Molecular Evidence for the Occurrence of *Abutilon mosaic virus*, A New World Begomovirus in India

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Abstract During an investigation in the year 2010, on the weed reservoir of begomovirus, *Abutilon pictum* showing bright yellow mosaic symptoms was observed in Udhagamandalam, Tamil Nadu, India. The complete bipartite genome of a begomovirus was cloned and sequenced which revealed association of *Abutilon mosaic virus* (AbMV). *Nicotiana benthamiana* plants inoculated biolistically with the concatemers generated through rolling circle amplification of the cloned DNAs were asymptomatic; however three out of nine plants showed presence of viral DNA A. A recombination event in the ORF BC1 with ToLCNDV DNA B (HM989846) was detected. This is the first molecular evidence of AbMV in India.

Keywords *Malvaceae* · *Abutilon pictum* · New world begomovirus · *Abutilon mosaic virus*

Geminiviruses (family *Geminiviridae*) are plant viruses with circular, single-stranded DNA (ssDNA) encapsidated in geminate icosahedral capsids. Most of the geminiviruses characterized belong to the genus *Begomovirus*, infect dicotyledonous plants and are transmitted by whitefly

Bemisia tabaci Genn. [1]. Begomoviruses are either mono or bipartite genomes each of ~2.7 kb in length. DNA A encodes the coat protein and genes involved in viral replication while DNA B is involved in systemic viral movement, symptom expression and host range determination [2–4]. Considering the importance of weeds as reservoirs of begomoviruses, a study was undertaken to identify begomovirus species in Indian weed plants.

Abutilon mosaic virus (AbMV) was one of the first viruses described in scientific literature [5] and is spread world-wide due to the interest of horticulturists for the infected ornamental plants having bright yellow mosaic symptoms. Graft transmissible nature of the etiologic agent suggestive of viral origin was proposed as early as 1904 [5] while association of a bipartite begomovirus with abutilon was first established by Abouzid and Jeske [6]. Abutilon, a decorative garden plant, is associated with different begomoviruses, such as AbMV (X15983, AM886130), *Abutilon mosaic virus-Hawaii* (AbMV-HW) (U51137); *Abutilon mosaic Bolivia virus* (AbMBoV) (HM585445), *Abutilon mosaic Brazil virus* (AbMBV) (FN434438), *Sida micrantha mosaic virus* (SimMV) (AJ557450, AJ557451), *Sida leaf curl virus* (SiLCV) (DQ641707).

Although weeds do not appear to be significant reservoirs of crop-infecting viruses, they are found to be natural reservoirs for some of the agriculturally significant begomoviruses [7–10]. Considering the importance of weeds harboring many begomoviruses [11, 12], during the year 2010, a study was undertaken for the identification and characterization of the begomovirus species which are associated with native weeds. Abutilon is one of the favored in-door and garden decorative plants. Within the genus *Abutilon*, 13 species are known to occur throughout India, of which seven species are commonly seen in southern peninsular region [13]. Three species of Abutilon are wide

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spread in India: *Abutilon indicum*, a common weed in agricultural fields, *Abutilon theophrasti* with velvety leaves in and around agricultural fields in northern and northwestern India, and *Abutilon pictum* cultivated as an ornamental in gardens for its desired flowers. The non-ornamental species of *Abutilon* occur abundantly as weeds throughout India, in and around the agricultural fields, cultivating crop plants and vegetables. It was felt necessary to study whether the *Abutilon* plants serve as reservoir for begomovirus infecting crop plants. The present communication attempts to study the begomovirus if any infecting *Abutilon* naturally in India. Here, we determine and analyze the full length genome sequence of AbMV; a new world (NW) begomovirus causing yellow mosaic disease in *Abutilon* sps. in Southern India. We also attempted to prove infectivity using concatemers generated by rolling circle amplification (RCA).

Leaf samples were randomly collected from plants of different *Abutilon* species found in and around the fields of cotton/okra/chilli/tomato which were infected with begomoviruses. Samples were also collected from *Abutilon* plants grown as ornamentals in botanical gardens. Identification at species level was done based on floral morphology and number of leaf lobes [13]. Fifty seven samples were collected from Punjab, Haryana, Rajasthan, Maharashtra and Tamil Nadu states of India, of which 38 belong to *A. indicum*, 3 of *A. theophrasti*, and 16 of *A. pictum* (*A. straitum*). Among these, one plant of *A. pictum* (Red vein *Abutilon*) (Fig. 1a) showed typical yellow mosaic symptoms of begomovirus in Udhagamandalam, Tamil Nadu, and two out of five samples collected from Abohar, Punjab, had yellow vein mosaic symptoms.

Total genomic DNA was extracted from six symptom free and three symptomatic leaf samples using the GEM-CTAB method [14]. Preliminary detection of begomovirus was performed by amplification with PAL1v1960/

PAR1c722 primer pair [15]. Amplification with $\beta 01/\beta 02$ primers [16] did not yield any amplicon which suggested absence of betasatellite. Presence of begomovirus was further confirmed by nucleic acid spot hybridization (NASH) test using radiolabelled probe to coat protein gene of *Tomato leaf curl New Delhi virus*-[India:New Delhi:JLD:2010] (ToLCNDV-[IN:ND:JLD:10] HM989845) prepared by random priming method [17]. PCR and NASH positive result for begomovirus infection was seen only in *A. pictum* leaves (Fig. 1b, c) collected from Udhagamandalam (11°24'46" North and 76°42'40" East), Southern India and no amplification or positive hybridization was seen in the *Abutilon* leaf samples collected from the plants in botanical gardens nearby or from Punjab (showing yellow vein mosaic symptoms), Haryana and Rajasthan. In order to characterize the genomic components of the *begomovirus*, 70 ng of total nucleic acid extracted from PCR positive sample was subjected to RCA [18] using Ø29 DNA polymerase (MBI Fermentas, Luthiunia, USA).

The rolling circle amplified viral DNA was restricted with several restriction endonucleases. Apparently full-length (~2.7 kb) fragments released (using *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I or *Xba*I restriction enzymes) were gel purified, cloned into the vector pUC18 linearized with the respective enzymes. Eighteen full length recombinant clones screened were found to have two distinct restriction profiles. The clones having one restriction site each for *Xba*I, *Hind*III and *Eco*RI but no *Bam*HI, and the clones which does not have either *Hind*III or *Eco*RI but have only one *Bam*HI and two *Xba*I restriction sites. Based on the restriction pattern, ten clones JIE1, JAE8, JAK1, JIK5, JAB1, JAX4, JAX9, JAX10, JAX11 and JIX12 were sequenced partially (ABI automated sequencer, South Campus, Delhi University, India) and nucleotide similarity searches were performed using BLAST at NCBI (www.ncbi.nlm.nih.gov). Seven

Fig. 1 (a) *Abutilon pictum* plant showing yellow mosaic symptoms observed in Udhagamandalam, Tamil Nadu (b) Agarose gel electrophoresis of randomly selected nine samples showing preliminary detection of begomovirus using Rojas primers. Lane 2 indicated in

circle shows the positive response (~1.1 kb amplicon) (c) Nucleic acid spot hybridization of the same nine samples using radiolabeled probe to coat protein gene of ToLCNDV-[IN:ND:JLD:10] (HM989845) showing positive hybridization in one sample

Fig. 2 Phylogenetic dendrograms of (a) DNA A and (b) DNA B for the isolates detected in this study aligned with nucleotide sequences of selected begomoviruses from NCBI GenBank database using

tomato leaf curl viruses as out group and neighbor-joining method with 1,000 bootstrap replicates aligned using Clustal W and MEGA 5.01. Isolate of the present study were highlighted

DNA A clones (JIE1, JAE8, JAK1, JIK5, JAB1, JAX11 and JIX12) and three DNA B clones (JAX4, JAX9, and JAX10) were found to have highest nucleotide identity with AbMV.

Complete nucleotide sequence of the clones JIX12, JIK5, JIE1 and JAX4 were determined and the sequences are available in GenBank (Acc No. HQ588901, HQ588900, HQ588899 and JN236209 respectively). DNA A clones, 2,637 nt in length having 95 % nucleotide sequence identity with *Abutilon mosaic virus*-[Germany] AbMV-[DE] (X15983) followed by 88 % nucleotide identity with *Sida golden mosaic virus* (GQ357649) while the DNA B clone, JAX4 (2,609 nt) shows 93 % nucleotide sequence identity with the DNA B component of the same isolate AbMV-[DE] followed by *Sida yellow mottle virus*-[Cuba:Sancti Spiritus 159:2009] (SiY-MoV-[Cuba:09] (HQ822124). Based on the threshold level of 89 % sequence identity for begomovirus species according to ICTV *Geminiviridae* study group guidelines [1], the present isolate can be considered to be an isolate of AbMV and the name *Abutilon mosaic virus*-[India:Udhagamandalam:JA:2011] (AbMV-[IN:Udg:JA:11]) is proposed.

Analysis of sequences shows the genome organization of typical NW bipartite begomoviruses, with the absence of ORF AV2 and only one ORF on viral sense strand, the ORF

AV1 encoding coat protein (CP). A PWRSMPTG motif identified in the deduced amino acid sequence of CP which is conserved in all AbMV isolates was found to be the same in the present isolate which could be a factor of making AbMV a non-vector-transmissible virus (Supplementary Fig. 1) whereas it is PWRSMAGT in other NW begomoviruses and it is totally different in old world (OW) begomoviruses. The putative Rep binding sequence, iteron is predicted to be ATTGGAGC (Supplementary Fig. 2) which occurs to be as direct tandem repeat upstream TATA motif as in the other AbMV isolates. To assess the relationship of the isolate under study with other begomoviruses, the genome sequences of the viruses which infect *Abutilon* spp. from the NW, the most closely related viruses identified using BLAST; *Corchorous golden mosaic virus* (CoGMV) which is a NW virus but reported to be occurring in OW [19, 20]; and some viruses of Indian origin were included for phylogenetic analysis (Supplementary Table 1). Isolate of the present study is clustering with West Indian isolate (AbMV-[DE]) and Hawaii (AbMV-[US:Haw]) as shown in (Fig. 2a, b). While the other two abutilon infecting viruses i.e., AbMBoV and AbMBV cluster with *Tomato golden mosaic virus* and *Squash leaf curl virus* respectively.

Keeping in view of emergence of recombinant viruses, putative recombination events for isolate of the present study were analyzed using RDP3 Beta42 [21] program with default *P* value cut off = 0.05. No significant recombination event was observed for DNA A, while four regions were detected as hotspots for DNA B (Supplementary Table 2) where one of the events was with an OW begomovirus, ToLCNDV (HM989846) as a minor parent for the event in the segment 1770–1886 nt identified by Chimaera method. It was a significant observation in the context of emergence of recombinant viruses infecting crop plants.

Based on the *in planta* cloning strategy suggested by Jeske et al. [22] which was proved to be an efficient tool, the infectivity study of the present isolate was carried out by enriching the viral genome by RCA of the clones JIX12 (DNA A) and JAX4 (DNA B). The full-length genomes were released from pUC18, gel-purified and subjected to self-ligation to form a circular molecule of the full-length viral genome. RCA was performed to the self-ligated DNA A and DNA B components, and the resultant concatemeric forms were used for inoculation. Twenty-one day's old seedlings of *Nicotiana benthamiana* were inoculated with the purified RCA product by biolistic delivery of 5 µg of the template [23, 24] using particle gene gun (PDS1000/HE; Bio-Rad, Munich, Germany) using helium at a pressure of 3 kgf/cm²; distance between particle plate and sample, 6–9 cm; amount of particles per bombardment, 0.03 mg; amount of DNA per bombardment, 0.2 µg; time of bombardment, 0.025 s; and vacuum of the chamber using a 1.0UM rupture disk at 1100psi. Inoculation was performed in two replicates with nine plants for each trial.

Inoculated seedlings were transferred to soilrite mix and kept at 26–28 °C, 16 h/8 h photoperiod at National Phytotron Facility, New Delhi and symptoms were recorded. *N. benthamiana* plants inoculated biolistically

with the concatemers generated through rolling-circle amplification of the cloned DNAs were showed asymptomatic infection at 45 dpi. DNA was extracted as described by Rouhibakhsh et al. [14] from 100 mg leaf tissue of 45 dpi and checked for non-symptomatic infection if any. RCA products of this DNA were digested by endonucleases *Bam*HI, *Hind*III, *Kpn*I, *Xba*I, *Dra*I, *Sau*3AI and *Rsa*I. Restriction profile (Fig. 3a) generated was similar to that of RCA product of original infected samples of *Abutilon* and of the clones used for inoculation. Hybridization of RCA products from inoculated plant genomic DNA with radiolabelled probe to CR region of the DNA B clone (224 bp fragment (Fig. 3c), restricted with *Sac*I (2,547 nt)-*Nsp*I (162 nt)) further confirming the presence of viral DNA (Fig. 3b). However, percentage of infectivity was low at ~30 % (three out of nine agroinoculated plants in two different experiments). The infectivity assay and the restriction profile akin to DNA from original leaf samples together demonstrating that the clones in the present study are biologically active. The results were in accordance with the previous report of AbMV infectivity to *N. benthamiana* [25]. Ten years later, Wege et al. [26] back transmitted the virus via *Malva parviflora* to its original host, *Abutilon* by grafting. It is proposed to confirm the infectivity in *Abutilon* by bombarding concatemers generated by RCA.

Detection of NW viruses in OW and vice versa in recent years is attributed to increase in global trade of agricultural products and prized variegated ornamental plants [20]. Introduction of the monopartite begomovirus *Tomato yellow leaf curl virus* from the Middle East/Mediterranean region to the NW [27]; the NW virus AbMV in UK [28] and New Zealand [29] are the few evidences how begomoviruses migrate across continents through propagules. In this context, our report on occurrence of AbMV in

Fig. 3 (a) Agarose gel electrophoresis showing restriction and PCR profile of the RCA product obtained from the biolistically inoculated sample of *Nicotiana benthamiana* Lane M 1 kb DNA Marker, lane 1 and 6 PCR amplified with Rojas primer (~1.1 kb), lane 2–5 restriction with different endonucleases representing DNA A (2.7 kb)-lane 2 *Bam*HI, lane 3 *Hind*III, lane 4 *Eco*RI, lane 5 *Xba*I; lane 5 *Xba*I—1.5 and 1.1 kb representing DNA B as predicted from fig. 3c; (b) Dot blot hybridization of the nine *N. benthamiana*

biolistically inoculated plants showing asymptomatic infection at 45 dpi (c) Genome organization of *Abutilon mosaic virus*-[India:Udhagamandalam:JA:2010] AbMV-[IN:Udg:JA:10]. The position and orientation of the virion and complementary sense genes are shown by arrows; position of restriction sites of selected enzymes by their genome positions starting from the putative nicking site within the nonanucleotide TAATATT↓AC

Southern India is significant. Contrary to the opinion on recent introduction of NW viruses into OW, Rybicki [30] suggested that perhaps NW viruses had their origin in OW and evolved differently after continental drift. Study by Lefeuvre et al. [31] also describes that NW begomoviruses have an Asiatic origin based on the estimation of GRD integration times. It is possible to detect remnant of such viruses such as report of CoGMV in India [19], and in Vietnam [20].

The limited objective of identifying the virus infecting ornamental *Abutilon* in India has been achieved in this study. Active spread of this virus and the possibility of genetic recombination with the indigenous begomoviruses need to be studied in future.

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