Molecular Characterization of a Begomovirus, α -Satellite, and β -Satellite Associated with Leaf Curl Disease of *Parthenium hysterophorus* in India

Susheel Kumar, Plant Molecular Virology Laboratory, Centre for Plant Molecular Biology Division, Council of Scientific and Industrial Research-National Botanical Research Institute (CSIR-NBRI), Lucknow-226001, U.P., India; Ashish Srivastava, Department of Plant Molecular Biology, University of Delhi South Campus; and Plant Molecular Virology Laboratory, CSIR-NBRI, Lucknow-226001, U.P., India; Meraj Jaidi and Puneet Singh Chauhan, Division of Plant-Microbe Interactions, CSIR-NBRI, Lucknow-226001, U.P., India; and S. K. Raj, Plant Molecular Virology Laboratory, CSIR-NBRI, Lucknow-226001, U.P., India

Abstract

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Parthenium hysterophorus plants exhibiting severe leaf curl and stunting symptoms were observed near agriculture fields in Lucknow, India. The association of a begomovirus, β -satellite, and α -satellite with these symptoms of a *Parthenium* disease was investigated by sequence analyses of virus and satellite DNA amplified by rolling circle amplification and polymerase chain reaction. The highest sequence identities and closest phylogenetic relationships for the begomovirus, β -satellite, and α -satellite detected in *P. hysterophorus* plants were to *Tomato leaf curl virus* (ToLCV), papaya leaf curl β -satellite (PaLCuB), and *Ageratum* yellow vein India α -satellite (AYVIA), respectively. These findings identified the virus and satellites infecting the

Parthenium sp. as ToLCV, PaLCuB, and AYVIA, respectively. *P. hysterophorus* and tomato seedlings infected with cloned ToLCV, PaLCuB, and AYVIA by agroinoculation developed leaf curl symptoms, whereas plants infected with ToLCV alone or with ToLCV and AYVIA developed mild yellowing. The results show that this complex infects and causes disease in *P. hysterophorus* and tomato. *P. hysterophorus* is an invasive weed commonly found around agricultural fields and along roadsides in India. These results indicate that *P. hysterophorus* plants infected with ToLCV and associated satellite DNA act as an alternate host (reservoir), and that could lead to increased incidence of tomato leaf curl disease.

Parthenium hysterophorus (family Asteraceae), commonly known as carrot grass or congress grass in India, is an annual or short-lived perennial plant that is native to the American tropics (<http://www.cabi.org/isc/datasheet/45573>). It is an invasive species in India and spread is of almost epidemic proportions, with reports from all states of India (Gnanavel 2013) and with crop production, livestock, and human health affected (Akhtar et al. 2010; Morin et al. 2009; Sharma et al. 2005; Singh et al. 2003). Few viruses have been reported to infect *P. hysterophorus*. *P. hysterophorus* has been reported as a symptomless carrier of *Tobacco streak virus* (TSV) in Australia by Sharman et al. (2009). In southern India, it has also been reported as a symptomless carrier of TSV and to play a major role in the spread of TSV in groundnut in (Prasada-Rao et al. 2003). However, infection of *P. hysterophorus* with a begomovirus has not been reported previously.

Viruses in the family *Geminiviridae* have circular, single-stranded genomes encapsidated in twinned icosahedral (geminata) virions. Geminiviruses transmitted by the whitefly *Bemisia tabaci* (Gennadius) are in the genus *Begomovirus* and infect a wide range of crops, including tomato (Lapidot et al. 2014; Moffat 1999). Begomoviruses have either a monopartite or bipartite genome organization (Fauquet et al. 2003). Monopartite begomoviruses have genomes consisting of a single component that encodes six genes: two in the virion sense (V1 [capsid protein] and V2 [precapsid protein]) and four in the complementary sense (C1 [replication-associated protein], C2 [transcription

activator protein], C3 [replication enhancer protein], and C4 [C4 protein]). Monopartite begomoviruses may associate approximately 1.4 kb (half the genome size of the helper begomovirus) single-stranded DNA molecules known as β -satellites and α -satellites (Briddon et al. 2000; Huang et al. 2013; Mansoor et al. 2003). The β -satellites are symptom-modulating and are widespread across the Old World (Briddon et al. 2008). The β C1 gene encoded by the β -satellites has important roles in symptom induction and suppression of transcriptional and posttranscriptional gene silencing, and can affect jasmonic-acid-responsive genes (Zhou 2013). The α -satellites are self-replicating satellite-like molecules that are dependent on a helper virus for movement in and between plants. The α -satellites occur with monopartite begomoviruses and β -satellites in the Old World (Briddon et al. 2004; Nawaz-ul-Rehman et al. 2012) and with bipartite begomoviruses (but no β -satellites) in the New World (Paprotka et al. 2010). The precise function of α -satellites is unclear (Zhou 2013). However, Nawaz-ul-Rehman et al. (2010) showed that α -satellites may be involved in overcoming RNA-mediated host defense and attenuating symptoms of the helper virus. The work of Nawaz-ul-Rehman et al. (2010) also showed that some α -satellites suppress posttranscriptional gene silencing (host defense).

Here, we report the occurrence of a tomato-infecting begomovirus (*Tomato leaf curl virus* [ToLCV]) together with papaya leaf curl β -satellite (PaLCuB) and *Ageratum* yellow vein India α -satellite (AYVIA) in *P. hysterophorus* plants with severe leaf curl and stunting symptoms. We further confirm that the cloned DNA of ToLCV and PaLCuB cause leaf curl disease in *P. hysterophorus*.

Materials and Methods

Sample collection and detection of a begomovirus. A survey was conducted of weeds growing near agricultural fields around Lucknow in the year 2012 and leaf curl disease symptoms were observed on *P. hysterophorus* plants. Leaf samples were collected from 15 randomly selected symptomatic and 15 nonsymptomatic plants. For detection of *Begomovirus* spp., total genomic DNA was isolated using GenElute Plant genomic DNA miniprep kit (Sigma-Aldrich) and polymerase chain reaction (PCR) was performed with *Begomovirus*-specific degenerate primers PALiv1978 and PARic496 following the

Corresponding authors: S. K. Raj; E-mail: skraj2@rediffmail.com; and S. Kumar, E-mail: susheelnbri@gmail.com

S. Kumar and A. Srivastava equally contributed for this study.

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described conditions (Rojas et al. 1993). All these samples were also checked for the presence of a begomovirus DNA B component with the primers BC1F and BC1R (Padidam et al. 1995), β -satellite DNA with primers Beta 01 and Beta 02 (Briddon et al. 2003), and α -satellite DNA with the primers DNA101 and DNA202 (Bull et al. 2003) following the recommended conditions. All PCR products were analyzed by electrophoresis in 1.0% agarose gels with the 1-kb DNA ladder (Thermo Fisher Scientific Inc.) as size markers.

Complete genome sequencing and sequence analysis. The full-length begomovirus genome was amplified from the DNA isolated from 15 symptomatic samples by the rolling circle amplification (RCA) method using an Illustra TempliPhi Amplification kit (GE Healthcare). The resulting products were digested with *Bam*HI, *Xba*I, *Dra*I, and *Xho*I and analyzed by electrophoresis in 1.5% agarose gels to identify enzymes to clone full-length genomic DNA. Putative full-length (approximately 2.7 kb) amplicons from three randomly selected samples were cloned into the pCambia1300 vector digested with the appropriate enzyme. The cloned begomovirus genome and approximately 1.4-kb DNA fragments obtained by PCR using α -satellite- and β -satellite-specific primers were sequenced and deposited in GenBank.

The sequences were compared with those publically available in the National Center for Biotechnology Information sequence database by BLASTn analysis for sequence identities. The open reading frames (ORF) located in the genome and their predicted amino acid sequences were analyzed with the ORF finder (www.ncbi.nlm.nih.gov/orffinder/) and ExPasy (<http://www.expasy.org/resources/search/keywords:translation>) tools, respectively. The species demarcation tool (Muhire et al. 2014) was used with default parameters to obtain the specific nucleotide sequence identity of the begomovirus, α -satellite, and β -satellites identified here with respective sequences of DNA molecules. The amino acid identities of protein-coding ORF of these molecules were obtained by the DiAlign tool (<http://www.genomatix.de/cgi-bin/dialign/dialign.pl>). The evolutionary history was inferred using the maximum-parsimony method and the tree was obtained using the Close-Neighbor-Interchange algorithm in MEGA v6.1 program (Tamura et al. 2013).

Preparation of clones for agroinoculation. Constructs for the infectivity of the begomovirus were produced by digesting the clone with *Bam*HI and *Hind*III and ligating the resulting 2,694-bp fragment into the pCambia1300 vector. Then, the full-length genomic 2,760-bp *Bam*HI fragment was ligated at the *Bam*HI site in the sense orientation. For preparation of infectious clones of the α -satellite and β -satellite, the RCA-amplified DNA were partially digested with *Sma*I and *Eco*RI, respectively, which produced an approximately

1.4-kb monomer and approximately 2.8-kb dimer. The dimers of approximately 2.8 kb recovered from both digestions were eluted independently and ligated at their respective cloning sites of pCambia1300 vector. The constructs for the infectivity of the begomovirus, β -satellite, and α -satellite will henceforth be referred to as Par-A, Par- β , and Par- α , respectively. pCambia1300 constructs were mobilized into *Agrobacterium tumefaciens* (GV3101) and agroinoculated by the syringe infiltration method (in combination or separately) into leaf lamina of *P. hysterophorus* and *Solanum lycopersicum* ('Pusa Ruby') seedlings at the four- to six-leaf stage. The infiltrated plants were kept in glasshouse conditions (natural illumination with temperature of $25 \pm 2^\circ\text{C}$) and examined regularly for appearance of symptoms. The experiments were repeated three times with 10 plants of each species for each inoculum.

Results

Disease symptoms and begomovirus detection. During the survey conducted in 2012, an outbreak of leaf curl disease symptoms was observed on a number of *P. hysterophorus* plants growing in the vicinity of tomato fields and along roads in Lucknow, India. The disease incidence was about 25% and diseased plants exhibited severe leaf curling, enation, twisting of leaves, shortening and swelling of internodes, and stunting symptoms (Fig. 1). The disease symptoms in these plants were similar to those in other plants induced by begomoviruses. Whiteflies were also observed on *P. hysterophorus* plants (with and without symptoms). Based on typical begomovirus-like (leaf curl) symptoms, the causal agent was suspected to be a *Begomovirus* sp.

Begomoviruses were detected by PCR using begomovirus-specific primers. PCR with begomovirus-specific primers resulted in amplification of approximately 1.2-kb DNA fragments from all 15 symptomatic samples, suggesting begomovirus infection, whereas no fragment was amplified from any of the 15 nonsymptomatic plants. All samples were also assessed for the presence of a DNA-B component by PCR with specific primers. Because no DNA fragments were amplified from any of the 15 samples, this suggested the begomovirus was monopartite.

Characterization of the begomovirus associated with *P. hysterophorus*. RCA products obtained from all 15 symptomatic leaf samples were digested with *Bam*HI, *Xba*I, *Dra*I, and *Xho*I. The expected approximately 2.7-kb DNA fragment was observed in all samples following digestion with *Bam*HI only. The approximately 2.7-kb fragments from three randomly selected samples were cloned and sequenced. The sequences of three isolates were identical to each other; therefore, the 2,760-nucleotide (nt) sequence of one isolate (from *P. hysterophorus* isolate SKR11) was deposited in the GenBank database under accession number JX524172. Analysis of the sequence revealed that the begomovirus characterized here contains six open reading frames—V1 and V2 in the virion sense and C1, C2, C3, and C4 in the complementary sense—and an intergenic region.

BLASTn analysis of the sequence of the SKR11 isolate revealed highest nucleotide identity (97%) with various isolates of ToLCV (HM007094, JX987088, HM803118, and HM851186) reported from India. Pairwise sequence comparison of the SKR11 isolate with ToLCV and other close begomovirus members (appeared in BLASTn) was also performed (Table 1). The isolate shared the highest sequence identity (97.3%) with ToLCV-[In:Ban:Chi:08] (HM007094) reported on *Capsicum* spp. from India and between 97.2 and 91.7% sequence identity with other ToLCV isolates reported from India (Table 1).

To determine the phylogenetic relationship of the SKR11 isolate with other close begomoviruses, phylogeny was done using the MEGA v6.1 program. The SKR11 isolate showed the closest relationship with isolate of ToLCV-[In:Luc:Zin:12] (JX987088) from *Zinnia elegans* in India and close relationships with other ToLCV isolates considered for this study (Fig. 2). Based on high sequence identity, close phylogenetic relationships, and begomovirus species demarcation criteria of >91% identity recommended by the International Committee on Taxonomy of Viruses (ICTV) *Geminiviridae* study group (Brown et al. 2015), the begomovirus isolate SKR11 was identified as an isolate of ToLCV, for which the isolate descriptor ToLCV-[In:Lko:Par:12] is proposed.

Fig. 1. Naturally infected *Parthenium hysterophorus* showing **A**, severe leaf curl, shortening and swelling of internodes, and stunting symptoms as compared with **B**, a healthy plant.

Characterization of satellite molecules associated with *P. hysterophorus*. Because monopartite begomoviruses are reported to be associated with satellites, PCR analyses with α -satellite- and β -satellite-specific primers were performed with three DNA samples

of infected *P. hysterophorus* (from which the begomovirus genome was amplified). Both primer pairs directed the amplification of approximately 1.4-kb DNA fragments from all three infected samples, which indicated the association of both an α -satellite and a β -satellite with

Table 1. Percent sequence identity of *Tomato leaf curl virus* (ToLCV)-[In:Lko:Par:12] (JX524172) isolate with other selected begomoviruses at the level of complete genome (nucleotide sequence identity) and encoded proteins (amino acid sequence identity)

Accessions	Acronyms (isolate descriptors) ^a	Host	Location	DNA genome	V2	V1	C3	C2	C1	C4
HM007094	ToLCV-[In:Ban:Chi:08]	<i>Capsicum</i> spp.	India	97.3	100	99.6	97.8	97.0	95.0	96.9
JX987088	ToLCV-[In:Luc:Zin:12]	<i>Zinnia elegance</i>	India	97.2	98.3	99.2	92.5	91.8	93.6	96.9
HM803118	ToLCV-[In:ND:Tom:10]	<i>Solanum lycopersicum</i>	India	96.7	100	100	97.8	98.5	94.2	89.7
HM851186	ToLCV-[In:ND:Tom:10]	<i>S. lycopersicum</i>	India	96.6	99.2	99	97.8	98.5	93.9	90.7
U38239	ToLCV-[In:Ban:Tom:02]	<i>S. lycopersicum</i>	India	96.3	100	99.2	97.0	95.5	92.2	95.9
JX678965	ToLCV-[In:Kar:SF:12]	<i>Helianthus annuus</i>	India	95.1	98.3	99.2	98.5	95.5	89.8	86.6
FJ914798	ToLCV-[In:Pin:Men:11]	<i>Mentha viridis</i>	India	93.3	92.4	95.7	94.8	97.0	95.6	97.9
AY754812	ToLCV-[In:Jan:Tom:06]	<i>S. lycopersicum</i>	India	91.7	97.5	99.2	97.0	94.8	86.4	87.6
GU199584	TbCSV-[CN:Mir:09]	<i>Mirabilis jalapa</i>	China	87.6	95.8	96.5	85.1	84.3	90.3	80.4
HG003650	TbCSV-[CN:Yun:Age:13]	<i>Ageratum conyzoides</i>	China	87.4	94.8	97.7	85.1	84.3	89.8	78.4
KF551584	TbCSV-[CN:Mir:09]	<i>M. jalapa</i>	China	87.1	93.2	96.9	87.3	88.1	80.8	76.8
GU199583	TbCSV-[CN:Alt:09]	<i>Alternanthera philoxeroides</i>	China	87.0	92.2	96.1	84.3	81.3	89.2	77.3
KF551575	TLCKeV-[In:ND:Tom:13]	<i>S. lycopersicum</i>	India	86.2	92.4	96.5	93.3	92.5	88.4	47.9
EU910141	ToLCKeV-[In:Ker:Tom:05]	<i>S. lycopersicum</i>	India	86.1	92.4	94.9	93.3	93.3	79.7	46.9
GQ268327	AEV-[In:Gor:Par:08]	<i>Trichosanthes dioica</i>	India	86.1	96.5	97.3	85.8	84.3	82.3	50.6
AJ437618	AEV-[NP:Ager:05]	<i>Ageratum conyzoides</i>	Nepal	86.1	96.9	99.5	85.8	82.8	83.4	50.6
AM701770	AEV-[PK:Fai:Bra:10]	<i>Brassica rapa</i>	Pakistan	86.0	93.9	97.3	89.6	85.1	83.4	51.8
AF336806	ChiLCV-[PK:Mul:Chi:07]	<i>Capsicum</i> spp.	Pakistan	85.5	85.6	98.4	83.6	85.1	85.1	76.0
AJ875159	ChiLCV-[Ba:Gaz:Tom:06]	<i>S. lycopersicum</i>	Bangladesh	85.3	89.8	96.5	82.1	88.1	84.8	72.2
GU136803	ChiLCV-[IN:Amr:Pap:09]	<i>Carica papaya</i>	India	85.1	89.0	98.8	77.6	86.6	82.2	75.3
FN543099	AEV-[IN:HP:Zin:09]	<i>Zinnia elegance</i>	India	85.1	98.3	97.7	89.6	85.1	81.4	50.6
FJ177030	JYMINV-[In:MP:Jat:08]	<i>Jatropha gossypifolia</i>	India	85.0	92.4	94.9	65.7	55.2	80.6	59.8
DQ852623	ToLCBaV-[In:Ker:Tom:05]	<i>S. lycopersicum</i>	India	84.8	71.3	92.2	87.3	82.8	85.3	85.6
HM149260	AEV-[In:UP:Pap:14]	<i>Papaver somniferum</i>	India	84.7	95.7	95.2	88.7	86.6	80.6	50.6
FJ345402	ChiLCV-[In:Pon:Ken:07]	<i>Hibiscus cannabinus</i>	India	84.7	88.0	96.5	82.1	88.1	84.2	70.1
DQ673859	ChiLCV-[In:Pun:Chi:07]	<i>Capsicum</i> spp.	India	84.3	91.5	96.5	77.6	85.8	85.6	77.3
AF165098	ToLCBaV-[In:Ban:03]	Not available	India	84.1	73.0	89.5	83.6	85.5	84.8	85.6
KF663698	ToLCBaV-[In:Kar:Tom:13]	<i>S. lycopersicum</i>	India	83.8	70.4	90.2	85.1	83.6	86.7	81.4
AF295401	ToLCBaV-[In:Ban:Cot:05]	<i>Gossypium</i> spp.	India	82.7	69.6	91.8	83.6	84.3	81.7	70.1
Z48182	ToLCBaV-[In:05]	Not available	India	82.3	70.4	90.2	85.8	83.6	81.9	72.2

^a Abbreviations: AEV = *Ageratum enation virus*, TbCuSV = *Tobacco curly shoot virus*, ChiLCV = *Chilli leaf curl virus*, ToLCBaV = *Tomato leaf curl Bangalore virus*, ToLCKeV = *Tomato leaf curl Kerala virus*, and JYMINV = *Jatropha yellow mosaic India virus*.

Fig. 2. Phylogenetic tree showing relationship of begomovirus isolate (JX524172) from *Parthenium hysterophorus* (indicated by ■) with other begomoviruses. The tree was constructed using MEGA v6.1 tool and using the neighbor-joining method with 1,000 bootstrap replicates.

ToLCV in *P. hystrophorus*. The approximately 1.4-kb α -satellite and β -satellite components obtained from the SKR11 sample were sequenced and deposited in GenBank under accession numbers JX570736 (SKR11- α -satellite) and JX987089 (SKR11- β -satellite).

The 1,389 nt of the SKR11- α -satellite encodes for a replication-associated protein (Rep) that is similar to the master Rep protein encoded by the genomic components (DNA-R) of nanoviruses (Zhang et al. 2012). It also has an A-rich region and a predicted hair-pin structure. The nucleotide sequence analysis of the SKR11- α -satellite isolate showed 87.2 to 97.0% identity with isolates of AYVIA (JQ012793, AJ512948, and AJ512958) (Table 2). High amino acid sequence identities (94.0 to 98.7%) were also observed for the

α -Rep of SKR11- α -satellite with those of AYVIA isolates (Table 2). The phylogenetic analysis also showed the closest relationships of SKR11- α -satellite with isolates of AYVIA (JQ012793, AJ512948, LK054802, and AJ512958) (Fig. 3). Owing to the high sequence identity and close relationships, the SKR11- α -satellite was identified as AYVIA, for which the isolate descriptor AYVIA-[In:Lko:Par:12] is proposed.

Sequence analysis of the SKR11- β -satellite revealed 1,367 nt that encode for a conserved β C1 ORF in the complementary-sense orientation as well as the satellite conserved region and an A-rich region. The pairwise sequence identity of the complete SKR11- β -satellite sequence revealed 83.5 to 85% nucleotide identities with PaLCuB

Table 2. Percent nucleotide sequence identity (Seq ID) and amino acid sequence identity of *Ageratum* yellow vein India α -satellite (AYVIA)-[In:Lko:Par:12] (JX570736) and the encoded α -replication-associated protein, respectively, with selected α -satellite sequences from the databases

Accessions	Acronyms (isolate descriptors)	Seq ID (%) ^a	
		nt	aa
JQ012793	<i>Ageratum</i> yellow vein India α -satellite, AYVIA-[In:Lko:Ama:13]	97.0	98.7
AJ512948	<i>Ageratum</i> yellow vein India α -satellite, AYVIA-[PK:Age:04]	95.1	97.8
AJ512958	<i>Ageratum</i> yellow vein India α -satellite, AYVIA-[In:Age:04]	95.0	97.8
FN543100	<i>Ageratum</i> yellow vein India α -satellite, AYVIA-[In:HP:Zin:09]	91.6	96.2
LK054802	<i>Ageratum</i> yellow vein India α -satellite, AYVIA-[In:HP:Mir:14]	90.7	98.7
HE861941	<i>Ageratum</i> yellow vein India α -satellite, AYVIA-[In:HP:Gly:12]	88.9	94.6
AJ512950	<i>Ageratum</i> yellow vein India α -satellite, AYVIA-[PK:Hib:04]	88.4	94.3
AJ512951	<i>Ageratum</i> yellow vein India α -satellite, AYVIA-[PK:Age:04]	87.9	–
AM930246	<i>Ageratum</i> yellow vein India α -satellite, AYVIA-[PK:Fai:Son:10]	87.4	93.7
AM930244	<i>Ageratum</i> yellow vein India α -satellite, AYVIA-[PK:Fai:Son:10]	87.2	94.0
AM930248	<i>Ageratum</i> yellow vein India α -satellite, AYVIA-[PK:Fai:Son:10]	87.2	94.0
AJ579359	Tomato yellow leaf curl Thailand α -satellite, ToYLCuTA-[Th:Tom:03]	83.1	89.5
AJ579356	Tomato yellow leaf curl China α -satellite, ToYLCuCNA-[Chi:Yun:Tom:03]	82.6	91.4
AJ579347	Tobacco curly shoot α -satellite, TbCuSA-[CN:Yun:Tob:03]	82.2	91.7
FN806782	Sida yellow vein α -satellite, SYVA-[CN:Yun:Sid:10]	82.1	90.8
EU384636	<i>Gossypium darwinii</i> symptomless α -satellite, GDSA-[PK:Mul:Cot:09]	81.3	88.6
FN658730	Cotton leaf curl Burewala α -satellite, CoLCuBA-[In:Raj:Cot:12]	81.2	88.6
AM236765	Malvastrum yellow mosaic α -satellite, MYMA-[CN:Mal:08]	82.1	91.1
AM050734	Sida leaf curl α -satellite, SLCuA-[CN:Hai:Sid:06]	80.7	91.4
AJ238493	<i>Ageratum</i> yellow vein α -satellite, AYVA-[Sin:Age:99]	80.1	89.8
EU384654	<i>Gossypium davidsonii</i> symptomless α -satellite, GDSA-[PK:Mul:Cot:09]	67.2	57.6
FM164939	Tomato leaf curl Pakistan α -satellite, ToLCuPA-[PK:Fai:Eup:09]	66.7	47.3
FJ218494	<i>Gossypium mustelinum</i> symptomless α -satellite, GMSA-[PK:Cot:12]	66.4	56.8
AJ416153	<i>Ageratum</i> yellow vein Singapore α -satellite, AYVSA-[Sin:Age:02]	61.8	40.5

^a Abbreviations: nt = nucleotide, aa = amino acid, and – = sequences were smaller; therefore, identity could not be obtained in the species demarcation tool.

Fig. 3. Phylogenetic tree showing relationship of α -satellite (JX570736) isolated from *Parthenium hysterophorus* (indicated by ■) with other α -satellite sequences. The tree was constructed using MEGA v6.1 tool and using the neighbor-joining method with 1,000 bootstrap replicates. The *Ageratum* yellow vein α -satellite (AYVA-[Sin:Age:99], AJ238493) reported from Singapore (shown by ►) is also considered for analysis. The tree is rooted on *Ageratum* yellow vein Singapore α -satellite (AYVSA-[Sin:Age:02], AJ416153).

sequences (AY438557, AY244706, AY230138, and DQ118862), and lower identities with other selected β -satellites (Table 3). The β C1 ORF of the SKR11- β -satellite showed 97.5 to 100% amino acid sequence identity with corresponding ORF of PaLCuB isolates (Table 3). The phylogenetic analysis showed the close relationships of SKR11- β -satellite with isolates of PaLCuB, and these isolates

clustered together in a monophyletic group (Fig. 4). Because of the high sequence identity and close relationships with PaLCuB, the SKR11- β -satellite was identified as isolate of PaLCuB, for which isolate descriptor PaLCuB-[In:Lko:Par:12] is proposed.

Infectivity assays. The infectivity assay was conducted with agro-infectious clones of ToLCV-[In:Lko:Par:12], PaLCuB-[In:Lko:Par:

Table 3. Percent nucleotide sequence identity (Seq ID) and amino acid sequence identity of Papaya leaf curl β -satellite (PaLCuB)-[In:Lko:Par:12] (JX987089) and the encoded β C1, respectively, with selected β -satellite sequences from the databases

Accessions	Acronyms (isolate descriptors)	Seq ID (%) ^a	
		nt	aa
DQ118862	Papaya leaf curl β -satellite, PaLCuB-[IN:Chi:05]	85.0	100.0
AY230138	Papaya leaf curl virus β -satellite, PaLCuB-[IN:Jab:03]	84.1	99.2
AY244706	Papaya leaf curl virus β -satellite, PaLCuB-[IN:ND:03]	83.3	98.3
AY438557	Papaya leaf curl virus β -satellite, PaLCuB-[IN:Mb:05]	83.5	97.5
AJ542490	Tomato leaf curl β -satellite, ToLCB-[IN:ND:02]	65.2	66.1
AY765255	Tomato leaf curl β -satellite, ToLCB-[IN:Lud:04]	64.8	65.0
AJ316036	Tomato leaf curl β -satellite, ToLCB-PK:RYK:97]	64.3	65.3
AY728263	Tomato leaf curl β -satellite, ToLCB-[IN:CP:04]	65.6	63.6
AJ704610	Tomato leaf curl China β -satellite, ToLCCNB-[CN:Gx16:02]	60.9	46.6
AJ704609	Tomato leaf curl China β -satellite, ToLCCNB-[CN:Gx14:02]	59.9	45.8
AJ704611	Tomato leaf curl China β -satellite, ToLCCNB-[CN:Gx17:02]	60.8	46.6
AJ704612	Tomato leaf curl China β -satellite, ToLCCNB-[CN:Gx18:02]	61.6	48.3
AJ704613	Tomato leaf curl China β -satellite, ToLCCNB-[CN:Gx22:02]	60.9	48.3

^a Abbreviations: aa = amino acid and nt = nucleotide.

Fig. 4. Phylogenetic tree showing the relationship of the β -satellite isolated from *Parthenium hysterophorus* (JX987089, indicated by ■) with selected other β -satellites. The tree was constructed using MEGA v6.1 tool and using the neighbor-joining method with 1,000 bootstrap replicates.

Table 4. Infectivity assay using seedlings of *Parthenium hysterophorus* and *Solanum lycopersicum* and agroinfectious clones of *Tomato leaf curl virus* (ToLCV)-[In:Lko:Par:12] (Par-A), Papaya leaf curl β -satellite (PaLCuB)-[In:Lko:Par:12] (Par- β), and *Ageratum* yellow vein India α -satellite (AYVIA)-[In:Lko:Par:12] (Par- α) DNA

Seedling, agroinfectious clones	Symptoms ^b	Symptomatic/inoculated plants			Detection of ^a		
		Experiment 1	Experiment 2	Experiment 3	ToLCV	PaLCuB	AYVIA
<i>P. hysterophorus</i>							
Par-A	Mild leaf curl	9/10	10/10	9/10	28/30	0/30	0/30
Par-A+Par-β	Severe leaf curl	10/10	9/10	9/10	28/30	28/30	0/30
Par-A+Par-β+Par-α	Severe leaf curl	10/10	10/10	9/10	29/30	29/30	29/30
Par-A+Par-α	Mild leaf curl	7/10	8/10	8/10	23/30	0/30	23/30
Par-β	No symptoms	0/10	0/10	0/10	0/30	0/30	0/30
Par-α	No symptoms	0/10	0/10	0/10	0/30	0/30	0/30
<i>S. lycopersicum</i>							
Par-A	Mild yellowing	8/10	9/10	7/10	24/30	0/30	0/30
Par-A+Par-β	Yellowing & leaf curl	9/10	9/10	10/10	28/30	28/30	0/30
Par-A+Par-β+Par-α	Yellowing & leaf curl	10/10	10/10	9/10	29/30	29/30	29/30
Par-A+Par-α	Mild yellowing	8/10	9/10	8/10	25/30	0/30	25/30
Par-β	No symptoms	0/10	0/10	0/10	0/30	0/30	0/30
Par-α	No symptoms	0/10	0/10	0/10	0/30	0/30	0/30

^a Fourth systemically infected leaf (from the top) was used for polymerase chain reaction (PCR) detection. Amplifications during PCR were obtained using degenerate primers of begomovirus, β -satellite, and α -satellite DNA (detailed in Materials and Methods).

^b Symptoms by 35 days postinoculation.

12], and AYVIA-[In:Lko:Par:12] DNA. *P. hystrophorus* and *S. lycopersicum* plants agroinoculated with the Par-A+Par-β+Par-α and Par-A+Par-β DNA developed prominent leaf curl and leaf curl accompanied with yellowing (only in tomato) symptoms, respectively, by 35 days postinoculation (dpi) (Table 4; Supplementary Fig. S1). The symptoms that developed in experimentally infected *P. hystrophorus* plants were indistinguishable from the symptoms observed in *P. hystrophorus* plants in the field. Plants inoculated with Par-A alone and in combination with Par-α (Par-A+Par-α) developed mild symptoms, whereas no symptoms were observed when plants were inoculated either with Par-β or Par-α alone, or mock inoculated (Table 4). This satisfies Koch's postulates for ToLCV with PaLCuB causing leaf curl disease of *P. hystrophorus*.

The presence or absence of ToLCV, AYVIA, and PaLCuB DNA in the leaves developing after inoculation of both *P. hystrophorus* and *S. lycopersicum* (agroinoculated plants) were assessed by PCR with the respective specific primers. The ToLCV DNA was detected in newly emerged leaves of plants agroinoculated with Par-A alone as well as in combinations with Par-A+Par-β and Par-A+Par-α. PaLCuB and AYVIA DNA were also detected in *P. hystrophorus* and *S. lycopersicum* plants, respectively, agroinoculated with Par-A+Par-β+Par-α (Table 4; Supplementary Fig. S2). These results indicated that ToLCV can replicate and move independently or in combinations with PaLCuB and AYVIA in inoculated plants. The mild symptoms in the plants inoculated with AYVIA together with ToLCV suggested that the α-satellite is not required for symptom development in *P. hystrophorus* as well as in *S. lycopersicum*.

Discussion

The study presented here has, for the first time, shown infection of the weed *P. hystrophorus* with ToLCV, PaLCuB and AYVIA in India. Koch's postulates were satisfied by showing that agroinfiltration of the complex of ToLCV, PaLCuB, and AYVIA could induce leaf curl and stunting in *P. hystrophorus* (Table 4) which were indistinguishable from the symptoms seen in naturally infected plants in the field (Fig. 1). Moreover, severe leaf curl symptom in *P. hystrophorus* plants was observed when ToLCV together with PaLCuB was inoculated, whereas ToLCV alone or in combination with AYVIA induced only mild leaf curl. Our finding shows that the βC1 protein of PaLCuB is a dominant symptom determinant. The βC1 gene encoded by β-satellite has been well documented as a symptom modulator (Mansoor et al. 2003; Zhou 2013). The α-satellites have no obvious contribution to symptoms induced by begomovirus-β-satellite disease complexes and appear to affect β-satellite replication but do not affect helper virus replication (Nawaz-ul-Rehman et al. 2010). Nawaz-ul-Rehman et al. (2010) observed that the α-Rep proteins encoded by two nonpathogenic α-satellites (*Gossypium darwinii* symptomless α-satellite and *Gossypium mustelinum* symptomless α-satellite) associated with *Cotton leaf curl Multan virus* can interact with *Cotton leaf curl Rajasthan virus* (CLCuRaV) Rep proteins and affect β-satellite replication but do not affect helper virus replication.

The infectivity of these agroinfecting-cloned DNA was also assessed in tomato (*S. lycopersicum* Pusa Ruby, the host from which ToLCV was first identified), where a complex of ToLCV, PaLCuB, and AYVIA induced severe leaf curl symptoms (Table 4). Agroinoculation of ToLCV alone in tomato induced mild yellowing only, whereas plants showed leaf curl when inoculated together with PaLCuB, strengthening the observations that PaLCuB exacerbates the severity of ToLCV whereas AYVIA has no role in symptom modulation. The infections of ToLCV in tomato have been reported from India (Chakraborty et al. 2003; Chatchawankanphanich and Maxwell 2002; Chatchawankanphanich et al. 1993; Gaikwad et al. 2011; Kumar et al. 2012; Muniyappa et al. 2000; Shelat et al. 2014). A study carried out by Chatchawankanphanich and Maxwell (2002) suggested that particle bombardment of ToLCV-[In:Ban:Tom:02] alone in tomato ('Flora Dade', 'Hayslip', and 'Geneva 80') may induce leaf curl and yellowing symptoms, though it takes a longer time (45 dpi) to develop such symptoms. In our case, only mild yellowing was observed in Pusa Ruby tomato plants inoculated with ToLCV-[In:Lko:Par:12] alone; however, symptoms developed

within 35 dpi and remained mild throughout the plant's life. The variation in symptoms may be due to differences in tomato cultivar, as was observed in case of okra varieties by Venkataravanappa et al. (2013), as well as sequence diversity of both ToLCV isolates, because ToLCV-[In:Ban:Tom:02] (Chatchawankanphanich and Maxwell 2002) shares 96.3% nucleotide identity with our ToLCV isolate. In addition to the report of ToLCV infection in tomato, association of ToLCV sunflower leaf curl disease in India (Vanitha et al. 2013) and with different β-satellites (cotton leaf curl Multan β-satellite) causing leaf deformity and stunting in mentha (Borah et al. 2010) is reported. Moreover, unpublished reports on the occurrence of PaLCuB associated with *Papaya leaf curl virus* in Delhi (AY244706), *Mungbean yellow mosaic India virus* in Gujarat (DQ118862), and ToLCV in Jabalpur (AY230138) and Andhra Pradesh (AY438557) in India are available in the GenBank database. However, occurrence of the complex of ToLCV, PaLCuB, and AYVIA which is being reported here is not known.

Weeds have been reported to be hosts of crop-infecting begomoviruses, which can lead to increased virus incidence and losses in agricultural crops grown (Bedford et al. 1998; Kashina et al. 2003; Tavares et al. 2012; Barreto et al. 2013). Here, using the infectious clones of ToLCV-[In:Lko:Par:12] and PaLCuB-[In:Lko:Par:12], we also established that this complex induces severe leaf curl symptoms in tomato, and it is possible that *P. hystrophorus* acts as a reservoir for ToLCV in tomato. Therefore, *P. hystrophorus* infected with ToLCV could lead to increased virus incidence and losses in adjacent tomato fields. The removal of this weed may be recommended to reduce inoculum pressure.

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