

Molecular identification and biological characterisation of a begomovirus associated with okra enation leaf curl disease in India

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Abstract An unusual symptom was noted in okra originating from Haryana state (India) consisting of leaf curl associated with enations. These were distinct from the leaf curl and/or vein yellowing symptoms usually shown by okra. PCR-mediated amplification was used to show the presence of a begomovirus and the complete genome sequences were determined for seven isolates. The sequences showed high levels of nucleotide sequence identity (91.9–99.1 %). In comparison to begomovirus sequences available in the databases the okra sequences showed the highest levels of nucleotide sequence identity (84.5 to 87.1 %) with Mesta yellow vein mosaic virus (MeYVV) and thus were classified as belonging to a

novel begomovirus species, tentatively named Okra enation leaf curl virus. In common with the majority of begomoviruses, Okra enation leaf curl virus (OELCuV) was shown to have a recombinant origin. Analysis of the transmission characteristics of a wild-type virus isolate by *Bemisia tabaci* showed the minimum acquisition access period to be 1 h and the minimum inoculation access period to be 30 min, with female insects transmitting with a greater efficiency than male insects. Under controlled conditions the host range of the virus was shown to be very narrow, limited to two species in the family Malvaceae, okra (*Abelmoschus esculentus*) and hollyhock (*Althaea rosea*), and seven in the family Solanaceae.

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Introduction

The geminiviruses (family *Geminiviridae*) are plant-infecting viruses characterized by their unique geminate particle morphology and circular single-stranded (ss) DNA genomes that are transmitted by arthropod vectors. Collectively the geminiviruses have a broad host range and are responsible for economically significant losses in crops worldwide (Harrison and Robinson 1999; Moffat 1999).

The majority of characterized geminiviruses belong to the genus *Begomovirus*. Begomoviruses are transmitted by the whitefly *Bemisia tabaci* and infect dicotyledonous plants (Lazarowitz 1992). With one recently identified exception (Melgarejo et al. 2013; Sánchez-Campos et al. 2013), begomoviruses native to the New World have genomes consisting of two components, which are referred to as DNA-A and DNA-B, each of 2.6–2.8 kb. Although a few bipartite begomoviruses have been identified in the Old World (OW), most have genomes consisting of only a single component, homologous to the DNA-A component of the bipartite viruses (Brown et al. 2012). The genomes of monopartite begomoviruses (and the DNA-A components of bipartite begomoviruses) originating from the OW encode six genes, two in the virion-sense and four in the complementary-sense. The virion-sense genes encode the coat protein (CP), which encapsidates/protects the virus genome and is involved in virus movement within and between plants, and the V2 protein, which is involved in virus movement in plants as well as being involved in overcoming host defences triggered by double-stranded RNA (RNA silencing; Amin et al. 2011). The complementary-sense genes encode the replication-associated protein (Rep; the only virus encoded protein required for virus replication, which is a rolling-circle replication initiator protein), the transcriptional activator protein (TrAP; involved in up-regulating late, virion-sense encoded genes, overcoming RNA silencing and up-regulating host transcription), the replication enhancer protein (REn; that interacts with Rep to provide a cellular environment suitable for virus replication) and the C4 protein (that may be involved in overcoming RNA silencing and may be a pathogenicity determinant; Rojas et al. 2005; Bisaro 2006).

Transcription, for all geminiviruses, is bidirectional with the major transcripts originating from a non-coding intergenic region (IR) that contains a hairpin structure encompassing the near ubiquitous sequence TAATAT TAC (known as the nonanucleotide sequence). The nonanucleotide sequence, together with repeated sequence motifs (known as “iterons”) adjacent to the TATA box of the Rep promoter, form the origin of virion-strand DNA replication. The iterons are sequence specific Rep binding sequences, which differ between geminivirus species. The Rep from one species will, in most cases, not initiate the replication of the genome of a second (Argüello-Astorga et al. 1994; Argüello-Astorga & Ruiz-Medrano 2001). A majority of the monopartite begomoviruses associate with a class of ssDNA satellites known as betasatellites (formerly known as DNA β). Betasatellites are approximately half the size of their helper begomoviruses which they require for replication, insect transmission and movement in plants (Saunders et al. 2000; Briddon et al. 2003; Zhou et al. 2003; Jose and Usha 2003; Cui et al. 2004; Li et al. 2005).

Together with okra yellow vein disease and okra leaf curl disease, okra enation leaf curl disease (OELCuD) causes severe losses in cultivated okra (*Abelmoschus esculentus* (L.) Moench) in India. The disease initially causes small pin-head enations on the under surface of leaves. This is followed by a warty and rough texture of leaves, with later leaves curling upwards. Affected plants show a twisting of the stem and lateral branches with leaves becoming thick and leathery. The curling and enations are more prevalent on leaves that develop soon after infection than in later leaves and plants are severely stunted with fruit being small, deformed and unfit for marketing (Singh 1996). Preliminary investigations in the field showed infected plants to be associated with heavy infestations of the whitefly *Bemisia tabaci*, the vector of begomoviruses. Here a distinct begomovirus is shown to be associated with OELCuD.

Materials and methods

Virus source

Leaf samples from seven okra plants exhibiting enation leaf curl symptoms were collected from three locations (farmer fields) in and around Munthal and Sonapat, Haryana state, India (Fig. 1). For all the samples virus was transmitted to okra plants (cv.1685) using the

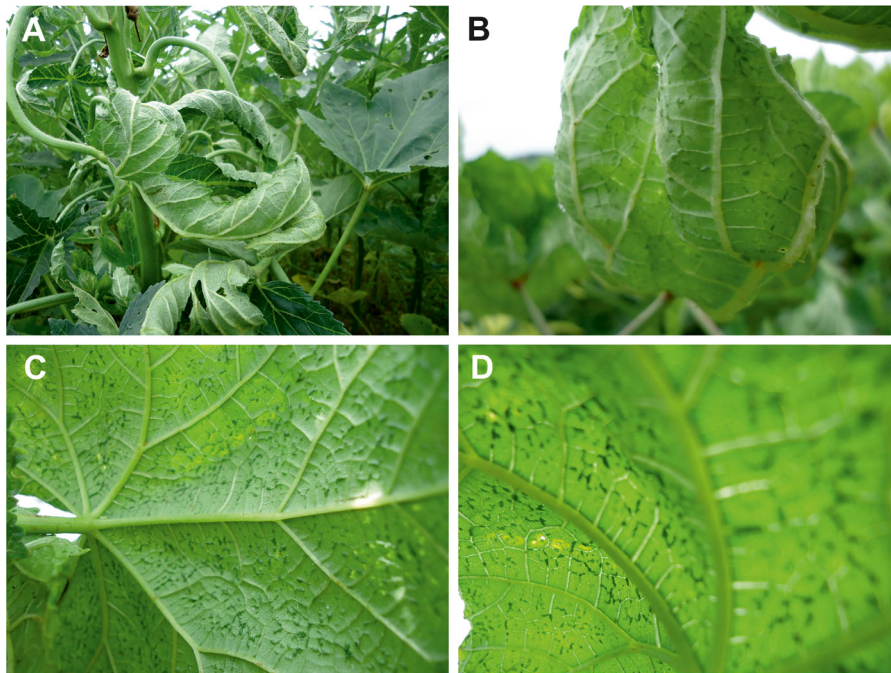


Fig. 1 Symptoms exhibited by Okra enation leaf curl virus-infected okra (*Abelmoschus esculentus*) plants in the field. Shown are the initial severe upward leaf curling **A** and associated vein

swelling and small enations on minor veins **B** and **C**. The swelling and enations are darker than surrounding tissues **D**

whitefly *B. tabaci*. Insect transmission was repeated three times under control conditions and enation leaf curl disease symptoms were exhibited following each transmission to okra plants (cv.1685). This ensures the absence of viruses not transmitted by *B. tabaci*. Duplicate leaf samples were stored at -80°C .

DNA isolation, PCR-mediated amplification and sequencing

Total nucleic acids were isolated from leaf samples by the method of Doyle and Doyle (1990). The full length genomes of begomoviruses were PCR amplified using the three sets of degenerate primers (synthesized by Sigma Aldrich) designed to produce overlapping fragments of the genomes (or DNA-A components) of OW begomoviruses (Venkataravanappa et al. 2012). In order to rule out mixed infections, the primers were designed in such way that the amplified product overlapping (approximately 200 bp) between the fragments amplified. PCR reactions were carried out in a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, CA) thermocycler. PCR reactions were carried out in a volume of 25 μL containing 100 ng of DNA template 0.5U Taq DNA polymerase (Fermentas,

Germany), 2 mM MgCl_2 (Fermentas, Germany), 0.16 mM dNTPs (Fermentas, Germany) and 0.3 μM of each primer. The thermo cycler was set for 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C to 58°C for 45 s and extension at 72°C for 90 s. Runs included an initial denaturation at 94°C for 3 min and a final extension at 72°C for 15 min. PCR products were electrophoresed on 0.8 % agarose gels stained with ethidium bromide (10 mg/ml) and were viewed in a gel documentation system (Alpha Innotech, USA). PCR reactions to amplify DNA-B component were carried out with the degenerate universal primers described by Rojas et al. (1993). Similarly, alphasatellites and betasatellites were amplified using universal primer pair UN101/UN102 (Bull et al. 2003) and Beta01/Beta02 (Bridson et al. 2002) respectively.

Amplification products were cloned into the pTZ57R/T vector (Fermentas, Germany) and transformed into DH5 α competent *Escherichia coli* cells (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Three clones from each transformation were sequenced. Automated sequencing was carried out on an ABI PRISM 3730 (Applied Biosystems) automated sequencer at Anshul Biotechnologies DNA sequencing facility (Hyderabad, Andhra Pradesh, India).

Sequence analysis

Multiple alignments were produced using Clustal-X (Jeanmougin et al. 1998). Sequence identity matrices were generated using the Bioedit Sequence Alignment Editor (version 5.0.9) (Hall 1999) and phylogenetic trees were generated using the neighbour-joining method in MEGA 5.0 (Tamura et al. 2011) with 1000 bootstrap replications. Split-decomposition trees were constructed with 1,000 bootstrap replicates based on parsimony splits as implemented in SplitsTree version 4.11.3 with default settings (Huson and Bryant 2006). Recombination analysis was carried out using the Recombination detection program (RDP), GENECOV, Bootscan, Max Chi, Chimara, Si Scan, 3Seq which are integrated in RDP3 (Martin et al. 2010).

Insect transmission

One reference okra isolate (EL38) among the seven collected from the field was transmitted by whitefly (*B. tabaci* Genn.), with a 12 h acquisition access period (AAP) and 12 h inoculation access period (IAP), to healthy okra plants (cv.1685) in an insect proof glasshouse. Resulting infected plants were tested by direct antigen coating (DAC)-ELISA (using ACMV polyclonal antibodies [DSMZ, Germany] and an alkaline phosphatase labeled anti-rabbit IgG secondary antibody [Sigma-Aldrich Chemie GmbH, Germany] and nucleic acid (dot-blot) hybridization using specific probes for the CP gene of the virus. ELISA plates were read at 405 nm using an EL310E ELISA reader (BIO-TEK Instruments, USA). Symptomatic okra plants were then maintained in the glasshouse and used for further analysis of the virus-vector relationship.

The minimum number of *B. tabaci* adults required for virus transmission was determined by allowing whiteflies a 12 h AAP on an infected okra plant (cv. 1685) and then transferring whiteflies to 10 day old healthy okra seedlings (cv1685) for a 12 h IAP. The minimum AAP and IAP for transmission of the virus were determined using 10 whiteflies per plant. To determine the minimum AAP, insects that had been maintained on a non-infected okra plant were transferred to an infected okra plant and given a set time period (0, 5, 10, 15, 20, 30 min and 1, 4, 8, 6, 12, 16 and 24 h) to feed. Insects were then transferred to young, healthy okra seedlings and allowed to feed for 12 h before the insects were killed by spraying

with 0.01 % imidacloprid (DuPont). To determine the minimum IAP the insects were given a 12 h AAP before being transferred and maintained for set time periods (same as minimum AAP determination) on healthy seedlings. After being sprayed with insecticide, plants were maintained in insect-proof cages and monitored weekly for the appearance of symptoms.

The effect of plant age (7, 10, 15, 20, 25 and 30 days after germination) on the efficiency of transmission of the virus by insects was determined using 10 viruliferous *B. tabaci* per plant. Insects were given a 24 h AAP and IAP each and 10 plants were tested in each age group. The effect of whitefly gender on virus transmission was carried out as described by Simmons et al. (2009). Male and female adult whiteflies were collected in separate collection bottles and allowed to feed separately on infected okra plants for a 24 h AAP and then transferred in groups of 10 to healthy okra seedlings (40 seedlings for each gender) for a 24 h IAP. After IAP, plants were sprayed with imidacloprid and maintained in insect-proof cages.

In all cases the transmission rates (the number of infected plants divided by the number of inoculated plants) and estimated transmission rate for single vector were calculated. The transmission rate of a single whitefly was calculated using the formula of Gibbs and Gower (1960).

$$P^* = 1 - (1 - T)^{1/I} \quad (1)$$

Where, P^* =estimated transmission rate for a single aphid; T =transmission rate calculated as $T=R/N$;

R =number of infected plants; N =number of receptor plants; I =number of whiteflies per receptor plant.

Seed transmission

Mature seeds were collected from symptomatic okra (cv. 1685) plants infected at the seedling stage by whitefly transmission, and from healthy (cv. 1685) plants that were maintained in an insect-proof glasshouse. The seed was treated with 2 % (v/v) sodium hypochlorite for 2 min and rinsed with water several times. Three sets of 25 seeds each from healthy and infected plants were sown in soil, sand and compost (2:1:2 w/w) mixture in separate earthen pots, kept in a glasshouse for 1 month and monitored for the appearance of symptoms. The seedlings were sprayed with imidacloprid (0.05 %) at

10 day intervals, to avoid insect transmission, and finally the presence of virus in seedlings was determined by indirect ELISA and PCR, as described above.

Determination of virus host range

Healthy seedlings of 59 plant species, representative of the families Leguminosae, Malvaceae, Euphorbiaceae, Solanaceae, Chenopodiaceae, Amaranthaceae, Asteraceae, Apocyanaceae, Cucurbitaceae and Caricaceae were raised in a glasshouse. Seedlings at the first leaf stage were transplanted into polythene bags containing a mixture of soil and farmyard manure (2:1). Individual seedlings were inoculated with 20 viruliferous whiteflies after a 24 h AAP on infected okra plants. After a 24 h IAP, the whiteflies were removed and the plants were sprayed with imidacloprid (0.05 %). The inoculated plants were kept in an insect-free glasshouse and monitored weekly for the appearance of symptoms of infection.

Results

Cloning and sequencing

The sequences of the genomes of seven viruses isolated from field collected okra plants exhibiting enation leaf curl symptoms (Fig. 1) were determined. This included one isolate (EL 38) that was maintained in the glasshouse for virus transmission studies. Virus genomes were amplified as overlapping fragments and sequenced to yield the complete genome sequence for each isolate. There was no amplification from non-symptomatic okra plants collected in the field or from plants raised in an insect-free glasshouse and lacking symptoms. The sequences ranged from 2722 to 2724 nt in length and are available in the nucleotide sequence databases under the accession numbers listed in Table 1. PCR amplifications with DNA-B primers were uniformly negative. All seven samples gave amplification with betasatellite primers (the sequences of betasatellites from four of the isolates here have been reported; Venkataravanappa et al. 2011b) and alphasatellite primers (data not shown). Alignment of the seven begomovirus isolates obtained here showed these to have 91.9–99.1 % nucleotide sequence identity, indicative of them being isolates of a single species (based on the 89 % species demarcation threshold for begomoviruses; Fauquet et al. 2008). All

isolates showed the genome organization typical of monopartite begomoviruses with two conserved genes encoded in the virion-sense (encoding the CP and the V2 protein) and four genes in the complementary-sense (encoding the Rep, C2, REn and C4; Table 1).

Comparisons with begomovirus sequences available in the nucleotide sequence databases (the isolates used are listed in Supplementary Table 1) showed the sequences obtained from okra to have the highest levels of nucleotide sequence identity with isolates of MeYVMV (84.5–87.2 %) followed by *Bhendi yellow vein mosaic virus* (BYVMV; 82.9–84.5 %; Table 2). These percent identities, being below the species demarcation threshold for begomoviruses, indicate that the seven begomovirus isolates sequences are representative of a previously unknown species of begomovirus. This is supported by a phylogenetic analysis (Fig. 2) that shows the seven isolates to segregate with, but to be distinct from, isolates of MeYVMV and the recently identified *Bhendi yellow vein Bhubhaneswar virus* (BYVBhV) (Venkataravanappa et al. 2013b). The name Okra enation leaf curl virus (OELCuV) is proposed for this newly identified species.

The sequences from okra encode the six conserved genes typical of the genomes of monopartite (or DNA-A components of bipartite) begomoviruses originating from the OW. The positions and coding capacities of the genes for each of the OELCuV sequences are indicated in Table 1. A pairwise comparison of the predicted amino acid sequences of the gene products of the OELCuV isolates with other begomoviruses showed the V2, CP and C4 regions to have the highest levels of identity with MeYVMV (Table 3). For the Rep protein, two isolates (EL10 and EL14) showed the highest levels of identity with BYVMV, whereas the remaining five isolates (EL32, EL37, EL38, EL39 and EL41) had the highest levels of identity with MeYVMV. TrAP showed the highest levels of amino acid identity with *Cotton leaf curl Bangalore virus* (CLCuBaV), with the exception of isolate EL10, which had the highest levels of identity to the TrAP of OYVMV. For the REn protein the OELCuV isolates showed highest levels of identity with either BYVMV or OYVMV (Table 3).

The non-coding, IR of the OELCuV isolates ranged from 273 to 274 nt in length and showed highest levels of nucleotide sequence identity (93.4 to 93.7 %; Table 2) to an isolate of BYVMV originating from Pakistan (AJ002453). The IR of the OELCuV isolates encompass

Table 1 Origin of virus isolates and features of the begomovirus clones obtained from okra

Isolate descriptor	Origin	Associated betasatellite	Accession no.	Size (nt)	Genes#	[coordinates of start/stop codons, number of amino acids (predicted coding capacity in kDa)]						
						CP	V2	Rep	C2	REn	C4	
[IN: Sonipet EL10:2006]	Sonipat	BYVB*	GU111996	2723	281–1039/250	121–486/121	2570–1482/362	1585–1133/150	1440–1036/134	2416–2114/100		
[IN: Sonipet EL14:2006]	Sonipat	OLCuB (KJ437507)	GU111997	2722	281–1033/250	121–486/121	2570–1482/362	1585–1133/150	1440–1036/134	2416–2114/100		
[IN: Sonipet EL32:2006]	Sonipat	OLCuB (KJ437508)	GU112000	2737	281–1033/250	121–486/121	2570–1482/362	1585–1133/150	1440–1036/134	2416–2114/100		
[IN: Munthal EL37:2006]	Munthal	OLCuB (KJ437509)	GU111998	2725	281–1033/250	121–486/121	2570–1482/362	1585–1133/150	1440–1036/134	2416–2114/100		
[IN: Munthal EL38:2006]	Munthal	OLCuB*	GU112002	2723	281–1033/250	121–486/121	2571–1483/362	1586–1134/150	1441–1037/134	2417–2115/100		
[IN: Munthal EL39:2006]	Munthal	BYVB*	GU112001	2723	281–1039/252	121–486/121	2570–1482/362	1585–1133/150	1440–1036/134	2416–2114/100		
[IN: Munthal EL41:2006]	Munthal	OLCuB*	GU111999	2723	281–1033/250	121–486/121	2570–1482/362	1585–1133/150	1440–1036/134	2416–2114/100		

* The betasatellite species associated OEnL-CuV were determined previously for four virus isolates (Venkataravanappa et al. 2011b). For three isolates the betasatellites have recently been sequenced. The betasatellites are Bendi yellow vein betasatellite (BYVB) and Okra leaf curl betasatellite (OLCuB)

Genes are indicated as coat protein (CP), replication-associated protein (Rep), transcriptional-activator protein (TrAP), and the replication enhancer protein (REn). The products encoded by genes V2 and C4 have yet to be named

Table 2 Pairwise percent nucleotide sequence identities for comparisons between the complete sequences and intergenic regions (IR) of the virus isolates from okra obtained here with selected other begomoviruses available in the databases

OELCV [IN: Sonipet EL10:06]	OELCV [IN: Sonipet EL14:06]	OELCV [IN: Sonipet EL32:06]	OELCV [IN: Sonipet EL37:06]	OELCV [IN: Munthal EL38:06]	OELCV [IN: Munthal EL39:06]	OELCV [IN: Munthal EL41:06]	Begomovirus species [#]					
Genome IR	Genome IR	Genome IR	Genome IR	Genome IR	Genome IR	Genome IR						
77.0	51.8	76.6	51.5	77.5	77.6	51.8	77.3	51.8	77.5	51.8	BYVBhV (1)*	
71.9	64.6	71.5	64.2	72.3	72.3	64.6	72.0	64.6	72.3	64.6	BYVHaV (1)*	
82.6	66.3	75.7	65.9	76.4	76.6	66.3	76.0	66.3	76.3	65.9	BYVMaV (1)*	
70.0–81.7	54.9–93.7	72.8–82.4	54.9–93.4	71.9–81.6	54.9–93.7	71.9–81.3	54.9–93.4	71.3–81.1	54.9–93.4	71.7–81.1	54.6–93.4	BYVMV (35)*
81.1–81.2	71.2–73.5	82.1–84.0	71.2–73.2	83.5–86.1	71.2–73.5	83.6–86.2	71.2–73.9	83.3–85.8	71.2–73.5	83.2–85.8	70.9–73.2	CLCuBaV (2)*
77.2–78.5	53.0–90.2	78.4–79.5	53.2–89.4	79.0–80.8	53.0–90.2	78.9–80.8	52.8–90.2	78.4–80.5	53.0–90.2	78.4–80.5	52.7–89.8	CLCuMuV (7)*
77.1–77.2	66.5–67.2	78.2–78.7	66.2–67.5	79.7–80.2	66.5–67.2	79.7–80.2	66.8–67.5	79.3–79.8	66.5–67.9	79.3–79.8	66.2–67.5	CLCuSHV (4)*
80.5–84.4	58.6–69.1	78.3–85.7	58.6–75.6	79.2–87.2	58.6–69.1	79.2–87.7	58.9–69.5	78.7–87.0	58.6–69.1	78.7–87.0	58.2–68.8	MeYVMV (11)*
79.3	69.5	79.2	69.2	79.8	79.8	69.5	69.5	79.3	69.5	79.2	69.2	OYVMV (1)*
68.7–69.2	53.3–58.5	68.7–69.5	52.9–57.8	694–70.3	53.3–58.5	69.3–70.2	53.3–58.5	69.1–69.7	53.3–58.5	69.1–69.7	53.6–58.9	ToLCNDV (6)*

*Numbers of sequences from the databases used in the comparisons

[#]The species are indicated as *Bhendi yellow vein Bhubaneswar virus* (BYVBhV) [acc. no. FJ589571], *Bhendi yellow vein Haryana virus* (BYVHaV) [FJ561298], *Bhendi yellow vein Maharashtra virus* (BYVMaV) [EU482411], *Bhendi yellow vein mosaic virus* (BYVMV), *Cotton leaf curl Bangalore virus* (CLCuBaV), *Cotton leaf curl Multan virus* (CLCuMuV), *Cotton leaf curl Shahdampur virus* (CLCuSHV), *Mesta yellow vein mosaic virus* (MeYVMV), *Okra yellow vein mosaic virus* (OYVMV) and *Tomato leaf curl New Delhi virus* (ToLCNDV). For each column the highest value is underlined

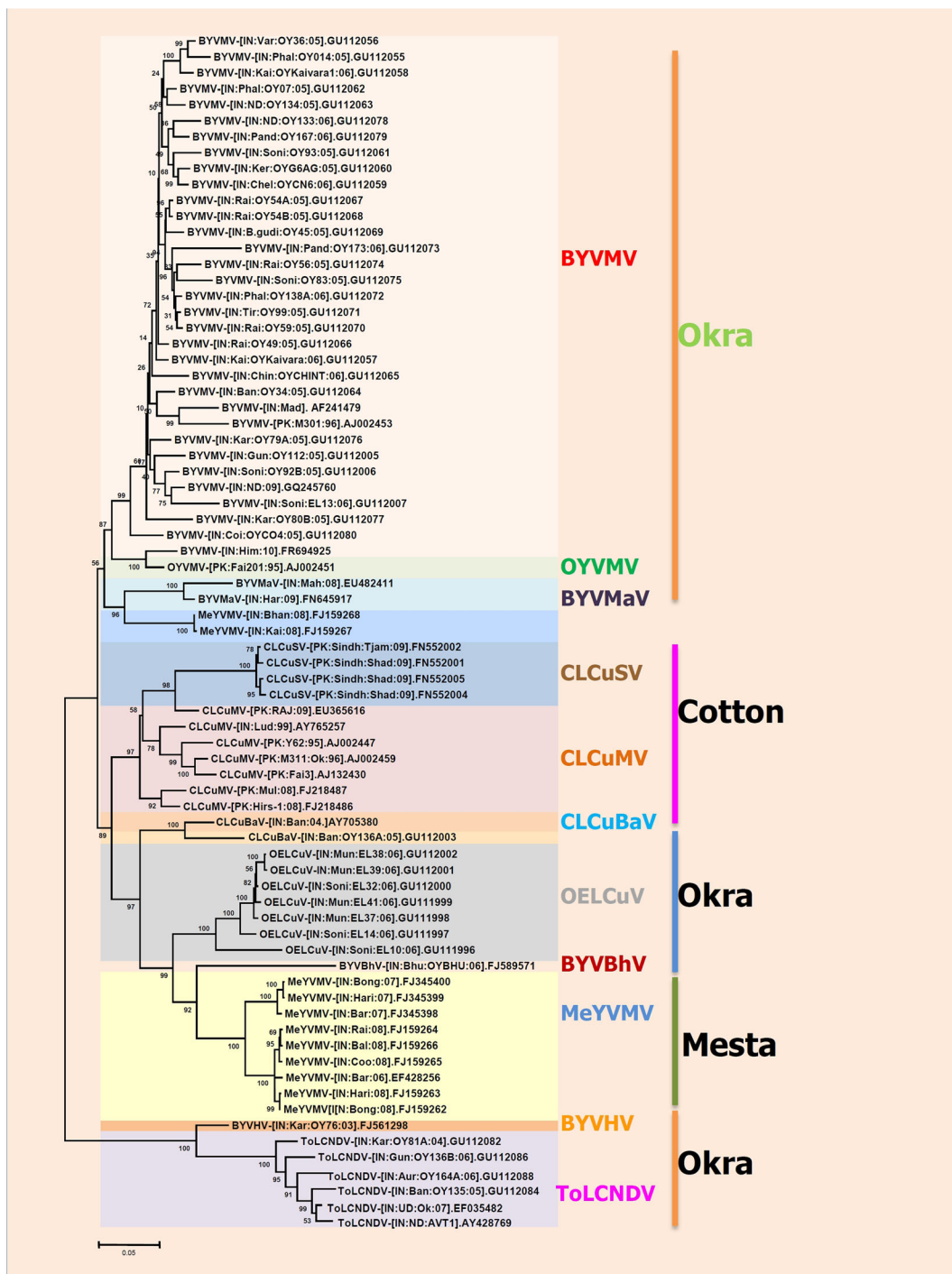


Fig. 2 Phylogenetic tree based upon an alignment of the complete nucleotide sequences of OELCuV isolates with the genomes (or DNA-A components) of selected begomoviruses obtained from the databases. For each sequence the database accession number is given. The tree was generated using the neighbour-

joining algorithm in MEGA 5. Horizontal distances are proportional to calculated mutation distances, vertical distances are arbitrary. The tree is unrooted. The numbers at nodes represent percentage bootstrap values (1000 replicates). The begomovirus sequences used are listed in Supplementary Table 1

Table 3 Highest and lowest percentage amino acid identities for pair-wise comparisons of the predicted gene products of OELCuV with selected other begomoviruses

Gene	OELCV- [IN: Sonipet EL10:06]	OELCV- [IN: Sonipet EL14:06]	OELCV- [IN: Sonipet EL32:06]	OELCV- [IN: Munthal EL37:06]	OELCV- [IN: Munthal EL38:06]	OELCV- [IN: Munthal EL39:06]	OELCV- [IN: Munthal EL41:06]	Begomoviruses*
CP	84.1	85.9	85.9	85.9	84.7	84.1	85.5	BYVBhV (1)*
	74.4	76.5	76.5	76.5	75.0	74.4	75.7	BYVHaV (1)*
	75.5	77.7	77.7	77.7	76.1	75.5	76.9	BYVMaV (1)*
	71.3–77.0	73.4–79.2	73.4–79.2	73.4–79.2	71.8–77.6	71.3–77.0	72.6–78.4	BYVMV (35)*
	76.7–77.1	78.9–79.2	78.9–79.2	78.9–79.2	77.3–77.7	76.7–77.1	78.1–78.5	CLCuBaV (1)*
	66.6–75.1	67.5–77.3	67.9–77.3	67.9–77.3	67.1–75.7	66.6–75.1	67.1–76.5	CLCuMuV (7)*
	74.8–75.5	76.9–77.7	76.9–77.7	76.9–77.7	75.3–76.1	74.8–75.5	76.1–76.9	CLCuShV (4)*
	73.2–91.4	75.3–92.9	75.3–92.9	75.3–92.9	73.8–92.1	73.2–91.4	74.6–92.1	MeYVMV (11)*
	74.8	76.9	76.9	76.9	75.3	74.8	7.61	OYVMV (1)*
	73.6–76.3	75.3–78.5	75.3–78.5	75.3–78.5	74.2–76.9	73.6–76.3	74.6–77.7	ToLCNDV (6)*
AV2	69.4	69.4	69.4	69.4	69.4	69.4	69.4	BYVBhV (1)*
	71.0	71.0	71.9	71.0	71.0	71.0	72.7	BYVHaV (1)*
	72.7	72.7	73.5	72.7	72.7	72.7	72.7	BYVMaV (1)*
	51.2–72.7	51.2–72.7	50.4–75.2	51.2–72.7	51.2–72.7	51.2–72.7	51.2–74.3	BYVMV (35)*
	81.8–85.9	81.8–85.9	80.1–85.1	81.8–85.9	81.8–85.9	81.8–85.9	80.9–85.9	CLCuBaV (1)*
	61.1–72.7	61.1–72.7	61.9–73.5	61.1–72.7	61.1–72.7	61.1–72.7	62.8–74.3	CLCuMuV (7)*
	58.6–63.6	58.6–63.6	58.6–64.4	58.6–63.6	58.6–63.6	58.6–63.6	58.6–64.4	CLCuShV (4)*
	73.5–89.2	73.5–89.2	74.3–87.6	73.5–89.2	73.5–89.2	73.5–89.2	75.2–88.4	MeYVMV (11)*
	71.0	71.0	71.9	71.0	71.0	71.0	72.7	OYVMV (1)*
	56.1–84.0	56.1–84.0	55.3–84.0	56.1–84.0	56.1–84.0	56.1–84.0	56.1–84.0	ToLCNDV (6)*
Rep	83.4	82.9	86.5	85.9	85.9	86.2	86.2	BYVBhV (1)*
	74.6	74.6	77.6	76.8	77.1	77.4	77.6	BYVHaV (1)*
	92.2	80.7	81.5	80.9	80.9	81.2	80.9	BYVMaV (1)*
	80.1–93.3	80.9–90.8	81.8–90.8	81.2–90.3	81.2–90.3	81.5–90.6	81.2–90.3	BYVMV (35)*
	86.1–85.6	85.9–88.3	91.7–95.5	91.1–95.0	91.1–95.0	91.4–95.3	91.1–95.0	CLCuBaV (1)*
	80.6–85.9	80.6–84.5	86.1–90.3	85.6–89.2	85.6–89.7	86.4–90.0	85.6–89.7	CLCuMuV (7)*
	84.8–86.1	84.8–85.9	90.3–91.7	89.2–90.6	89.7–91.1	90.0–91.4	89.7–91.1	CLCuShV (4)*
	85.3–88.6	84.5–86.1	90.8–91.9	90.3–91.4	90.3–91.4	90.6–91.7	90.3–91.4	MeYVMV (11)*
	85.9	84.8	87.6	87.0	87.0	87.3	87.0	OYVMV (1)*
	68.3–76.0	69.1–76.8	71.0–79.8	70.2–79.0	70.5–79.3	70.7–79.6	71.0–79.8	ToLCNDV (6)*
TrAP	65.3	66.6	66.6	68.6	66.0	66.0	65.3	BYVBhV (1)*
	64.0	58.0	58.0	59.3	57.3	57.3	59.3	BYVHaV (1)*
	96.6	83.3	83.3	84.6	82.6	82.0	82.0	BYVMaV (1)*
	86.0–97.3	78.6–87.3	78.6–87.3	80.0–88.6	78.0–86.6	77.3–86.0	77.3–86.0	BYVMV (35)*
	78.6–85.3	84.0–96.0	84.0–96.0	85.3–97.3	83.3–95.3	82.6–94.6	82.0–94.0	CLCuBaV (1)*
	78.0–79.3	80.0–82.0	80.0–82.0	81.3–82.0	79.3–81.3	78.6–80.6	78.0–80.0	CLCuMuV (7)*
	78.0–79.3	80.6–81.3	80.6–81.3	82.0–82.6	80.0–80.6	79.3–80.0	78.6–79.3	CLCuShV (4)*
	78.6–94.0	80.6–86.6	80.6–86.6	82.0–86.6	80.0–86.6	79.3–86.0	79.3–84.6	MeYVMV (11)*
	87.3	82.6	82.6	84.0	82.0	81.3	81.3	OYVMV (1)*
	53.3–56.0	48.0–52.0	48.0–52.0	48.6–52.6	48.0–52.0	48.0–52.0	48.6–52.6	ToLCNDV (6)*
REn	71.6	67.3	67.1	68.6	66.4	65.6	68.6	BYVBhV (1)*
	79.4	77.8	76.4	77.9	75.7	75.0	77.9	BYVHaV (1)*

Table 3 (continued)

Gene	OELCV- [IN: Sonipet EL10:06]	OELCV- [IN: Sonipet EL14:06]	OELCV- [IN: Sonipet EL32:06]	OELCV- [IN: Munthal EL37:06]	OELCV- [IN: Munthal EL38:06]	OELCV- [IN: Munthal EL39:06]	OELCV- [IN: Munthal EL41:06]	Begomoviruses*
	95.5	89.8	88.8	90.2	88.0	87.3	90.2	BYVMaV (1)*
	86.5–95.5	83.3–91.3	82.0–90.3	83.5–91.7	81.3–89.5	80.5–88.8	83.5–91.7	BYVMV (35)*
	82.0–86.5	78.9–87.6	79.8–88.8	81.3–90.2	79.1–88.0	78.3–87.3	81.3–90.2	CLCuBaV (1)*
	32.0–87.3	30.4–83.3	31.3–85.8	31.3–87.3	31.3–84.3	31.3–84.3	31.3–87.3	CLCuMuV (7)*
	85.0–86.5	80.4–81.8	82.8–84.3	84.3–85.8	82.0–83.5	82.0–82.8	84.3–85.8	CLCuShV (4)*
	70.5–94.7	70.6–89.1	70.5–88.0	70.5–89.5	69.8–87.3	69.1–86.5	71.9–89.5	MeYVMV (11)*
	88.8	85.5	84.3	85.8	83.5	82.8	85.8	OYVMV (1)*
	63.9–66.9	63.0–63.7	63.9–67.9	64.7–66.1	63.9–67.1	63.2–66.4	64.7–66.4	ToLCNDV (6)*
C4	50.0	69.0	67.0	69.0	69.0	69.0	69.0	BYVBhV (1)*
	55.0	51.0	50.0	51.0	51.0	51.0	51.0	BYVHaV (1)*
	81.0	43.0	45.3	43.0	43.0	44.0	44.0	BYVMaV (1)*
	25.3–79.4	35.0–85.0	34.5–84.0	35.0–85.0	35.0–85.0	35.5–86.0	35.5–85.0	BYVMV (35)*
	59.0–62.0	77.0–81.0	76.0–80.0	77.0–81.0	77.0–81.0	78.0–82.0	78.0–81.0	CLCuBaV (1)*
	55.0–59.0	70.0–80.0	73.0–79.0	70.0–80.0	70.0–80.0	75.0–81.0	71.0–80.0	CLCuMuV (7)*
	53.9–54.0	76.0–81.0	75.0–80.0	76.0–81.0	76.0–81.0	77.0–82.0	77.0–82.0	CLCuShV (4)*
	46.4–57.8	64.2–81.3	63.3–80.3	64.2–81.3	64.2–81.3	65.1–82.3	65.1–82.3	MeYVMV (11)*
	57.0	75.0	74.0	75.0	75.0	76.0	76.0	OYVMV (1)*
	36.0–58.0	35.0–55.0	36.0–53.0.	35.0–55.0	35.0–55.0	35.0–55.0	35.0–55.0	ToLCNDV (6)*

* Numbers of sequences from the databases used in the comparisons

a predicted stem-loop sequence containing, within the loop, the conserved nonnucleotide sequence (TAATATTAC) that is found in the majority of geminiviruses characterized to date and marks the origin of virion-strand DNA replication (Heyraud et al. 1993). Also within the IR are perfectly repeated iterons, with the sequence GGGTCTC, adjacent to the TATA box of the Rep promoter. These sequences are Rep binding motifs and are recognised by Rep in a sequence specific manner during the initiation of rolling-circle replication (Argüello-Astorga et al. 1994). The iteron sequence of OELCuV is shared only with BYVMV and MeYVMV.

Analysis for recombination

A neighbour-net analysis of sequences using the Split Tree program revealed an extensive networked relationship of OELCuV isolates with other begomoviruses, indicative of recombination (Fig. 3). This shows a reticulated network structure

indicative of phylogenetic incongruence, suggesting parts of the sequences have different origins due to recombination. Nevertheless, the tree agrees with the phylogenetic analysis shown in Fig. 2 that OELCuV is distinct from all other Asian begomoviruses isolated from okra and *Tomato leaf curl New Delhi virus* (ToLCNDV) included in the analysis. ToLCNDV is not frequently identified in malvaceous species, being instead a virus usually identified in tomato (Padidam et al. 1995).

A comprehensive analysis for recombination using RDP3, based on the alignment of all OELCuV sequences and selected begomoviruses from the databases is summarized in Fig. 4, with details of the detected recombination events given in Supplementary Table 2. The analysis showed evidence for recombination in all OELCuV isolates, with most of the sequences originating from BYVMV, BYVBhV and MeYVMV. For all OELCuV isolates, the sequences containing the origin of replication originate from BYVMV, which is consistent with OELCuV isolates sharing

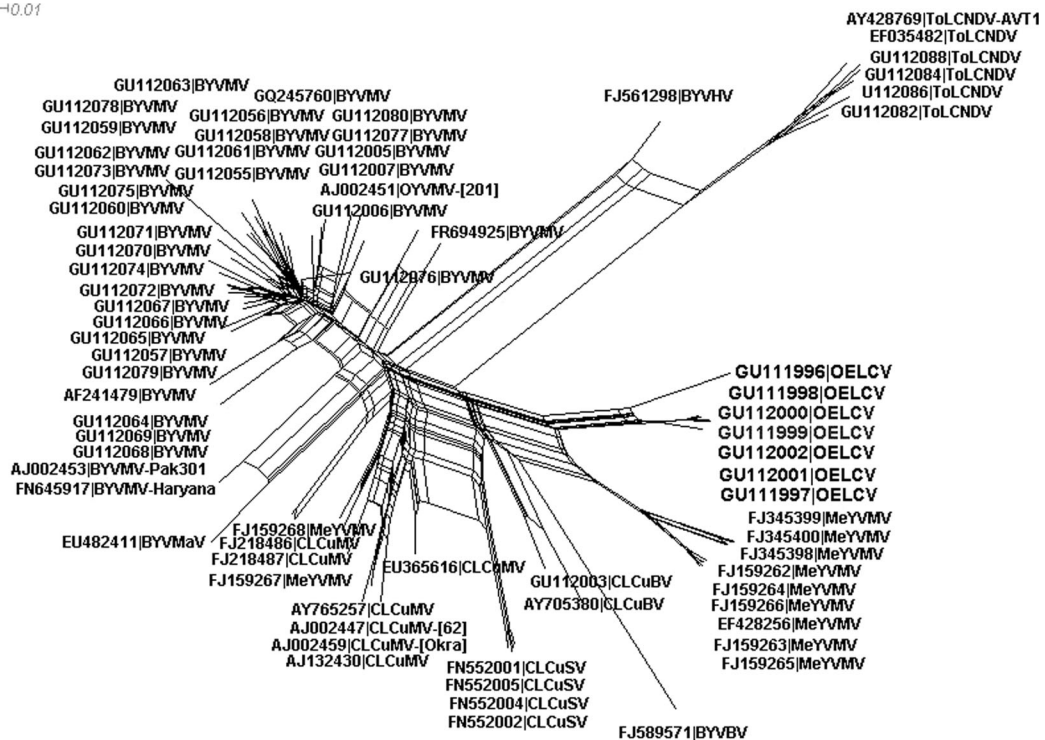


Fig. 3 A neighbour-net constructed from an alignment of the OELCuV sequences with the sequences of the genomes (or DNA A components) of selected other begomoviruses isolated from okra. The begomovirus sequences used are listed in Supplementary Table 1

iteron sequences with this species. OELCuV isolate EL10 (GU111996) was distinct from the other isolates in that it contains a small fragment derived from ToLCNDV.

Virus-vector relationship and seed transmission

The insect transmission experiments conducted showed a minimum of two whiteflies per okra plant to be effective for virus transmission, with typical symptoms appearing after a minimum incubation period of 10–12 days under controlled conditions. The transmission rate (T) and estimated transmission rate for a single whitefly (P*) increased with an increase in number of whiteflies per plant used in for transmission (Table 4). The minimum AAP and IAP for virus transmission by *B. tabaci* were 1 h and 30 min, respectively. The T and P* values showed a direct correlation with AAP and IAP periods, reaching a maximum at 24 h (Table 5).

Adult female whiteflies transmitted the virus more efficiently (higher T and P*) than male whiteflies

(Table 6). The age of okra plants had a profound effect on the of virus transmission by the vector (Table 7). The efficiency of transmission was highest (maximum T and P* -,1.00) when 7 day old seedlings were inoculated and decreased as the age of seedlings increased (only 50 % transmission for 25 day old plants). For all transmission assays, plants exposed to non-viruliferous whiteflies did not develop symptoms and no virus was detected by PCR.

Of the 75 seedlings grown from seed collected from symptomatic, infected okra plants, none either developed symptoms or were found to harbour virus using either ELISA or PCR diagnostic techniques.

Determination of host range

The host range of OELCuV isolate EL 38 was determined by whitefly transmission to 59 plant species (Table 8). Of the species investigated, only two species in the family Malvaceae, *Abelmoschus esculentus* (L.) Moench (okra) and *Althaea rosea* Cav. (holly-hock)), and seven in the family Solanaceae proved



Fig. 4 RDP analysis for recombination of the OELCuV isolates. For each of the OELCuV isolates recombinant fragments are shown as shaded bars with the origin (parental virus species)

indicated where it could be determined. The orientation and approximate position of genes are shown as arrows at the top of the diagram

susceptible to OELCuV by whitefly transmission. All the susceptible plant species produced symptoms

similar to those in okra, consisting of leaf curling and enations.

Table 4 Transmission efficiency of virus OELCuV by *Bemisia tabaci*

Number of Whiteflies used (I)	Plants infected (R)/Plants inoculated (N)	Transmission Rate (T=R/N)	Estimated Transmission rate for single whitefly (P*)
0	0/10	0.00	–
1	0/10	0.00	0.00
2	1/10	0.10	0.05
4	2/10	0.20	0.05
6	5/10	0.50	0.11
8	6/10	0.60	0.11
10	8/10	0.80	0.15
12	10/10	1.00	1.00
14	10/10	1.00	1.00
20	10/10	1.00	1.00

AAP and IAP of 24 h was given

Discussion

OELCuD is an emerging problem for okra cultivation and was first reported from Karnataka (Bangalore) in southern India in the early 1980s (Singh and Dutta 1986; Singh 1996). The disease can cause significant yield losses, ranging from 30 to 100 %, depending upon the age of the plant at the time of infection. Diseases associated with begomoviruses are an increasing problem for okra production on the Indian sub-continent. A number of factors are likely to contribute to this, including the introduction of whitefly biotype(s) that are more efficient vectors, a reduction in the genetic diversity of the crop and intensification in agriculture to feed an ever increasing population (Seal et al. 2006a). Additionally, the propensity of begomoviruses to evolve/adapt by recombination and component exchange is likely to play a part (Seal et al. 2006b). In common with the majority of dicotyledonous crops grown on the sub-continent, okra has been shown to be affected by a number of distinct begomoviruses, which include the species BYVMV (Jose and Usha 2003), *Okra yellow vein mosaic virus* (Zhou et al. 1998), CLCuBaV (Venkataravanappa et al. 2013a), *Cotton leaf curl Alabad virus* (CLCuAV) (Venkataravanappa et al. 2011a), *Bhendi yellow vein Haryana virus* [FJ561298] and *Bhendi yellow vein Maharashtra virus* [EU482411] (Brown et al. 2012) and the proposed species BYVBhV (Venkataravanappa et al. 2013b). In the majority of cases, for okra diseases of begomovirus etiology occurring on the sub-continent, betasatellites have been identified where efforts have been made to detect them (Bridson et al. 2003; Venkataravanappa et al. 2011b).

Recently, however, apparently bipartite begomoviruses have been isolated from okra. The bipartite ToLCNDV (V. Venkataravanappa, unpublished results) and a newly identified bipartite species closely related to ToLCNDV, for which the name Bhendi yellow vein Delhi virus has been proposed, were isolated from okra (Venkataravanappa et al. 2012). Here a further monopartite begomovirus has been identified in okra, adding to the known complexity of begomoviruses associated with this vegetable crop in southern Asia.

Recombination is a major driving force in the evolution of geminiviruses (Seal et al. 2006a, b) and the evidence suggests that recombination has played a part in the origin of OELCuV. The evidence further suggests that, for the most part, sequences making up OELCuV have originated from other malvaceous begomoviruses; CLCuBaV, MeYVMV and BYVMV. This is consistent with the apparently narrow host range of OELCuV, being confined to a few plant species in the family Malvaceae. CLCuBaV was first identified in cotton, specifically *Gossypium barbadense*, in southern India (Chowda Reddy et al. 2005) but has not been encountered again until recently. The results here, showing CLCuBaV to be a donor of sequences of OELCuV, and the identification of CLCuBaV in okra (Venkataravanappa et al. 2013a) suggests that, rather than being a virus associated with cotton leaf curl disease (CLCuD), it is an okra virus which occasionally infects cotton. Also, the identification of a small fragment of sequence likely originating from ToLCNDV (for OELCUV-[IN: Sonipet EL10:2006] acc. no. GU111996) indicates that, irrespective of whether the ToLCNDV infections of okra cause the disease or are

Table 5 Determination of minimum acquisition access period and minimum inoculation access period for insect transmission of OELCuV between okra plants

Determination of minimum AAP*				Determination of minimum IAP#							
AAP*	IAP#	Transmission	Plants infected (R)/inoculated (N)	Transmission Rate (R/N)	Estimated Transmission rate for single whitefly (P*)	AAP*	IAP#	Transmission	Plants infected (R)/plants inoculated (N)	Transmission Rate (R/N)	Estimated Transmission rate for single whitefly (P*)
0 min	12 h	0/10	0/10	0.00	0.00	12 h	0 min	0/10	0/10	0.00	0.00
5 min	0/10	0/10	0/10	0.00	0.00		5 min	0/10	0/10	0.00	0.00
10 min	0/10	0/10	0/10	0.00	0.00		10 min	0/10	0/10	0.00	0.00
15 min	0/10	0/10	0/10	0.00	0.00		15 min	0/10	0/10	0.00	0.00
20 min	0/10	0/10	0/10	0.00	0.00		20 min	0/10	0/10	0.00	0.00
30 min	0/10	0/10	0/10	0.00	0.00		30 min	1/10	1/10	0.10	0.01
1 h	2/10	2/10	2/10	0.20	0.02		1 h	2/10	2/10	0.20	0.02
4 h	4/10	4/10	4/10	0.40	0.05		4 h	5/10	5/10	0.50	0.07
8 h	6/10	6/10	6/10	0.60	0.09		8 h	6/10	6/10	0.60	0.09
12 h	7/10	7/10	7/10	0.70	0.11		12 h	7/10	7/10	0.70	0.11
16 h	8/10	8/10	8/10	0.80	0.15		16 h	9/10	9/10	0.90	0.21
24 h	10/10	10/10	10/10	1.00	1.00		24 h	10/10	10/10	1.00	1.00

* Acquisition access period

Inoculation access period

In each case 10 whiteflies per plant were used

Table 6 Effect of sex on transmission of OELCuV by *B. tabaci*

Sex of whitefly	Number of whiteflies/ plant (I)	Plants infected (R)/plants inoculated (N)	Transmission Rate (T=R/N)	Estimated Transmission rate for single whitefly (P*)
Female	10	25/40	0.625	0.093
Male	10	18/40	0.450	0.058

AAP and IAP of 24 h was given

opportunistic, this bipartite begomovirus is contributing to viruses infecting okra by recombination. Additionally, the finding that the sequences spanning the Rep promoter of OELCuV likely originate from BYVMV is consistent with these two species sharing common iterons.

A previous analysis looking at betasatellite diversity in okra, which included some of the plant samples analyzed here, identified two distinct betasatellites in the OELCuV infected plants; Bhendi yellow vein betasatellite (BYVB) and Okra leaf curl betasatellite (OLCuB) (Venkataravanappa et al. 2011b). For the other three isolates the betasatellite associated has been shown to be OLCuB (Table 1; V. Venkataravanappa, unpublished results). This would seem to indicate that OELCuD may be caused by one virus (OELCuV) in association with at least two distinct betasatellites. For the majority of betasatellite-associated diseases, such as CLCuD and Ageratum yellow vein disease, the betasatellite encodes the dominant symptom determinant (Qazi et al. 2007; Saunders et al. 2004). The results here (a disease caused by more than one betasatellite associated with one virus) might suggest that, for OELCuD, the virus encodes the dominant symptom determinant. For CLCuD in Asia it has been suggested that although the betasatellite determines symptoms, this must be carried to the correct plant tissues by the

virus for *bona fide* disease symptoms to ensue (Qazi et al. 2007). It is equally possible that the betasatellites associated with OELCuV encode the dominant symptom determinant but that the virus has a tissue specificity that is distinct from other okra begomoviruses in Asia. Studies with infectious clones will be needed to investigate the contribution that each component (virus and betasatellite) makes to symptoms in plants.

The whitefly transmission characteristics determined for OELCuV are in overall agreement with the results for other begomoviruses and what is known about the mechanism of transmission of geminiviruses. Geminiviruses have a circulative, non-propagative mechanism of transmission (Gray and Banerjee 1999). This mechanism is associated with a latent period (meaning there is a period between acquisition and the ability to transmit) due to the virus needing to pass through the body of the insect from the gut to the salivary glands. Typical of begomoviruses, the time required for OELCuV acquisition is longer than the period required for transmission. This is due to acquisition having to occur from specific plant cells, usually those associated with the phloem, whereas transmission occurs from insect salivation during the probing to reach the phloem – there may be numerous aborted instances of probing but relatively few successful ingestion events

Table 7 Effect of age of the seedlings on transmission of OELCuV by *B. tabaci*

Age of the seedlings in days after germination	Number of whiteflies used (I)	Plants infected (R)/plants inoculated (N)	Transmission Rate (T=R/N)	Estimated Transmission rate for single whitefly (P*)
7	10	10/10	1.00	1.00
10	10	10/10	1.00	1.00
15	10	9/10	0.90	0.21
20	10	7/10	0.70	0.11
25	10	5/10	0.50	0.07
30	10	4/10	0.40	0.05

AAP and IAP of 24 h was given

Table 8 Determination of the host range of OELCuV by insect transmission

Species	Infected/Inoculated ^{a, b}	Transmission (%)	Latent period (days)	Symptoms	Dot Blot
Family: Amaranthaceae					
<i>Amaranthus viridis</i>	0/20	0.00	–	–	–
Family: Apocyanaceae					
<i>Catheranthus roseus</i>	0/20	0.00	–	–	–
Family Asteraceae					
<i>Ageratum conyzoides</i>	0/20	0.00	–	–	–
Family : Caricaceae					
<i>Carica papaya</i>	0/20	0.00	–	–	–
Family: Cucurbitaceae					
<i>Cucumis sativus</i>	0/20	0.00	–	–	–
<i>Cucurbita moschata</i>	0/20	0.00	–	–	–
<i>Cucurbita pepo</i>	0/20	0.00	–	–	–
Family: Chenopodiaceae					
<i>Chenopodium amaranticolor</i>	0/20	0.00	–	–	–
Family: Euphorbiaceae					
<i>Croton bonplandianum</i>	0/20	0.00	–	–	–
<i>Crotonbon plandianum</i> Bail	0/20	0.00	–	–	–
<i>Euphorbia geniculata</i>	0/20	0.00	–	–	–
<i>Euphorbia hirta</i>	0/20	0.00	–	–	–
<i>Phyllanthus niruri</i>	0/20	0.00	–	–	–
Family: Legumisoae					
<i>Arachis hypogea</i> L.	0/20	0.00	–	–	–
<i>Cajanus cajan</i>	0/20	0.00	–	–	–
<i>Cajanus cajan</i>	0/20	0.00	–	–	–
<i>Cicer arietinum</i>	0/20	0.00	–	–	–
<i>Glycine max</i>	0/20	0.00	–	–	–
<i>Glycine max</i> (L.) Merrill	0/20	0.00	–	–	–
<i>Lablab purpureus</i>	0/20	0.00	–	–	–
<i>Phaseolus vulgaris</i>	0/20	0.00	–	–	–
<i>Phaseolus vulgaris</i> L.	0/20	0.00	–	–	–
<i>Pisum sativum</i> L.	0/20	0.00	–	–	–
<i>Psopocarpus tetragonolobus</i>	0/20	0.00	–	–	–
<i>Vigna mungo</i>	0/20	0.00	–	–	–
<i>Vigna mungo</i> (L.) Hepper	0/20	0.00	–	–	–
<i>Vigna radiate</i> (L.) Wilezek	0/20	0.00	–	–	–
<i>Vigna umbellate</i>	0/20	0.00	–	–	–
<i>Vigna umbellate</i>	0/20	0.00	–	–	–
<i>Vigna unguiculata</i>	0/20	0.00	–	–	–
Family: Malvaceae					
<i>Abelmoschus esculentus</i> (L.)	20/20	100.00	8–10	leaf curl	++
<i>Acalypha indica</i>	0/20	0.00	–	–	–
<i>Althaea rosea</i> (L.) Cav.	19/20	95.00	20–25	leaf curl	++
<i>Gossypium herbaceum</i> L.	0/20	0.00	–	–	–
<i>Gossypium hirsutum</i>	0/20	0.00	–	–	–

Table 8 (continued)

Species	Infected/Inoculated ^{a, b}	Transmission (%)	Latent period (days)	Symptoms	Dot Blot
<i>Hibiscus rose-sinensis</i>	0/20	0.00	–	–	–
<i>Malvastrum coromandelianum</i>	0/20	0.00	–	–	–
<i>Sida cordifolia</i>	0/20	0.00	–	–	–
Family: Solanaceae					
<i>Capsicum annuum</i> L.	0/20	0.00	–	–	–
<i>Datura stramonium</i>	3/20	15.00	30–35	leaf curl	++
<i>Solanum lycopersicum</i>	0/20	0.00	–	–	–
<i>N. clevelandii</i>	16/20	80.00	30–32	leaf curl	++
<i>N. glutinosa</i>	19/20	95.00	30–35	leaf curl	++
<i>N. occidentalis</i>	16/20	80.00	30–32	leaf curl	++
<i>N. sylvestris</i>	16/20	80.00	30–35	leaf curl	++
<i>N. tabacum</i> cv. Anand	14/20	70.00	30–32	leaf curl	++
<i>N. tabacum</i> cv. Jayashree	18/20	90.00	30–32	leaf curl	++
<i>N. tabacum</i> cv. Riwaka-1	18/20	90.00	30–32	leaf curl	++
<i>N. tabacum</i> cv. Samsun	14/20	70.00	30–32	leaf curl	++
<i>N. tabacum</i> cv. White burley	14/20	70.00	30–32	leaf curl	++
<i>N. tabacum</i> cv. Xanthi	18/20	90.00	30–33	leaf curl	++
<i>N. tabacum</i> L.	19/20	95.00	30–33	leaf curl	++
<i>Nicotiana benthamiana</i>	19/20	95.00	30–32	leaf curl	++
Weeds					
<i>Acanthospermum hispidum</i>	0/20	0.00	–	–	–
<i>Bidens pilosa</i>	0/20	0.00	–	–	–
<i>Galinsoga parviflora</i>	0/20	0.00	–	–	–
<i>Parthenium hysterophorus</i>	0/20	0.00	–	–	–
<i>Stachytarpetta indica</i>	0/20	0.00	–	–	–
<i>Tridax procumbens</i>	0/20	0.00	–	–	–

^a 10 *B. tabaci* per seedling^b AAP and IAP -24 h each

during which virus is actually acquired (Stafford et al. 2012). For the number of whiteflies used for transmission, and for increasing AAP or IAP there was a positive correlation with efficiency of transmission (increasing T and P* values) whereas the age of the seedlings used for transmission showed a negative correlation with the efficiency of transmission. These vector relations are typical of begomoviruses (Muniyappa et al. 2000; Caciagli et al. 1995). Further, transmission efficiency varied with the sex of whiteflies. Generally female whiteflies transmit begomoviruses with greater efficiency than males (Muniyappa et al. 2000; Czosnek et al. 2001), as is the case here, although for *Squash leaf curl virus* (a bipartite begomovirus) this was not the case (Polston et al. 1990). The reason for the differing ability

of male and female insects to transmit begomoviruses remains unclear

The lack of seed transmission of OELCuV in okra is in agreement with previous studies which have shown geminiviruses not to be vertically transmitted (Brown et al. 2012; Fargette et al. 1996).

It is becoming evident that there are a number of distinct begomoviruses associated with diseases of okra in southern Asia. Only for one, BYVMV with its betasatellite BYVB, have Koch's postulates in okra been satisfied (Jose and Usha 2003). There is now a need to satisfy Koch's postulates for the disease caused by the other viruses, including OELCuV. The availability of infectious clones of all these viruses (and associated satellites) would be an invaluable tool for screening

okra germplasm for natural (host-plant) resistance, as a means of controlling losses. Additionally, further studies are required to determine the actual geographical extent of each of the viruses. These will be the focus of future studies.

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