is relatively closer to the members of *Tritimovirus* genus than to any other genera of the *Potyvirida*e family (Subba Reddy 2009).

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