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Infectivity of okra enation leaf curl virus and the role of its V2 protein in pathogenicity

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ABSTRACT

Cotton crop has been severely affected by multiple begomoviruses in Pakistan and India. In our previous study, we found okra enation leaf curl virus (OELCuV), cotton leaf curl Multan betasatellite (CLCuMuB) and cotton leaf curl Multan alphasatellite (CLCuMuA) infecting cotton in Pakistan. The current study was designed to investigate the infectivity of OELCuV and its ability to trans-replicate non-cognate CLCuMuB. Agro-infectious clones containing the partial tandem repeats of OELCuV and CLCuMuB were constructed and the infectivity assays were carried out through *Agrobacterium* mediated transformation in the model host species *Nicotiana benthamiana* under controlled conditions. The results showed that in the inoculated plants OELCuV alone can cause downward curling and yellowing of leaves with thickened veins. However, when co-inoculated with the non-cognate CLCuMuB it could functionally trans-replicate CLCuMuB resulting in a more severe phenotype. The expression of Pre-coat/V2 protein in the *N. benthamiana* plants through the potato virus X (PVX) system caused localized cell death after severe leaf curling in the infiltrated leaves. The tissue tropism of the virus was associated with the systemic development of a hypersensitive response (HR), which ultimately lead to the plant death. The results indicated the involvement of V2 protein in the pathogenicity of OELCuV and its ability to trigger the host defense machinery. This study also demonstrated the ability of OELCuV to trans-replicate CLCuMuB resulting in typical leaf curl disease symptoms in *N. benthamiana*.

Geminiviruses are arthropod-transmitted plant viruses characterized by circular and single-stranded DNA (cssDNA) genomes, encapsidated in two joined icosahedrons (Brown et al., 2012). The family *Geminivirdae* has been classified into nine genera *Becurto-, Begomo-, Capula-, Curto-, Eragro-, Grablo-, Mastre-, Topocu- and Turncurto-virus* (Zerbini et al., 2017). The whitefly borne genus *Begomovirus* encompasses > 388 accepted species, infecting dicotyledonous plants (Zerbini et al., 2017). Mostly, the New World (NW) begomoviruses have genome size ~5 kb, which is equally distributed into two components known as DNA-A and DNA-B. While, most of the Old World (OW) begomoviruses have monopartite genome approximately 2.8 kb in size, which is a homolog of the bipartite begomovirus DNA-A. Begomoviruses from the OW are distinct from the NW begomoviruses, having an additional pre-coat protein (V2) (conserved for OW begomoviruses) (Nawaz-ul-Rehman et al., 2009). The OW monopartite begomoviruses genome encompasses six open reading frames (ORFs) in the opposite orientation, all of which are transcribed by a common bidirectional promoter present in the conserved region (CR). The virion-sense coat protein (CP) is responsible for insect transmission and facilitates *in planta* viral movement in cooperation with the V2 protein. The V2 protein is also involved in transcriptional- and post-transcriptional gene silencing (PTGS) suppression by the begomovirus (Wang et al., 2014; Saeed et al., 2015). It also helps to sustain high viral accumulation in the plants during infection (Fondong, 2013). The replication-associated protein (Rep), transcriptional activator protein (TrAp), replication enhancer protein (REn) and C4 protein are transcribed from the complementary-sense strand. The begomoviruses initiate their rolling circle replication (RCR) by the Rep protein. The TrAp commences the upregulation of the late virion-sense genes, PTGS suppression and also overcome virus induced hypersensitive cell death (Mubin et al., 2010). The REn triggers elevated accumulation of the viral DNA and C4 protein may act as a symptom determinant (Hanley-Bowdoin et al., 2013).

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Nevertheless, in bipartite begomoviruses the DNA-B encoded nuclear shuttle protein (NSP) and movement protein (MP) accomplish *in planta* systemic spread of the virus genome and virus shuttling across the nucleus, respectively (Hanley-Bowdoin et al., 2013).

The monopartite begomoviruses in the OW are often found in association with small and cssDNA satellites, which may have no or little sequence homology with their helper begomovirus (Mayo et al., 2005). Three types of DNA-satellite molecules including alpha-, beta- and delta-satellites have been identified so far. The non-pathogenic alphasatellites have the only ORF encoded on the virion-sense strand and thus, are capable of autonomous replication (Nawaz-Ul-Rehman et al., 2010). The betasatellites and deltasatellites have recently been classified into the genus *Betasatellite* and *Deltasatellite* in the sub-viral family *Tolecusatellitidae* (Lozano et al., 2016). The complementary-sense protein βC1 of the betasatellites play vital role in efficient monopartite begomoviruses infection. Besides, betasatellites comprised of an Adenine-rich (A-rich) region and a satellite conserved region (SCR) followed by a stem loop with nona-nucleotide sequence (TAATATTAC) (Briddon and Stanley, 2006). Betasatellites enhance viral pathogenicity by suppressing the host defenses, leading to the systemic infection and symptom development. Although majority of the satellite associated begomoviruses can cause infection alone however, the presence of a betasatellite induces more severe symptoms (Nawaz-ul-Rehman et al., 2009). Nevertheless, the betasatellite molecules are quite flexible for trans-replication by a diverse range of non-cognate begomoviruses (Nawaz-ul-Rehman et al., 2009; Saeed et al., 2005; Sivalingam and Varma, 2012) and even mastreviruses (Kumar et al., 2014). The newly characterized deltasatellites from the NW do not encode any protein and are not indispensable for begomovirus infection (Fiallo-Olivé et al., 2016).

The plants immune responses to the invading pathogens are triggered by the pathogen-encoded avirulence (*avr*) protein products and their recognition by the resistance (R) genes (Pumplin and Voinnet, 2013). Such responses are often lead to the pathogen halt as a result of rapid and localized cell death or hypersensitive response (HR) and the blockage of systemic infection (Lam et al., 2001). Like animal viruses, plant viruses also manipulate the HR response of the host plant. The *V2* gene of many monopartite begomoviruses has been empirically shown to elicit HR in the model plant *Nicotiana benthamiana* (Mubin et al., 2010; Sharma and Ikegami, 2010). However, according to Matić et al. (2016) a similar HR was elicited by the C2 protein of tomato yellow leaf curl virus (TYLCV) in *N. benthamiana* and *Arabidopsis* plants (Matić et al., 2016). Similarly, the NSP of bipartite begomoviruses can also induce a cell death response (Hussain et al., 2007). However, the induction of an HR is usually suppressed by viral-encoded proteins (Mubin et al., 2010; Matić et al., 2016) thus, HR cannot be seen in the infected plants.

Okra enation leaf curl virus (OELCuV) is a typical monopartite begomovirus causing leaf curling, pin headed enations on the abaxial side of the leaves and twisting of main stem (Venkataravanappa et al., 2015). In our previous study, we have identified a field infection of OELCuV in cotton plants associated with cotton leaf curl Multan betasatellite (CLCuMuB) and cotton leaf curl Multan alphasatellite (CLCuMuA) in Pakistan (Hameed et al., 2014). The cotton crop in Pakistan has been infected by a complex of multiple monopartite begomoviruses in association with single betasatellite species CLCuMuB and occasionally alphasatellites (Sattar et al., 2013, 2017). The present study was aimed to investigate the ability of OELCuV to trans-replicate CLCuD-associated CLCuMuB and to decipher the role of V2 protein in the OELCuV pathogenicity.

A complete monomeric genome of OELCuV DNA-A (HF567945) was previously cloned at *HindIII* site in pGem3Zf + vector as pGOE (Hameed et al., 2014). A ~0.2 mer partial tandem repeat of OELCuV was excised from the monomeric construct pGOE and subsequently ligated into the binary vector pGreenII 0000 (Hellens et al., 2000) as ~550bp *HindIII/XhoI* fragment to produce pGOE-0.2 recombinant construct. The OELCuV monomer was digested from pGOE using *HindIII*, gel-purified and ligated into the similarly digested pGOE-0.2 clone to construct a partial tandem repeat construct pGOE-1.2. The orientation of the partial tandem repeat was verified using *HindIII* restriction digestion, releasing a ~2.7 kb fragment in tandem orientation. Similarly, a partial tandem repeat construct pGMB-1.4 was constructed using a 0.4 mer *SalI/KpnI* partial fragment of CLCuMuB using previously constructed monomeric construct of CLCuMuB (HF567946). The V2 protein of OELCuV and its deletion mutant (mV2) were PCR amplified from the clone pGOE using primer pairs OEV2F/OEV2R and MuOEV2F/OEV2R, respectively (supplementary Table 1). To commence deletion analysis point mutations were introduced at the N-terminal of the primer MuOEV2F to surpass the V2 start codon (supplementary Table 1). The amplified V2 ORF and its mutated derivative mV2 were cloned at *ClaI/SmaI* site in a PVX-based vector (pgR107) (Chapman et al., 1992) to obtain pgR-V2 and pgR-mV2, respectively. All the constructs were prepared to be transformed into the *Agrobacterium* strain LBA4404 (having pSoup as a helper plasmid to replicate pGreenII binary vector) through electroporation. The agrobacterium cultures containing recombinant PVX vectors were infiltrated into the *N. benthamiana* plants following Just et al. (2017). Equal number of plants were used in each set of experiments and each experiment was repeated twice (Table 1) under controlled conditions. The systemic top leaves of the infiltrated plants were used for total genomic DNA isolation using CTAB method (Doyle and Doyle, 1990). The infectivity of OELCuV and CLCuMuB was confirmed by PCR using CLCV1/CLCV2 (Briddon and Markham, 1994) and Beta01/Beta02 (Briddon et al., 2002) primers (Supplementary Table 1) and Southern blot hybridization, respectively. Total genomic DNA (~10 µg) was electrophoresed on 1.2% agarose gel for Southern blot hybridization using Hybond nylon membrane. The membranes were hybridized with digoxigenin (DIG) (Roche Diagnostics, Switzerland) labelled PCR products using the primers CLCV1/CLCV2 and Beta01/Beta02, respectively as described by Nahid et al. (2011). Total RNA was extracted from the plants inoculated with pgR-V2 and pgR-mV2 and cDNA was synthesized using Reverted H Minus First strand cDNA synthesis kit (ThermoFisher Scientific). To confirm the accumulation of V2 and mV2 transcripts, the cDNA was employed into reverse transcriptase polymerase chain reaction (RT-PCR) in Thermal cycler (Bio-Rad c1000 Touch) using respective primers (Supplementary Table 1).

Table 1
Infectivity analysis of OELCuV and CLCuMuB in *Nicotiana benthamiana* plants.

Inoculations	Plants infected/ inoculated		RT/PCR	Southern blot hybridization	Symptoms[a]	Latent period (dpi)
	Exp 1	Exp 2				
OELCuV	8/10	7/10	+	+	MDC, VT	12
CLCuMuB	10/10	8/10	+	+	SDW, VT	11
pgR-V2	10/10	10/10	+	N/A	LY, DC, Nc,	5
pgR-mV2	5/5	5/5	+	N/A	MVY, VT	8
pgR107	1/1	1/1	–	N/A	MVY, VT	8

[a] R/T PCR: Reverse transcriptase polymerase chain reaction, Symptoms are mentioned as MDC: Mild downward curling, DC: Downward curling, VT: Vein thickening, SDW: Severe downward curling, LY: leaf yellowing, Nc: necrosis, MVY Mild vein yellowing, N/A: Not applicable.

The plants inoculated with OELCuV alone started showing mild downward curling of leaves, vein thickening and leaf yellowing symptoms comparing to the control plants at 10–12 days of post inoculation (dpi) (Fig. 1A,B). Nevertheless, the plants showed enhanced symptom severity with OELCuV in combination with CLCuMuB showing severely curled and crumpled leaves with thickened veins (Fig. 1C). After the symptoms were fully developed, total genomic DNA was extracted at 25

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