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EVALUATION OF THE RESISTANCE AGAINST BEGOMOVIRUSES IMPARTED BY THE SINGLE-STRANDED DNA BINDING PROTEIN VirE2

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Begomoviruses belonging to the family *Geminiviridae* are plant-infecting distinct single-stranded (ss)-DNA viruses causing significant reductions in crop yield. VirE2 is ss-DNA binding protein and can be used to obtain resistance against ss-DNA viruses. We have utilized VirE2 from *Agrobacterium tumefaciens* to get resistance against begomoviruses. Resistance was evaluated against both mono- and bipartite begomoviruses using *Agrobacterium*-mediated inoculation technique. *Nicotiana benthamiana* plants were inoculated with infectious clones of *Cotton leaf curl Kokhran virus* (CLCuKoV)/Cotton leaf curl Multan betasatellite (CLCuMuB) and *Tomato leaf curl New Delhi virus* (ToLCNDV) alone as well as along with VirE2 construct. The plants inoculated with infectious clones showed typical virus symptoms while delayed or milder symptoms were observed in plants inoculated with viruses along with VirE2 construct, furthermore, 68% resistance against CLCuKoV/CLCuMuB and 56% against ToLCNDV was observed. The visual observations were confirmed through PCR and Southern hybridization. The results have also shown that *VirE2 gene* was able to reduce the replication of both viruses. Therefore, inhibition of viral DNA replication by VirE2 could be adapted to protect economically important crops against begomoviruses.

Keywords: VirE2, begomoviruses, monopartite, bipartite, resistance, non-pathogen derived resistance

INTRODUCTION

Geminiviruses are plant infecting circular single-stranded DNA (ss-DNA) viruses belonging to family *Geminiviridae* and are characterized by small, isometric, twinned, icosahedral virion particles (Jeske, 2009). The family *Geminiviridae* comprises of seven genera namely *Mastrevirus*, *Curtovirus*, *Begomovirus, Topocuvirus*, *Becurtovirus, Turncurtovirus, Eragrovirus* based upon insect vector, host range and genome organization (Adams *et al*., 2013). *Begomovirus* is the largest genus of this family consisting of either mono- or bipartite genome transmitted by whitefly (*Bemisia tabaci*) and infect many dicotyledonous plants including cotton, cassava, beans, tomato, pepper etc. causing great economic losses to worldwide agricultural production (Rojas *et al*., 2005). The majority of monopartite begomoviruses are found associated with DNA satellites called betasatellite and alphasatellite (Briddon *et al*., 2006). The ss-DNA genome of monopartite begomoviruses prevalent in the old world encodes six open reading frames (ORFs), four on the complementary-sense strand namely C1, C2, C3 and C4, which encode replication-associated protein, transcriptional activator protein, replication enhancer protein and C4 protein, respectively. Two ORFs are present on the virion-sense strand (V1 and V2) that encodes viral coat protein (CP) and movement related proteins, respectively (Fondong, 2013). In bipartite begomoviruses viral genome consist of two components DNA-A and DNA-B, each of size 2.8 kb. DNA-A encodes all the proteins necessary for viral replication, gene expression and encapsidation whereas component B encodes viral movement proteins (Stanley *et al*., 2005). Natural resistance sources of geminiviruses are limited (Maule *et al*., 2007) and resistance against these ss-DNA viruses is a most important problem worldwide (Yousaf *et al*., 2013).

The diversity of begomoviruses can be estimated by the escalating number of its reported species. Additionally, only a single crop is attacked by diverse range of viruses for example tomato is being attacked by several mono- as well as bipartite viruses (Melgarejo *et al*., 2013), likewise a complex of begomoviruses is attacking cotton and causing CLCuD (Sattar *et al*., 2013), and cassava-infecting begomoviruses causing Cassava mosaic disease in Africa (Patil and Fauquet, 2009). Most of the begomoviral species have emerged during the past 10 years (Briddon *et al*., 2010; Fauquet *et al*., 2008). This huge diversity and emergence of begomoviruses can be attributed to a number of factors which include random mutations, recombination, pseudo-recombination, mixed infections, emergence of invasive

whitefly biotypes, long distance movement, and acquiring novel viral components (Maria *et al*., 2008). These scenarios necessitate exploring sequence independent and broad-spectrum resistance strategy for rapidly evolving diverse begomovirus disease complexes.

Genetically engineered resistance strategies are either pathogen derived resistance (PDR) or non-pathogen derived resistance (NPDR). The PDR involves the addition of a part or a complete viral gene into the plant which later on hinder in the replication process of the viral pathogen. But PDR is not found highly effective against ss-DNA viruses due to the problem of virus induced gene silencing and it is not broad-spectrum as well (Ilyas *et al*., 2011). Whereas, NPDR employs some host resistance genes or genes from any other source involved in adaptive host development induced on pathogen attack (Shepherd *et al*., 2009). NPDR for geminiviruses is not much illustrated in the past nevertheless only some non-pathogen genes have been attempted to get resistance against these phytopathogens (Edelbaum *et al*., 2009; Hanley-bowdoin *et al*., 2012; Lopez-Ochoa *et al*., 2006; Padidam *et al*., 1999; Sunitha *et al*., 2011). Previously it has been shown that a ssDNA-binding (SSB) protein, G5 complemented ss-DNA accumulation and interfered with viral movement (Padidam *et al*., 1999). VirE2 is a 60 kDa (556 amino acids) SSB encoded by Ti plasmid of *Agrobacterium tumefaciens* which binds to ssDNA in a co-operative and sequence non-specific zipper-like manner. It possesses two nuclear localization signals. Deletion of C-terminal 37 residues of VirE2 resulted in complete loss of ssDNA binding function (Sen *et al*., 1989). Further mutational analysis showed that ssDNA binding activity is related with the C-terminus, whereas cooperativity was found to be related with N-terminus. The mutation in central region reduced the tumorigenicity but did not affect ssDNA binding (Dombek and Ream, 1997). VirE2 expressing transgenic plants showed the complementation for the virulence showing formation of T-complex in plant cell (Citovsky *et al.*, 1992; Gelvin, 1998; Sunitha *et al.*, 2011). VirE2 expressing transgenic plants showed *in planta* functioning of VirE2 and no negative effect on plant growth (Gelvin, 2012). VirE2 reduced *Mungbean yellow mosaic India virus* (MYMIV) DNA accumulation (Sunitha *et al*., 2011). In this study, utilizing NPDR based upon the concept of SSB, we have studied the potential of VirE2 to control both monopartite and bipartite begomoviruses in *N. benthamiana* plants by transient assay. Future implications of using VirE2 for broad-spectrum resistance against begomoviruses and other ss-DNA viruses are discussed.

MATERIALS AND METHODS

N. benthamiana plants grown in greenhouse with a photoperiod of 16-hr light/8-hr dark at 26±2°C were used for the propagation of virus and assaying the resistance. The resistance assay was carried out using infectious clone of a monopartite begomovirus named *Cotton leaf curl Khokhran virus* (CLCuKoV)/*Cotton leaf curl Multan betasatellite* (CLCuMuB) (Mansoor *et al*., 2003) and a bipartite *Tomato leaf curl New Delhi virus* (ToLCNDV) (Saeed, 2008; Padidam *et al*., 1995). Total genomic DNA of *Agrobacterium tumefaciens* strain C58 was extracted using CTAB (Brake *et al*., 2001). Primers were designed to the VirE2 sequence [Genbank: NC_003065.3] which allow the amplification of full length gene. The sequence of primer set was 5′-GATAAGCTTATGGATCCGAAGGCCGAAGGCAATG-3′ and 5′-AATGTCGACCTACAGACTGTTTACGGTTGGGCC G-3′. These primers introduced *Hind*III and *Sal*I restriction sites, respectively which were used for cloning of VirE2 gene. PCR amplified VirE2 was cloned in a TA-cloning vector (pTZ57R/T; Fermantas). Cloned VirE2 was sequenced and then sub-cloned in plant expression vector pJIT163 (Guerineau *et al*., 1992) at *Hind*III and *Sal*I sites to produce p35SVirE2. p35SVirE2 was then cloned into pGreen0029 (Hellens *et al*., 2000) at *Kpn*I and *Xho*I restriction sites. The confirmed clone pGreen0029-VirE2 was transformed into *Agrobacterium tumefaciens* (strain LBA4404) by electroporation and pVirE2 was obtained. A colony of recombinant plasmid pVirE2 was cultured along with appropriate antibiotics. Infectious clones of CLCuKoV/CLCuMuB and ToLCNDV were also cultured similarly. The cells were collected by centrifugation (3000g, 10min) and re-suspended to a final concentration of OD600 of 1.0 in a solution containing 10mM $MgCl_2$, 10mM MES and 100µM acetosyringone. The *Agrobacterium* cultures of pVirE2 and infectious clones were mixed in equal (v/v) ratios separately. The mixture was kept at room temperature 2-3 hours or overnight before agroinfiltration. *N. benthamiana* plants of 5-6 leaf-stage were agroinfiltrated by delivering the mixture on the lower side of the leaf with a 5 ml needle-less syringe. For each plant 3-4 leaf were inoculated twice at different locations. All plants were photographed and leaf tissues were taken when control plants were fully symptomatic. Total genomic DNA extraction from newly developing *N. benthamiana* leaf tissues was carried out by CTAB method (Doyle *et al*., 1990). Presence of infiltrated CLCuKoV was checked by PCR using primer set CLCV1 and CLCV2 as described by (Shahid *et al*., 2007) while primers β01 and β02 (Briddon *et al*., 2002) were used for the detection of betasatellite. Moreover, ToLCNDV was amplified using a primer set; (5′-GCAAATCGATATGGCGAAGCGACCAG-3′ and 5′-GTCGACTATTAATTTGTGGCCGAATC-3′) to amplify the CP region of ToLCNDV. Virus titer was determined by Southern blot analysis and hybridization was carried out using DIG-labeled probes (Roche) of CLCuKoV, CLCuMuB and ToLCNDV DNA-A by following manufacturer's instructions. The incidence of disease was checked by visual observation of five different levels of

symptoms 0, 1, 2, 3 and 4 for symptoms exhibiting no symptoms (NS), very mild (VM), mild (M), sever (S) and very sever (VS) symptoms, respectively. The percentage of incidence of disease was calculated on the bases of results of PCR using the formula; %I = (PCR positive plants/total plants)*100. The percentage of symptomatic severity was calculated by using the formula; S = (Σi/N [VM])*100 where S = percentage of symptom severity, Σ = sum of observed values, N = total number of observed plants, VM = value of full-scale (Medina-Hernandez *et al*., 2013; Vander Plank, 1963).

RESULTS

To study the potential of VirE2 to block the replication of attacking viruses, *N. benthamiana* plants were inoculated with either CLCuKoV/CLCuMuB or ToLCNDV along with pVirE2 by agroinfiltration. The replication of virus was compared with control *N. benthamiana* plants which were inoculated with either CLCuKoV/CLCuMuB or ToLCNDV alone. Three independent biological replicates were studied and the results are shown in Table 1. Plants were monitored for the typical symptoms of the disease after every two days and assessed for the disease severity. *N. benthamiana* plants inoculated with CLCuKoV/CLCuMuB showed the symptoms of downward leaf curling; vein swelling and darkening on newly emerging leaves of all plants at 21 days post inoculation (dpi) were taken as positive control plants (Fig.1A) whereas non-inoculated *N. benthamiana* served as negative controls (Fig.1B). Plants inoculated with CLCuKoV/CLCuMuB showed infection efficiency of 100% as symptoms were observed in all control plants. As a result of three independent experiments, in average, 68.33% plants co-inoculated with pVirE2 and CLCuKoV/CLCuMuB remained symptomless like healthy negative control plants (Fig.1C). However remaining 31.66% plants showed 5-6 days delay in symptom onset as compared to control plants. Furthermore, these plants showed mild symptoms as compared to control plants qualitatively. Plants co-inoculated with pVirE2 and CLCuKoV/CLCuMuB were kept in green house and observed at 40 and 60 dpi, visually no symptoms were observed in these plants and they remain healthy. However, in average 61.66% incidence of virus was observed on these plants as shown by the presence of virus in the systemic leaves of plants when subjected to PCR at 21dpi while many asymptomatic plants were PCR negative (Table 1). Thus an incidence reduction of 38.34% was observed. All PCR positive plants when subjected to Southern hybridization using CLCuKoV and CLCuMuB DNA probe; virus accumulation in these plants was detected. However, there was a significant decrease in the virus titer when it was compared with control *N. benthamiana* plants (Fig.1D,E). These results indicate that VirE2 is playing a role in the reduction of CLCuKoV and CLCuMuB accumulation in the systemic leaves.

Inoculation of ToLCNDV in *N. benthamiana* resulted in the typical symptoms of upward leaf curling, vein thickening and plant stunting at 14 dpi. Infection efficiency was 100% as all control plants showed symptoms (Fig.2A) with comparison to non-inoculated healthy *N. benthamiana* (Fig.2B). The majority of *N. benthamiana* plants co-inoculated with pVirE2 and ToLCNDV did not show severe symptoms. On average, 56.66% plants remained symptomless and healthy like negative control and showed a resistance response even after 40 and 60 dpi (Fig.2 C). As far as symptom severity is concerned, in this case 43.33% plants got infected but 3-4 days delayed in symptom development was observed (Table 1). However, in 71.66%

Table 1. Resistance evaluation of *N. benthamiana* plants co-inoculated with VirE2 and infectious clones of CLCuKoV/ CLCuMuB and ToLCNDV.

Inoculum for Agroinfiltration	Expt. No.	Total No. of plants	Plants showing symptoms at 14-21 dpi	% Symptomatic Plants	Symptoms severity			Molecular Analyses	
					Sever	Mild	Delay in Latent period (days)	PCR	Southern Analysis
non-inoculated plants	in all	3	0	0	NA	NA	NA	–	– – –
CLCuKoV/ CLCuMuB	I	10	10	100	10	-	-	+ 10/10	+++
	II	10	10	100	10	-	-	+10/10	+++
	III	20	20	100	20	-	-	+20/20	+++
CLCuKoV/ CLCuMuB + pVirE2	I	10	4	40	1	3	5-6	+5/10	+
	II	10	3	30	1	4	5-6	+6/10	+
	III	20	5	25	2	3	5-6	+15/20	+
ToLCNDV	I	10	10	100	10	-	-	+10/10	+++
	II	10	10	100	10	-	-	+10/10	+++
	III	20	20	100	20	-	-	+20/20	+++
ToLCNDV + pVirE2	I	10	5	50	1	4	3-4	+7/10	+
	II	10	5	50	2	3	3-4	+6/10	+
	III	20	6	30	1	5	3-4	+17/20	+

*Virus presence by PCR; + present, - absent. Virus titer by Southern analysis; ++++ strong signals, ++ weak signals, + very weak signals, – – No signals

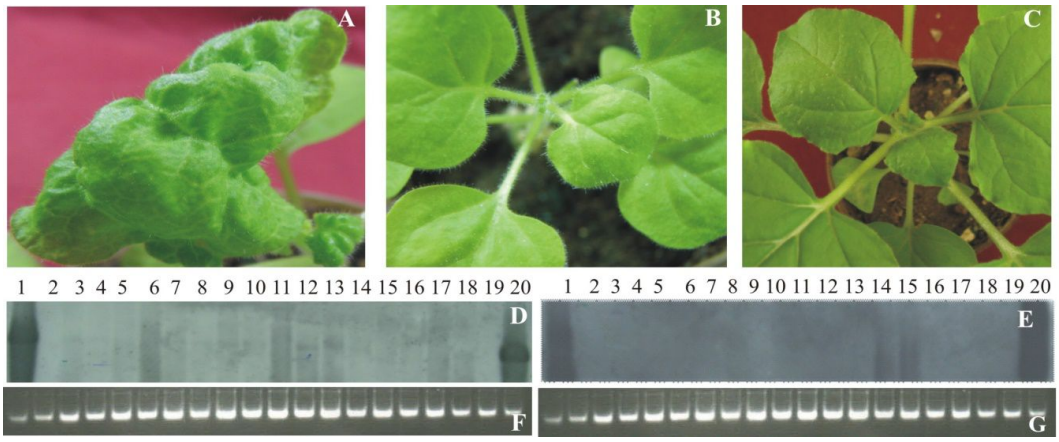

Figure 1. Transient expression of VirE2 in N. benthamiana resulted in milder symptoms against a monopartite begomovirus and associated betasatellite.

(A) *N. benthamiana* control plants inoculated with infectious clones of CLCuKoV/CLCuMuB (B) Non-inoculated healthy *N. benthamiana*(C) Plants co-inoculated with infectious clones of CLCuKoV/CLCuMuB + pVirE2 (D) Southern blot showing CLCuKoV inhibition by transiently expressed VirE2. Lane 1 and 20 are positive control plants showing higher level of CLCuKoV. Lane 2 and 19 are negative control plants of healthy *N. benthamiana*. Lanes 3-18 are showing level of CLCuKoV in *N. benthamiana* plants co-inoculated with CLCuKoV/CLCuMuB and pVirE2. (E) Southern blot showing CLCuMuB inhibition by transiently expressed VirE2. Lane 1 and 20 are positive control plants showing higher level of CLCuMuB-DNA. Lane 2 and 19 are negative control plants of healthy *N. benthamiana*. Lanes 3-18 are showing level of CLCuMuB in *N. benthamiana* plants co-inoculated with CLCuKoV/CLCuMuB and pVirE2. (F & G) DNA from plant leave tissues was extracted at 21 dpi and approximately 10 µg genomic DNA was loaded in each well.

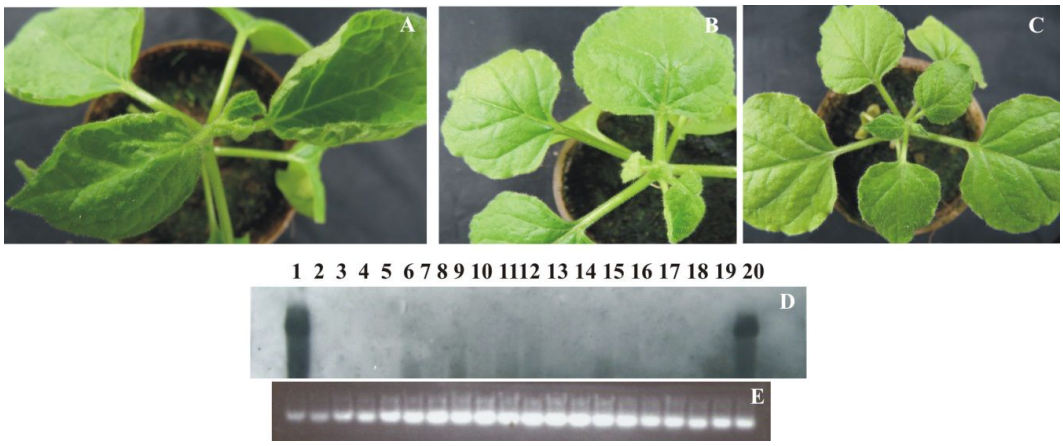

Figure 2. Transient expression of VirE2 in *N. benthamiana* against bipartite begomovirus infectivity.

(A) Control plants inoculated with infectious clone of ToLCNDV (B) Non-inoculated healthy *N. benthamiana* (C) Plant inoculated with infectious clone of ToLCNDV + pVirE2 showing no symptoms after 14 dpi (D) Southern blot showing ToLCNDV inhibition by transiently expressed VirE2. Lane 1 and 20 are positive control plants showing higher level of ToLCNDV. Lane 2 and 19 are negative control plants of healthy *N. benthamiana*. Lanes 3-18 are showing level of ToLCNDV in *N. benthamiana* plants co-inoculated with ToLCNDV and VirE2in *N. benthamiana* plants. (E) DNA from plant leave tissues was extracted at 14 dpi and approximately 10 µg genomic DNA was loaded in each well.

co-inoculated plants ToLCNDV was detectable by PCR showing the presence of virus and an incidence reduction of 28.33% was found. Southern hybridization results showed a considerable decrease in the level of virus titer in all the co-inoculated plants either mild symptomatic or non-symptomatic plants as compared to positive control plants (Fig. 2D). These results indicated that although ToLCNDV is present in the pVirE2+ToCLNDV inoculated plants but its level was not enough to produce symptomatic infection possibly be due to sequestering of ssDNA-binding by VirE2.

DISCUSSION

NPDR strategies against plant viral pathogens control the

spread of viruses by interfering at any developmental stage of its life cycle. In the present study we checked the resistance potential of VirE2 against a monopartite virus associated with betasatellite CLCuKoV/CLCuMuB and a bipartite virus ToLCNDV. Padidam *et al*. (1999) showed the effectiveness of another SSB to interfere in the replication of ToLCNDV while Sunitha *et al*. (2011) have shown the inhibition of MYMIV replication by VirE2. Another SSB, G5, imparted resistance to diverse cassava-infecting begomoviruses; *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV) and *Sri Lankan cassava mosaic virus* (SLCMV) (Patil and Fauquet, 2009). This study complements the finding of previous study suggested that SSB may perhaps be suitable target to control diverse begomoviruses. It would be interesting to see whether SSB based strategies can be used against nanoviruses (another class of ssDNA plant viruses). VirE2 protein plays an important role in T-DNA transfer, considering how agrobacterium interacts with plants, it may be imagined that the VirE2 protein should be expressed by agrobacteria during the interplay between agrobacterium and plants and the over-expression of the VirE2 protein from an additional cassette may have certain effect on the T-DNA transfer. VirE2 is an important protein for the infectivity of agrobacterium. So the possibility that overexpression of VirE2 would interfere the virulence of agrobacterium which carry the begomovirus infectious clone may also exist and some transgenic lines of VirE2 inoculated through mechanical or whitefly inoculation may provide better assessment of the target gene.

In the present study we have provided the results of transient expression of the genes. Although final conclusion could not be drawn from the transient expression as required protein expresses within the inoculated patch of the leaf tissue and hence over expression studies are imperative for concrete results. But the transient expression system is a quick method to check working of genes into plant tissue without regenerating transgenic plants by tissue culture technique and has been extensively used to assess many transgenes into plants and it is a highly adaptable analyzing technique (Orzaez *et al*., 2006; Voinnet *et al*., 2003). Recently using transient expression technique Medina-Hernández and associates (2013) have evaluated the outcome of homologous and heterologous constructs sequence derived from *Pepper golden mosaic virus* (PepGMV) and concluded that both constructs were functional and induced transient resistance against PepGMV. Here also the results of transient expression of VirE2 in plants have been proved as an effective strategy for assaying the infectivity of begomoviruses and resistance response of plants. Moreover, we have obtained reproducible results using higher inoculum dose by taking equal (v/v) amount of *Agrobacterium* cultures (infectious clone and recombinant agrobacterium) during agroinfiltration. Transient expression of *VirE2 gene* has controlled the spread of high levels of CLCuKoV/CLCuMuB and ToLCNDV in *N. benthamiana* by agroinfiltration. However, the development of transgenic cotton and tomato plants is in progress to check their resistance against geminiviruses provided by this gene.

We found less amount of inoculated virus in the systemic leaves of transiently expressing VirE2 plants as compared to the control plants. Results of Southern blot analysis showing the decrease of CLCuKoV/CLCuMuB and ToLCNDV DNA (Fig. 1,2) are similar to the decrease of MYMIV as described by Sunitha *et al*. (2011). The percentage of the plants showing resistance may be calculated on the basis of the number of the plants that accumulate less amount of virus measured by Southern blot analysis, however since almost all of the plants showed less amount of virus titer by Southern analysis (Fig. 1,2), so resistance was calculated by considering symptomatic and non-symptomatic plants. Infectivity data showed that *Agrobacterium tumefaciens*-mediated transient expression of VirE2 resulted in 68% resistance against CLCuKoV/CLCuMuB and 56% against ToLCNDV, thus SSB can be used in transgenic plants for obtaining resistance against ss-DNA viruses and associated satellites as well. Unlike PDR, VirE2 binding is sequence non-specific thus it could be used as a broad-spectrum resistance against all genera of geminiviruses and also against nanoviruses.

***Conclusion*:** The single-stranded DNA binding protein VirE2 is an efficient candidate gene for the control of begomoviruses.

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