Complex nature of infection associated with yellow vein mosaic disease in Bhendi (*Abelmoschus esculentus*)

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Abelmoschus esculentus **(Bhendi) is a traditional vegetable crop widely cultivated and consumed commonly in India. Yellow vein mosaic disease (YVMD) caused by Bhendi yellow vein mosaic virus (BYVMV) is a major constraint for Bhendi cultivation in India. To study the nature of infection in the field, we collected leaves which showed typical YVMD in Madurai (9 plants) and carried out rolling circle amplification (RCA) for plant #6. Intriguingly, the digestion of RCA product did not yield expected fragments. This suggests that there may be new viruses due to recombination or mutation or mixed infection. However, on digesting the RCA product of plant #1 with *Sac*I, monomers of 2.7 and 1.3 kb were released and each was cloned into the pOK12 vector. Sequenced RCA digested products showed mixed infection by BYVMV DNA A, Okra enation leaf curl virus (OELCuV) DNA A, beta, alphasatellite with the nanovirus origin of replication. Consequently, mixed infection was also confirmed by Southern hybridization and qPCR in all the analysed plant samples. In the mixed infection of BYVMV and OELCuV, we examined high level of OELCuV DNA A accumulation.**

Keywords: Alphasatellite, BYVMV, mixed infection, OELCuV.

GEMINIVIRUSES encompass a large group of plant viruses with single stranded DNA (ssDNA) genome encapsidated in twinned icosahedral particles, capable of infecting both monocot and dicot plants[1]. They replicate through rolling circle mechanism in the nuclei of infected plant cells[2]. Based on genome organization, host range and insect vector, geminiviruses are classified into seven genera: *Mastrevirus*, *Topocuvirus*, *Curtovirus*, *Becurtovirus*, *Turncurtovirus*, *Eragovirus* and *Begomovirus*[3]. Begomoviruses are the largest among the entire genus and infect economically important crop plants from diverse families such as Malvaceae, Solanaceae, Rosaceae and Fabaceae.

Abelmoschus esculentus (lady's finger or bhendi), a member of the family Malvaceae is an important vegetable crop, rich in fibre and vitamins. India contributes about 70% of its global production[4]. Bhendi is used as a regular diet in India and its cultivation is constrained by the occurrence of yellow vein mosaic disease (YVMD). YVMD was first reported in 1924 from Bombay[5], India. The infection is characterized by persistent vein clearing, followed by yellowing and reduces the yield up to 96% (ref. 6). YVMD is caused by the Bhendi yellow vein mosaic virus (BYVMV), a whitefly-transmitted monopartite begomovirus associated with a betasatellite[7]. DNA A encodes for seven open reading frames (ORFs) required for virus replication, encapsidation and movement, whereas betasatellite encodes for a single ORF βC1. Extensive studies have been carried out on BYVMV genes. It has been shown that the C2 of BYVMV is localized in the nucleus, acts as a transactivator of the virion-sense promoter and as a weak suppressor of post-transcriptional gene silencing (PTGS)[8–10]. The C4 of BYVMV is a suppressor and its ectopic expression in *Nicotiana benthamiana* produced mild leaf curl suggesting it as a symptom determinant[8]. The lone ORF C1 of betasatellite is essential for the symptom production and suppressor of PTGS[8,10].

The outbreak of new begomoviruses and their natural adaptability to infect non-host plants shows that the viruses can evolve by recombination and mutation to be more devastating and pathogenic[11–15]. In order to address the above and to study the present nature of infection in the field, samples were collected from Madurai and Chennai of Tamil Nadu and rolling circle amplification (RCA) was performed. Cloning and sequencing results further demonstrate that there is a mixed infection by the BYVMV and Okra enation leaf curl virus (OELCuV) along with the alpha and betasatellites. Based on these studies, we report here a mixed infection in bhendi fields and suggest the essentiality of continuous monitoring of the field for the control and management of the disease.

Bhendi leaf samples of 10 plants varying in degree of symptoms of typical yellow vein mosaic disease were collected from a farmer's field in Madurai (Plants #1–9) and Chennai (Plant #10). While collecting the samples, care was taken to pluck only the symptomatic leaves from each individual plant and the samples were placed into appropriately labelled covers.

Viral DNA from infected bhendi plants was extracted by the citrate method[16] followed by the alkali lysis method for its enrichment. RCA was performed *in vitro* using φ29 DNA polymerase to amplify the viral circular DNA. The reaction mixture consisted of 1× reaction buffer of φ29 DNA polymerase, 1 mM dNTP mix and 50 µM exo resistant random hexamer. After adding 20–50 ng of template DNA, it was made up to a final volume of 20 µl with nuclease-free water. It was then denatured at 95°C for 3 min and cooled to room temperature. Subsequently, 5 units of φ29 DNA polymerase (Fermentas, EU) and 0.02 U of pyrophosphatase were added and incubated at 30°C for 18–20 h. Finally, the enzyme was inactivated by incubation at 65°C for 10 min. Amplification of the RCA product was confirmed on 0.8% agarose gel.

RCA product was digested with *Sac*I (plant #1) to release the viral components of sizes 2.7 and 1.3 kb

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Table 1. List of primers used for sequencing and real-time PCR analysis

Name of primers	Sequence	Co-ordinates
Seq. P1 OELCuV DNA A MDU	F: 5′-CTCGCTGACTTAAGCTCC-3′	993–1013
BYVMV C2 MDU	F: 5′-AGATCTTTAGAGATATTTGAGGAC-3′ R: 5′-AAGCTTATGCAGCATTCGTCTTTC-3′	1170–1189 1601–1583
BYVMV βC1 MDU	F: 5′-CTGCAGTTAAATTATTATCTTATTATCAAATAG-3′ R: 5′-TGTACATGAAAATATCTATACATTTCATC-3′	180–207 602–578
OELCuV CP (DNA A) MDU RT	F: 5′-GCACCCCCTACGATTTCCAG-3′ R: 5′-ACAAGCATACTGTCCTCCTG-3′	565–584 704–685
BYVMV Rep (α Sat) MDU RT	F: 5′-GGTTAAGGCACTCTTTGGTG-3′ R: 5′-AGCCATTCTCACAGGACTTC-3′	688–707 883–864
OELCuV Rep (α Sat) SURAT RT	F: 5′-GACGAAACTAGGGTTTCAGG-3′ R: 5′-CTGATGTGACTCCTTCGTCA-3′	406–423 570–551
BYVMV V2 (DNA A) MDU RT	F: 5′-GCAACTTTTGTCGCAGGATT-3′ R: 5′-ATAGGCCTGTTTGTCCATGC-3′	462–481 693–674
BYVMV βC1 (β Sat) MDU RT	F: 5′-CGCGCGGTAAATGGTAAATA-3′ R: 5′-CTACGACGCGCGATATAACT-3′	1017–1036 1177–1158

which were cloned into the respective site of pOK12. Upon digestion of various restriction enzymes, differential digested clones (4 clones) were selected and sequenced. The confirmed clones were then named as pVK1 (OELCuV DNA A), pVK2 (BYVMV DNA A), pVK3 (BYVMV alphasatellite) and pVK4 (BYVMV betasatellite). An additional sequencing primer was designed from the known sequences to cover the full-length 2.7 kb DNA (Table 1).

Full-length nucleotide sequences of DNA A and satellite DNAs were assembled using EMBOSS explorer (http://bioinfo.nhri.org.tw/cgi-bin/emboss/merger) and compared to DNA and protein sequence databases using BLASTn and BLASTp. The ORFs present in the virion and complementary senses were predicted using Open Reading Frame finder tool available at NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Full-length nucleotide sequences of both the DNA satellites were submitted to NCBI and the accession number for pVK3 and pVK4 are KR068484 and KR068483.

Alphasatellite sequences (n = 433) were retrieved from NCBI database. With the aid of the cyclic DNA sequence aligner tool (http://kdbio.inesc-id.pt/software/csa/), the sequences were formatted to begin with the start codon before performing multiple sequence alignment using T-coffee.

Viral DNA was quantified using a fluorescent dye, Hoechst 33258 and the equal concentration of DNA from all the plant samples were loaded on 0.8% agarose gel and electrophoresed at 50 V in 1× TNE buffer (40 mM Tris-acetate, pH 7.5, 20 mM sodium acetate, 2 mM EDTA)[17]. The electrophoresed DNA samples were then transferred from the gel onto a Biobond™ Nylon transfer membrane and then hybridization, labelling, detection, stripping and reprobing were carried out according to the instructions mentioned in Alkphos Direct™ labelling and detection system (GE healthcare). The blot was then hybridized with five different probes based on sequencing results and the available begomovirus genome sequence in the database (BYVMV DNA A, BYVMV βC1, OELCuV DNA A, BYVMV alphasatellite and alphasatellite Rep-Surat) to detect the DNA A and satellite components.

Absolute quantification was performed using the standard curve method to determine the copy number of viruses in the infected samples. Standards were prepared using the plasmid containing the viral constructs and serially diluted to give 10-fold dilutions from 10^4 to 10^9. The reaction mixture consisted of 1× SYBR Green master mix (ABI) and specific primers (forward and reverse) at the concentration of 10 pM; 50 ng of template DNA was added to the reaction mixture making up the total volume to 20 µl with nuclease-free water. Real-time PCR was done with ABI prism 7000 with the cycling parameters of 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 3 min and 40 cycles each consisting of 95°C for 15 s (denaturation) followed by 60°C for 1 min (annealing and extension). A dissociation protocol was performed after amplification with a gradual rise in temperature from 60°C to 95°C to check the specificity of the reaction. The copy number of the virus in each plant sample was calculated on basis of the threshold C_t value. All the standards, samples and the control were performed in triplicates and the data was analysed by ABI prism 7000 qPCR SDS software.

Out of all the 10 plants collected, plant #6 was archetypically analysed. It was initially confirmed by PCR using BYVMV C2 and βC1 specific primers and RCA

Table 2. Expected and observed digested fragments by the respective enzymes in sample number 6

Enzymes	Fragments expected: BYVMV BYVMV DNA A (kb)	Fragments expected: OELCuV OELCuV DNA β (kb)	Fragments expected: DNA A (kb)	Fragments expected: DNA α (kb)	Fragments obtained (kb)
*Hin*dIII	2.7	–	2.7	1.35	ND
*Sac*I	–	1.35	2.7	–	1.35
*Kpn*I	2.7	1.35	2.7	1.35	1.35
*Eco*RV	2.7	0.94, 0.22, 0.18	2.7	1.35	1.3, 1.2
*Mlu*I	2.7	–	–	–	D
*Cla*I	–	–	2.7	–	ND
*Xho*I	–	–	2.7	–	1.4
*Pst*I	1.7, 1.02	–	1.7, 1.02	1.35	1.4, 1.35
*Nde*I	–	–	–	1.7, 0.18	1.35
*Ssp*I	1.65, 0.89, 0.19	1.35	1.65, 0.65, 0.39, 0.03	1.1, 0.24	1.8, 1.35, 1.4, 1.1, 0.8, 0.6, 0.55, 0.39

D, Degraded; ND, Not digested.

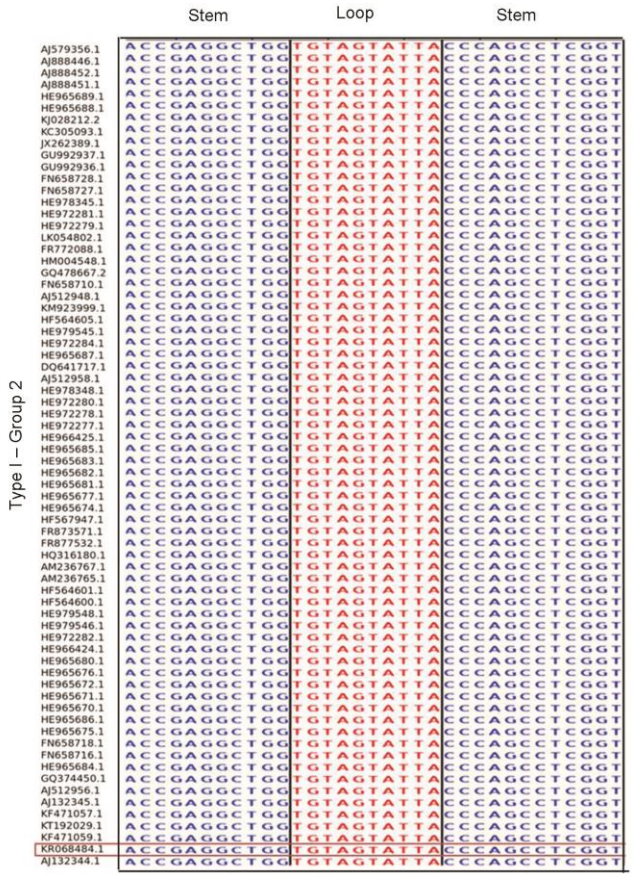

Figure 1. Alignment of hairpin sequences of alphasatellites belonging to Type I – Group 2 from NCBI database. Alphasatellite reported from this study (KR068484.1) is boxed in the figure. Stem and loop positions of alphasatellites are indicated.

was carried out only for the sixth plant. RCA product was digested on the basis of the restriction pattern of the already available sequence of BYVMV (Accession No. AF241479 [DNA A] and AJ308425 [DNA β]) Madurai. All the digestions did not yield expected products for the DNA A and betasatellite as depicted in Table 2. We also retrieved the OELCuV DNA A (Accession No. KC342220) and DNA alphasatellite-Surat (Accession No. HF546575) isolate in the NCBI database and digested with various enzymes and observed similar results (Table 2).

On the contrary, the RCA product of the plant #1 released the expected monomers (2.7 kb or 1.3 kb) upon *Sac*I digestion, subsequently cloned into pOK12 at the corresponding site. The clones were confirmed by various restriction enzyme digestions, after observing differential patterns upon digestions, four clones were selected and sent for sequencing. Bioinformatics analyses of the sequences showed the presence of mixed infection with OELCuV and BYVMV DNA A, alpha and betasatellite.

The 1370 bp long alphasatellite had nanovirus origin of replication (TAGTATTAC) and showed 97% identity with Okra virus alphasatellite–Haryana isolate. It is categorized as Type I – Group 2 by Xie *et al.*[18] (Figure 1). On the other hand, the 1346 bp long betasatellite had a nonanucleotide sequence (TAATATTAC) and it showed 96% identity with BYVMV betasatellite–Raichur isolate. Partial sequence of DNA A of OELCuV (2.6 kb) showed 98% identity with OELCuV–Gandhinagar isolate, while BYVMV DNA A (2.4 kb) showed 95% identity with BYVMV–Varapampatti isolate. From the sequence analyses, the similarity between two satellites (alpha and beta) was less than 45%, while Rep of BYVMV showed 60.5% identity with alphasatellite Rep. The alphasatellite with nanovirus origin had 58.4% identity with alphasatellite–Surat isolate (Accession No. HF546575)[19].

The observation of mixed infection was also confirmed by Southern hybridization. Viral DNA (8 µg) for all the samples was blotted onto the nylon membrane. It was hybridized with various probes such as β C1 (0.43 kb) gene for the BYVMV betasatellite, 1.37 kb of BYVMV alphasatellite, 0.615 kb of *Rep* gene for the OELCuV alphasatellite–Surat isolate and all the plants were found to have the satellite component to a varied degree of

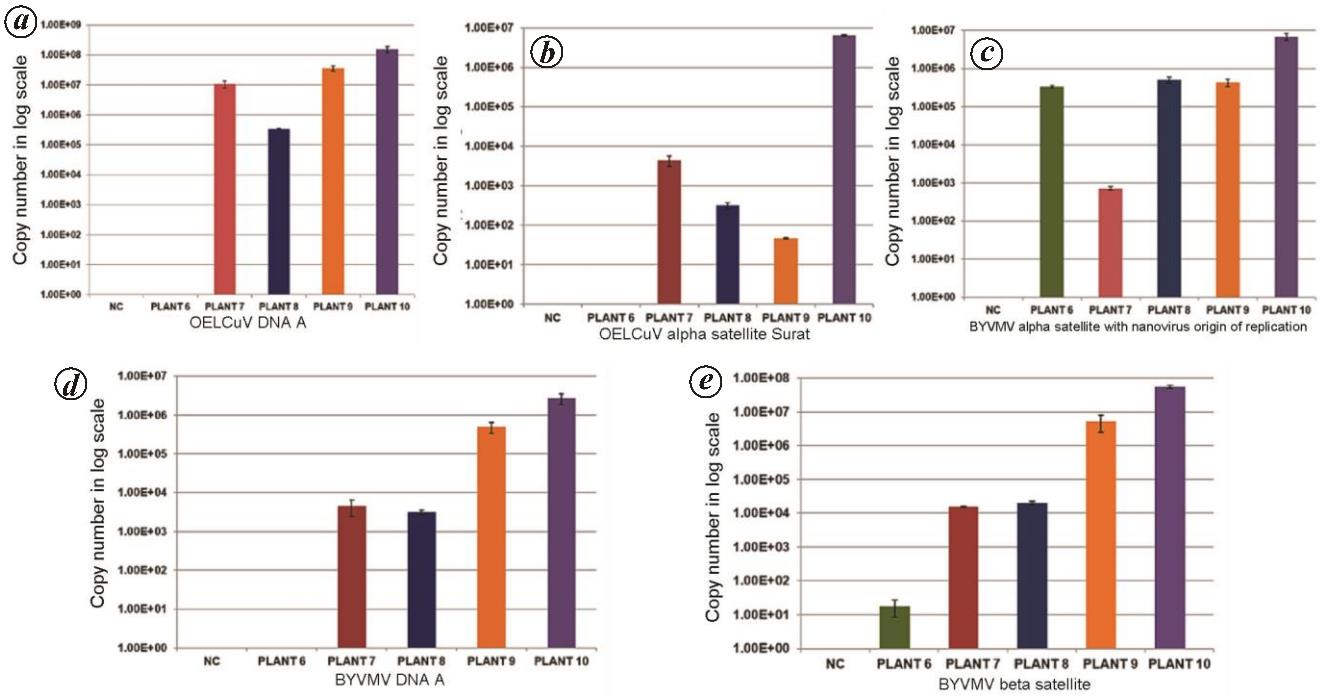

Figure 2 ***a–e.*** Copy number quantification of DNA A of OELCuV, BYVMV and their satellites by qPCR. About 50 ng of viral DNA (plant number 6–10) was used along with the SYBR Green master mix. The copy number of OELCuV DNA A (***a***), alphasatellite-SURAT isolate (***b***), alphasatellite with nanovirus origin (***c***), BYVMV DNA A (***d***) and betasatellite (***e***) component in each plant was quantified on the basis of C_t value of the established standard. In the graph, NC indicate negative control (DNA of healthy plant).

accumulation (Figure S1 *a–e*; see Supplementary Information online). BYVMV and OELCuV DNA A were detected when their respective DNA A was used as probe. Due to the 86% similarity between both the DNA A, cross-hybridization is possible while using DNA A as probe.

In order to substantiate that there is mixed infection in the field and to rule out the possibility of cross-hybridization for both DNA A of BYVMV and OELCuV in Southern hybridization, specific primers were designed for BYVMV and OELCuV from the V2 and V1 regions respectively (Table 1). Initially, PCR was performed to ensure that there is no cross-amplification for two DNA A and satellites component (Figure S2 *a–f*; see Supplementary Information online). After ensuring that there is no cross-amplification by PCR, quantitative PCR was carried out for plants #6–10 to determine the copy numbers by absolute quantification method (Figure 2 *a–e*). Interestingly, we found more predominant accumulation of OELCuV than BYVMV in typical yellow vein symptomatic plants.

Begomoviruses are well known for synergistic relationship and their high recombinant rate results in the emergence of new pathogenic strains to broaden the range of their hosts with more than one virus to produce a mixed infection that circulates in the field[11,20–23]. Previously, a mixed infection by Groundnut bud necrosis virus (GBNV) and OYVMV in okra from Nasik district of Maharashtra, India[24] was detected. Similar phenomena were observed in pepper by Pepper huasteco yellow vein virus and Pepper golden mosaic virus in Mexico, Southern US[25] and in cassava with African cassava mosaic virus and East African cassava mosaic virus in Cameron[26]. Mixed infection was also reported from other crops such as radish, chilli pepper, cashew and tomato[27,28]. Similarly, Taqman-based qPCR was successfully used to quantify the viral titre of Squash leaf curl virus in cucurbit[29]. Subsequently, single and multiplex RT-PCR has been developed to detect mixed infection by Cucurbit chlorotic yellow virus and Cucurbit yellow stunting disorder virus which induce similar symptoms in cucurbit[30]. In line with previous observations, we also found a mixed infection of BYVMV, OELCuV and its associated satellites in bhendi which was confirmed by sequencing, Southern hybridization and qPCR. Based on the copy number quantification, we examined more predominant accumulation of OELCuV than BYVMV. Our studies speculate a synergism between BYVMV and OELCuV to induce yellow vein mosaic symptoms. Mixed infection may also result in the synergistic, additive or antagonistic interactions in plants. A detailed study is necessary to prove the dominance of a symptom of one virus over others in the fields.

Begomovirus has become a serious threat to crop plants which is evident from the present study. Begomoviruses may evolve into a more virulent form than expected hence, we suggest the need for frequent periodical

studies to evaluate the nature of begomoviruses circulating in the field.

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