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

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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 mustelinium 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 in 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 begomoviruses 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 alongwith 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).

Table 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.

| Percent Identity | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  | 16  | 17  | 18  |
|------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1                |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 2                |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 3                |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 4                |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 5                |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 6                |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 7                |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 8                |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 9                |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 10               |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 11               |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 12               |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 13               |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 14               |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 15               |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 16               |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 17               |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 18               |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |

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 nt 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 mustilinum, the symptomless alphasatellites from Pakistan. For the alphasatellite sequence KJ843307, a nucleotide identity of 91.4% was observed with an isolate of Gossypium darwinnii, 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.
Figure 3: The pairwise nucleotide identity percentage matrix of the cloned alphasatellite sequences (KJ614231, KJ843304, KJ843305, KJ843306, KJ843307 and KM108329) produced in this study and selected alphasatellite sequences obtained from the database.

Figure 4: Maximum likelihood tree was depicted from cloned alphasatellite sequences produced in this study (labeled with black diamond) and selected alphasatellite sequences obtained from the database under TN93 model (BIC = 14638.95; INL = -7124.56; AIC = 1427.27; freqA = 0.296; freqT = 0.284; freqC = 0.195; freqG = 0.223; R = 1.235). The tree is unrooted and the number on nodes represent percentage bootstrap values (1000 replicates).
Table 1: The accession number of the begomovirus sequences obtained from GenBank database used for analysis in this study.

<table>
<thead>
<tr>
<th>Begomoviruses</th>
<th>Location</th>
<th>Abbreviation</th>
<th>Accession number</th>
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<tr>
<td>Bhendi yellow vein Bhubhaneshwar virus</td>
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<td>BYV BhV-[IN-Ori-03]</td>
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<td>Madurai, India</td>
<td>BYV VM-IN[IN-Mad]</td>
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<tr>
<td>Bhendi yellow vein mosaic virus</td>
<td>Maharashtra, India</td>
<td>BYV VM-[IN-Mah- NOL751]</td>
<td>EU589392</td>
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<td>Bhendi yellow vein mosaic virus</td>
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<td>BYV VM-Har[IN-Har-07]</td>
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<td>Bhendi yellow vein mosaic virus</td>
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<td>BYV VM-[PK-Fai201-95]</td>
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<td>BYV VM-TN[IN-Coi4-04]</td>
<td>FJ179372</td>
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<td>Bhendi yellow vein mosaic virus</td>
<td>Thanagan, India</td>
<td>BYV VM-Tha[IN-Tha-05]</td>
<td>FJ176235</td>
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<td>Mesta yellow vein mosaic Bahraiich virus</td>
<td>Bahraiich, India</td>
<td>MeYVMBaV-[IN-Bah-07]</td>
<td>EU360303</td>
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<tr>
<td>Mesta yellow vein mosaic virus</td>
<td>Amadalavalasa, India</td>
<td>MeYVM-And[IN- Ama27-08]</td>
<td>FJ159269</td>
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<td>Mesta yellow vein mosaic virus</td>
<td>Changa Manga, Pakistan</td>
<td>MeYVM-[PK-CM-09]</td>
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<td>Mesta yellow vein mosaic virus</td>
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<td>MeYVM-Ben[IN-Bon- LC-07]</td>
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<td>Okra enation leaf curl virus</td>
<td>Sonipat, India</td>
<td>OEL CuV-[IN-SonEL10-06]</td>
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<td>Brazil</td>
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<td>Okra yellow crinkle virus</td>
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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 davidii symptomless alphasatellite (GDarSLA) and Gossypium mustelinium 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.

<table>
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<th>Alphasatellite</th>
<th>Abbreviation</th>
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<td>Sida yellow vein disease associated DNA 1 complete sequence</td>
<td>SiYVCNA-[China:Y340:sida]</td>
<td>FN806782</td>
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<tr>
<td>Sida yellow vein China alphasatellite clone J25.16, complete</td>
<td>SiYVCNA-[Jp:tom:J25.16]</td>
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<td>Ageratum conyzoides symptomless alphasatellite complete sequence</td>
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<td>HG518790</td>
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<td>Okra leaf curl alphasatellite</td>
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<td>HE966420</td>
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<td>Bhendi yellow vein mosaic virus-associated alphasatellite</td>
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<td>GDavSLA-[Pak:Must- alphaB-1B]</td>
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<td>GMusSLA-[Pak:Punc- alphaB-9]</td>
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<td>Gossypium darwinii symptomless alphasatellite isolate Rh-4</td>
<td>GDarSLA[Ind:Gossy:Rh-4]</td>
<td>KM103525</td>
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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. Therefore, study of the identity, distribution and molecular variability of satellite molecules has become very important to obtain successful management of the begomoviruses. The identification of alphasatellites produced in this study will be helpful in approaches being used for the development of strategies against the begomoviruses and their associated satellite molecules.

7. ACKNOWLEDGEMENT
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8. REFERENCES


