Iqbal Z (2024)
Notulae Botanicae Horti Agrobotanici Cluj-Napoca
Volume 52, Issue 4, Article number 14222
DOI:10.15835/nbha52414222
Research Article

CP's differential role in mono- and bipartite begomovirus movement and its role in phloem access for monopartite begomoviruses

Zafar IQBAL*

King Faisal University, Central Laboratories, P.O. Box 31982, Al-Ahsa, Kingdome of Saudi Arabia;
*zafar@kfu.edu.sa (*corresponding author)*

Abstract

Begomoviruses (family *Geminiviridae*), the most wide-spread and devastating single-stranded DNA viruses, rely heavily on their coat protein (CP) for essential functions including encapsidation, DNA binding (both single-stranded and double-stranded), *in planta* movement, insect transmission, and virus transport inside the insect. This study aimed to assess whether the continuous provision of CP is an essentiality in the begomovirus movement. To do so, the CP genes of two monopartite begomoviruses (pedilanthus leaf curl virus and cotton leaf curl Kokhran virus), and a bipartite begomovirus (tomato leaf curl New Delhi virus), were mutated by introducing a stop codon in the N-terminal region, non-overlapping region to the *(A)V2* gene, to disrupt CP function. To ensure continuous CP provision (expression) at the site of inoculation and within the phloem, the CPs of these three begomoviruses were cloned under the cauliflower mosaic virus 35S promoter and within potato virus X vector. The CP mutant viral clones were agro-infiltrated into *Nicotiana benthamiana* plants to verify the objectives. The results demonstrated that CP mutation abolished the viral infectivity and spread in monopartite begomoviruses but not in bipartite begomoviruses. Notably, transient expression of CP, particularly through PVX and 35S promoter, significantly increased the presence of viral DNA in newly developing leaves, suggesting improved viral movement. This suggests that the continuous presence of CP is necessary for efficient spread, particularly in monopartite begomoviruses, likely by facilitating plasmodesmatal access to phloem cells for systemic infection. However, these findings are preliminary and require further investigation to draw definitive conclusions. Nevertheless, they have significant implications for understanding begomovirus biology and developing novel control strategies.

Keywords: begomovirus; coat protein; cotton leaf curl Kokhran virus; expression; movement; *Nicotiana benthamiana*; pedilanthus leaf curl virus; tomato leaf curl New Delhi virus

Introduction

Begomoviruses, a most wide-spread and destructive genus within the Geminiviridae family, are single-stranded (ss) ~2.7-5.2 kb genome size DNA viruses transmitted by whitefly (*Bemisia tabaci*) in a circulative and persistent manner (Czosnek *et al*., 2017). These phytopathogens pose a significant threat to dicot plant species worldwide. Based on the genomic structure, begomoviruses are divided into two main types, monopartite and bipartite begomoviruses. The former group comprises of a single genome component that can

Received: 22 Oct 2024. Received in revised form: 01 Dec 2024. Accepted: 09 Dec 2024. Published online: 11 Dec 2024.
From Volume 49, Issue 1, 2021, Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

independently cause a symptomatic infection. In contrast, the latter group comprises of two genome components, DNA A and DNA B, and require both components for a symptomatic infection. Nonetheless, in some cases, DNA A alone from bipartite begomoviruses can move systemically within the plant and cause asymptomatic infections with low viral titre (Klinkenberg and Stanley, 1990; Briddon and Markham, 2001; Saunders *et al*., 2002; Fontenelle *et al*., 2007; Iqbal *et al*., 2017). Monopartite begomoviruses encode six genes on both strands: *V1/CP*, *V2*, *C1/Rep*, *C2/TrAP*, *C3/REn*, and *C4*. Bipartite begomoviruses encode these genes on DNA-A and all are essential for encapsidation, movement, replication, regulating transcription, and enhancing replication, respectively (Fondong, 2013). Nonetheless bipartite begomoviruses encode two additional movement proteins, NSP (BV1) and MP (BC1), on DNA-B. Both types share a common region (CR) with a hairpin structure (TAATATT/AC) and iterons, to which Rep binds to induce nick to commence replication (Hanley-Bowdoin *et al*., 2013).

Movement of begomoviruses is very intriguing and is achieved by the complex interplay between viral proteins and host cellular machinery. In bipartite begomoviruses, the two open reading frames (ORFs) encoded by DNA B—nuclear shuttle protein (NSP; BV1) and movement protein (MP; BC1)—are responsible for both cell-to-cell (local) and long-distance (systemic) movement of the virus from initially infected cells to newly emerging leaves (Etessami *et al*., 1988; Sanderfoot and Lazarowitz, 1995). Two primary models, the relay race model (RRM) and couple skating models (CSM), have been suggested for the movement of begomoviruses. In the RRM, as they traverse through plant tissues, NSP initially binds and transports dsDNA from the nucleus to the cytoplasm, followed by MP-mediated movement through plasmodesmata (Noueiry *et al*., 1994). In the CSM, NSP escorts ssDNA to the cytoplasm, where MP aids in its transport. Both NSP and MP are essential for intracellular movement, as mutations in either disrupt cell-to-cell viral DNA movement (Sudarshana *et al*., 1998). Notably, the MP protein redirects NSP to the plasma membrane in fission yeast (Frischmuth *et al*., 2007). In *Nicotiana benthamiana*, transgenic expression of the different virus encoded MPs localized around epidermal cells (Radhakrishnan *et al*., 2008) and to both the plasma membrane and plasmodesmata, further emphasizing its role in coordinating NSP movement (Kleinow *et al*., 2009).

In contrast to bipartite begomoviruses, monopartite begomoviruses rely primarily on the pre-coat protein (V2), CP, and C4 for viral movement (Rojas *et al*., 2005; Chowda-Reddy *et al*., 2008). Although the exact mechanism of CP-mediated cell-to-cell and systemic movement is not fully understood, studies have proposed models based on transient expression assays of CP and C4 proteins. This model suggests that CP transports viral dsDNA out of the nucleus, aiding its delivery to neighbouring cells and systemic tissues. CP localizes at the cell periphery, facilitating the handoff of viral DNA to C4, which then uses its N-terminal myristylation domain to mediate local (cell-to-cell) movement by increasing plasmodesmata size (Rojas *et al*., 2001). V2 and CP expression in insects and *N. benthamiana* cells showed that V2 localizes at periphery of the cell while CP remains in the nucleus, with both proteins interacting to enable viral movement (Priyadarshini *et al*., 2011). Additionally, CP may contribute to the swift dissemination of begomoviruses through the phloem, either as nucleoprotein complexes or virions.

In monopartite begomoviruses, the CP is indispensable for systemic infection and movement (Rojas *et al*., 2001; Iqbal *et al*., 2012), while in bipartite begomoviruses, CP is dispensable, as the NSP is believed to take over CP's movement function (Haq *et al*., 2011; Iqbal *et al*., 2017). However, if NSP is mutated, CP can sometime compensate for its role (Guevara-González *et al*., 1999; Unseld *et al*., 2004). This functional overlap suggests that CP and NSP may share a common evolutionary origin (Kikuno *et al*., 1984; Boulton *et al*., 1991). CP localization in secondary plasmodesmata during infection supports its involvement in geminiviruses movement (Dickinson *et al*., 1996). In the case of tomato yellow leaf curl virus (TYLCV), CP localizes at the nuclear membrane and nucleolus, where it involves in the nuclear movement of viral DNA (Rojas *et al*., 2001). During infection, as begomoviruses replicate within the nucleus, their movement requires the translocation of viral DNA across both the plasma membrane and nuclear envelope. CP and NSP, targeting the nucleus, aid in

shuttling viral DNA between these compartments (Rojas *et al*., 2001; Unseld *et al*., 2004), by binding to both ssDNA and double-stranded DNA (dsDNA) in a sequencing-independent manner (Lazarowitz and Beachy, 1999).

To investigate the assumption that CP plays some role in virus movement, particularly in accessing the phloem, the CP of three different begomoviruses was knocked down: two monopartite begomoviruses; cotton leaf curl Kokhran virus (Ko) and pedilanthus leaf curl virus (Pe), and a bipartite begomovirus, tomato leaf curl New Delhi virus (To). The CP of each virus was mutated by introducing a premature stop codon near the 5' end in the non-overlapping region of (A)V2 gene. These CP-mutated viruses were then co-inoculated into *N. benthamiana* plants along with a construct expressing CP under the 35S promoter and Potato virus X (PVX). The effect of heterologous CP expression on systemic virus movement was assessed, allowing insights into the functional role of CP in viral movement across different begomovirus types.

Materials and Methods

Construction of the CP gene mutant infectious clones of the viruses

CP gene mutants were generated using PCR-mediated mutagenesis with designed abutting oligonucleotide primers in the non-overlapping region near the 5' end of the CP (Table 1).

For Ko, a clone (accession number AJ496286) was used to create the CP gene mutant of Ko ($Ko^{\Delta CP}$). Initially, a full-length Ko construct harbouring CP gene mutation was amplified using specifically designed primers. The resultant amplicon was cloned into pTZ57R/T vector and then digested with *Kpn*I–*Not*I to yield a ~650 bp fragment, which was cloned in a binary vector, pGreen0029 (Hellens *et al*., 2000). Then a complete monomeric insert of pTZ57R/T vector was excised as a *Kpn*I fragment and inserted into the unique *Kpn*I site of the pGreen0029 construct, to yield a tandem partial repeat harbouring mutant CP gene, as described earlier (Iqbal *et al*., 2012).

For Pe, a clone (accession number AM712436) was used and a dimeric construct bearing mutation in the CP of Pe ($Pe^{\Delta CP}$) was generated. The monomeric PCR amplicon, harbouring CP gene mutation, was restricted with an introduced *Xho*I site, cleaned, circularized, and restricted again with *BamH*I. The mutated fragment was then ligated into pGREEN0029. The resultant binary vector clone (monomeric repeat of $Pe^{\Delta CP}$ in pGREEN0029) was linearized with *Xho*I, dephosphorylated, and then a monomeric insert of pTZ57R/T vector was introduced to yield the dimeric construct ($Pe^{\Delta CP}$).

Similarly, for To, a clone of the DNA A (TA; accession number U15015) was used to generate a dimeric CP gene mutant ($TA^{\Delta CP}$). The monomeric PCR amplicon, harbouring CP gene mutation, was restricted with an introduced *Mlu*I site, cleaned, circularized, and restricted again with *Xba*I and cloned in the binary vector pGREEN0029. The resultant binary vector clone (monomeric repeat of $TA^{\Delta CP}$ in pGREEN0029) was linearized with *Mlu*I, dephosphorylated, and then a monomeric insert of pTZ57R/T vector was introduced to yield the dimeric construct ($TA^{\Delta CP}$). The construction of CP gene mutant has been previously described (Iqbal *et al*., 2017).

To confirm the successful introduction of the desired mutation and the absence of unintended mutations in other ORFs, all infectious clones harbouring CP gene mutations were sequenced in their entirety.

Table 1. The designed set of primers used in the mutagenesis study

Primer	Sequence (5' – 3')	Comments[$]
Ko-mCPF	GGTACCGTAGTTTGAGTCaCAGACATGATATTCAG	^Mut CP Ko
Ko-mCPR	CGGTACCTTACATGGACCTTCACATCCTCTAG	
Pe-mCPF	CTCGAGGAACTGGGCTGACCCATAG	*Mut CP Pe
Pe-mCPR	CTCGAGAAAGGACTGGACCTTACATG	
TA-mCPF	ATCGATTAGGGTAGCGATTCTaGTGTG	^Mut CP TA
TA-mCPR	ATCGATCGCGATGTGTGAGTCCAGTTC	
Ko-CP35sF	GGTCGACGAATTATGTCGAAGCGACCAG	Amp CP of Ko
Ko-CP35sR	GGATCCAATTCAATATCTATTAATTTGTCACG	
Ko-CPPVXR	ATCGATAATTCAATATCTATTAATTTGTCACG	
Pe-CPPVXF	GTCGACATTATGTCGAAGCGACCAG	Amp and det of CP of Pe
Pe-CPPVXR	CCCGGGATTTATTAATTTGTCACGGAATC	
TA-CPPVXF	GCAAATCGATATGGCGAAGCGACCAG	Amp CP of TA
TA-CPPVXR	GGTCGACTATTAATTTGTGGCCGAATC	
ToLNC4pvx/35F	CGTCGACAAGATGGGTCTCCGC	Det of TA
ToLNV2pvx/35sR	CCCGGGCTTCTATACATTCTGTAC	

[$]Abbreviations: Amp (amplification), CLCuKoV (Ko), Mut (mutagenesis), Det (detection), Ko (cotton leaf curl Kokhran virus), Pe (pedilanthus leaf curl virus), and TA (ToLCNDV DNA A).

*Unique mutant created by nucleotide deletion to induce a frameshift.

^Underlined nucleotides indicate changes made to introduce a stop codon, while lowercase letters represent additional nucleotides that induce a frameshift.

Construction of the CP constructs for transient expression

Unique oligonucleotide primers were designed to express the CPs of Ko, Pe, and To, constitutively through the CaMV 35S promoter in pJIT163 vector (Guerineau and Mullineaux, 1993) and PVX vector (Chapman *et al*., 1992) (Table 2). To achieve downstream directional cloning, unique restriction sites (*Sal*I and *BamH*I) were introduced in the primers.

The pJIT163 construct, containing the 35S promoter and terminator, was transferred to the pGreen0029 at *EcoR*V-*Kpn*I sites. All PCR reagents, restriction enzymes, and cloning materials were obtained from Thermo Fisher Scientific (Arlington, Canada).

Table 2. Strategies to design infectious clone of CP mutant of three begomoviruses

Virus	Mutant	ORF position	Changed nucleotides (position)	Introduced restriction site	Stop codon/no. of nucleotides deleted or added[%]	Partial repeat strategy	Partial repeat size (bp)
Ko	$Ko^{\Delta CP}$	287-1057	530-532	*Kpn*I	TAA/ +1	RSP - *Kpn*I PRCS - *Kpn*I & *Not*I FLCS - *Kpn*I	~ 650
Pe	$Pe^{\Delta CP}$	309-1079	560-562	*Xho*I	TAA/+1	RSP - *Xho*I FLCS - *Bam*HI	Dimer
To	$TA^{\Delta CP}$	292-1062	517-519, 528	*Mlu*I	TAG/-1	RSP - *Mlu*I FLCS - *Xba*I	dimer

Abbreviations: restriction site introduced in primers (RSP), partial repeat cloning site (PRCS), full-length component cloning site (FLCS).

[%] An extra nucleotide was added (+1) or deleted (-1) to introduce a frame-shift mutation.

Agrobacterium-mediated inoculations and plants maintenance

The pGREEN0029 binary constructs were transformed into Agrobacterium tumefaciens (strain GV3101) via electroporation. Positive transformants were confirmed by colony PCR and their Inocula were prepared to a final OD_{600} of 0.6 and infiltrated into *N. benthamiana* plants using sterile syringe, as described earlier (Hussain *et al.*, 2005). Infiltrated plants were kept in an insect-free growth room under controlled conditions: 65% relative humidity, 25/22 ◦C Day/night temperature, 16 h photoperiod (200 µ/m^2/s light intensity). Symptom development was monitored daily, and at 25-30 days post-inoculation (dpi) and were recorded with a digital camera. Newly emerging leaf tissues, developing subsequent to the inoculated leaves, were obtained for DNA isolation and subsequent PCR and Southern blot analyses, as previously described (Iqbal *et al.*, 2012).

DNA extractions and PCR-mediated analysis of the infectivity

Total genomic DNA was isolated from the collected leaf tissues of inoculated and control plants (Doyle and Doyle, 1990). To confirm the presence of the viral constructs, specific primers (Table 1) targeting the CP gene of each virus (Ko, Pe, and TA) were used for PCR-based detection.

Southern blot analysis

For Southern blotting, 10 µg of DNA extracted from plants was electrophoresed on a 1.5% agarose gel and transferred to a nylon membrane (Hybond XL, Amersham) by capillary transfer. TA and Pe were detected using DIG-labeled PCR probes targeting the IR (primers ToLNC4pvx/35R and ToLNV2pvx/35R) and CP (primers Pe-CPPVXF/Pe-CPPVXR) regions, respectively. Hybridization was performed at 50 °C for 16 h, and signals were detected using CDP-Star, as described previously (Iqbal *et al*., 2017). Whereas Ko viral DNA was detected using a radioactively labelled CP gene amplicon by the random primer method using the "Rediprime II DNA Labeling System" (Amersham), as described earlier (Iqbal *et al*., 2012). Hybridization signals were detected using a phosphorimager (Bio-Rad Personal FX Phosphorimager).

Results

Effects of $Ko^{\Delta CP}$ on infectivity and symptoms

Plants inoculated with the wild-type Ko exhibited upward leaf curl symptoms at 12 days dpi and systemic viral spread in the newly developed leaves following inoculation, as confirmed by both PCR and Southern blotting (Table 3). Whereas plants inoculated with the $Ko^{\Delta CP}$ remained asymptomatic, and no viral DNA was not detected in newly emerging leaves, by either PCR or Southern blotting (Figure 2). These findings indicate that the CP mutation rendered the virus non-infectious in *N. benthamiana*, abolishing its ability to establish systemic infection.

To investigate the potential for CP complementation, constitutive expression of the CP through 35S-CP^{Ko} failed to induce symptomatic infection but resulted in asymptomatic infection. PCR mediated analysis detected viral DNA in four of 15 inoculated plants, but Southern blot hybridization was negative (Figure 1).

None of the plants inoculated with the control constructs, including the mock and 35S-CP^{Ko}, exhibited any symptoms. These plants remained symptomless and were indistinguishable from the non-inoculated control group, demonstrating that the inoculation procedures and control treatments had no impact on plant health.

These results suggest that CP expression in inoculated tissues partially restored the virus's ability to spread to newly emerging leaves, enabling limited systemic movement. However, it did not fully restore the virus's ability to induce symptomatic infection, indicating that the CP plays a critical but non-exclusive role in symptom development.

Figure 3. Infectivity analysis of CLCuKoV and its CP gene mutant on *N. benthamiana* plants

Inoculum	Infectivity				Phenotype	Latent period (days)
	Diagnostic PCR (Plants infected/ inoculated)			Southern blotting		
	Expt.I	Expt.II	Expt.III			
	Ko	Ko	Ko			
Ko	5/5	5/5	5/5	+ + +	ULC	12
$Ko^{\Delta CP}$	0/5	0/5	0/5	ND	NS	--
$Ko^{\Delta CP}$ and 35S-CP^{Ko}	1/5	1/5	2/5	ND	NS	--
35S-CP^{Ko}	0/3	0/3	0/3	NI	NS	--
Mock	0/3	0/3	0/3	NI	NS	--
Healthy (non-inoculated)	0/3	0/3	0/3	ND	NS	--

*Abbreviation: cotton leaf curl Kokhran virus (Ko), Ko with the mutated CP ($Ko^{\Delta CP}$), upward leaf curling (ULC), no symptoms (NS), not detected (ND), and not included in the analysis (NI).

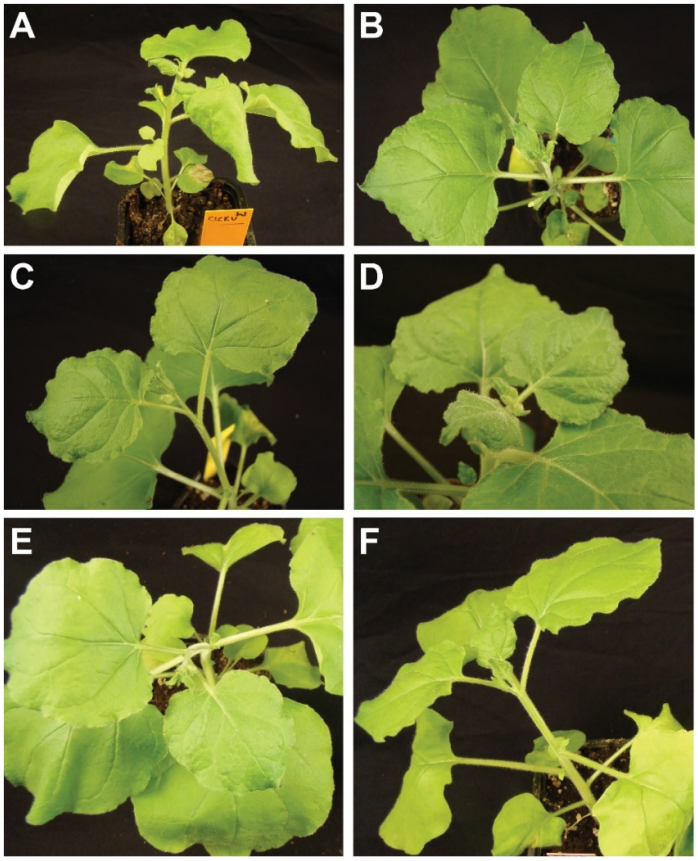

Figure 1. Effect of CP mutation on CLCuKoV-induced symptoms in *N. benthamiana* plants
Plants were either with wild type Ko (A), or $Ko^{\Delta CP}$ (B), $Ko^{\Delta CP}$ and 35S-CP^{Ko} (C), 35S-CP^{Ko} (D), mock (E), and non-inoculated healthy control (F)

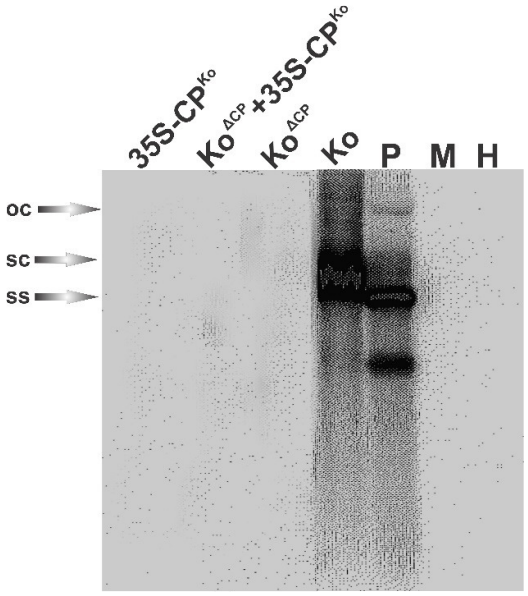

Figure 2. Detection of CLCuKoV and its CP gene mutant in *N. benthamiana* through Southern blot hybridization

The DNA samples resolved on the gels were isolated from leaves of a non-inoculated (healthy) plant (H), mock-inoculated plant (M), Ko plasmid (control; P), wild type Ko, or $Ko^{\Delta CP}$, $Ko^{\Delta CP}$ and 35S-CP^{Ko}, and 35S-CP^{Ko}. Approximately 10 µg of total DNA extract was loaded onto each lane. Viral DNA forms were identified as single-stranded (ss), supercoiled (sc), and open-circular (oc). The additional, unlabeled band below the viral DNA forms is likely composed of defective viral variants.

Effects of $Pe^{\Delta CP}$ on infectivity and symptoms

The wild-type Pe clone demonstrated high infectivity in *N. benthamiana* plants, with all inoculated plants developing characteristic symptoms. By 12 dpi, infected plants exhibited pronounced upward curling of leaves, which progressively intensified, leading to significant stunting compared to non-inoculated, control plants (Figure 3). In diagnostic PCR, Pe was detected in all symptomatic plants and Southern blot confirmed the presence of Pe DNA in tested plants (Table 4).

Figure 3. Effect of CP mutation on CLCuKoV-induced symptoms in *N. benthamiana* plants

Plants were either with wild type Ko (A), or $Ko^{\Delta CP}$ (B), $Ko^{\Delta CP}$ and 35S-CP^{Ko} (C), 35S-CP^{Ko} (D), mock (E), and non-inoculated healthy control (F)

Table 4. Infectivity analysis of Pe and its CP gene mutant on *N. benthamiana* plants

Inoculum	Infectivity				Symptoms	Latent period
	PCR diagnostics (Plants infected/ inoculated)			Southern blotting		
	Expt.I	Expt.II	Expt.III			
	Pe	Pe	Pe			
Pe	5/5	5/5	5/5	+ + +	ULC	12
$Pe^{\Delta CP}$	0/5	0/5	0/5	ND	NS	--
$Pe^{\Delta CP}$ and 35S-CP^{Pe}	2/5	1/5	1/5	ND	NS	
$Pe^{\Delta CP}$ and CP^{Pe}-PVX	2/5	1/5	1/5	ND	VY	8
35S-CP^{Pe}	0/3	0/3	0/3	NI	--	
CP^{Pe}-PVX	0/3	0/3	0/3	NI	VY	8
PVX	0/3	0/3	0/3	NI	VY	8
Mock	0/3	0/3	0/3	ND	--	
Healthy (non-inoculated)	0/3	0/3	0/3	ND	NS	

*Abbreviation: Pe - pedilanthus leaf curl virus, $Pe^{\Delta CP}$ - pedilanthus leaf curl virus with the CP gene mutated, upward leaf curling (ULC), vein yellowing (VY), no symptoms (NS), not detected (ND), and not included in the analysis (NI).

In contrast, inoculation of plants with the CP mutant of Pe ($Pe^{\Delta CP}$) failed to induce any symptoms or asymptomatic infection (Figure 3). Viral DNA was undetectable in the newly emerged leaves of all 15 inoculated plants, as confirmed by both Southern blotting and PCR (Figure 4). The findings demonstrate that the CP gene mutation renders Pe incapable of systemic plant infection.

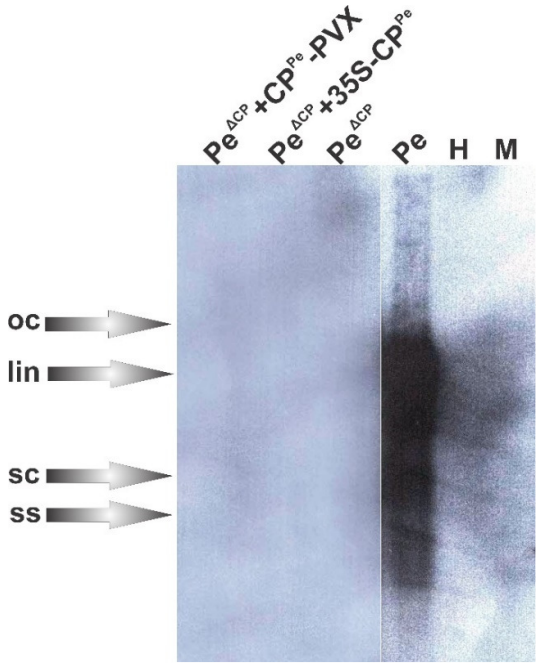

Figure 4. Detection of Pe and its CP gene mutant in *N. benthamiana* through Southern blot hybridization
The DNA samples resolved on the gels were isolated from leaves of a non-inoculated (healthy) plant (H), mock-inoculated plant (M), wild type Pe, or $Pe^{\Delta CP}$, $Pe^{\Delta CP}$ and 35S-CP^{Pe}, and $Pe^{\Delta CP}$ and CP^{Ko}-PVX. Approximately 10 µg of total DNA extract was loaded onto each lane. Viral DNA forms were identified as open0circular (oc), linear (lin), single-stranded (ss), and supercoiled (sc)

Co-inoculation of $Pe^{\Delta CP}$ and 35S-CP^{Pe} successfully, but partially complemented the CP mutation, leading to asymptomatic infection in 4 out of 15 plants. This infection was detectable only by PCR, as Southern blotting did not detect viral DNA (Figure 4).

Similarly, inoculation of $Pe^{\Delta CP}$ with PVX-CP^{Pe} (a PVX vector expressing Pe CP) also led to infection in 4 out of 15 plants. Notably, these plants exhibited symptoms typical of PVX infection, such as vein yellowing, at 8 dpi, distinct from Pe symptoms and were comparable to those observed in plants inoculated with CP^{Pe}-PVX alone. Again, the Pe DNA was detectable only by PCR and not by Southern blotting (Figure 4).

Control plants inoculated with mock or 35S-CP^{Pe} constructs remained symptomless and indistinguishable from non-inoculated plants (Figure 3), confirming that the inoculation procedures and control treatments had no adverse effects on plant health.

Effects of mutation of ToLCNDV-encoded CP on infectivity and symptoms

Upon inoculation with the wild-type ToLCNDV (TA and TB components), *N. benthamiana* plants exhibited severe leaf curling by 12 dpi. These symptoms progressed further, and plants showed deformation of stems and stunting in the growth (Figure 5). PCR and Southern blot hybridization confirmed the presence of viral DNA (TA) components, further validating successful infection (Figure 6).

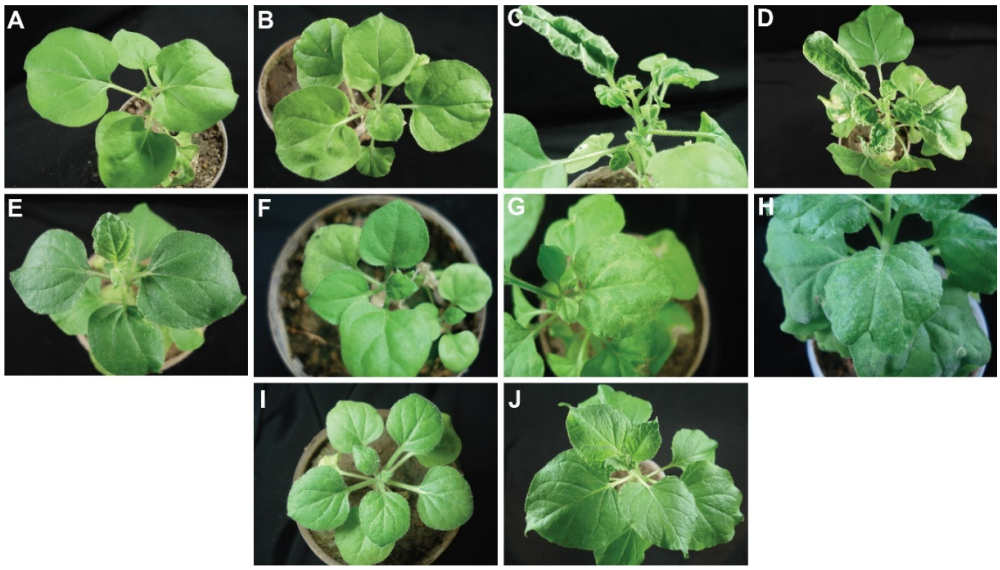

Figure 5. Effect of CP mutation on ToLCNDV-induced symptoms in *N. benthamiana* plants
Plants were either with wild type TATA alone (A), $TA^{\Delta CP}$ (B), TA and TB (C), $TA^{\Delta CP}$ and TB (D), $TA^{\Delta CP}$ and 35S-CP^{TA} (E), $TA^{\Delta CP}$ and CP^{TA}-PVX (F), 35S-CP^{TA} (G), CP^{TA}-PVX (H), mock (I), and non-inoculated healthy control (J)

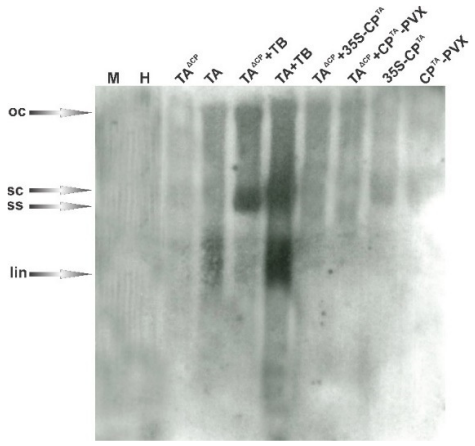

Figure 6. Detection of TA and its CP gene mutant in *N. benthamiana* through Southern blot hybridization

The DNA samples resolved on the gels were isolated from leaves of a non-inoculated (healthy) plant (H), mock-inoculated plant (M), $TA^{\Delta CP}$, TA, $TA^{\Delta CP}$ and TB, TA and TB, $TA^{\Delta CP}$ and 35S-CP^{TA}, $TA^{\Delta CP}$ and CP^{TA}-PVX, 35S-CP^{TA}, and CP^{TA}-PVX. Approximately 10 µg of total DNA extract was loaded onto each lane. Viral DNA forms were identified as open0circular (oc), linear (lin), single-stranded (ss), and supercoiled (sc)

Inoculation of plants with ToLCNDV harboring CP gene mutation ($TA^{\Delta CP}$ and TB) led to symptoms identical to those caused by ToLCNDV, although a slightly extended latent period of 13-14 days was observed compared to the 12-day latent period of the wild-type ToLCNDV (Figure 5). Both PCR and Southern blotting detected viral DNA in symptomatic plants, with levels comparable to those in wild-type infections (Figure 6).

Plants inoculated with TA alone led to asymptomatic infection in 4 out of 15 inoculated plants, detectable only by PCR but not Southern blotting (Figure 6). In contrast, plants inoculated with $TA^{\Delta CP}$, a mutant lacking the CP gene, exhibited asymptomatic infection in only one out of 15 plants (Table 5). Again, this infection could only be detected by PCR but not Southern blotting.

Table 5. Infectivity analysis of ToLCNDV and its CP gene mutant on *N. benthamiana* plants

Inoculum	Infectivity				Phenotype	Latent period (days)
	PCR diagnostics (Plants infected/ inoculated)			Southern blotting		
	Expt.I	Expt.II	Expt.III			
	TA	TA	TA			
TA	1/5	2/5	1/5	ND	NS	--
TA and TB	5/5	5/5	5/5	+ + +	ULC	12
$TA^{\Delta CP}$	0/5	1/5	0/5	ND	NS	--
$TA^{\Delta CP}$ and TB	5/5	5/5	4/5	+ +	ULC	12-13
$TA^{\Delta CP}$ and 35S-TA^{CP}	0/5	0/5	0/5	ND	NS	--
$TA^{\Delta CP}$ and CP^{TA}-PVX	2/5	1/5	1/5	ND	VY	10
35S-CP^{TA}	0/3	0/3	0/3	NI	--	
CP^{TA}-PVX	0/3	0/3	0/3	NI	VY	8
PVX	0/3	0/3	0/3	NI	VY	8
Mock	0/3	0/3	0/3	NI	--	--
Healthy (non-inoculated)	0/3	0/3	0/3	ND	NS	--

* Abbreviation: TA – tomato leaf curl New Delhi virus DNA A, TB - tomato leaf curl New Delhi virus DNA B, $TA^{\Delta CP}$ - tomato leaf curl New Delhi virus DNA A with the CP gene mutated, upward leaf curling (ULC), vein yellowing (VY), not included (NI), no symptoms (NS), and not detected (ND).

Co-inoculation of $TA^{\Delta CP}$ and 35S-CP^{TA} (a construct expressing ToLCNDV CP under 35S promoter) showed no symptomatic infection, and viral DNA was undetectable by either PCR or Southern blotting (Figure 6).

Similarly, inoculation of $TA^{\Delta CP}$ with a CP^{TA}-PVX did not induce symptoms (Figure 5). Although Southern blotting failed to detect viral DNA, PCR analysis confirmed its presence in 4 out of 15 inoculated plants (Figure 5). These plants displayed vein yellowing, a characteristic symptom of PVX infection.

Discussion

The findings presented in this study elucidate the intricate role of the CP in begomovirus movement, systemic infection, and symptom development. By meticulously examining the effects of CP gene mutations in three distinct begomoviruses—Ko, Pe, and ToLCNDV - valuable insights have been gained into the diverse functions of CP within the context of begomovirus biology. The results are consistent with previous studies, confirming the critical but nuanced role that CP plays in viral movement and infection. Begomovirus CP plays a pivotal role in begomovirus evolution, driving virus fitness, host range, and adaptation. Its multifunctionality—spanning encapsidation, DNA binding, *in planta* movement, and insect transmission—highlights its evolutionary significance (Zhou, 2013; Hanley-Bowdoin *et al*., 2013). Although CP is conserved across begomoviruses, New World (NW) viruses show greater sequence conservation than Old World (OW) viruses, likely due to lower recombination rates. NW CP genes also have fewer nuclear localization sequences and are under strong purifying selection, in contrast to their OW counterparts (Mondal, 2019). Moreover, differences in CP dependency between monopartite and bipartite begomoviruses reflect evolutionary pressures favoring distinct strategies for host colonization and systemic spread (Rojas *et al*., 2005).

Monopartite begomoviruses rely exclusively on their CP for systemic infection and movement. In this study, mutation of the CP genes of Ko and Pe completely abolished their ability to spread systemically in *N. benthamiana* plants, as confirmed by Southern blot and PCR analysis. These findings corroborate previous studies highlighting the CP's indispensable role in viral movement and infectivity across various geminiviruses (begomo-, curto-, and mastrevirus) species (Boulton *et al*., 1989; Briddon *et al*., 1989; Rigden *et al*., 1993; Wartig *et al*., 1997; Iqbal *et al*., 2012; Iqbal *et al*., 2017). Beyond its role in viral movement (shuttling viral DNAs across the cell and nuclear membrane), CP also functions as a protective shield for the viral DNA during systemic spread in the phloem (Hipper *et al*., 2013). It is thus vague whether the CP's critical function is tied specifically to "long-distance viral spread" or "nuclear shuttling", or yet unidentified aspects. In monopartite begomoviruses, CP facilitates the transport of newly synthesized viral DNA from the nucleus to the cytoplasm by binding to viral ssDNA, safeguarding it from degradation, and providing nuclear localization signals (Palanichelvam *et al*., 1998; Rojas *et al*., 2001; Pitaksutheepong *et al*., 2007; Sharma and Ikegami, 2009; Iqbal *et al*., 2017). CP can shuttle replicated DNA back to the cytosol via nuclear pores. The N-terminal domain of CP binds to ssDNA, enabling its interaction with nuclear machinery for import and export (Pitaksutheepong *et al*., 2007; Poornima and Savithri, 2009). Additionally, CP-mediated nuclear localization is likely important during the early stages of infection, when viral proteins are not yet expressed, and the virus relies on the CP for nuclear entry. In bipartite begomoviruses, CP in nuclear entry may be less apparent due to the presence of the NSP, encoded by DNA B (Gafni and Epel, 2002).

The ToLCNDV mutant ($TA^{\Delta CP}$) revealed a unique contrast to monopartite begomoviruses. While bipartite begomoviruses rely on NSP and MP encoded by DNA B for movement (Noueiry *et al.*, 1994). In this study, it was revealed that ToLCNDV-encoded CP plays a less critical role in systemic movement compared to NSP. The $TA^{\Delta CP}$ mutant failed to induce symptomatic infection or systemic movement, supporting earlier findings that, while NSP is the primary driver of viral movement, CP can occasionally compensate for NSP deficiencies (Martins *et al*., 2020). This suggests some redundancy in the movement functions of CP and NSP,

potentially due to their shared evolutionary origins (Kikuno *et al*., 1984). Interestingly, transient expression of CP in the $TA^{\Delta CP}$ mutant partially restored systemic movement, implying that CP plays a role in the virus movement – potentially by facilitating the virus's access to phloem tissues. This observation aligns with studies showing that CP localizes at plasmodesmata, aiding in the transport of viral nucleic acids across the plant's vasculature (Rojas *et al*., 2005). *Agrobacterium*-mediated inoculation experiments with *N. benthamiana* plants demonstrated that DNA A alone can move to distal tissues, albeit with asymptomatic infection and low DNA titre (Briddon and Markham, 2001). However, this independent movement of DNA A required the presence of CP (Briddon and Markham, 2001; Iqbal *et al*., 2017), suggesting its involvement in long-distance viral movement, either as virions or nucleoprotein complexes, through the phloem.

The functional redundancy between CP and NSP in bipartite begomoviruses demonstrates the virus's adaptability in movement mechanisms. While NSP is primarily responsible for nucleocytoplasmic transport and plasmodesmata targeting in bipartite begomoviruses (Sudarshana *et al*., 1998), CP can compensate for NSP mutations, ensuring systemic infection, of a monopartite begomovirus, TYLCV (Rojas *et al*., 2001). CP likely plays multifaceted role: assisting in viral movement by shuttling viral DNA inside the cell (between the nucleus and cytoplasm), and function as a protective shield for viral DNA during systemic (long-distance) transport through the phloem, either as virions or nucleoprotein complexes (Briddon and Markham, 2001; Gafni and Epel, 2002). This aligns with previous studies emphasizing the crucial role of CP in viral movement and infectivity across various begomovirus species (Harrison *et al*., 2002; Iqbal *et al*., 2012; Iqbal *et al*., 2017; Iqbal *et al*., 2022).

Interestingly, transient expression of CP under the 35S promoter (35S-CP^{Ko} and 35S-CP^{Pe}) did not fully restore symptom development but enabled asymptomatic systemic movement of the virus, as confirmed by PCR. This suggests that CP is crucial for viral spread, it may not be solely responsible for symptom expression (Unseld *et al*., 2004). CP may be essential for accessing the phloem for long-distance movement or re-establishing infection in young tissues. Moreover, a sustained supply of CP appears crucial for maintaining a productive infection cycle, implying its role in maintaining infection in development tissues, even if the virus is not actively replicating there. While the 35S promoter offers strong and constitutive expression, it may lead to overexpression of the CP, potentially altering its natural expression levels compared to viral infection conditions. This could affect the protein's localization, interactions with host cellular machinery, or viral movement, which might not fully mimic the physiological conditions during natural infection. Nonetheless, PVX-mediated expression of CP led to detection of virus in one plant, supporting the idea that PVX, though phloem-limited like many begomoviruses, could deliver CP where required to facilitate movement. So, contrasting to continuous supply of CP, it is speculated that full replication and symptom expression likely depend on the coordinated activity of other viral proteins, such as V2 and C4, particularly in monopartite begomoviruses (Priyadarshini *et al*., 2011).

This study provides a foundational understanding of the role of CP in begomovirus movement in *N. benthamiana,* a model for plant which may not fully reflect represent virus behavior in other host (like tomato and cotton) species. So, future research can be expanded on validating these results in different host plants to gain a more comprehensive understanding of CP's role in natural infection settings. Additionally, futuristic studies should focus on unraveling the precise mechanisms by which the CP facilitates plasmodesmatal access and phloem loading in monopartite begomoviruses, as well as exploring why bipartite begomoviruses exhibit reduced CP dependency. Advanced molecular and structural studies are needed to dissect CP interactions with host cellular machinery and viral components. Investigating CP dynamics in diverse host plants and under varying environmental conditions can provide broader insights. Additionally, leveraging these findings to design targeted antiviral strategies, such as CP inhibitors or RNA interference-based approaches, holds promise for developing effective begomovirus control methods in agricultural settings.

Conclusions

Conclusively, these results highlight the differential roles of CP in monopartite and bipartite begomoviruses. In monopartite viruses, CP is indispensable for movement, while in bipartite viruses, its role is more supportive, complementing NSP in certain conditions. Transient CP expression restored limited viral movement but not symptom development, suggesting that CP is indispensable for systemic transport but works in concert with other viral proteins for full pathogenicity. However, the partial restoration of movement in CP-knockout mutants suggests that CP still contributes to efficient viral spread and may have functional overlap with NSP in bipartite begomoviruses. Differences in CP dependency between monopartite and bipartite begomoviruses underline the evolutionary pressures shaping their infection strategies. Future studies should explore CP's precise interactions in other plant species, like tomato, with host machinery and other viral factors to elucidate its role in infection dynamics. These insights contribute to our understanding of viral movement and may inform future strategies for controlling begomovirus infections in crop plants.

Authors' Contributions

The author read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

Author is highly grateful to the Deanship of Scientific Research, King Faisal University, Al-Ahsa, Saudi Arabia, for providing funds to publish this work (KFU242710).

Conflict of Interests

The author declares that there are no conflicts of interest related to this article.

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