

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

Gurpreet Kaur¹, Navraj Kaur Sarao¹, Abhishek Sharma^{2*} and Irfan Khan³

¹School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana, Punjab, India

²Department of Vegetable Science, Punjab Agricultural University, Ludhiana, Punjab, India

³Department of Plant Pathology, Punjab Agricultural University, Ludhiana, Punjab, India

gurpreet.sandhu0508@gmail.com , abhishek@pau.edu

ABSTRACT

The young leaf samples of disease affected okra plant were collected from okra growing regions of Punjab, India. The full genome of the virus causing the disease was amplified using Rolling Circle amplification (RCA) and cloned. The full sequence of the virus was determined after sequencing. Sequence analysis and nucleotide (ntd) comparison revealed that the viral genome is 2,741 bp in length and showed the highest nucleotide identities of 91-99% with *Okra enation leaf curl virus* (OELCV) which is severely affecting okra in India. The plants possessing OELCV was also shown to contain the alphasatellites. Four out of six alphasatellites associated with the virus showed the similarity with Sida yellow vein China alphasatellite which was earlier reported in Nepal and China. One alphasatellite showed the 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. Due to high content of tryptophan and lysine, okra plays a significant role in balanced diet. The immature fruits are used as salads, soups and stews [1]. It has been reported that okra is vulnerable to at least 19 plant viruses [2,3] and these viruses pose serious constraints to okra production. Among these viruses *yellow vein mosaic virus* (YVMV) and *okra enation leaf curl virus* (OELCV) causes significant losses in the okra production.

Kulkarni reported the occurrence of yellow vein mosaic disease for the first time in 1924 in Bombay province [4] while the okra enation leaf curl disease (OELCD) was first reported from Bangalore in early 1980's. Based on the stage of developmental stage of plant, about 50 to 90 per cent drop in the yield has been observed [5, 6]. The typical symptoms of OELCD include curling of leaves, thickening of vein and reduction in leaf size [7]. The virus causing the disease belongs to a species of genus Begomovirus, family Geminiviridae [8]. The genome of viruses in the genus Begomovirus is either a bipartite (having two genomic components known as DNA-A and DNA-B) or a monopartite (having a single component DNA-A). The DNA-A and DNA-B have equal size (~2.8 kb). Monopartite begomoviruses, in addition to main helper genome DNA-A, contain additional circular ssDNA satellite molecules known as beta satellite and alphasatellite having half the genome size of DNA-A. These satellite molecules do not show any similarity to the genome of helper virus but rely on the helper virus for replication [9]. Beta satellites play role in pathogenicity and symptom induction but the role of alphasatellites in this aspect is not clear [10,11]. The association of alphasatellites with a yellow vein disease in *Ageratum conyzoides* was first reported in 1999 [12]. Alphasatellite genomes are approximately 1,375 nts and have an A-rich region and a predicted hairpin structure. It also encodes an ORF (alpha-Rep) which translates into amino acid protein having the molecular weight of ~37 kDa. This encoded protein bears resemblance to Rep protein of nanovirus. Although the function of alphasatellites is not known but in early stages of infection, they can reduce the disease symptoms caused by begomovirus-beta satellite complexes [13]. The aim of this study was to identify and characterize the alphasatellite molecules associated with Okra enation leaf curl virus causing okra OELCD of okra from Punjab (India).

2. MATERIAL AND METHODS

Sample Collection

Leaves samples of six symptomatic okra plants showing leaf curling, typical yellowing and thickening of veins were collected from distinct locations and genomic DNA was isolated using the modified cetyl trimethyl ammonium bromide (CTAB) method [14] and stored in -20°C.

RCA and PCR-mediated amplification

The samples were initially analysed by PCR with a pair of degenerate primers AV494 and AC1048 corresponding to the coat protein region (CP) [15] showed the presence of a begomovirus while for the amplification of the alphasatellites, a specific pair of primers DNA101/DNA102 [16] was used. PCR reaction was carried out in a reaction volume of 25 µl. In all PCR reactions 100ng of DNA and 0.2 µM of primers were used along with 1X PCR buffer, 0.2 mM dNTPs and 1.5 mM MgCl₂ and 1 unit of Taq DNA polymerase. All PCR amplifications were carried out in a programmable DNA thermocycler (Mastercycler Gradient-ependorf™) with an initial denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 1 min, 58°C for 2 min and 72°C for 3 min followed by final extension at 72°C for 10 min. The PCR amplified fragments were resolved on 1% agarose gel and visualized in a gel documentation system (Alpha Imager HP, USA) after staining with ethidium bromide. The samples which failed to show any amplification were subjected to RCA [17] with φ29 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.

Cloning

PCR products of both CP (~570 bp) and alphasatellites (~1.4 kb) were purified from the gel using the Nucleospin Gel and PCR clean-up kit (Macherey-Nagel GmbH & Co. Germany). The products after the purification of gel were cloned into the pGEM-T Easy vectors which were then sequenced. For the full genome cloning, the virus genome was amplified by RCA. The RCA product was then digested with EcoRV for 3 h at 37°C with 5 U of the enzyme. The resultant product was analysed on a 1% agarose gel, which indicated the presence of ~2.7 kb fragment which was then purified and cloned into pJET1.2 vector. Restriction digestion was done to confirm the clone. 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) [18]. The similarity of nucleotide sequences was initially depicted by using the BLAST program available at the National Centre for Biotechnology Information (NCBI) (<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. To calculate the pairwise nucleotide sequence identity percentage, the Clustal W algorithm [19] available in MegAlign (Lasergene, DNASTAR, Madison, WI, USA) was used. For the phylogenetic analysis, the best model that fit the data was tested for full genome and alphasatellite sequences using Mega 7 software [20]. To describe the best substitution pattern, out of maximum likelihood fits of 24 different nucleotide substitution models, the model with lowest BIS score (Bayesian Information Criterion) was adopted and by using one thousand bootstrap iterations phylogram was developed. The best base substitution model selected for full genome was HKY (Hasegawa-Kishino-Yano) and for the alphasatellites TN93 (Tamura-Nei) was used. The ORFfinder tool available in NCBI was used to find ORF in the sequences (<http://www.ncbi.nlm.nih.gov/orffinder/>).

4. RESULTS

PCR detection and sequencing

PCR with specific primers (AV494 and AC1048) in all the six plants resulted in the amplification of fragments of size ~570 bp which were cloned and sequenced. The partial sequencing indicated that all the sequences were identical (results not shown). Therefore, only one sample was selected for the cloning of full genome. The full genome was cloned and sequenced. The size of the genome was determined to be 2,741 ntd and is submitted in the databases under the accession number KP208672.

Full genome analysis

The nucleotide comparisons showed that the full genome is 91-99% identical to all the OELCV nucleotide sequences present in the NCBI database with the highest similarity to an OELCV isolate emerged from Gandhinagar, India (KC019308). The organization of the genome has characteristic

features of a begomovirus. The 2.7 kb genome consists of six open reading frames (ORFs). It encodes two ORFs (AV1 and AV2) in virion sense and four ORFs (AC1, AC2, AC3 and AC4) in complementary sense orientation. The coat protein (CP) and V2 proteins are encoded by virion-sense ORFs whereas replication enhancer protein, transcription activator protein, Rep and C4 protein are encoded by complementary sense strand. An intergenic region (IR) which contains a nonanucleotide sequence “TAATATTAC” that form a stem-loop structure is present between the virion- and complementary-sense ORFs. This non-coding nonanucleotide motif plays role in replication of the genome [21]. The full nucleotide sequence of the clone produced in this study was aligned with selected nucleotide sequences of DNA-A of begomoviruses available in the databases (Table 1). The pairwise nucleotide identity percentage matrix (Figure 1) and a phylogenetic tree constructed from the alignment shows that the virus obtained from the okra segregates with the OELCV sequence used in alignment, justifying it as an isolate of OELCV (Figure 2).

Figure 1: The pairwise nucleotide identity percentage matrix of the cloned DNA-A sequence (KP208672) produced in this study and selected begomovirus sequences obtained from the database.

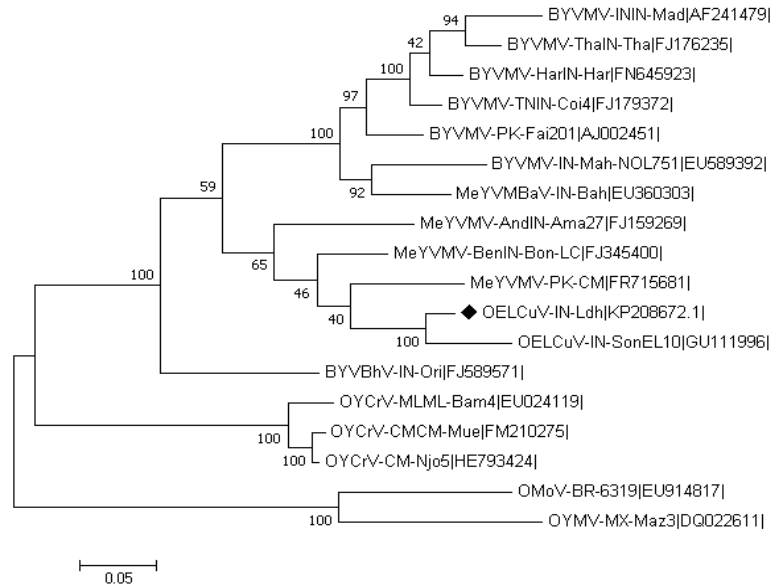


Figure 2: Maximum likelihood tree was depicted from cloned DNA-A sequence produced in this study (labeled with black diamond) and selected begomovirus sequences obtained from the database under HKY model (BIC = 40821.280; INL = -20207.59; AIC = 40491.247; freqA = 0.263; freqT = 0.301; freqC = 0.201; freqG = 0.233; R = 1.093). The tree is unrooted and the number on nodes represent the percentage bootstrap values (1000 replicates).

Alphasatellites analysis

The six alphasatellites obtained from okra infected with OELCV, were determined to be 1,319-1,374 ntd, in length. The four alphasatellite sequences [KJ614231, KJ843304, KJ843305 and KM108329] showed 94.1% to 98.1% nucleotide sequence identity with isolates of Sida yellow vein China alphasatellite (SiYVCNA) (FN806782) originating from China and SiYVCNA originating from Nepal [22]. These alphasatellites shared 98.5%-100% sequence identity with themselves. This inferred that the alphasatellites are isolates of SiYVCNA. These sequences show the typical organization of alphasatellites, having a single ORF which encodes Rep protein (315 amino acids) in the virion-sense, an A-rich region with 51% adenine composition and a hairpin loop having nonanucleotide sequence "TAGTATTAC" characteristic of nanoviruses [23]. The alphasatellite KJ843306 showed 89% similarity with *Gossypium mustelinum*, the symptomless alphasatellites from Pakistan. For the alphasatellite sequence KJ843307, a nucleotide identity of 91.4% was observed with an isolate of *Gossypium darwinii*, the symptomless alphasatellite (GDarSLA) (KM103525), from Pakistan. The Rep of this alphasatellite (295 AA) contained 20 amino acid residues less than the other alphasatellites. The pairwise nucleotide identity percentage matrix (Figure 3) and a phylogenetic tree (Figure 4) constructed from the alignment of the alphasatellite sequences produced in this study with alphasatellite sequences from the database (Table 2) confirmed that these molecules are the isolates of previously identified alphasatellites.

Table 1: The accession number of the begomovirus sequences obtained from GenBank database used for analysis in this study.

Begomoviruses	Location	Abbreviation	Accession number
Bhendi yellow vein Bhubhaneswar virus	Orissa, India	BYVBhV-[IN-Ori-03]	FJ589571
Bhendi yellow vein mosaic virus	Madurai, India	BYVMV-IN[IN-Mad]	AF241479
Bhendi yellow vein mosaic virus	Maharashtra, India	BYVMV-[IN-Mah- NOL751]	EU589392
Bhendi yellow vein mosaic virus	Haryana, India	BYVMV-Har[IN-Har-07]	FN645923
Bhendi yellow vein mosaic virus	Faisalabad, Pakistan	BYVMV-[PK-Fai201-95]	AJ002451
Bhendi yellow vein mosaic virus	Coimbatore, Tamil Nadu, India	BYVMV-TN[IN-Coi4-04]	FJ179372
Bhendi yellow vein mosaic virus	Thanagan, India	BYVMV-Tha[IN-Tha-05]	FJ176235
Mesta yellow vein mosaic Bahaich virus	Bahaich, India	MeYVMBaV-[IN-Bah-07]	EU360303
Mesta yellow vein mosaic virus	Amadalavalasa, India	MeYVMV-And[IN- Ama27-08]	FJ159269
Mesta yellow vein mosaic virus	Changa Manga, Pakistan	MeYVMV-[PK-CM-09]	FR715681
Mesta yellow vein mosaic virus	Bongaon, Bengal, India	MeYVMV-Ben[IN-Bon- LC-07]	FJ345400
Okra enation leaf curl virus	Sonipat, India	OELCuV-[IN-SonEL10-06]	GU111996
Okra mottle virus	Brazil	OMoV-[BR-6319-08]	EU914817
Okra yellow crinkle virus	Mue, Cameroon	OYCrV-CM[CM-Mue-08]	FM210275
Okra yellow crinkle virus	Njombe, Cameroon	OYCrV-[CM-Njo5-07]	HE793424
Okra yellow crinkle virus	Bamako, Mali	OYCrV-ML[ML-Bam4-06]	EU024119
Okra yellow mosaic Mexico virus	Mazatepec, Mexico	OYMV-[MX-Maz3-04]	DQ022611

5. DISCUSSION

Okra is an important vegetable crop in India. It has high commercial value due to its nutritional properties. The productivity of this crop is susceptible to several biotic and abiotic stresses. The *Okra enation leaf curl virus* is one of the biotic stresses effecting cultivation of okra worldwide. The OELCV is forming complexes with other satellite molecules which alter the symptoms. According to a study by Chandran [24] 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 typical enation, reduction in size and leaf curling in okra plants. Previously Hameed and coworkers [25] found the association of OELCV with Cotton leaf curl Multan alphasatellite infecting cotton in Pakistan (FN658728). Similarly SiYVCNA has been found associated with *Tomato Yellow Leaf Curl Virus* and *Ageratum Yellow Vein Virus* in Japan [26]. The function of the alphasatellites is not clear till date but in some cases these satellites have been shown to decrease the symptoms produced by virus infection by reducing the level of beta satellites. The beta satellites help the helper virus in the symptom induction while alphasatellites down-regulate the activity of the beta satellites by decreasing their replication activity. Thus allows the virus

infected plant to live for long time and in turn provides more time for vector-mediated spread [27]. In another study it has been observed that the Rep protein involved in the post-transcriptional gene silencing, which overcome the plant host defence mechanism. Recently, the Rep of *Gossypium darwinii* symptomless alphasatellite (GDarSLA) and *Gossypium mustelinum* symptomless alphasatellite (GMusSLA), have been shown to suppress RNA silencing [13]. These two alphasatellites were previously found associated with cotton leaf curl virus. It is therefore difficult to say what would be the effect of the introduction of an alphasatellite into the agroecosystem of Punjab (India). 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
Ageratum conyzoides symptomless alphasatellite complete sequence	SiYVCNA-[Pak:Okra:Neo25]	HG518790
Okra leaf curl alphasatellite	OLCA-[Pak:Gossy:KL-68]	HE966420
Ageratum conyzoides associated symptomless virus alphasatellite	AgCSVA-[Pak:Hol:HYB-3]	FR772085
Bhendi yellow vein mosaic virus-associated alphasatellite	BYVVA-[Ind:okra:10]	JX183091
Gossypium mustelinum symptomless alphasatellite	GMusSLA-[Pak:Hol:YBAPA-3]	FR772087
Gossypium davidsonii symptomless alphasatellite	GDavSLA-[Pak:Must- alphaB-1B]	EU384655
Gossypium mustelinum symptomless alphasatellite[Gossypium lobatum]	GMusSLA-[Pak:Gossy:Lob-1]	FJ218496
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

6. Conclusion

There is large diversity among the helper virus and their associated satellite molecules. This association of helper virus is helping them to generate new complex disease symptoms. The phenomenon of recombination contributes towards the evolution of such different complexes. For the study of the recombination events the knowledge of the molecules participating with the virus is must. Therefore, study of the identity, distribution and molecular variability of satellite molecules associated with the viruse has become very important.

7. ACKNOWLEDGEMENT

We thank the University Grant Commission, Government of India, for funding this study.

8. REFERENCES

1. Salameh NM. Genetic diversity of okra (*Abelmoschus esculentus* L.) landraces from different agro-ecological regions revealed by AFLP analysis. American-Eurasian J Agric Environ Sci. 2014; 14:155–60.
2. Brunt A, Crabtree K, Gibbs A. Viruses of Plants–Description and lists from the VIDE Database. CAB International (Wallingford, UK). 1990; 233–34.
3. Swanson MM, Harrison BD. Serological relationships and epitope profiles of isolates of okra leaf curl geminivirus from Africa and the Middle East. Biochimie. 1993; 75: 707-11.
4. Kulkarni CS. Mosaic and other related diseases of crops in the Bombay Presidency. Poona Agri Coll Mag; 1924.
5. Sastry KSM, Singh SJ. Effect of yellow vein mosaic virus infection on growth and yield of okra crop. Indian Phytopathol. 1974; 27: 294-97.
6. Sinha SN, Chakrabartha AK. Effect of yellow vein mosaic virus infection on okra seed production. Seed Sci Res. 1978; 6: 67-70.
7. Sanwal SK, Singh M, Singh B and Naik PS. Resistance to yellow vein mosaic virus and okra enation leaf curl virus: challenges and future strategies. Curr Sci. 2014. 106: 470–71.
8. Fauquet CM, Stanley J. Revising the way we conceive and name viruses below the species level: a review of geminivirus taxonomy calls for new standardized isolate descriptors. Arch Virol. 2005; 150: 2151-79.
9. Murrant AF, Mayo MA. Satellites of plant viruses. Annu Rev Phytopathol. 1982; 20: 49-70.
10. Mansoor S, Khan SH, Bashir A, Saeed M, Zafar YM, Briddon RW, Stanley J, Markham PG. Identification of a novel circular single-stranded DNA associated with cotton leaf curl disease in Pakistan. Virol. 1999; 259: 190-99.
11. Saunders K, Briddon RW, Stanley J. Replication promiscuity of DNA-beta satellites associated with monopartite begomoviruses; deletion mutagenesis of the Ageratum yellow vein virus DNA-beta satellite localizes sequences involved in replication. J Gen Virol. 2008;

89: 3165-72.

12. Saunders K, Stanley J. A nanovirus-like DNA component associated with yellow vein disease of *Ageratum conyzoides*: evidence for interfamilial recombination between plant DNA viruses. *Viol.* 1999; 264:142-52.
13. Nawaz-Ul-Rehman MS, Nahid N, Mansoor S, Briddon RW, Fauquet CM. Post-transcriptional gene silencing suppressor activity of two non-pathogenic alphasatellites associated with a begomovirus. *Viol.* 2010; 405: 300-08.
14. Doyle JJ, Doyle JL. Isolation of plant DNA from fresh tissue. *Focus* 2. 1990; 13-15.
15. Wyatt SD, Brown JK. Detection of subgroup III geminivirus isolates in leaf extracts by degenerate primers and polymerase chain reaction. *Phytopathol J.* 1996; 86: 1288-93.
16. Bull SE, Briddon RW, Markham PG. Universal primers for the PCR-mediated amplification of DNA 1: a satellite-like molecule associated with begomovirus-DNA β complexes. *Mol Biotechnol.* 2003; 23: 83-86.
17. Inoue-Nagata AK, Albuquerque LC, Rocha WB, Nagata T. A simple method for cloning the complete begomovirus genome using the bacteriophage phi29 DNA polymerase. *J Virol Methods.* 2004; 116: 209-11.
18. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucl Acid S.* 1999; 41: 95-98.
19. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 1994; 22: 4673-80.
20. Tamura S, Stecher G and Kumar K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Biol Evol.* 2016; 33: 1870-74.
21. Orozco BM, Hanley-Bowdoin L. A DNA structure is required for geminivirus replication origin function. *J Virol.* 1996; 7: 148-58.
22. Shahid MS, Yoshida S, Khatri-Chhetri GB, Briddon RW, Natsuaki KT. Complete nucleotide sequence of a monopartite begomovirus and associated satellites infecting *Carica papaya* in Nepal. *Virus Genes.* 2013; 46: 581-84.
23. Briddon RW, Bull SE, Amin I, Mansoor S, Bedford ID, Rishi N, Siwatch SS, Zafar Y, Abdel-Salam AM, Markham PG. Diversity of DNA 1: A satellite-like molecule associated with monopartite begomovirus-DNA β complexes. *Viol.* 2004; 324: 462-74.
24. Chandran SA, Packialakshmi RM, Subhalakshmi K, Prakash C, Poovannan K, Nixon Prabu A, Gopal P, Usha R. First report of an alphasatellite associated with *Okra enation leaf curl virus*. *Virus Genes.* 2013; 46(3): 585-87.
25. Hameed U, Rehman M, Herrmann HW, Haider MS and Brown JK. First Report of *Okra enation leaf curl virus* and Associated Cotton leaf curl Multan betasatellite and Cotton leaf curl Multan alphasatellite Infecting Cotton in Pakistan: A New Member of the Cotton Leaf Curl Disease Complex. *Plant Dis Notes.* 2014. 98:1447.

26. Shahid MS, Ikegami M, Waheed A, Briddon RW and Natsuaki KT. Association of an Alphasatellite with *Tomato Yellow Leaf Curl Virus* and *Ageratum Yellow Vein Virus* in Japan is Suggestive of a Recent Introduction. *Viruses*. 2014. 6: 189–200.
27. Idris AM, Shahid MS, Briddon RW, Khan AJ, Zhu JK, Brown JK. An unusual alphasatellite associated with monopartite begomoviruses attenuates symptoms and reduces beta satellite accumulation. *J Gen Virol*. 2011; 92: 706–17.