The role of a geminiviral DNA β satellite in viral pathogenicity and movement

by

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to my father

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Abstract

Geminiviruses (family *Geminiviridae*) have circular single-stranded genomes encapsidated in twinned quasi-isometric particles and are responsible for major crop losses worldwide. The largest genus, *Begomovirus*, comprises viruses transmitted by the whitefly *Bemisia tabaci*. Most begomoviruses have bipartite genomes, termed DNA A and DNA B. The DNA A component encodes proteins required for viral DNA replication and encapsidation whereas the DNA B encodes two proteins that are essential for systemic movement. A small number of begomoviruses have a monopartite DNA genome that resembles the DNA A of bipartite begomoviruses. This DNA carries all gene functions for replication and pathogenesis. Specific small circular single-stranded DNA satellites containing a single open reading frame (ORF), termed DNA β, have recently been found in association with certain monopartite begomovirus infections. They comprise about 1350 nucleotides and require a helper begomovirus for replication and encapsidation. DNA β contributes to the production of symptoms and enhanced helper virus accumulation in certain hosts. This study examines the role of DNA β satellite in viral pathogenicity and movement in the host plant.

Infectivity analysis of *Tomato leaf curl virus* and DNA β having mutation in the C1 and V1 ORF indicated that the complementary-sense ORF, βC1, is responsible for inducing disease symptoms in *Nicotiana tabacum*. An ORF present on the plus strand, βV1, appeared to have no role in pathogenesis. Tobacco plants transformed with the βC1 ORF under the control of the C*auliflower mosaic virus* 35S promoter, or with a dimeric DNA β exhibited severe disease-like phenotypes, while plants transformed with a mutated version of βC1 appeared normal. Northern blot analysis of RNA from the transgenic plants using strand-specific probes identified a single complementary-sense

transcript. The transcript carried the full βC1 ORF encoding a 118 amino acids product. It mapped to the DNA β nucleotide (nt) position 186-563 and contained a polyadenylation signal 18 nt upstream of the stop codon. A TATA box was located 43 nt upstream of the start codon. These results indicate that βC1 protein is responsible for DNA β induced disease symptoms.

Tomato leaf curl New Delhi virus (ToLCNDV) is a bipartite begomovirus in which both DNA A and DNA B are required for systemic infection. Inoculation of tomato plants with ToLCNDV DNA A alone induced local but not systemic infection whereas co-inoculation with DNA A and the DNA β resulted in systemic infection. The presence of both DNA A and the DNA β in systemically infected tissues and the absence of DNA B was confirmed by probe hybridization. DNA β containing a disrupted βC1 ORF did not mobilize the DNA A for systemic infection. Co-inoculation of plants with DNA A and a construct of βC1 ORF, under the control of the *Cauliflower mosaic virus* 35S promoter, resulted in the systemic movement of the DNA A. βC1 fused to GFP accumulated around and inside the nucleus, at the periphery of tobacco and onion epidermis cells and co-localized with the endoplasmic reticulum. This distribution would be consistent with βC1 mediating intra cellular transport from the nucleus to the plasma membrane. These results showed that the βC1 protein can replace the functions of DNA B in allowing the systemic movement of a bipartite geminivirus DNA A.

Declaration

This work contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Publications

Sections of this thesis have been published (see Appendix 1) or in preparation for publication, as follows.

Saeed, M., Behjatnia, S. A., Mansoor, S., Zafar, Y., Hasnain, S. & Rezaian, M. A. (2005). A single complementary-sense transcript of a geminiviral DNA beta satellite is determinant of pathogenicity. *Mol Plant Microbe Interact* **18**, 7-14.

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Abbreviations

AbMV	*Abutilon mosaic virus*
ACMV	*African cassava mosaic virus*
AYVV	*Ageratum yellow vein virus*
AP	alkaline phosphatase
AYVV	*Ageratum yellow vein virus*
BCTV	*Beet curly top virus*
BDMV	*Bean dwarf mosaic virus*
BGMV	*Bean golden mosaic virus*
bp	base pair
BYVMV	*Bhendi yellow vein mosaic virus*
CaMV	*Cauliflower mosaic virus*
cDNA	complementary DNA
ChiLCuV	*Chili leaf curl virus*
CLCuAV	*Cotton leaf curl Alabad virus*
CLCuGV	*Cotton leaf curl Gezira virus*
CLCuMV	*Cotton leaf curl Multan virus*
CLCuRV	*Cotton leaf curl Rajasthan virus*
CP	coat protein
C	complementary-sense
CSR	complementary-strand replication
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dpi	days post-inoculation
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA

EDTA	ethylenediamine-*tetra*-acetic acid
ER	endoplasmic reticulum
EpYVV	*Eupatorium yellow vein virus*
g	gram(s)
g	relative centrifugal force
GFP	green fluorescent protein
GUS	ß-glucuronidase
h	hour(s)
HoLCrV	*Hollyhock leaf crumple virus*
HYVMV	*Honeysuckle yellow vein mosaic virus*
ICMV	*Indian cassava mosaic virus*
IR	intergenic region
kb	kilobase pairs
L	litre(s)
LB	Luria broth
M	molar
min	minute(s)
MOPS	3-N-Morpholinopropanesulfonic acid
MP	movement protein
mRNA	messenger RNA
miRNA	micro-RNA
MSV	*Maize streak virus*
MYVV	*Malvastrum yellow vein virus*
MYVYV	*Malvastrum yellow vein Yunnan virus*
NLS	nuclear localisation signal
NSP	nuclear shuttle protein

NTP	nucleoside triphosphate
nt	nucleotide
ORF	open reading frame
ori	origin of replication
OYVMV	*Okra yellow vein mosaic virus*
PaLCuV	*Papaya leaf curl virus*
PCR	polymerase chain reaction
Pd	Plasmodesmata
PM	Plasma membrane
PTGS	post-transcriptional gene silencing
RCR	rolling circle replication
RDR	recombination-dependent replication
REn	replication-enhancer protein (encoded by *AC3/C3*)
Rep	replication-associated protein
RF	replicative form
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
s	second(s)
SDS	sodium dodecyl sulphate
SiYVV	*Sida yellow vein virus*
siRNA	small interfering RNA
SLCMV	*Sri Lankan cassava mosaic virus*
SqLCV	*Squash leaf curl virus*
SSC	standard sodium citrate
ssDNA	single-stranded DNA

ssRNA	single-stranded RNA
TbCSV	*Tobacco curly shoot virus*
TBE	tris-borate-EDTA
TbLCYV	*Tobacco leaf curl Yunnan virus*
TGMV	*Tomato golden mosaic virus*
TGS	transcriptional gene silencing
ToLCNDV	*Tomato leaf curl New Delhi virus*
ToLCJV	*Tomato leaf curl Java virus*
ToLCV	*Tomato leaf curl virus* (Australian isolate)
Tris	tris(hydroxymethyl)aminomethane
TPCTV	*Tomato pseudo-curly top virus*
TYLCV	*Tomato yellow leaf curl virus*
TYLCCNV	*Tomato yellow leaf curl China virus*
TYLCTHV	*Tomato yellow leaf curl Thailand virus*
V	volt(s)
VIGS	virus-induced gene silencing
V	Virion-sense
WT	wild-type
YFP	yellow fluorescent protein
ZiLCV	*Zinnia leaf curl virus*

Chapter 1 - General Introduction

1.1 Introduction

Geminiviruses (family *Geminiviridae*) have circular single-stranded (ss) DNA genomes encapsidated in twinned quasi-isometric particles and are responsible for major crop losses worldwide (Moffat, 1999). Geminiviruses have been divided into four genera, *Mastrevirus*, *Begomovirus*, *Curtovirus*, and *Topocuvirus*, based on genome organization, insect vector and host ranges (Figure 1.1). The names of these genera are derived from that of the type member of each genus which are *Maize streak virus* (MSV), *Bean golden mosaic virus* (BGMV), *Beet curly top virus* (BCTV), and *Tomato pseudo-curly top virus* (TPCTV). The family *Geminiviridae* is the second largest among the plant viruses with 133 officially recognized species. The genus *Begomovirus* is the largest in the family comprising 117 species transmitted by the whitefly *Bemisia tabaci* (Stanley et al., 2005). This reflects the economic importance and enormous diversity of geminiviruses resulting from widespread distribution and host adaptation. Most begomoviruses have bipartite genomes, termed DNA A and DNA B. DNA A and DNA B share a region of ~ 200 nucleotides (nt) within the intergenic region called the common region. This contains the conserved stem-loop and nonanucleotide sequence (NS), a highly conserved loop sequence TAATATTAC. The DNA A component encodes proteins required for viral DNA replication and encapsidation (Sunter et al., 1987) whereas the DNA B encodes two proteins that are essential for systemic movement and symptom expression (Brown & Nelson, 1988, Noueiry et al., 1994, Pascal et al., 1993). A number of begomoviruses associated with several diseases of tomato from the Old World have a monopartite DNA genome that resembles the DNA A of bipartite begomoviruses. This DNA carries all gene functions for replication and pathogenesis (Dry et al., 1993, Kheyr-Pour et al., 1991, Navot et al., 1991). Recently,

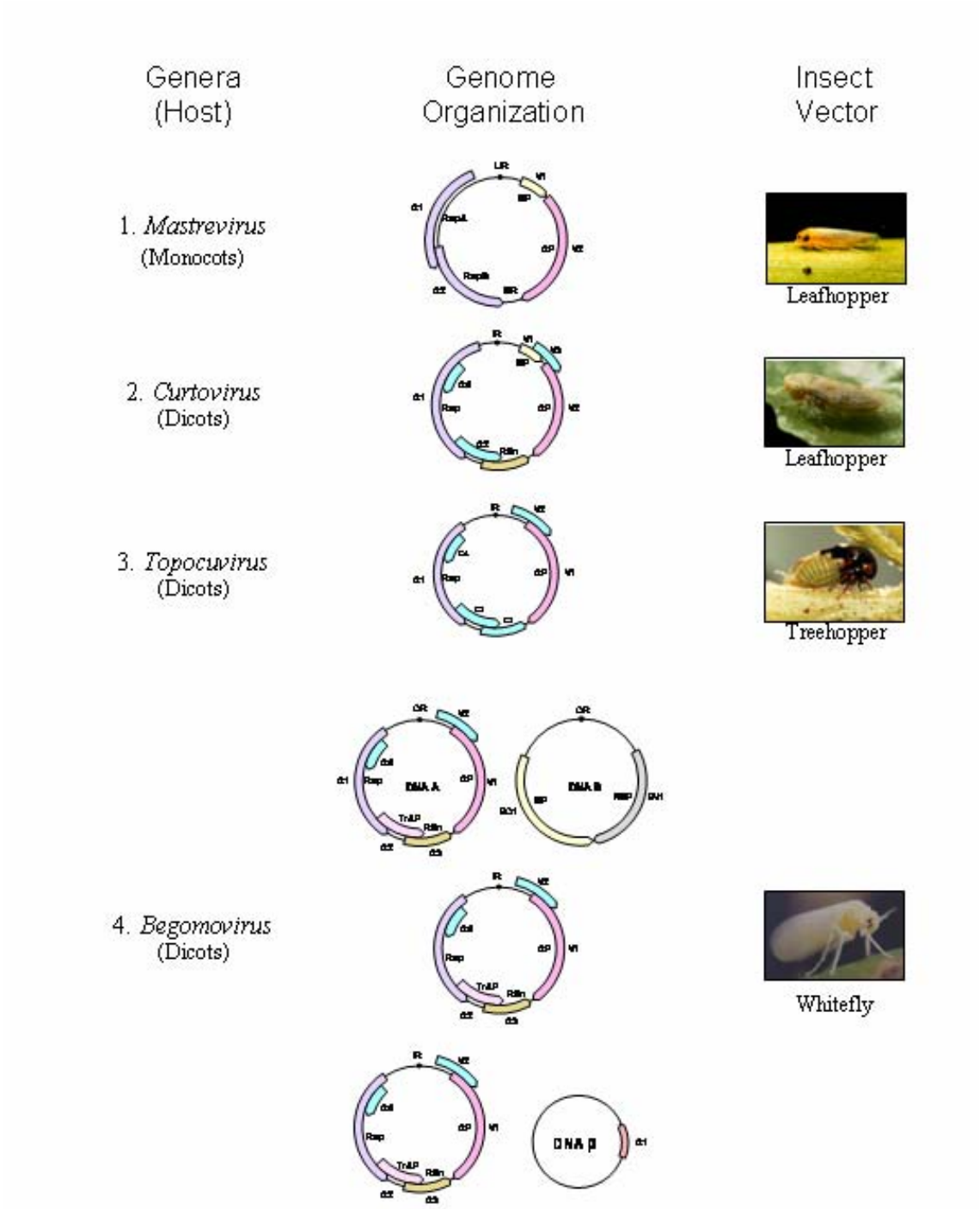

Figure 1.1 Genome organization of geminiviruses. ORFs are designated as either being encoded on the virion (V) or complementary (C) strand. Gene functions are shown where these are known. The position of the stem-loop containing the conserved nonanucleotide (TAATATTAC) is indicated. CP/V1, coat protein; MP/V2, movement protein Rep/C1, replication associated protein; TrAP/C2, transcriptional activator protein; REn/C3, replication enhancer protein; pathogencity determinat/C4: MP/BC1, movement protein; NSP/BV1, nuclear shuttle protein; LIR, large intergenic region; SIR, small intergenic region; IR, inter-genic region; CR, common region.

certain monopartite begomoviruses including *Ageratum yellow vein virus* (AYVV), *Cotton leaf curl Multan virus* (CLCuMV), some begomoviruses infecting tomato and tobacco in China, and *Bhendi yellow vein mosaic virus* (BYVMV) in India have been found to require a satellite molecule called DNA β for induction of disease symptoms in some host plants (Briddon et al., 2001, Jose & Usha, 2003, Saunders et al., 2000, Zhou et al., 2003).

1.2 Begomovirus-DNA β satellite diseases

1.2.1 Genome organization of begomoviruses

Begomovirus genomes have either one (monopartite) or two (bipartite) DNA components ranging from 2.5 to 2.8 Kb in size. These viruses replicate in the host cell nucleus via a double-stranded (ds) DNA intermediate, termed replicative form (RF). The RF is used as a template for transcription as well as replication. Both strands code for viral proteins (Hanley-Bowdoin et al., 1999). The DNA A of bipartite begomoviruses and monopartite begomoviruses have a very similar genome organization and encode 5-6 overlapping open reading frames (ORFs). The virion-sense strand (V) of DNA A encodes the coat protein (CP, AV1/V1) that encapsidates the viral ssDNA. The DNA A of Old World begomoviruses encodes an additional ORF AV2/V2 that has been implicated in virus movement (Padidam et al., 1996, Rigden et al., 1993). The DNA A complementary-sense (C) strand encodes the replication-associated protein (Rep, AC1/C1), a transcriptional activator protein (TrAP, AC2/C2), and a replication enhancer protein (REn, AC3/C3). TrAP is involved in the control of both viral and host gene expression. Some DNA A of bipartite viruses and all monopartite viruses encodes AC4/C4 that participates in cell-cycle control (Briddon & Stanley, 2006). The DNA B encodes two ORFs, a virion-sense nuclear shuttle protein (NSP, BV1) and a

complementary-sense movement protein (MP, BC1). The DNA A and DNA B share no homology except for a ~ 150-250 nt common region which contains the origin of replication and regulatory regions for bi-directional transcription. This region which contains a conserved nonanucleotide sequence (TAATATTAC) located in the stem loop structure. Monopartite geminiviruses also contain similar non-coding inter-genic region (Hanley-Bowdoin et al., 1999).

1.2.2 DNA β satellites

Satellites are viruses or nucleic acids that depend on a helper virus for replication but lack nucleotide sequence homology to the helper virus. Satellite viruses code for their own coat protein, whereas RNA satellites are packaged in coat protein encoded by the helper virus (Mayo et al., 2005). Many plant RNA viruses have RNA satellites associated with them. The majority of satellites interfere with the replication of helper virus resulting in attenuated symptoms. Some satellites exacerbate disease symptoms induced by helper virus or produce a novel symptom which is usually not associated with helper virus infection. (Roossinck et al., 1992).

The first viral satellite DNA was found to be associated with *Tomato leaf curl virus* (ToLCV) from Australia (Dry et al., 1997). This 682 nt DNA depends on ToLCV for its replication and encapsidation, but its replication can also be supported by other geminiviruses. It has no discernible effects on viral replication or on symptoms caused by ToLCV. In contrast, DNA β satellites associated with some begomovirus infections are required for the induction of disease symptoms in some host plants (Briddon et al., 2001, Jose & Usha, 2003, Saunders et al., 2000, Zhou et al., 2003). These DNA β satellites are about 1350 nt long and have some sequence similarity to ToLCV satellite DNA and carry potential ORFs. Like ToLCV sat-DNA, DNA β satellites require a helper virus for replication and encapsidation (Mansoor et al., 2003b). Despite their

recent discovery, DNA β satellites may have existed for many centuries. The DNA β satellite associated with *Eupatorium yellow-vein virus* (EupYVV) has been linked to disease symptoms described about 1250 years ago (Saunders et al., 2003). The ToLCV satellite DNA that lacks any ORF has been suggested to be a remnant of a DNA β that was at one time associated with this monopartite begomovirus (Saunders et al., 2000).

DNA β satellites have a conserved genome organization consisting of a single complementary-sense ORF (βC1), an adenine rich region, and a satellite-conserved region (SCR) (Figure 1.2). The SRC contains a stem loop sequence TAA/GTATTAC which is similar to sequence in the origin of replication of geminiviruses (Briddon et al., 2003). Besides full-length molecules, naturally occurring DNA β deletion mutants of 660 to 896 nt have been reported (Briddon et al., 2003). The majority of deletions encountered were downstream of the hairpin structure, including all or part of the βC1 coding region. These deletion mutants did not induce DNA β associated symptoms in cotton or ageratum (Briddon et al., 2003).

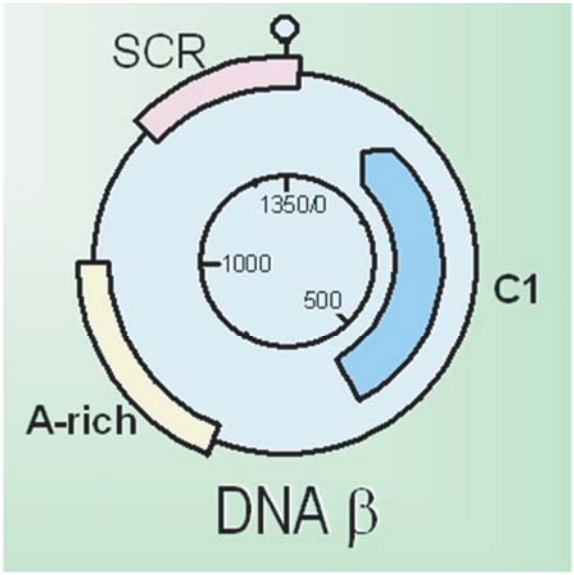

Figure 1.2 Genome organization of DNA ß satellite. The position and orientation of the conserved C1 gene is shown as an arrow within the circle. The relative position of the satellite conserved region (SCR) and A-rich region are shown as shaded slices.

The DNA β satellites contribute to the production of symptoms and enhanced levels of helper virus accumulation in certain hosts. Hence, it has been proposed that DNA β might affect the replication of helper virus by either facilitating their spread in host plants, or by suppressing the host gene silencing (Mansoor et al., 2003b, Saunders et al., 2000). DNA βs encode a protein, βC1, that is a determinant of pathogencity and suppressor of gene silencing (Cui et al., 2004, Saeed et al., 2005, Saunders et al., 2004).

Currently there is some confusion about the number of DNA β satellites that have been reported. Some recent reviews have noted that as many as 130 full-length DNA β sequences are available in the GeneBank database (Briddon & Stanley, 2006, Rojas et al., 2005). Analysis of these sequences indicates that many of them have over 96% sequence similarity with those that have been reported. A list of distinct DNA β satellites is presented in Table 1.1 which is based on sequence diversity of satellite, status of helper virus as a species, and characterization published in scientific journals.

DNA 1 components are satellite-like molecules associated with many begomovirus-DNA β disease complexes. Sequence comparison of DNA 1 components shows that they have a conserved genome organization containing a single virion-sense Rep ORF, an A-rich region, and a hairpin structure. DNA 1 components are capable of self replication but require helper virus for encapsidation and movement within the host plant. DNA 1 components have no effect on symptom expression (Briddon et al., 2004).

1.3 Replication of begomoviruses and DNA satellite

Geminiviruses do not encode DNA polymerase and thus depend upon many host gene functions. These viruses replicate in differentiated cells that are in G phase and have shut down most of their DNA replication activities. Geminivirus infection reactivates the replication and converts the cell back to S phase of cell-cycle (Hanley-Bowdoin et al., 1999). Geminiviruses use both rolling circle (RCR) and recombination dependent

replication (RDR) mechanisms for the replication of their genome. Upon entry into a plant cell, the virus particles are targeted to the nucleus where replication occurs and probably involves CP (Hull, 2002). RCR is a two step process. The first step is the synthesis of complementary-sense synthesis using the virion-sense strand as template, to produce a ds RF intermediate. This process is thought to be primed by a short RNA molecule (Hanley-Bowdoin et al., 1999). Supporting this hypothesis is the isolation of oligo-ribonucleotides complementary to the 3′ intergenic region from several mastreviruses, which can be extended by DNA polymerase in vitro (Hayes et al., 1988). The host DNA polymerase completes the synthesis of complementary-sense strand synthesis. The synthesis of virion-sense strand is mediated by Rep nicking within TAATATTAC. The geminivirus Rep proteins is a site-specific endonuclease that nicks and ligates the viral strand at the same position in vitro (Laufs et al., 1995). The nonanucleotide sequence together with small iterated flanking sequences (called iterons) and intervening sequences form the origin of virion-sense DNA replication. The Rep proteins show specificity for the replication of their cognate genomes. Trans-replication of the DNA B component by DNA A encoded Rep is achieved by Rep-iteron specificity. Because of this Rep specificity, most DNA A components are unable to trans-replicate heterologous DNA B (Hanley-Bowdoin et al., 1999).

Table 1.1

Begomovirus-DNA β satellite disease complexes

Name of helper virus specie	DNA β Accession number*	Reference
1. Tomato leaf curl disease satellite (ToLCV)	U74627§	(Dry et al., 1997)
2. Ageratum yellow vein virus (AYVV)	AJ252072	(Saunders et al., 2000)
3. Cotton leaf curl Multan virus (CLCuMV)	AJ298903	(Briddon et al., 2001)
4. Tomato yellow leaf curl China virus (TYLCCNV)	AJ421621	(Zhou et al., 2003)
5. Malvastrum yellow vein virus (MYVV)	AJ421482	(Zhou et al., 2003)
6. Tobacco curly shoot virus (TbCSV),	AJ421484	(Zhou et al., 2003)
5. Bhendi yellow vein mosaic virus (BYVMV)	AJ308425	(Jose & Usha, 2003)
6. Eupatorium yellow vein virus (EpYVV)	AJ438938	(Saunders et al., 2003)
7. Okra yellow vein mosaic virus (OYVMV)	AJ316039	(Briddon et al., 2003)
8. Chili leaf curl virus (ChiLCuV)	AJ316032	(Briddon et al., 2003)
9. Hollyhock leaf crumple virus ((HoLCrV)	AF397214¶	(Briddon et al., 2003)
10. Tobacco leaf curl Yunnan virus (ToLCYV)	AJ536621-2†	(Cui et al., 2004)
	AJ536628†	
11. Tomato yellow leaf curl Thailand virus (TYLCTHV)	AJ566746-8†	(Cui et al., 2004)
	AJ316044†	
12. Honeysuckle yellow vein mosaic virus ((HYVMV)	AJ316040	(Bull et al., 2004)
13. Zinnia leaf curl virus (ZiLCV)	AJ542499	(Bull et al., 2004)
14. Cotton leaf curl Gezira virus (CLCuGV)	AY077798	(Idris et al., 2005)
15. Sida yellow vein virus (SiYVV)	AJ810093	(Xiong et al., 2005)
16. Cotton leaf curl Rajasthan virus (CLCuRV)	AY083590	(Stanley et al., 2005)
17. Malvastrum yellow vein Yunnan virus (MYVYNV)	AJ786712	(Jiang & Zhou, 2005)
18. Tomato leaf curl Java virus (ToLCJV)	AB162142‡	(Kon et al., 2006)

*In many examples more than one closely related DNA β sequence was reported but in subsequent studies only one isolate was used.

†Where infectivity data is not available, accession numbers of all DNA β sequences reported are given.

‡ToLCJV is not an ICTV recognized species; it has been recently characterized and is likely to be included in the ICTV list.

¶The DNA satellites associated with HoLCrV are probably defective DNA β having 95% sequence similarity with DNA β associated with Okra yellow vein disease in Africa.

§ToLCV is a monopartite begomovirus and not a begomovirus-DNA β satellite disease. However, it is included in the list because it was the virus in which DNA satellite was first reported.

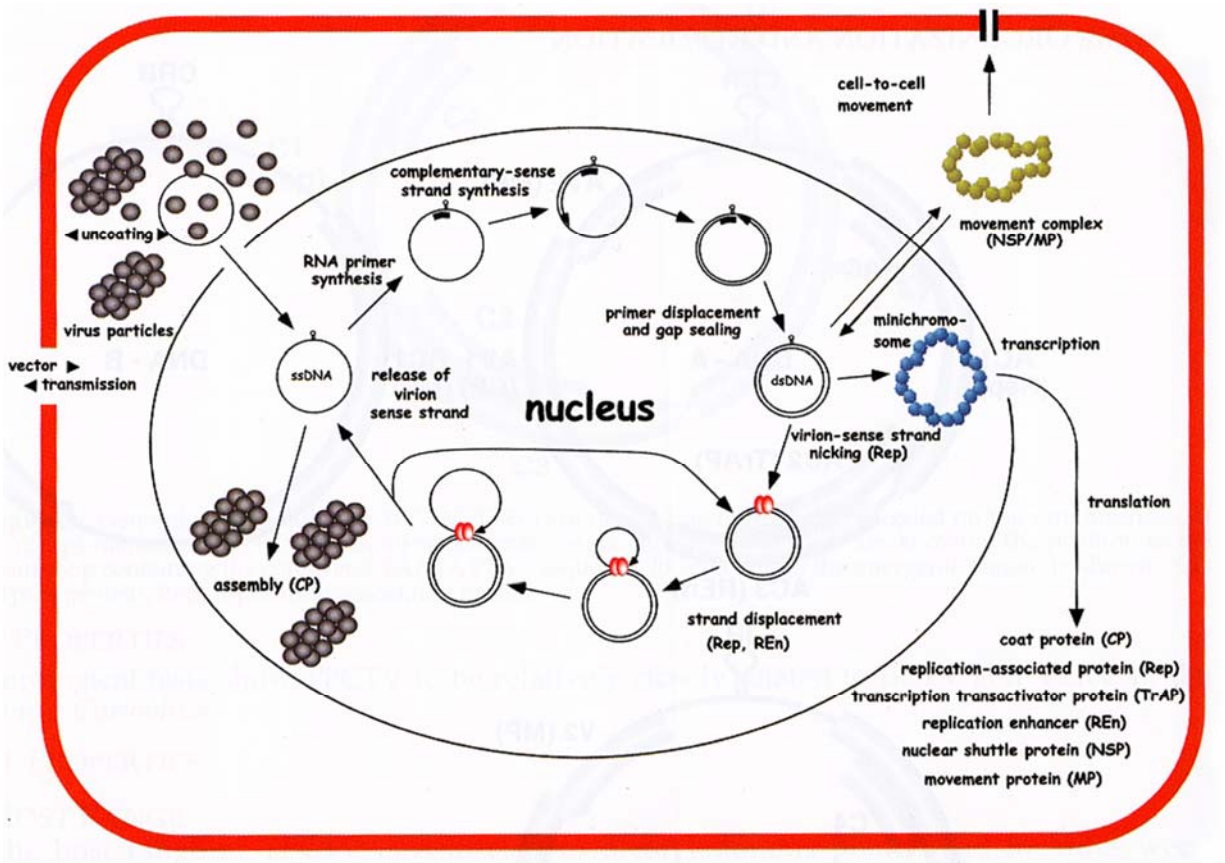

Figure 1.3 Diagram of the begomovirus replication strategy. Virus particles are introduced into the cell during whitefly feeding and the uncoated ssDNA is replicated in the nucleus. The mechanism of initial entry into the nucleus is not yet understood. A dsDNA intermediate is synthesised from the ssDNA template by host factors alone. ssDNA is synthesised from the dsDNA template by a rolling circle mechanism involving Rep and REn viral proteins in association with host factors, and is assembled into virus particles that accumulate in the nucleus. dsDNA adopts a transcriptionally active minichromosome form. Conflicting reports have suggested the involvement of either dsDNA (shown here) or ssDNA in nuclear transport and cell-to-cell movement, mediated by NSF and MP (Stanley et al., 2005).

Begomoviral DNA satellites can be trans-replicated by diverse helper viruses (Mansoor et al., 2006). It has been shown that DNA β satellites with a deletion in the βC1 ORF and A-rich region can be trans-replicated by the helper virus (Briddon, 2003, Qian & Zhou, 2005). Thus satellite conserved region seems to be essential for replication. This is located adjacent to the stem-loop structure, TAA/GTATTAC. By analogy to geminivirus, it may be the site where Rep introduces a nick during replication of virion-sense strand synthesis. Recently, Albert et al. (2005) reported that like geminiviruses, the ToLCV satellite and a DNA β use both RDR and RCR for

replication of their ssDNA molecules. In spite of their similarity with geminivirus for replication, these DNA satellites do not have precise high affinity Rep binding motifs that should be necessary for trans-replication. Somehow the interaction between begomovirus and DNA β is relaxed in comparison to the specific interaction between DNA A and DNA B components. This less stringent requirement may account for the observed replicational promiscuity of the geminiviral DNA satellites (Briddon & Stanley, 2006).

1.4 Viral pathogenicity

Viral pathogenicity is an outcome of the virus attempting to establish an infection and the host attempting to resist it. Therefore, all viral proteins involved in replication and spread contribute towards disease symptoms. However, some viral proteins have more drastic effects than the others and are thus known as pathogenicity proteins (Hull, 2002). Numerous studies have shown that MP encoded by DNA B of bipartite begomoviruses is the pathogenicity determinant with few exceptions like ToLCNDV where NSP is the pathogenicity determinant (Hussain et al., 2005, Ingham et al., 1995, Pascal et al., 1993). For monopartite begomoviruses and curtoviruses, the C4 protein is the pathogenicity determinant (Latham et al., 1997, Rigden et al., 1994). Transgenic plants expressing these proteins alone produce virus-like symptoms (Krake et al., 1998, Latham et al., 1997, Pascal et al., 1993).

The helper virus associated with the begomovirus-DNA β satellite complexes can induce symptoms in *N. benthamiana*. However, AYVV and CLCuMV infection produces an asymptomatic infection with low virus accumulation in their respective host plants. Co-inoculation of these helper begomoviruses with their cognate DNA βs results in symptoms resembling those in field infected plants (Briddon et al., 2001; Saunders et al., 2000). Recently, research from three laboratories has shown that DNA β

satellites encode a single protein which is the determinant of viral pathogenicity for begomovirus-DNA β satellite complexes (Cui et al., 2004, Saeed et al., 2005, Saunders et al., 2004).

1.4.1 Silencing suppression and viral pathogenicity

RNA silencing is the suppression of gene expression through sequence specific interaction mediated by RNA. It is well established that dsRNA or hairpin RNA induces a post- transcriptional gene silencing (PTGS) mechanism in which the dsRNA is cleaved by Dicer, an RNaseIII-type enzyme, into small interfering RNAs (siRNA) of 21-26 nt. The production of siRNAs by Dicer is an ATP-dependent step and involves interactions with other proteins, including an argonaute-like protein, a dsRNA binding protein, and an RNA helicase. siRNA are intermediate in the gene silencing pathway, serving as guides to form an RNA-induced silencing complex that destroys cognate ssRNA (Waterhouse & Fusaro, 2006). Plant viruses which have an RNA genome produce dsRNA intermediates (Hull, 2002). Bi-directional transcription of geminiviruses produces an overlapping dsRNA transcript (Hanley-Bowdoin et al., 1999). Plant viruses thus trigger PTGS. PTGS is a plant defence mechanism against viruses and other nucleic acid invaders. To escape viral infection, plants have evolved an RNA silencing system, an antiviral defence mechanism. RNA silencing is non-cell-autonomous which may reflect the antiviral nature of the process.

MicroRNAs (miRNA) are ~21 nt long, non-coding RNAs that regulate host gene expression. Many miRNAs down-regulate expression of transcriptional factors involved in plant development. PTGS and miRNA pathways share many components of RNA silencing pathways (Waterhouse & Fusaro, 2006). Over-expression of viral silencing suppressors from many plant viruses result in abnormal development. This suggests that

viral pathogencity may result from perturbation of host miRNA pathways by the expression of viral silencing suppressors (Mallory & Vaucheret, 2006).

Many begomoviruses and curtoviruses encode a AC2/C2 protein that acts as a suppressor of RNA silencing (Voinnet et al., 1999). The AC2/C2 of begomoviruses also acts as a trans-activator protein for CP and NSP. Recently, it has been shown that in certain begomoviruses such as *African cassava mosaic virus* (ACMV) and *Sri Lankan cassava mosaic virus* (SLCMV) the AC4 protein is the suppressor of gene silencing (Vanitharani et. al., 2004). Transgenic plants expressing AC2 show enhanced susceptibility to diverse viruses; however, phenotypically these plants are normal in appearance. A recent study has provided direct evidence that geminiviral silencing suppressors may be largely responsible for pathogencity. Transgenic plants expressing the *AC4* gene encoded by ACMV and SLCMV displayed developmental abnormalities. This protein is a miRNA binding protein that down regulates a class of miRNA involved in plant development. Thus the host gene expression is up-regulated because of the non-availability of miRNA. Therefore, normal development is disturbed and this leads to developmental abnormalities (Chellappan et al., 2005).

The DNA β satellite encodes a single protein, βC1, which is a pathogencity determinant (Cui et al., 2004, Saeed et al., 2005, Saunders et al., 2004). Cui et al., (2005) have demonstrated that the βC1 also acts as a silencing suppressor. Expression of the *βC1* gene in transgenic plants also induces developmental abnormalities. Hence, the βC1 and AC4/C4 proteins share characteristics of pathogenicity and silencing suppression. It is likely that the βC1 protein may be involved in the regulation of miRNA involved in plant development.

1.5 Virus movement

Plant virus movement is a two stage process, cell-to-cell movement through plasmodesmata (Pd) and long distance transport through phloem. Plant viruses mainly

use two principal strategies for cell-to-cell movement. One involves binding of MP or MP complexes with the viral genome, which is either RNA or DNA, and increasing size exclusion limit of Pd. The other is dependent on tubule formation (Lucas, 2006).

Monopartite and bipartite begomoviruses vary considerably with respect to their movement in host plants. CP is not required for systemic infection by bipartite begomoviruses indicating that virus DNA may be the principal form of transport in host plants (Gardiner et al., 1988, Sudarshana et al., 1998). In bipartite begomoviruses NSP encoded by DNA B can substitute for the function of CP in transport (Ingham et al., 1995). NSP like CP is targeted to the nucleus. Besides NSP, these viruses encode MP to mediate cell-to-cell movement. NSP and MP function cooperatively in cell-to-cell movement, NSP exports viral DNA to the cytoplasm whereas MP interacts with this complex and targets the Pd (Gafni & Epel, 2002). In a recent study using electron microscopic analysis, it has been shown that ds DNA assembles with NSP and MP to form a conspicuous structure (Hehnle et al., 2004).

In all monopartite begomoviruses CP is an absolute requirement for systemic movement (Briddon et al., 1989, Lazarowitz et al., 1989). Thus monopartite begomoviruses either move as virions or nucleo-protein complexes. CP is a DNA binding protein and localizes to the cell nucleus. CP of *Tomato yellow leaf curl virus* (TYLCV) has been shown to bind the labelled DNA in tobacco epidermis cells and move them to the nucleus. Thus the CP of monopartite viruses carries out functions performed by NSP. Besides CP, these viruses encode two additional proteins, C4 and V2 that are involved in viral movement (Rojas et al., 2001). A mutation in V2 ORF results in asymptomatic infection with reduced virus accumulation (Padidam et al., 1996, Rigden et al., 1993). Subcellular localization studies of the V2 ORF of TYLCV have shown that V2 protein localizes around the nucleus and at the cell periphery and colocalizes with endoplasmic reticulum (ER). At the nuclear periphery V2 may help to

transport nucleoprotein complex to the Pd possibly through ER (Rojas et al., 2001). The C4 ORF is a determinant of pathogenicity and may have a role in virus movement (Jupin et al., 1994, Rigden et al., 1994). Subcellular localization studies of the C4 ORF of TYLCV have shown that C4 protein is targeted to the cell periphery. At the cell periphery, C4 may act in the delivery of viral DNA to the neighbouring cell. The TYLCV C4-mutant is capable of systemic movement in a permissive host like *N. benthamiana* but unable to infect tomato plants (Jupin et al., 1994). However, ToLCV is closely related to the TYLCV where a mutation in C4 ORF resulted in mild symptoms without affecting virus accumulation (Rigden et al., 1994). Both C4 and V1 protein could mediate cell-to-cell movement of fluorescently labelled dyes to the adjacent cell (Rojas et al., 2001).

1.5.1 Begomovirus-DNA β satellite

All known DNA β satellites are associated with monopartite begomoviruses. The helper viruses' infection causes symptoms in *N. benthamiana*. However, these viruses accumulate at low levels in their natural host and cause asymptomatic infection (Briddon et al., 2001, Jose & Usha, 2003, Saunders et al., 2000, Zhou et al., 2003). It is therefore assumed that the satellite may have a direct role in viral movement by facilitating viral DNA movement throughout the plant, thereby amplifying the helper virus levels (Saunders et al., 2000). Alternatively, it may have an indirect role in viral movement as βC1 protein has been shown to suppress gene silencing (Cui et al., 2005). Because these helper viruses are monopartite begomoviruses, it is likely that they may have similar requirement for viral movement in the host plants. The role of βC1 protein in viral movement is unclear.

1.6 Cotton leaf curl disease in Pakistan

Since early 1990s, cotton leaf curl disease (CLCuD) in Pakistan has been more devastating than any other pathogen or insect pest. The earliest record of CLCuD was in 1967 when some plants in cotton fields were reported (Hussain & Ali, 1975). However, the disease remained unnoticed until 1988 when it was reported in a block of 60 ha in the province of Punjab, which produces around 80% of the total yield. The disease became economically important in 1992 when the majority of the cotton crop in the Punjab province became affected. Yields decreased from 1.938 million metric ton in 1991 to 1.105 million metric ton in 1993. During 1992-97, the total yield losses were worth US$5 billion. The economy of Pakistan is heavily dependent on the cotton crop which accounts for 60% of foreign exchange. During 1995, CLCuD was reported from Indian Punjab state, and since then it has also become a significant problem in India. During 1997 the disease was reported in Sindh province, which is the second most important province for the cotton crop in Pakistan (Briddon & Markham, 2000).

The symptoms of disease are vein-thickening, upward or downward leaf curling, stunting, enations which usually develop into a leaf-like structure on the underside of the leaf (Briddon et al., 2001). The experimental host range of CLCuD includes cotton, tobacco, tomato, okra, hibiscus, and ageratum. Leaf curl symptoms occur on many herbaceous and woody species including cotton, tobacco, hibiscus, okra, sunflower, hemp, and many weeds in the field (Briddon & Markham, 2000).

The yield losses depend upon time and severity of the infection. Early infection results in total crop failure. Initial attempts at creating resistant/tolerant varieties through breeding against CLCuD were partially successful. However, since 2001 the incidence of a “resistance breaking strain” of the disease has been reported in the Burewala district. All resistant varieties have been shown to be susceptible to the new strain and it has spread to the major cotton belt in the Punjab province (Amin et al., 2006).

1.6.1 Aetiology of the disease

The whitefly *Bemisia tabaci* was identified as the vector of CLCuD in 1992. Because of the symptoms and the fact that the disease was not seed-transmissible, it was suggested that the disease might be caused by a begomovirus. Mansoor et al. (1993) showed that a begomovirus was associated with the disease. Zhou et al. (1998) reported the incidence of four distinct variants of the virus which was named *Cotton leaf curl virus*. Mansoor et al. (1999) showed that a novel satellite-like component called DNA 1 was associated with CLCuD. In a subsequent study by Briddon et al. (2000) the inoculation of cotton plants with the *Cotton leaf curl virus* (later renamed *Cotton leaf curl Multan virus* (CLCuMV) resulted in asymptomatic infection, suggesting that some additional agents might be associated with the disease. Meanwhile, it was reported that a newly identified DNA satellite named DNA β and AYVV were responsible for ageratum yellow vein disease in Singapore (Saunders et al., 2000). Soon, the causal agent of CLCuD was identified as a begomovirus component, CLCuMV together with a DNA β component (Briddon et al., 2001). A later study reported that CLCuD can be caused by a combination of a DNA β and either *Cotton leaf curl Alabad virus* (CLCuAV), CLCuMV, *Cotton leaf curl Khokhran virus* (CLCuRV) or *Papaya leaf curl virus* (PaLCuV) (Mansoor et al., 2003a).

1.7 Aims of this study

This thesis examines some aspects of the interaction of CLCuD DNA β with helper viruses and the host plant. The broader objectives of the work described in this thesis are:

1) To determine the role of DNA β in viral pathogencity by:
    a) mutagenesis,
    b) expression in transgenic plants, and

c) transcript mapping

2) To determine the role DNA β in viral movement using:

a) infectivity analysis with a helper begomovirus impaired in viral movement

b) confocal laser section microscopic location technique to identify the subcellular location of DNA β encoded ORF, C1.

Chapter 2 - General Materials and Methods

2.1 Materials

2.1.1 Solutions

All chemicals used were analytical or molecular biology grade. Solutions were prepared with Nanopure® water (Syborn/Barnstead, Boston, USA) and autoclaved where appropriate. The solutions used in the study are described in Table 2.1.

2.1.2 Bacterial Strains

Escherichia coli strain XL1-Blue (Stratagene, La Jolla, USA) was used for routine cloning work. *Agrobacterium tumefaciens* strain LBA 4404 (Invitrogen, Carlsbad, USA) was used to transform plants whereas strain C58 was used to inoculate plants with various geminiviruses and DNA β satellite.

Table 2.1 Solutions and their compositions

Solutions	Compositions
Agarose gel loading buffer DNA (10x)	78% glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 10 mM EDTA
Agarose gel loading buffer RNA (10x)	50% glycerol, 200 mM EDTA, 0.08% (w/v) bromophenol blue
Denaturing gel loading buffer RNA (5x)	70% deionised formamide, 10% (v/v) formaldehyde, 6% (v/v) agarose gel loading buffer, 14% (v/v) MOPS/EDTA buffer

Hybridization buffer	0.25 M sodium phosphate buffer (pH 7.2), 7% (w/v) SDS, 1 mM EDTA
LB broth	1% (w/v) bacto-trypton, 0.5% yeast extract, 1% (w/v) NaCl, pH 7.0
LB agar	1% (w/v) bacto-trypton, 0.5% yeast extract, 1% (w/v) NaCl, 1.2 % (w/v) bacto-agar, pH 7.0
MOPS/EDTA buffer (10x)	200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0
Murashige & Skoog (MS) salt	4.33 g MS basal salt mixture/L, pH 5.7-5.8
DNA extraction buffer	50 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 100 mM NaCl, 1% (w/v) SDS, 2-mercaptoethanol (v/v) 1.5%
SSC (1x)	150 mM NaCl, 15 mM tri-sodium citrate, pH 7.0
TBE buffer (10X)	90 mM Tris-borate, I mM EDTA
STET plasmid isolation buffer	8% (w/v) sucrose, 5% (v/v) Triton X-100, 50 mM EDTA (pH 8.0), 50 mM Tris-HCl (pH 8.0)
Non-selection plates	MS basal salts, 0.01% (w/v) Gamborg vitamins, 0.8% (w/v) agar, 3% (w/v) sucrose, 0.54 µM NAA, 3.58 µM BA
Selection plates	Ingredients for non-selection plates with 250 µg/ml Kanamycin and 500 µg/ml Claforan

2.2 Methods

Molecular biology methods were as described in Sambrook & Russell (2001) or according to the manufacturer's instructions. Methods which have been significantly modified from their published form are described here and specific methods are described in relevant chapters.

2.2.1 Dephosphorylation of DNA 5′

Following restriction enzyme digestion of DNA, 5′ phosphate groups were removed using alkaline phosphatase (Roche, Basel, Switzerland). The dephosphorylated DNA was purified with a Qiagen (Valencia, CA, USA) PCR purification kit.

2.2.2 Converting 5′ or 3′ overhangs to blunt end

To convert 5′ or 3′ overhangs to blunt ends, 100-500 ng of DNA digested with appropriate restriction enzymes was mixed with 1x T4 DNA polymerase buffer, 100 µM of dNTPs, 0.1 mg/ml BSA, and 5 units of T4 DNA polymerase (Fermentas Vilnius, Lithuania). The reaction volume was 50 µl and incubation was at 37 ^{o}C for 5 minutes.

2.2.3 Preparation of electro-competent *E. coli* cells

500 ml of LB was inoculated with a 5 ml overnight culture of the *E. coli* and grown at 37 ^{o}C at 200-225 rpm. When an optical density OD_{600} of 0.5-0.8 was reached, cells were chilled on ice for 15-20 min and centrifuged at 4000 rpm at 4 ^{o}C for 15 min. The cells were washed with one vol (500 ml) ice-cold sterile water and centrifuged again. The cells were washed again with 0.5 vol (250 ml) ice-cold sterile water and centrifuged. The pellet was washed with 0.06 vol (30 ml) ice-cold sterile 10% glycerol and transferred to a 50 ml Falcon tube for centrifugation. The cells were finally suspended

in 0.004 vol (2 ml) of 10% ice-cold glycerol. Aliquots of 25-50 µl were placed into ice-cold eppendorf tubes, snap-frozen in liquid nitrogen, and stored at -80 ^{o}C.

2.2.4 Transformation of bacteria with recombinant plasmids

Electro-competent *E. coli* XL Blue, *A. tumefaciens* C58 and LBA 4404 cells were transformed by electroporation using a Gene-Pulser^{TM} apparatus (Biorad, Hercules, CA, USA). Approximately 1 ng of plasmid DNA or 1 µl of ligation mix was added to 50 µl of electro-competent cells and transferred to an ice-cold cuvette (Invitrogen, Carlsbad, USA) with a 0.1 cm electrode gap. The cold cuvette was placed into the Gene-Pulser^{TM} electroporator (Biorad, Hercules, CA, USA) set at 1.8 kV, 25 µFD, and 200 Ohms and was given a single pulse. The cells were immediately mixed with 500-600 µl of LB media and incubated at 37 ^{o}C for one hour. 20-200 µl of transformation mix was spread on 1.2% LB agar plates with appropriate antibiotic selection and incubated at 37 ^{o}C overnight.

2.2.5 Preparation of bacterial plasmid DNA

Plasmid DNA for sensitive protocols such as biolistic inoculation and sequencing was prepared from 1-5 ml of overnight culture; 10 ml overnight culture was used for the isolation of low copy number plasmid using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA).

Plasmid DNA for routine restriction digestion and *E. coli* transformation was prepared using a miniprep boiling method. 1.5 ml of overnight culture was centrifuged at 13000 rpm for 1 min and cells were resuspended in 350 µl of STET (Table 2.1) buffer. After adding 12.5 µl of lysozyme (20 mg/ml) the cells were incubated in boiling water for 1 min and centrifuged at 13000 rpm for 10-15 min. The pellet was removed with a sterile tooth pick. DNA in the solution was precipitated with 40 µl of 3 M sodium

acetate (pH 5.2) and centrifuged for 5-10 min at 13000 rpm. The resulting pellet was washed with 70% ethanol and vacuum dried before suspension in 40 µl of sterile water.

2.2.6 Preparation of bacterial glycerol stocks

Glycerol stocks of *E. coli* or *A. tumefaciens* were prepared by adding 750 µl of 40% sterile glycerol to 750 µl of overnight culture, snap-frozen in liquid nitrogen and stored at -80 ^{o}C.

2.2.7 Total nucleic acid extraction

100-150 mg of fresh leaf tissue was frozen in liquid nitrogen and ground briefly. 200-300 µl of extraction buffer (Table 2.1) and a small amount of sterile sand was added before grinding for one min. 200-300 µl of phenol/chloroform (4:1) was added and the mixture was vortexed for 15 s and placed on ice till all samples were prepared. The samples were centrifuged for 10 min at 13000 rpm. The supernatant was extracted twice with 200-300 µl of phenol/chloroform (1:1) and once with an equal volume of chloroform. DNA was precipitated with 1/10 vol of sodium acetate (pH 5.2) and 2.5 vol of absolute ethanol and kept at -20 ^{o}C for 30 min before centrifugation. The final DNA pellet was washed with 70% ethanol, dried and resuspended in 100 µl of sterile water.

2.2.8 Dot blot hybridization analysis

Dot blot hybridization was used as a quick and reliable method to assess for geminivirus infection. 50 mg of leaf tissue was ground in the presence of sterile sand and 200 µl of 0.5 M NaOH for one min and centrifuged at 13000 rpm for 5 min. 5 µl of supernatant was dotted on nylon membrane and washed twice with chloroform for 20 s and UV cross-linked using a Stratalinker® apparatus **(**Stratagene, La Jolla, USA). Nucleic acids were hybridized using standard protocols (Sambrook & Russell 2001).

2.2.9 Tobacco transformation

An overnight culture of *Agrobacterium* strain LBA 4404 carrying the desired gene with OD_{600} of 0.2-0.3 was centrifuged at 500 rpm. The pellet was resuspended and adjusted to OD_{600} 0.3 in MS liquid medium. Just before the start of co-cultivation, the cells were diluted 10 times in MS medium. Young fully expanded tobacco leaves were trimmed by removing 0.5 cm from top and bottom, and cut into 4-6 pieces with 4 cut edges. The leaf pieces were floated upside down on MS liquid media. Six leaf pieces were placed into diluted culture and incubated for 3 min with occasional gentle mix. Leaf pieces were transferred to sterile 3MM for few sec to remove excessive culture. Leaf strips were placed onto non-selection medium (Table 2.1) and patted gently down with a sterile spatula. They were kept in growth room in dark for two days. Next, leaf pieces were washed in 15 ml of MS liquid containing 500 µg/ml Claforan for two min with gentle mixing. Leaf pieces were gently pressed on the selection media (Table 2.1) using a sterile spatula and put in the growth room. After 4-6 weeks, transgenic shoots were transferred to selection media containing 250 µg/ml of kanamycin for root development. Transgenic seedlings approx 6 cm in height were transferred to the glass house for hardening.

Chapter 3 - A single complementary-sense transcript of a geminiviral DNA β satellite is determinant of pathogenicity

3.1 Introduction

CLCuD is a major constraint to the production of cotton in Pakistan. The disease has been reported to be caused by a begomovirus-DNA β satellite complex (Briddon et al., 2001). Analysis of DNA β sequences has shown that it contains a single complementary-sense ORF (βC1) which is conserved in size and location. However, sequence analysis based on codon usage predicts another virion-sense ORF (βV1) in the DNA β associated with CLCuD and AYVV (Saunders et al., 2000). This chapter describes the functional analysis of βC1 and βV1. Evidence is presented implicating βC1 ORF as the pathogenicity determinant of DNA β as it causes disease-like symptoms when expressed in transgenic plants. A single polyadenlated transcript encoding this ORF was identified and mapped on the DNA β satellite.

3.2 Material and Methods

3.2.1 Construction of clones

A full-length *Kpn*I monomeric DNA β was released from plasmid pCLCβ02 (Briddon et al. 2001) and ligated at the *Kpn*I site of pBluescript II SK+ (Stratagene, La Jolla, CA) in both virion-sense and complementary-sense orientations, to produce pBS-β to use as s template for synthesis of strand-specific probes.

A 280 bp *Sna*BI/*Kpn*I fragment of DNA β was cloned into the binary transformation vector pBin20 (Hennegan and Danna, 1998) to produce pBin-0.2β. An infectious head-to-tail 1.2-mer of DNA β was constructed by ligating the *Kpn*I monomer into pBin-0.2β to produce pBin-1.2β that contains a single copy of the βC1

and βV1 ORFs (Figure 3.1B). The identity of clones was confirmed by restriction analysis. Agroinfectious constructs of ToLCV (Dry et al., 1993) and CLCuMV (Briddon et al., 2001) have been described previously.

3.2.2 Mutagenesis of C1 and V1 ORFs of DNA β

Site-directed mutagenesis of βC1 was carried out by PCR using two flanking oligonucleotide primers (P1-V and P3-C; Table 3.1) to amplify a 290 bp fragment (nt 195 to nt 484; Figure 3.1B). The mutant fragment containing a premature stop codon at amino acid (aa) position 41 was digested with *Sna*BI and *Bgl*II and the resulting 252 bp DNA was introduced into pBS-β clone from which the corresponding wild-type (WT) fragment had been removed, to produce pBS-βC1Mut1. Another stop codon was introduced at codon position 9 by amplifying a 93 bp fragment (nt 504 to nt 596, Figure 3.1B) using the flanking oligonucleotides P5-V and P7-C (Table 3.1). This fragment was digested with *Sal*I and *Sph*I and used in PCR as a mutant mega primer along with the P1 primer (Table 3.1) to amplify the 391 bp *Sna*BI/*Sph*I fragment from pBS-βC1Mut1 (Figure 3.1B). The amplified DNA containing two introduced stop codons was digested with *Sna*BI and *Sph*I and introduced into pBS-β from which the corresponding WT fragment had been removed, to produce pBS-βC1Mut2 (Figure 3.1B).

A βV1 frame shift mutant at nt position 586 was produced by digesting the pBS-β with *Sph*I, removing the 3′ overhang by treatment with T4 DNA polymerase followed by blunt end re-ligation to produce pBS-βV1FS. In addition, the ATG initiation codon of the V1 ORF was changed to ATA by PCR using two flanking oligonucleotide primers (P1-V and P4-C; Table 3.1) to amplify a 290 bp DNA (nt 195 to nt 484; Figure 3.1B). This fragment was digested with *Sna*BI and *Bgl*II and the resulting 252 bp DNA was introduced into pBS-βV1FS from which the corresponding

WT sequence had been removed to produce pBS-βV1Mut (Figure 3.1B). All constructs were sequenced to verify the mutations introduced. All mutant constructs that were initially produced as monomer clones were released and ligated into pBin-0.2β and introduced into *A. tumefaciens* strain C58.

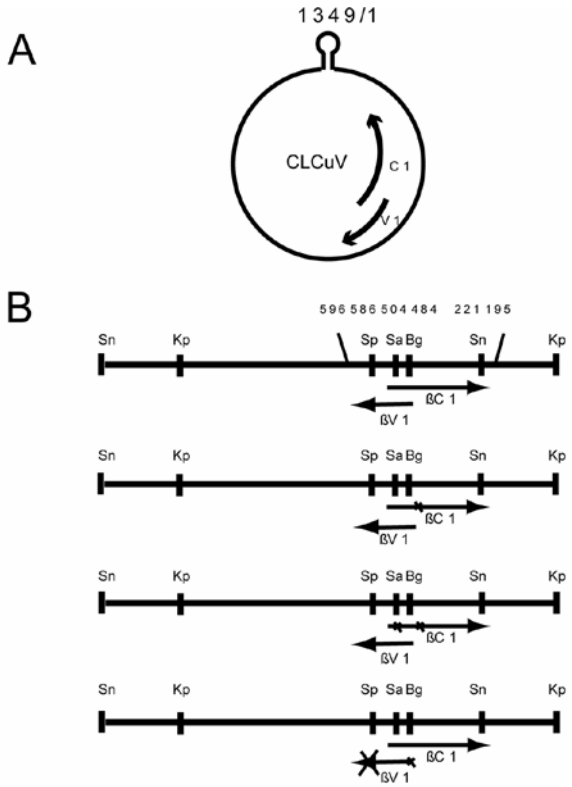

Figure 3.1 Circular (A) and linear (B) maps of CLCuV and its mutant constructs. ORFs are shown by arrows and the position of mutation indicated by a cross (X). Restriction sites are: Sn, SnaBI; Kp, KpnI; Sp, SphI; Sa, SalI and Bg, BglII. Numbering of residues is as in Briddon et al. 2001. Linear map shows the complemmentry-sense strand

Table 3.1: DNA β-specific oligonucleotide primers

Primers[a]	Size (nt)	Nucleotide Position[b]	Sequence (5' to 3')[c,d]
P1-V	24	195-209	GGCAAGCTTTTAAACGGTGAACTT
P2-V	24	472-486	CGACTCGAGATGAAGATCTTCATA
P3-C	60	425-484	TGAAGATCTTCATTCACATGAGGATACTATCCA CAAAGTCACCATCGCTAATC**T**AGTATG
P4-C	23	459-484	TGAAGATCT**T**TATTCACATGAGG
P5-V	33	504-533	AACGTCGACTATGAACCTGACTCCCT**A**CTTGTT
P6-C	24	536-551	TAACTCGAGATGACACCGAGCGGA
P7-C	25	573-596	TCTATAAATGCATGCACACTAAAC
P8-C	24	606-621	ACCTCTAGA*CTA* CACAAAATATCT

[a] V, virion-sense; C, complementary sense

[b] Nucleotide position of CLCuDβ as in Briddon et al. (2001)

[c] Bold and enlarged characters show mutations

[d] Restriction sites are underlined

3.2.3 Whole-plant infectivity assays

A. tumefaciens cultures harbouring a tandem-repeat construct of either ToLCV in pBin19 or a partial-repeat construct of DNA β in pBin20 were grown for 36-48 h, mixed in equal proportions and inoculated into the axillary buds of *Nicotiana tabacum* plants as described previously (Rigden et al., 1996). Developing leaves were sampled 21-28 days after inoculation. The presence of viral DNAs was detected by dot blot analysis using the full length ^{32}P-labelled ToLCV or DNA β probes as described (Behjatnia et al. 1996).

3.2.4 Plant transformation

The entire βC1 ORF was amplified from clone pBS-β with oligonucleotides P1-V and P6-C (Table 3.1). The 363 bp PCR product was digested with *Xho*I and *Hin*dIII and cloned into the plant expression vector pART7 (Gleave, 1992), to produce pART7-βC1.

The expression cassette containing the *Cauliflower mosaic virus* (CaMV) 35S promoter, βC1 ORF and the octopine synthase gene *(ocs)* 3′-untranslated region, was released by digestion with *Not*I and ligated into the binary transformation vector pART27 (Gleave, 1992), to produce pART27-βC1. pART27-βC1Mut, containing a mutant βC1 was produced similarly using clone pBS-βC1Mut2 as the DNA template in PCR. The integrity of the βC1 and βC1Mut ORFs was confirmed by sequencing.

A head-to-tail dimeric clone of DNA β was obtained directly using a 10:1 molar ratio of insert to vector (pBS SK+) in the ligation reaction to produce pBS-2β. This construct was cloned into the *Hin*dIII site of the binary vector pBin19 to produce pBin-2β.

The pART27-βC1, pART27-βC1Mut and pBin-2β constructs were introduced into *A. tumefaciens* strain LBA4404 by electroporation. Tobacco (*N. tabacum* cv. *Samsun*) was transformed with the constructs as described (Horsch et al., 1985, chapter 2). Transgenic shoots (2β, 35S:βC1 and 35S:βC1Mut) were selected on 0.7% bacto-agar plates containing culture medium (0.43% MS salts, 3% sucrose, 0.01% Gamborg vitamins) supplemented with 250 μg ml^{-1} kanamycin and grown at 25 to 27 ^{o}C under artificial light (150μE . s^{-1} . m^{-2}) with a 16 hour photoperiod. Transgenic plants were transferred to potting soil and maintained in the glasshouse under natural light at 25 to 30 ^{o}C.

3.2.5 Northern blot analysis of RNA from transgenic plants

RNA was isolated from 2β, 35S:βC1 and 35S:βC1Mut transgenic lines using Plant RNA Reagent (Invitrogen, Carlsbad, CA). Samples of RNA (20 μg) were electrophoresed in a 1.5% formaldehyde agarose gel and blotted onto nylon membrane (BioRad, Hercules, CA). Blotted RNA was hybridized overnight at 68 ^{o}C (Sambrook &

Russell, 2001) with strand-specific ^{32}P UTP-labelled full-length DNA β probes produced using a T7 RNA polymerase kit (Promega, Madison, WI) and the full-length DNA β DNA template in pBS (pBS-β).

3.2.6 Mapping the termini of the DNA β transcript

The 3′ end of DNA β-encoded RNA was mapped using the 3′ RACE procedure (Frohman et al., 1988). Poly A^+ template RNA was prepared from a 2β transgenic line using mRNA Isolation Systems (Promega, Madison, WI). First strand cDNA was synthesized using an oligo-dT primer. The cDNA was used as a template in PCR with oligo-dT primer and a gene-specific primer (P2-V or P6-C; Table 3.1) to amplify either βC1 or βV1 putative transcripts, using One Step RT-PCR System (Invitrogen, Carlsbad, CA). The product was cloned into the pGem-T Easy Vector (Promega, Madison, WI) and sequenced. Similarly, the 5′ end of DNA β-encoded RNA was mapped using the 5′ RACE procedure (Frohman et al., 1988). First strand cDNA was synthesized by reverse transcriptase using oligo-dT primer (Invitrogen, Carlsbad, CA) and tailed with dC using terminal transferase. An oligo-dG primer and the gene specific primer for either the βC1 or βV1 ORF (P1-V or P8-C; Table 3.1) were used to amplify the cDNA. The DNA product was cloned into the pGem-T Easy Vector (Promega, Madison, WI) and sequenced.

3.3 Results

3.3.1 ToLCV can transreplicate DNA β

Inoculation of tobacco plants with a ToLCV infectious clone produced a mild mosaic (Figure 3.2B) 18-21 days post inoculation (dpi), while inoculation with CLCuMV did not produce any symptoms. Co-agroinoculation of tobacco with DNA β and either ToLCV or CLCuMV caused more severe symptoms indistinguishable from each other.

The symptoms were downward curling of leaf margins, swelling and darkening of the veins and the formation of small enations on the veins (Figure 3.2C). Dot blot analysis of DNA extracted from the inoculated plants revealed that CLCuMV replicated in tobacco but did not produce symptoms. Furthermore DNA β could replicate in tobacco in the presence of either CLCuMV or ToLCV (results not shown). The frequency of co-infection of DNA β with CLCuMV in tobacco plants was low (3/30), compared to co-infection with ToLCV (10/10). Therefore in subsequent experiments ToLCV was used as the helper virus.

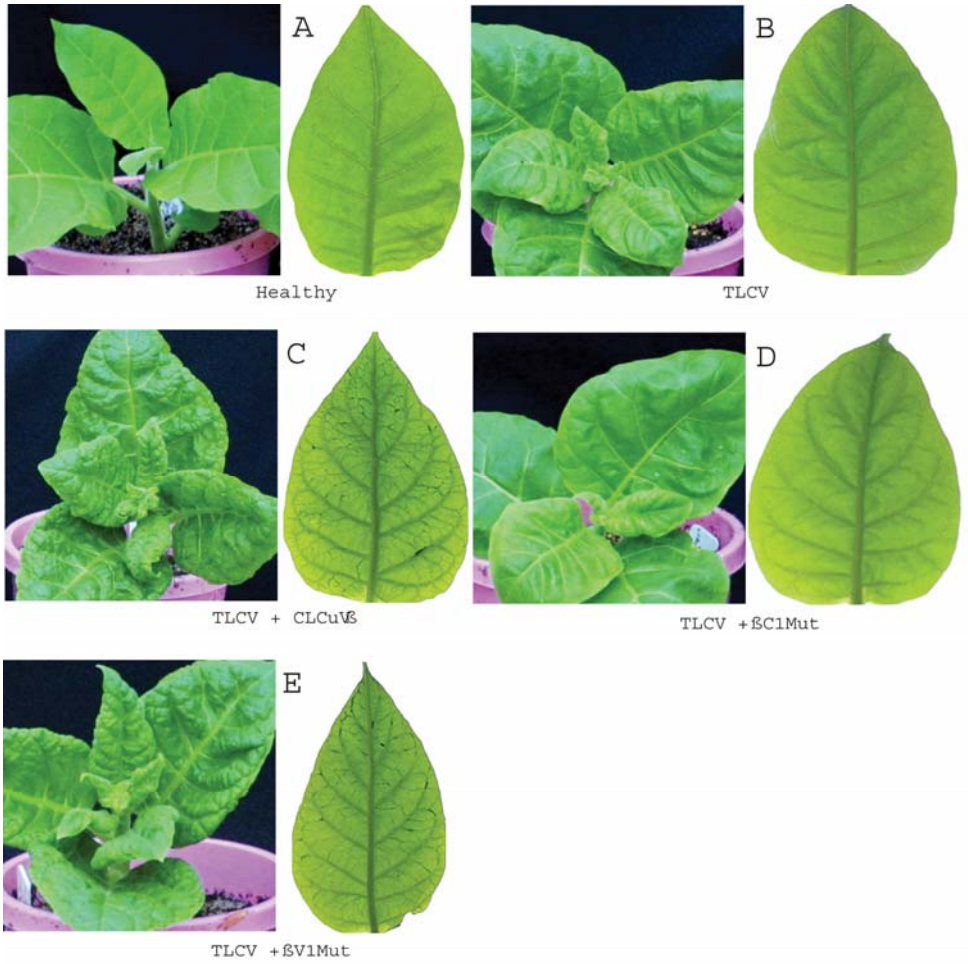

Figure 3.1. Disruption of the ßC1 ORF eliminates the DNA ß associated phenotype. (A) Uninfected tobacco plant. (B) Mild mosaic in tobacco plants agroinoculated with TLCV and (D) TLCV plus C1 mutant compared to characteristic symptoms in plants co-agroinoculated with TLCV and either (C) CLCuVß or (E) V1 mutant . The leaves photographed with transmitted light are shown to highlight CLCuVß associated vein-swelling phenotype.

3.3.2 Disruption of βC1 ORF eliminates the DNA β associated phenotype

Tobacco plants (9/9) co-agroinoculated with ToLCV and either pBin-βC1Mut1 or pBin-βC1Mut2 (Figure 3.1D) developed typical ToLCV symptoms at 18-21 dpi. The same number of plants co-agroinoculated with ToLCV and WT DNA β (pBin-1.2β construct) showed the more severe symptoms characteristic of DNA β co-infection (Figure 3.2C). These symptoms were indistinguishable from those plants co-inoculated with ToLCV and pBin-βV1Mut (Figure 3.2E and 3.1B), indicating that the βV1 does not have a role in symptom severity. Dot blot analysis of DNA extracted from inoculated plants showed the presence of DNA β in all plants, irrespective of whether they were inoculated with WT or mutant DNA β construct. Moreover, the plants inoculated with ToLCV together with either WT type or mutant DNA β showed comparable levels of viral DNA accumulation. These results indicated that the βC1 ORF is required for inducing severe disease symptoms in *N. tabacum* and that βC1 ORF does not encode a product essential for DNA β replication.

3.3.3 Expression of the βC1 gene in transgenic plants results in growth abnormalities

Transgenic tobacco plants were generated to assess the effects of βC1 expression under the control of either its own putative promoter or the CaMV 35S promoter. During transformation, it was observed that some of the shoots generated were severely deformed and failed to develop into plantlets. Regeneration frequency from the 35S:βC1 construct (pART27-βC1) was extremely low and only two transgenic lines could be recovered after a tenfold increase in the number of explants for transformation. One of the 35S:βC1 transgenic lines exhibited a severe phenotype. The plant was stunted and developed deformed leaves that were extremely small in size and had a rigid appearance (Figure 3.3A). Another 35S:βC1 transgenic line showed less severe effects,

grew to maturity and produced seeds. In this transgenic line some leaves showed vein swelling in small patches on the lower side of the leaves that was similar to the vein-swelling symptom produced in tobacco and cotton plants co-infected with CLCuMV and DNA β.

Figure 3.3 Transgenic tobacco plants containing 35S:βC1 and 2β displayed growth abnormalities. Examples of 35S:βC1, 2β, and 35S:βC1 is shown.

Tobacco plants were also transformed with a tandem repeat of DNA β. The pBin-2β construct was designed to derive βC1 ORF expression by the putative promoter present in the DNA β satellite. As the linear transgene, carrying tandem repeat of DNA β, contains an un-interrupted unit length copy of DNA β, it is anticipated that expression from this linear template would be similar to that from the circular satellite DNA. The frequency of transformation with the pBin-2β construct was higher and eight transgenic lines were recovered. The majority of these plants (six out of eight) showed a severe phenotype (Figure 3.3B). They grew slowly, did not produce flowers, and their leaves were rigid and elongated. One of the transgenic lines showed a less severe phenotype, grew to maturity and produced seeds. Several transgenic lines carrying the 35S:βC1Mut (pART27-βC1Mut) construct were also produced, and all appeared normal

(Figure 3.3C). These results confirmed that the βC1 ORF product is responsible for the induction of a virus-like phenotype in the transgenic tobacco plants.

3.3.4 Replicating DNA β rescued from a dimeric β transgenic plant following ToLCV infection

Two independent 2β transgenic tobacco lines were clonally propagated and three plants of each line were inoculated with ToLCV. Southern blot analysis of DNA from the ToLCV-infected 2β transgenic tobacco plants at 21 dpi revealed the presence of ToLCV replicating DNA and DNA β (Figure 3.4A) of the same size as present in non-transgenic tobacco plants co-inoculated with ToLCV and DNA β. No replicating form of DNA β was observed in non-infected 2β transgenic plants (Figure 3.4A). These results indicate that replicating DNA β was released from the transgenic plant chromosome and was amplified in the presence of ToLCV.

After ToLCV-infection, the 2β plants appeared to undergo a change of phenotype (Figure 3.4B). Three weeks post inoculation the transgene-associated phenotype began to diminish in severity and within 10 weeks post inoculation the symptoms of these plants were similar to the viral symptoms of non-transgenic tobacco plants co-inoculated with ToLCV and DNA β. In contrast, there was no noticeable change of phenotype in the control non-infected 2β plants. This observation suggested that the presence of replicating DNA β in 2β plants lead to a remission of the βC1 phenotype.

ToLCV-infected and non-infected T_0 plants of one of the two 2β transgenic tobacco lines that produced seeds were selected for further studies. The seeds were germinated on MS salts culture medium containing kanamycin. It was observed that progeny T_1 plants from non-infected 2β transgenic tobacco showed the parental

transgene phenotype, whereas T_1 progeny from ToLCV infected 2β plants produced the normal phenotype of non-transgenic healthy plants (Figure 3.5). The progeny plants lacked ToLCV infection, as the virus is not seed-transmitted (Seemanpillai et al. 2003). These results raised the possibility that the βC1 transgene expression was silenced by the replicating DNA β. A similar heritable silencing has been reported for ToLCV infected transgenic plants carrying GUS ORF driven by the ToLCV promoters (Seemanpillai et al. 2003).

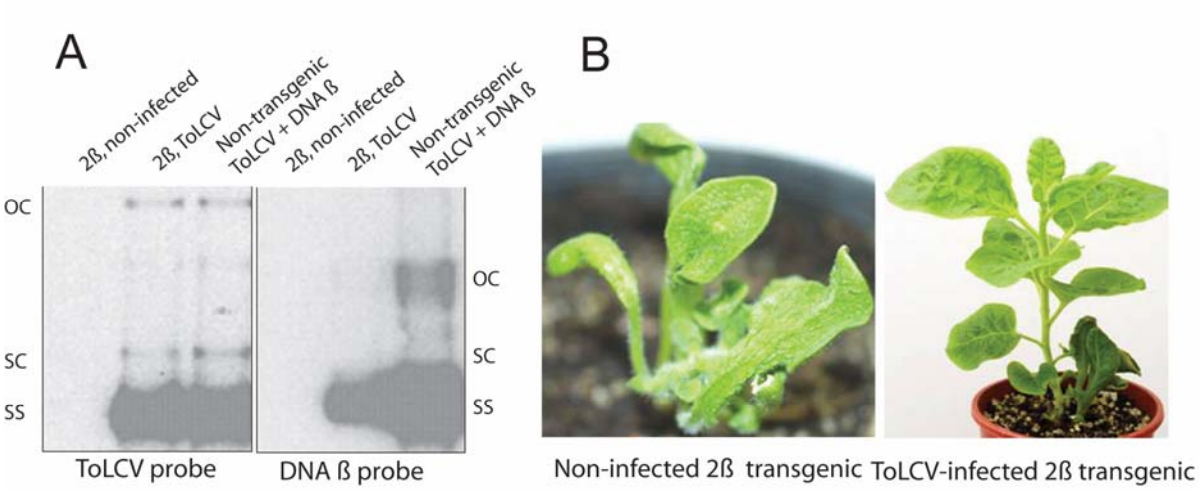

Figure 3.4 Replicating DNA β rescued from a dimeric β transgenic plant following ToLCV infection. (A) Southern blot analysis showing replicating ToLCV and DNA β in the transgenic plants infected with ToLCV. DNA sample from a 2β transgenic line is shown which is representative of the two 2β transgenic lines analysed. The blots were hybridised with either the ToLCV or DNA β probe. The position of single-stranded (SS), super coiled (SC), and open-circular (OC) DNA forms are indicated. (B) Non-infected transgenic plant shows severe phenotype compared to milder symptoms in ToLCV-infected transgenic plant.

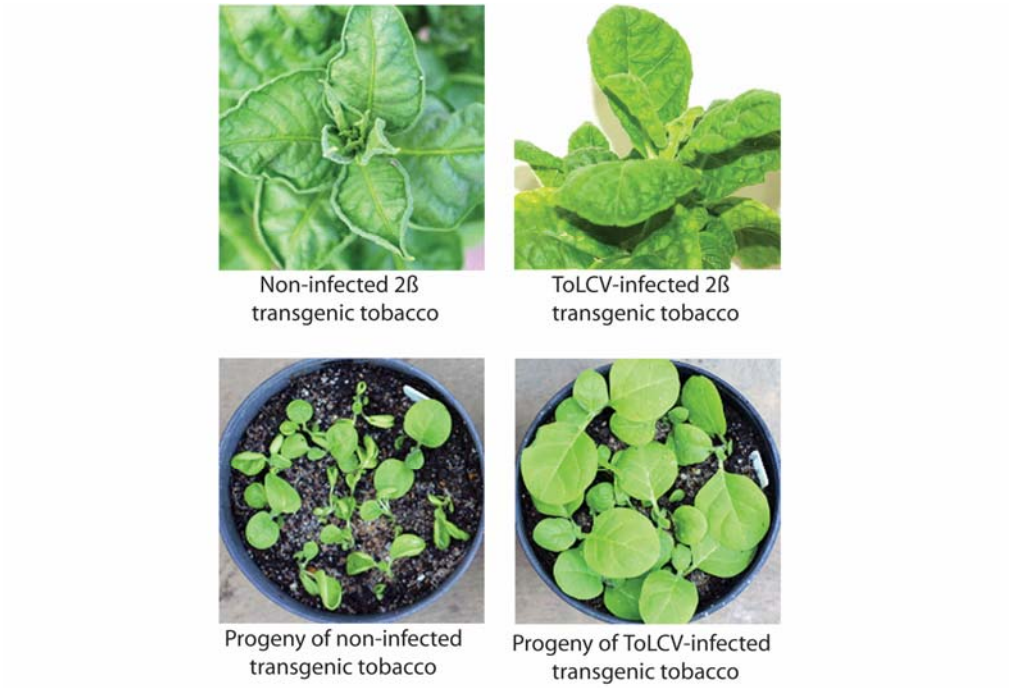

Figure 3.5 Phenotypic remission of ToLCV infected 2β transgenic plants was heritable. Progeny of a non-infected 2β transgenic plant showed inheritance of abnormal phenotype, whereas the progeny of ToLCV infected 2β transgenic plant showed a normal phenotype.

3.3.5 A single transcript encompasses the βC1 ORF

Using a strand-specific probe for the full-length DNA β molecule, northern blot analysis of the 35S:βC1 line showing a severe phenotype and four 35S:βC1Mut transgenic lines, revealed the presence of two complementary-sense transcripts of approximately 400 and 500 nt in size. In contrast, a single transcript of approximately 400 nt was detected in each of the two 2β transgenic plants tested (Figure 3.6). No transcript was detected with a plus strand probe, even after prolonged exposure of the blot to X-ray film (results not shown). There was a significant difference in the level of βC1 mRNA accumulation in the transgenic plants containing the three constructs. The highest level of RNA accumulation was in plants expressing mutated βC1 under the control of CaMV 35S promoter, followed by those containing the 2β transgene and WT βC1 driven by the CaMV 35S promoter (Figure 3.6). These results indicate that DNA β contains a

transcription unit whose product has a similar size and function to the βC1 transcript expressed by the CaMV 35S promoter.

Amplification of the cDNA corresponding to the 5′ region of the βC1 ORF by 5′-RACE from a 2β transgenic line gave a DNA product of about 400 bp. Sequencing of seven independent clones of this cDNA revealed two clusters of 5′ ends at the DNA β coordinates 559 and 563 (Figure 3.7). A promoter element TATAAAT was identified at nt position 588-594, 43 nt upstream of the start codon. Amplification of cDNA corresponding to the 3′ region of the βC1 ORF produced a DNA product of about 400 bp. Sequencing of five independent cDNA clones revealed 3′ ends at DNA β coordinates 186 and 190 (Figure 3.7). A polyadenylation signal AATAAA was found within the βC1 coding region at nt 209 to nt 214, 18 nt upstream of the stop codon. The position of the DNA β-encoded transcript determined in this study and the potential regulatory elements are summarized in Figure 3.7. Attempts were made for the RACE PCR using the βV1 specific primers, but no consistent DNA products were amplified. These results indicate that DNA β encodes a functional transcript that contains the βC1 ORF.

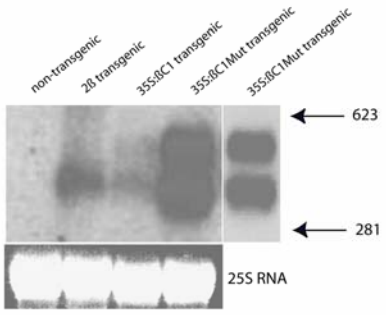

Figure 3.6 Northern blot analysis of DNA β transcripts in plants transformed with either a dimeric β construct (2β transgene), βC1 (35S:βC1 transgene) or βC1 mutant (35S:βC1Mut transgene). The 25S rRNA band following ethidium bromide staining indicates the relative quantities of RNA analysed. The positions of RNA markers are indicated. A shorter exposure for RNA sample from the 35S:βC1Mut transgenic plant is shown to clearly illustrate the presence of two transcripts.

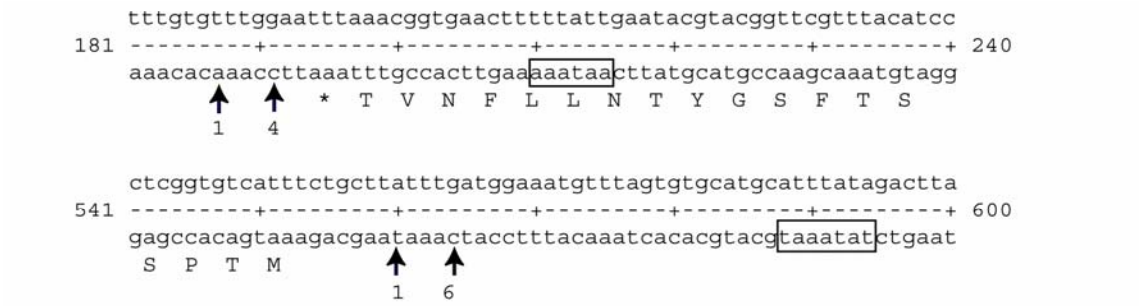

Figure 3. 7 Mapping of 3' and 5' ends of the ßC1 transcript. The nucleotide sequence of CLCuV between coordinates 181-240 and 541-600 and the C1 amino acid sequence in these regions are shown. The beginning of the C1 ORF is indicated by an arrow (↑) and the end by a star (*). Putative promoter TATA element and polyadenylation signal are boxed. The position and number of independent RACE clones mapping to the 3' and 5' termini are indicated↑.

3.4 Discussion

The DNA β satellite associated with cotton leaf curl disease has been shown to be supported for replication by four distinct cotton-infecting monopartite begomoviruses (Mansoor et al., 2003). This study showed that DNA β can also use ToLCV as a helper virus. Like the ToLCV sat-DNA, the DNA β satellite appears to be capable of using the Reps from a diverse range of begomoviruses (Lin et al., 2003). Given the high specificity of the geminiviral origin of DNA replication for the cognate Rep protein (Fontes et al., 1994), it remains unclear how the geminivirus DNA satellite can achieve such promiscuity.

Comparison of 28 DNA β species has shown that the position and size of the βC1 ORF is conserved (Briddon et al., 2003). Furthermore, a potential virion-sense ORF (βV1) encoded by CLCuD and AYVV DNA βs has been predicted (Saunders et al., 2000). Therefore attempts were made to analyse the possible role of both ORFs.

In this study tobacco used as the host plant for mutagenesis studies. Cotton, the natural host of DNA β was symptomless when inoculated with CLCuMV alone but showed drastic symptoms when co-inoculated with DNA β (Briddon et al. 2001). However, inoculation efficiency of CLCuMV in cotton was extremely low and it was impractical for this study. *N. benthamiana* was also tested but showed a severe symptom when inoculated with CLCuMV with or without DNA β.

Evidence for the involvement of the βC1 ORF in modulation of symptom expression has been provided before. Zhou et al. (2003) characterized a few DNA β species associated with tomato and tobacco-infecting begomoviruses and found that in-frame mutation of the βC1 initiation codon resulted in loss of symptom severity in *N. benthamiana*. They raised the possibility that mild symptoms could be attributed to leaky expression of the βC1 gene from an in-frame start codon. Recently Saunders et al. (2004) have demonstrated that disruption of βC1 ORF prevented infection of the AYVV-satellite complex in ageratum and altered their phenotype in *N. benthamiana* to that produced by AYVV alone. Infectivity of two βC1 mutant constructs was tested, one with a stop codon at aa position 41, another carrying two stop codons at positions 9 and 41 and found that both resulted in loss of pathogenicity in tobacco plants when co-inoculated with ToLCV. These results indicate that the βC1 ORF is involved in pathogenicity and that the expression of its N-terminal 40 aa is not sufficient for its function. In contrast to the βC1 ORF, DNA β carrying a mutation in the putative βV1 ORF produced a disease-like phenotype in tobacco plants indistinguishable from the WT DNA β when co-inoculated with ToLCV. Therefore the βV1 ORF has no apparent role in determining symptom severity in tobacco.

The observation of virus-like phenotypes in tobacco plants expressing βC1 either under the control of the CaMV 35S promoter or from a DNA β promoter, was consistent with the results of βC1 mutagenesis. In contrast, the transgenic plants

generated from mutant βC1 were indistinguishable from non-transgenic plants. These results confirm that βC1 is responsible for pathological symptoms associated with DNA β infections. In similar studies Saunders et al (2004) and Cui et al (2004) transformed *N. benthamiana* plants with the DNA β and observed severe developmental abnormalities, vein-greening, and cell proliferation in the vascular bundles. The strong phenotype exhibited by βC1 protein expression therefore appears to be a common feature of DNA β satellites.

The virus-like phenotype developed by βC1 transgenic plants were more severe than viral symptoms produced in non-transgenic tobacco plants co-infected with ToLCV and DNA β. A possible explanation for this is that the βC1 transgene is expressed in all tissues, whereas its expression is limited to phloem cells during the normal ToLCV infection, where the virus is localized (Rasheed et al., 2006). Another explanation is that the βC1 may be down-regulated by viral-encoded proteins during normal infection (Hanley-Bowdoin et al., 1999), hence not resulting in the drastic effects observed in transgenic plants. As yet no experimental evidence for the possible regulation of βC1 is available.

Seemanpillai et al (2003) reported transcriptional silencing of geminiviral promoter-driven transgenes following homologous virus infection. Consistent with these results, the severe virus-like phenotype exhibited by 2β transgenic tobacco plants was reduced following inoculation with ToLCV, to symptoms typical of those produced by co-inoculation with ToLCV and DNA β in non-transgenic tobacco plants. While the methylation status of the 2β trasngene or the fate of the βC1 transcript was were not studied, the silencing effect and its heritability closely resembled the ToLCV induced silencing of GUS transgenes driven by the viral promoters that involve transgene methylation (Seemanpillai et al. 2003).

Geminiviral genes conferring disease-like phenotypes on transgenic plants have been reported before. The BC1 (MP) ORFs of a number of bipartite begomoviruses have been implicated in symptom development and their expression in their respective hosts produced phenotypes mimicking viral disease symptoms (Pascal et al., 1993). The C4 ORF of curtovirus BCTV (Stanley & Latham, 1992) and monopartite begomovirus ToLCV (Rigden et al., 1994) has been implicated as a determinant of symptom severity. Expression of C4 protein of these viruses under the control of the CaMV 35S promoter produced virus-like symptoms in transgenic plants (Latham et al., 1997). βC1 is another geminivirus encoded gene that confers a virus-like phenotype on transgenic plants. The exact roles of these proteins in geminiviral pathogenesis remain unknown.

Northern blot analysis of transgenic lines expressing WT and mutant βC1 revealed the existence of two βC1 mRNA transcripts of approximately 400 and 500 nt in size. No plus-strand transcript of DNA β was detected. The lack of virion-sense transcript is consistent with the finding that the virion sense ORF (βV1) is not involved in either DNA β replication or in symptom expression. A single transcript was found in the dimeric DNA β transgenic plants analysed. In addition to a transcript of similar size, a longer transcript was also found in plants expressing either WT βC1 or mutant βC1 under the control of the CaMV 35S promoter. A possible reason for this is the presence of two polyadenylation signals in the 35S:βC1construct, one in the βC1 (Figure 3.7) and another in the termination sequence of the expression cassette.

Significant differences were observed in the levels of βC1 mRNA accumulation among the plants transformed with WT βC1 and mutant βC1. The level of expression in mutated βC1 transgenic plants was several-fold higher compared to the WT βC1 transgenic plants (Figure 3.6). In studies with *Squash leaf curl virus* (SqLCV) BC1/MP

expressing transgenic plants (Pascal et al. 1993) and with ToLCV C4 expressing transgenic plants (Krake et al. 1998), comparable levels of accumulation of WT and mutant ORFs expressed from the CaMV 35S promoter were observed. It is possible that constitutive expression of βC1 from the CaMV 35S promoter is lethal to tobacco plants. This is consistent with the extremely low frequency of regeneration of 35S:βC1 transgenic plants observed here. In this regard, βC1 resembles the Rep genes (C1) of ToLCV (Dry et al., 2000) or the closely related *Begomovirus*, TYLCV (Bendahmane & Gronenborn, 1997) whose expression in transgenic plants has not been attained, possibly because Rep induces a hypersensitive response (Selth et al., 2004).

The βC1 transcript identified has a promoter TATA element (Breathnach & Chambon, 1981), 43 nt upstream of the putative start codon. The presence of two distinct 5' ends in close proximity to a putative promoter element has been observed previously in transcripts of plant and geminivirus genes (Baulcombe et al., 1987; Mullineaux et al., 1993). Mapping of the 3' end also revealed two distinct 3' terminus. The 3' region of the βC1 mRNA contained a consensus polyadenylation signal (Figure 3.7; Joshi, 1987). In contrast results, the 3' ends of transcripts encoded by AYVVβ mapped uncharacteristically to several different locations downstream of the putative termination codon (Saunders et al. 2004).

In conclusion, DNA β encodes an ORF, βC1, which modulates geminiviral symptom severity. The mechanism by which βC1 induces disease symptoms remains unknown and may include changes in virus spread, replication of helper virus or specific interaction with host proteins.

Chapter 4 - A begomovirus DNA satellite encoded βC1 protein provides the DNA B encoded movement functions for infection

4.1 Introduction

Specific small circular single-stranded DNA satellites containing a single open reading frame (ORF), termed DNA β, have recently been found in association with certain monopartite begomovirus infections. They comprise about 1350 nucleotides and require a helper begomovirus for replication and encapsidation (Mansoor et al., 2006). DNA β contributes to the production of symptoms and enhanced helper virus accumulation in certain hosts. Briddon & Stanley (2006) have reported that the DNA β encoded protein, βC1, is the determinant of both pathogencity and suppression of gene silencing. This chapter shows βC1 also has a role in movement of the helper virus. Evidences provided to show that DNA β satellite associated with CLCuD can replace the movement functions of the DNA B of a bipartite begomovirus.

4.2 Materials and methods

4.2.1 Infectious clones and transient expression constructs

Infectious clones of CLCuD DNA β, DNA A and DNA B of ToLCNDV, and transient expression constructs of the *βC1* gene and the ToLCV encoded *C4* gene under the transcriptional control of 35S CaMV promoter have been described (Briddon et al., 2001, Krake et al., 1998, Padidam et al., 1995; Saeed et al., 2005).

4.2.2 Inoculation of plant and analysis of viral DNA

Tomato plants were inoculated by biolistic bombardment with partial tandem dimers of ToLCNDV DNA A in pBluescript SK (-), ToLCNDV DNA B in pGem-7zf (+) (Padidam et al., 1995), DNA β in pBluescript SK (+), 35S:βC1 in pART7 (Saeed et. al.,

2005), and 35S:C4 in pJit163 (Briddon et al., 2001, Krake et al., 1998, Padidam et al., 1995, Saeed et al., 2005). DNA was deposited onto gold particles (0.5 µg DNA and 250 µg gold particles per plant) (Selth et al., 2004). After bombardment, the plants were grown at 25^{o}C to 27^{o}C under artificial light (150µE . s^{-1} . m^{-2}) with a 16 hour photoperiod.

Viral DNAs were detected by dot blot or Southern blot analysis using ^{32}P-labelled DNA probes to DNA A (*Eco*RV and *Pst*I fragment, nt 307-2113), DNA B (*Spe*I and *Pst*I fragment, nt 370-2068), and DNA β (*Kpn*I monomer) were prepared as described previously (Dry et al. 1993). Plants inoculated with DNA A alone were tested by PCR using DNA A specific primers (798V: 5′-GCATCGTGATCGTTATCAAGTCTTA -3′ and 1838RC: 5′-TGATCTGAGCCCAAAGGTCTATA-3′).

Graft transmission was done by top grafting (Crete et al., 2001).The complete nucleotide sequence of ToLCNDV DNA A in the systemically infected plants was verified using the BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA).

4.2.3 Detection of βC1 and C4 mRNA by reverse transcription-PCR

Total RNA from tomato leaf was prepared using Plant RNA Reagent (Invitrogen, Carlsbad, CA). First strand cDNA was synthesized using an oligo-dT primer. The cDNA was used as a template in PCR with oligo-dT primer and a gene-specific primer (βC1:5′-TAACTCGAGATGACACCGAGCGGA-3′ or ToLCV-C4:5′-TAACTCGAGA TGAGAATGGGGAGC-3’) to amplify either βC1 or C4 putative transcripts using the RT-PCR System (Invitrogen, Carlsbad, CA).

4.2.4 Subcellular localization of GFP fusion proteins

pART7-N´gfp, a variant of the shuttle vector pART7 containing the GFP ORF without an initiation codon down stream of the multiple cloning site (Selth et al., 2005), was used to transiently express βC1:GFP fusion proteins in onion (*Allium cepa*) and tobacco (*Nicotiana tabacum*) tissue. Full-length βC1 was amplified from the clone pBS-β (Saeed et al., 2005) with primer pair P01 (5'-TAACT CGAGATGACACCGAGCGGA-3')/ and P02 (5'-GCTCTAGAAACGGTGAACTTTTTAT-3'). The amplified product was digested with *Xho*I and *Xba*I and cloned into similarly digested pART7-N´gfp. This generated N-terminal fusions with GFP. pART7-ATG:GFP which expresses free GFP, and pBI121-H2B:YFP, which expresses Arabidopsis Histone 2B fused to the GFP yellow variant YFP (Boisnard-Lorig et al., 2001), were used as controls. Fully expanded *Nicotiana tabacum* leaves were cut into 1.5 X 1.5 cm squares and kept on moistened filter paper in Petri dishes. Plasmid DNA was delivered by biolistic bombardment (Selth et al., 2004). Tissue pieces were kept in the dark for 24 h and GFP expression was visualized using a Bio-Rad Radiance 2100 confocal laser scanning microscope system (Biorad, Hercules, CA, USA). Endoplasmic reticulum was identified with aqueous rhodamine B hexyl ester stain (100 µg/ml) (Molecular Probes, Carlsbad, CA). Tobacco leaves bombarded with βC1:GFP construct were cut into pieces of 0.5x0.5 cm squares and kept on filter paper moistened with rhodamine B for 30 minutes. Excitation wavelengths of 488 nm and 543 nm were used for GFP and rhodamine B, respectively. Images were corrected for brightness and contrast and further manipulated with Photoshop (Adobe Systems, Mountain View, CA).

4.3 Results

4.3.1 DNA β satellite can substitute for DNA B of a bipartite virus for infection

ToLCNDV is a bipartite begomovirus that requires both DNA components for systemic infection (Padidam et al., 1995). Twenty tomato plants inoculated with DNA A and

DNA B developed downward leaf curling 6-7 days post inoculation (dpi) and by 21 dpi showed pronounced reduction in height and leaf size. In contrast, fifty tomato plants inoculated with DNA A alone remained symptomless (Figure 4.1A and 4.IB, Table 4.1). The plants inoculated with the DNA A alone lacked detectable levels of DNA A in the distal leaves when tested by dot blot hybridization (data not shown). Twelve of the DNA A inoculated plants were further analysed by Southern blotting. The inoculated leaves accumulated replicative forms of DNA A (Figure 4.2A, lane 1) but no DNA A could be detected in the newly developing leaves (lane 2). When leaf samples were tested by PCR for accumulation of DNA A, no products were obtained from extracts of distal leaves, while products of the expected size were obtained from inoculated leaves (Figure 4.2C). Thus ToLCNDV DNA A alone was able to replicate at the site of bombardment but did not spread systemically.

To test whether a DNA β satellite could influence the pattern of DNA A infection, fifty tomato plants were inoculated with a mixture of DNA A and the DNA β associated with CLCuD (Briddon et al., 2001). Eleven plants showed leaf curling 13-16 dpi and by 28 dpi had developed pronounced leaf curling, stunting, vein thickening and enations (Figure 4.1C and 4.1C1, Table 4.1). New leaves of all fifty inoculated plants were tested by dot blot analysis. The new symptomatic leaves of the eleven plants were found to contain both DNA A and DNA β, whereas non-symptomatic plants lacked a detectable level of either DNA (data not shown). Southern blot hybridization confirmed the presence of replicative forms of both DNA A and DNA β in the distal leaves of these plants (Figure 4.2A and 4.2C, lanes 5-6). However, the level of viral DNA was lower in these plants compared to the level of viral DNA in the plants infected with DNA A and DNA B (lanes 3-6). These results indicated that the DNA β can mobilize ToLCNDV DNA A from sites of bombardment to the distal tissues, presumably by providing the movement functions normally provided by DNA B.

Table 4.1

Infectivity analysis of *Tomato leaf curl New Delhi virus* DNA A showing that DNA β satellite, or the transient expression of either *βC1* or *Tomato leaf curl virus* encoded *C4* gene mediates its systemic infection in tomato plants.

Inoculum	Infectivity (infected*/inoculated) Experiment 1	2	3	Total	Symptom type
DNA A	0/30	0/30	0/40	0/100	None
DNA A+ DNA B	14/14	3/3	3/3	20/20	Severe leaf curling
DNA A+ DNA β	5/18	4/16	2/16	11/50	Severe leaf curling
DNA A+ DNA βMut	0/20	0/15	0/15	0/50	None
DNA A+ 35S:βC1	2/18	3/16	2/16	7/50	Mild leaf curling
DNA A+ 35S:βC1Mut	0/18	0/16	0/16	0/50	None
DNA A + 35S:C4	2/18	1/16	1/16	4/50	Mild leaf curling
DNA A + 35S:C4Mut	0/18	0/16	0/16	0/50	None

* Infected plants identified by dot blot hybridization using a ToLCNDV DNA A specific probe

The DNA β associated with CLCuD encodes a pathogenicity protein, βC1 (Saeed et al., 2005). To determine whether this protein was responsible for the spread of DNA A from the site of bombardment, fifty tomato plants were co-bombarded with constructs of DNA A and a DNA β mutant that had two stop codons introduced in the βC1 ORF (Saeed et al., 2005). No symptoms were observed in any of the fifty inoculated plants (Figure 4.1D, Table 4.1). None of these plants accumulated a detectable level of either DNA A or mutant DNA β in systemic leaves when tested by Southern blot hybridization (Figure 4.2A and 4.2C, lane 8). However, DNA A and the mutant DNA β accumulated in the inoculated leaves of twelve plants tested by Southern blotting (lane 7). These results indicate that the ability of DNA β to mediate systemic spread of DNA A was dependent on βC1 expression.

4.3.2 Transient expression of *βC1* gene enhances local accumulation of ToLCNDV DNA A and permits its systemic movement

It has been shown previously that co-inoculation with *Tomato golden mosaic virus* (TGMV) DNA A and transient expression constructs of the movement proteins BC1 and BV1 encoded by TGMV DNA B, under the control of the CaMV 35S promoter, enhances local accumulation of TGMV DNA A and permits its systemic spread (Jeffrey et al., 1996). When tomato plants were co-inoculated with ToLCNDV DNA A and the transient expression construct 35S:βC1, a proportion showed mild leaf curling symptoms at 13-16 dpi (Figure 4.3C, Table 1). Control tomato plants inoculated with either DNA A alone or with a 35S:βC1Mut construct, developed no symptoms (Figure 4.3B).

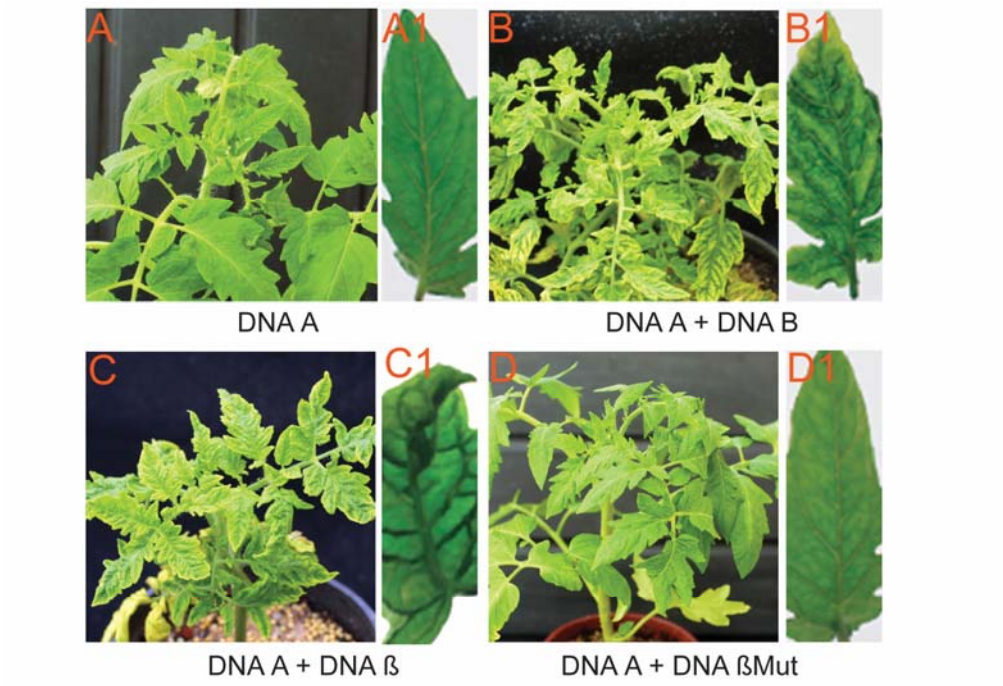

Figure 4.1 DNA can substitute for DNA B of a bipartite begomovirus in producing symp-toms for infection. Lack of symptoms on tomato plants bombarded with ToLCNDV DNA A alone (A) compared with symptomatic plants co-bombarded with either DNA A and DNA B (B) or DNA A and DNA (C). Plants bombarded with DNA A and DNA C1-mutant remain symptomless (D). The excised leaves are shown to highlight DNA associated vein-thickening. Symptoms shown at 28 days post inoculation.

To test whether transient expression of the *βC1* gene could influence the level of accumulation of ToLCNDV DNA A at the site of inoculation, total nucleic acids from tomato leaves which had been co-inoculated with DNA A and the 35S:βC1 construct

were assayed at 13-16 dpi. Higher levels of DNA A accumulated (Figure 4.4A, lanes 3-4) than in leaves inoculated either with the DNA A alone, or with DNA A and the 35S:βC1Mut construct (lanes 1-2 and lanes 5-6). This suggests that localized expression of the *βC1* gene increases local accumulation of ToLCNDV DNA A in a manner analogous to that which occurs with DNA B in the TGMV system.

To test whether transient expression of the *βC1* gene at the site of inoculation could also mediate the systemic movement of ToLCNDV DNA A, newly developed leaves of the inoculated plants were assayed at 13-16 dpi. DNA A accumulated in symptomatic systemic leaves of tomato plants that had been co-inoculated with DNA A and the 35S:βC1 construct (Figure 4.4B, lanes 3-4), whereas systemic leaves of asymptomatic plants lacked detectable levels of DNA A (lane 5-6). The level of DNA A accumulation in these plants was similar to the level observed in the plants infected with DNA A and DNA β (Figure 4.4B, lanes 3-4 and lanes 11-12). These results were reproduced in three independent experiments and there was no evidence for the presence of either DNA B or DNA β in these plants when analysed by Southern hybridization (results not shown). Localized expression of βC1 therefore mediates systemic spread of ToLCNDV DNA A.

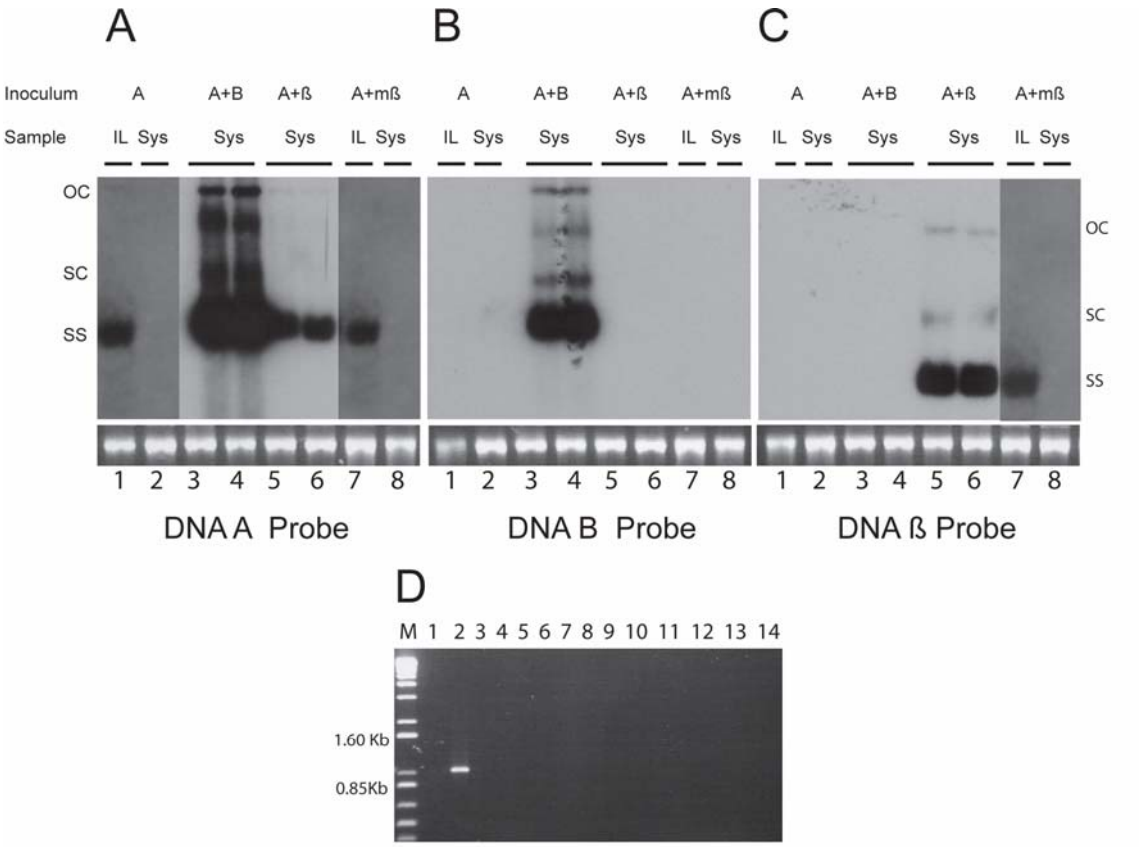

Figure 4.2 Southern blot analysis of plants inoculated with a combination of Tomato leaf curl New Delhi virus (ToLCNDV) DNA A and DNA β. The positions of single-stranded (SS), super coiled (SC), and open-circular (OC) forms are indicated. Each lane was loaded with 5 µg of DNA. Ethidium bromide-stained DNA is shown as loading control. IL; inoculated leaves, Sys; systemically infected leaves. DNA A specific shows PCR (D) from healthy leaf (lane 1), inoculated leaf (lane 2), and newly developed leaves from the tomato plants inoculated with DNA A alone (lanes 3-14). M, DNA size markers.

4.3.3 Transient expression of the ToLCV *C4* gene also enhances local accumulation of ToLCNDV DNA A and permits its systemic movement

The C4 protein encoded by begomoviruses has some functional similarities to the βC1 protein, particularly in pathogencity determination and as a suppressor of host gene silencing (Vanitharani et al., 2005). TLCV is a monopartite begomovirus that requires the C4 ORF for wild-type symptoms (Rigden et al., 1994). We therefore tested whether the localized expression of the TLCV *C4* gene could enhance local accumulation of

ToLCNDV DNA A and mediate its systemic spread using the transient expression system described above. Tomato plants were inoculated with either DNA A alone or together with a transient expression construct of C4. A small proportion of tomato plants inoculated with the infectious construct of DNA A together with the 35S:C4 construct showed mild leaf curling symptoms at 13-16 dpi (Figure 4.3C, Table 1). In contrast, none of the control plants inoculated with DNA A and the 35S:C4Mut construct developed symptoms.

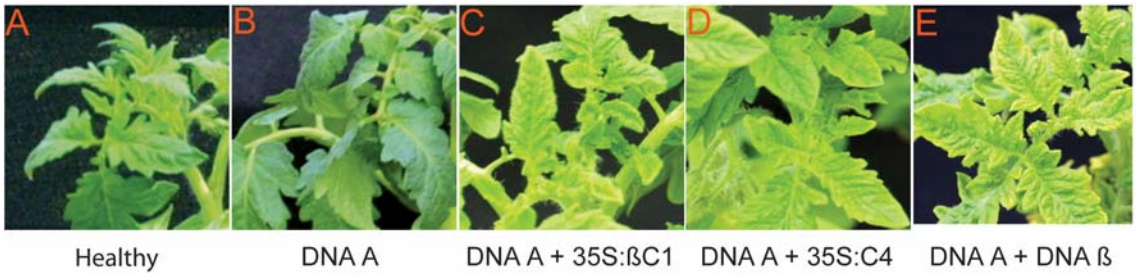

Figure 4.3 Co-bombardment of tomato plants with a construct of ToLCNDV DNA A and transient expression constructs of either βC1 or the C4 ORF induces mild leaf curl symptoms. Non-symptomatic tomato plants bombarded with DNA A alone (B), compared to plants showing mild symptoms induced by co-infection of DNA A with either 35S:βC1 (C) or 35S:C4 (D). A control plant bombarded with DNA A and DNA β (E) is shown for comparison. Symptoms shown at 35 days post inoculation.

To determine whether localized expression of the TLCV *C4* gene could influence the accumulation of ToLCNDV DNA A in inoculated leaves, leaves of tomato plants that were inoculated with either DNA A alone or together with the 35S:C4 construct were analysed by Southern blotting at 13-16 dpi. A noticeably higher level of DNA A was observed when it was co-inoculated with the 35S:C4 construct (Figure 4.4A, lanes 7-8) than in leaves inoculated with the DNA A alone, or DNA A with the 35S:C4Mut construct (lanes 1-2 and lanes 9-10). These results show that localized expression of the *C4* gene enhances the accumulation of ToLCNDV DNA A in inoculated leaves.

To investigate whether transient expression of the *C4* gene at the site of inoculation could also mediate the systemic movement of ToLCNDV DNA A to the new leaves, at 13-16 dpi newly developed leaves of the inoculated plants were tested by Southern blot analysis. DNA A was detected in newly developing leaves of the symptomatic tomato plants that were inoculated with DNA A and the 35S:C4 construct (Figure 4.4B, lanes 7-8), whereas asymptomatic plants lacked detectable levels of DNA A (lane 9-10). The level of systemic DNA A accumulation in these plants (Figure 4.4B, lanes 3-4) was similar to the levels observed in plants co-infected with DNA A and DNA β (lanes 3-4 and lanes 11-12). These results show that transient expression of the C4 protein can lead to systemic movement of DNA A, as was with βC1.

4.3.4 The wild-type ToLCNDV only requires movement functions at the site of inoculation for systemic infection

We were interested to know whether tomato plants in which DNA A was mobilized from local sites of inoculation by transient expression of either βC1 or the C4 ORF, could sustain systemic infection. Dot blot analyses of symptomatic plants co-inoculated with DNA A and either 35S:βC1 or 35S:C4 showed that DNA A accumulation in these plants at 60 dpi was similar to the DNA A accumulation at 13-16 dpi (results not shown). No βC1 or C4 transcripts were detected in distal tissues by RT-PCR whereas control plants inoculated with DNA A and DNA β contained the ~375 bp product expected from a βC1 transcript (Figure 4.4C). Sequence analysis of the DNA A from systemically infected leaves showed that it was identical to that used as inoculum, and therefore that it had not mutated or recombined with the 35S:βC1 or the 35S:C4 construct.

When scions from these tomato plants were tip-grafted onto healthy tomato plants, mild leaf curling was observed on re-growth from the rootstocks 15-18 days later. Subsequent removal of the scions did not affect the continuing symptom development on the stocks which remained symptomatic in the glasshouse for up to one year, and DNA A was retained throughout this period (data not shown). These results indicate that once ToLCNDV DNA A has spread systemically, it provides the gene functions required for infection.

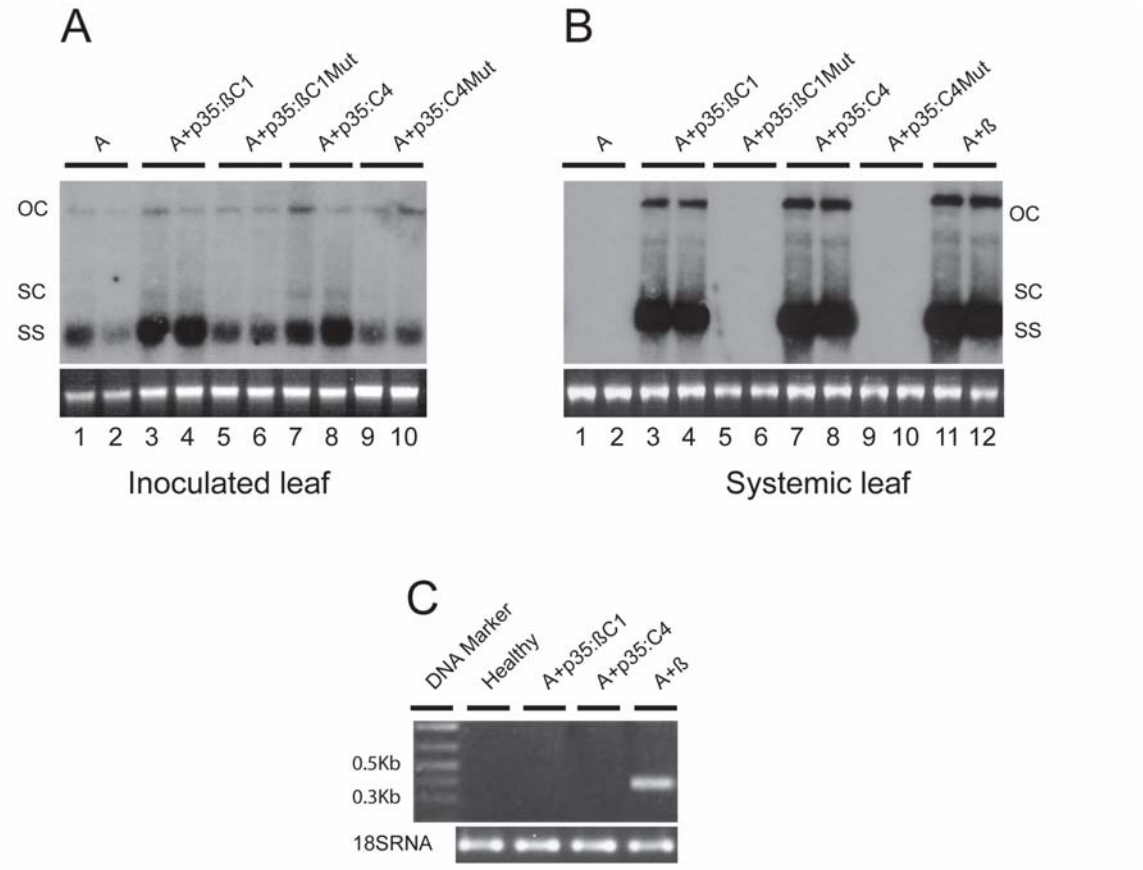

Figure 4.4 Transient expression of βC1 or the C4 allows movement of Tomato leaf curl New Delhi virus (ToLCNDV) DNA A. Nucleic acid extracts from inoculated (A) or systemic (B) leaves were analysed by Southern blotting using a DNA A specific probe. The position of single-stranded (SS), super coiled (SC), and open-circular (OC) DNA forms are indicated. Each lane was loaded with 5 µg of DNA. Ethidium bromide-stained DNA is shown as loading control. Detection of βC1 and C4 transcript by RT-PCR (C). 18S rRNA served as an RT-PCR control.

4.3.5 The βC1 protein accumulates mainly at the cell periphery and co-localizes with the endoplasmic reticulum

The βC1 protein encoded by the DNA satellite associated with *Tomato yellow leaf curl China virus* (TYLCCNV) has been reported to localize to the cell nucleus (Cui et al., 2005). Given the role of βC1 protein from the CLCuD DNA β in movement, its subcellular localization was studied. A construct having a transcriptional fusion of βC1 to GFP downstream of the CaMV 35S promoter was delivered by biolistic bombardment to tobacco and onion epidermal cells and transient expression was analyzed by confocal microscopy. The control free GFP was distributed in both the cytoplasm and the nucleus of bombarded cells (Figure 4.5A and 4.5H). In contrast, the βC1:GFP fusion protein was distributed mainly at the cell periphery with a weak florescence inside and around the nucleus (Figure 4.5B and 4.5I). The Arabidopsis histone 2B:YEP (Boisnard-Lorig et al., 2001) was used as a control for nuclear localization, and found the fusion protein in the nucleus exclusively (Figure 4.5D and 4.5K). These results suggested that βC1 is localized around the nucleus and at the cell periphery.

To distinguish between accumulation of the βC1 to the cell wall and at plasma membrane, the onion epidermal cells expressing GFP:βC1 were subjected to plasmolysis by mannitol treatment and were observed under the microscope. After plasmolysis, fluorescent microscopy indicated that βC1:GFP-containing bodies appeared within the cytoplasm but not at cell walls (Figure 4.5M).

To determine whether the βC1 protein was colocalized with the endoplasmic reticulum, tobacco leaf transformed with βC1:GFP was stained with rhodamine B hexyl ester, which labels endomembranes such as those of the endoplasmic reticulum (Grabski et al., 1993). The tobacco epidermal cells expressing βC1:GFP and stained with rhodamine B hexyl ester, were observed under the confocal microscope. The

images were simultaneously collected for GFP and rhodamine fluorescence. An overlay of GFP (Figure 4.5E and 4.5N) and rhodamine fluorescence (Figure 4.5F and 4.5O) clearly indicated that βC1 colocalizes with endoplasmic reticulum (Figure 4.5G and 4.5P).

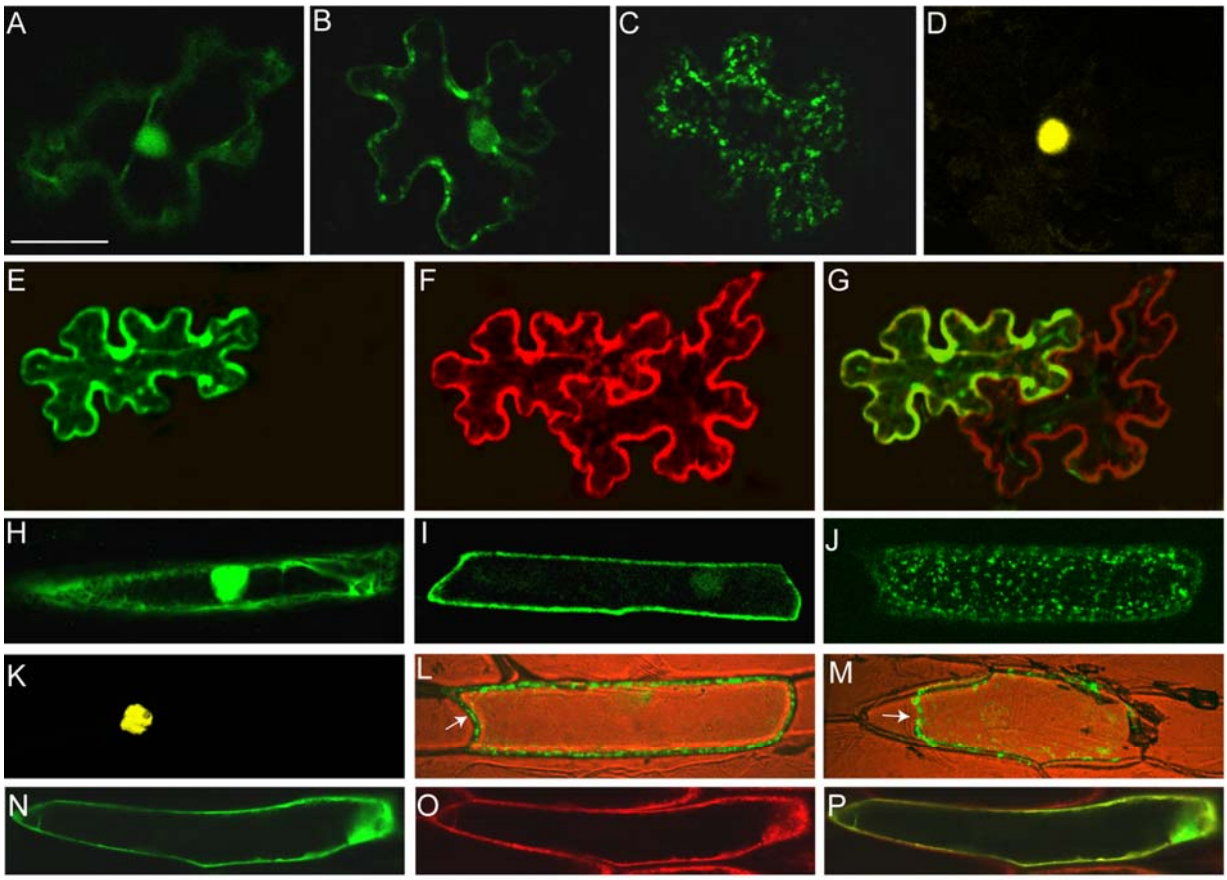

Figure 4.5 βC1 is localized around the nucleus and at the periphery of a plant cell. Cells are shown as single CLSM image.Subcellular localization of βC1:GFP construct in tobacco (B-G) and onion epidermis cells (I-M). Bar represent 50 μM. Free GFP was distributed throughout the cell (A and H). βC1:GFP was distributed around the nucleus and at cell periphery (B and I) or in association with punctate bodies in the cell cytoplasm (C and J). A positive control for nuclear localization, H2B:YFP is also shown (D and K). Green fluorescence associated with βC1:GFP (E) and the red fluorescence of the endoplasmic reticulum stain, rhodamine B-hexyl ester, (F). Merging patterns of green (E and N) and red fluorescence (F and O) shows colocalization with the endoplasmic reticulum (G and P). Localization of βC1:GFP at the cell periphery, before (L) and after plasmolysis (M) of an onion epidermis cell. Arrows indicate the position of the plasma membrane.

4.4 Discussion

This study reports that the DNA β satellite could substitute for DNA B of a bipartite begomovirus in providing the movement function. While DNA A of bipartite begomoviruses alone was capable of independent replication and encapsidation (Rojas et al., 2005), the reported incidence of their infection is inconsistent. For example, the biolistic bombardment of TGMA DNA A failed to produce a systemic infection (Jeffry et. al. 1996) but it caused systemic infection by *Agrobacterium*-mediated inoculation (Briddon and Markham 2001). However, extensive infectivity of ToLCNDV DNA A has failed to show any systemic spread of the DNA A from sites of bombardment even when detection was carried out by PCR (Padidam et al., 1995, Padidam et al., 1996); Figure 4.2A and 4.2D).

The replication of the DNA β satellite associated with CLCuD is supported by four distinct cotton-infecting monopartite begomoviruses (Mansoor et al., 2003) and ToLCV (Saeed et al., 2005). In this study we found that this DNA β satellite not only can use ToLCNDV DNA A as a helper virus but also provides movement function for its systemic infection. However, the level of viral DNA was lower in these plants compared to the level of viral DNA in the plants infected with DNA A and DNA B. A possible explanation for this difference may be that more cells may be infected with DNA A and DNA B infection compared to the DNA A and DNA β infection. A DNA β satellite associated with AYVV has been shown to substitute for the DNA B associated with SLCMV for infection (Saunders et al., 2002). However, SLCMV DNA A alone can cause systemic infection in *N. benthamiana*. Saunders et al (2002) therefore proposed that SLCMV DNA A has biological characteristics of a monopartite begomovirus and that the virus probably acquired a DNA B component from the *Indian cassava mosaic virus* (ICMV).

Compared to the higher rates of infection by DNA A and DNA B, about one fifth of inoculated tomato plants became infected following biolistic bombardment of DNA A and DNA β. Briddon et al (2001) also reported similarly low levels of successful inoculation (10-16%) with CLCuMV and DNA β in *N. benthamiana* and cotton using biolistic bombardment.

In addition to its role in pathogenicity (Cui et al., 2004, Saeed et al., 2005, Saunders et al., 2004) and virus movement (this study), βC1 has been reported to be a suppressor of gene silencing (Cui et al., 2005). As yet there is no direct evidence for the involvement of the ToLCV encoded C4 protein in the suppression of gene silencing. However, the C4 protein homologues, encoded by ACMV and SLCMV, have been identified as suppressors of gene silencing (Vanitharani et. al., 2004). Many of the plant virus-encoded suppressors of silencing were initially identified as pathogenicity determinants involved in systemic invasion of host plants (Scholthof, 2005). Whether suppression of host defence by the βC1 or the C4 protein has a role in the systemic infection of ToLCNDV DNA A remains unclear.

The subcellular localization study has shown that the βC1 fused to GFP localized around and inside the nucleus, and at the periphery of tobacco epidermis cells. While its peri-nuclear and the cell periphery distribution would be consistent with βC1 mediating intra-cellular transport from the nucleus to the plasma membrane. The significance of the βC1 protein accumulating inside the cell nucleus is unclear. It is likely that the accumulation βC1:GFP fused protein inside the nucleus may reflect the small size of fusion protein (approx. 43 kDa), which is small enough to diffuse passively through the large nuclear pore complex (Gafni & Epel, 2002). Alternately, nuclear localization βC1 protein may be consistent with its function such as suppression

of gene silencing or transport of DNA into or outside the nucleus. Subcellular localization of βC1 resembled to that of the *Maize streak virus* (MSV) and TYLCV V1 protein, which had been implicated in virus movement (Kotlizky et al., 2000; Rojas et al., 2001). However, these results are inconsistent with the report by Cui et. al. (2005a) that the βC1 encoded by the Y10β is targeted to the cell nucleus. The Y10-βC1 protein has a nuclear localization signal (NSL), ^{45}PALAKKK^{51}, where mutation resulted in the loss of nuclear localization (Cui et al., 2005a). However, βC1 encoded by CLCuD DNA β lacks this sequence. Whether such a discrepancy is related to the difference in NLS of these two proteins remains unclear.

Using biolistic bombardment, it was shown that DNA As of *Bean dwarf mosaic virus* (BDMV) and *Abutilon mosaic virus* (AbMV) DNA were delivered only to the outer epidermal and cortical layers of bean hypocotyl tissues, and their further movement to the vascular tissue required DNA B encoded gene functions (Levy & Czosnek, 2003; Seo et al., 2004; Sudarshana et al., 1998, Wang et al., 1999). It is therefore likely that ToLCNDV DNA A might have also been delivered to the epidermal tissues where it could replicate but was unable to spread to the neighbouring cells (Figure 4.2A and 4.4A). The localized expression of either βC1 or C4 may have provided movement functions to spread DNA A to the vascular tissues. This hypothesis is supported by a recent study that the βC1 protein can bind both ss and ds DNA in a size and sequence independent manner (Cui et al., 2005).

The observation that once the DNA A had spread to the vascular tissue, it provided all gene functions for a steady infection was supported by graft transmission studies. These results suggest that βC1 ORF product is not involved in systemic spread within vascular tissues of the infected plant.

Based on these observations and subcellular localization of the βC1, it is proposed that βC1 protein has a role in cell-to-cell movement of the helper virus, ToLCNDV DNA A. A similar movement function has been reported for the C4 and V1 proteins encoded by TYLCV (Rojas et al., 2001). However, it remains unclear how βC1 protein increases the accumulation of cognate helper virus in cotton plants.

Chapter 5 - General discussion and concluding remarks

Despite their recent discovery, more than 130 DNA β satellite sequences have now been deposited in GenBank (Rojas et al., 2005). They are associated with monopartite begomoviruses in a wide variety of vegetable and fibre crops, ornamental plants and weeds, mainly throughout Asia and some in Africa (Mansoor et al., 2003b). The DNA β associated with CLCuD is responsible for symptom expression of a devastating disease in Pakistan. At the start of the study presented here, it was know that the DNA βs contribute to the production of symptoms and enhanced helper virus accumulation in certain hosts (Briddon et al., 2001, Saunders et al., 2000). It was considered that DNA β may contribute directly to the helper virus replication. Alternatively, it may have an indirect role by producing a cellular environment which is conducive for viral DNA replication, or by facilitating viral DNA spread throughout the plant, thereby enhancing helper virus accumulation. Another possibility is that DNA β may encode a pathogencity determinant that serves to suppress the host gene silencing mechanism (Saunders et al., 2000). It is also possible that DNA β may encodes a protein that serves as a pathogencity determinant, RNA silencing suppressor and MP as has been reported for several viral proteins encoded by RNA viruses (Scholthof, 2005). With this information, the broad aims of this study were to investigate the role of DNA β associated with CLCuD in pathogencity and movement in plants.

Mutagenesis, transcript mapping, and expression in transgenic plants were used to investigate the DNA β encoded functional ORF(s). Evidences such as abolishing DNA β associated phenotype by disruption of βC1 ORF, induction of growth abnormalities by the expression of βC1 gene in transgenic plants results, and detection of a single transcript by Northern blot analysis showed that DNA β encodes a single complementary-sense ORF, βC1 whose product functions as a pathogenicity determinants (Saeed et al., 2005). Meanwhile, research from two other laboratories has

shown that DNA β satellites encode a single protein which is the determinant of viral pathogenicity for begomovirus-DNA β satellite complexes (Cui et al., 2004; Saunders et al., 2004). Further research may help to understand the precise role of βC1 protein in symptom development.

Plant virus movement is a two stage process, cell-to-cell movement through plasmodesmata (Pd) and long distance transport through phloem. The ability of βC1 protein to mobilize ToLCNDV DNA A from sites of inoculation to produce systemic infection indicates that βC1 mediates cell-to-cell movement function. Further evidence is provided by sub-cellular localization studies of βC1:GFP fused protein, where it accumulated around and inside the nucleus, at the plasma membrane and in association with punctate bodies at the cell periphery. Importantly, the βC1 fused showed protein co-localization with the endoplasmic reticulum. Co-localization of the βC1 with ER would be consistent with having a role in intra cellular transport from the nucleus to the cell periphery and eventually to the neighboring cell. However, it remains unclear how βC1 protein increases the accumulation of helper begomovirus (Section 1.5.1) in cotton plants.

The DNA β has been shown to interact with four distinct cotton-infecting monopartite begomoviruses for infection (Mansoor et al., 2003a). In this study, tt was found that ToLCV, a monopartite begomovirus and DNA A of a bipartite begomovirus can trans-replicate DNA β to produce systemic infection in host plants. An exciting challenge would be to understand the mechanisms involved in begomovirus Rep-mediated trans-replication of DNA β satellites.

There are some functional similarities between βC1 and AC4/C4 ORF encoded by begomoviruses: (a) both proteins act as pathogencity determinant (Rigden et al., 1994, Saeed et al., 2005, Saunders et al., 2004) (b) their expression in transgenic plants produces developmental abnormalities (Chellappan et al., 2005, Krake et al., 1998,

Latham et al., 1997, Saeed et al., 2005, Saunders et al., 2004), (c) βC1 and some AC4 encoded by DNA A of bipartite begomoviruses act as a silencing suppressor (Chellappan et al., 2005, Cui et al., 2005a). (d) both proteins have been shown to induce hyperplasia in infected plants (Cui et al., 2005b, Latham et al., 1997), and (e) the C4 ORF encoded by TYLCV, has been implicated in viral spread (Jupin et al., 1994, Rojas et al., 2001). The present study extends the similarities between the C4 and the βC1 protein by demonstrating that βC1 has a role in helper virus movement.

βC1 protein has been identified as a multi-functional protein behaving as both pathogenicity and movement protein. This protein is also a suppressor of gene silencing (Mansoor et al., 2003b; unpublished results). Therefore, βC1 has functional similarities to many plant RNA viral proteins which encode proteins such as HC-Pro, P19, 2b (Scholthof, 2005).

Virus-induced gene silencing (VIGS) is a homology dependent mechanism used for silencing of a transgene or endogene. It is becoming an increasing valuable tool in the field of genomics to study the function of a gene by down-regulating its expression and analyzing the resulting phenotype (Carrillo-Tripp et al., 2006). Silencing of the severe virus-like phenotype exhibited by 2β transgenic tobacco plants following ToLCV infection (Chapter 3) demonstrated the potential of DNA β for VIGS. The observation that DNA β satellites with a deletion in the βC1 ORF have been isolated from various begomovirus-DNA β diseases (Briddon et al., 2003) suggests that the βC1 protein is not essential for DNA β replication. The results described in this study also showed that βC1 protein was not required for replication. Therefore DNA βs could be manipulated to remove the βC1 coding region. In fact a DNA β satellite associated with TYLCCNV has been successfully used as an efficient vector for VIGS (Tao & Zhou, 2004). However, there is a need to improve inoculation efficiency of cotton plants with the helper virus and DNA β for VIGS. It is unlikely that the biolistic inoculation can be an

effective inoculation tool as reported before (Briddon et al., 2001). Extensive biolistic inoculation with ToLCNDV DNA A and DNA β could result only in 20% of the inoculated plants being systemically infected whereas 100% of the plants inoculated with DNA A and DNA B were systemically infected (Chapter 4). Cotton is a host plant for *Agrobacterium*; therefore agroinoculation would be an attractive alternative method for inoculation. This would allow utilization of cotton-infecting begomoviruses such as CLCuMV and DNA β as a vector for VIGS. Moreover, an efficient inoculation would be a valuable tool that may be used for the screening of CLCuD resistant varieties of cotton.

Apart from understanding the role of DNA β in viral pathogencity and movement, this study has both epidemiological and pathological implications. Diseases caused by begomovirus-DNA β have been expanding rapidly both in geographical distribution and in host range. Given the presence of a large number of begomoviruses throughout Asia and Africa (Briddon & Stanley, 2006), the promiscuity of DNA β for helper viruses, and the ability of DNA β to substitute for DNA B, the probability exists that new diseases such as cotton leaf curl may emerge from bipartite begomoviruses in the form of a monopartite virus associated with a DNA β satellite. As suggested by Saunders et. al. (2002), monopartite and bipartite begomovirus do not seem to be separated by a rigid boundary.

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Appendix 1 - Publications arising from this work

Saeed, M., Behjatnia, S. A., Mansoor, S., Zafar, Y., Shahida, H., and Rezaian, M. A. 2005. A single complementary-sense transcript of a geminiviral DNA β satellite is determinant of pathogenicity. Mol. Plant-Microbe Interact. 18, 7-14.

Saeed, M. Randles, J. W. Zafar, Y. and Rezaian, .M. A. A monopartite begomovirus-associated DNA β satellite substitutes for the DNA B of a bipartite begomovirus to permit systemic infection (Accepted, J. Gen. Virol.)

Saeed, M., Akbar Behjatnia, S.A., Mansoor, S., Zafar, Y. Hasnain, S. and Ali Rezaian, M. (2005): A single complementary-sense transcript of a geminiviral DNA β satellite is determinant of pathogenicity.
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