

Original Research Article

**Molecular identification and characterization of
alphasatellites associated with Okra enation leaf curl
virus**

ABSTRACT

The leaf samples from okra plants showing the yellow vein mosaic disease symptoms were collected in Punjab, India. The genome of the virus causing the disease was amplified, cloned and sequenced. Sequence analysis revealed that the viral genome is 2,741 bp in length. Further nucleotide (nts) sequence comparisons showed that the genome has the highest sequence identities of 91-99 % with Okra enation leaf curl virus (OELCV) infecting okra in India and Indian subcontinent. The plants harboring OELCV was also shown to contain the alphasatellites. Three out of six alphasatellites associated with the virus was shown to be Sida yellow vein China alphasatellite which has previously been identified in the Yunnan Province of China and Nepal. Two alphasatellites showed highest identity with *Gossypium darwinii* symptomless alphasatellite (GDarSLA) and one with *Gossypium mustelinum* symptomless alphasatellite (GMusSLA).

Keywords: Aphasatellites, Begomovirus, Disease, Phylogenetic analysis.

1. INTRODUCTION

India is the most important okra producing country in the world. Okra is susceptible to at least 19 plant viruses [1,2] and these viruses pose serious constraints to okra production. Among these viruses yellow vein mosaic virus (YVMV) causes significant losses in the okra production. In India, occurrence of this disease was first reported by Kulkarni [3] in Bombay province. The yield loss in infected plants ranges between 50 to 90 per cent depending on stage of growth at which infection occurs [4,5]. Okra yellow vein mosaic disease is caused by YVMV, which is a species of genus *Begomovirus*, family *Geminiviridae* [6]. The genome of viruses in the genus *Begomovirus* consists of either two genomic components, bipartite (known as DNA-A and DNA-B) of about equal size (~2.8 kb) or a single component, monopartite, homologous to the DNA- A component of bipartite viruses. Monopartite begomoviruses are associated with additional circular ssDNA molecules, known as beta satellite or alphasatellite that are about half the size of DNA-A. These molecules depend on a helper virus for replication, are dispensable for the replication of the helper virus, and lack sequence homology to the helper virus' genome [7]. Beta satellites have been involved in pathogenicity but alphasatellites have no known function and are certainly not involved in symptom induction [8,9]. Alphasatellites were first

reported to be associated with a yellow vein disease in *Ageratum conyzoides* in 1999 [10]. Alphasatellite genomes are approximately 1,375 nts and encode a single ORF (*alpha-Rep*). They have an A-rich region and a predicted hairpin structure. The *alpha-Rep* ORF encodes a 315 amino acid protein of ~37 kDa that resembles nanovirus Reps. Some alphasatellites can attenuate disease symptoms caused by begomovirus-beta satellite complexes in the early stages of infection [11]. The objective of this study was to identify and characterize the alphasatellite molecules associated with *Okra enation leaf curl virus* causing YVMD of okra from Punjab (India).

2. MATERIAL AND METHODS

Sample Collection

Leaves of six symptomatic okra plants showing typical yellow vein mosaic symptoms were collected from two distinct locations and genomic DNA was extracted using modified cetyl trimethyl ammonium bromide (CTAB) method [12] and stored at -20°C.

Rolling circle amplification (RCA) and PCR-mediated amplification

Initial analysis by PCR with a pair of degenerate primers AV494 and AC1048 corresponding to coat protein region (CP) [13] showed the presence of a begomovirus. The samples which failed to show any amplification were subjected to Rolling circle amplification (RCA) [14] with ϕ 29 DNA polymerase using the TempliPhi DNA amplification Kit (GE Healthcare, Buckinghamshire, United Kingdom). The resultant RCA product was diluted with distilled water to the ratio 1:5 which was then used as template in PCR with the primers DNA101/DNA102 [15].

Cloning

PCR products of both CP and alphasatellites were purified from the gel using the Nucleospin Gel and PCR clean-up kit (Macherey-Nagel GmbH & Co. Germany). The gel-purified PCR products were cloned into the vectors pGEM-T Easy and the DNA sequence for the clones were determined. For full genome cloning the high molecular weight ϕ 29 DNA polymerase RCA product was digested with *EcoRV*. Digestion was carried out for 3 h at 37°C with 5 U of enzyme. The product was run on a 1% agarose gel, which showed the presence of ~2.7 which was purified and cloned into pJET1.2 vector. The clone was confirmed by restriction digestion. Three clones for each fragment were subjected to sequencing.

Sequence analysis

The sequences (both forward and reverse) were assembled using Bioedit Sequence Alignment Editor software (version 5.09) [16]. The similarity of genomic sequences was initially analyzed by using the BLAST program available at the National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>). The representative sequences were downloaded from GenBank database so that the comparisons could be made between the sequences found in this study with similar sequences from different hosts and geographical regions. The Clustal W algorithm [17] available in MegAlign (Lasergene, DNASTAR, Madison, WI, USA) was used to calculate pairwise nucleotide and protein identities. Phylogenetic analysis was carried out using the neighbor-joining algorithm and the Kimura two-parameter model available in Mega 6 [18] and one thousand bootstrap iterations. The ORF on complementary sense orientation, were identified using the ORF finder tool

available at NCBI (<http://www.ncbi.nlm.nih.gov/>).

3. RESULTS

PCR detection, sequencing and organization of full-length begomoviral genomes

PCR amplification with specific primers (AV494 and AC1048) in all the six plants yielded a single product of the expected size (~570 bp) which were cloned and sequenced. The partial sequencing revealed them to have near identical sequences (results not shown). For this reason only one sample was selected for complete sequencing. The complete sequence was determined to be 2,741 nt and is available in the databases under the accession number KP208672.

Full genome analysis

Sequence comparisons showed the sequence to be closely related to isolates of *Okra enation leaf curl virus* (OELCV) (91-99% nucleotide sequence identity to all OELCV sequences available in the databases) with the highest to an OELCV isolate originating from Gandhinagar, India (KC019308). The sequence has a genome organization typical of begomoviruses (~2.7 kb) with two genes encoded in the virion-sense, the coat protein (CP) and V2 protein, and four genes encoded in the complementary-sense, Rep, the transcriptional activator protein (TrAP), the replication enhancer protein (REn) and the C4 protein (Table 1). Between the virion- and complementary-sense genes lies a non-coding, intergenic region (IR) that contains a predicted stem-loop structure with the nonanucleotide sequence "TAATATTAC" forming part of the loop. This motif forms part of the origin of virion-strand DNA replication [19]. A phylogenetic tree, based on an alignment of the complete nucleotide sequence of the clone with selected genomes (or DNA- A components) of begomoviruses from the databases (Table 1), shows the virus from okra to segregate with the isolates of OELCV included in the analysis and to be distinct from other begomoviruses, confirming its identification as an isolate OELCV (Figure 1).

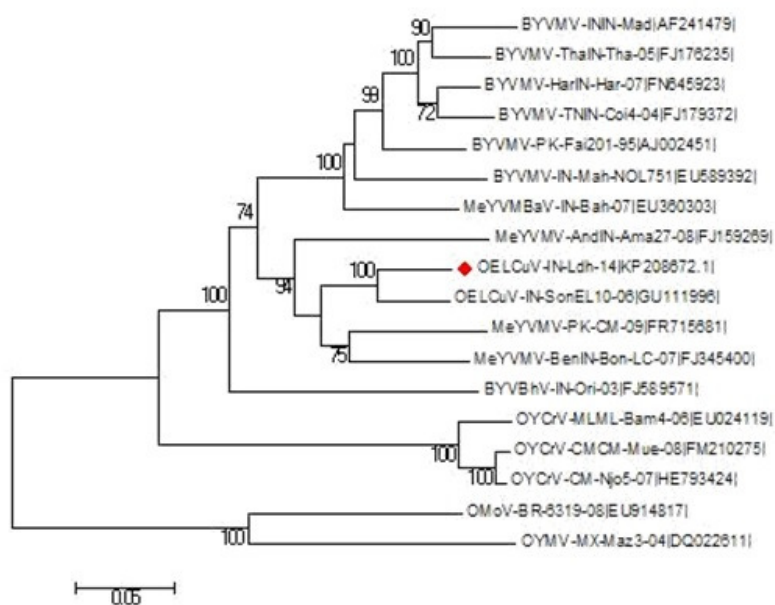


Figure 1: Phylogenetic tree based upon an alignment of the complete nucleotide of the cloned DNA-A produced in this study (labeled with diamond) and the selected begomovirus sequences obtained from the database. The tree is unrooted and the number at nodes represent percentage bootstrap values (1000 replicates).

Alphasatellites analysis

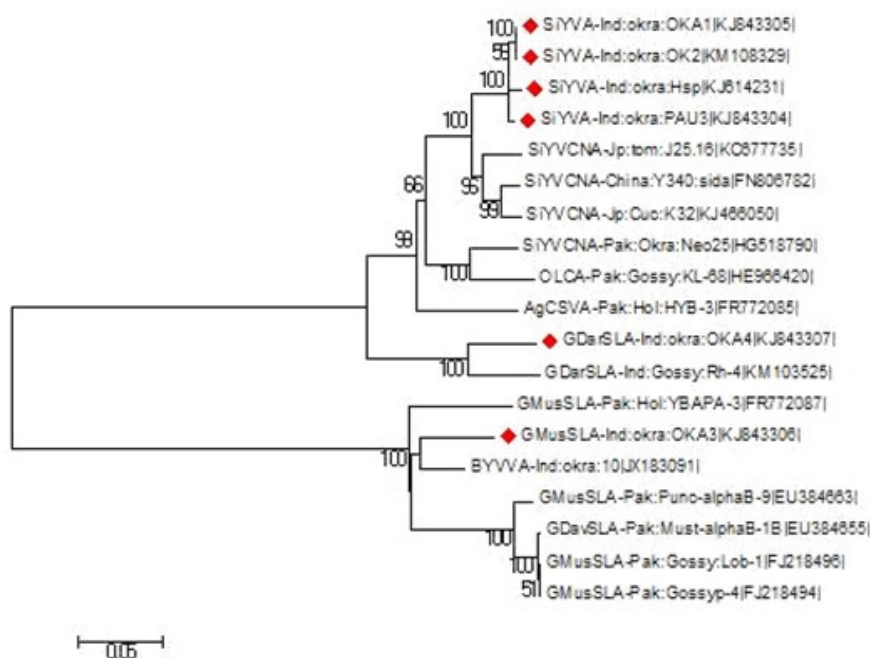
The six alphasatellite clones obtained, isolated from okra infected with OELCV, were determined to be 1,319-1,374 nt, in length. The alphasatellite sequences represented three distinct genotypes. The type 1 alphasatellites [KJ614231, KJ843304, KJ843305 and KM108329] showed the highest levels of nucleotide sequence identity (94.1% to 98.1%) with isolates of Sida yellow vein China alphasatellite (SiYVCNA) available in the databases with the highest levels of identity to an isolate (FN806782) originating from China and SiYVCNA originating from Nepal [20]. The type 1 alpha shared 98.5%-100% sequence identity with themselves. This indicates that the alphasatellites identified here are isolates of SiYVCNA. These sequences show the typical arrangement of alphasatellites, containing a single large open reading frame with a coding capacity of 315 amino acids in the virion-sense (coordinates 80-1027 for both), a region of sequence rich in adenine with 51% adenine content and a predicted hairpin structure containing the nonanucleotide sequence motif TAGTATTAC, within the loop, typical of nanoviruses [21]. The type 2 alphasatellites showed highest similarity (89%) with Gossypium mustelinum symptomless alphasatellite isolate from Pakistan (FR772087). With the type 3 alphasatellite sequences, a maximum sequence identity of 91.4% was noted with an isolate of Gossypium darwinii symptomless alphasatellite (GDarSLA) (KM103525), from Multan, Pakistan. The Rep of type 3 (295 AA) contained 20 amino acid residues less than type 1, and type 2. A phylogenetic analysis, based upon an alignment of the complete nucleotide sequences of the alphasatellites identified here with alphasatellite sequences from the database (Table 2) is shown in Figure 2. In the tree the alphasatellite isolates segregate with the previously characterized alphasatellite isolates.

Table 1: GenBank accession numbers of selected begomovirus sequences used in this study for analysis.

Begomoviruses	Abbreviation	Accession number
Bhendi yellow vein Bhubhaneswar virus - [India- Orissa-2003]	BYVBhV-[IN-Ori-03]	FJ589571
Bhendi yellow vein mosaic virus - India [India- Madurai]	BYVMV-IN[IN-Mad]	AF241479
Bhendi yellow vein mosaic virus - [India- Maharashtra-NOL751]	BYVMV-[IN-Mah-NOL751]	EU589392
Bhendi yellow vein mosaic virus - Haryana [India- Haryana - 2007]	BYVMV-Har[IN-Har-07]	FN645923
Bhendi yellow vein mosaic virus - Pakistan[Pakistan-Faisalabad 201-1995]	BYVMV-[PK-Fai201-95]	AJ002451
Bhendi yellow vein mosaic virus - Tamil Nadu[India-Coimbatore 4-2004]	BYVMV-TN[IN-Coi4-04]	FJ179372

Bhendi yellow vein mosaic virus - Thanagan [India-Thanagan-2005]	BYVMV-Tha[IN-Tha-05]	FJ176235
Mesta yellow vein mosaic Bahraich virus - [India- Bahraich-2007]	MeYVMBaV-[IN-Bah-07]	EU360303
Mesta yellow vein mosaic virus - Andhra Pradesh[India-Amadalavalasa 27-2008]	MeYVMV-And[IN-Ama27-08]	FJ159269
Mesta yellow vein mosaic virus - [Pakistan-Changa Manga-2009]	MeYVMV-[PK-CM-09]	FR715681
Mesta yellow vein mosaic virus - Bengal [India- Bongaon-Leaf curl-2007]	MeYVMV-Ben[IN-Bon-LC-07]	FJ345400
Okra enation leaf curl virus - [India-Sonipat EL10-2006]	OELCuV-[IN-SonEL10-06]	GU111996
Okra mottle virus - [Brazil-6319-2008]	OMoV-[BR-6319-08]	EU914817
Okra yellow crinkle virus - Cameroon [Cameroon- Muea-2008]	OYCrV-CM[CM-Mue-08]	FM210275
Okra yellow crinkle virus - [Cameroon-Njombe 5-2007]	OYCrV-[CM-Njo5-07]	HE793424
Okra yellow crinkle virus - Mali [Mali-Bamako 4-2006]	OYCrV-ML[ML-Bam4-06]	EU024119
Okra yellow mosaic Mexico virus - [Mexico- Mazatepec 3-2004]	OYMV-[MX-Maz3-04]	DQ022611

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4. DISCUSSION

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According to a study by Chandran [22] the association of Okra enation leaf curl alphasatellite

(OELCA) with OELCV was discovered. On the other hand, the alphasatellites identified in this study shows the association of alphasatellites other than OELCA with OELCV which causes enation and leaf curling in okra plants. Some alphasatellites have been shown to reduce the symptoms induced by virus infection by specifically down-regulating beta satellite levels, thus possibly allowing the infected plant to survive longer and allowing greater time for vector-mediated spread [23]. Another study has shown that at least some alphasatellites encode a Rep protein which can suppress post-transcriptional gene silencing, indicating that they are involved in overcoming plant host defences [11]. It is therefore difficult to say what would be the effect of the introduction of an alphasatellite into the agroecosystem of Punjab (India). The role of alphasatellites in the etiology of begomoviral diseases is largely unclear [24,25], but recently, the Rep of two alphasatellites, *Gossypium darwinii* symptomless alphasatellite (GDarSLA) and *Gossypium mustelinum* symptomless alphasatellite (GMusSLA), have been shown to suppress RNA silencing [11]. It has been demonstrated that they can reduce the accumulation of the helper virus [26], possibly by competing for cellular resources, which as a result attenuates the severity of the disease, playing a role similar to that of the defective interfering DNAs [24]. The identification of alphasatellites produced in this study will be helpful in different approaches being used for the development of strategies against the begomoviruses and their associated satellite molecules.

Table 2: GenBank accession numbers of selected alphasatellite sequences used in this study for analysis.

Alphasatellite	Abbreviation	Accession number
Sida yellow vein disease associated DNA 1 complete sequence	SiYVCNA-[China:Y340:sida]	FN806782
Sida yellow vein China alphasatellite clone J25.16, complete	SiYVCNA-[Jp:tom:J25.16]	KC677735
Sida yellow vein China alphasatellite isolate K32	SiYVCNA-[Jp:Cuc:K32]	KJ466050
<i>Ageratum conyzoides</i> symptomless alphasatellite complete sequence	SiYVCNA-[Pak:Okra:Neo25]	HG518790
Okra leaf curl alphasatellite	OLCA-[Pak:Gossy:KL-68]	HE966420
<i>Ageratum conyzoides</i> associated symptomless virus alphasatellite	AgCSVA-[Pak:Hol:HYB-3]	FR772085
Bhendi yellow vein mosaic virus-associated alphasatellite	BYVVA-[Ind:okra:10]	JX183091
<i>Gossypium mustelinum</i> symptomless alphasatellite	GMusSLA-[Pak:Hol:YBAPA-3]	FR772087
<i>Gossypium davidsonii</i> symptomless alphasatellite	GDavSLA-[Pak:Must- alphaB-1B]	EU384655
<i>Gossypium mustelinum</i> symptomless	GMusSLA-[Pak:Gossy:Lob-1]	FJ218496

alphasatellite[Gossypium lobatum]		
Gossypium mustelinum symptomless alphasatellite [Gossypium gossypoides]	GMusSLA-[Pak:Gossyp-4]	FJ218494
Gossypium mustelinum symptomless alphasatellite isolate	GMusSLA-[Pak:Punc- alphaB-9]	EU384663
Gossypium darwinii symptomless alphasatellite isolate Rh-4	GDarSLA[Ind:Gossy:Rh-4]	KM103525

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