SHORT COMMUNICATION

Invasion of previously unreported dicot plant hosts by chickpea chlorotic dwarf virus in Pakistan

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Abstract Members of the genus, *Mastrevirus* (family, *Geminiviridae*) transmitted by leafhopper vectors infect monocotyledonous or dicotyledonous plants, and infection of agricultural crops results in reduced yield and quality. During 2012, a study was undertaken in the Punjab and Sindh Provinces in Pakistan to determine the identity of suspect geminiviruses associated with symptomatic cotton and vegetable plants exhibiting foliar enations, leaf curling, mosaic, and stunting reminiscent of geminivirus infection. To determine suspect geminiviral identity, fifteen apparently full-length mastrevirus genome (~ 2600 base pairs) were amplified by rolling-circle amplification, digested, cloned into the plasmid vector, pGEM-3Zf+, and sequenced from cucumber, *Gossypium arboreum* L., *Gossypium hirsutum* L., okra and tomato. The mastrevirus full-length genome sequences obtained shared their highest pairwise nucleotide sequence identity, at 97.3–98.6%, with previously reported C and L strains of *Chickpea chlorotic dwarf virus* (CpCDV) from chickpea and cotton in Pakistan, respectively. However, CpCDV has not been previously identified from cucumber, *G. arboreum*, okra, or tomato. The association of CpCDV with four previously unreported plant hosts suggests that CpCDV strains C and strain L have a broader than expected host range, and

therefore may be found to negatively affect vegetable crops, particularly, when grown in proximity to cotton.

Keywords *Chickpea chlorotic dwarf virus* · *Gossypium arboreum* · *Mastrevirus*

Geminiviruses are among the most economically important group of DNA viruses in cotton-vegetable cropping systems, worldwide. Geminiviruses have a circular, single-stranded (ss) DNA genome of 2.5–3.2 kb in size. Each ssDNA molecule is encapsidated in an icosahedral-twinned particle [12]. Geminiviruses replicate through a double-stranded (ds) DNA intermediate, and transcription occurs in the plant nucleus [25].

The *Geminiviridae* is divided into the nine genera: *Begomovirus*, *Becurtovirus*, *Capulavirus*, *Curtovirus*, *Eragrovirus*, *Grablovirus*, *Mastrevirus*, *Topocuvirus*, and *Turncurtovirus* on the basis of host range, genome organization, phylogenetic relationship and type of insect vector [3, 30, 31]. Members of mastreviruses infect either monocotyledonous or dicotyledonous plants and are transmitted by leafhoppers vector in a circulative persistent manner. The monopartite ssDNA genome of mastreviruses ranges in size from 2500 to 2700 nucleotides (nt) [18]. Their genome is composed of four open reading frames (ORFs) that encode the movement protein (MP) and coat protein (CP) on the virion strand, whereas, the replication-associated protein (RepA) and replication protein (Rep) genes are transcribed on the complementary strand. The genome also contains two non-coding regions comprising a long intergenic region (LIR) and short intergenic region (SIR), which are located nearly opposite of one another on the viral genome [26].

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Based on the International Committee on Taxonomy of Viruses (ICTV), there are 29 species assigned to the genus, *Mastrevirus* [20]. According to the proposed classification, there are seven dicot-infecting mastrevirus species, including *Tobacco yellow dwarf virus* (TbYDV), *Chickpea chlorosis virus* (CpCV), *Chickpea chlorosis Australia virus* (CpCAV), *Chickpea yellows virus* (CpYV), *Chickpea red leaf virus* (CpRLV), *Chickpea yellow dwarf virus* (CpYDV), and *Chickpea chlorotic dwarf virus* (CpCDV) [8, 16, 20, 28].

During 2012 field surveys were conducted to determine the identity and prevalence of geminiviruses associated with cotton-vegetable crop agroecosystems in the Punjab and Sindh Provinces of Pakistan. Virus-like symptoms reminiscent of geminivirus infection, including leaf curling, vein thickening, leaf yellowing, mosaic, necrotic spots, and overall stunting of plants were observed in commercial cotton (*G. hirsutum*), cucumber (*Cucumis sativus* L.), tomato (*Lycopersicon esculentum* Mill.), and okra (*Abelmoschus esculentus* L.) fields in the Vehari and Multan districts (Fig. 1). The incidence of disease symptoms ranged between 20 and 70%. Leaf samples were collected from the newly developing leaves of symptomatic plants and from asymptomatic desi cotton *G. arboreum* (Central Cotton Research Institute, Multan), transported to the laboratory on ice, and stored at -20°C. Total nucleic acids were isolated from the leaves using the cetyltrimethylammonium bromide (CTAB) method [4]. Rolling circle amplification (RCA) (TempliPhi kit; GE Healthcare, USA) was used to enrich circular DNA. The RCA products were digested with the restriction endonucleases *EcoRI*, *PstI*, and *HindIII* to linearize suspect geminiviral dsDNA replication intermediates, yielding fragments of ~2.7 kbp in size. The linearized 2.7-kbp

fragments were ligated into the plasmid vector pGEM-3Zf+ (Promega, Madison, WI) and transformed in competent *E. coli* DH5alpha cells. The resultant plasmids were isolated and purified using standard molecular biology methods [7], and plasmids bearing inserts of the expected size were sequenced bi-directionally using Sanger DNA sequencing at the USDA-ARS, MSA Genomics Laboratory. The DNA sequences were assembled using EditSeq and SeqMan software (DNASTar Inc., Madison, WI, USA), and provisionally annotated using the BLASTn algorithm (<http://www.ncbi.nih.gov/BLAST/>) [2], available at the GenBank database.

All fifteen apparently full-length geminivirus sequences recovered shared their highest sequence similarity to isolates of CpCDV. The percentage coverage ranged 100%, with similarity scores of 98–99%. The sequence for the top 10 or more hits obtained from BLASTn searches were downloaded and used as reference sequences. Fifteen complete representative CpCDV sequences were submitted to GenBank and assigned the respective Accession number (Table 1).

To determine the relationship to other previously reported CpCDV isolates, the sequences determined herein, were subjected to pairwise nucleotide (nt) analysis using the Sequence Demarcation Tool, SDT v1.2 [20]. The SDT results indicated that all 15 CpCDV sequences shared 97.4–98.6% pairwise nt identity with previously reported strains C or L of CpCDV. Eleven isolates from cucumber, *G. hirsutum*, okra, and tomato shared their highest pairwise nt sequence identity, at 97.6–98.6, with the CpCDV strain C isolated from chickpea (*Cicer arietinum*) from Faisalabad Pakistan [AM849097] (Fig. 2 and Fig S1). According to guidelines for species identification [20], the Pakistan sequences represented new isolates of CpCDV strain C. Previously, CpCDV strain C has been identified in lentil and chickpea plants from Pakistan [16], but not from cotton, cucumber, okra, or tomato plants. All CpCDV isolates identified from *G. hirsutum* were found to be present in plants also co-infected with begomoviruses, based on PCR amplification, cloning, and sequencing of the CP [9] (data not shown), as has been previously reported for this host in Pakistan [19]. While the four sequences recovered from *G. arboreum* and *G. hirsutum* shared their highest nt identity, at 97.3–98.3%, with a previously reported L strain of CpCDV reported from *G. hirsutum* [19], there has been no previous report of CpCDV from *G. arboreum*. Akhtar et al. [1] demonstrated infecting using graft-inoculation of *G. arboreum* with *Cotton leaf curl Kokhran virus*-Burewala strain (CLCuKoV-Bu) and several susceptible *G. hirsutum* lines. The *G. arboreum* genotypes were reported to develop latent viral infections, but visible symptoms did not develop on the grafted plants, despite confirmation of CLCuKoV-Bu in inoculated plants by PCR amplification.

Fig. 1 Plants infected with the mastrevirus (CpCDV) exhibiting characteristic geminivirus-like symptoms, in Pakistan **a** cucumber, curling and mosaic; **b** cotton *G. arboreum*, no symptoms; **c** okra, foliar chlorosis and thickening; **d** tomato plant, leaf curling

Table 1 Plant host, year and location of collection, genome size, and GenBank Accession number for mastrevirus field isolates

Field isolate	Host plant	Collection year	Location of district in Pakistan	Genome size (nt)	Genbank Accession number	Virus isolate
496	Cotton (<i>G. hirsutum</i>)	2012	Multan	2585	KY676359	CpCDV-C
501	Cotton (<i>G. hirsutum</i>)	2012	Multan	2585	KY676352	CpCDV-C
509	Cotton (<i>G. hirsutum</i>)	2012	Multan	2624	KY676357	CpCDV-C
516	Cotton (<i>G. hirsutum</i>)	2012	Multan	2582	KY676353	CpCDV-C
518	Cotton (<i>G. hirsutum</i>)	2012	Multan	2582	KY676358	CpCDV-C
520	Cotton (<i>G. hirsutum</i>)	2012	Multan	2586	KY676351	CpCDV-L
520-A	Cotton (<i>G. hirsutum</i>)	2012	Multan	2588	KY676354	CpCDV-L
520-B	Cotton (<i>G. hirsutum</i>)	2012	Multan	2589	KY676355	CpCDV-L
528	Cotton (<i>G. hirsutum</i>)	2012	Multan	2582	KY676356	CpCDV-C
154	Cotton (<i>G. arboreum</i>)	2012	Multan	2585	KT634301	CpCDV-L
349	Cucumber (<i>Cucumis sativus</i>)	2012	Vehari	2588	KT719388	CpCDV-C
349-A	Cucumber (<i>C. sativus</i>)	2012	Vehari	2598	KT719389	CpCDV-C
349-B	Cucumber (<i>C. sativus</i>)	2012	Vehari	2594	KT719390	CpCDV-C
364	Okra (<i>Abelmoschus esculentus</i>)	2012	Vehari	2586	KT719391	CpCDV-C
330	Tomato (<i>Lycopersicon esculentum</i>)	2012	Vehari	2586	KP881605	CpCDV-C

Also, Rahim ullah et al. [29] graft-inoculated *G. hirsutum* plants using a scion from *G. arboreum*-CLCuKoV-Bu infected plants and demonstrated viral accumulation, albeit only mild symptoms developed in inoculated *G. hirsutum* plants, suggesting a low-level of replication by CLCuKo-Bu in *G. arboreum*. Thus, this is the first report of natural infection of *G. arboreum* by a geminivirus, and of infection of this cotton species by the mastrevirus, CpCDV.

The phylogenetic analysis was carried out using maximum-likelihood (ML) for 1000 bootstrap iterations, at > 70% support, implemented in MEGA 6 [27]. The ML analysis included 15 full-length sequences of newly determined CpCDV genomes representing different host plants and locations in Pakistan, and 20 representative mastrevirus isolates available in the GenBank database, representing different strains, respectively (Fig. 2). The CpCDV isolates originating from cotton (determined herein) clustered in two distinct clades. One group clustered with strain C together with all of the isolates from vegetable hosts e.g. cucumber, okra, and tomato, whereas, the second group clustered with previously reported strain L isolates from cotton (Fig. 2).

To confirm that a biologically-viable mastrevirus was associated with the CpCDV strain C-infected field plants, the apparently full-length genome cloned isolate 349 isolated from cucumber (*C. sativus*) from Vehari, Pakistan, was used for construction of a 1.2-mer infectious clone. The 1.2-mer was constructed by ligating the PCR-amplified fragment (2397 bp) with a 799 bp fragment restricted from

original clone of CpCDV using *HindIII* and *EcoRI*, into the binary plasmid vector pGII0229 [10]. The infectious clone was transformed into competent *Agrobacterium tumefaciens* LBA 4404 cells. The expected size was confirmed by restriction digestion, and viral identity was determined by confirmatory DNA sequencing e.g. 800 bp of each end, and the clone was used for agro-inoculation of tobacco (*Nicotiana tabacum*) plants [21]. The agro-inoculum was infiltrated into young plants at the 3–4 true leaf stage using a 5-ml sterile syringe outfitted with a 31-gauge needle. The plants were held at 30 °C in a well-lit growth room and observed periodically for symptoms development, for 6 weeks. The CpCDV 1.2-mer construct was found to be infectious to *N. tabacum*, based on the development of characteristic geminivirus symptoms in newly-emerging leaves 3-weeks post-inoculation, with 13 out of 15 (86.6%) tobacco plants showing symptoms by 5–6 weeks post-inoculation. Symptoms in plants inoculated with the CpCDV 1.2-mer consisted of leaf curling and crumpling, and overall stunting of plants. Symptoms were not observed in mock-inoculated (non-transformed, *A. tumefaciens* LBA 4404 suspension) tobacco plants by 6wks post-inoculation. The symptomatic plants (13 of 13) were positive for CpCDV presence using PCR amplification with CpCDV-specific primers, whereas, total nucleic acids purified from leaves of 2 selected mock-inoculated tobacco plants contained no amplicon, as was also observed for the PCR amplified, no template control (double-distilled water) (Fig. 3).

Fig. 2 Maximum likelihood (ML) phylogenetic tree reconstructed for the complete genomes of *Chickpea chlorotic dwarf virus* determined here, with representative sequences of 18 CpCDV sequences (GenBank), and two other species of dicot infecting mastrevirus (CpRLV and CpCV-A). Mastreviruses used for this

analysis are *Chickpea chlorosis virus* (CpCV-A), *Chickpea chlorotic dwarf virus* (CpCDV) and *Chickpea redleaf virus* (CpRLV). The ML tree (1000 iterations) was rooted with *Cotton leaf curl Kokhran virus-Burewala strain* (CLCuKoV-Bu) (FR750321). The bootstrap values of are shown for nodes, at > 70% for 1000 replicates

Recently, mastreviruses have increased in prevalence and in distribution worldwide, posing an increased threat to agricultural crops [15]. Although until recently, most mastreviruses studied were known to infect monocotyledonous plants, those infecting dicotyledonous species have become increasingly recognized as important viral pathogens. Thus far, two dicot-infecting mastreviruses, CpYDV and CpCDV, have been reported outside of Australia, where they were first described in Pakistan and India respectively [13, 16]. For example, CpYDV has been identified in symptomatic chickpea plants in Faisalabad, Pakistan [16], and several strains of CpCDV has been detected from legumes in the Africa [11], the Indian sub-continent, including Pakistan [16], and in the Middle East [17]. Most characteristically, CpCDV has been identified as a pathogen of primarily legumes, such as chickpea, faba

bean, and lentil, which develop symptoms of foliar yellowing, stunting, and reduced seed production [14, 22, 23]. Recently and somewhat unexpectedly, CpCDV has been identified in a number of previously unreported hosts, none of which are legumes, and include cotton [19], papaya [24], pepper [1], squash [5], sugar beet [6], tomato, and watermelon [32].

The results reported herein demonstrate that CpCDV is not only more widely distributed than previously thought, it has a broad host range that includes an extensive number of different dicotyledonous plants in at least six families, e.g. Caricaceae, Chenopodiaceae, Cucurbitaceae, Fabaceae, Malvaceae, and Solanaceae, and seems to be increasingly adapted to cotton-vegetable cropping systems in Pakistan, affected species already infected with whitefly-transmitted geminiviruses. The co-infection of two CpCDV strains, C

Fig. 3 Agro-inoculation of tobacco (*Nicotiana tabacum* L.) plants. A, Mock-inoculated, negative control plant; B and C, tobacco plants inoculated with a 1.2 mer infectious clone of CpCDV, showing leaf curl symptoms that developed post-inoculation

and L with a number of begomoviruses, in many plant host species underscores the potential of these emergent geminiviruses to adversely affect cotton and food production with increasing prevalence, and the widespread distribution of CpCDV, in vegetable crops, is itself alarming. The invasion of a number of botanically diverse, previously unreported mastreviruses in Pakistan (and elsewhere) highlights the importance of increased surveillance for the presence of these viruses in cultivated and wild host species acting as mixing vessels for these viruses leading to emergence of new strains and species, which can be a potential threat to the sustainability of agricultural and crop productivity.

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