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Recombination analysis and *in silico* structural characterization of βC1 protein gene from Okra leaf curl betasatellite

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Abstract

Okra leaf curl and yellow vein mosaic disease caused by begomovirus and satellite DNA complexes is a major constraint of okra (*Abelmoschus esculentus* L.) production in India. We performed recombination analysis, *in silico* structural characterization of βC1 protein gene from okra leaf curl betasatellites isolated from Hyderabad and discussed the recombination events, hot spots and break points. The results of recombination and *in silico* structural characterization indicates that the virus associated with Okra leaf curl disease in Southern India could be a variant of Bhendi yellow vein mosaic virus associated betasatellite. Six major and minor inter-specific recombination events, hot spots and breakpoints were identified in Okra leaf curl betasatellites, Hyderabad isolate. The closest major and minor recombination was observed with Bhendi yellow vein betasatellite-Muthupatti and Malvastrum yellow vein betasatellite-China. Recombination was also observed with Cotton leaf curl betasatellite-Rajasthan and Cotton leaf curl Gezira betasatellite-Sudan. The predicted secondary structure indicates a mixed class with helices (29.7%), extended sheets (33.9%) and loops (36.4%).The predicted tertiary model of Okra leaf curl betasatellite has three α helix: one short helix, H1 (37-41) and two long helices, H2 (62-74) & H3 (84-97). This is the first structural characterization for the βC1 protein gene associated with Okra leaf curl disease.

Keywords: Begomovirus; Okra leaf curl disease; βC1gene; betasatellites; recombination analysis; structure characterization.
Abbreviations: OLCD_Okra leaf curl disease; OLCuB_Okra leaf curl betasatellite; CLCuB_Cotton leaf curl betasatellite; BYVMB_Bhendi yellow vein mosaic betasatellite; CLCuGB_Cotton leaf curl Gezira betasatellite; RDP3_Recombination detection programme 3.

Introduction

Okra (*Abelmoschus esculentus* L.) is an important vegetable crop belonging to family *Malvaceae*. It is widely cultivated in various parts of the world not only for human consumption but also in industrial use as fiber. India ranks world largest producer of okra with around 3.5 million tones as per Food and Agriculture Organization report. Begomovirus causes yellow vein mosaic and leaf curl disease considered as the most serious disease threatening okra production in India. Naturally infected plants develop prominent symptoms like yellow vein mosaic, reduced fruit size, either upward or downward leaf curling with stunted plant growth. The association of both mono-partite as well as bipartite begomoviruses with yellow vein mosaic and leaf curl disease in okra has been reported in India (Jose and Usha, 2000; Jose and Usha, 2003; Ghosh et al., 2008;Venkataravanappa et al., 2011; Kumar et al., 2012; Sayed et al., 2013; Venkataravanappa et al., 2013). The bipartite begomovirus genome has two ssDNA molecules (~2.7 kb), known as DNA-A and DNA-B while monopartite begomovirus has only DNA-A with satellite molecule known as betasatellites. Betasatellites are small (~1.4kb), highly diverse with circular, ssDNA associated with monopartite begomoviruses, their replication, movement and transmission between plants are mediated by a helper virus (Briddon et al., 2003; Mansoor et al., 2003; Leke et al., 2013; Briddon and Stanley 2006; Briddon et al., 2008). Currently, several betasatellites and alfasatellites have been identified associated with okra yellow vein mosaic and leaf curl disease not only in India but also from South Western Cameroon and Oman (Venkataravanappa et al., 2011; Venkataravanappa et al., 2012; Chandran et al., 2013; Leke et al., 2013; Sayed et al., 2013; Akhtar et al., 2014). An association of recombinant Cotton leaf curl Bangalore virus was reported recently causing yellow vein and leaf curl disease in India. On the basis of recombination analysis result it was expected that this new begomovirus strain infecting okra might have been emerged via exchange of genetic material of Bhendi Yellow vein mosaic virus and Cotton leaf curl Multan virus (Venkataravanappa et al., 2013). The betasatellites has only one βC1 gene with highly conserved region (A-Rich) designated as satellite conserved region with ~150 base pair long size (Briddon et al., 2003). The βC1 gene has been

known for various functions like pathogenicity determinant (Saunders et al., 2004, Cui et al., 2005), binding DNA sequences in non-specific manner (Cui et al., 2005),virus movement (Saeed et al., 2007), RNA silencing suppressor (Kon et al., 2007), interaction with various host factors, disease symptoms inducer (Yang et al., 2008; Eini et al., 2009) and interfering with host gene expression (Andleeb et al., 2010; Cheng et al., 2011). Due to frequent recombination and mutation, multiple new strains of begomovirus have emerged during the last 50 years, causing complex disease in new hosts (Bigarre et al., 2001). Recently, a begomovirus causing Okra leaf curl disease (OLCD) in Mali, West Africa has been identified as a recombinant isolate of Cotton leaf curl Gezira virus and finally designated as Cotton leaf curl Gezira betasatellite (CLCuGB) (Kon et al., 2009; Leke et al., 2013). The genetic diversity and recombination analysis results of Cotton leaf curl Gezira virus and CLCuGB associated with OLCD indicated the interspecies recombination origin of CLCuGB isolates with close parents like Hollyhock leaf crumple virus and Tomato leaf curl Diana Virus (Tiendrebeogo et al., 2010). Recombination analysis and *in silico* structural characterization of βC1 gene from Okra leaf curl betasatellite (OLCuB) isolated from Hyderabad will help to find out the origin of recombination hot spots and possible emergence of new strains to cause disease not only in okra but also in other new hosts. So far, no structural information is available for βC1 protein gene of OLCuB. The recombination analysis and predicted structure will help to get valuable information about pathogenicity and structural function relationship. Based on the published information and nature of complex disease, this study was conducted for recombination analysis and *in silico* structural characterization of βC1 protein gene of OLCuB from Hyderabad, India.

Results

Recombination analysis of βC1 protein gene

Comprehensive recombination analyses of selected βC1 protein gene sequences were done using RDP3 (Martin et al., 2010). Six major and minor inter-specific recombination hot spots and breakpoints were identified in OLCuB Hyderabad isolate (Table 1, Fig. 1). The majority of βC1 protein gene sequences showed evidence of recombination. The major and minor recombination events were observed with Bhendi yellow vein mosaic betasatellite-Muthupatti (GenBank: NC_003405) and Malvastrum yellow vein betasatellite-China (GenBank: AJ744882) followed by Okra yellow vein mosaic betasatellite-Aurangabad (GenBank: GU233520) and Bhendi yellow vein betasatellite-Varanasi (GenBank: HM590506). Recombination was also observed in Cotton leaf curl virus betasatellite-Rajasthan (CLCuB-GenBank: HM146307) and Cotton leaf curl Gezira betasatellite-Sudan (GenBank: AY044142). Malvastrum yellow vein betasatellite-China (GenBank: AJ744882) showed expected recombination evidence with other unrelated betasatellites. In contrast, OLCuB exhibited a close recombination pattern with BYVMB which served as major parent and BYVMB or CLCuB could be major isolate in emergence of OLCuB in Hyderabad, Southern India.

Molecular modeling and structural analysis of βC1 protein gene of OLCuB and CLCuB

We did molecular modeling of βC1 protein gene from OLCuB and CLCuB to identify the structure based evolutionary relationship of OLCuB from their probable ancestor. Predicted secondary structure, constructed topology diagram and superimposed stereo image of βC1 protein gene OLCuB and CLCuB are shown in Fig. 2 A, B, C. The results indicated that there were no Cys residues in OLCuB but one Cys residues were observed in CLCuB without any disulphide bonds. The predicted secondary structure showed mixed class with helices (29.7%), extended sheets (33.9%) and loops (36.4%). The predicted tertiary model of OLCuB have three α helix: one short helix (H1: 37-41 and two long helices H2:62-74, H3:84-97). CLCuB also have three helices (H1: 34-39, H2: 72-75, H3: 80-96) and two anti-parallel β-sheets (Fig. 3). The total solvent accessible surface area was 7670Å^2 and 7836Å^2 respectively, computed for βC1 protein gene from OLCuB and CLCuB. Two nests were suggested by ProFunc Nest analysis in OLCuB, located at Asp 47-Tyr 50 and Gln 21-Gln 23. ProFunc analysis of CLCuB also suggested two nests; primary located at Asn71 (A), Met72 (A), and Phe73 (A) while secondary at Arg39 (A), Lys40 (A) and Ser41 (A). However, these nests are probably not accessible to solvent and no cleft formation observed. No known hits were found when Expasy's ScanProsite was run in the above sequence to find motifs and domain. Superimposition of βC1 protein gene from OLCuB and CLCuB model showed 32.2% identity and 57.6% similarity between them. The output of 3D2GO server showed the oxidoreductase activity as possible function for CLCuB and OLCuB with a confidence level of 0.61% and 0.12% respectively (Table 2).

Discussion

In India, Okra leaf curl and yellow vein mosaic disease have emerged as major constraint causing significant yield loss. It is reported that begomoviral genome sequences exchanged with other resulted in emergence of new strains causing complex disease on new hosts and posing serious threat to crop production. To reveal evolutionary links, explicit role of begomovirus in causing the leaf curl disease and possible roles of βC1 protein gene from OLCuB Hyderabad isolate with its previously reported ancestor (CLCuB), we have made an attempt using structural biology approach. Using the βC1 protein gene as a target, the computational analysis of structural and functional analysis of the gene was subjected to in depth i*n silico* analysis for mapping of recombinant points within gene and other functional domains. The present study reports the recombination analysis and predicted tertiary structure of βC1 protein gene associated with leaf curl disease of okra in Hyderabad, Southern India. In recombination analysis, six major and minor interspecific recombination events and hot spots and breakpoints were identified with OLCuB Hyderabad isolate which indicates that OLCuB could be a recombinant derivative of BYVMB or CLCuB causing leaf curl disease in Southern India. The modeled structure of βC1 protein gene predicts the oxidoreductase activity which is the most important function among DNA viruses. Oxidoreductase is a fundamental constitutive enzyme used in many biochemical pathways including central intermediary metabolism, cell survival and defense mechanisms. They catalyze oxidation or reduction reactions where electrons are transferred from one molecule to another molecule. Oxidoreductases were known to be coded by large dsDNA viruses (Kho et al., 2003). On the basis of structural analysis and molecular modeling of βC1 protein gene, possible evolutionary relationships and homology was observed in OLCuB and CLCuB. Our analysis result indicates that BYVMB and CLCuB could be the

Table 1. Recombination analysis of OLCuB Hyderabad isolate with selected betasatellites.

Recombinant Events	Parental sequences (% similarity)		Breakpoints (Nucleotide position)		*P*- Value	Recombination score
	Major	Minor	Start	End		
1	BYVB-NC_003405 (92.4)	MaYVB-AJ744882 (NA)	855	867	$7.67X10^{-6}$	0.393
2	OYVB-GU233520 (90.8)	BYVB-HM590506 (95.7)	1084	1224	$1.09X10^{-26}$	0.570
3	BYVB-NC_003405 (92.4)	CLCuGB-AY044142 (NA)	666	691	$2.87X10^{-6}$	0.460
4	BYVB-HM590506 (90.3)	MaYVB-AJ744882 (65.9)	874	914	$5.47X10^{-5}$	0.429
5	BYVB-GU111969 (90.2)	SYVB-AJ810094 (NA)	702	846	$4.34X10^{-14}$	0.510
6	OLCuB-GU111961 (86.8)	CLCuB-HM146307 (NA)	924	1053	$8.79X10^{-12}$	0.660

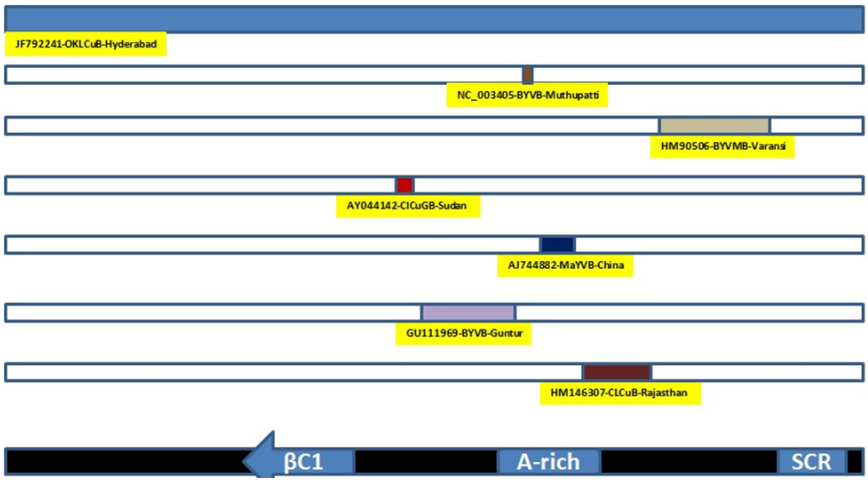

Fig 1. Analysis of Recombination for betasatellites isolated from okra: The bars represents the sequences of betasatellites with the likely origins of sequences shown in color, as indicated in the box. Only interspecific recombination, that is recombination between distinct betasatellites are shown. The betasatellites acronyms given are Okra Leaf Curl betasatellite (OLCuB), Bhendi yellow vein betasatellite (BYVB), Bhendi yellow vein mosaic betasatellite (BYVMB), Cotton Leaf Curl Gezira betasatellite (CLCuGB), Malvestrum yellow vein betasatellite (MaYVB), Cotton Leaf Curl betasatellite (ClCuB), The box bellow at the bottom of the diagram indicates the approximate position of the βC1 gene, the A-rich region and the satellite conserved region (SCR) features that are common to all betasatellites.

major betasatellites served as a source for the emergence of recombinant strain causing okra leaf curl disease in India. It is reported that this disease was more geographically distributed in Northern India and Pakistan (Venkataravanappa et al., 2011) But, recently the occurrence of okra leaf curl and yellow vein mosaic disease from Southern India has been reported (Sayed et al., 2013; Venkataravanappa et al., 2013). The association of begomovirus causing disease in okra have been published in many reports (Kon et al., 2009; Tiendrebeogo et al., 2010; Shih et al., 2009; Leke et al., 2013; Akhtar et al., 2014). In the Old World, monopartite begomovirus and associated betasatellite are affecting okra and other Malveaceous plants (Briddon et al., 2003; De La et al., 2006; Paprotka et al., 2010;Hernandez-Zepeda et al., 2007). While, in the New World recent reports suggests the association of bipartite begomovirus with okra disease in Northern India (Venkataravanappa et al., 2012). Recently, the associations of betasatellites and alphasatellites with begomoviruses have been reported (Leke et al., 2013; Akhtar et al., 2014). The betasatellite was identified as CLCuGB while alphasatellites (designated as Alpha-1 and Alpha-2) showed highest homology with Cotton leaf curl Gezira alphasatellite and Okra leaf curl Burkina Faso alphasatellite. This information itself emphasizes the begomovirus-betasatellite diversity level infecting okra in Western Africa (Leke et al., 2013).The geographical origin of okra and its associated diseases is still debatable, with the majority favoring a South Asian origin over a North African origin (Hamon et al., 1995). It has been reported that different betasatellites in India can cause disease in okra (Venkataravanappa et al., 2011; Venkataravanappa et al., 2013). Recently, an association of alphasatellites DNA with Okra enation leaf curl virus in okra plants has been identified for the first time from India (Chandran et al., 2013). Our results showed higher diversity among βC1 protein gene of betasatellites associated with OLCD in India than Africa. On the basis of published information it is expected that Southeast Asia could be the center of origin for begomoviruses associated betasatellites (Nawaz-ul-Rehman et al., 2009). There are many reports highlighting the presence of nonanucleotide (TAATATTAC) in the satellite conserved region of betasatellite (Guo et al., 2008; Tahir and Mansoor 2011; Singh et al., 2012; Venkataravanappa et al., 2012). Few distinct betasatellites from okra have been identified without any established correlation with functions and symptom appearance (like yellow vein or leaf curl/enation). This may be accredited to other betasatellites, helper virus, cultivars and varieties of okra, co-infections and

Table 2. Predicted functions for protein OLCuB and CLCuB using 3D2GO.

OLCuB			CLCuB		
GO Term	Description	Confidence	GO Term	Description	Confidence
GO:0005737	cytoplasm	0.16	GO:0016491	oxidoreductase activity	0.61
GO:0016020	membrane	0.14	GO:0009055	electron carrier activity	0.34
GO:0005886	plasma membrane	0.12	GO:0055114	oxidation reduction	0.33
GO:0016663	oxidoreductase activity, acting on other nitrogenous compounds as donors, oxygen as acceptor	0.12	GO:0051540	metal cluster binding	0.24
GO:0016661	oxidoreductase activity, acting on other nitrogenous compounds as donors	0.12	GO:0050662	coenzyme binding	0.20
			GO:0048037	cofactor binding	0.20

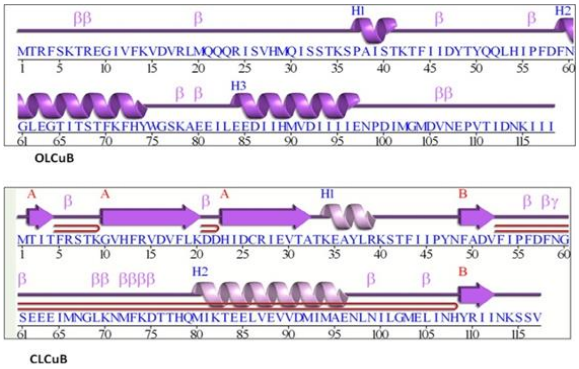

Fig 2. (A) Secondary structure βC1 protein gene.

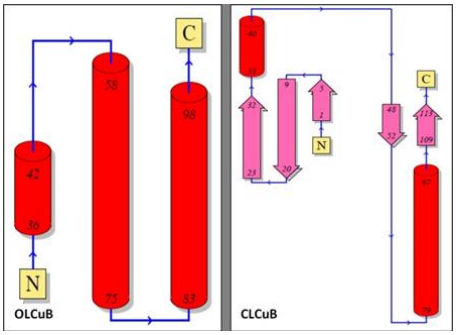

Fig 2 .(B). Topology diagram βC1 protein gene.

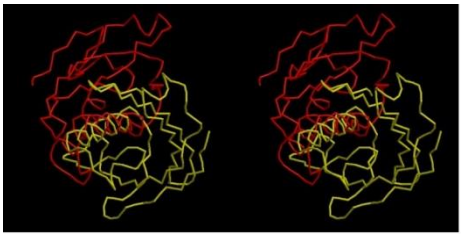

Fig 2. (C).Superimposed stereo images of βC1 protein gene of OLCuB (red) and CLCuB (Yellow).

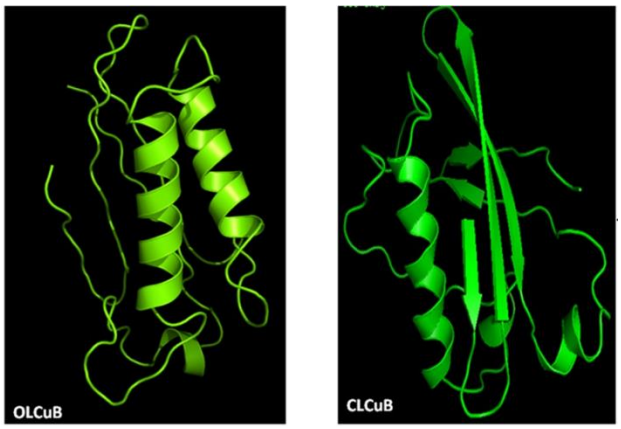

Fig 3. Tertiary structure image of βC1 protein gene of OLCuB and CLCuB drawn using PyMol.

presence of alphasatellites (Nawaz-ul-Rehman et al., 2010; Idris et al., 2011). Recombination is the most important molecular mechanism for the emergence of new viral strains. Presumably different strains can simultaneously infect host cells and exchange their genetic material. A recombinant virus infecting okra causing leaf curl symptoms from South Western Cameroon has been recently identified (Leke et al., 2013). Recombination is known to occur in geminiviruses and is probably the most important molecular mechanism for developing genetic changes that allow exploitation of new ecological niches. The RDP results clearly indicate that the OLCuB emerged as a recombinant from its major and minor parents CLCuB and BYVMB respectively. The above discussed genetic event can be validated well at the protein level, i.e. whether the OLCuB and CLCuB proteins have similarity at structural level or not. The structure of βC1 protein gene of CLCuB belongs to α/β mixed fold class whereas all-α topology is predominant in OLCuB. Evolutionary, the α/β fold class is the common structural ancestor of the rest major fold classes (α, β and α+β) (Orengo et al., 1999; Chandonia and Kim 2006). During the evolution of begomoviruses, inter-specific recombination has resulted in remarkable structural and functional diversity and is the major cause of new strains/species emergence causing disease complex in tropical and subtropical regions. Currently, there is no NMR or X-ray crystallographic structure available for βC1 protein gene and this is the first report about the predicted tertiary structure model of βC1 protein gene from okra leaf curl betasatellite to illuminate their evolutionary relationship.

Materials and methods

Plant material, PCR amplification, Cloning and Sequencing of betasatellites

Field survey was conducted during the cropping seasons in the major okra growing areas of Hyderabad and twenty symptomatic young plant leaves were collected from naturally infected okra exhibiting severe leaf curling followed by stunted plants growth. Total nucleic acid was isolated from symptomatic okra plants exhibiting typical leaf curl disease following published protocol (Jose and Usha 2000) and subjected to amplification by PCR using specific primers to identify the causal virus and betasatellites.. The primer sequence used for betasatellite amplification was 5'-GGT ACC ACT ACG CTA CGC AGC AGC C 3'(Forward) and 5'GGT ACC TAC CCT CCC AGG GGT ACA C-3'(Reverse). The PCR amplified product was gel purified, ligated into pGEMT-easy vector and transformed into competent cell of *E.coli* (strain DH5α) according to manufacturer's instruction (Promega). The recombinant clone was sequenced from both the directions using automated DNA analyzer (ABI Prism, Perkin Elmer) at JK Agri Genetics Ltd, Hyderabad, India; assembled and submitted in GenBank (GenBank: JF792241) and designated as OLCuB Hyderabad isolate (Sayed et al., 2013).

Recombination analysis

Recombination analysis was performed by using a set of selected βC1 protein gene sequences to exhibit the genetic diversity by using recombination detection program (RDP3) (darwin.uvigo.es/rdp/rdp.html) for probable recombinant detection and possible recombination breakpoints and hot spots. The cutoff for *P-value* was set at 0.05 with standard Bonferroni corrections (Martin et al., 2010).

Molecular modeling and structural analysis of βC1 protein gene of OLCuB

Till date, no 3D structural information is available for βC1 protein gene of OLCuB Hyderabad isolate. As an alternate to homology modeling, we used threading approach of I-TASSER for structure prediction, as low sequence homology was found for both OLCuB and CLCuB (Zhang, 2008; Roy et al., 2010). For βC1 protein gene structure prediction, from OLCuB and CLCuB amino acid sequences were retrieved from NCBI (GenBank: OLCuB: JF792241; GenBank: CLCuB: NC_013637; Expasy's UniProtKB: D1GZI5). Evaluation of the predicted three-dimensional models, the stereo-chemical properties was done with PROCHECK (Laskowski et al., 1993). Structural visualization and analysis were performed by PyMOL Version 1.3 (De Lano, 2005). Expasy's ScanProsite was run to find motifs and domain, whereas EBI's ProFunc was used for their probable biochemical function of a protein from its three-dimensional structure (Laskowski et al., 2005). Secondary structure (topology diagram) of βC1 protein gene from OLCuB and CLCuB was done by PDBSum; and for better understanding and comparison, structural alignment was done by using SuperPose, which calculates protein superposition's using a modified quaternion approach (Maiti et al., 2004). Protein function prediction server 3D2GO was used for both the modeled structures to predict their functions. On the basis of recombination analysis, it is expected that the causal organism of OLCD identified in Hyderabad could be a variant of BYVMB biologically validated in future.

Conclusion

We made a sincere effort to analyze the diversity, recombination and structure prediction of βC1 protein gene from OLCuB associated with okra leaf curl disease from Hyderabad, South India. The present findings will help in quick, reliable and efficient detection, designing and development of leaf curl disease resistant okra varieties. This will also elucidate the diversity and evolutionary relationship and possible emergence of variant virus strains of begomoviruses in Southeast Asia. During evolution of begomoviruses, inter-specific recombination has resulted in remarkable structural and functional diversity and is the major cause of the emergence of new strains/species causing varied diseases in tropical and subtropical regions. Recombinant viral isolates with significantly changed biological properties gains an ability to adopt and sustain in different environmental conditions to cause new disease with extended host range. It is concluded that the okra leaf curl disease reported in Hyderabad is caused by a variant of Bhendi yellow vein mosaic virus, a monopartite begomovirus associated with betasatellites. Our *in silico* analysis highlights the origin and complex etiology of okra leaf curl disease in India, however, recombination events could be biologically validated in future.

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Competing Interests

The authors have declared that no competing interest exists.

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