Role of betasatellite in begomovirus pathogenesis

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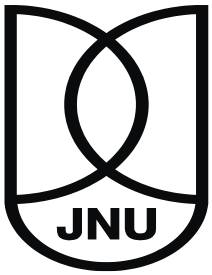

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CERTIFICATE

This is to certify that the research work embodied in this thesis entitled, **"Role of betasatellite in begomovirus pathogenesis"** submitted for the award of degree of Doctor of Philosophy has been carried out by **Ms. Neha Gupta** under the guidance and supervision of **Prof. Supriya Chakraborty** at Molecular Virology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi, India. The work is original and has not been submitted in part or full for any degree or diploma in this or any other university or institute.

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Abbreviations

1	TLCV	Tomato leaf curl virus
2	ToLCPaB	Tomato leaf curl Patna betasatellite
3	ToLCPaV	Tomato leaf curl Patna virus
4	ToLCGV	Tomato leaf curl Gujrat virus
5	ToLCNDV	Tomato leaf curl New Delhi virus
6	TGS	Transcriptional gene silencing
7	PTGS	Post-transcriptional gene silencing
8	JA	Jasmonic acid
9	PS-II	Photosystem II
10	SLCMV	Sri Lankan Cassava mosaic virus
11	CMD	Cassava mosaic disease
12	TYLCD	Tomato yellow leaf curl disease
13	TYLCV	Tomato yellow leaf curl virus
14	MSV	Maize streak virus
15	AYVV	Ageratum yellow vein virus
16	CR	Common region
17	ORFs	Open reading frames
18	TrAP	Transcription activator protein
19	REn	Replication enhancer protein
20	CP	Coat protien
21	MP	Movement protein
22	NSP	Nuclear shuttle protein
23	SCR	Satellite conserved region
24	IR	Intergenic region
25	RBR	Retinoblastoma related protein
26	PCNA	Proliferating cell nuclear antigen
27	RFC	Replication factor C
28	MCM2	Minichromosome maintenance 2
29	RPA	Replication protein A
30	ChiLCV	Chilli leaf curl virus
31	UBC2	Ubiquitin-conjugating enzyme2
32	PI4K	Phosphatidylinositol 4-kinase
33	SLCMV-Col	Sri Lankan cassava mosaic virus-Columbia
34	TAD	Transcriptional activation domain
35	CLE	Conserved late elements
36	TGMV	Tomato golden mosaic virus
37	ACMV	African cassava mosaic virus
38	TYLCCNV	Tomato yellow leaf curl China virus
39	CaLCuV	Cabbage leaf curl virus
40	ADK	Adenosine kinase

41	CSN5	COP9 signalosome 5
42	CUL1	Cullin 1
43	HR	Hypersensitive response
44	PaLCuV	Papaya leaf curl virus
45	ToLCKeV	Tomato leaf curl Kerala virus
46	ToLCV	Tomato leaf curl virus
47	BGMV	Bean golden mosaic virus
48	PYMV	Potato yellow mosaic virus
49	BSCTV	Beet severe curly top virus
50	CKIs	Cyclin-dependent kinase inhibitors
51	CLV1	CLAVATA 1
52	BAM1	BARELY ANY MERISTEM 1
53	BIN2	Brassinoids inhibitor 2
54	SPLCV	Sweet potato leaf curl virus
55	FLS2	FLAGELLIN SENSING 2
56	BRI1	BRASSINOSTEROID INSENSITIVE 1
57	TLCYnV	Tomato leaf curl Yunnan virus
58	EACMV	East African cassava mosaic virus
59	MYMIV	Mung yellow mosaic India virus
60	DRM2	Domain rearranged methyltransferase 2
61	PVX	Potato virus X
62	ToLDeV	Tomato leaf deformation virus
63	SLCCNV	Squash leaf curl China virus
64	TYLCSaV	Tomato yellow leaf curl Sardinia virus
65	AbMV	Abutilon mosaic virus
66	EACMCV	East African cassava mosaic Cameroon virus
67	SGS3	Suppressor of gene silencing 3
68	HDA6	Histone deacetylase 6
69	RDR1	RNA-dependent RNA polymerase 1
70	NIG	NSP-interacting GTPase
71	NIK	NSP interacting kinas
72	PERK	Proline-rich extension-like receptor kinase
73	TCrLYV	Tomato crinkle leaf yellows virus
74	BDMV	Bean dwarf mosaic virus
75	NISP	NSP-interacting syntaxin domain-containing protein
76	SYTA	Synaptotagmin
77	ToMoV	Tomato mottle virus
78	TYLCV-OM	Tomato yellow leaf curl virus-Oman
79	BYVMV	Bhendi yellow vein mosaic virus
80	SAHH	S-adenosyl homocysteine hydrolase
81	CaM	Calmodulin-like protein
82	CLCuMuB	Cotton leaf curl Multan betasatellite
83	UBC3	Ubiquitin-conjugating enzyme 3

84	TYLCCB	Tomato yellow leaf curl China betasatellite
85	PDF1.2	PLANT DEFENSIN1.2
86	PR4	PATHOGENESIS RELATED4
87	CORI3	CORONATINE INSENSITIVE13
88	OEC	Oxygen-evolving complex
89	GRAB	Geminivirus Rep-A binding,
90	RPT4a	RP Triple-A ATPases
91	EML1	EML1-EMSY-LIKE 1
92	VSRs	Viral suppressors of RNA silencing
93	GRIK-1	Geminivirus Rep interacting kinase
94	SnRK1	SUCROSE NON-FERMENTING1-related protein kinase 1
95	AS1	ASYMMETRIC LEAVES 1
96	ATG	Autophagy-related genes
97	LIMYB	L10-interacting MYB domain-containing protein
98	DAMP	Danger-Associated Molecular Patterns
99	ssDNA	Single-stranded DNA
100	CLCuMuB	Cotton leaf curl Multan betasatellite
101	MAPK	Mitogen-activated protein kinase
102	CLCuMuB	Cotton leaf curl Multan betasatellite
103	ATP	Adenosine triphosphate
104	CLCuKV-Dab	Cotton leaf curl Kokhran virus-Dabawali
105	SeMV	Sesbania mosaic virus
106	CroYVMB	Croton yellow vein mosaic betasatellite
107	ToLCJoB	Tomato leaf curl Joydebpur betasatellite
108	ToLCBB	Tomato leaf curl Bangladesh betasatellite
109	TYLCTHB	Tomato yellow leaf curl Thailand betasatellite
110	PCR	Polymerase chain reaction
111	RaLCB	Radish leaf curl betasatellite
112	BYVB	Bhindi yellow vein betasatellite
113	IPTG	Isopropyl β-D-thiogalactoside
114	HRP	Horse radish peroxidase
115	DAB	3,3'- diaminobenzidine
116	ToLCGV	Tomato leaf curl Gujrat virus
117	3-AT	3-amino-1, 2, 4-triazole
118	ToLCV	Tomato leaf curl virus
119	EACMCV	East African cassava mosaic Cameroon virus
120	PepGMV	Pepper golden mosaic virus
121	PHYVV	Pepper huasteco yellow vein virus
122	TYLCMLC	Tomato yellow leaf curl Mali virus
123	CLCuGB	Cotton leaf curl Gezira betasatellite
124	BiFC	Bimolecular Fluorescence comp
125	RaLCV	Radish leaf curl virus
126	SAHH	S-adenosyl homocysteine hydrolase

127	NBT	Nitro blue Tetrazolium Chloride
128	ROS	Reactive oxygen species
129	RbohB	Respiratory burst oxidase homologues B
130	GR	Glutathione reductase
131	Cat	Catalase
132	TSWV	Tomato spotted wilt virus
133	BDMV	Bean dwarf mosaic virus
134	%	Percent
135	°C	Degree Celsius
136	cDNA	Complementary DNA
137	DAPI	4, 6-diamidino-2-phenylindole
138	DO	Drop out
139	DNA	Deoxyribonucleic acid
140	dpi	Days-post inoculation
141	dNTPs	Deoxy nucleotide triphosphates
142	dsDNA	Double stranded DNA
143	EDTA	Ethylene diamine tetra acetic acid
144	EtBr	Ethidium bromide
145	g	Gram
146	ICTV	International Committee on Taxonomy of Viruses
147	IR	Intergenic region
148	LiAc	Lithium acetate
149	kb	Kilo base pairs
150	kDA	Kilo Dalton
151	LB	Luria-Bertani medium
152	M	Molar
153	min	Minutes
154	ml	Millilitre
155	mm	Millimeter
156	mM	Millimolar
157	NaCl	Sodium chloride
158	NaOH	NaOH Sodium hydroxide
159	ng	Nano gram
160	OD	Optical density

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Chapter 1

Introduction

1. Introduction

During the past few decades, more than half of the diseases in plants have been due to viruses (Anderson et al., 2004). *Geminiviridae* represents the largest family of plant viruses, constituting 520 species of geminivirus belonging to different genera (Zerbini et al., 2017). Geminiviruses have emerged as a significant threat to agricultural productivity and the global economy. The widespread distribution and diversification of geminiviruses caused huge crop loss worldwide, especially in tropical and subtropical regions. The heavy economic distress caused due to geminiviral infections for tomatoes in Florida was USD 140 million (Moffat, 1999), for cotton in Pakistan as USD 5 billion (Briddon et al., 2000), for legumes in India as USD 300 million. While in Africa, USD 1300-2300 million was lost due to infection in Cassava (Thresh et al., 1998). Many economically important vegetable, fiber, and ornamental crops get affected severely every year.

Geminiviruses are typified by small, single-stranded, circular DNA molecules which are encapsidated into twinned icosahedral capsids (Zhang et al., 2001; Hesketh et al., 2018). These viruses are phloem limited and transmit through the hemipterous insect vectors. The International Committee on Taxonomy of Viruses (ICTV) has categorized the *Geminiviridae* family into fourteen genera depending on phylogeny, host range, genome organization, and type of insect vector (Zerbini et al., 2017). They are *Becurtovirus, Begomovirus, Curtovirus, Mastrevirus, Capulavirus, Eragrovirus, Grablovirus, Topocuvirus, Turncurtovirus Citlodavirus, Maldovirus, Mulcrilevirus, Opunvirus* and *Topilevirus* (Fiallo-Olivé et al., 2021). *Begomovirus* constitutes the broadest genera that infect nearly 445 dicotyledonous plant species. The devastating diseases caused by begomoviruses are transmitted through *Bemisia tabaci* Genn. (whiteflies) (De Barro et al., 2011).

Begomovirus consists of either monopartite or bipartite genome. The typical bipartite begomovirus contains DNA-A and DNA-B genomic components (Lazarowitz and Shepherd, 1992). Each is approximately 2.7 kb in length and encodes proteins in virion (positive) and complementary (negative) sense strands. A common region called intergenic region (IR) of around 200-250 nucleotides that contains a stem-loop structure and embedded nonanucleotide sequence is essential for DNA-A and DNA-B replication. Monopartite begomoviruses are composed of a single circular genome that is homologous to DNA-A component of bipartite counterparts. Occasionally, monopartite begomoviruses are accompanied by additional satellite DNA molecules, either alphasatellite, betasatellite, or deltasatellite (Dry et al., 1997; Briddon and Stanley, 2006; Fiallo-Olivé et al., 2012). These satellites rely on a helper virus for replication (except alphasatellites), movement,

encapsidation, and vector transmission (Briddon et al., 2004; Zhou et al., 2013). Betasatellite (DNA-β) encodes two proteins, βV1 and βC1, that play an indispensable role in symptom induction, host defense suppression, HR induction, and insect transmission (Gnanasekaran et al., 2019a; Hu et al., 2020; Gupta et al., 2021). Besides, betasatellites contain a non-coding region that includes satellite conserved region (SCR) and an adenine-rich region, important for betasatellite replication and size maintenance, respectively (Briddon et al., 2003; Reddy et al., 2020).

Betasatellites do not share significant sequence similarity with the helper virus and lack conserved Rep binding iterons sequences present at the origin of replication in geminiviral DNA (Briddon and Stanley, 2006). This relaxes the stringency of Rep binding onto the Rep binding motifs (RBM) of SCR and favors the replication promiscuity for betasatellites by distinct helper begomoviruses. Experimental evidence supports transreplication of betasatellite associated with cotton leaf curl disease by tomato leaf curl virus (ToLCV) (Saeed et al., 2005). Tomato leaf curl Patna betasatellite which is associated with tomato leaf curl Patna virus (ToLCPaV) can be trans-replicated by DNA-A of tomato leaf curl Gujrat virus (ToLCGV) and tomato leaf curl New Delhi virus (ToLCNDV) (Kumari et al., 2010). Alarmingly, betasatellites have been found in association with several bipartite begomoviruses (Sivalingam et al., 2012; Kumar et al., 2015). The expeditious adaptation of betasatellites with multiple begomoviral components increases the chances of recombination, which generates a diversity of geminivirus-betasatellite complexes and promotes the probability of disease occurrence in new hosts (Nawaz-ul-Rehman et al., 2009; Gnanasekaran et al., 2019a).

In Asia, particularly China and the Indian subcontinent is concerned with emerging begomovirus-betasatellite disease complexes in a wide range of economically important crops like *Ageratum conyzoides* (Xiong et al., 2007), *Abelmoschus esculentus* (Venkataravanappa et al., 2011), Althea rosea (Briddon et al., 2003), *Capsicum annuum* (Senanayake et al., 2012), *Capsicum frutescens* (Kumar et al., 2011), *Cucurbita moschata* (Namrata et al., 2010), *Crotalaria juncea* (Kumar et al., 2010), *Cardiospermum microcarpum* (Salim and Thushari, 2010), *Glycine max* (Ilyas et al., 2010), *Gossypium hirsutum* (Mansoor et al., 2003), *Hibiscus spp* (Das et al., 2008), *Langenaria siceraria* (Sohrab et al., 2010), *Luffa cylindrical* (Sohrab et al., 2003), *Mimosa spp* (Ha et al., 2008), *Mentha arvensis* (Borah et al., 2010), *Nicotiana tabacum* (Singh et al., 2011), *Raphanus sativus* (Singh et al., 2012; Bhattacharya et al., 2015), *Solanum lycopersicum* (Kumari et al., 2010), *Solanum tuberosum* (Mubin et al., 2009). *Vigna mungo* (black gram), *Phaseolus vulgaris* (French bean), and *Vigna radiate* (mung bean) are essential legumes that get significantly affected (Varma and Malathi, 2003).

Countless reports from the last two decades proved the vital role of betasatellite in pathogenesis. Typical symptoms associated with begomovirus-betasatellites complexes range from mild symptom such as vein yellowing to severe symptoms like chlorosis, downward leaf curling, enations, and stunting, which damage crops adversely (Saunders et al., 2003; Kumar et al., 2015; Bhattacharya et al., 2015). Besides having a profound effect on symptom development, betasatellite encoded βC1 protein also assists in viral movement intracellularly and systemically, which enhances pathogenesis. βC1 protein suppresses various host defense response pathways like transcriptional gene silencing (TGS) (Yang et al., 2011; Zhong et al., 2017), post-transcriptional gene silencing (PTGS) (Cui et al., 2005; Li et al., 2014), suppresses jasmonic acid (JA) mediated responses, ubiquitin-proteasomal pathway and regulates autophagy-mediated defense responses (Gnanasekaran et al., 2019a; Gupta et al., 2021). βC1 protein also inhibits photosynthesis by disintegrating oxygen-evolving complex embedded in the photosystem II and damaging the chloroplast structure (Bhattacharyya et al., 2015). This may lead to suppression of defense hormones and a favorable microenvironment for viral proliferation. Recently, βC1 protein has been reported to suppress the expression of defense-related genes by regulating mitogen activated protein kinase (MAPK) pathway (Hu et al., 2019). The major pathogenicity determinant protein βC1 plays numerous significant roles in pathogenesis by interacting with various host factors.

However, the mechanical details of regulation between betasatellite and geminivirus are still unknown. The promiscuous behavior of betasatellites due to transreplication makes the investigation on begomovirus-betasatellite complexes challenging. To interrupt the increasing diversity of virus-betasatellite complexes, it is important to deeply understand the underlying detailed molecular interactions between virus and betasatellite. Furthermore, understanding the role of proteins encoded by betasatellites will provide insight into developing antiviral strategies. Based on the current knowledge about betasatellites, the following objectives have been proposed.

1. To study biochemical activities of βC1 encoded by different betasatellites.
2. To elucidate the role of betaC1 in pathogenesis.
3. To understand the role of betaV1 in pathogenesis.

Chapter 2

Review of Literature

2. Review of Literature

Viruses are obligate parasites of cellular systems. Although considered acellular, the debate over the living or non-living nature of viruses based on multiple theories has always been controversial. Viruses are the most abundant entities, exist at all the planet's places where life forms are present. They hijack the host cellular machinery for its proliferation and pathogenesis. Effective viral replication and propagation involve coordinated molecular and cellular reprogramming that regulates host-virus interaction. Viruses cause serious illness in plants and animals and have been responsible for many pandemics and epidemics in the past hundred years. Several cultivated crop plants, which are essential to feed humankind and livestock, ornamental plants, fiber, and medicines generating plants, are affected globally due to viral disease pandemics (Jones, 2021). Global economic impact due to viral pandemic has been estimated as approximately USD 30 billion annually (Sastry and Zitter, 2014). In the late 1980s, Africa faced severe food shortages and loss of livelihood among rural inhabitants in many regions like Uganda due to the cassava mosaic disease (CMD) epidemic (Legg et al., 2006). The South Asian countries have been threatened by the Sri Lankan Cassava mosaic virus (SLCMV; genus *Begomovirus*; family *Geminiviridae*) that causes destructive CMD (Siriwan et al., 2020). Another pandemic arose due to tomato yellow leaf curl disease (TYLCD) epidemics in the tropical and subtropical countries that impacted the whole world (Moriones and Navas-Castillo, 2000). TYLCD is caused by the tomato yellow leaf curl virus (TYLCV), a begomovirus that causes up to total yield loss.

Geminiviruses are one of the most devastating plant viruses belonging to the largest family *Geminiviridae*. Characterized by the circular, single-stranded DNA genome, they cause devastating diseases in plants, faring as a prominent reason for global crop loss and compromised food security. Geminiviruses are phloem-limited viruses and are transmitted by hemipterous insect vectors. Their unique virion includes a twinned icosahedral structure enclosing the circular genomic DNA (Figure 2.1) (Zhang et al., 2001; Hesketh et al., 2018). DNA replication occurs through the rolling circle and recombination-dependent mechanism (Jeske et al., 2001). In differentiated host cells, geminiviruses reprogram the cell cycle and transcriptional events (Hanley-Bowdoin et al., 2013), making the microenvironment suitable for its own replication. Inside the infected plant cell, host DNA polymerases convert the viral

ssDNA into dsDNA. Host nucleosomes pack the dsDNA forming minichromosomes that reside in the host nucleus and act as a template for virus transcription (Abouzid et al., 1988). Early transcription events drive the genes essential for virus replication and transcription, followed by late genes required for encapsidation and movement. By altering the host gene expression profile and regulating the host cell signaling pathways, geminiviruses induce severe diseases in plants which manifest as leaf curling, veinal swelling, chlorosis, growth stunting, stem bending, and smalling of leaves, etc. (Mansoor et al., 2006; Bhattacharyya et al., 2015).

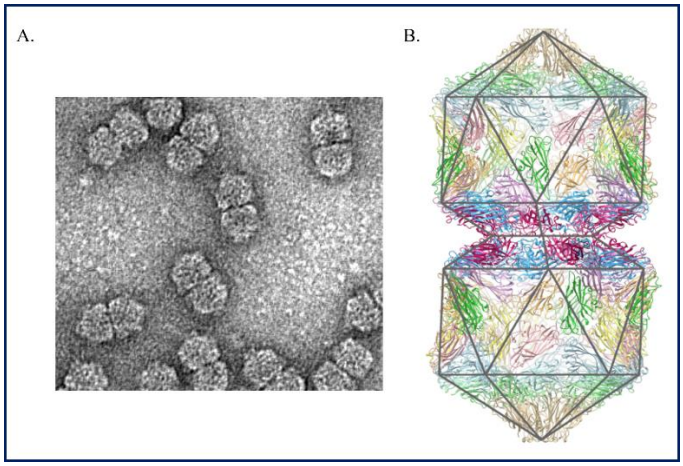


Figure 2.1 Icosahedral geminate virion particle.

(A) Twinned icosahedral subunit isolated from Maize streak virus (MSV) (Zhang et al., 2001; Fiallo-Olivé et al., 2021). (B) Atomic model containing 110 subunits of coat protein (CP) in Ageratum yellow vein virus (AYVV) capsid (adopted form Hesketh et al., 2018).

Based on their phylogenetic relationships, genome organization, host range, and insect vectors, geminiviruses are categorized into fourteen genera- *Becurtovirus, Begomovirus, Capulavirus, Citlodavirus, Curtovirus, Eragrovirus, Grablovirus, Maldovirus, Mastrevirus, Mulcrilevirus, Topocuvirus, Opunvirus, Topilevirus, Topocuvirus,* and *Turncurtovirus* (Fiallo-Olivé et al., 2021). These fourteen genera constitute 520 species in total. Among these, begomovirus constitutes the largest genus with 445 species that are predominantly transmitted by whitefly [*Bemisia tabaci* Genn.] vector. Members of the genera *Becurtovirus, Curtovirus, Mastrevirus, Turncurtovirus,* and *Mulcrilevirus* are transmitted by insect vector leafhoppers. Grablovirus and Topocuvirus are transmitted through treehoppers, while aphids are responsible for Capulavirus transmission. Genera *Becurtovirus*, *Begomovirus, Curtovirus*, *Capulavirus, Grablovirus*, *Topocuvirus*, *Turncurtovirus,Citlodavirus*, *Mulcrilevirus*, *Opunvirus,* and *Topilevirus* infect dicot plants. However, the members of genera *Eragrovirus and Mastrevirus and a species of*

Maldovirus infect monocots. (Varsani et al., 2014; Chen and Gilbertson, 2009; Brown et al., 2015; Rojas et al., 2005; Roumagnac et al., 2015; Loconsole et al., 2012; Bahder et al., 2016; Muhire et al., 2013; Fontenele et al., 2020; Lu et al., 2021; Briddon et al., 1996; Fiallo-Olivé et al., 2021).

While most classified genera comprise a monopartite genome, begomoviruses can contain either monopartite or bipartite genome (Figure 2.2). Based on their geographical distributions and genetic diversities, begomoviruses are grouped into Old world (Africa, Europe, Australia, and Asia) and New World categories (America) (Nawaz-ul-Rehman and Fauquet, 2009). The New World begomoviruses mostly have a bipartite genome, while the Old-World ones contain both mono and bipartite genomes. The genome of a bipartite begomovirus contains two separately encapsidated DNA molecules, known as DNA-A and DNA-B, of sizes ranging from 2600-2800 nt (Nawaz-ul-Rehman and Fauquet, 2009). Monopartite begomovirus have a genome of one DNA molecule, which is structurally and genetically similar to DNA-A of bipartite begomoviruses. Both DNA A and DNA B include a common region (CR) of 200-250 nucleotides that encompasses a conserved stem-loop structure and the sequence TAATATTAC. The DNA-A component contains open reading frames (ORFs) encoding five to seven proteins, while DNA-B codes for two proteins. Two of the proteins in DNA-A of bipartite begomovirus and the monopartite virus are encoded in the virion sense strand and four in the complementary sense strand. The complementary sense strand proteins are replication-associated rep protein (REP; AC1), transcription activator protein (TrAP; AC2), replication enhancer protein (REn; AC3), and AC4 protein. Coat protein (CP; AV1) and pre-coat proteins (AV2) are encoded in the virion sense strand. However, the AV2 ORF is absent in new world bipartite begomoviruses (Fondong, 2013). DNA-B contains ORFs BC1 and BV1, encoding movement protein (MP) and nuclear shuttle protein (NSP), respectively. The geminivirus proteins work in coordination to facilitate replication, movement, and anti-defense response to establish a successful infection process (Hanley-Bowdoin et al., 2013; Kumar, 2019).

In the infection establishment process, the subviral components of begomoviruses play important roles. Known as alphasatellite, betasatellite, deltasatellites or non-coding satellites, these satellite molecules depend on the helper virus for their replication and propagation, but some of them are adapted in modulating the biological properties of helper viruses (Mansoor et

al., 2006; Lozano et al., 2016). While alphasatellites are self-replicating and depend on the helper virus for encapsidation, movement, and transmission, betasatellites are transreplicated by helper begomovirus and mastrevirus (Saunders et al., 2008; Saunders and Stanley, 1999; Kumar et al., 2014). All betasatellites have the following regions in their genome: an A-rich region, satellite conserved region (SCR), and a single ORF on the complementary sense strand encoding βC1 protein (Briddon et al., 2003). Recently, some betasatellites have been found to have another ORF, overlapping with βC1 ORF, in virion-sense strand DNA that encodes βV1 protein (Hu et al., 2019). SCR is a conserved 150-200 nucleotides sequence containing a hairpin loop structure with TAATATTAC, a conserved motif indispensable for betasatellite replication. The size and position of βC1 ORF are conserved throughout betasatellites with a start and stop codons located between 195-209 and 544-570, respectively. In natural conditions, plants can be infected by multiple viruses, and the stringency of betasatellites associated with their helper virus is very less, which increases the diversity of geminivirus-betasatellite complexes and enhances the probability of disease occurrence in new hosts. Furthermore, the high evolutionary rate of geminiviruses enables them to adapt to new hosts.

2.1 Begomovirus DNA-A encoded ORFs

2.1.1 AC1/C1

The complementary-sense strand ORF, AC1 in bipartite or C1 in monopartite begomovirus encodes for a replication-associated protein (Rep). Highly conserved with respect to sequence and function, Rep protein is necessary for the replication of geminiviral DNA (Hanley-Bowdoin et al., 1990). The expression of Rep is controlled by a bidirectional promoter present in the intergenic region (IR) (Hanley-Bowdoin et al., 1999). Rep binds to geminiviral DNA in a sequence-specific manner and possesses nicking and joining activities required for the rolling circle replication mechanism (Orozco and Hanley-Bowdoin, 1996; Gutierrez, 1999). Additionally, it oligomerizes and can hydrolyze ATP (Desbiez et al., 1995). Rep has three domains: the N-terminal domain involved in DNA binding and nicking activity, the central domain for oligomerization, and the C-terminal domain for ATP hydrolysis activity (Desbiez et al., 1995; Orozco et al., 1997; Krenz et al., 2011). The autoregulatory role of Rep is also reported (Sunter et al., 1993). It binds to the iteron sequences in the common region of the genome and represses its own transcription (Eagle et al., 1994). In some cases, Rep also negatively regulates

Figure 2.2 Genomic organization of different genera of family *Geminiviridae*.

V and C represent virion-sense and complementary-sense strand. IR-intergenic region; LIR-long intergenic region; SIR, short intergenic region; Rep- replication-associated protein; CP-coat protein; NSP- nuclear shuttle protein; MP- movement protein; TrAP- transcriptional activator protein; Ren- replication enhancer protein (Adopted from Fiallo-Olivé et al., 2021)

complementary-sense strand gene expression (Shivaprasad et al., 2005). This multitasking protein Rep reprograms host replication machinery by interacting with many host factors related to the replication process. Few of them are cell cycle regulator-retinoblastoma related protein (RBR); a sliding clamp for DNA polymerase-proliferating cell nuclear antigen (PCNA); catalytic factor of PCNA loading- replication factor C (RFC); pre-replication complex component-minichromosome maintenance 2 (MCM2); a protein involved in recruiting replication apparatus – replication protein A (RPA) (Arguello-Astorga et al., 2004; Egelkrout et al., 2001; Rizvi et al., 2015; Loor et al., 1997). Rep modulates sumoylation by interacting (SUMO)-conjugating enzyme (SCE1) for creating favorable situations for viral proliferation (Castillo et al., 2004). Chilli leaf curl virus (ChiLCV) encoded Rep regulates viral transcription positively by interacting with histone monoubiquitination 1 (NbHUB1) and ubiquitin-conjugating enzyme 2 (NbUBC2) (Kushwaha et al., 2017). Rep interferes with the methylation cycle to suppress RNA silencing machinery, thereby regulating pathogenesis (Rodríguez-Negrete et al., 2013). ChiLCV-Rep enhances pathogenesis by controlling phosphatidylinositol 4-kinase (PI4K) relocalization (Mansi et al., 2019). The pathogenicity of Rep is attributed to the seven amino acid stretch embedded in the C-terminal region of Rep in a virulent strain of Sri Lankan cassava mosaic virus-Columbia (SLCMV-Col) (Wang et al., 2020).

2.1.2 AC2/C2

AC2/C2 ORF present in the complementary-sense strand of DNA-A encodes for transcriptional activator protein (TrAP). The monodirectional promoter that lies within the coding region of AC1 expresses dicistronic transcript that translates both AC2 and AC3 protein together (Shivaprasad et al., 2005). TrAP plays a significant role in viral infection and pathogenesis. AC2 is nearly 15kDa protein, composed of N-terminal region (basic domain) that has nuclear localization signal, nonclassical Zinc finger containing central region (DNA-binding domain) and a C-terminal sequence (acidic region, with transcriptional activation domain (TAD)) (Trinks et al., 2005; Babu et al., 2018). A typical nuclear-localized protein, AC2, can shuttle between the cytoplasm and the nucleus during infection through interacting karyopherin-α (Chandran et al., 2012). The non-phosphorylated form of AC2 has been detected in the nucleus and cytosol (Wang et al., 2003). The role of AC2 in gene activation, gene silencing suppression, and pathogenesis are well characterized. Being a viral transcription factor, AC2 mediates the transactivation of rightward promoter transcribing late ORFs AV1/2 in DNA-A and BV1 in DNA-B (Shivaprasad

et al., 2005). AC2-mediated transactivation involves conserved late elements (CLE) present in the rightward promoter of DNA-A and DNA-B (Argüello-Astorga et al., 1994; Shivaprasad et al., 2005; Trinks et al., 2005). Tomato golden mosaic virus (TGMV) encoded AC2 protein interacts with PEAPOD transcription factor for activating the CP promoter (Lacatus and Sunter, 2009). Furthermore, the deletion of AC2 from TGMV resulted in a drastic reduction in AV1 encoded CP expression (Sunter et al., 1990). African cassava mosaic virus (ACMV) and tomato yellow leaf curl China virus (TYLCCNV) encoded AC2 protein has been implicated in the suppression of RNA silencing pathway (Van Wezel et al., 2002; Voinnet et al., 1999). The AC2 protein of cabbage leaf curl virus (CaLCuV) and TGMV reduces cytosine methylation globally as it inhibits adenosine kinase (ADK) that regulates S-adenosyl methionine synthesis (Buchmann et al., 2009). It employs multiple mechanisms to regulate RNA silencing machinery negatively.

AC2 determines pathogenicity by regulating various defense mechanisms employed by the host during infection. C2 from tomato yellow leaf curl Sardinia virus (TYLCSV) targets components of ubiquitination machinery to imbalance phytohormonal homeostasis. The interaction between AC2 and COP9 signalosome 5 (CSN5) compromises the network between CSN5 and Cullin 1 (CUL1) that is important for SCF-based ubiquitin E3 ligases activity (Lozano-Durán et al., 2011). This signal pathway controls the responses of many plant hormones like auxins, abscisic acid, jasmonates, ethylene, and gibberellins. TYLCSV encoded AC2 also induces a hypersensitive response (HR) through the hypervariable region in its C-terminal domain. However, AC2 of ToLCNDV inhibits NSP induced HR (Hussain et al., 2007). Likewise, C2 of papaya leaf curl virus (PaLCuV) negatively regulates HR induced by V2 of PaLCuV (Mubin et al., 2010). Taken together, AC2 protein is considered as one of the important pathogenicity determinants of geminiviruses.

2.1.3 AC3/C3

ORF AC3 encodes for replication enhancer protein (REn). As the name suggests, AC3 enhances the replication process of viral DNA and symptom development during begomoviral infection (Sung and Coutts, 1995). The monodirectional promoter that expresses AC2 also regulates AC3 expression through generating dicistronic transcript (Shivaprasad et al., 2005). Like other geminiviral proteins AC1 and AC2, REn also oligomerizes, reported for tomato leaf curl Kerala virus (ToLCKeV) (Pasumarthy et al., 2010). ToLCKeV encoded REn has also been reported to

interact with Rep and enhance its ATP hydrolysis activity (Pasumarthy et al., 2010). REn interacts with geminiviral rep protein and host protein PCNA and enhances viral titer (Castillo et al., 2003). In addition, it also interacts with the NAC1 transcription factor and enhances viral replication in host cells (Selth et al., 2005).

2.1.4 AC4/C4

AC4/C4 ORF is the smallest among other geminiviral ORF and is completely embedded in the AC1 ORF. The diverse functionality of AC4/C4 protein is attributed to its least conservation in nature. C4 has been known to be involved in developing symptoms during infection. Mutation in the start codon of C4 encoded by ToLCV reduced the symptom development in the host but did not affect the viral titer (Rigden et al., 1994). C4 mutation in beet severe curly top virus (BSCTV) affected the symptom expression in plants and inhibited the movement of virus in systemic leaves, suggesting the role of C4 in viral movement (Teng et al., 2010). AC4 protein of TGMV, bean golden mosaic virus (BGMV), and potato yellow mosaic virus (PYMV) are also involved in the movement of the virus (Pooma and Petty, 1996; Hoogstraten et al., 1996; Sung and Coutts, 1995).

The vein-swelling phenotype development during the manifestation of BCTV in the host plant has been ascribed to C4 as the overexpression of C4 led to ectopic cell division in *Nicotiana benthamiana* (Stanley et al., 1986; Stanley and Latham, 1992; Latham et al., 1997; Fondong, 2019). Expression of C4 gene of BSCTV in *Arabidopsis* induces Ring finger protein RKP1, the protein like human KPC1. RKP1 acts as a ubiquitin E3 ligase and interacts with Cyclin-dependent kinase inhibitors (CKIs), thus lowering the protein level of CKIs during the infection with the effect of continued cell cycle progression (Lai et al., 2009). AC4/C4 protein functions like a human oncogenic factor as induction of the protein lead to tumorigenic callus formation and hyperplasia (Mills-Lujan et al., 2015). It sequesters a negative regulator of cell cycle, SKη kinase, and enables G1 to S phase cell cycle transition (Mei et al., 2018a). A receptor kinase, CLAVATA 1 (CLV1), regulates WUSCHEL gene expression and helps in maintaining the meristem undergoes binding by the S-acylated form of BSCTV C4 protein; an interaction leading to anomalous siliques development in *Arabidopsis* (Li et al., 2018). As localized to the plasmodesmata and the plasma membrane, BARELY ANY MERISTEM 1 (BAM1), another RLK helps in expanding the systemic movement of RNAi signals and thus obstructing the spread

of the virus to other cells. However, TYLCV C4 protein binds to BAM1 and inhibits the propagation of silencing signals (Rosas-Diaz et al., 2018). *Arabidopsis* Shaggy-like kinase protein AtSK21, also known as AtBIN2 (Brassinosteroid inhibitor 2), negatively regulates brassinosteroid signaling. During the sweet potato leaf curl virus (SPLCV) infection, viral C4 protein targets AtBIN2, inducing anomalous development, including male sterility in *Arabidopsis* (Bi et al., 2017). C4 physically interacts with RLKs, FLAGELLIN SENSING 2 (FLS2), and BRASSINOSTEROID INSENSITIVE 1 (BRI1). It affects the downstream pathways as the interaction reduces the time of apoplastic ROS burst without influencing downstream marker genes expression (Garnelo Gómez et al., 2019).

Highly divergent AC4/C4 proteins have a common conserved myristoylation motif in the N-terminal region responsible for the binding of AC4 protein to the membranes. C4 of tomato leaf curl Yunnan virus (TLCYnV) undergoes SKη mediated phosphorylation followed by myristoylation, which is necessary for nucleocytoplasmic shuttle of protein (Mei et al., 2018b). The myristoylation motif in the sequence overlaps with the chloroplast transit peptide in the case of TYLCV. The relocalization of C4 protein from the plasma membrane to the chloroplast is responsible for inhibiting salicylic acid related defense responses (Medina-Puche et al., 2020). AC4 protein is also involved in suppressing RNA silencing machinery. ACMV encoded AC4 protein binds to small interfering RNAs and microRNAs and suppresses post-transcriptional gene silencing (Chellappan et al., 2004). It also inhibits PTGS synergistically with AC2 of East African cassava mosaic virus (EACMV), thereby increasing EACMV accumulation (Vanitharani et al., 2004). Interaction of ToLCV-C4 with shaggy-like kinase is important for silencing the suppression activity of C4 (Dogra et al., 2009). Besides this, AC4 also suppresses HR and confers drought stress tolerance in plants. (Mei et al., 2019; Corrales-Gutierrez et al., 2020).

2.1.5 AC5/C5

AC5/C5 protein has not been well characterized yet. However, in the mung yellow mosaic India virus (MYMIV), AC5 was reported to be necessary for viral replication (Raghavan et al., 2004). The N-terminal portion of MYMIV-AC5 is implicated in suppressing PTGS, while suppression of domain rearranged methyltransferase 2 (DRM2) by AC5 inhibits TGS. AC5 also induces HR when expressed through potato virus X (PVX). Symptom development was affected in the AC5 deletion mutant of tomato leaf deformation virus (ToLDeV) (Melgarejo et al., 2013).

2.1.6 AV1/V1

AV1/V1 ORF encodes for coat protein (CP), the only structural protein involved in assembly and viral DNA packaging. The deletion of CP has resulted in increased exposure of ssDNA to nucleases and thereby reduced ssDNA accumulation (Padidam et al., 1996; Briddon et al., 1989; Sunter et al., 1990). CP aids in nucleo-cytoplasmic shuttling and systemic movement in all the viruses, including bipartite begomoviruses that have separate proteins dedicated for movement function (Liu et al., 2001; Padidam et al., 1996; Ingham et al., 1995; Seo et al., 2004). The tomato leaf curl Java virus encoded CP contains a stretch of arginine-rich amino acid at 16-20: KVRRR, 52-55: RKPR at the N-terminal region, and a hydrophobic stretch 245 -250: LKIRIY at the C-terminal region (Sharma and Ikegami, 2009). These stretches are responsible for nucleo-cytoplasmic shuttling. CP interacts with many host proteins like karyopherin-α and importin-α for nucleo-cytoplasmic trafficking (Guerra-Peraza et al., 2005; Kunik et al., 1999). CP residues determine the insect vector specificity as T147S mutation in squash leaf curl China virus (SLCCNV) affects the whitefly transmission (Pan et al., 2020). Several amino acid residues have been identified in CP of TYLSV and abutilon mosaic virus (AbMV) responsible for vector-mediated viral transmission (Kuno et al., 1998; Höhnle et al., 2001). CP is also known to interact with vector proteins to mediate efficient viral transmission.

2.1.7 AV2/V2

The AV2 ORF overlaps with the AV1 ORF at the 3'end and encodes AV2 protein or pre-coat protein. This ORF is absent in bipartite begomoviruses belonging to the New World (Padidam et al., 1996). In many geminiviruses, AV2 protein displays a pathogenicity determinant role. AV2 mutated ToLCV, and Tomato leaf curl New Delhi (ToLCNDV) infected plants showed lower viral DNA accumulation (Padidam et al., 1996; Rigden et al., 1993). The AV2 protein of the East African cassava mosaic Cameroon virus (EACMCV) has a protein kinase C domain that plays a role in pathogenesis (Chowda-Reddy et al., 2008). Additionally, AV2 protein is also important for movement function (Rothenstein et al., 2007). The lower viral DNA accumulation in plants infected with AV2 mutant virus was not observed in the protoplasts system (Padidam et al., 1996). AV2 protein interacts with various proteins of TGS and PTGS pathways like a suppressor of gene silencing 3 (SGS3), histone deacetylase 6 (HDA6), and AGO4 protein to suppress RNA silencing machinery (Glick et al., 2008; Wang et al., 2018; Wang et al., 2019; Wang et al., 2020).

ToLCNDV encoded AV2 protein suppresses RNA-dependent RNA polymerase 1 (RDR1) mediated recovery phenomena in *N. tabacum* infected with ToLCNDV, thereby negatively regulating host defense pathways (Basu et al., 2018).

2.2 Begomovirus DNA-B encoded ORFs

2.2.1 BV1

BV1 ORF is present in the virion-sense strand DNA and encodes nuclear shuttle protein (NSP). NSP has a high affinity for nucleic acids, possesses a nuclear localization signal, and is involved primarily in the viral DNA shuttling between the nucleus and cytoplasm (Noueiry et al., 1994; Pascal et al., 1994). The interaction between the viral NSP and histone 3 (H3) leads to the export of viral DNA from the nucleus (Zhou et al., 2011). In addition, NSP-interacting GTPase (NIG) redirects the NSP nuclear-cytoplasmic movement and facilitates NSP interaction with MP in the cytoplasm (Carvalho et al., 2008). NSP induces AS2 expression in the infected cells that enhance decapping activity of DCP2, accelerating the mRNA turnover and hindering siRNA accumulation and host RNA silencing (Ye et al., 2015). The host NSP interacting kinase (NIK) mediated resistance against geminiviruses is limited by the NSP. It interacts with NIKs and suppresses its indirect regulation on ribosomal protein gene expression (Fontes et al., 2004). The plants deficient with *nik*-exhibited enhanced susceptibility to begomoviruses infection (Fontes et al., 2004). RLK Proline-rich extensin-like receptor kinase (PERK) like protein is exploited by the viral machinery to positively regulate viral protein NSP and enhance the infection of tomato crinkle leaf yellows virus (TCrLYV) and TGMV (Florentino et al., 2006). ToLCNDV NSP induces a hypersensitive response in *N. tabacum* and tomato through the N-terminal region of the protein. In contrast, the same protein induces leaf curling symptoms similar to that of viral symptoms in *N. benthamiana* (Hussain et al., 2005). ToLCNDV-NSP overexpression produces typical leaf curling symptoms and induces HR (Hussain et al., 2005). Similarly, bean dwarf mosaic virus (BDMV)-NSP elicits HR response in BDMV resistant bean cultivar Pinto bean cvs. Othello (Garrido-Ramirez et al., 2000).

2.2.2 BC1

This complementary-sense strand ORF encodes MP. It facilitates cell-cell viral movement through increasing plasmodesmata size exclusion limit (Noueiry et al., 1994). NSP-interacting syntaxin domain-containing protein (NISP) transports NSP-NIG-viral DNA complex to the

endosomes. From there, the connection between the MP and host protein, synaptotagmin (SYTA) that is a regulator of endosomes recycling, promotes viral genome transportation to plasmodesmata (Martins et al., 2020; Lewis and Lazarowitz, 2010). MP shows varied affinity and preferences to ssDNA, dsDNA, and other forms (Rojas et al., 1998). BDMV and tomato mottle virus (ToMoV) MP overexpressing plants develop abnormal phenotypes like viral infected plants, suggesting the role of MP in pathogenicity (Hou et al., 2000).

2.3 DNA Satellites

2.3.1 Alphasatellites

Alphasatellites are ssDNA molecules with approximately 1375 nucleotides. It possesses a hairpin loop having a conserved nonanucleotide sequence essential for replication, an A-rich region, and an alpha-Rep encoding ORF (Xie et al., 2010). Alphasatellites do not depend on helper viruses for their replication. Therefore, they are not strict satellites. The Rep protein of a few alphasatellites have PTGS suppression activity and is known to interact with the Rep of helper begomoviruses (Nawaz-ul-Rehman et al., 2010). Alphasatellite found associated with tomato yellow leaf curl virus-Oman strain (TYLCV-OM) along with betasatellite was reported to reduce symptoms and betasatellite accumulation (Idris et al., 2011). They also negatively regulate the transmission of helper viruses by whiteflies (Mar et al., 2017).

2.3.2 Betasatellites

Betasatellites are the additional satellite molecules found associated with monopartite begomoviruses. The first betasatellite was found in association with AYVV that enhanced the pathogenicity of AYVV to a greater extent (Saunders et al., 2000). βC1 complements the function of DNA-B as, in some cases, it is involved in the local and systemic movement of viruses (Saeed et al., 2007). βC1 interacts with CP of bhendi yellow vein mosaic virus (BYVMV) and karyopherin-α, a nuclear import protein (Usha et al., 2006). Being the major symptom and pathogenicity determinant in monopartite begomovirus infection, βC1 interacts with several host factors to suppress defense and establish disease (Gnanasekaran et al., 2019a).

βC1 protein interacts with SAHH (S-adenosyl homocysteine hydrolase), which is responsible for maintaining the methyl cycle during TGS and dampens TGS (Yang et al., 2011). An endogenous RNAi suppressor, Calmodulin-like protein (CaM), is upregulated by βC1 protein, triggering an

interaction cascade that leads to degradation of SGS3 and suppression of RDR6 activity, eventually affecting the antiviral RNA silencing process (Li et al., 2014a; Li et al., 2017). Cotton leaf curl Multan betasatellite (CLCuMuB) βC1 expressing transgenic plants showing increased expression of AGO1 and DCL1 underscore the nuanced anti-PTGS process in play (Eini, 2017). NtRFP1, a tobacco RING-finger protein, which functions as a ubiquitin E3 ligase interacts with βC1 protein and mediates βC1 ubiquitination, attenuating betasatellite mediated symptom expression (Shen et al., 2016). βC1 protein interrupts SKP1 and CUL1 interaction during CLCuMuV infection disrupting the proteasomal degradation pathway and altering plant hormonal signaling cascades (Jia et al., 2016). *Solanum lycopersicum* E2 enzyme UBC3 (Ubiquitin-conjugating enzyme 3) activity is also blocked by βC1, with the aftermath of decreased level of total polyubiquitinylated protein and increased symptom severity (Eini et al., 2009). *In vivo* and in vitro experiments showed that CLCuMuB βC1 protein interacts with autophagy-related protein NbATG8 through its ATG8 interacting motif (LVSTKSPSLIK) and directs it for degradation. Disruption of βC1-ATG8 interaction by a point mutation (V32A) in the ATG8 interaction motif promotes the virus replication and disease symptoms. Tomato yellow leaf curl China betasatellite (TYLCCB) encoded βC1 regulates Nbrgs-CaM, which induces degradation of NbSGS3 with the help of ATG factors (Li et al., 2017). βC1 protein CLCuMuB disrupts the interaction between a negative autophagic regulator and ATG3 protein to induce autophagy in *N. benthamiana* (Ismayil et al., 2020). SnRK1 phosphorylates TYLCCB encoded βC1 protein at serine 33 and threonine 78 residues, which negatively impacts the titers of both helper virus and betasatellite as well as disease development in *N. benthamiana* (Zhong et al., 2017). βC1 protein interacts with MKK2 and MAPK4 inhibiting the kinase activity and limiting the antiviral activity of MPK (Hu et al., 2019). Although betasatellite encoded βC1 protein does not have a direct impact on JA biosynthetic genes, it represses JA downstream marker genes such as *PLANT DEFENSIN1.2* (*PDF1.2*), *PATHOGENESIS RELATED4* (*PR4),* and *CORONATINE INSENSITIVE13* (*CORI3*), thus hampering the hormonal defense suppression mechanisms (Yang et al., 2008). As βC1 interacts with MYC2, the MYC2-mediated JA responses get suppressed (Li et al., 2014b). CLCuMuB βC1, when interacting with the DELLA protein, represses its degradation, affecting the GA response pathway. βC1 protein encoded from TYLCCB associated with TYLCCV inhibits terpene synthesis by interacting with MYC2 transcription factor (Li et al., 2014b).

Radish leaf curl betasatellite (RaLCB) encoded βC1 protein disturbs chloroplast organization, photosynthetic efficiency, and causes veinal chlorosis (Bhattacharyya et al., 2015). A recent finding suggests PsbP (photosystem II subunit P), an extrinsic protein of oxygen-evolving complex (OEC), plays a defensive role against geminiviruses (Gnanasekaran et al., 2019b). *PsbP*-silenced plants had higher virus replication levels than the control plants, and *PsbP* overexpression lines showed reduced disease symptoms evidenced by lower virus replication at early stages of infection. However, during later phases of infection, RaLCB-βC1 interacts with PsbP and permits successful virus replication (Gnanasekaran et al., 2019b). Recently βC1 encoded from ToLCPaB has been found to possess ATP hydrolyzing activity that regulates the betasatellite and viral DNA accumulation (Gnanasekaran et al., 2021).

2.4 Host defense strategies

Due to the coevolution of plants and pathogenic viruses, plants acquired multiple strategies to defend and counter viral infection and pathogenesis. However, viruses co-evolve to overcome such resistance responses (Kumar, 2019; Gupta et al., 2021). Plants have evolved to develop very complex defense strategies against geminiviral infection. RNA silencing machinery remains to be one of the prominent mechanisms. While TGS carries out viral genome methylation, consequently leading to the repression of viral pathogenicity proteins, PTGS mediates the degradation of the viral mRNAs, thereby suppress the viral infection. Several chromatin remodelers have evolved in plants that carry out repressive modifications on the host genome, divert to target the viral genome. MicroRNAs (miRNAs) have emerged as effective tools for achieving broad host defense regulatory mechanisms like autophagy, ubiquitination, hormonal signalling, protein kinases also play a significant role in guarding and shielding the host from geminivirus by providing the ammunition to the host to act against geminivirus. Against these wide arrays of defense mechanisms, various suppressor proteins are evolved, and sophisticated strategies are deployed by the geminiviruses that emphasize the dynamic relationship between the host and the geminiviruses in driving the coevolution. Figure 2.3 represents the overview of plant immune strategies against geminiviruses.

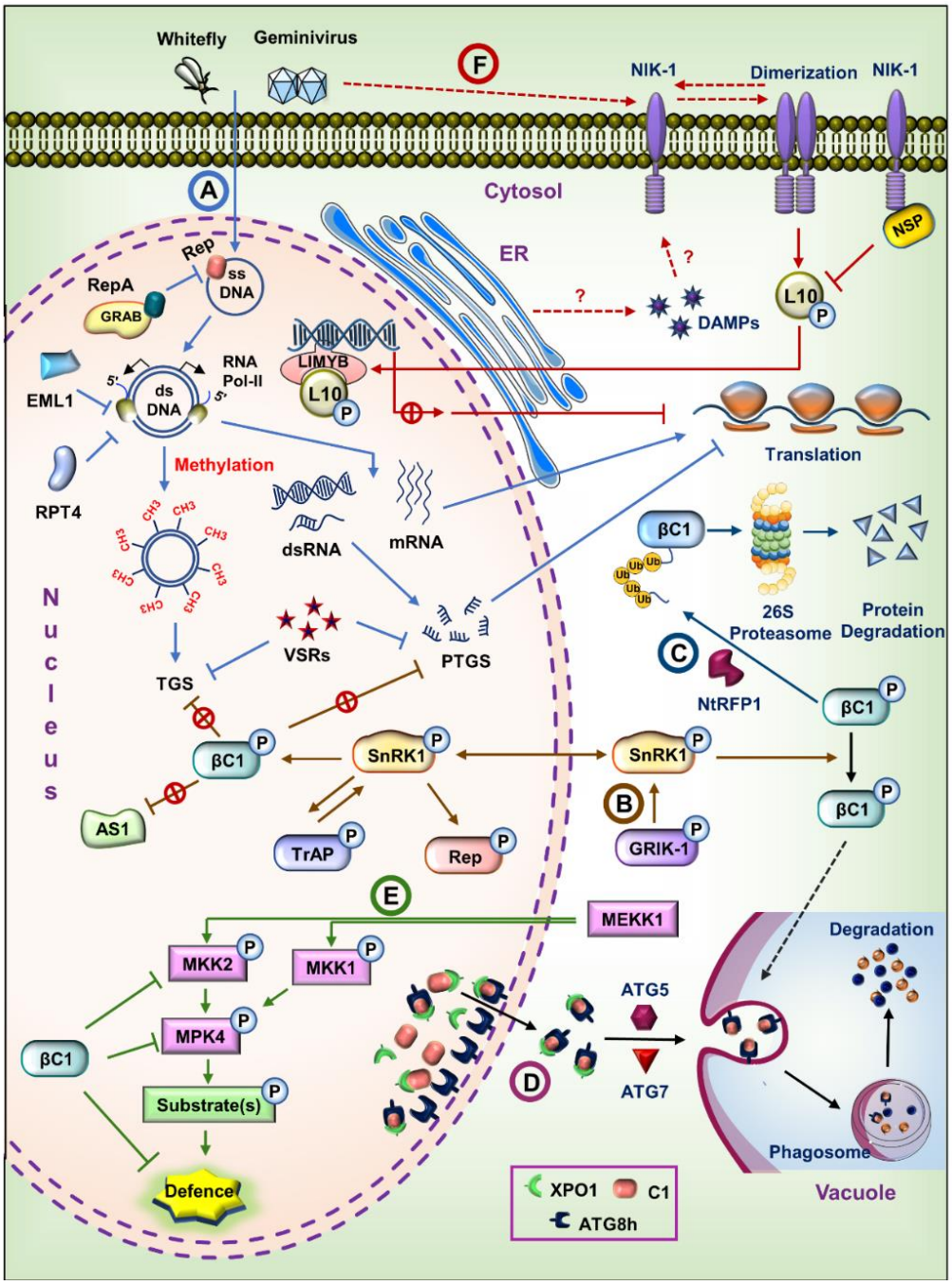


Figure 2.3 Schematic overview of plant immune strategies against geminiviruses.

Geminivirus infection initiates withthe release of viral ssDNA into the nucleus, subsequently leading to the replication, transcription and translation of viral genome. (A) Plants counteract geminivirus genetic life cycle via multiple host factors. GRAB interacts with RepA and interferes with the replication. RPT4a and EML1 hamper the geminivirus active transcription by obstructing the RNA Pol-II on virus euchromatin. Additionally, host induces RNAi via TGS and PTGS to suppress the viral gene expression. Virus-encoded VSRs potentially

suppress the RNAi. (B) Geminivirus induced GRIK1 autophosphorylates and activates SnRK1 which interact and phosphorylates the viral Rep, TrAP (AL2/C2) and βC1 protein. Phosphorylation of Rep and TrAP impedes Rep binding and causes a delay in the infection, respectively. βC1 phosphorylation hampers the TGS and PTGS suppressor functionalities and attenuates symptom expression via suppression of AS1-βC1 mediated downstream responses. Phosphorylated βC1 may also direct to autophagy. (C) Tobacco RFP1 interacts with βC1 and prompts the βC1 degradation via ubiquitin-mediated 26S proteasomal pathway and causes the symptom attenuation. (D) ATG8h interacts with nuclear C1 and translocates to cytosol XpoI dependent manner. The ATG8h-C1 complex is then recruited into autophagosomes with the aid of ATG5 and ATG7 for vacuolar degradation. (E) Defence regulated MEKK1-MKK1/MKK2-MPK4 module induced, activated by geminivirus infection, and exerts the basal defense response. However, βC1 protein directly interacts with MKK2 and MPK4, thereby suppressing the broad spectrum of downstream defense reactions. (F) NIK-1 from plasma membrane activated upon the geminivirus infection triggers dimerization and autophosphorylation. Alternatively, PTI induced DAMPs secreted from ER in response to virus attack may cause NIK-1 activation. Active NIK-1 phosphorylates and translocate L10 into the nucleus where it binds to LIMYB to block the transcription of ribosomal biosynthesis genes which affects the global translation and prevents the translation of viral genes (Adopted from Gupta et al., 2021).

Earlier attempts, extensive and laborious, were made to control the insect vector spread in the fields through pesticides and rouging infected plants. Later, the emergence of genetically modified (GM) crops with heightened antiviral activity had provided a promising approach to deal with various virus-induced stresses in plants. However, GM crops faced a considerable challenge and criticism regarding risk assessment towards consumers and the environment.

In this vast array of cellular pathways, the principal task is to identify the mechanisms where plant growth does not get compromised. Current efforts focus on using a precise gene-editing tool for providing a broad range of resistance against viruses. Various laboratories worldwide are standardizing the CRISPR-cas9 system for providing a broad range of adaptive immunity and resistance against geminiviruses. Lower viral titer and symptoms have been observed when different coding and non-coding regions of geminiviral DNA were targeted (Ali et al., 2016).
Extensive work is being undertaken to decipher mechanisms at the genetic and molecular levels in the context of geminiviral pathogenesis and defense. Molecular studies carried out to elucidate the viral strategies involving the characterization of putative targets in the cellular transcriptome, proteome, metabolome in the background of geminivirus interaction. Geminiviral diseases are increasing at an accelerated pace due to high evolution rate enlarging their geographical barrier

and host range. Although various techniques ranging from conventional methods to molecular approaches have been adopted to control the geminiviral infections, due to mixed virus infections the success is limited. To combat such situations, more efforts need to be channelized to find out the suitable host factors involved in the resistance during plant-geminivirus interaction.

Chapter 3

Objective 1

3. To study biochemical activities of βC1 encoded by different betasatellites

3.1 Introduction

Geminiviruses are twinned, non-enveloped, incomplete, icosahedral particles which possess circular, single-stranded DNA genomes. *Geminiviridae*, the most prominent family of plant viruses, comprises more than 500 species, infects numerous economically essential plants in the tropical and subtropical region of the world (Zerbini et al., 2017). Considering host range, type of transmission vector, genome organization, and phylogenetic relationship as criteria for classification, the family *Geminiviridae* is subdivided into fourteen genera, namely: *Becurtovirus, Begomovirus, Capulavirus, Citlodavirus, Curtovirus, Eragrovirus, Grablovirus, Maldovirus, Mastrevirus, Mulcrilevirus, Topocuvirus, Opunvirus, Topilevirus, Topocuvirus,* and *Turncurtovirus* (Fiallo-Olivé et al., 2021). The whitefly (*Bemisia tabaci* Genn.) transmitted begomovirus comprises the largest group of plant viruses that are most numerous and geographically widespread (Kumar et al., 2015). Begomoviruses possess genomes that are either bipartite, comprising two molecules of circular single-stranded DNA (ssDNA) termed DNA-A and DNA-B, or monopartite, containing a single DNA-A-like genomic component (Lazarowitz and Shepherd, 1992). Monopartite begomoviruses are primarily associated with single-stranded satellite DNA molecules, namely, alphasatellites, betasatellites, and deltasatellites (Saunders et al., 2000; Nawaz-ul-Rehman and Fauquet, 2009; Kumar et al., 2017). The majority of the old world's begomoviruses are monopartite and require betasatellite for their efficient infection and pathogenesis. Moreover, the association and maintenance of betasatellite with bipartite begomoviruses has also been reported (Sivalingam et al., 2010).

Betasatellites are small, circular, single-stranded DNA molecules of approximately 1.3 kb (Saunders et al., 2000). Betasatellites exacerbate the symptoms induced by its helper virus and cause enhanced accumulation of helper virus in the infected host plants. The typical genome of betasatellite contains a satellite conserved region (SCR), an adenine-rich region, and an ORF in a complementary sense strand that encodes the βC1 protein (Saunders et al., 2004). A new protein, βV1 encoded by virion sense strand of betasatellites, has recently been identified (Hu et al., 2020). Despite lacking substantial sequence similarities with its associated helper viruses,

betasatellite relies on its helper virus for replication, encapsidation, insect transmissibility, cell to cell, and long-distance movement (Zhou, 2013). Origin of replication of betasatellite encompasses stem-loop structure, SCR region, and part of the intergenic region immediately upstream of SCR (Briddon et al., 2001). Earlier studies demonstrated that betasatellite trans-replication could be achieved by distinct begomoviruses, indicating the more relaxed specificity of its origin of replication (Nawaz-ul-Rehman et al., 2012; Zhou, 2013).

The βC1 protein, the symptom determinant of TYLCCNB, interacts with ASYMMETRIC LEAVES 1 (AS1) protein in the molecular disguise of ASYMMETRIC LEAVES 2 (AS2), thereby alters the leaf polarity and suppresses the jasmonic acid response (Yang et al., 2008). βC1 protein interacts with an MYC2 transcription factor to repress the jasmonic acid-mediated plant resistance against whiteflies (Li et al., 2014b). CLCuMuB encoded βC1 protein elicits the betasatellite specific symptoms by interacting with a ubiquitin-conjugating enzyme, SlUBC3 (Eini et al., 2009). The tobacco RING E3 ligase NtRFP1 attenuates the TYLCCNB-βC1 induced symptoms by 26S proteasomal ubiquitination of βC1 (Shen et al., 2016). The βC1 protein functions as a suppressor of gene silencing and is required for symptom induction and effective virus infection (Cui et al., 2005; Li et al., 2014a). The βC1 protein also suppresses the host antiviral RNA silencing defense by upregulating the expression of host calmodulin-like protein named NbRgs-CaM (Li et al., 2014a). The tomato SUCROSE-NONFERMENTING1-related kinase (SlSnRK1) attenuates the symptom expression and geminivirus infection by interacting and phosphorylating the TYLCCNB-βC1 protein (Shen et al., 2011). TYLCCNB-βC1 regulates MAPK (Mitogen-activated protein kinase) cascade to suppress host defense responses and promote infection (Hu et al., 2019). RaLCB encoded βC1 interacts with chloroplast oxygen-evolving enhancer protein 2 (PsbP) and interferes with PsbP-mediated antiviral defense in plants (Gnanasekaran et al., 2019b). The βC1 protein encoded by CLCuMuB induces autophagy in host cells to enhance pathogenesis (Ismayil et al., 2020). Multifaceted biological functions of betasatellites encoded βC1 protein to influence the pathogenesis and accumulation of helper viruses and betasatellite in the infected plants necessitates comprehensive research.

Adenosine triphosphate (ATP) is an essential high-energy molecule that drives many metabolic reactions in a living cell. ATP acts as a substrate for kinase activities, signals several biological events, and plunges energy for motor proteins. Enzymes of several biological pathways commonly possess ATPase (ATP hydrolyzing) activity to utilize ATP (Fawaz et al., 2011).

ATPases are a class of enzymes that catalyze the hydrolysis of ATP to adenosine diphosphate (ADP) and free inorganic phosphate (Pi). Being the most abundant protein in the cell, ATPase is associated with several functions such as ATP synthase, ions transport, protein degradation, protein folding, DNA helicases, and gene regulation (Rappas et al., 2004; Islam et al., 2014). Almost all the biochemical pathway and transport of the cell is driven by energy released during the hydrolysis of ATP and other abundant cellular NTP; GTP, CTP, and TTP. NTPases domain of several viral proteins encompasses consensus phosphate-binding loop (Walker A motif, G/AXXXXGKT/S) required for ATP binding, Walker B motif (XXXXD) required for ATP hydrolysis, and motif C (conserved Arg residue) required for binding to γ-phosphate of ATP (George et al., 2014; Walker et al., 1982).

Geminivirus encoded replication-associated protein (Rep) possesses consensus Walker A motif, Walker B motif, and motif C to exhibit ATPases activity needed for its DNA-helicase motor function (George et al., 2014). However, several non-canonical ATPase do not possess consensus sequences for binding and hydrolyzing ATP. ATP-grasp fold enzymes superfamily is a group of proteins that bind to ATP with its typical ATP-grasp fold domain (Fawaz et al., 2011). In addition to the involvement of canonical and non-canonical motif/domain in ATPase activity, few viral proteins are reported to exhibit ATPase activity in their natively unfolded form. The natively unfolded C4 protein of cotton leaf curl Kokhran virus-Dabawali (CLCuKV-Dab) exhibits ATPase activity, and it does not contain canonical ATPase domain (Guha et al., 2013). Similarly, sesbania mosaic virus (SeMV) encoded polyprotein 2a domain P10 possesses ATPase activity and does not encompass the canonical ATPase domain (Nair and Savithri, 2010).

Recently, a novel ATPase activity has been found in the ToLCPaB encoded βC1 protein (Gnanasekaran et al., 2021). However, in which aspect of the begomovirus-betasatellite-host interaction, the ATPase activity of ToLCPaB-βC1 is involved, has not been explored yet. Therefore, present study has been carried out with the scope of exploring the significance of this molecular function of the βC1 and conserveness.

3.2 Materials and methods

3.2.1 Bioinformatics analysis

The weblogo tool (WebLogo - About (berkeley.edu) was used to study the conservation of amino acids amongst βC1 proteins. Clustal Omega program carried out multiple sequence alignment (Clustal Omega < Multiple Sequence Alignment < EMBL-EBI). The standard default threshold values were used for both tools used in this study.

3.2.2 Plasmid construction

Construction of wild-type and mutant protein expression clones

βC1 proteins encoded by croton yellow vein mosaic betasatellite (CroYVMB, GenBank accession number. FJ593630) isolate Pataudi referred to as CroYVMB-βC1, tomato leaf curl Joydebpur betasatellite (ToLCJoB, GenBank accession number. JN663863) isolate Nagpur referred to as ToLCJoB-βC1, tomato leaf curl Bangladesh betasatellite (ToLCBB, GenBank accession number. JN663876) isolate Vellanad referred to as ToLCBB-βC1 and Tomato yellow leaf curl Thailand betasatellite- India: Dhanbad (TYLCTHB, GenBank accession number. EU573713.1) referred to as TYLCTHB-βC1 were amplified by polymerase chain reaction (PCR) from respective monomer clones of CroYVMB, ToLCJoB, ToLCBB and TYLCTHB using specific primer pairs (Table. 3.1) and cloned into a pJET1.2 cloning vector. Further, the pGEX-6p2-βC1 expression construct was generated by cloning the βC1 ORF in frame with GST of pGEX-6p2 vector (GE Healthcare, Piscataway, NJ, USA) at *BamH*I/*Sal*I restriction sites for CroYVMB-βC1 and ToLCBB-βC1 and *Not*1/*Sal*1 for ToLCJoB-βC1 and TYLCTHB-βC1. All βC1 mutant protein expression constructs were ascertained by DNA sequencing. The protein expression constructs were transformed into *Escherichia coli* ArcticExpress (DE3) strain (Agilent technologies, New York, USA). In addition to the betasatellites mentioned above, other satellites used in this study are Tomato leaf curl Patna betasatellite (ToLCPaB), RaLCB, and Bhendi yellow vein betasatellite (BYVB).

Construction of wild-type and mutant proteins for Yeast two-hybrid (Y2H)

The βC1 ORF of ToLCPaB (Kumari et al., 2010) was PCR amplified from an infectious clone of ToLCPaB with a specific primer pair (ToLCPaBFP / ToLCPaBRP) and cloned into a pJET1.2 cloning vector. Further, the pGADC1- ToLCPaB-βC1 and pGBDC1- ToLCPaB-βC1 constructs

were generated by cloning the βC1 ORF in pGADC1 and pGBDC1 vectors at *BamH*I and *Sal* I restriction site. Similarly, pJET-ToLCPaB-βC1K49A, pJET- ToLCPaB-βC1R69A and pJET-ToLCPaB-βC1R91A constructs, cloned at *Cla*I and *Sal*I restriction sites were used to generate pGADC1-ToLCPaB-βC1K49A, pGBDC1-ToLCPaB-βC1K49A, pGADC1-ToLCPaB-βC1R69A, pGBDC1-ToLCPaB-βC1R69A, pGADC1-ToLCPaB-βC1R91A, and pGBDC1-ToLCPaB-βC1R91A. All the above-mentioned pJET-βC1 constructs were confirmed by PCR, restriction digestion, and DNA sequencing.

3.2.3 Expression and purification of wild-type and mutant βC1 protein

The expression conditions of GST-βC1 recombinant fusion protein were standardized as induction with 0.2mM isopropyl β-D-thiogalactoside (IPTG) induction followed by incubation at 12ºC, 200rpm for 24 hours. The protein expression strain of *E. coli*, ArcticExpress (DE3) expressing GST-βC1 was harvested by centrifugation at 4ºC, 5000 rpm for 5 minutes. The pelletized cells were suspended in lysis buffer containing 50mM Tris-HCl (pH 7.5), 100mM NaCl, 10% glycerol, 5mM β-mercaptoethanol, 1mM PMSF and 0.05% TritonX-100. The cell lysate was sonicated and pelletized at 4ºC, 13,000 rpm for 30 minutes. The supernatant obtained after centrifugation was used to purify the GST-βC1 fusion protein.

The purification of GST-βC1 protein was achieved by affinity chromatography using Glutathione resin (G-Biosciences, St Louis, USA). The protein-bound glutathione beads were washed with wash buffer containing 50mM Tris-HCl (pH 7.5), 500mM NaCl, 10% glycerol, 5mM β-mercaptoethanol, and 0.05% TritonX-100. After equilibration, the GST-βC1 fusion protein was eluted using buffer containing 25mM Tris-HCl (pH8.0), 100mM NaCl, 10% glycerol, 5mM β-mercaptoethanol, 0.05% TritonX-100 and 50mM reduced glutathione. GST protein was purified following the same procedure from *E. coli* ArcticExpress (DE3) strain transformed with the pGEX-6p2 vector. The purified GST-βC1 was further purified by anion-exchange chromatography using diethylaminoethyl (DEAE)-Sepharose beads (Sigma-Aldrich, St Louis, USA). The GST-βC1 bound to DEAE-Sepharose beads was eluted with Tris buffers containing 100mM, 150mM, 200mM, 250mM, 300mM, 350mM, 400mM, 450mM NaCl as final concentration. The expression and purification of all βC1 proteins (GST-ToLCJoB-βC1 GST-ToLCBB-βC1, GST-CroYVMB-βC1, GST-TYLCTHB-βC1, GST-BYVB-βC1, GST-RaLCB-βC1, and GST-ToLCPaB-βC1) were carried out using the above protocol.

Table 3.1 List and sequences of primers used in chapter 3.

S.No.	Primer	Sequence
1	TLCJBeC1 FP	GTCGACTATGTTTCACCTCACAGAATTAATC
2	TLCJBeC1 RP	GCGGCCGCTCATACATGTGCCATTTATATC
3	ToLCBBeC1 FP	GGATCCATGCAAATGACGATCAAGTATAC
4	ToLCBBeC1 RP	GTCGACTCACACACACACATTCGTACAG
5	TYLCTBeC1 FP	GTCGACTATGACGATCAAATACAAGAAC
6	TYLCTBeC1 RP	GCGGCCGCTTAAACAGATGAGTTCG
7	CYVBeC1FP	GGATCCATGACGATCATATATCAGAATG
8	CYVBeC1FP	GTCGACTTACACATTTACATATTTAGAC
9	ToLCPaBFP	GGATCCCAATGTTTAGACCCATAGAATTAAC
10	ToLCPaBRP	GTCGACTACACTTGCACGCGTATACACAC

3.2.4 ATPase assay

ATPase assays were performed as previously described (Islam et al., 2014; George et al., 2014). The desired amount of proteins and 0.2µCi of [γ-32P] ATP (6000 Ci mmol-1) was used in this study. The indicated protein was incubated in a buffer containing 20mM Tris-HCl (pH 8.0), 1 mM MgCl2, 100 mM KCl, 8 mM DTT, 80 µg/ml bovine serum albumin, 100 µM cold ATP, and [γ-32P] ATP in a total reaction volume of 10µl and incubated at 37˚C. After incubation, 1 µl of the reaction was spotted on a polyethyleneimine thin layer chromatography (TLC) plate (Sigma-Aldrich, St Louis, USA) and air-dried. Thin-layer chromatography was carried out with running solvent containing 0.5M LiCl and 1M HCOOH. Subsequently, the TLC plates were autoradiographed to detect the [γ-32P] ATP and cleaved [γ-32P] inorganic phosphate. An equal amount of purified GST protein was used as a negative control.

3.2.5 Western blotting

All βC1 proteins were loaded together in a single gel and resolved by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then electrophoretically transferred to the nitrocellulose membrane through semidry transfer apparatus

running at 5V, 60mA for an hour. Following a blocking by 5% BSA for 2 h at room temperature, the membrane was washed and probed with a primary anti-GST antibody (Sigma, St. Louis, USA). The membrane was then washed and probed with a secondary antibody, which is conjugated to a horseradish peroxidase enzyme (HRP). The substrate 3,3'-diaminobenzidine (DAB) was used for colorimetric detection of proteins.

3.2.6 Virus inoculation and DNA blotting

The *Agrobacterium tumefaciens* strain EHA105 carrying infectious partial tandem dimer of CroYVMB, ToLCJoB, ToLCBB, TYLCTHB, BYVB and RaLCB, and ToLCPaB were inoculated along with tomato leaf curl Gujrat virus (ToLCGV) referred to as VA in *N. benthamiana* plants as described previously (Kumari et al., 2010). Total genomic DNA was extracted from the uppermost symptomatic leaves (Singh et al., 2012). Detection of viral DNA from the isolated total DNA was carried out by southern blotting analysis following standard procedure. Total genomic DNA was run on 0.8% agarose gel and transferred to the Hybond-N^+ membrane. These membranes were probed with [α-^{32}P] dCTP-labeled DNA-probe specific for ToLCGV-Rep and autoradiographed with the phosphoimager.

3.2.7 Yeast Two-Hybrid assay

Saccharomyces cerevisiae strain AH109 cells were cotransformed by the required combination of Yeast two-hybrid constructs. To find out the oligomerization of ToLCPaB-βC1 protein and mutants ToLCPaB-βC1K49A, ToLCPaB-βC1R69A, ToLCPaB-βC1R91A, the plasmid combination pGADC1-ToLCPaB-βC1+pGBDC1-ToLCPaB-βC1, pGADC1-ToLCPaB-βC1K49A+pGBDC1-ToLCPaB-βC1K49A, pGADC1-ToLCPaB-βC1R69A+pGBDC1-ToLCPaB-βC1R69A and pGADC1- ToLCPaB-βC1R91A+pGBDC1- ToLCPaB-βC1R91A were cotransformed into AH109 cells. Likewise, the plasmid combination for negative control were pGADC1-ToLCPaB-βC1+pGBDC1, pGADC1+pGBDC1-ToLCPaB-βC1, pGADC1+pGBDC1-ToLCPaB-βC1K49A, pGBDC1+pGADC1-ToLCPaB-βC1K49A, pGADC1+ pGBDC1-ToLCPaB-βC1R69A, pGBDC1+pGADC1-ToLCPaB-βC1R69A, pGADC1+pGBDC1-ToLCPaB-βC1R91A, pGBDC1+ pGADC1-ToLCPaB-βC1R91A and pGADC1+pGBDC1. The interaction was checked by the ability of the transformants to grow on synthetic dropout media lacking Leu, Trp, His and containing 2 mM 3-amino-1, 2, 4-triazole (3DO + 3-AT) at 30ºC. Combination pGADT7-Rep+pGBKT7-Rep was used as a positive control.

3.2.8 Circular Dichroism Spectroscopy of wild-type and mutant proteins of ToLCPaB-βC1

Circular dichroism spectrum measurements of wild-type and mutant proteins of GST-βC1 of ToLCPaB were obtained at 25°C in a Quartz cell with a 1 mm light path with Chirascan Model of Applied Photosystem CD spectrophotometer (Greenfield 2006). An average of 3 scans was corrected for buffer contributions. The secondary structure content of the proteins was predicted by using K2D and CDNN software.

3.3 Results

3.3.1 Analysis of amino acids conservation in different βC1 proteins

The conservation of amino acid residues at the 49th, 69^{th,} and 91st positions which are crucial for ATP hydrolysis activity of ToLCPaB-βC1 was analyzed among βC1 proteins encoded by 66 distinct members of betasatellite using the weblogo tool (https://weblogo.berkeley.edu/). The result revealed the presence of conserved Lys/Arg residue at 49th and 91st positions of βC1 encoded by distinct betasatellites (Figure 3.1). Although Arg-69 of ToLCPaB-βC1 is important for its ATPase activity, this was found to be non-conserved among distinct βC1 proteins.

Considering the variations in the conservation of lysine/arginine residue at positions 49 and 91, βC1 proteins encoded by CroYVMB, ToLCJoB, ToLCBB, TYLCTHB, BYVB, and RaLCB were selected (Table. 3.2).

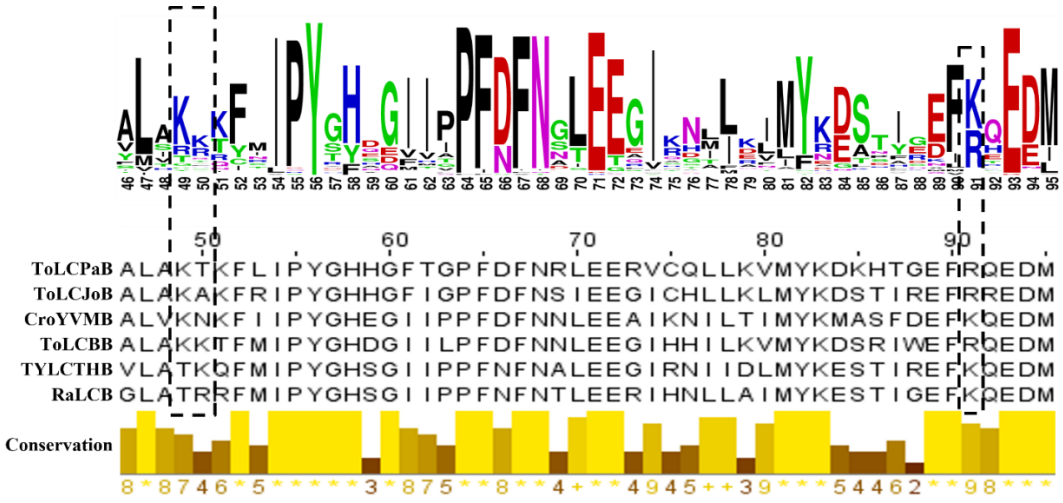


Figure 3.1 Sequence analysis of βC1 proteins obtained from diverse betasatellites.

Weblogo-generated depiction of the degree of conservation of amino acid residues of βC1 proteins encoded by 66 distinct betasatellites (top) and multiple-sequence alignment of βC1

proteins encoded by ToLCPaB, ToLCJoB, CroYVMB, ToLCBB, TYLCTHB, and RaLCB (bottom).

Table 3.2 Selected betasatellites and their amino acid differences at the 49th, 69th, and 91st positions.

Betasatellite	49	69	91
ToLCPaB	K	R	R
ToLCJoB	K	S	R
CroYVMB	K	N	K
ToLCBB	K	N	R
TYLCTHB	T	A	K
RaLCB	T	T	K

3.3.2 Cloning of different βC1 ORFs for protein expression and purification

3.3.2.1 Cloning of βC1 ORF in pJET1.2 cloning vector

All the βC1 ORFs – ToLCJoB-βC1, ToLCBB-βC1, TYLCTHB-βC1, and CroYVMB-βC1 were PCR amplified and cloned into a pJET1.2 cloning vector. PCR amplified product was extracted using gel extraction kit and ligated into pJET1.2 vector proceeded with the transformation of ligation product into *E. coli* DH5α competent cells. The transformed colonies were screened on an antibiotic selection plate containing 100µg/ml ampicillin. The pJET1.2-βC1 clones were confirmed by PCR and restriction digestion of the plasmid DNA. The PCR and restriction digestion confirmed clones were further confirmed by DNA sequencing analysis.

ToLCJoB-βC1 ORF was PCR amplified using primers TLCJBeC1FP and TLCJBeC1RP. Full length βC1 of approximately 381bp was observed. The product was cloned into the pJET1.2 vector and confirmed by PCR and restriction digestion (Figure 3.2).

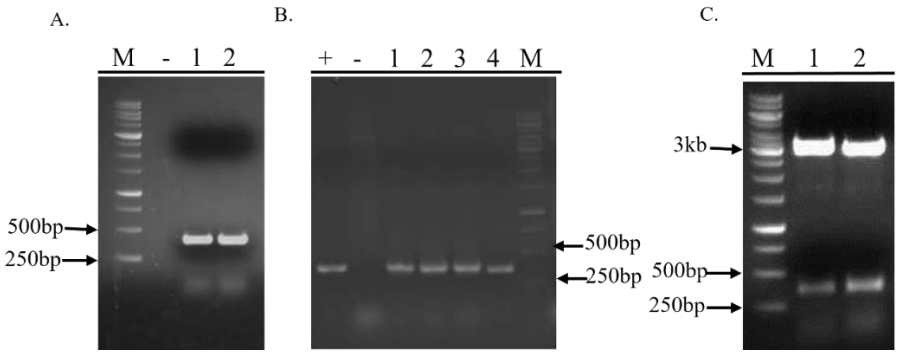


Figure 3.2 Cloning of ToLCJoB-βC1 in pJET1.2 vector.

(A) Agarose gel electrophoresis showing the amplification of ToLCJoB-βC1. (B) pJET-ToLCJoB-βC1 clone was confirmed by PCR using primers TLCJBeC1FP and TLCJBeC1RP. (C) Confirmation of pJET1.2-ToLCJoB-βC1 clone by restriction digestion with *Not*1 and *Sal*1 which shows release of approximately 381bp.

ToLCBB-βC1 ORF was amplified using primers ToLCBBeC1FP and ToLCBBeC1RP. Approximately 363bp amplified product representing full-length βC1 was observed. Figure 3.3 shows amplification of ToLCBB-βC1 ORF and confirmation of pJET1.2-ToLCBB-βC1 by PCR and digestion.

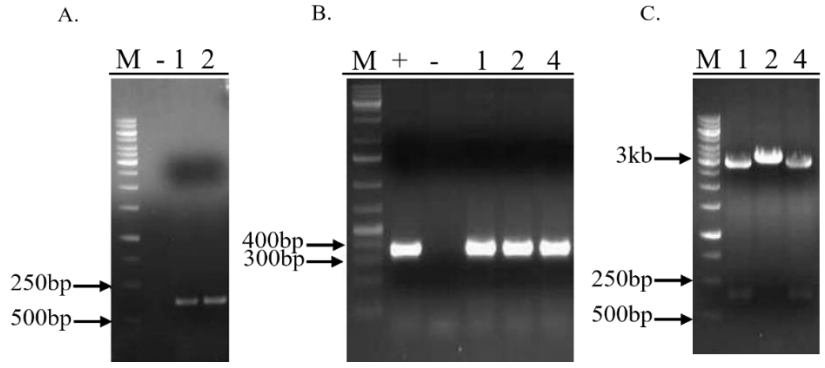


Figure 3.3 Cloning of ToLCBB-βC1in pJET1.2 vector.

(A) Agarose gel electrophoresis showing the amplification of ToLCBB-βC1. (B) pJET-ToLCBB-βC1 clone was confirmed by PCR using ToLCBBeC1FP and ToLCBBeC1RP primers. (C) Confirmation of pJET1.2-ToLCBB-βC1 clone by restriction digestion with *BamH*1 and *Sal*1 shows release of approximately 363bp.

TYLCTHB-βC1 ORF was PCR amplified using TYLCTBeC1FP and TYLCTBeC1RP primers pair. Approximately 357bp amplified product representing full-length βC1 was observed. The pJET1.2-TYLCTHB-βC1 clone was confirmed both by PCR and digestion (Figure 3.4).

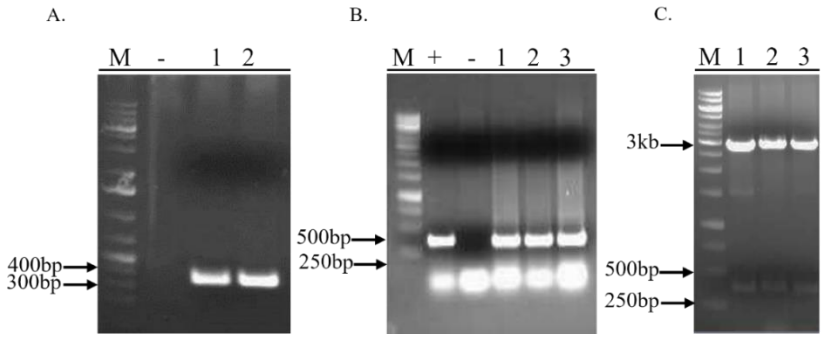


Figure 3.4 Cloning of TYLCTHB-βC1 in pJET1.2 vector.

(A) Agarose gel electrophoresis showing the amplification of TYLCTHB-βC1. (B) pJET1.2-TYLCTHB-βC1 clone was confirmed by PCR using TYLCTBeC1FP and TYLCTBeC1RP. (C) Confirmation of pJET1.2-TYLCTHB-βC1 clone by restriction digestion with *Not*I and *Sal*I shows the release of approximately 357bp.

Similarly, CroYVMB-βC1 ORF was confirmed by PCR and restriction digestion using CYVBeC1FP and CYVBeC1RP primer pair. Approximately 357bp amplified product representing full-length βC1 was observed (Figure 3.5).

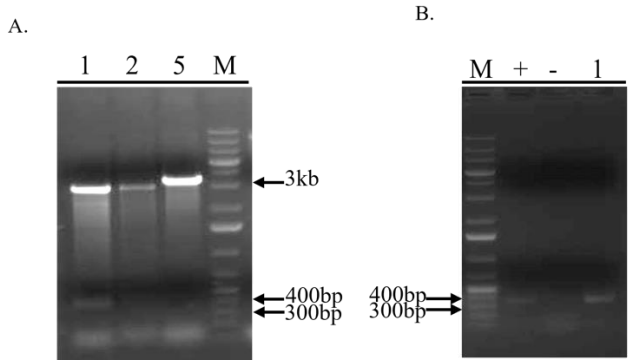


Figure 3.5 Cloning of CroYVMB-βC1 in pJET1.2 vector.

(A) Confirmation of pJET1.2 CroYVMB-βC1 clone by restriction digestion with *BamH*I and *Sal*I shows the release of approximately 357bp. (B) pJET-CroYVMB-βC1 clone confirmation was done by PCR.

3.3.2.2 Cloning of βC1 ORF in pGEX-6p2 vector

The pJET1.2-ToLCBB-βC1 confirmed plasmid DNA was digested with restriction enzymes, *BamH*1 and *Sal*1, and run-on agarose gel electrophoresis. Approx. 363bp fallout on agarose gel electrophoresis was extracted and cloned in frame to the pGEX-6p2 vector previously digested with *BamH*1 and *Sal*1 enzymes. The pGEX-6p2-ToLCBB-βC1 clones were confirmed by ToLCBB-βC1 specific PCR and restriction digestion with *BamH*1 and *Sal*1 (Figure 3.6). The pGEX-6p2-ToLCBB-βC1 expression constructs were transformed into ArcticExpress (DE3) protein expression strain. The ArcticExpress (DE3) strain harboring GST-ToLCBB-βC1 was confirmed by βC1 specific PCR.

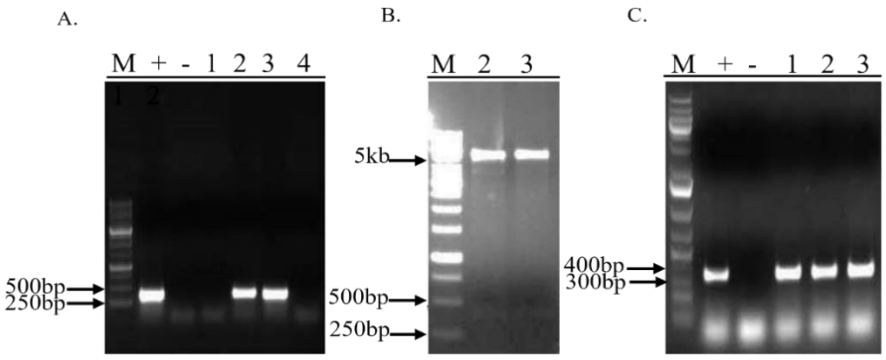


Figure 3.6 Confirmation of pGEX6p2-ToLCBB-βC1 clone.

(A) pGEX6p2-ToLCBB-βC1 clone confirmation by PCR using primers specific for ToLCBB-βC1. (B) Confirmation by restriction digestion with *BamH*1 and *Sal*1 showing release of approximately 363bp. (C) ToLCBB-βC1 specific PCR confirmation of plasmid isolated from ArcticExpress (DE3) *E. coli* strain transformed with pGEX6-ToLCBB-βC1expression construct.

The pJET1.2-ToLCJoB-βC1confirmed plasmid DNA was restriction digested with *Not*I and *Sal*I and resolved on agarose gel electrophoresis. Approx. 381bp fallout on agarose gel electrophoresis was extracted and cloned in frame to the pGEX-6p2 vector restriction digested with *Not*I and *Sal*I enzymes. The pGEX-6p2-ToLCJoB-βC1 clones were confirmed by ToLCJoB-βC1 specific PCR and restriction digestion with *Not*I and *Sal*I (Figure 3.7). The pGEX-6p2-ToLCJoB-βC1 expression constructs were transformed into an ArcticExpress (DE3) protein expression strain of *E. coli*. ArcticExpress (DE3) strain harboring GST-ToLCJoB-βC1 was confirmed by βC1 specific PCR.

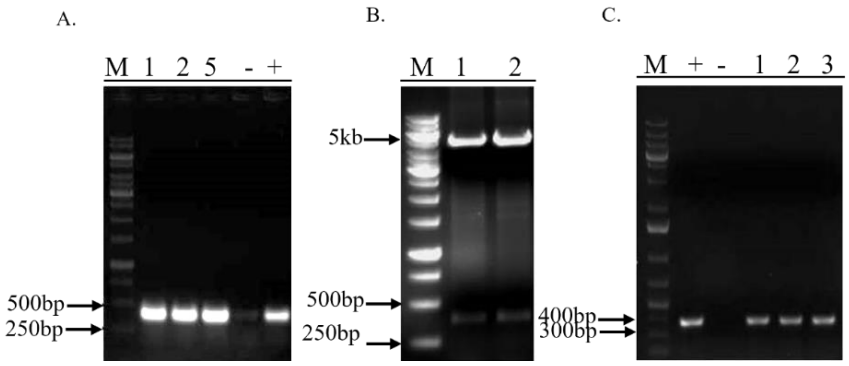


Figure 3.7 Confirmation of pGEX6p2-ToLCJoB-βC1 clone.

(A) PCR confirmation of pGEX6p2-ToLCJoB-βC1 clone using primers specific for ToLCJoB βC1. (B) Confirmation by restriction digestion with *Not*1 and *Sal*1 showing release of approximately 381bp. (C) ToLCJoB-βC1 specific PCR confirmation of plasmid from *E. coli* strain ArcticExpress (DE3) transformed with pGEX6p2-ToLCJoB-βC1 expression construct.

The pJET1.2-CroYVMB-βC1 confirmed plasmid DNA was restriction digested with *BamH*I and *Sal*I and resolved on agarose gel electrophoresis. Approx. 357bp fallout on agarose gel was extracted and cloned in frame to the pGEX-6p2 vector restriction digested with *BamH*I and *Sal*I enzymes. The pGEX-6p2-CroYVMB-βC1 clones were confirmed by CroYVMB-βC1 specific PCR and restriction digestion with *BamH*I and *Sal*I. The pGEX-6p2-CroYVMB-βC1 expression construct was transformed into *E. coli* stain ArcticExpress (DE3). The ArcticExpress (DE3) strain harboring GST-CroYVMB-βC1 was confirmed by βC1 specific PCR (Figure 3.8).

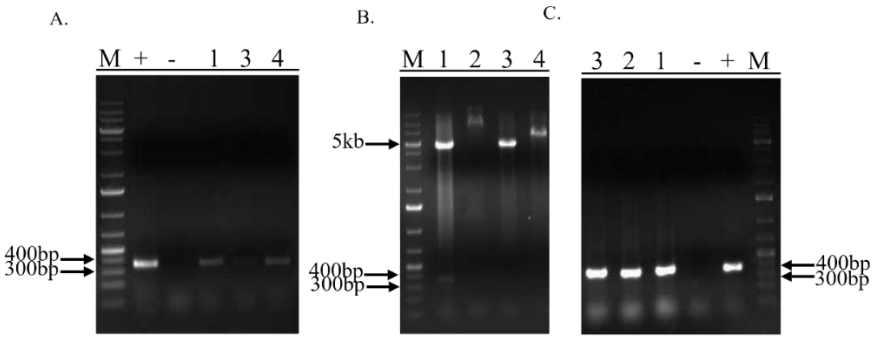


Figure 3.8 Confirmation of pGEX6p2-CroYVMB-βC1 clone.

(A) PCR confirmation of pGEX6p2-CroYVMB-βC1 clone using primers specific for CroYVMB βC1. (B) Confirmation by restriction digestion with *Sal*1 and *BamH*1 indicating release of approximately 357bp. (C) CroYVMB-βC1 specific PCR confirmation of ArcticExpress (DE3) strain of *E. coli* transformed with pGEX6-CroβC1 expression construct.

The pJET1.2-TYLCTHB-βC1 confirmed plasmid DNA was digested with *Not*I and *Sal*I and resolved on agarose gel electrophoresis. Approx. 357 bp fallout on agarose gel electrophoresis was extracted and cloned in frame to the pGEX-6p2 vector previously digested with *Not*I and *Sal*I enzyme. The pGEX-6p2-TYLCTHB-βC1 clones were confirmed by CroβC1 specific PCR and restriction digestion with *Not*I and *Sal*I (Figure 3.9). The pGEX-6p2-TYLCTHB-βC1 expression construct was transformed into ArcticExpress (DE3) protein expression strain of *E. coli*. The ArcticExpress (DE3) strain harboring GST-TYLCTHB-βC1 was further confirmed by TYLCTHB-βC1 specific PCR.

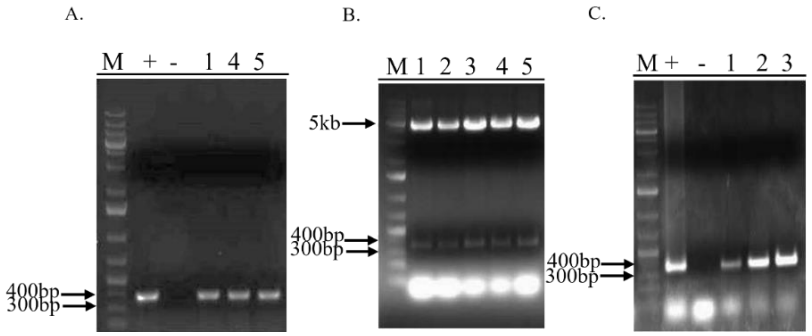


Figure 3.9 Confirmation of pGEX6p2-TYLCTHB-βC1 clone.

(A) Confirmation of clone by PCR using primers specific for DhanβC1. (B) Confirmation by restriction digestion with *Not*1 and *Sal*1 shows release of approximately 357bp. (C) TYLCTHB-βC1 specific PCR confirmation of *E. coli* ArcticExpress (DE3) strain transformed with pGEX6-TYLCTHB-βC1 expression construct.

3.3.3 Purification GST-βC1 fusion protein encoded by diverse betasatellites

E. coli strain ArcticExpress (DE3) harboring GST-RaLCB-βC1, expression construct was induced to express the GST-RaLCB-βC1 fusion protein. The GST-RaLCB-βC1 protein of approx. 40kDa was purified by affinity chromatography using glutathione sepharose beads (Figure 3.10A). The affinity purified GST-RaLCB-βC1 protein was further purified by ion exchange chromatography using DEAE Sepharose beads (Figure 3.10B).

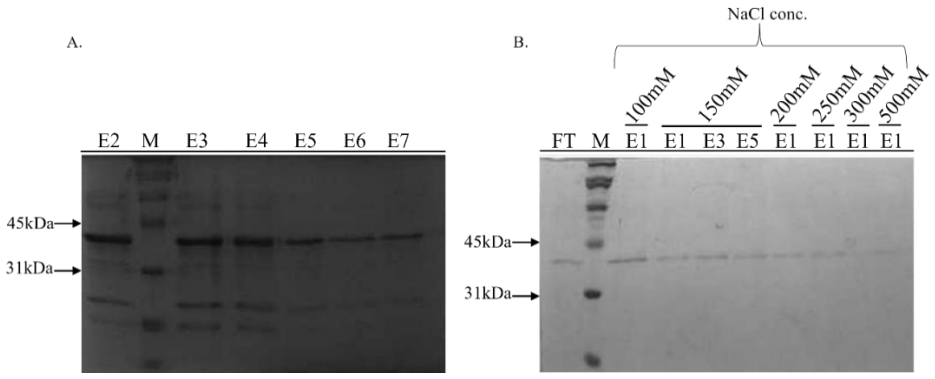


Figure 3.10 Purification of GST tagged RaLCB-βC1 protein.

(A) Purification initially by affinity chromatography, (B) further by ion exchange chromatography using DEAE Sepharose beads.

E. coli strain ArcticExpress (DE3) harboring GST-ToLCPaB-βC1 expression construct was induced to express the GST-ToLCPaB-βC1fusion protein. The GST-ToLCPaB-βC1 protein of approx. 40kDa was purified by affinity chromatography using glutathione sepharose beads

(Figure 3.11A). The affinity purified GST- ToLCPaB-βC1 protein was further purified by ion exchange chromatography using DEAE Sepharose beads (Figure 3.11B).

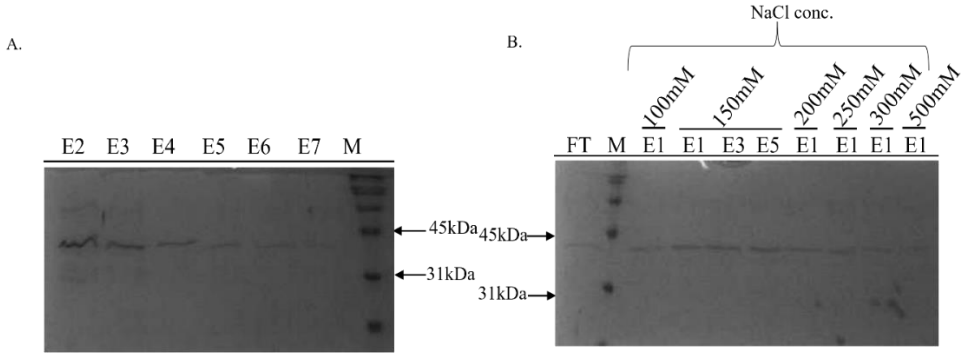


Figure 3.11 Purification of GST tagged ToLCPaB-βC1 protein.

(A) Purification initially by affinity chromatography, (B) followed by ion exchange chromatography.

E. coli strain ArcticExpress (DE3) harboring GST-ToLCJoB-βC1 expression construct was induced to express the GST-ToLCJoB-βC1 fusion protein. The GST-ToLCJoB-βC1 protein of approx. 40kDa was purified by affinity chromatography using glutathione sepharose beads (Figure.3.12A). The affinity purified GST-ToLCJoB-βC1 protein was further purified by ion exchange chromatography using DEAE Sepharose beads (Figure 3.12B).

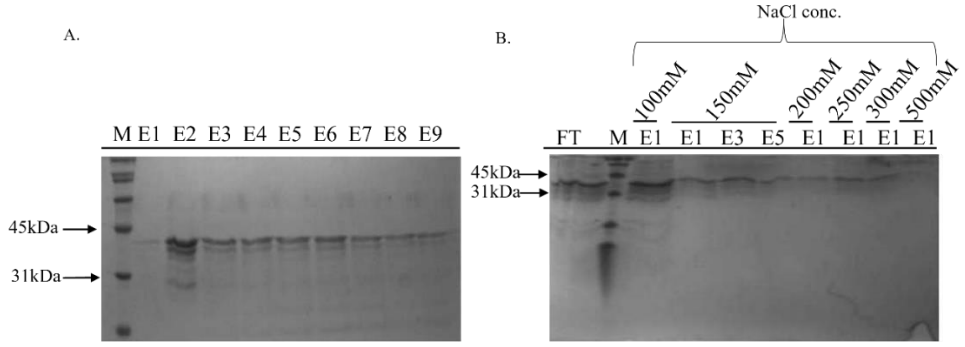


Figure 3.12 Purification of GST tagged ToLCJoB-βC1 protein.

(A) Purification initially by affinity chromatography, (B) followed by ion exchange chromatography.

The ArcticExpress (DE3) strain of *E. coli* harboring GST-TYLCTHB-βC1 expression construct was utilized to express the GST-TYLCTHB-βC1 fusion protein. The GST-TYLCTHB-βC1 protein of approx. 40kDa was purified by affinity chromatography using glutathione sepharose

beads. The affinity purified GST-TYLCTHB-βC1 protein was further purified by ion exchange chromatography using DEAE Sepharose beads.

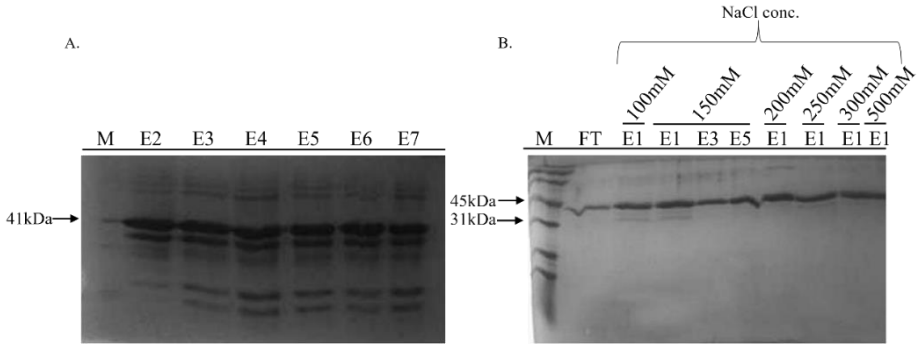


Figure 3.13 Purification of GST tagged TYLCTHB-βC1 protein.

(A) Purification initially by affinity chromatography, (B) followed by ion exchange chromatography.

The ArcticExpress (DE3) strain of *E. coli* harboring GST-CroYVMB-βC1 expression construct was induced to express the GST-CroYVMB-βC1 fusion protein. The fusion protein of approx. 40kDa was purified by affinity chromatography using glutathione sepharose beads (Figure 3.14A). The affinity purified GST- CroYVMB-βC1 protein was further purified by ion exchange chromatography using DEAE Sepharose beads (Figure 3.14B).

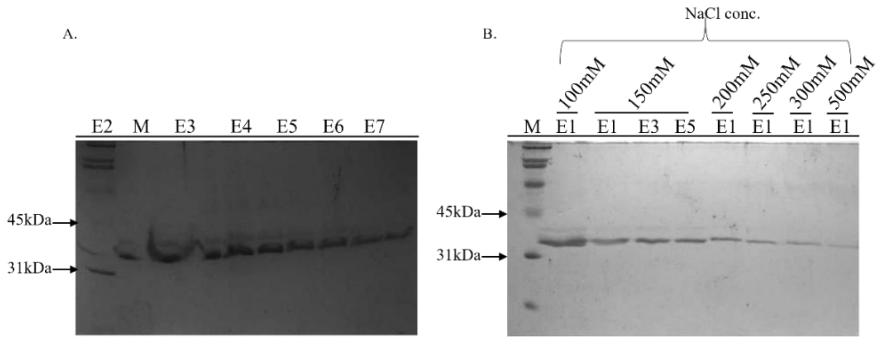


Figure 3.14 Purification of GST tagged CroYVMB-βC1 protein.

(A) Purification initially by affinity chromatography using glutathione Sepharose beads, (B) followed by ion exchange chromatography.

The ArcticExpress (DE3) strain of *E. coli* harboring GST-TYLCTHB-βC1 expression construct was induced to express the GST-TYLCTHB-βC1 fusion protein. The GST-TYLCTHB-βC1 protein of approx. 40kDa was purified by affinity chromatography using glutathione sepharose

beads (Figure 3.15A). The affinity purified GST-TYLCTHB-βC1 protein was further purified by ion exchange chromatography using DEAE beads (Figure 3.15B).

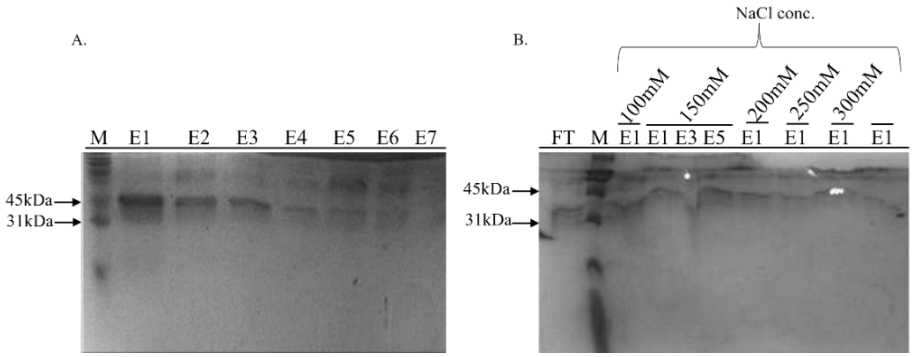


Figure 3.15 Purification of GST-TYLCTHB-βC1 fusion protein.

(A) Purification of GST tagged TYLCTHB-βC1 protein by affinity chromatography
(B) Purification of affinity purified GST-TYLCTHB-βC1 protein.

3.3.4 Analysis of GST-βC1 fusion proteins reveals purity and specificity of βC1 proteins

To test the specificity of the βC1 proteins purified by affinity chromatography followed by ion exchange chromatography, a western blotting assay was carried out. All selected elutes of different βC1 proteins were loaded together in a single gel and resolved by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Using semidry transfer apparatus, purified proteins were transferred to the nitrocellulose membrane. The membrane was probed with a primary antibody (anti-GST), proceeded by probing with a secondary antibody tagged with horseradish peroxidase enzyme (HRP). Detection of proteins was carried out by using DAB as a substrate in the presence of hydrogen peroxide solution. HRP precipitated DAB and brown color bands observed at around 40kDa, indicating specific GST-tagged βC1 proteins (Figure 3.16). Purified GST protein was used as a control.

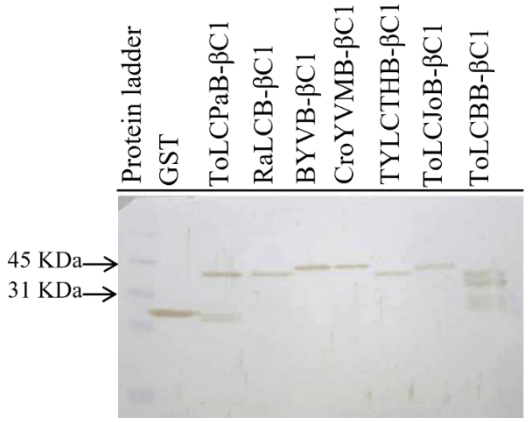


Figure 3.16 Western blot assay using an anti-GST antibody for analyzing GST-βC1 proteins.

All purified βC1 proteins were analyzed for specificity. Lane 1 shows the protein ladder. Purified GST protein (28kDa) was used as a control (Lane 2).

3.3.5 ATPase activity is ubiquitously displayed by diverse βC1 proteins

ATPase assays were carried out using the equal amount of purified selected βC1 proteins and ToLCPaB-βC1 protein indicated the positive control for the assay. All the tested βC1 proteins were indicated, according to these ATPase assays, to be able to hydrolyze ATP, whereas purified GST protein used as negative control did not. The ToLCPaB-βC1, RaLCB-βC1 and ToLCJoB-βC1 proteins showed slightly less ATP hydrolysis activity compared to others. The CroYVMB-βC1, ToLCBB-βC1, and ToLCJoB-βC1 proteins, each of which was found to have the conserved Lys/Arg residues at positions 49 and 91 similar to ToLCPaB-βC1, also showed ATPase activity (Figure 3.17). Surprisingly, TYLCTHB-βC1 and RaLCB-βC1, each lacking the Lys/Arg residue at position 49, nevertheless also showed ATPase activity. These proteins, however, were observed to have the Lys/Arg residue at position 50 (Figure 3.17). These results taken together suggested the presence of ATPase activity for all the tested βC1 proteins. In addition, from these results, it was concluded that conserved Lys/Arg residues at positions 49/50 and 91 are associated with the ATPase activity of the βC1 proteins.

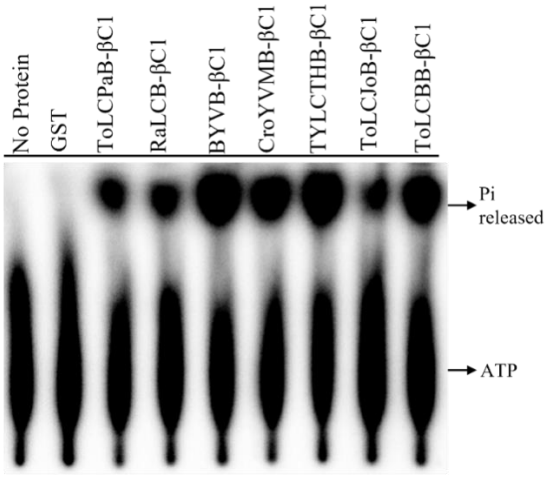


Figure 3.17 Autoradiograph of ATPase assay performed with purified βC1 proteins.

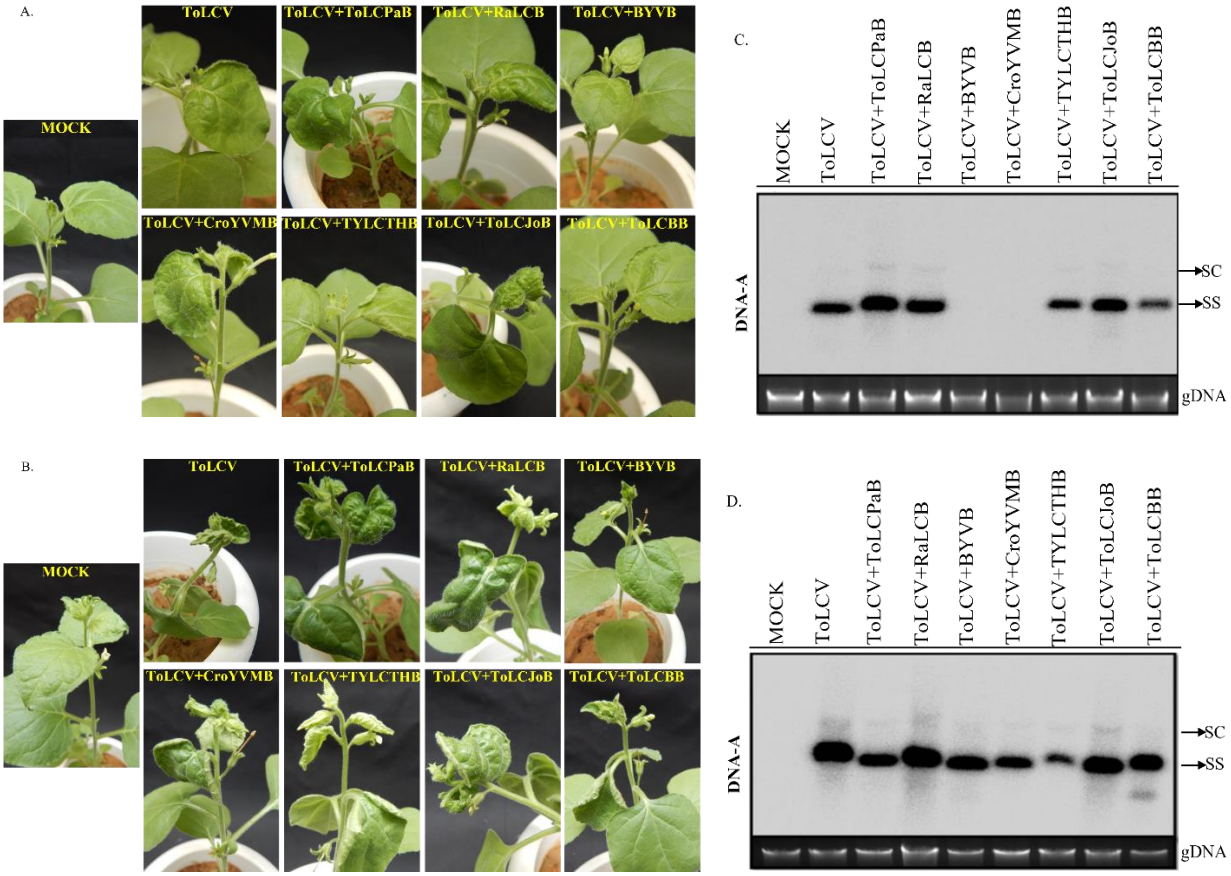
ATPase assay performed with equal amounts of purified βC1 proteins encoded by different betasatellites. The purified GST protein was used as a negative control. Pi indicates the released free inorganic phosphate, and ATP indicates the non-hydrolyzed [γ-32P] ATP.

3.3.6 ATPase activity of βC1 negatively regulates the accumulation of helper begomovirus at an early stage of infection

To identify the biological significance of the ATPase activity of βC1 from the perspective of viral pathogenesis, it was necessary to understand the effect of all these betasatellites on viral pathogenesis. For this purpose, *N. benthamiana* plants were inoculated with mock, ToLCGV (referred as A) alone, A+ToLCPaB, A+ToLCJoB, A+CroYVMB, A+ ToLCBB, A+TYLCTHB, A+RaLCB, A+BYVB. *N. benthamiana* plants inoculated with A alone showed typical symptoms such as upward leaf curling, vein thickening, and stunted plant growth (Figure 3.18). ToLCPaB, ToLCJoB, and RaLCB co-inoculated with A, showed typical betasatellite associated symptoms like downward leaf curling, vein thickening, and stunted plant growth. A+ToLCJoB and A+RaLCB also exhibited veinal chlorosis. Interestingly, all these three betasatellite encoded βC1 proteins- ToLCPaB-βC1, RaLCB-βC1, and ToLCJoB-βC1 showed lower ATP hydrolysis activity compared to others. However, *N. benthamiana* plants inoculated with A+CroYVMB, A+ToLCBB, A+TYLCTHB, and A+BYVB did not exhibit typical betasatellite associated symptoms, instead displayed DNA-A associated symptoms like upward leaf curling, stunted growth and thickening of vein as shown by plants infected with only A alone (Figures 3.18A, B). Likewise, it was observed that these betasatellites encoded βC1 proteins- CroYVMB-βC1,

ToLCBB-βC1, and TYLCTHB-βC1 showed increased ATPase activity. These observations suggested that the ATPase activity of βC1 protein may be associated with the ability of βC1 to regulate DNA-A associated symptoms.

The accumulation of the viral DNA from the uppermost systemically symptomatic leaves of infected plants was assessed using Southern blotting analysis. Here, the accumulation of helper virus in plants inoculated with A + ToLCPaB or A+ToLCJoB or A+ RaLCB, βC1 proteins of these betasatellites showed less ATP hydrolysis activity, was found to be more comparable with that in A alone infected *N. benthamiana* plants at early dpi (14 dpi) (Figure 3.18C). The accumulation of helper virus in plants inoculated with A+ToLCBB, A+TYLCTHB was found to be less compared to that of DNA-A alone infected plants at 14dpi. However, at later dpi, all combinations showed increased accumulation of helper virus infected plants (Figure 3.18D). These findings indicated that the ATPase activity of βC1 may negatively regulate the accumulation of helper begomovirus DNA.



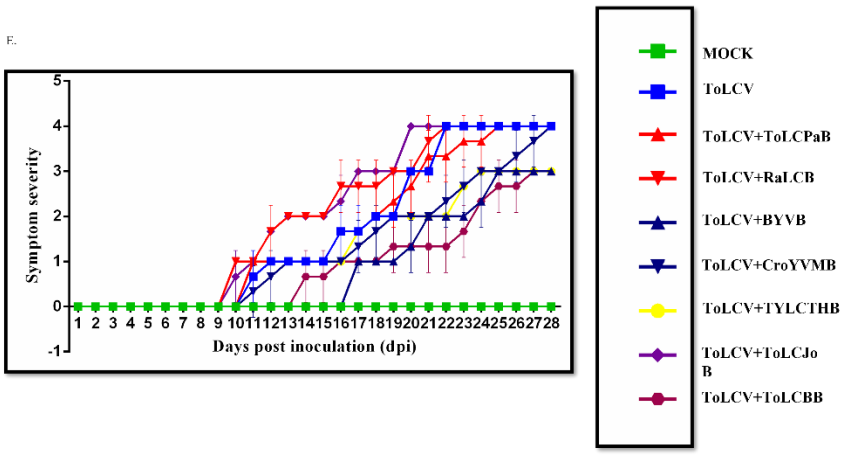


Figure 3.18 ATPase activity of βC1 negatively regulates helper begomovirus accumulation.

(A, C) Photographs of *N. benthamiana* plants infected with ToLCGV along with different betasatellite at 14 dpi and 21dpi, respectively. (B, D) Southern blotting analyses showing the comparative levels of the DNA of helper virus (ToLCGV) in the indicated infected *N. benthamiana* plants at 14 dpi (B) and 21dpi (D) Viral DNA forms are indicated as SC (supercoiled) and SS (single-stranded). An ethidium bromide (EB)-stained gel showing plant genomic DNA (gDNA) served as a loading control. (E) Disease progression graph showing symptom severity.

3.3.7 ATPase deficient mutants of ToLCPaB encoded ToLCPaB-βC1 protein maintains structural integrity

K49, R69, and R91 amino acid residues were found to be crucial for the ATPase activity of the ToLCPaB encoded ToLCPaB-βC1 protein (Gnanasekaran et al., 2021). Mutated ToLCPaB-βC1 proteins with an alanine substitution at these residues couldn't hydrolyze ATP. βC1 protein forms a large multimeric complex *in vivo* and *in vitro* (Cheng et al., 2011). To test the structural integrity of each of the strongly ATPase-altering βC1 mutants, circular dichroism (CD) and yeast two-hybrid assay was carried out. The CD spectra acquired of the purified GST-βC1 mutant proteins K49A, R69A or R91A were found to be similar to that of the GST-βC1 wild-type (Figure 3.19).

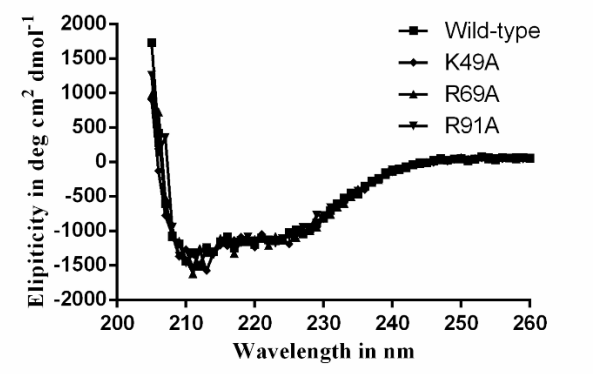
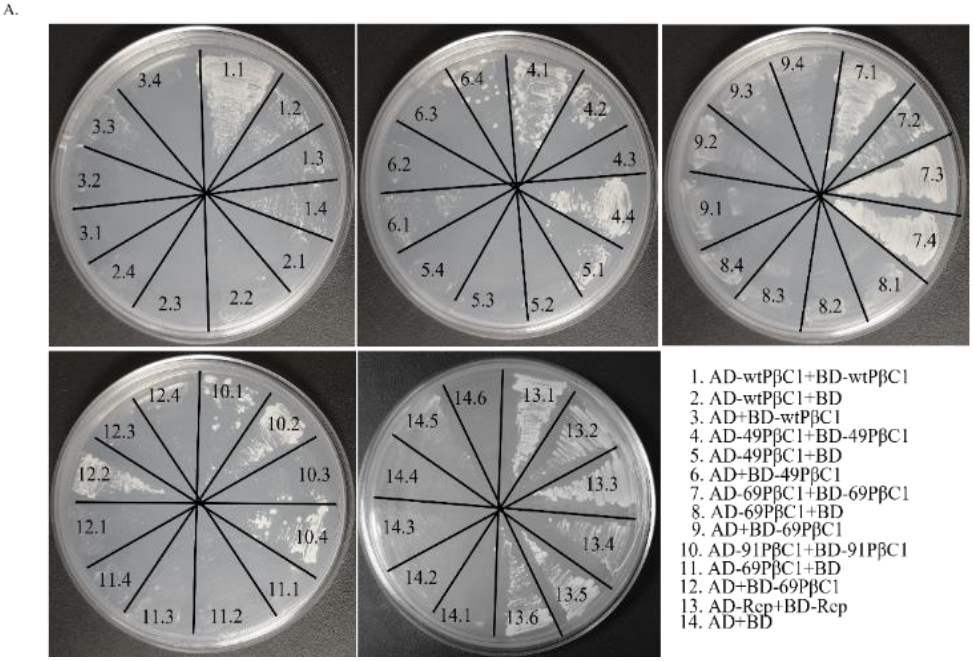


Figure 3.19 CD spectra of the wild-type and mutant βC1 proteins.

Furthermore, the results of yeast two-hybrid assays also indicated that ToLCPaB-βC1 (wild type) and mutant proteins ToLCPaB-βC1K49A, ToLCPaB-βC1R69A, and ToLCPaB-βC1R91A can interact with itself, therefore oligomerizes (Figure 3.20). Yeast with co-transformation of pGADC1-ToLCPaB-βC1+pGBDC1-ToLCPaB-βC1, pGADC1-ToLCPaB-βC1K49A+pGBDC1-ToLCPaB-βC1K49A, pGADC1-ToLCPaB-βC1R69A+pGBDC1+ToLCPaB-βC1R69A and pGADC1-ToLCPaB-βC1R91A+pGBDC1-ToLCPaB-βC1R91A grew on the selection medium supplemented with 2 mM 3-amino-1,2,4-triazole (3-AT) while the negative controls did not grow (Figures 3.20 A, B). Both CD and yeast two-hybrid assay conclude that mutations did not alter global conformation and structural integrity of βC1 proteins.



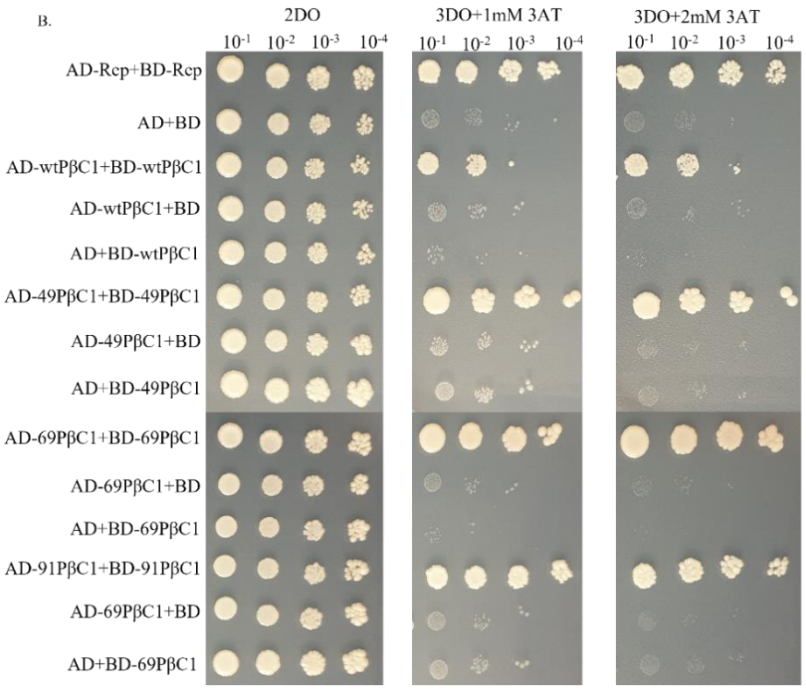


Figure 3.20 Yeast two-hybrid assay confirms oligomerization of ToLCPaB-βC1 protein and its ATPase deficient mutants.

(A) Growth of AH109 yeast cells after co-transformation in selection media with 2mM (3-AT) selection. (B) Growth of diluted cotransformed AH109 yeast cells in D-Leu-Trp-His-Ade (2DO) and D-Leu-Trp-His (3DO) selection media with 1.5mM and 2mM (3-AT) selection media.

3.4 Discussion

The pathogenicity determinant βC1 protein plays a pivotal role in viral pathogenesis by contributing to geminivirus virulence and mitigating the plant defense response. Earlier studies investigating the associations of the biological functions of βC1 in pathogenesis revealed that βC1 protein interacts with various host proteins to reinforce begomovirus virulence and suppress the host defense response. Recently, ToLCPaB-βC1 protein in its natively purified protein form has been reported to hydrolyze γ-phosphate of the substrate (Gnanasekaran et al., 2021). The current study demonstrates that this novel ATP hydrolysis activity exhibited by ToLCPaB-βC1 protein is conservative and has pathological implications during infection. All the betasatellites - CroYVMB, ToLCJoB, ToLCBB, TYLCTHB, BYVB, and RaLCB encoded βC1 proteins can hydrolyze ATP in the presence of divalent cation. The amino acid residues Lys-49/50, Arg-69, and Arg-91, which are crucial for ATP hydrolysis activity of ToLCPaB-βC1, are conserved in most of the βC1 protein encoded from distinct betasatellites. Even though the βC1 protein

sequence does not include the canonical ATPase motif, all the natively purified βC1 proteins could hydrolyze γ-phosphate of the substrate. Earlier findings revealed that the βC1 protein can bind DNA (Cui et al., 2005). Several studies have indicated that ATP binding and hydrolysis activity of DNA-binding proteins lead to changes in the conformations of these proteins that positively or negatively affect their ability to bind DNA. It is also reported that ToLCPaB-βC1 protein could bind ATP, and ATP binding controls the DNA binding activity of βC1 protein (Gnanasekaran et al., 2021). In some of the proteins like T7 gene 4-encoded helicase, NTP binding, and oligomerization are prerequisites for DNA binding property of the protein (Lee et al., 2008). The yeast two-hybrid analysis revealed that ToLCPaB-βC1 can oligomerize, which may favor the DNA binding property of βC1 protein. The ATPase deficient proteins where residues K49, R69, and R91 of ToLCPaB-βC1 protein were substituted with alanine do interact with itself, and therefore oligomerize. CD-spectroscopy also revealed the intact global structural integrity of all ToLCPaB-βC1 and its mutant proteins. It further strengthens findings of Gnanasekaran et al., 2021 that altered DNA bindings by mutant βC1 proteins in the presence of ATP shown was due to the absence of ATP binding in mutant protein, not due to any conformational change after mutation.

Transient expression of the βC1 protein has been found to induce leaf curling symptoms similar to those of betasatellite-infected plants (Cui et al., 2004; Saeed et al., 2005). A betasatellite mutant lacking the βC1 open reading frame (ORF) exhibited a typical symptom of DNA-A infection, and this observation corroborated the indispensable role of the βC1 protein in betasatellite symptom development. Nevertheless, betasatellites whose βC1 proteins exhibited lower ATPase activity were able to produce typical betasatellite associated symptoms in infected plants while others did not. It is important to note that increased accumulation of helper begomovirus was observed upon inoculation with each betasatellite encoding βC1 with lower ATP hydrolysis activity at an early phase of infection. This finding suggested that ATPase activity of the βC1 protein perhaps negatively regulates the replication of helper begomovirus to maintain an optimum level of viral titer.

Viral ATPase activity is mainly associated with biological motor functions, such as DNA packaging and translocation. In 2004, Cui et al. reported that a betasatellite associated with TYLCCNV enhances the DNA accumulation of helper begomovirus. Although the βC1 protein is dispensable for its replication, the replication/proliferation of betasatellite is highly

compromised (70%) in the absence of the βC1 protein (Saunders et al., 2008). However, mutual regulation of helper begomovirus and betasatellite replication in the infected plant remains unexplored.

ATPase activity has been shown to induce conformational changes in motor proteins to power the packing of viral DNA into the procapsid (Kreft and Jetz, 2007). Further, an ATP-hydrolysis-induced conformational rearrangement of amino acid residues of the baculovirus-encoded P143 protein has been shown to dissociate the DNA-protein complex (McDougal and Guarino, 2001). In conclusion, βC1 proteins encoded by different betasatellites were able to display novel ATPase activity in the presence of $MgCl_2$. The βC1 protein, despite lacking the Walker A and Walker B motifs and instead does so by using novel non-canonical motifs. Amino acid residues Lys-49/50, Arg-69, and Arg-91 of βC1, which are important for ATPase activity, are present in all βC1 proteins. The ATPase activity of βC1 negatively correlates with the accumulation of helper begomovirus and betasatellite.

Chapter 4

Objective 2

4. To elucidate the role of betaC1 in pathogenesis

4.1 Introduction

The increasing diversity of begomoviruses globally, with overlapping host range and vector specificity, expanded the scenario of mixed viral infections in the same plant. Distinct viral species infecting the same plant may result in additive, synergistic or antagonistic interactions (Moreno & López-Moya, 2020). Frequently, mixed infection under natural conditions has significant pathological, biological, and epidemiological consequences. In the 20th century, the outbreak of cassava mosaic disease led to heavy economic loss due to synergistic interaction between East African cassava mosaic virus- Uganda strain (EACMV-UG) and ACMV (Legg and Fauquet, 2004; Zinga et al., 2013). Synergism between Cameroon strain of ACMV (ACMV-[CM]) and East African cassava mosaic Cameroon virus (EACMCV) also led to increased symptom severity and DNA accumulation in tobacco and cassava. The combinatorial effect of the pepper golden mosaic virus (PepGMV) and pepper huasteco yellow vein virus (PHYVV) in multiple hosts also represents a typical model for mixed infection (Rentería-Canett et al., 2011). Likewise, many virus species associated with tomato leaf curl disease coexist together. Both TYLCV and TYLCSV can infect tomatoes together and are present in a single nuclei (Morilla et al., 2004). Reassortment of tomato yellow leaf curl Mali virus (TYLCMV) with cotton leaf curl Gezira betasatellite (CLCuGB), a subviral agent during mixed infection conditions, led to severe disease in tomatoes in Mali (Chen et al., 2009). The synergistic interaction between begomovirus and betasatellites has also been found to break down the resistance in chilli cultivar that is resistant to begomoviruses (Singh et al., 2016).

Begomovirus, the largest genus in the family, *Geminiviridae*, comprises 445 species out of approximately 520 species of geminiviruses identified so far (Fiallo-Olivé et al., 2021). They infect a broad range of economically important dicotyledonous plants worldwide and transmit by the insect vector, whitefly (Brown et al., 2015). Most of the begomoviruses that belong to the New World, such as TGMV, ACMV, PepGMV, are bipartite. They possess two DNA molecules, DNA-A and DNA-B, each approximately 2.7 kb in length. However, both monopartite and bipartite begomoviruses are prevalent in the Old World. Monopartite begomoviruses have a single genomic component that is homologous to the DNA-A molecule of bipartite begomoviruses. The DNA-A component contains open reading frames (ORFs) encoding five to

seven proteins, while DNA-B codes for two proteins. The complementary sense strand proteins encoded by DNA-A are replication-associated rep protein (REP; AC1), transcription activator protein (TrAP; AC2), replication enhancer protein (REn; AC3), and AC4 protein, while the virion sense strand encodes for coat protein (CP; AV1) and pre-coat protein (AV2). The New World bipartite begomoviruses lack AV2 ORF (Fondong, 2013). DNA-B has two ORFs - BC1 and BV1, that code for MP and nuclear shuttle protein (NSP). Geminiviral proteins coordinate to facilitate replication and movement and circumvent a plant's defense response to establish a successful infection process (Hanley-Bowdoin et al., 2013; Kumar, 2019).

During most monopartite begomovirus infections, a subviral component called betasatellite plays an essential role (Saunders et al., 2001; Cui et al., 2005). Betasatellites rely on the helper begomoviruses for proliferation, movement, encapsidation, and insect transmission (Zhou, 2013). With a genome of nearly 1350nt, and two proteins βV1 and βC1 encoded in virion and complementary sense strand, respectively, the betasatellites are indispensable for symptom induction, inhibition of host defense responses, and insect transmission (Zhou, 2013; Gnanasekaran et al., 2019; Saeed et al., 2005; Hu et al., 2020). Besides, betasatellite contain a satellite conserved region (SCR) necessary for Rep mediated replication of betasatellite and an adenine-rich region required for the size maintenance of betasatellites (Briddon et al., 2003). Betasatellites do not share sequence homology with their cognate helper viruses except at hairpin structure in the SCR region which has conserved nonanucleotide sequence essential for replication (Saunders et al., 2008). Lack of sequence similarity and absence of Rep binding iterons in betasatellites compared to helper viruses facilitates the trans-replication of betasatellites by both cognate and non-cognate helper viruses (Zhang et al., 2016; Kumari et al., 2010). Consequently, mixed infections in the field increase the diversity of geminivirus-betasatellite complexes that enhance the probability of disease occurrence in new hosts. In a few cases, betasatellites also regulate the pathogenesis of bipartite begomoviruses (Sivalingam and Varma, 2012).

Such complexity of interactions during the begomoviral pathogenesis requires more attention. Furthermore, the disease etiology behind the mixed infections, including the begomoviral-betasatellite synergism, is unknown. Viruses are known to regulate infection by expanding the functionality of their proteome through combinatorial interactions between viral proteins (Wang

et al., 2021). In India, tomato cultivation is significantly affected by tomato leaf curl disease (ToLCD) caused by the most widespread begomovirus, TYLCNDV. The association of ToLCNDV with betasatellites has increased the severity and drastically limited tomatoes production (Sivalingam and Varma, 2012). The current study unfolds one of the interactive regulations between ToLCNDV DNA-A encoded AC2 protein and the non-cognate RaLCB encoded βC1 protein.

4.2 Material and methods

4.2.1 Agroinfiltration

Agrobacteriumtumefaciens containing an infectious partial tandem dimer of ToLCNDV and ToLCNDVΔAC2 mutant along with RaLCB was cultured in rifampicin (30 µg/ml) and kanamycin (50 µg/ml) containing LB media for 36-48 hours at 28°C. The culture was centrifuged at 5000 rpm for 5minutes, and the pellet was resuspended in agroinfiltration buffer (10mM MES, 10mM $MgCl_2$, and 100µM acetosyringone; pH 5.6-5.8). The OD of culture was set to 0.5 at 600nm. Dark incubated three-weeks-old *N. benthamiana* plants were infiltrated from the abaxial side using a 1ml syringe. Plants were observed for symptoms and photographed at 14 and 21dpi.

4.2.2 RNA isolation and cDNA preparation

Samples from infected *N. benthamiana* plants infiltrated with ToLCNDV, ToLCNDV+RaLCB, and ToLCNDVΔAC2+RaLCB were collected at 5^{th} day post-inoculation (dpi). RNA isolation was carried out following Singh et al. (2016). Leaf tissue (100mg) was homogenized in 1ml Trizol reagent using liquid nitrogen. Chloroform (200 µl) was added to the grounded tissue to remove protein impurities, and samples were centrifuged at 12000 rpm for 10 mins, preceded by incubation at room temperature. The supernatant was transferred in a fresh microcentrifuge tube, followed by RNA precipitation using isopropanol. The precipitated RNA was centrifuged at 13000 rpm at 4°C for 20 min. Pellet was washed to remove salt impurities with 70% ethanol and air dried at room temperature. The thoroughly dried pellet was dissolved in RNase-Free Distilled water.

Synthesis of complementary DNA (cDNA) was done using an iScript cDNA synthesis kit (BIO-RAD) following the protocol mentioned in the user manual. In a 20µl reaction, 1µg of RNA was

incubated with 4µl of 5X iScript mix, in addition to remaining RNAase-free water. The reaction conditions were: 5 min at 25^{o}C, 20 min at 46^{o}C, 1 min at 95^{o}C, and hold at 4^{o}C.

4.2.3 Quantitative real-time PCR (qRT-PCR) and analysis

For qRT-PCR, 10µl of 2X SSoAdvanced Universal SYBR Green Supermix (Biorad), 0.7µl of 10µM of forward and reverse primer, 2µl of 50ng cDNA, was added in a total volume of 20µl of nuclease-free water. Applied Biosystems 7500 fast real-time PCR was programmed for DNA denaturation at 95^{o}C for 30 sec, again 95^{o}C for 15sec (40 cycles), annealing and extension at 60^{o}C for 30 sec. Reference control used for normalization was protein phosphatase 2A. Relative gene expression was determined by the ΔCt method.

4.2.4 Yeast Two-Hybrid assay

To identify the viral interacting partner of βC1 protein, *Saccharomyces cerevisiae* strain AH109 cells were cotransformed by the desired combination of Yeast two-hybrid constructs. The plasmid combinations used for yeast two-hybrid analysis were pGBKT7-RlβC1+pGADT7-AC2, pGBKT7-RlβC1+pGADT7-AC4, pGBKT7-RlβC1+pGADT7-AV2 with their respective negative controls pGBKT7+pGADT7-AC2, pGBKT7+pGADT7-AC4, and pGBKT7+pGADT7-AV2. The other negative controls included were pGBKT7-RlβC1+pGADT7 and pGBKT7+pGADT7. pGADT7-Rep + pGBKT7-Rep combination was used as a positive control. The interaction was determined by the ability of the transformants to grow on synthetic dropout (SD) media lacking Leu, Trp, His and containing 2mM 3-amino-1, 2, 4-triazole (3DO + 3AT) at 30ºC.

4.2.5 Plasmid construction for Bimolecular Fluorescence complementation (BiFC) assay and subcellular localization

The AC2 ORF (420bp) of ToLCNDV DNA-A was PCR amplified from a monomeric clone of ToLCNDV-DNA-A with primer pair AttBAC2FP/AttBAC2RP and cloned into a gateway based pDONR vector for constructing entry clone. Similarly, RlβC1 encoded from RaLCB was cloned into the same vector using the primer pair attBRBetC1FP/attBRBetC1RP. For BiFC studies, pSITE-EYFP-N1: βC1 and pSITE-EYFP-C1:AC2 constructs were generated using pDONR-βC1 and pDONR-AC2 clones, respectively. All the clones were confirmed by PCR and sequencing. AC2 ORF was amplified from the confirmed pJET1.2-AC2 construct and cloned into the

pCAMBIA1302 vector at *NcoI* and *SpeI* sites for localization studies. Further, the clones for in planta expression were transformed into *A. tumefaciens* strain GV3101 and confirmed by PCR. All the primers used are mentioned in Table 4.1

Table 4.1 List and sequences of primers used in chapter 4.

S.No.	Primer	Sequence
1	AttBAC2FP	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGCAGTC TTCATCACACTC
2	AttBAC2RP	GGGGACCACTTTGTACAAGAAAGCTGGGTTAGGACCTGG GTTTTGAAGAC
3	attBRBetC1FP	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGACGAT CAAATACAAAAAC
4	attBRBetC1RP	GGGGACCACTTTGTACAAGAAAGCTGGGTTTACAGATGA ACGCGTATAC
5	NAC2LOFP	CCATGGGAATGCAGTCTTCATCACACTCGA
6	NAC2LORP	ACTAGTAGGACCTGGGTTTTGAAGACTCTC

4.2.6 Confocal microscopy

For BiFC, three-four weeks old *N. benthamiana* plants were infiltrated with *Agrobacterium* strain GV3101 harboring pSITE-EYFP-N1: βC1 and pSITE-EYFP-C1:AC2. The epidermal cells of infiltrated leaves were visualized for yellow fluorescence obtained from reconstituted yellow fluorescence protein (YFP) under TCS SP8 X confocal microscope (Leica Germany) after 48 hours post-infiltration. 4′,6-diamidino-2-phenylindole (DAPI) was used for nuclear detection with 340-380nm excitation and 435-485 nm emission filters. For the YFP signal, excitation and emission filters were set at 465-495nm and 520-540nm. Subcellular localization experiment was carried out by infiltrating the pCAMBIA1302-AC2 construct, followed by the same protocol mentioned above. For green fluorescent protein (GFP) signal, excitation and emission were at 465-495nm and 515-555nm, respectively.

4.3 Results

4.3.1 AC2 protein regulates symptom development associated with βC1 protein

Agrobacterium strain EHA105 carrying infectious partial tandem repeat of ToLCNDV DNA-A, ToLCNDVΔAC2, ToLCNDVΔAC4, ToLCVNDΔAV2 were infiltrated along with RaLCB in three-weeks-old *N. benthamiana* plants. Symptoms were observed until 21 dpi. At 14dpi, plants infiltrated with ToLCNDV DNA-A and RaLCB together exhibited downward curling of leaves, veinal chlorosis, stem bending, and stunted growth (Figure 4.1). In comparison, ToLCNDVΔAC2 and RaLCB coinfected plants showed mild leaf curling and enations that disappeared on later stage of infection. Plants infiltrated with ToLCNDVΔAC4 along with RaLCB showed typical symptoms associated with RaLCB. However, ToLCVNDΔAV2 with RaLCB and ToLCNDV DNA-A infected plants showed mild symptoms, and no symptoms were observed in mock plants. The absence of typical betasatellite associated symptoms like veinal chlorosis in plants infiltrated with ToLCNDVΔAC2 and RaLCB suggests that the AC2 ORF of ToLCNDV assists or regulates betasatellite associated symptom development. This observation suggests a possibility of synergistic interaction between AC2 and βC1 at DNA level via trans-activation or at protein level via AC2-βC1 interaction.

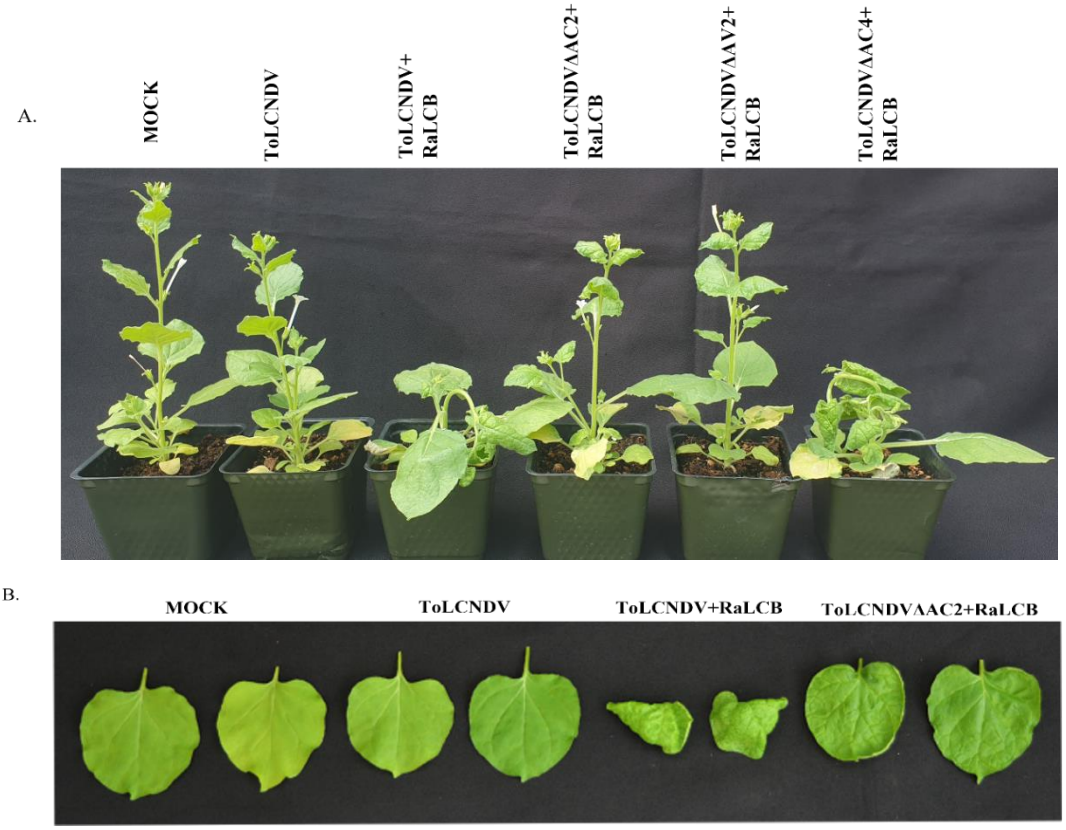


Figure 4.1 RaLCB associated symptom development on *N. benthamiana*.

(A) Symptoms induced on *N. benthamiana* plants infected with ToLCNDV and its mutants along with RaLCB at 14dpi. (B) Comparison of leaves of *N. benthamiana* plants infected with ToLCNDV+RaLCB and ToLCNDVΔAC2+RaLCB.

4.3.2 AC2 protein regulates transcript accumulation of βC1 during infection

Since the ToLCNDVΔAC2 + RaLCB coinfiltrated plants did not show symptoms associated with RaLCB, βC1 transcript accumulation was detected at 5dpi by qRT-PCR. Compared to the ToLCNDV+RaLCB infiltrated *N. benthamiana* plants, βC1 transcript accumulation was reduced drastically in plants coinfiltrated with ToLCNDVΔAC2+RaLCB. However, the transcript level of AC2 in plants infected with ToLCNDV+RaLCB compared to ToLCNDV DNA-A alone was not significantly altered (Figure 4.2). This suggests the role of AC2 protein in the transcript accumulation of βC1. Furthermore, on scanning the sequence of RaLCB, conserved late elements involved in the AC2 mediated transactivation of other ORFs and host genes were found.

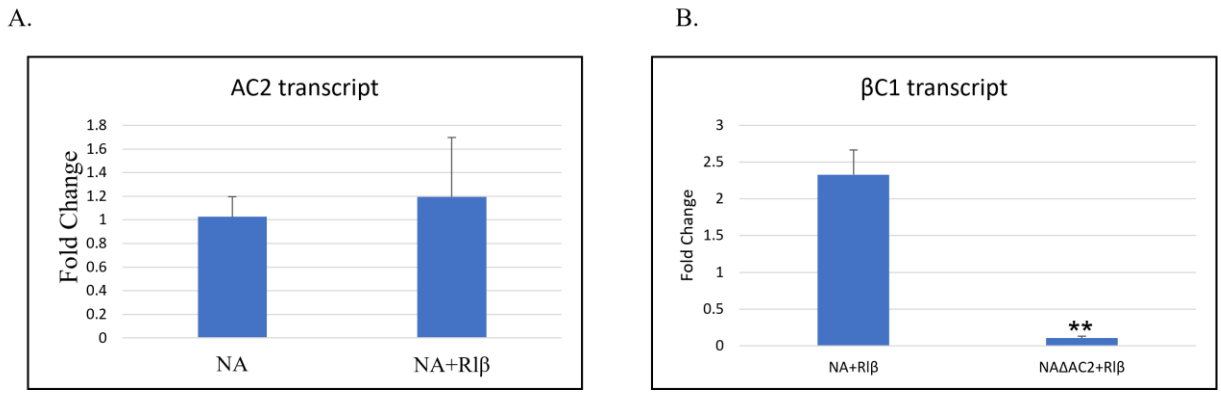


Figure 4.2 Transcript detection of AC2 and βC1 in infected plants.

(A) AC2 transcript accumulation in plants infected with ToLCNDV+RaLCB compared ToLCNDV. (B) Comparison of βC1 transcript in ToLCNDVΔAC2+RaLCB and ToLCNDV+RaLCB infected *N. benthamiana* at 5dpi.

4.3.3 Yeast two-hybrid analysis reveals the interaction between AC2 and βC1 proteins

The dependency of betasatellites on helper viruses suggests the possibility of protein-protein interactions among betasatellite and helper virus-encoded proteins. Here, we hypothesized that

βC1 protein might interact with ToLCNDV DNA-A encoded suppressor proteins (AC2, AC4, and AV2) and regulate symptom development. Therefore, the yeast two-hybrid assays were carried out to determine the viral interacting partner of βC1 protein. Interestingly, we found the interaction between the AC2 protein of ToLCNDV and βC1 protein encoded by RaLCB. The yeast cells AH109 which were cotransformed with pGADT7-AC2+pGBKT7-βC1 were grown on SD media lacking amino acids leucine, tryptophan, and histidine and having 5mM 3-AT (Figure 4.3A). Combination pGADC1-AC1+pGABDC1-AC1 was taken as a positive control, while vector combination, pGADC1+pGBDC1 was used for negative control.

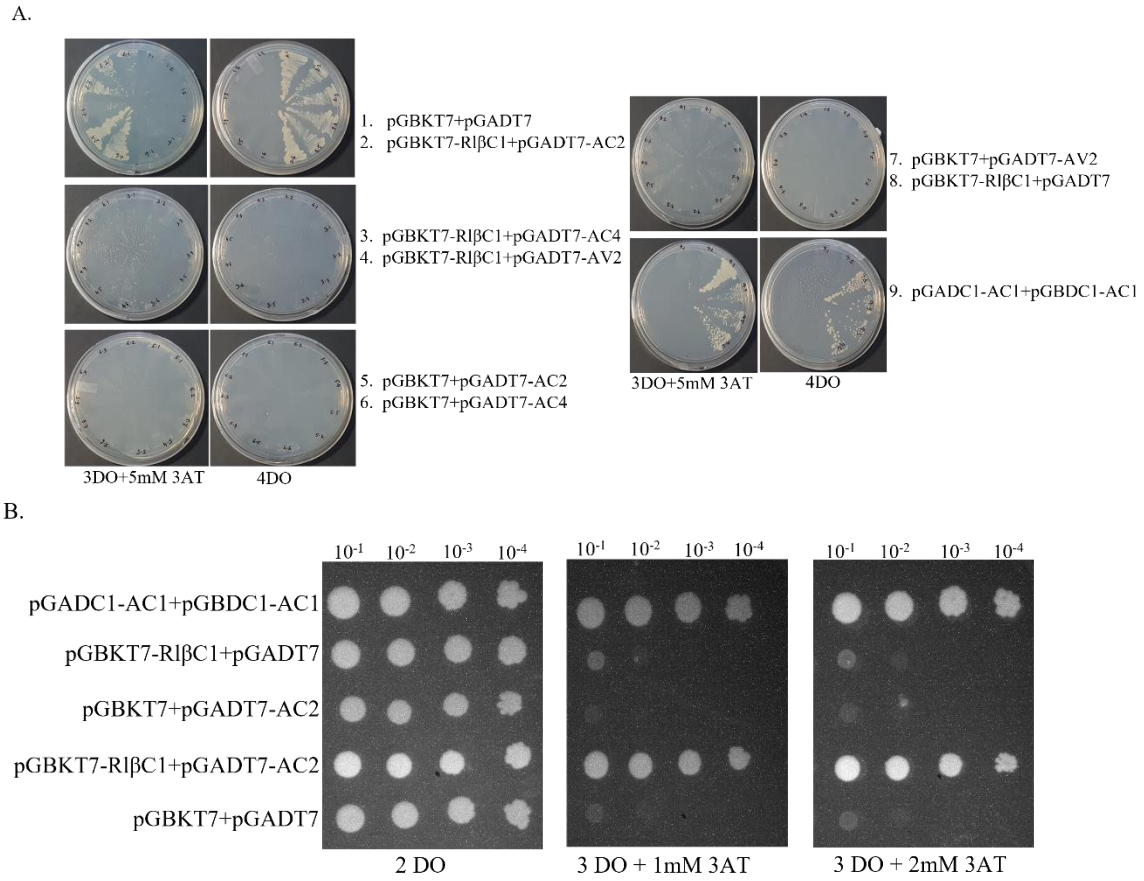


Figure 4.3 Yeast two-hybrid analysis between ToLCNDV encoded proteins and RaLCB-βC1.

(A) *S. cerevisiae* AH109 transformants harboring Y2H plasmid combination (as indicated in the right panel) grown on the selection medium SD-Leu-Trp-His supplemented with 2mM 3-amino-1,2,4-triazole. The yeast cotransformed with pGADT7-AC2 and pGBKT7-βC1 grew on the selection medium. (B) Growth of cotransformed yeast after serial dilutions from cultures of OD 1.0 at 600nm.

4.3.4 BiFC confirms the interaction between AC2 and βC1 proteins

The interaction between AC2 and βC1 proteins was further tested *in planta* by BiFC assay. The three-weeks old plants were infiltrated with pSITE-EYFP-N1: βC1 and pSITE-EYFP-C1:AC2, and epidermal leaf sections were analyzed by confocal microscopy after 48hours. The reconstituted yellow fluorescent protein signal was observed predominantly in the nucleus (Figure 4.4). Minor signal was also visible in the chloroplasts suggesting that the interaction between AC2 and βC1 occurs in the nucleus and chloroplasts. No fluorescence was detected in leaves infiltrated with EYFP-N1+EYFP-C1, EYFP-N1: βC1+EYFP-C1, and EYFP-N1+EYFP-C1:AC2.

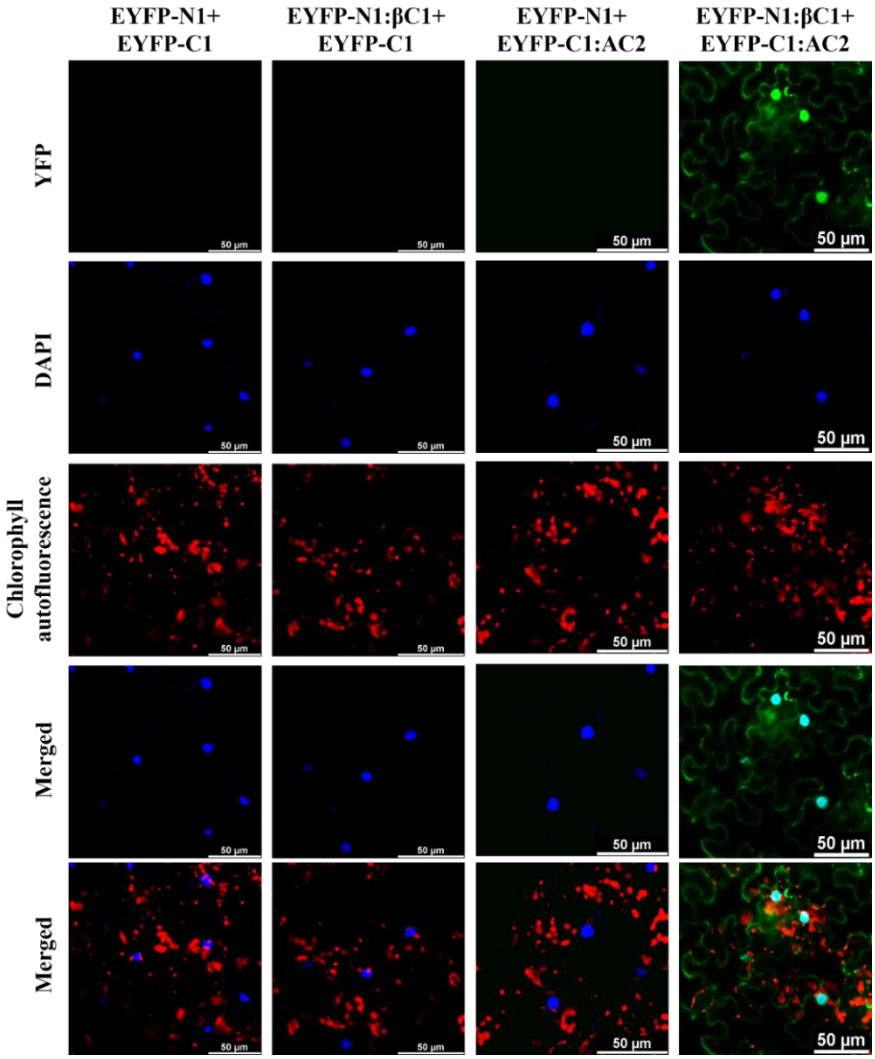


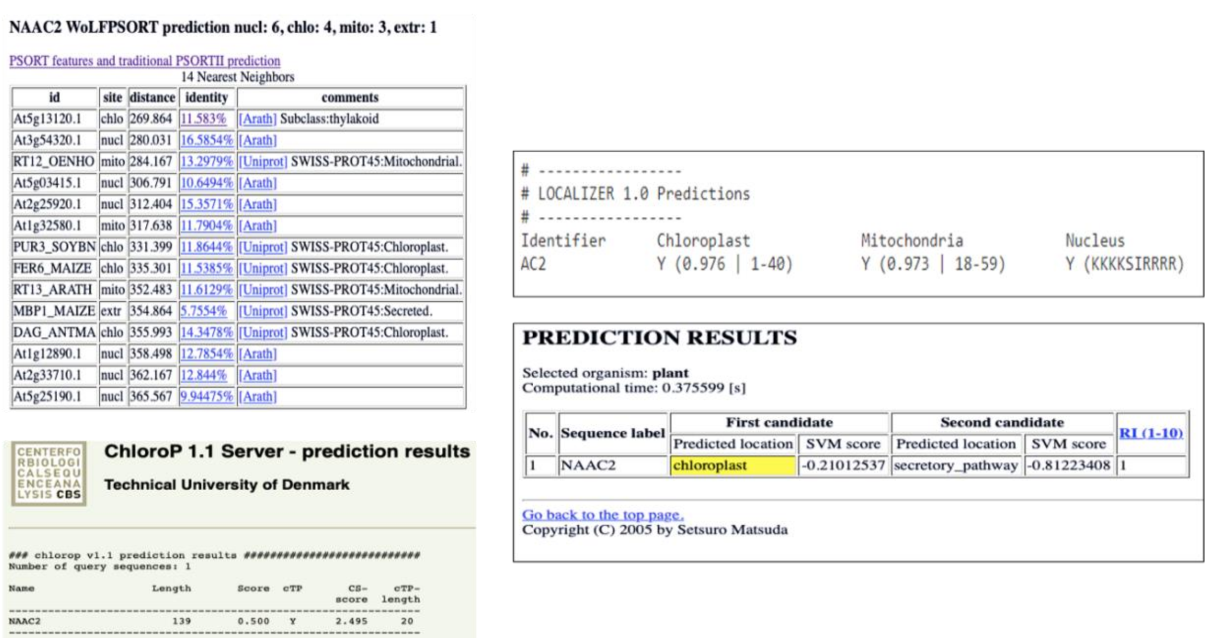
Figure 4.4 BiFC to study the interaction between AC2 and βC1.

Agrobacterium strain GV3101 harboring different combinations of BiFC constructs were coinfiltrated into *N. benthamiana* plants. Epidermal cells of leaves were visualized by confocal microscopy. The reconstituted YFP was observed in the nucleus and minor in the chloroplasts. Nucleus was stained with DAPI.

4.3.5 AC2 protein localizes both in nucleus and chloroplast

The mild interaction observed in the chloroplast led us to study the localization of AC2 protein (TrAP). *In silico* analysis to predict AC2 localization using various software predicted the putative presence of this protein in nucleus and chloroplast (WoLFPSORT) (Figure 4.5A). Further, LOCALIZER 1.0 and ChloroP 1.1 tools predicted the presence of chloroplast transit peptide of around 20 bp in the N-terminal region of the protein. The confocal microscopy experiment further confirmed the localization of AC2 protein in the nucleus and cytoplasm. Chloroplast autofluorescence and DAPI (for nuclear staining) merged with the green fluorescent protein signal obtained from infiltration of *Agrobacterium* harboring pCAMBIA1302-AC2 in *N. benthamiana* (Figure 4.5B).

A.



NAAC2 WoLFPSORT prediction nucl: 6, chlo: 4, mito: 3, extr: 1

PSORT features and traditional PSORTII prediction

14 Nearest Neighbors

id	site	distance	identity	comments
At5g13120.1	chlo	269.864	11.583%	[Arath] Subclass:thylakoid
At3g54320.1	nucl	280.031	16.5854%	[Arath]
RT12_OENHO	mito	284.167	13.2979%	[Uniprot] SWISS-PROT45:Mitochondrial.
At5g03415.1	nucl	306.791	10.6494%	[Arath]
At2g25920.1	nucl	312.404	15.3571%	[Arath]
At1g32580.1	mito	317.638	11.7904%	[Arath]
PUR3_SOYBN	chlo	331.399	11.8644%	[Uniprot] SWISS-PROT45:Chloroplast.
FER6_MAIZE	chlo	335.301	11.5385%	[Uniprot] SWISS-PROT45:Chloroplast.
RT13_ARATH	mito	352.483	11.6129%	[Uniprot] SWISS-PROT45:Mitochondrial.
MBP1_MAIZE	extr	354.864	5.7554%	[Uniprot] SWISS-PROT45:Secreted.
DAG_ANTMA	chlo	355.993	14.3478%	[Uniprot] SWISS-PROT45:Chloroplast.
At1g12890.1	nucl	358.498	12.7854%	[Arath]
At2g33710.1	nucl	362.167	12.844%	[Arath]
At5g25190.1	nucl	365.567	9.94475%	[Arath]

ChloroP 1.1 Server - prediction results

Technical University of Denmark

```
### chlorop v1.1 prediction results ##############################
Number of query sequences: 1

Name                   Length     Score  cTP       CS-   cTP-
                                                 score  length
----------------------------------------------------------------
NAAC2                     139     0.500   Y      2.495     20
----------------------------------------------------------------
```

```
# ----------------
# LOCALIZER 1.0 Predictions
# ----------------
Identifier     Chloroplast          Mitochondria          Nucleus
AC2            Y (0.976 | 1-40)     Y (0.973 | 18-59)     Y (KKKKSIRRRR)
```

PREDICTION RESULTS

Selected organism: **plant**
Computational time: 0.375599 [s]

No.	Sequence label	First candidate		Second candidate		RI (1-10)
		Predicted location	SVM score	Predicted location	SVM score	
1	NAAC2	chloroplast	-0.21012537	secretory_pathway	-0.81223408	1

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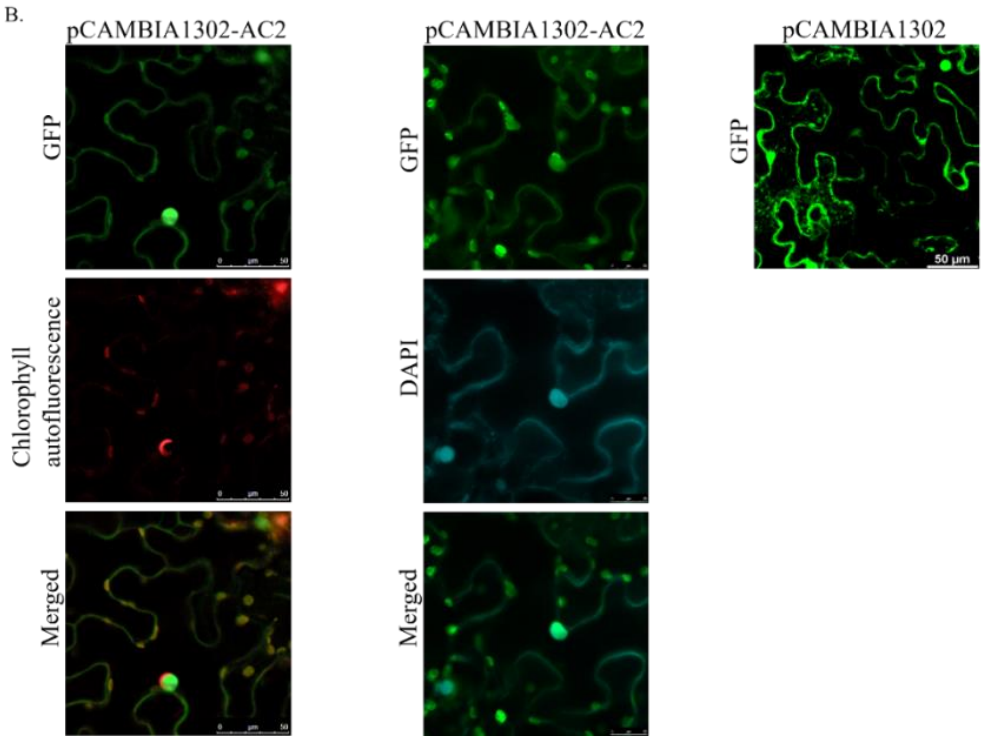


Figure 4.5 Localization of the AC2 protein (TrAP) of ToLCNDV.

(A) Bioinformatic analysis to study putative localization of AC2 protein. (B) *Agrobacterium* strain GV3101 harboring pCAMBIA1302-AC2 and pCAMBIA1302 were infiltrated into *N. benthamiana* plants. Epidermal cells of leaves were visualized by confocal microscopy. The GFP signal was observed both in the nucleus and chloroplasts in pCAMBIA1302-AC2 infiltrated plants, while pCAMBIA1302 infiltrated samples shows dispersed localization of GFP.

4.4 Discussion

In the Indian subcontinent, the cases of mixed geminiviral infections are rapidly increasing, which is attributed to the high recombination efficiency of viruses and the transreplicative behavior of betasatellites. Different combinations of viruses and virus-betasatellites lead to outcomes of disease development in other hosts. *N. benthamiana* plants infected with radish leaf curl virus (RaLCV) associated with RaLCB produce classic symptoms of betasatellite like downward leaf curling, enations, veinal chlorosis, stunted growth. However, no symptoms appeared with the same combination of the virus-betasatellite complex on tomato (Singh et al., 2012). Such differences in disease development are credited to the interactive regulation between host, helpervirus, betasatellite, and vector involved (Moreno and López-Moya, 2020).

This study found that the AC2 protein encoded by ToLCNDV has a vital role in developing betasatellite-associated veinal chlorosis symptoms. ToLCVNDΔAC2+RaLCB infected plants did not develop symptoms except mild leaf curling. In addition, in such plants, the transcript level of βC1 encoded by RaLCB was reduced significantly compared to ToLCVNDΔAC2+RaLCB infected plants. AC2 protein is known to involve in the transcriptional activation of geminiviral virion-sense strand ORFs AV1 and BV1, encoding CP and NSP, respectively (Sunter and Bisaro, 1992; Sung and Coutts, 1996; Shivaprasad et al., 2005). Different cis-elements have been suggested to regulate this AC2-mediated transactivation of viral genes (Argüello-Astorga et al., 1994; Cazzonelli et al., 2005). In Mungbean yellow mosaic virus (MYMV), a consensus conserved late element (CLE) "GTGGTCCC" in rightward promoter in DNA-A has been implicated in mediating transactivation by AC2 protein (Shivaprasad et al., 2005). In DNA-B of MYMV, abscisic acid-responsive elements (ABREs) have been found within 70 nucleotides upstream to BV1 promoter that aid in AC2 mediated transactivation. Surprisingly, both CLE and ABREs are present in the RaLCB genome, at nearly 380bp and 80bp upstream to βC1 transcription start site, respectively. Though not proven here, the presence of such elements and reduction in βC1 transcript in ToLCVNDΔAC2+RaLCB infected plants might speculate the possibility of transactivation of βC1 ORF by AC2 protein.

Further, an attempt was made to uncover one of the viral interacting partners of βC1 protein to understand the molecular interplay between cognate and non-cognate associations. Interestingly, yeast two-hybrid analysis reveals the interaction between the AC2 protein of ToLCNDV and βC1 protein encoded by RaLCB. Notably, strong interaction was observed in the nucleus, and slight was detected in the chloroplast, analyzed by BiFC assay. The interaction between AC2 and βC1 proteins in the chloroplast seems to regulate venial chlorosis symptom associated with betasatellite. Although RaLCB encoded βC1 does not have a chloroplast transit peptide, it localizes into the chloroplast and nucleus (Bhattacharyya et al., 2015). AC2 is a nuclear protein. However, the non-phosphorylated form of this protein has been detected in the cytoplasm (Trinks et al., 2005; Wang et al., 2005). Since mild interaction was observed in the chloroplast, it prompted us to study the localization of AC2 protein. Additionally, *in silico* analysis suggested putative localization of the protein in nucleus and chloroplast and disclosed the presence of chloroplast transit peptide in N-terminal domain of AC2 protein. Confocal microscopy results revealed the same. Since the nuclear-chloroplast molecular networking is important during plant-

pathogen interactions, it is possible that AC2 might be translocated from the nucleus to the chloroplast during viral pathogenesis. Many viral proteins are reported to relocalize from one cellular compartment to another to enhance their molecular networking. TYLCV encoded C4 protein relocalizes from the plasma membrane to chloroplast upon defense activation (Medina-Puche et al., 2020). Similarly, the C2 protein of TYLCV gets relocalized from the nucleus to nucleoplasm in the presence of CP (Wang et al., 2021).

Although more conclusive experimentation is required to unveil the scope of the interaction between ToLCNDV-encoded AC2 protein and βC1 protein of RaLCB, it is convincing that begomovirus-betasatellite associations in mixed infections involve combinatorial interactive proteome. Further investigation over mixed infections is needed to develop strategies against geminivirus outbreaks.

Chapter 5

Objective 3

5. To understand the role of betaV1 in pathogenesis

5.1 Introduction

Geminiviruses belong to the most prominent and the largest family of plant viruses, *Geminiviridae*, characterized by single-stranded, circular DNA molecules (Zerbini et al., 2017). Geminiviruses are encapsidated into twinned icosahedral virion particles and transmitted by hemipterous insect vectors. Based on the genomic composition, insect vector, host range, and phylogenetic analysis, geminiviruses are categorized into fourteen genera: *Becurtovirus, Begomovirus*, *Curtovirus*, *Mastrevirus, Capulavirus, Eragrovirus, Grablovirus, Topocuvirus, Turncurtovirus Citlodavirus, Maldovirus, Mulcrilevirus*, *Opunvirus*, and *Topilevirus* (Fiallo-Olivé et al., 2021). These viruses infect many dicots and monocots plants globally and cause tremendous damage to economically significant crops, especially in the tropical and sub-tropical regions. The largest and most widespread, whitefly transmitted genus, *Begomovirus*, constitutes 445 out of approximately 520 geminiviral species (Zerbini et al., 2017; Varsani et al., 2017). Begomoviruses can either be bipartite with two circular, single-stranded DNA molecules, DNA-A and DNA-B or monopartite, having a single DNA molecule, which shares homology with DNA-A of bipartite begomoviruses (Lazarowitz and Shepherd, 1992; Hanley-Bowdoin et al., 2000). Most monopartite begomoviruses have been spread across the Old World, while bipartite begomoviruses are prevalent in the New World (Rybicki, 1994; Nogueira et al., 2021). Both DNA-A and DNA-B are 2.7kb in size approximately and encode proteins essential for viral replication, transcription, movement, and encapsidation. The complementary sense strand of DNA-A encodes a replication-associated protein (Rep/AC1), transcriptional activator protein (TrAP/AC2), replication enhancer protein (REn/ AC3), and AC4 protein, while coat protein (CP/AV1) and AV2 protein are transcribed from the virion sense strand (Laufs et al., 1995; Sunter and Bisaro, 1991; Pooma and Petty, 1996; Hanley-Bowdoin et al., 1999). DNA-B encodes nuclear shuttle protein from the BV1 ORF present in the virion sense strand and MP from the BC1 ORF located in the complementary sense strand (Hanley-Bowdoin et al., 1999).

Monopartite begomoviruses typically are accompanied by single-stranded DNA molecules, either alphasatellite or betasatellite (Briddon and Stanley, 2006). Betasatellites are known to regulate the pathogenesis of helpervirus severely during infection (Cui et al., 2004). Betasatellites (1.3kb size) possess satellite conserved region (SCR), Adenine-rich (A-rich)

region, and an ORF encoding a prime pathogenicity factor, βC1 protein (Mansoor et al., 2003). Betasatellites do not share sequence homology with their helper viruses except at the SCR, which has a stem-loop structure containing conserved nonanucleotide sequence typical of geminivirus, which aids in Rep protein-mediated replication of betasatellite (Briddon et al., 2003). A-rich region helps in maintaining the size of betasatellite genome for effective encapsidation and may be involved in the viral movement (Saunders et al., 2000). Trans-replication of betasatellites by different helper viruses increases the diversity of begomovirus-betasatellite complexes, which brings about mixed infections in the fields (Nawaz-ul-Rehman et al., 2012). Betasatellite modulates helper virus replication, augments disease severity, suppresses host defense, and enhances the vector performance (Cui et al., 2005; Zhou, 2013). Betasatellite encoded βC1 protein conditions cellular machinery to facilitate the survival and proliferation of begomovirus-betasatellite complexes. It targets various host factors by interacting and modulating their functions. βC1 protein encoded by betasatellites associated with tomato leaf curl Java virus (ToLCJAV), bhendi yellow vein mosaic virus (BYVMV), and tomato yellow leaf curl China virus (TYLCCV), cotton leaf curl Multan virus (CLCuMV) are known to suppress RNA silencing pathway, which plays a significant role in defense against the geminiviruses (Kon et al., 2007; Gopal et al., 2007; Cui et al., 2005; Amin et al., 2011). Besides intensifying the infection, TYLCCNB encoded βC1 protein upregulates the endogenous suppressor of RNA silencing, Nbrgs-CaM that interacts with suppressor of gene silencing (SGS3) and promotes its degradation, thus drastically reducing posttranscriptional gene silencing activity (PTGS) against the virus (Li et al., 2014a; Li et al., 2017). It also inhibits methylation-mediated transcriptional gene silencing by inhibiting S-adenosyl homocysteine hydrolase (SAHH), an enzyme involved in methyl cycle maintenance during TGS (Yang et al., 2011). Phosphorylation mimic mutants of TYLCCNB-βC1 shows reduced suppression of TGS and PTGS during viral infection (Zhong et al., 2017). In addition, βC1 promotes whitefly transmission by directly interacting with MYC2 transcriptional factor whose dimerization regulates terpene synthesis (Li et al., 2014b). Over and above that, βC1 engages with various factors involved in the ubiquitination-proteasomal pathway to abstain ubiquitination of pro-viral proteins (Shen et al., 2016; Eini et al., 2009; Jia et al., 2016; Shen et al., 2011). Betasatellites also govern the proviral role of autophagy in geminiviral pathogenesis (Li et al., 2017). Furthermore, an important signaling cascades like SnRK1 mediated signaling and MAPK pathway also get regulated by βC1 protein. A single protein βC1

encoded by betasatellite known till recently displays jack-of-all-trades quality and efficiently hijacks the host cellular environment.

The evolutionary origin of betasatellites is uncertain. Host plants and recombination events between pre-existing DNA elements might have played a role in betasatellites evolution. However, many new viral proteins keep emerging either through modifications in existing non-coding ORF or originated de Novo (Sabnath et al., 2012).

The speculations were reported based on molecular studies and experimentation for the origin of other transcripts in betasatellite (Saunders et al., 2004). TESTCODE software program predicted a 5.3 kDa functional protein encoded from virion sense strand overlapping with βC1 ORF on the complementary strand in AYVV DNA betasatellite (Saunders et al., 2000). In CLCuV DNA-β, V1, V2, and V3 transcripts were predicted at the overlapping region of βC1 ORF (Briddon et al., 2001). In addition, mutation at the CLCuVβ-βV1 ORF showed no significant enhancement in symptom development and pathogenesis (Saeed et al., 2005). Further, the presence of βV1 ORF has also been detected in TYLCCNB in the virion sense strand (Hu et al., 2020). It plays an important role during the viral infection and induces hypersensitive response (HR)-type cell death (Hu et al., 2020). Similarly, a novel transcript βV1, generating from the virion sense strand, has been identified with 116 aa coding ORF between 234-584 coordinates of RaLCB (unpublished). Since βV1 is a novel protein identified recently, it is yet to be characterized. Keeping this background information, in this study, an attempt has been made to determine the significance of βV1 protein in modulating pathogenesis.

5.2 Material and methods

5.2.1 Bioinformatics analysis

The PSORT and its extension WoLF PSORT tools were used to predict the localization of βV1 protein in the cellular environment. Promoter analysis was carried out using the PLANTCARE software database. The βV1 topology and overall orientation were speculated through Phobius. To analyze the protein structure and function of βV1, I-TASSER server was used. The standard default threshold values were used for all tools used in this study.

5.2.2 Plasmid construction

The βV1 ORF (351bp) of RaLCB was PCR amplified from an infectious clone of RaLCB with the specific primer pair and cloned into a pJET1.2 vector at sites compatible with the vector. For overexpression studies in planta, the βV1 and βC1 ORFs were amplified using 106KV1FP/ 106KV1RP and RLBETA106FP/RLBETA106RP primer pairs, respectively, to obtain pJET1.2-βV1 and pJET1.2-βC1 clones. Then pGR106-βV1 and pGR106-βC1 constructs were generated by digesting pJET1.2 clone at *Cla*I and *Sal*I restriction site and ligated with the pGR106 vector at the same sites. Similarly, the βV1 ORF was cloned into the pCAMBIA1302 vector for localization study at *Nco*I and *Spe*I restriction sites and confirmed using RK11301FP/ RK11301RP primer pair. For yeast two-hybrid assay (Y2H), the pGBKT7-βV1 construct was generated by first obtaining pJET1.2-βV1 clone using primer pair KV1KT7FP/106KV1RP. The construct pJET1.2-βV1 was digested and ligated to linearized pGBKT7 vector at *BamH*I and *Sal*I restriction site. ORFs of ToLCNDV such as AC1, AC3, AV1, AV3 were amplified from a monomeric ToLCNDV DNA for cloning into pJET1.2 vector using specific primers (Table 5.1) and further cloned in frame into Y2H vector pGADT7 at compatible restriction sites as mentioned above. All the clones were ascertained by sequencing. Further, the clones for in planta expression were transformed into specific *Agrobacterium* strains.

Table. 5.1 List and sequences of primers used in chapter 5.

S.No.	Primer	Sequence
1	106KV1FP	ATCGATATGTCTATTACAGGAGCCTCTTC
2	106KV1RP	GTCGACTTAATGAGTGTTCATCATATATG
3	KV1KT7FP	GGATCCTAATGTCTATTACAGGAGCCTCTTC
4	NAAC1FP	CCCGGGTATGGCTTCGCCACGTCGTTTTAG
5	NAAC1RP	GGATCCTCAACTCGCCTCCTGCGAATGCTCTTC
6	NAAC3FP	CATATGATGATCACGGATTCACGCACAGGGG
7	NAAC3RP	GGATCCTTAATAAATATCCAGTTTTATATC
8	NAAV1FP	GAATTCATGGCGAAGCGACCAGCAGATATCA
9	NAAV1RP	GGATCCTTAATTTGTGGCCGAATCATAAA
10	NAAV3FP	CATATGATGAAATTCACGCTACATGGCCTAT

11	NAAV3RP	GAATTCTCATCGGCCTGTTGGTCCAG
12	NAAC3BIFCFP	CACCATGATCACGGATTCACGCACAGGG
13	NAAC3BIFCRP	ATAAATATCGAGTTTTATATCATATGAAG
14	RLBETAV1BIFP	CACCATGTCTATTACAGGAGCCTCTTC
15	RLBETAV1BIRP	ATGAGTGTTCATCATATATGAACAC
16	RK1301FP	CCATGGATATGTCTATTACAGGAGCCTCTTCC
17	RK1301RP	ACTAGTATGAGTGTTCATCATATATGAACACT
18	RLBETA106FP	ATCGATATGACGATCAAATACAAAAACCAG
19	RLBETA106RP	GTCGACTTATACAGATGAACGCGTATACA

5.2.3 PVX based protein expression

Agrobacterium-mediated virus infection was performed according to the method as suggested (Lee and Yang, 2006). *Agrobacterium tumefaciens* strain GV3101+ pJIC Sa_Rep harboring pGR106 vector alone, pGR106-βV1 and pGR106-βC1 were cultured in LB medium containing rifampicin (30 µg/ml) and kanamycin (50 µg/ml) at 28°C for 36-48 hours at 200 rpm. The culture was centrifuged and resuspended in agroinfiltration buffer consisting of 10mM MES buffer (pH 5.8), 10mM $MgCl_2$, and 100µM acetosyringone. Optical density (OD) was set to 0.5 at a wavelength of 600 nm, and the inoculum was incubated in the dark for 3 hours. Dark-adapted (2-3hrs), 3-weeks old *N. benthamiana* plants were infiltrated through the abaxial side of leaves with the aid of 1 ml needleless syringe. In each case, leaves were collected for staining purposes (5dpi and 7dpi) and RNA isolation procedure (5dpi).

5.2.4 3,3-diaminobenzidine (DAB) staining

Detection of hydrogen peroxide (H_2O_2) was done by using the DAB-uptake method as per Orozco-Cardenas and Ryan (1999) with slight modifications. *N. benthamiana* leaves were excised from petiole and immersed in 100ml of 1mg/mL 3,3-diaminobenzidine (DAB)-HCl solution-pH 3.8 (Sigma). After 10 hours of incubation in the dark at 25°C, the leaves were incubated overnight in absolute ethanol to remove excess stain and photographs were taken.

5.2.5 Nitro Blue Tetrazolium Chloride (NBT) staining

For detection of superoxide (O_2^-), 0.2gm NBT stain (Sigma Aldrich) was dissolved in 100ml of 50mM sodium phosphate buffer (pH 7.5) to make 0.2% NBT solution. Fresh leaves were collected and placed immediately into the solution and incubated on a shaker with low rpm for 10 hours in the dark for stain uptake. Later the leaves were immersed in absolute ethanol and photographed.

5.2.6 Trypan blue staining

Tissue cell death was visualized using a trypan blue stain. The stain solution was prepared by dissolving lactic acid (85% w: w): phenol (TE saturated pH 7.5-8.0): glycerol: H_2O in the ratio of 1:1:1:1 and 100mg trypan blue stain. Leaves were excised from petiole and immersed in 100ml of trypan blue stain for 10 hrs and transferred to absolute ethanol, and photography was carried out.

5.2.7 RNA isolation and cDNA preparation

Infiltrated leaves from infected *N. benthamiana* plants were collected at 5th dpi, and total RNA was isolated, according to Singh et al. (2016). Each leaf sample (100mg) was ground using liquid nitrogen and homogenized in 1ml Trizol reagent (Sigma Aldrich). The homogenized tissue was treated with 200 µl of chloroform for removing protein impurities and incubated at room temperature for 10 min followed by centrifugation at 12000 rpm for 10 min. The supernatant was collected in a fresh tube, and RNA precipitation was carried out by adding an equal volume of isopropanol. Spined down the precipitated RNA at 13000 rpm at 40°C for 20 min. Subsequently, the pellet was washed with 70% ethanol twice to remove the salt impurities and dried at room temperature. The RNA pellet was dissolved in UltraPure™ DNase/RNase-Free Distilled Water and quantified. 5µg RNA was treated with DNAase using TURBO DNA-free Kit (Ambion). RNA was incubated with 0.1 volume of 10X TURBO DNAase buffer and 1µl DNAase for 20min at 37^{o}C followed by the addition of 0.1 volume of DNAase Inactivation reagent. The reaction was incubated at 37^{o}C again for 5 min. Complementary DNA (cDNA) was synthesized using an iScript cDNA synthesis kit (BIO-RAD) following the protocol mentioned in the user manual. In brief, 4µl of 5X iScript mix was added to 1µg of DNAase treated RNA, and the final volume was made up to 20µl by nuclease-free water. Reaction conditions used in thermal cycler

were- Priming: 5 min at 25^{o}C, Reverse transcription (RT): 20 min at 46 ^{o}C, RT inactivation: 1 min at 95 ^{o}C followed by the hold at 4 ^{o}C.

5.2.8 Quantitative real-time PCR (qRT-PCR) and analysis

qRT-PCR was performed using SSoAdvanced Universal SYBR Green Supermix (Biorad). In a reaction volume of 20µl, 10µl of 2X SYBR Green Supermix was added to 2µl of 50ng cDNA and 0.7µl of 10µM of forward and reverse primer and remaining 6.6µl of nuclease-free water. The Applied Biosystems 7500 fast real-time PCR was programmed for DNA denaturation at 95 ^{o}C for 30 sec, again 95 ^{o}C for 15sec (40 cycles), annealing and extension at 60 ^{o}C for 30 sec. Reference control used for normalization was protein phosphatase 2A. Relative gene expression was determined by ΔCt method.

5.2.9 Confocal microscopy

A. tumefaciens strain GV3101 harboring pC1302-βV1 construct was infiltrated into three-weeks old *N. benthamiana* plants for localization. While for BiFC, pSITE-EYFP-N1: βV1 and pSITE-EYFP-C1:AC3 constructs were used. After 48 hours post infiltration, the epidermal cells of infiltrated leaves were visualized under TCS SP8 X confocal microscope (Leica, Germany), and images were acquired. For nuclear detection, DAPI was used. The excitation and emission filter used was 340-380nm and 435-485nm, respectively. For green fluorescent protein signal, excitation and emission were at 465-495nm and 515-555nm, respectively.

5.2.10 Yeast two-hybrid analysis (Y2H)

Yeast-two hybrid assay was carried out by expressing bait protein in fusion to GAL4 DNA binding domain (pGBKT7-βV1) and prey proteins fused to GAL4 activation domain (pGADT7-AC1, pGADT7-AC2, pGADT7-AC3, pGADT7-AC4, pGADT7-AV1, pGADT7-AV2, pGADT7-AV3) in AH109 strain of yeast. AH109 yeast strain was grown till OD reached 0.5. The yeast culture was pelletized at 3000rpm for 5min at room temperature and treated with 10X LiAc buffer. The appropriate plasmid combinations were cotransformed into the yeast culture. The transformed colonies were grown on synthetic defined (SD) double dropout media. Further, the interaction was confirmed by growing transformants on SD media lacking leucine, tryptophan, and histidine but having 2mM 3-AT.

5.3 Results

5.3.1 Computational analysis

5.3.1.1 Localization

The PSORT tool scanned βV1 protein for the presence of signal peptides and predicted the localization of βV1 either in vesicles of the secretory system or in the plasma membrane (Figure 5.1). The C-terminal amino acids 93-109 constitute the transmembrane signal, while the calculations projected the N-terminal tail (1-92) inside the cell. In addition, it reveals the presence of Dileucine motif in the N-terminal tail at position 62 of βV1 (Figure 5.1A). This dileucine motif is known to act as a sorting signal for targeting the protein to a vacuolar membrane (Larisch et al., 2012). WoLFPSORT prediction tool predicted a higher probability of βV1 being localized extracellularly or in the thylakoids of chloroplast (Figure 5.1B). It also projected vacuolar localization of βV1. Another tool, Virus-mPLoc under Cell-PLoc 2.0 software, shows localization of βV1 in host cellular membrane (Figure 5.1C). Overall, βV1 protein is hypothesized to be localized to the plasma membrane or in an organelle membrane.

A.

152092337418559 (116 aa)
PSG: a new signal peptide prediction method
N-region: length 0; pos.chg 0; neg.chg 0
H-region: length 27; peak value 1.43
PSG score: -2.98

GvH: von Heijne's method for signal seq. recognition
GvH score (threshold: -2.1): -5.24
possible cleavage site: between 49 and 50

>>> Seems to have no N-terminal signal peptide ALOM: Klein et al's method for TM region allocation
Init position for calculation: 1
Tentative number of TMS(s) for the threshold 0.5: 1
Number of TMS(s) for threshold 0.5: 1
INTEGRAL Likelihood = -4.73 Transmembrane 93 - 109
PERIPHERAL Likelihood = 0.85 (at 6)
ALOM score: -4.73 (number of TMSs: 1)

MTOP: Prediction of membrane topology (Hartmann et al.)
Center position for calculation: 100
Charge difference: -2.5 C(-0.5) - N(2.0)
N >= C: N-terminal side will be inside

>>> Single TMS is located near the C-terminus

>>> membrane topology: type Nt (cytoplasmic tail 1 to 92) MITDISC: discrimination of mitochondrial targeting seq
R content: 0 Hyd Moment(75): 1.13
Hyd Moment(95): 0.36 G content: 1
D/E content: 1 S/T content: 10
Score: -3.82

Gavel: prediction of cleavage sites for mitochondrial preseq
R-2 motif at 53 SRVILK

NUCDISC: discrimination of nuclear localization signals
pat4: none
pat7: none
bipartite: none
content of basic residues: 6.0%
NLS Score: -0.47

KDEL: ER retention motif in the C-terminus: none

ER Membrane Retention Signals: none

SKL: peroxisomal targeting signal in the C-terminus: none

PTS2: 2nd peroxisomal targeting signal: none

VAC: possible vacuolar targeting motif: none

RNA-binding motif: none

Actinin-type actin-binding motif:
type 1: none
type 2: none

NMYR: N-myristoylation pattern : none

Farnesylation/Geranylgeranylation motif: none

memYQRL: transport motif from cell surface to Golgi: none

Tyrosines in the tail: too long tail

Dileucine motif in the tail: found
LL at 62

checking 64 PROSITE DNA binding motifs: none

checking 1 PROSITE low specificity DNA binding motifs: none

checking 71 PROSITE ribosomal protein motifs: none

checking 33 PROSITE prokaryotic dna binding motifs: none

Final Results:

48.0 %: vesicles of secretory system 36.0 %: plasma membrane 16.0 %: cytoskeletal >> prediction for 152092337418559 is ves

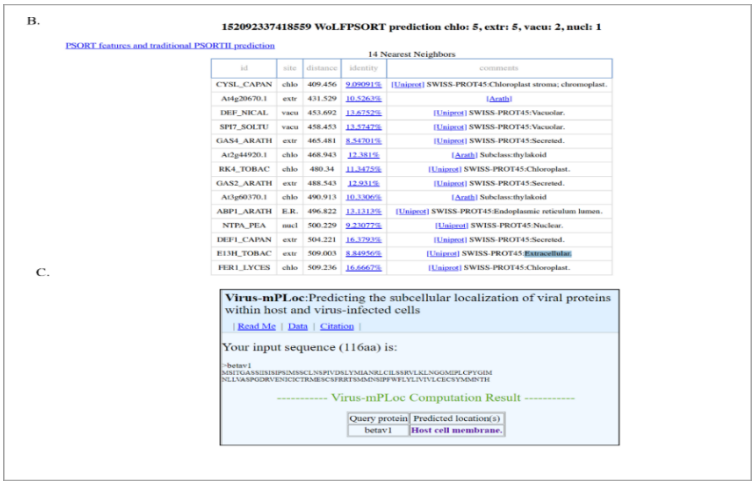


Figure 5.1 *In silico* localization of βV1 protein.

Protein localization tools (PSORT (A), WoLF PSORT(B), and plant-mPLoc (C)) predicted that βV1 protein might be targeted to the plasma membrane or organellar membrane.

5.3.1.2 Topology

Topological studies revealed the overall orientation of βV1 protein indicated that it might be a transmembrane protein with an N- terminal cytoplasmic sequence (1-93), a transmembrane helix (94-114), and a short C-terminal region outside the membrane. There is a signal peptide at the N terminal portion of a protein, suggesting the destination of βV1 into the secretory pathway or membrane (Figure 5.2 A, B, C). This putative data also corresponds to the PSORT output.

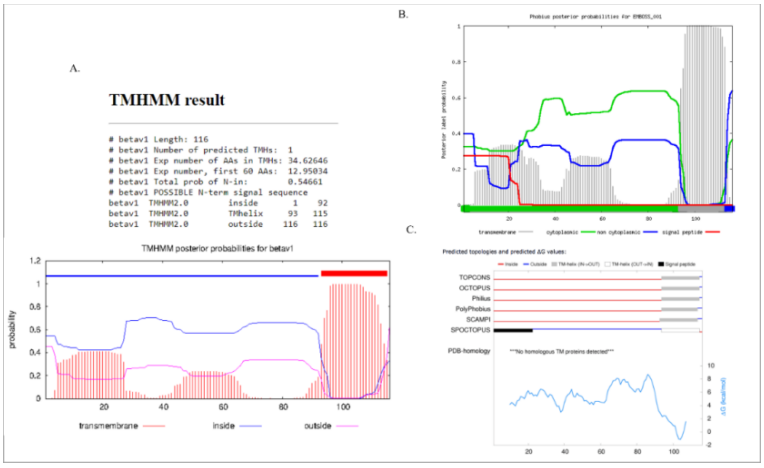


Figure 5.2 Prediction of the topology of βV1 protein.

(A) Output of Phobius program. (B, C) Comparison of output from different programs for deciphering topology of βV1.

5.3.1.3 Promoter analysis

The putative promoter region of βV1 was analyzed by PLANTCARE software to decode cis-acting elements involved in its regulation**.** Many cis-acting sequences and transcription factor binding sites are present both in the positive and negative sense strand, 500bp upstream to the initiation start site of βV1 transcription (Figure 5.3). Several TATA-box elements, which function as core promoter sequences and CAAT-box region for transcription regulation, present upstream to βV1 coding sequence. Additionally, many sites where transcription factor binds like MYB, ABRE, GT1, LTR are also found on the promoter sequence.

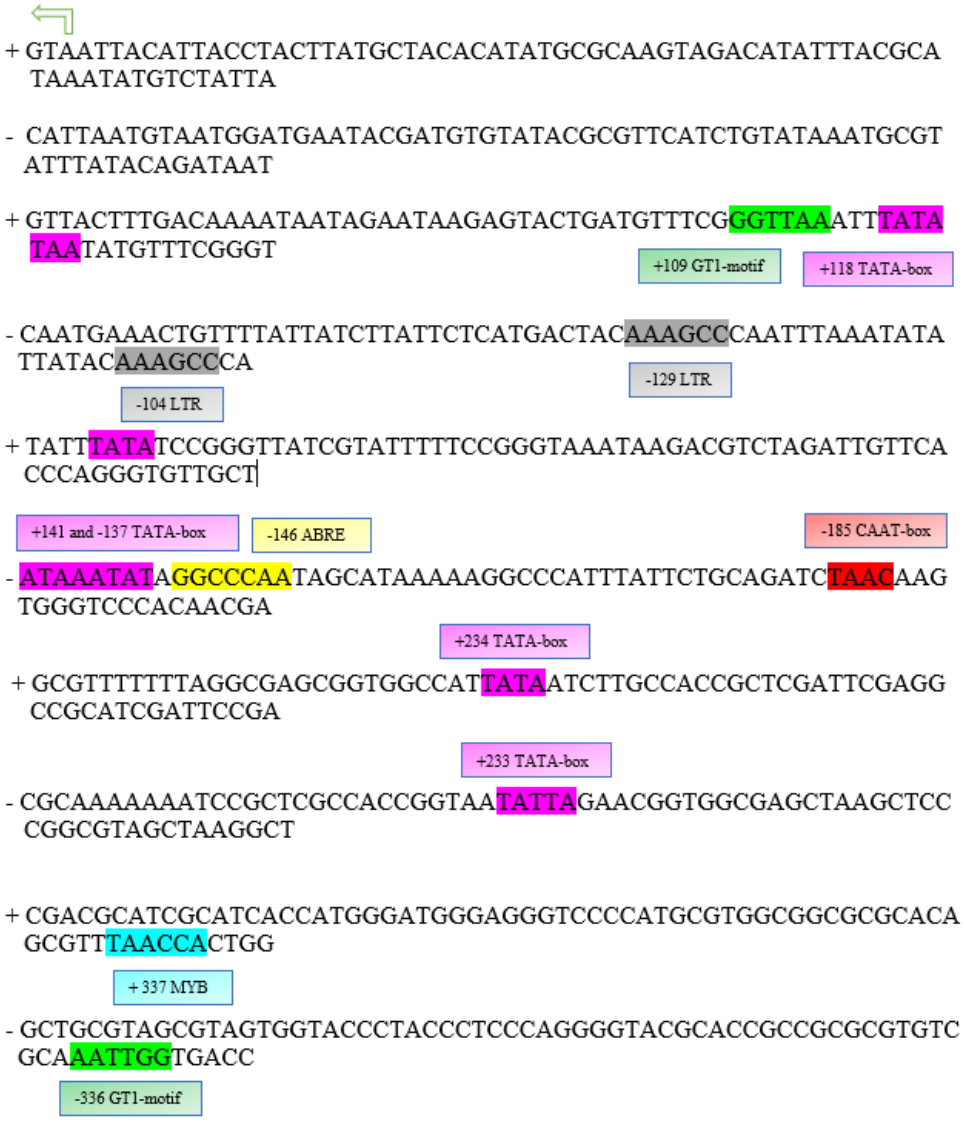


Figure 5.3 Promoter analysis of βV1 ORF.

Cis-acting regulatory sequences upstream of βV1 transcription start site (indicated through green arrow) scanned through PLANTCARE software. Distinct elements are represented by different colors. (+) and (-) denotesthe virion sense and complementary sense strand, respectively.

5.3.1.4 Structural and Functional prediction of βV1

To gain insight into structural and functional aspects of βV1, the amino acid sequence was submitted to the I-TASSER server program. The results predicted the secondary structure, solvent accessibility, molecular function of the βV1 protein. They provided the putative 3D model based on the confidence score (C-score) value estimated according to the alignment of threading templates. The βV1 protein was predicted to have three prominent helices and several sheets with many coils in between. There is a seemingly large number of residues in the protein that are buried inside, not exposed to the cytosolic aqueous environment (Figure 5.4A). This may be correlated to the membranous localization of βV1 protein, as predicted by PSORT. Probably, βV1 binds to an ion at the molecular level, which the program projected in terms of Gene Ontology score. Furthermore, I-TASSER generated the top five 3D structures of βV1 protein. Considering the highest C-score value with local highest accuracy, the best model was selected (Figure 5.4B)

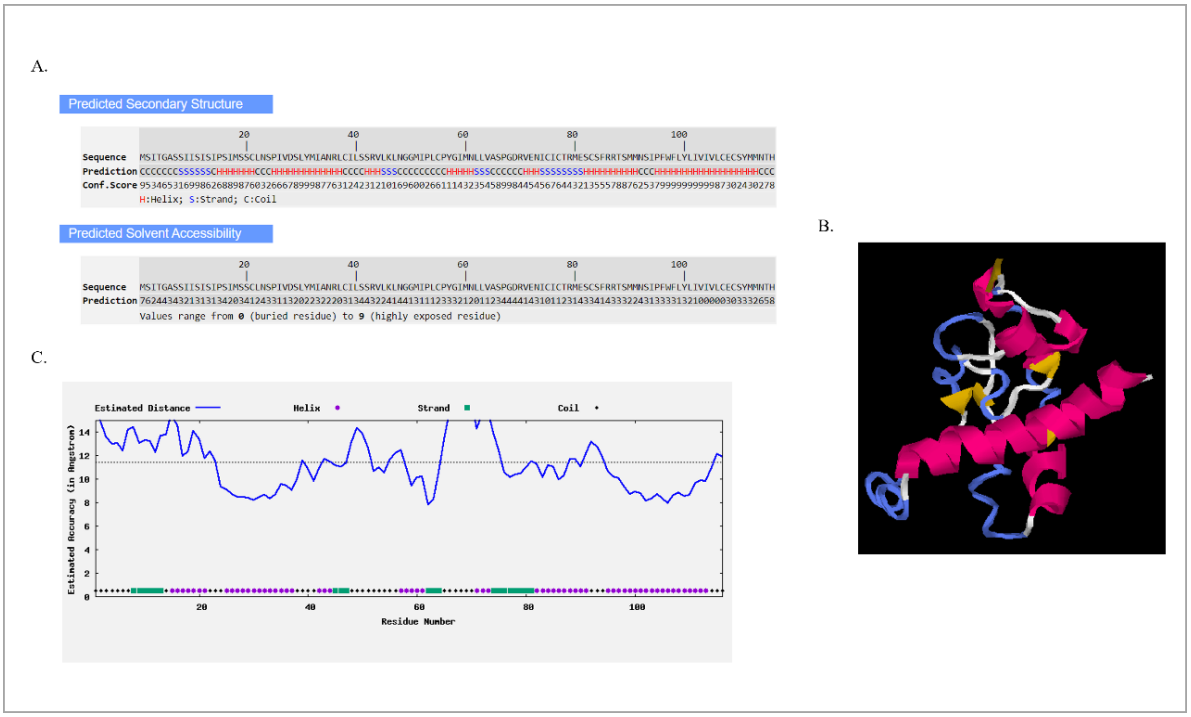


Figure 5.4 I-TASSER prediction of protein configurations.

(A) Estimation of secondary structure and solubility of βV1 protein. (B) Best 3D model prediction based on confidence score. (C) Estimated local accuracy of the model by the program.

5.3.2 Cloning of βV1 ORF in different vectors

The βV1 ORF from RaLCB was PCR amplified from a monomeric clone of RaLCB and cloned into a pJET1.2 cloning vector. PCR amplified product was extracted using gel extraction kit and ligated into pJET1.2 vector proceeded with the transformation of ligation product into *E. coli* DH5α competent cells. The transformed colonies were screened on an antibiotic selection plate containing 100µg/ml ampicillin. The pJET1.2-βV1 clones were confirmed by PCR and restriction digestion of the plasmid isolated. The PCR and restriction digestion confirmed clones were further confirmed by DNA sequencing analysis. The pJET1.2-βV1 confirmed plasmid DNA was restriction digested with *Cla*I and *Sal*I and run on agarose gel electrophoresis. Approx. 351 bp fallout on agarose gel electrophoresis was extracted and cloned in frame to the pGR106 vector, restriction digested with *Cla*I and *Sal*I enzymes. The pGR106-βV1 clone was confirmed by βV1 specific PCR and restriction digestion with *Cla*I and *Sal*I. The pGR106-βV1 construct was transformed into Agrobacterium strain GV3101 harboring pJIC Sa_Rep. The *Agrobacterium* strain carrying pGR106-βV1 was confirmed by βV1 specific PCR.

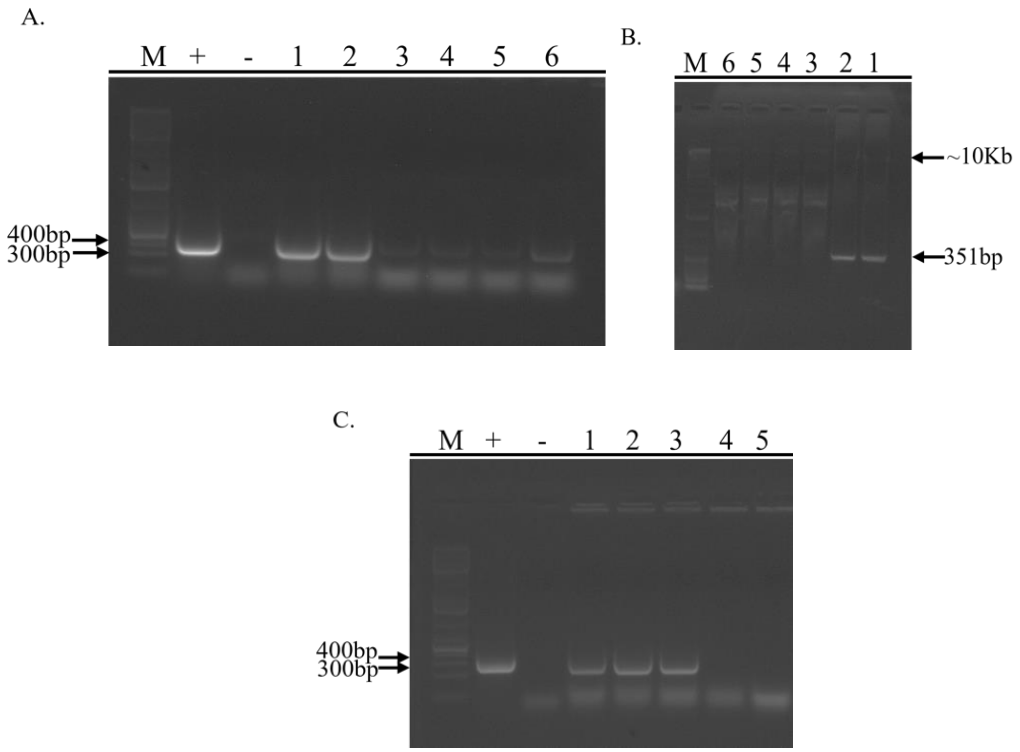
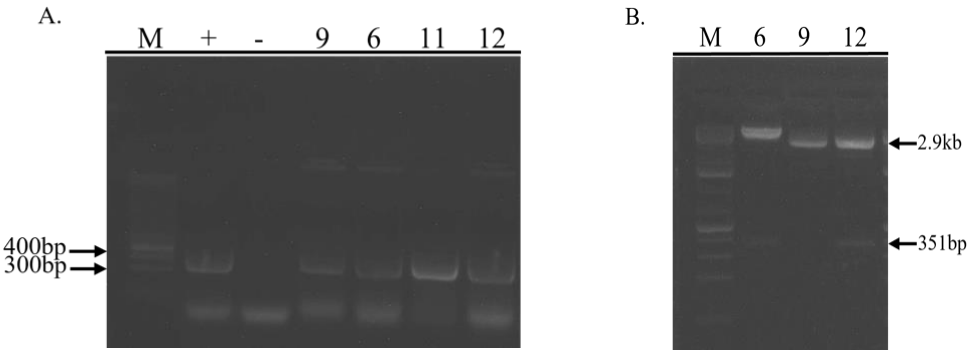


Figure 5.5 Confirmation of pGR016-βV1 clone.

(A) Confirmation by PCR using primers specific for βV1, (B) by restriction digestion with *Cla*I and *Sal*I showing the release of approximately 351bp. (C) Confirmation of pGR106- βV1 clone was further transformed into *Agrobacterium tumefaciens* strain GV3101+pJIC Sa_Rep and verified successfully transformed colonies by PCR.

Likewise, the pJET1.2-βV1 (clone constructed for Y2H cloning) confirmed plasmid DNA was restriction digested with *BamH*I and *Sal*I and run-on agarose gel electrophoresis. Approx. 351 bp fallout on agarose gel electrophoresis was extracted and cloned in frame to the pGBKT7 vector, restriction digested with *BamH*I and *Sal*I enzymes. The pGBKT7-βV1 clone was confirmed by βV1 specific PCR and restriction digestion with *BamH*I and *Sal*I (Figure 5.6)



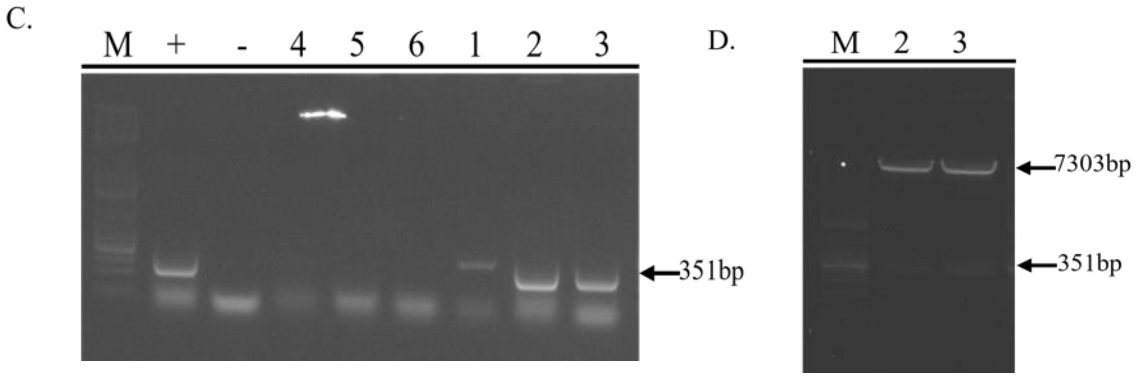


Figure 5.6 Confirmation of pJET-βV1 clone in pGBKT7 vector.

(A) Confirmation by PCR using primers specific for βV1, (B) by restriction digestion with *BamH*1 and *Sal1* showing the release of approximately 351bp. (C) Confirmation of pGBKT7-βV1 clone by PCR using primers specific for βV1. (D) Confirmation of pGBKT7- βV1 clone by restriction digestion with *BamH*1 and *Sal1* shows release of approximately 351bp.

Similarly, to study localization, βV1 was cloned into pCAMBIA1302 vector (Figure 5.7).

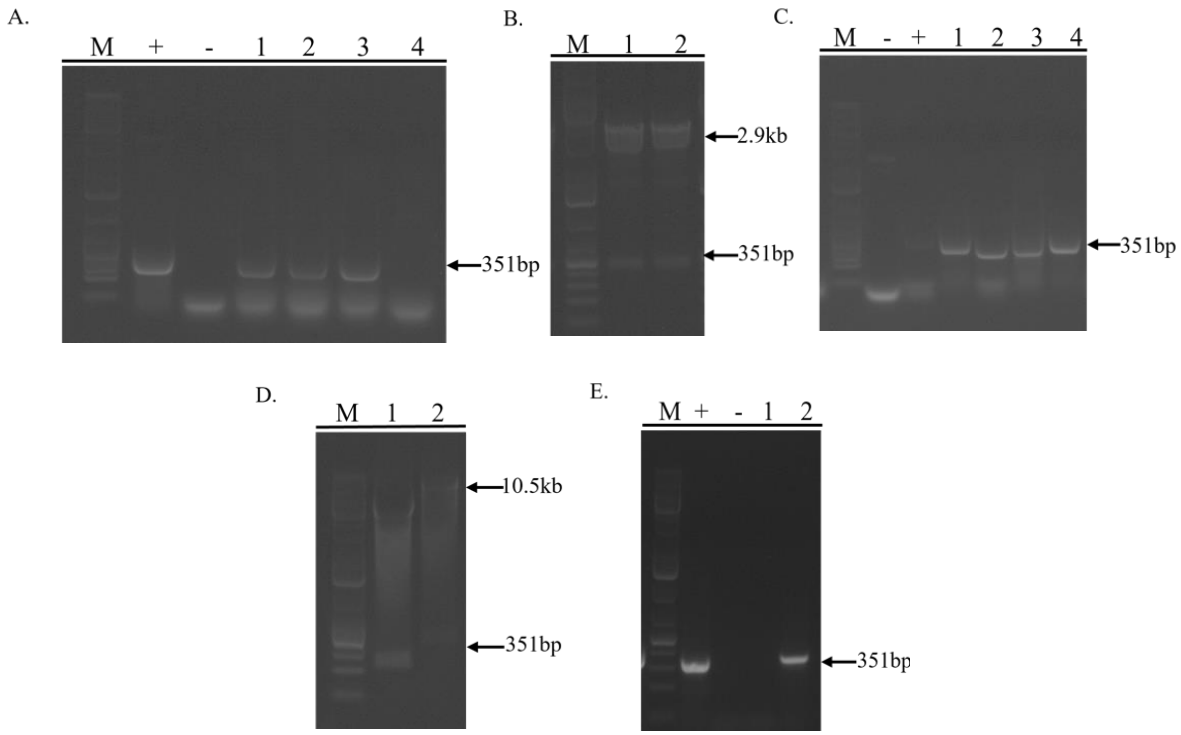


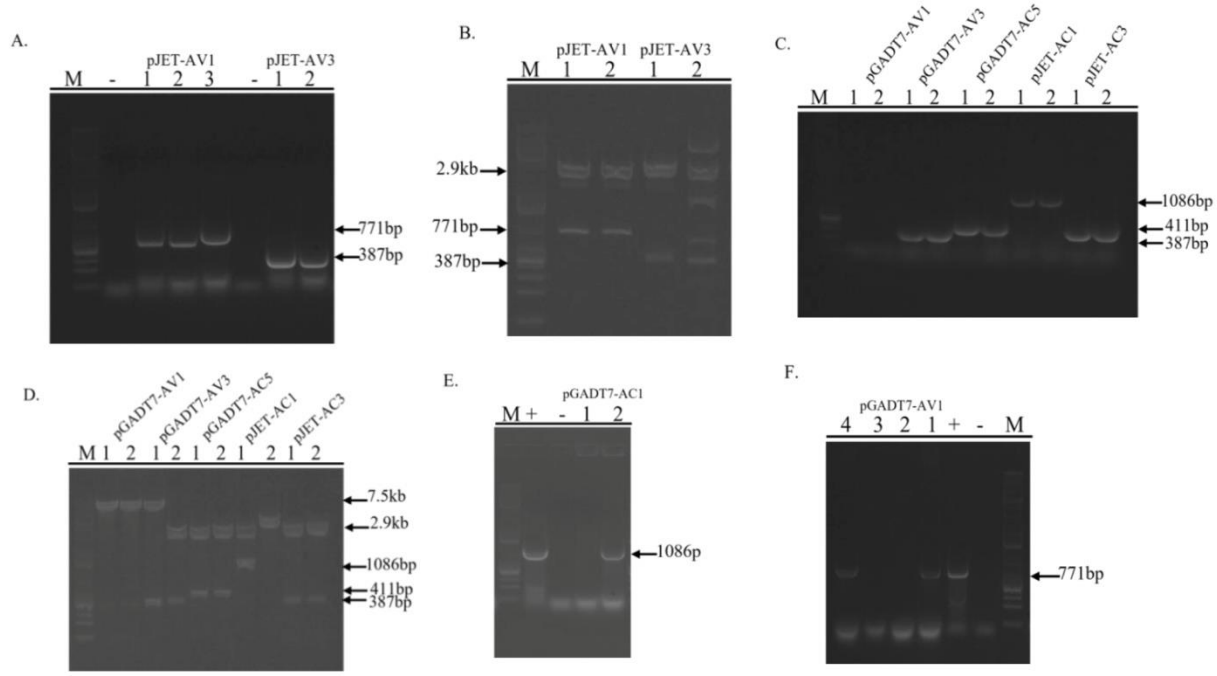
Figure 5.7 Confirmation of βV1 ORF in localization vector.

(A) Confirmation by PCR using primers specific for βV1 in pJET-βV1, (B) by restriction digestion with *Nco*I and *Spe*I showing release of approximately 351bp. (C) Confirmation of

pCAMBIA1302-βV1 clone by PCR using primers specific for βV1 ORF. (C) Confirmation of pCAMBIA1302-βV1 clone by restriction digestion with *Nco*I and *Spe*I shows release of approximately 351bp. (E) pCAMBIA1302- βV1 clone was further transformed into *Agrobacterium* GV3101and verified successfully transformed colonies by PCR.

5.3.3 Cloning of ToLCNDV DNA-A encoded ORFs in Y2H vector

ToLCNDV ORFs (AC1, AC3, AV1, AV3) were PCR amplified from the monomeric clone of ToLCNDV and cloned into a pJET1.2 cloning vector. PCR amplified product was extracted using gel extraction kit and ligated into pJET1.2 vector proceeded with the transformation of ligation product into *E. coli* DH5α competent cells. The transformed colonies were screened on an antibiotic selection plate containing 100µg/ml Ampicillin. The pJET1.2 clones were confirmed by PCR and restriction digestion of the plasmid isolated (Figure 5.8). The PCR and restriction digestion confirmed clones were further confirmed by DNA sequencing analysis. The pJET1.2 confirmed plasmid DNA was restriction digested with specific restriction sites and cloned in frame to the pGADT7 vector. The pGADT7 clones were confirmed by PCR and restriction digestion and sequencing.



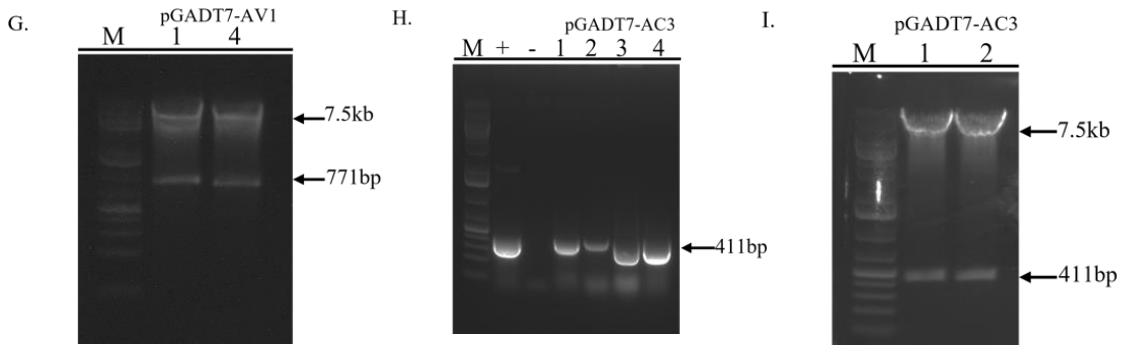


Figure 5.8 Confirmation of ToLCNDV encoded ORFs in Y2H vector.

(A) Confirmation by PCR using primers specific for AV1 and AV3. (B) by restriction digestion with *EcoR*I-*BamH*I and *Nde*I-*EcoR*I, respectively. (C) Confirmation of pGADT7-AV3, pJET-AC1, and pJET-AC3 clones by PCR using primers specific for each. (D) Confirmation of pGADT7-AV3, pJET-AC1 and pJET-AC3 clone by restriction digestion. (E) Confirmation of pJET-AC1 by PCR. (F) Confirmation of pGADT7-AV1 clone by PCR using primers specific for AV1. (G) Confirmation of pGADT7-AV1 clone by restriction digestion. (H, I) Confirmation of pGADT7-AC3 clone by PCR and digestion.

5.3.4 Potato Virus X (PVX) based overexpression of RaLCB encoded βV1 and βC1 in *N. benthamiana*

Agrobacterium harboring PVX, PVX-βV1, and PVX-βC1 were infiltrated into 3-weeks old *N. benthamiana* plants. In plants infiltrated with PVX alone (pGR106 vector), mild mosaic symptoms appeared on the leaves at around 7dpi. However, plants infected with PVX-βV1 showed severe necrotic lesions in addition to leaf curling, mild chlorosis, and mosaic symptom. The necrotic lesions were observed both at local tissue and at systemic leaves and resembles the hypersensitive response (HR) type cell death (indicated by red arrow, Figure 5.9A, B). This observation is consistent with the strong hypersensitive response mediated cell death in *N. benthamiana* during local transient expression of βV1. A similar response was observed during PVX based expression of TYLCCBencoded βV1 protein (Hu et al., 2020). PVX-βC1 expressing plants displayed typical betasatellite associated symptoms like leaf curling, veinal chlorosis, stunted growth (Figure 5.7B).

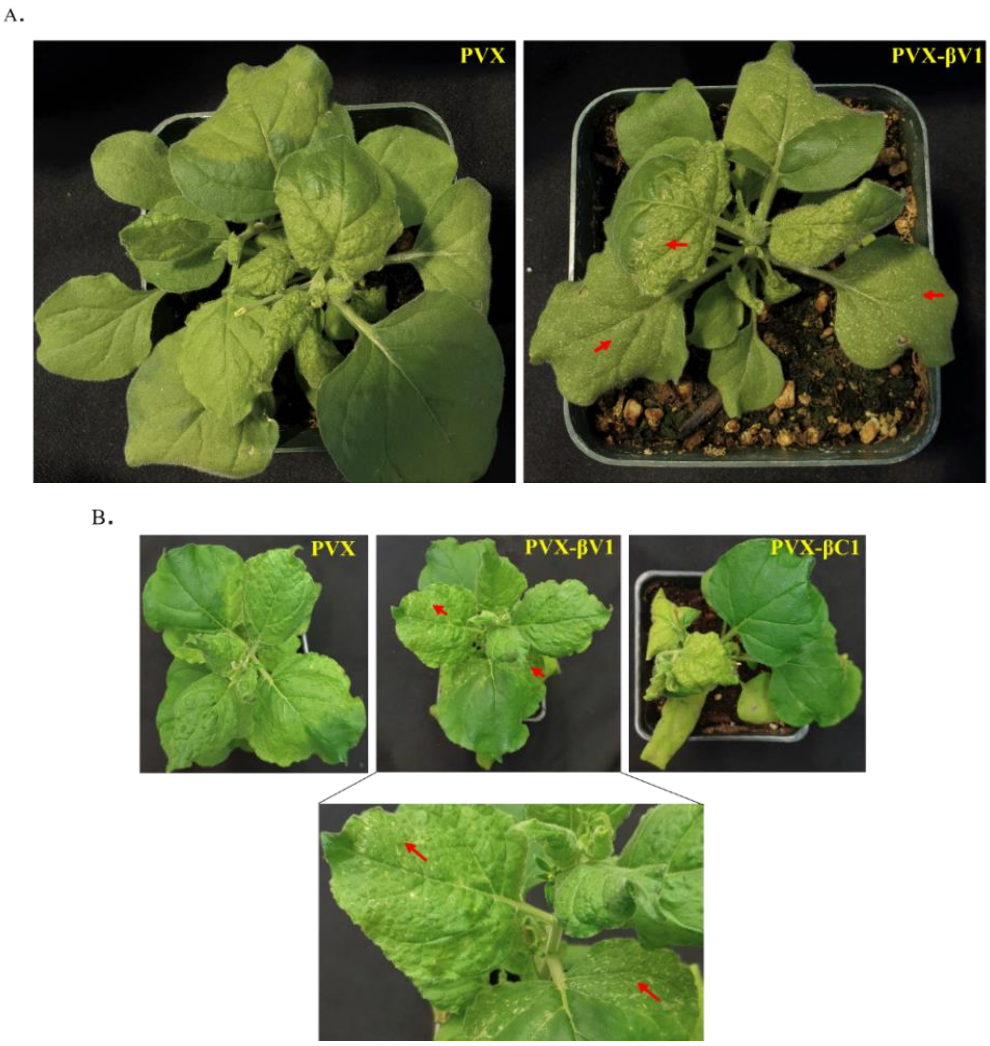


Figure 5.9 βV1 protein acts as inducer of HR response.

(A) Necrotic spots representing HR-like cell death observed in *N. benthamiana* plants infiltrated with PVX-βV1 at 7dpi, absent in plants infected with PVX alone. (B) HR response is clearly visible in systemic leaves of βV1 expressing plants while PVX-βC1 infiltrated plants showing typical leaf curling, stunted growth, and veinal chlorosis, but no visible HR.

The HR response is one of the earliest defense responses of the plant towards pathogen prior to cell death. The initial recognition of the avirulence factor or elicitor leads to the oxidative burst, resulting in the accumulation of reactive oxygen species (ROS), leading to cellular damage. Therefore, HR is preceded by reactive oxygen species (ROS) accumulation in the cells. To study ROS generation in PVX-βV1 infiltrated plants, leaves were stained with DAB and NBT to observe the presence of hydrogen peroxidases (H_2O_2) and superoxide anions (O_2^-) respectively

(Figure 5.10A) and, additionally trypan blue to visualize the dead cells. PVX-βV1 showed maximum accumulation of free radicals compared to the PVX-βC1 and PVX alone, which further supports the role of βV1 as an inducer of cell death. Mock plants did not produce any reactive oxygen species. βV1 induced cell death was further supported by staining of PVX-βV1 infiltrated plants with trypan blue. Figure 5.10B shows higher cell death in the case of βV1 expressing leaves.

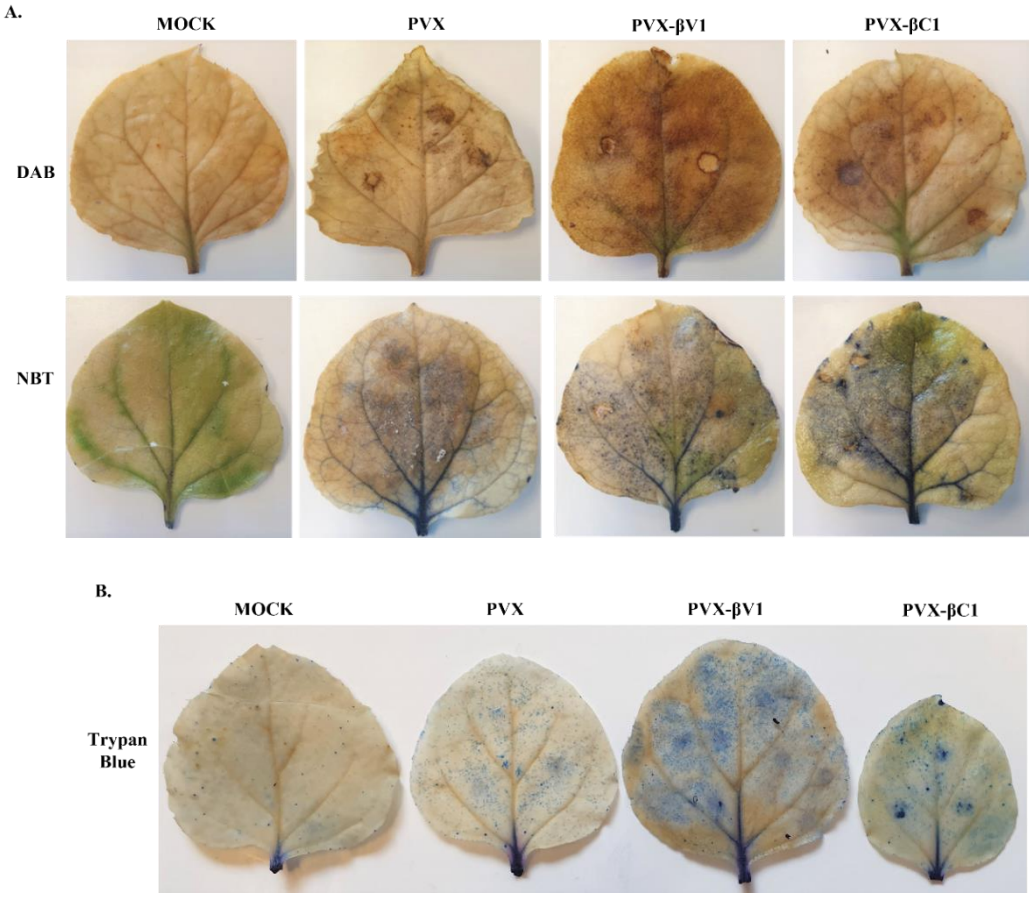
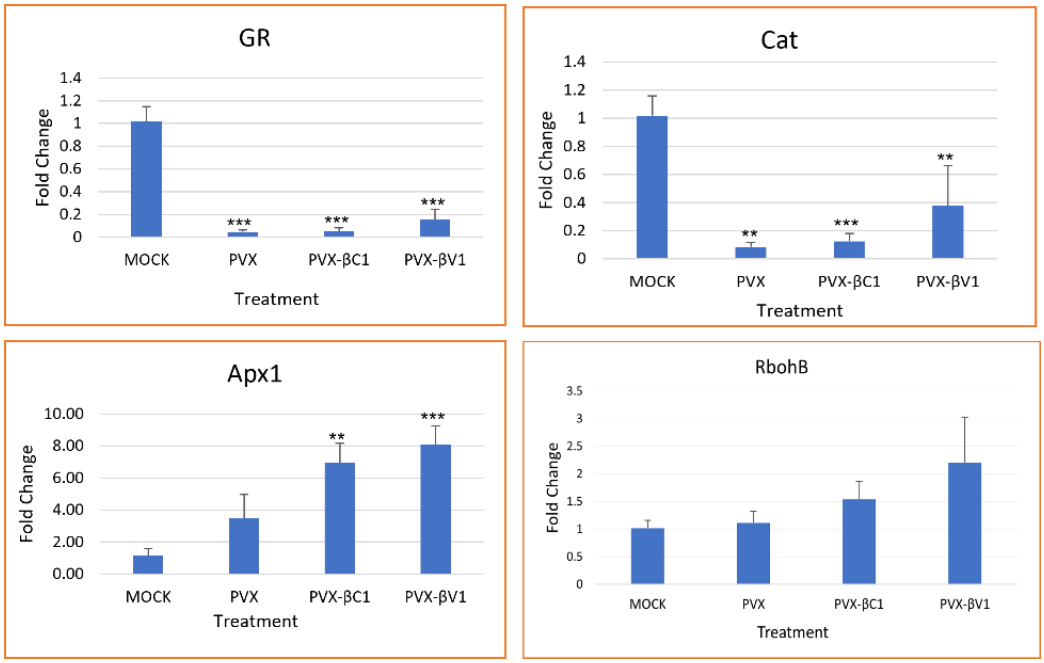


Figure 5.10 PVX-βV1 induction of HR preceded by the generation of ROS and free radicals.

(A) The relatively higher accumulation of ROS and free radicals were detected both in PVX-βV1 and PVX- βC1 infiltrated leaves samples collected at 7dpi by 3,3-Diaminobenzidine (DAB) and Nitro Blue Tetrazolium chloride (NBT) staining respectively. (B) Cell death examination by trypan blue staining shows significantly higher dead cells in PVX-βV1 infiltrated cells.

5.3.5 Expression analysis of hypersensitive response and pathogenesis-related transcripts during PVX based overexpression of βV1 and βC1

Oxidative burst during HR to check the elicitor involves the transcript accumulation of HR-related genes. Many transcripts regulating HR response like respiratory burst oxidase homologues B (RbohB), Glutathione reductase (GR), Catalase (Cat) and pathogenesis related transcripts like NPR1, PR1 and PDF1.2 were analyzed through Real-Time Quantitative Reverse Transcription PCR, performed with the samples collected from *N. benthamiana* plants infiltrated with PVX alone, PVX-βV1 and PVX-βC1 at 5th dpi (Figure 5.11). The reference gene used for normalization was protein phosphatase 2A (PP2A). RbohB, which plays a major role in hydrogen peroxide production in the cells, increased to 2.2-fold and 1.5-fold in PVX-βV1 and PVX-βC1, respectively. However, the fold increase was not significant compared to uninfected plants (Mock), and PVX alone infiltrated plants. Interestingly, the transcript level of glutathione reductase and catalase (ROS scavenging enzymes) which protects cells from damaging impact of H_2O_2 and other free radicals were significantly downregulated in all the treatments compared to mock plants. The transcript level of ascorbate peroxidase enzyme was increased to 8.0 and 6.9 folds in PVX-βV1 and PVX-βC1 inoculated plants, respectively. The expression of defense-related transcript NPR1 was not affected in the infected plants. Nonetheless, PR1 and PDF1.2 were significantly increased in PVX-βC1, and PVX-βV1 inoculated plants, respectively. However, plants infiltrated with PVX alone showed higher PDF1.2 levels compared to PVX-βV1, suggesting βV1 might have a role in negatively regulating PDF1.2 levels, thereby having indirect control on jasmonic acid responses during infection.



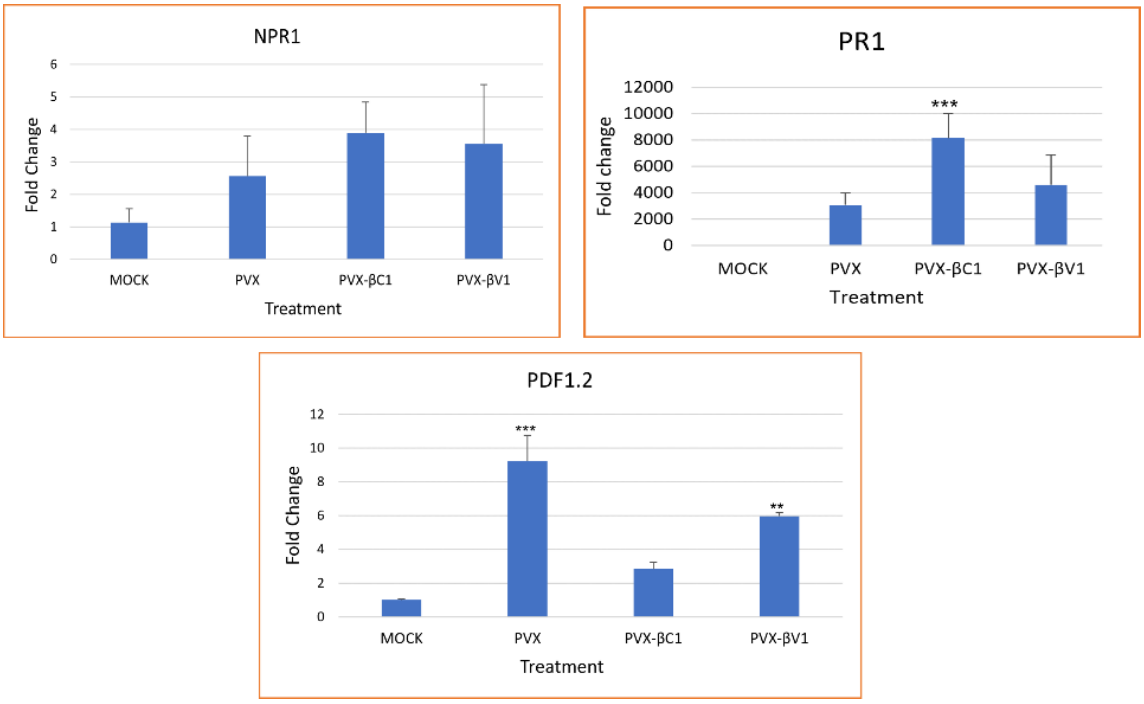


Figure 5.11 Transcript analysis through qRT-PCR.

Fold change expression of HR-related transcripts (RbohB, Cat, GR, and Apx1), and defense-related transcripts (NPR1, PR1, and PDF1.2) in infiltrated leaves of *N. benthamiana* infiltrated with PVX, PVX-βC1, and PVX-βV1 at 5dpi. The PP2A gene was used as a reference control.

5.3.6 Sub-cellular localization of βV1 protein

The presence of a transmembrane signal in βV1 based on the *in-silico* data analysis pointed to the localization of βV1 in the plasma membrane. The localization studies through confocal microscopy revealed the presence of βV1 protein in the cellular periphery or plasma membrane. GFP signal was observed throughout the cell in the case of pCAMBIA1302 vector infiltrated samples. However, GFP fluorescence in pAMBIA1302-βV1 infiltrated plants was present only at the cellular periphery (Figure 5.12).

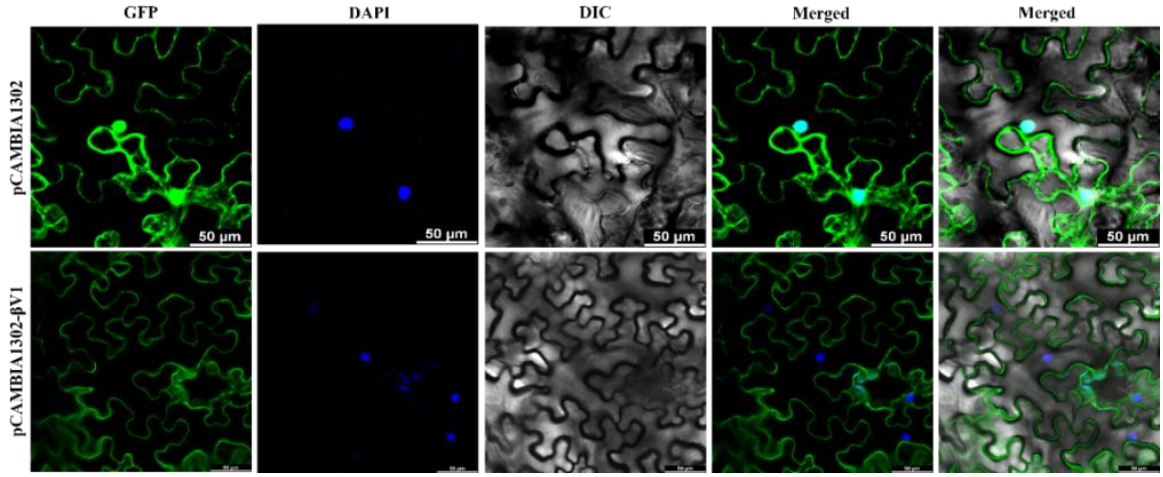


Fig 5.12 Localization of βV1 protein.

Confocal microscopy reveals the localization of βV1 protein in the plasma membrane. Lanes 1-3 show localization of GFP (upper panel) or βV1-GFP (lower panel), a nucleus stained with DAPI, DIC image of *N. benthamiana* epidermal cells. Lanes 4 and 5 represent merged images.

5.3.7 βV1 protein encoded by RaLCB interacts with ToLCNDV encoded AC3 protein

ToLCNDV DNA-A association with RaLCB produces a severe array of symptoms on *N. benthamiana* plants. The dependence of the helper virus on RaLCB for severe disease development suggests an intricate network of connections between the betasatellite proteins and viral proteins. After functional characterization of the βV1 protein at an individualistic level, it is important to unveil this connection. To study the interaction between βV1 and ToLCNDV encoded ORFs AC1, AC2, AC3, AC4, AV1, AV2 and AV3, yeast-two hybrid assay (Y2H) was conducted. Interestingly, yeast cells AH109 which were cotransformed with pGADT7-AC3 and pGBKT7-βV1 were grown on SD media having 2 mM 3AT but lacking amino acids leucine, tryptophan, and histidine (Figure 5.13). pGADC1-AC1 and pGABDC1-AC1 combination was taken as positive control, while combination pGADC1 and pGBDC1 was used for negative control.

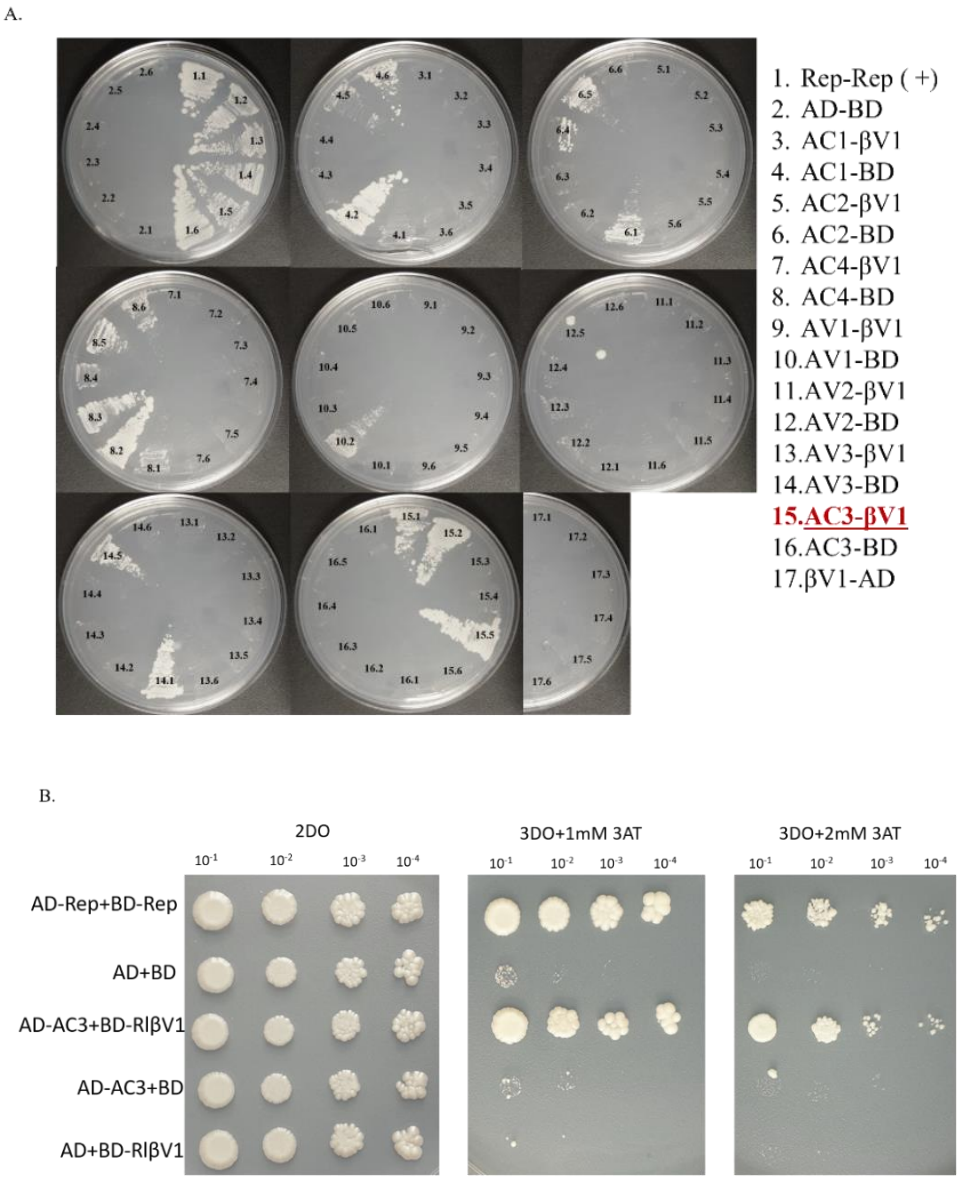


Figure 5.13 βV1 interacts with the AC3 protein of ToLCNDV.

(A) *S. cerevisiae* AH109 transformants harboring Y2H plasmid combination (as indicated in the right panel) grown on the selection medium SD-Leu-Trp-His supplemented with 2mM 3-amino-1,2,4-triazole. The yeastco-transformed with pGADT7-AC3 and pGBKT7-βV1 grew on the selection medium. interacts with helper virus encoded AC3protein. The growth was not observed in any other cotransformed combination except positive control. (B) Growth of cotransformed yeast after serial dilutions from cultures of OD 1.0 at 600nm. AD and BD represents pGADT7 and pGBKT7 respectively.

5.3.8 BiFC confirms the interaction between AC3 and βV1 proteins

The interaction between AC3 and βV1 proteins was further tested in planta by BiFC assay. The three-weeks old plants were infiltrated with pSITE-EYFP-N1: βV1 and pSITE-EYFP-C1:AC3 and epidermal leaf sections were analyzed by confocal microscopy after 48hours. The reconstituted yellow fluorescent protein signal was observed in the nucleus, suggesting that AC3 and βV1 interacts in the nucleus (Figure 5.14). No fluorescence was detected in leaves infiltrated with EYFP-N1+EYFP-C1, EYFP-N1: βV1+EYFP-C1, and EYFP-N1+EYFP-C1:AC3. During viral infection, βV1 might be relocating to the nucleus in the presence of AC3 protein for regulating the pathogenesis.

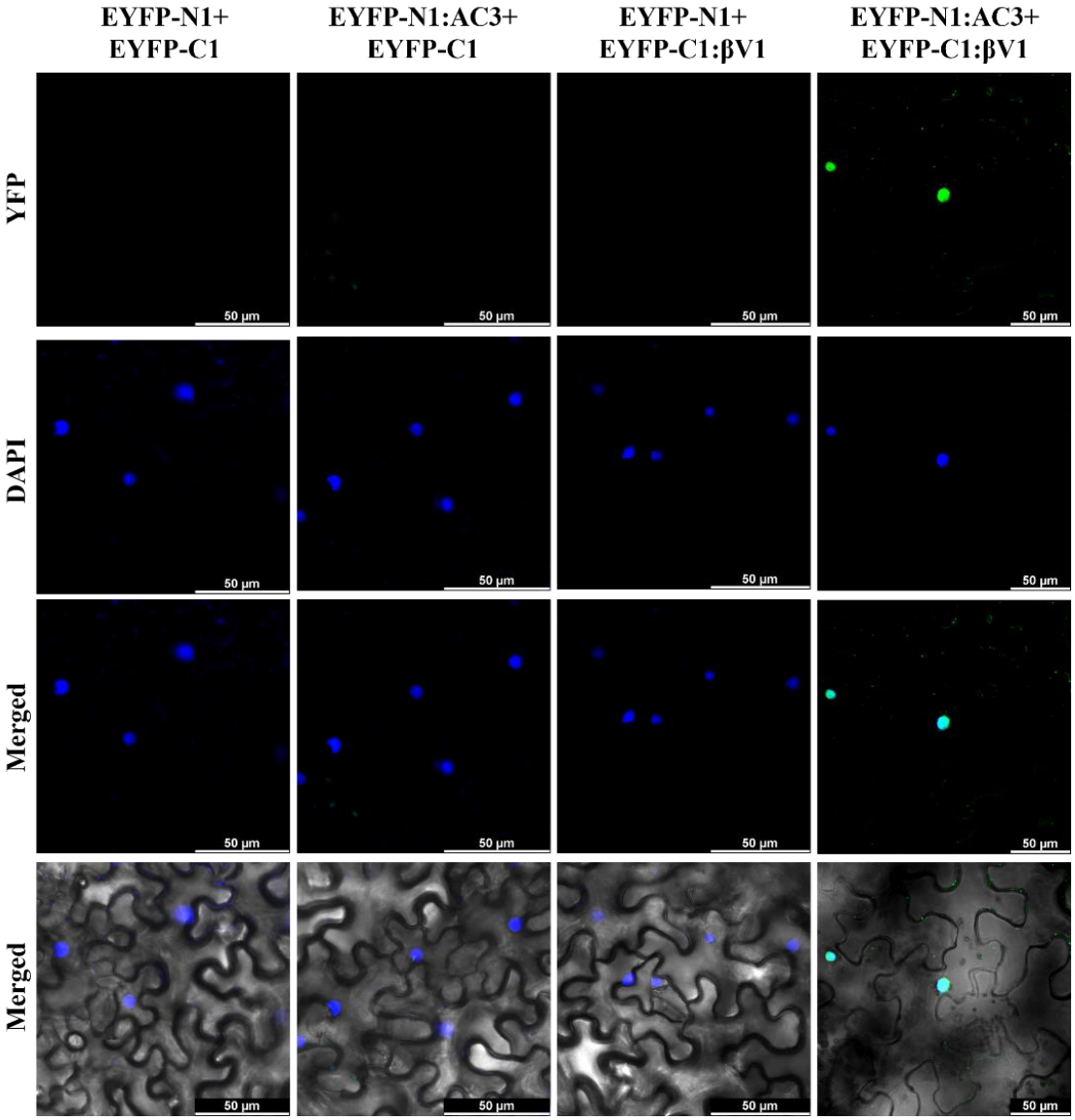


Figure 5.14 BiFC to study the interaction between AC3 and βV1.

Agrobacterium strain GV3101 harboring different combinations of BIFC constructs were coinfiltrated into *N. benthamiana* plants. Epidermal cells of leaves were visualized by confocal microscopy. The reconstituted YFP was observed in the nucleus. The nucleus was stained with DAPI.

5.4 Discussion

Viruses, because of their limited coding capacities, modulate the host cellular system by reprogramming the proteome and transcriptome of the host in a direction that proliferates the virus and enhances its pathogenesis. Monopartite begomoviruses with their 2.7kb genome and limited capacity of coding, depend on the betasatellites for symptom development, defense regulation, and movement. Until recently, betasatellite was known to encode a single protein βC1 of 13kDa, in the complementary sense strand, which plays a major role associated with betasatellites. The coevolution of cognate begomoviruses-betasatellites complexes might have grounded the existence of a new βV1 ORF.

Here, we characterized the function of βV1 protein, a novel protein encoded by the virion sense strand of RaLCB. βV1 protein, when transiently expressed using PVX based vector in *N. benthamiana*, was able to produce typical leaf curling symptom. Moreover, βV1 was found to induce HR-type cell death which is usually not observed during typical geminivirus infections. HR is a form of resistance response generated in the plant during the compatible interaction of resistance gene (R-gene) in the host and a pathogen-associated avirulence gene (Avr gene) during the infection (Flor, 1971; Dangl and Jones, 2001). This gene-for-gene relationship theory ascertains the outcome of interaction into a disease or resistance. The slight systemic necrosis observed in the PVX alone infected plants was clearly different from the HR response elicited by PVX-βV1 over-expressing plants, which was observed both at local tissue and the systemic leaves. It is possible the βV1 protein acts as a protein elicitor to generate hypersensitive response and may interact with any R-gene associated with the host, which required further detailed investigation. During natural geminiviral infection, the viral molecular network may regulate the expression of βV1 towards the lower level to bypass this defense response for successful pathogenesis. Similar HR induction was observed when the N protein of tomato spotted wilt virus (TSWV) was over-expressed in *Capsicum chinense* and reported as avr component of HR

(Lovato et al., 2008). Many viral proteins like CI, P1, P3N-PIPO encoded by potyviruses, citrus tristeza virus encoding triplet proteins p33, p18, and p13 act as viral elicitors and pathogenicity determinants (García & Pallás, 2015). NSP of BDMV, C2 of TYLCSVand V2 encoded by tomato leaf curl Java virus-A have been identified as avirulence factors (Zhou et al., 2007; Sharma and Ikegami, 2010; Matić et al., 2016). Though the present study has not elucidated the exact role of βV1 in viral pathogenesis, its role in HR induction has been proven beyond doubt. The generation of ROS and free radicals was also confirmed by DAB and NBT staining. Cell death induced by HR was further observed by trypan blue staining. Additionally, the alteration of HR and defense-related transcripts in all the infected plants favored the HR generation.

βV1 protein was predicted to be a transmembrane protein through the FFPred tool, which has N-terminal cytoplasmic region, one transmembrane helix, and a C-terminal cytoplasmic tail. In silico analysis revealed the localization of βV1 protein in membrane with a higher probability of transporter/cation transmembrane transport function. Interestingly, the presence of βV1 at the cellular periphery as observed by confocal microscopy establishes the protein to be membrane localized. Furthermore, calcium (Ca^{2+}) ions influx plays a significant role in elicitor perception that primes major signaling cascades during host-pathogen interaction (Garcia et al., 2006). Since βV1 have a greater likelihood of nucleoside/nucleotide/adenyl nucleotide/ATP binding/purine nucleoside binding or catalytic or ion channel activity (unpublished, Reddy KK et al.), there is a possibility that βV1 may mimic or regulate calcium ion transporter which, upon activation by protein kinases aids in the generation of reactive oxygen species and mitogen-activated protein kinase (MAPK) cascade signaling pathways for defense (Garcia et al., 2006). Although βC1 protein negatively interferes with the MAPK defense pathway, both βV1 and βC1 protein may act synergistically for regulating the host proteome to be functional towards severe pathogenesis (Hu et al., 2019; Hu et al., 2020).

In addition, any viral protein expands its functionality by interacting with other viral proteins or host proteins (Wang et al., 2021). The present study also identified that βV1 protein interacts with the REn encoded by helper virus ToLCNDV. The interaction in the nucleus suggests the relocalization of βV1 protein into the nucleus to regulate pathogenesis.

The interaction paved the way for another molecular study on the functional aspect of βV1 protein in viral pathogenesis. Further research and experimentation support will help us to find new mechanisms and strategies to encounter these viruses.

Chapter 6

Summary

6. Summary

Betasatellites play a significant role in geminiviral pathogenesis and cause detrimental effects on many valuable crops that impacts food availability and the global economy. βC1, a prime pathogenicity determinant, induces disease symptoms, suppresses RNA silencing, impairs host defense response, and aids virus movement. Many reports have revealed that βC1 interacts with the host proteome and conditions the host cellular environment to establish disease. Recently, ToLCPaB-βC1 protein has been reported for the first time to hydrolyze γ-phosphate of the substrate in its natively purified protein form (Gnanasekaran et al., 2021). The characterization of βC1 protein for its biochemical activities is important to understand the functional state of protein at the molecular level during disease development. The present study finds out that this novel ATP hydrolysis activity exhibited by ToLCPaB-βC1 protein is conservative and has a biological connection during the infection process.

- All the betasatellites - CroYVMB, ToLCBB, ToLCJoB, TYLCTHB, BYVB, and RaLCB encoded βC1 proteins can hydrolyze ATP in the presence of divalent cation.
- The amino acid residues Lys-49/50, Arg-69, and Arg-91, which are crucial for ATP hydrolysis activity of ToLCPaB-βC1, are conserved in all the βC1 protein encoded from distinct betasatellites.
- ToLCPaB encoded βC1 protein oligomerizes, and mutations at 49^{th}, $69^{th,}$ and 91^{st} residues do not affect protein oligomerization.
- The ATPase deficient mutants of ToLCPaB-βC1 protein maintain the structural integrity of the protein.
- The ATPase activity of βC1 negatively correlates with the accumulation of helper begomovirus.

In the past, various pandemics turned into epidemics due to the complex association of begomoviruses and betasatellites. Various new disease complexes are constantly emerging, where, in addition to associations of betasatellites with monopartite viruses, betasatellite-bipartite begomoviruses associations are also reported. The mechanism behind the disease etiology of such mixed infections has not been explored much. Here, we tried to investigate the possible regulation between the βC1 encoded from RaLCB and the viral proteins of ToLCNDV DNA-A,

apart from replication. This study found an interactive network existing between the ToLCNDV encoded AC2 and βC1 protein of RaLCB.

- AC2 protein influences the veinal chlorosis symptom associated with RaLCB.
- The presence of AC2 responsive elements in the RaLCB sequence and reduction in βC1 transcript in ToLCVNDΔAC2+RaLCB infected plants might suggest the possibility of transactivation of βC1 ORF by AC2 protein.
- Yeast two-hybrid assay revealed that the AC2 protein of ToLCNDV interacts with the βC1 protein encoded by RaLCB. The interaction occurs strongly in the nucleus, confirmed by BiFC. A mild interaction was also observed in the chloroplast.
- AC2 protein localizes both in the nucleus and the chloroplast.

Coevolution of betasatellites along the line with geminiviruses, leads to increase in subtle molecular mechanisms to enhance pathogenesis and generate anti-host defense responses. Earlier, betasatellites were speculated to have another ORF called βV1 based on bioinformatics and some molecular work. However, it has recently come into the picture when TYLCCNB encoded βV1 ORF was found to have an impact on pathogenesis (Hu et al., 2019). In our laboratory, a novel transcript βV1, with 116 amino acids coding ORF between 234-584 coordinates in the virion-sense strand of RaLCB, has been investigated (unpublished). The present study revealed the characteristic feature and pathogenicity of βV1 protein.

- βV1 protein has a single transmembrane signal and localizes to the plasma membrane.
- Structurally, protein has three prominent helices with many sheets and coils in between.
- Functional prediction of βV1 suggests that it may bind to an ion.
- Transient overexpression βV1 protein induces a hypersensitive response in *N. benthamiana.*
- ROS and free radical accumulation precede the hypersensitive response.
- βV1 alters the accumulation of transcripts related to HR and defense
- Y2H analysis reveals that the βV1 protein interacts with the AC3 protein encoded by ToLCNDV DNA-A.
- The BiFC assay confirms the interaction between the βV1 and AC3.
- The βV1 and AC3 interact in the nucleus, suggesting that βV1 might be translocated from the plasma membrane to the nucleus and may influence viral accumulation.

- Overall, βV1 protein may function as a protein elicitor that may interact with any host resistance factor to generate a defense response in the form of HR.

Taken together, the research carried out to study the biology of viral satellite molecules and their interaction with viral proteins will pave the way to understand disease development so that antiviral strategies can be designed in the future.

Chapter 7
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7. References

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Chapter 8

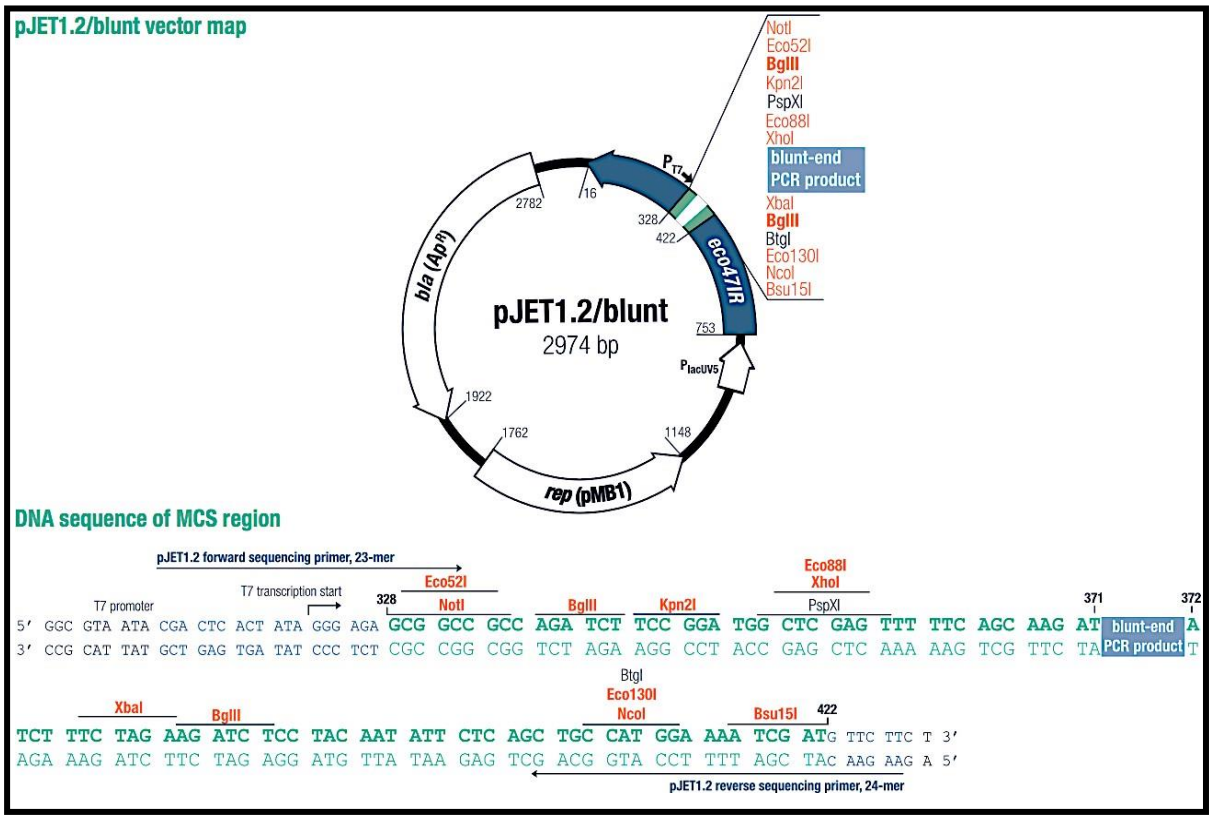
Appendix

8. Appendix

Vectors used in the study:

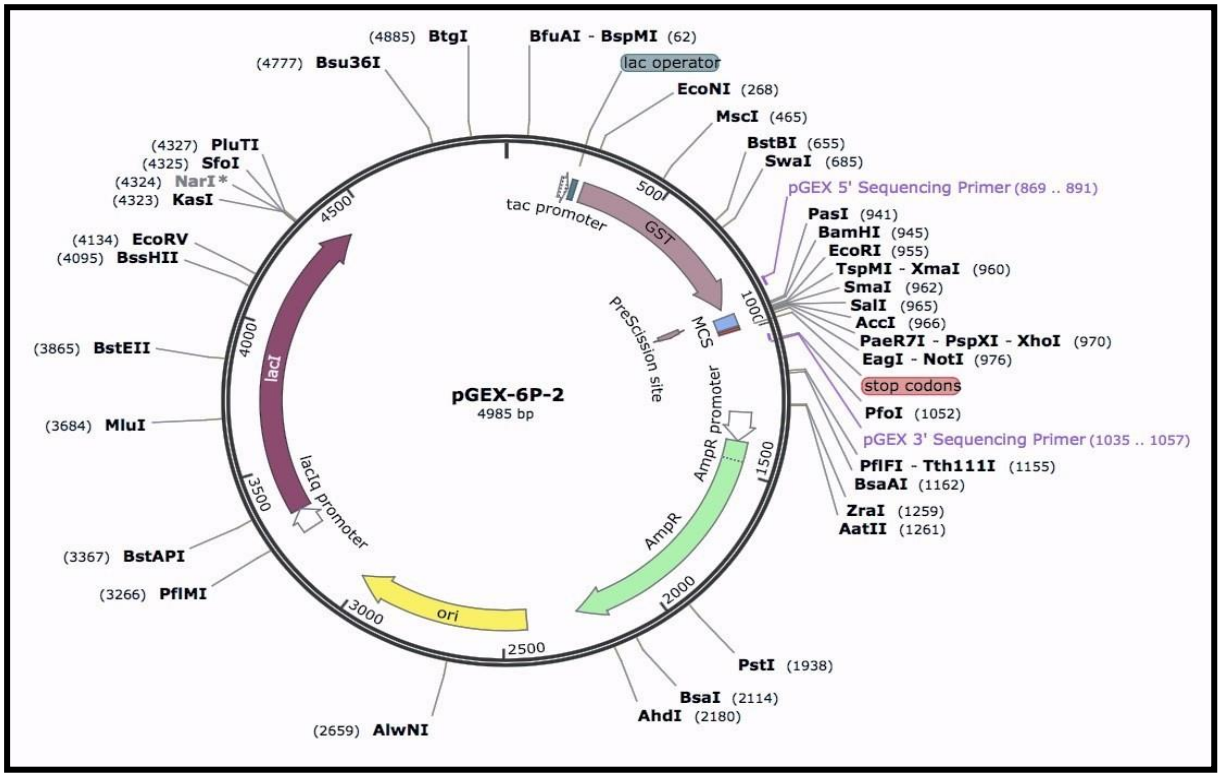
A. Cloning vector

1. pJET1.2vector



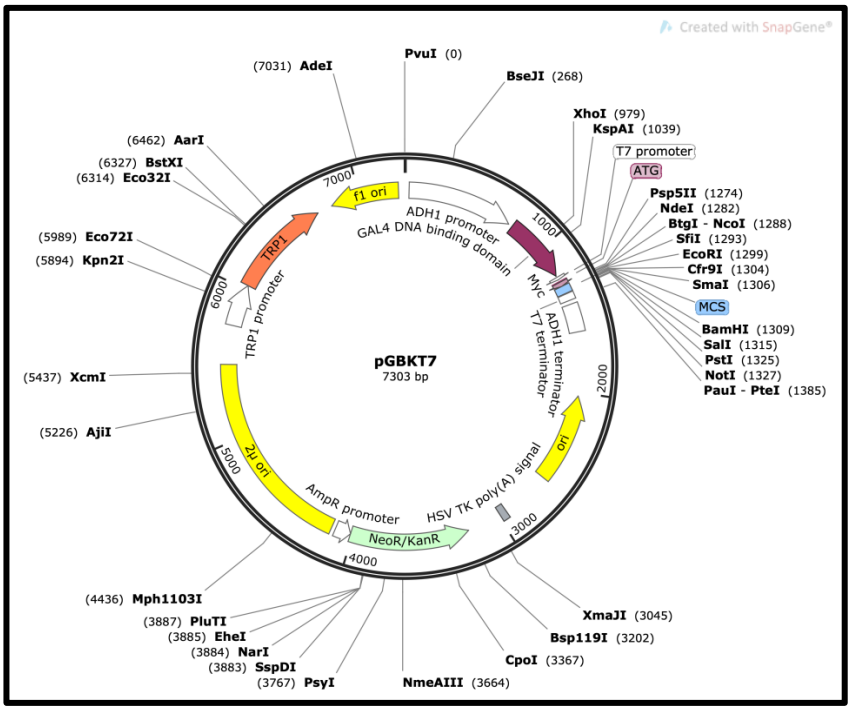
B. Bacterial protein expression vector

1. pGEX-6P-2vector

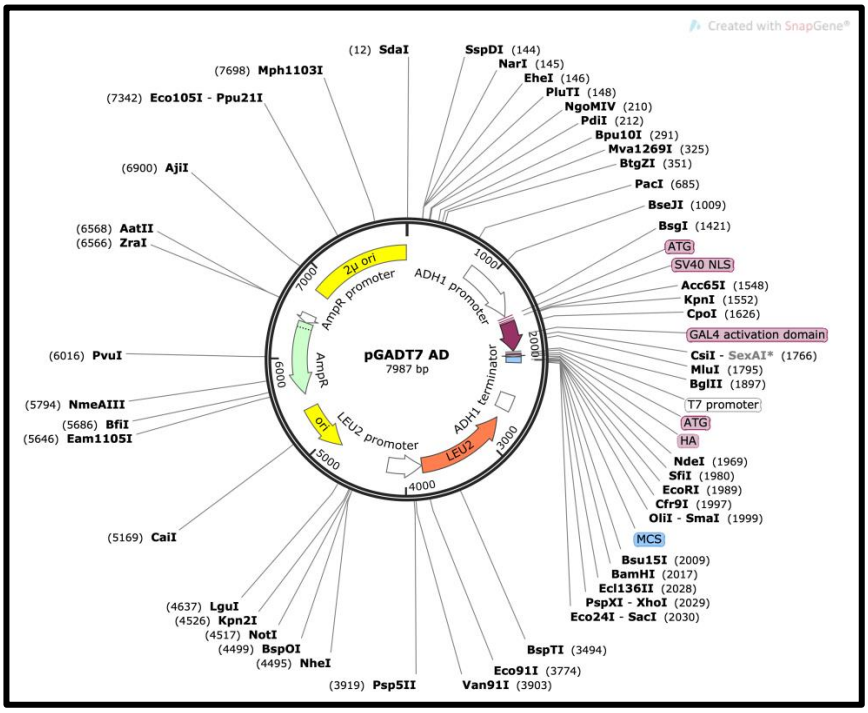


C. Yeast transformation vectors

1. pGBKT7vector

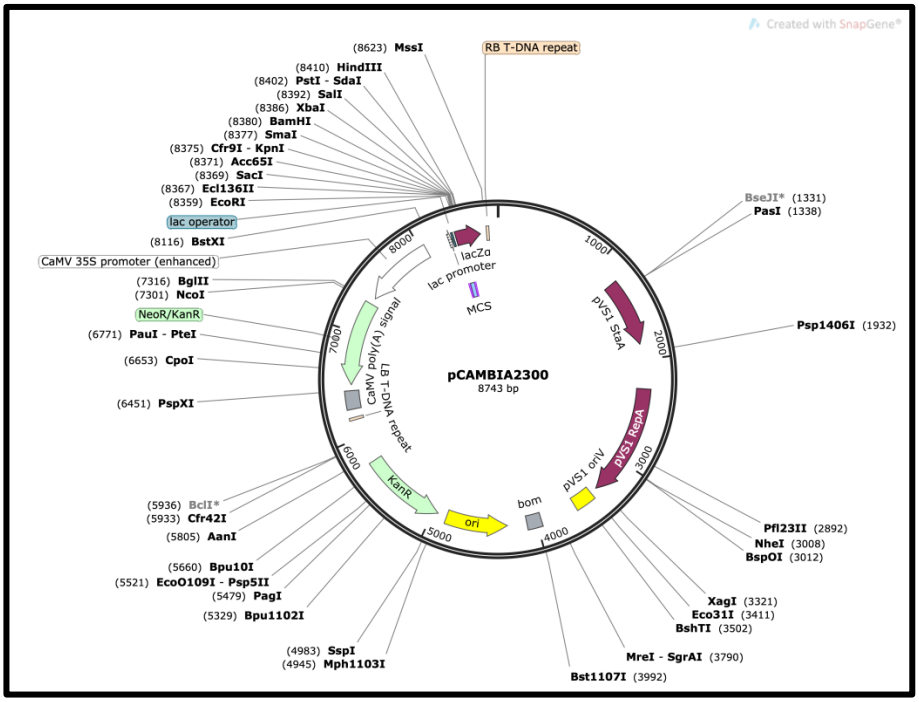


2. pGADT7vector

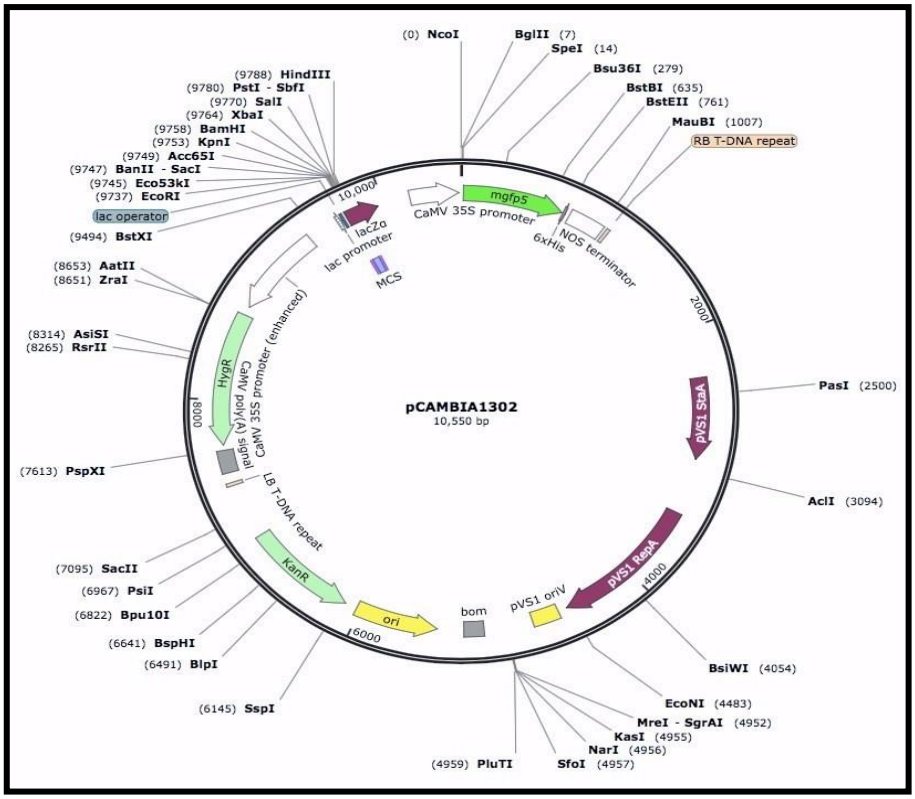


D. Plant expression vector

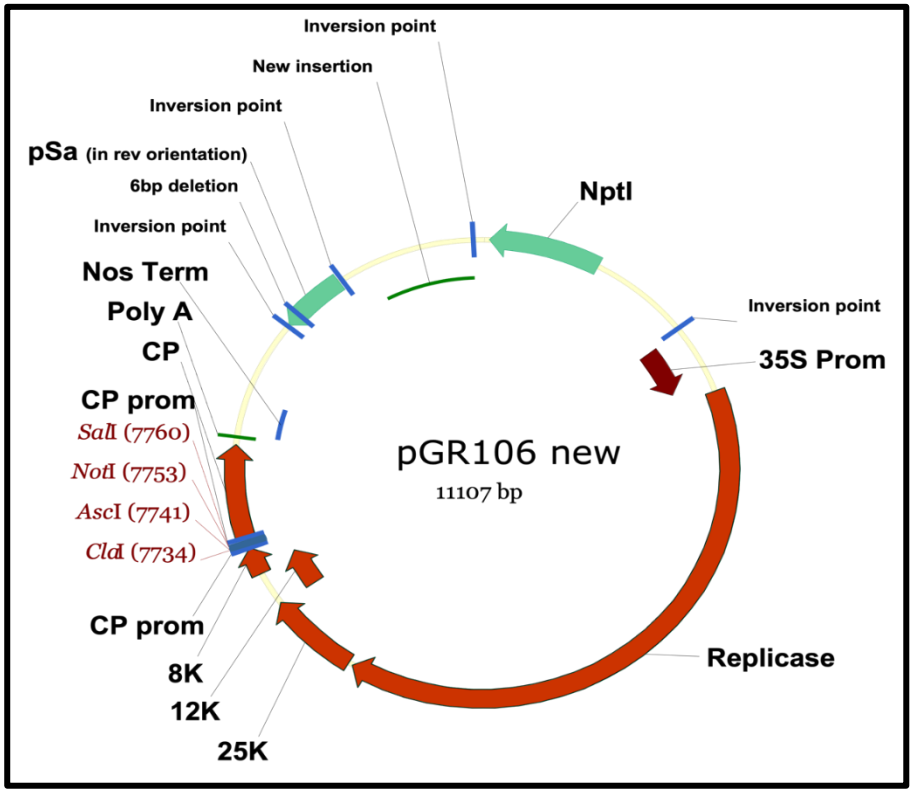
1. pCAMBIA2300



2. pCAMBIA1302

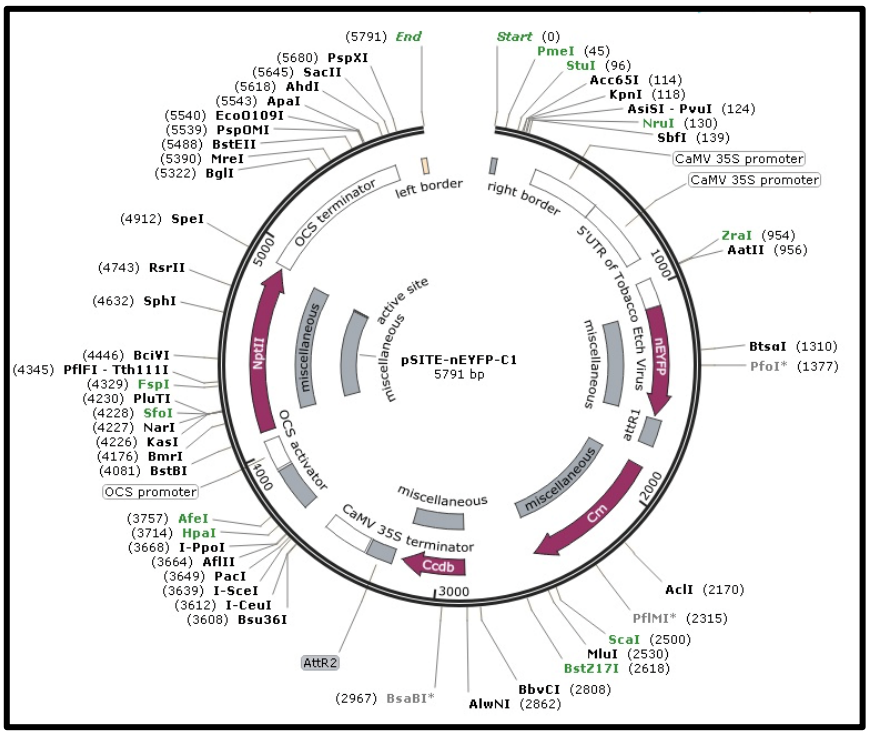


3. pGR106 vector

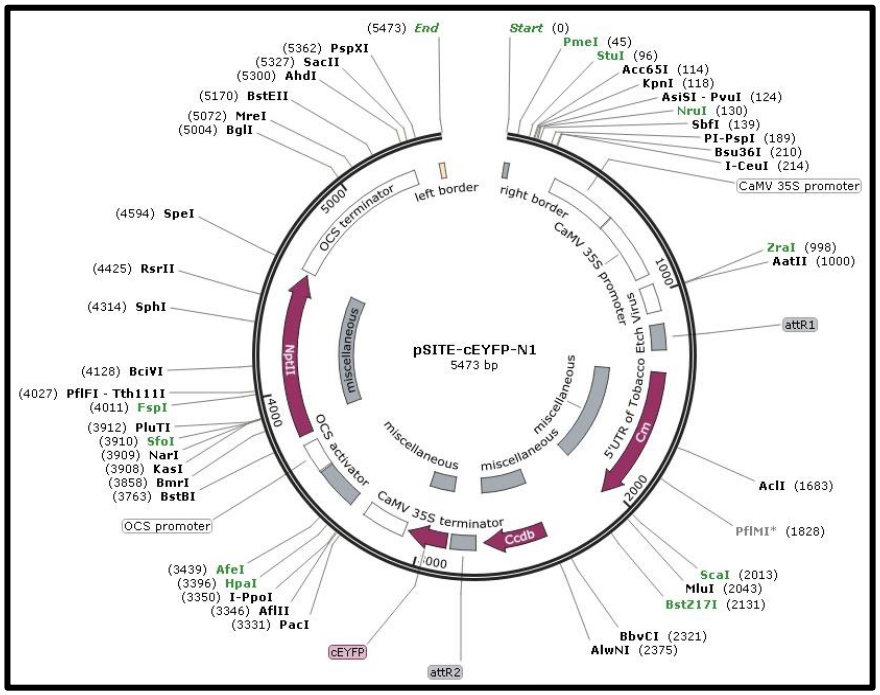


E. BiFC vectors

1. pSITE-nEYEp-N1



2. pSITE-cEYEp-N1



Preparation of reagents, buffers, and media

Buffer/Reagent/Media	**Method of preparation**
Commonly used solutions	
1M Tris-HCl (pH-8.0)	121.1g of Tris base was dissolved in 800ml of H_2O. The pH was adjusted to 8.0 by adding concentration HCl. The solution was allowed to cool at room temperature and the pH was adjusted. Then, volume of solution was adjusted to 1 litre with H_2O. This was aliquoted and sterilized by autoclaving.
NaOH (10N)	400 g of NaOH pellets were added to 800ml of water with continuous stirring. The volume was adjusted to one litre with distilled water upon the completed dissolution of the pellets in water. The solution was stored at room temperature.
SDS (10%w/v)	100g of electrophoresis-grade SDS was dissolved in 900ml of H_2O. It was heated to 68°C and stirred with magnetic stirrer to assist dissolution. The volume was adjusted to 1litre with sterile water and stored at room temperature.
Potassiumacetate (5M)	490.15g of potassium acetate was taken and dissolved in 800ml of distilled water. The volume was then made upto 1litre with distilled water.
EDTA (0.5M, pH 8.0)	186.1g of disodium EDTA.$2H_2O$ was added to 800ml of water. It was stirred vigorously on a magnetic stirrer and pH was adjusted to 8.0 with NaOH. It was dispensed into a liquots and sterilized by autoclaving.
Ethanol (70%)	This was made by mixing 70ml of absolute ethanol with 30ml of sterile distilled water.
Chloroform:Isoamyl alcohol (24:1)	The mixture was prepared by adding 24ml of chloroform and 1ml of isoamyl alcohol.

$CaCl_2$ (2.5M)	11g of $CaCl_2.6H_2O$ was dissolved in a final volume of 20ml of distilled water. The solution was sterilized by passing it through a 0.22µ filter. It was aliquots and stored at 4°C.

Buffers used for plasmid isolation	
Solution I	It was prepared by adding: 2.5ml of 1M Tris-HCl (25mM) - pH 8.0 4.5ml of 20% Glucose (50mM) 2.0ml of 0.5M EDTA (10mM) The final volume was made upto100ml with sterile distilled water.
Solution II	It was prepared freshly by mixing: 2.0ml of 10N NaOH (0.2N) 10.0ml of 10% SDS The volume was made upto100ml with sterile distilled water.
Solution III (pH 5.8)	It was prepared by adding: 5M Potassium acetate - 60ml Glacial acetic acid - 11.5ml Distilled water - 28.5ml The concentration of potassium acetate found in the final solution was 3M. The solution was autoclaved and stored at 4°C.
Reagents/Media used for cloning	
Ampicillin (100mg/ml)	1 g of ampicillin was dissolved in 10 ml of double distilled water, filter sterilized through 0.22µ filter and aliquoted in 1.5ml tubes and stored at -20^0C.
Kanamycin (50mg/ml)	0.5 g of kanamycin was dissolved in 10 ml of double distilled water, filter sterilized through 0.22µ filter and aliquoted in 1.5ml tubes and stored at -20^0C.

Luria broth media	1g of bacto-tryptone, 0.5g of yeast extract and 1g of NaCl was dissolved in 95 ml of water and the pH was adjusted to7.0 with 5N NaOH. The volume was made upto 100ml and sterilized by autoclaving.
Luria agar media	1g of bacto-tryptone, 0.5g of yeast extract and 1g of NaCl was dissolved in 95 ml of water and the pH was adjusted to 7 with 5N NaOH. The volume was adjusted to 100ml and 1.5g of bacto-agar was added. It was then sterilized by autoclaving.

100mM $MgCl_2$	2.03g of $MgCl_2.6H_2O$ was dissolved in distilled water and the volume was adjusted to100ml and sterilized by autoclaving.
100mM $CaCl_2$	1.47g of $CaCl_2.2H_2O$ was dissolved in distilled water, volume adjusted to100ml and sterilized by autoclaving.
Buffer used for total genomic DNA isolation	
Extraction buffer (100ml)	The buffer was prepared by the addition of 10 ml of 1M Tis-HCl pH 8.0, 10 ml of 0.5M of EDTA pH 8.0, 10 ml of 5N of NaCl and 500µl of β-mercaptoethanol. The volume was made up to 100 ml using sterile distilled water.
Solutions used for Southern hybridization	
SSC (20X)	It was prepared by adding 75.3 g NaCl, 88.2 g Sodium citrate (pH adjusted to 7.0 by adding 14N HCI) in 1L of distilled water.
Denaturation solution	It was prepared by adjusting final concentration of 1.5 M NaCl and 0.5M NaOH in distilled water.
Depuration solution	It was prepared by adding 2.8 ml of HCl in 197.2 ml of sterile distilled water.
Neutralization solution	It was prepared by adding 1.5M NaCl, 1.0 M Tris-HCl (pH 7.2).

Denhardt's solution (50X)	It was prepared by adding 1 % (W/V) ficol, 1% (W/V) polyvinylpyrrolidone, 1% (W/V) BSA and was dissolved in distilled water.
Herring sperm DNA	100 mg of DNA was dissolved in 10 ml of sterile distilled H_2O and mixed by vortexing, stored at -20°C.

Protein purification buffers	
Lysis buffer	It was freshly prepared by adding: 5 ml of 1M Tris-HCl (pH 8.0) 1ml of 5M NaCl 10ml of 100% glycerol 5ml of 100mM $MgCl_2$ 34 µl of 2-mercaptoethanol 1ml of 100mM EDTA 1ml of 100mM PMSF 500µl of Triton X-100 500µl of Tween 20 The final volume was made upto 100ml with sterile distilled water.
Wash buffer	It was freshly prepared by adding: 5 ml of 1M Tris-HCl (pH 8.0) 5ml of 5M NaCl 10ml of 100% glycerol 5ml of 100mM $MgCl_2$ 34 µl of 2-mercaptoethanol 1 ml of 100mM PMSF 500µl of Triton X-100 500µl of Tween 20 The final volume was made upto 100ml with sterile distilled water.

Equilibration buffer	It was freshly prepared by adding: 5 ml of 1M Tris-HCl (pH 8.0) 1ml of 5M NaCl 10ml of 100% glycerol 5ml of 100mM $MgCl_2$ 34 µl of 2-mercaptoethanol 500µl of Triton X-100 The final volume was made upto 100ml with sterile distilled water.

Elution buffer	It was freshly prepared by adding: 5 ml of 1M Tris-HCl (pH 8.0) 1ml of 5M NaCl 10ml of 100% glycerol 5ml of 100mM $MgCl_2$ 34 µl of 2-mercaptoethanol 500µl of Triton X-100 5ml of 1M reduced glutathione. The final volume was made upto100ml with sterile distilled water.
Yeast Transformation Solutions	
10X Lithium Acetate (LiAc)	A stock solution of 1M LiAc (Sigma Catalog #L6883) was prepared in double distilled water. pH 7.5 was adjust with dilute acetic acid. Finally, the solution was autoclaved and stored at room temperature.
50% (w/v) PEG3350	50g of PEG (Sigma Catalog #P3640, average molecular weight: 3350) was dissolved in dH_2O and the volume was made upto100ml. Finally, the solution was filter sterilized or autoclaved and stored at room temperature.

10X TE buffer	10× TE buffer was prepared by adding 100 mM Tris-HCl (pH 7.5) and 10 mM EDTA (pH 8.0) solutions which was then autoclaved and stored at room temperature.
1X TE Buffer	1X TE buffer was prepared by adding1 ml of 10X TE buffer in 9ml of dH_2O.
TE-LiAc Solution	A solution of TE–LiAc was prepared by adding1ml of 10X TE buffer and 1ml of 10X LiAc solutions in 8ml of sterile dH_2O.
TE-LiAc-PEG Solution	TE–LiAc–PEG Solution was prepared by adding 1ml of 10X TE buffer and 1ml of 10X LiAc in 8ml of 50%(w/v) PEG3350.

Yeast media	
YPAD Broth	20 g of Difco peptone and 10 g of yeast extract was added to 960 ml of deionized H_2O and the pH was adjusted to 5.8.40mg of adenine sulfate was added into it. The final solution was autoclaved and cooled to 55°C.2% (v/v) glucose was added to the solution which was prepared by adding 40ml of a 50% stock solution, filter sterilized or autoclaved separately.
YPAD Agar	20 g of Difco peptone, 10 g of yeast extract and 15–20 g of agar was added to 960ml of deionized H_2O and the pH was adjusted to 5.8.40 mg of adenine sulfate was added into it. The final solution was autoclaved and cooled to 55°C.2%(v/v) glucose was added to the solution which was prepared by adding 40ml of a 50% stock solution, filter sterilized or autoclaved separately.
Synthetic dropout media	6.7 g of Difco yeast nitrogen base without amino acids was added to 860ml deionized H_2O and the pH was adjusted to 5.8. The solution was then autoclaved and cooled to 55°C.100 ml of the appropriate 10X dropout solution and 40 ml of a 50% stock solution of glucose (which was filter sterilized or autoclaved separately) were then added into it.

Synthetic dropout Agar	6.7 g of Difco yeast nitrogen base (Difco Catalog #0919-15-3) without amino acids and 15–20 g of agar was added to 860 ml deionized H_2O and the pH was adjusted to 5.8. The solution was then autoclaved and cooled to 55°C. 100ml of the appropriate 10X dropout solution and 40 ml of a 50% stock solution of glucose were added and finally poured into100-150-mm petri dishes.
Agro infiltration	
Infiltration buffer	The infiltration buffer includes 10mM MES buffer, 10mM $MgCl_2$ and 0.1mM acetosyringone.

Chapter 9
Publications
and
conference proceedings

9. Publications and Conference proceedings

List of publications

1. **Gupta, N.,** Reddy, K., & Bhattacharyya, D. (2021). Plant responses to geminivirus infection: guardians of the plant immunity. *Virology Journal*, *18*(1), 1-25.
2. Gnanasekaran, P., **Gupta, N.,** Ponnusamy, K., & Chakraborty, S. (2021). Geminivirus betasatellite-encoded βC1 protein exhibits novel ATP hydrolysis activity that influences its DNA-binding activity and viral pathogenesis. *Journal of Virology*, JVI-00475.

List of abstracts presented in conferences

1. **Gupta N**, Gnanasekaran P, Chakraborty S. A novel role of betasatellite encoded βC1 protein in begomoviral pathogenesis. In: International Conference of Virology, VIROCON 2020, organised by Indian Virological Society at Indian Science Academy, New Delhi, India during February 18 – 20, 2020.
2. **Gupta N**, Gnanasekaran P, Chakraborty S. Betasatellite encoded βC1 protein can hydrolyse ATP and regulates pathogenesis. In: International Conference of Virology, INTERVIROCON 2018, organised by Indian Virological Society at PGIMER, Chandigarh, India during November 12 – 14, 2018**.**
3. **Gupta N**, Kumar R V, Singh Achuit K, Singh Ashish K, Singh, D and Chakraborty S. Emergence of chilli-infecting begomoviruses in India and the role of associated satellites in pathogenesis. In: the 8th International Geminivirus Symposium & the 6th International ssDNA Comparative Virology Workshop held at Vivanta by Taj and Jawaharlal Nehru University, New Delhi, India during November 7 – 10, 2016, P. 65.

Awards and Honours

1. Received **Best Oral Presentation Award** (1st prize) at the International Conference of Virology, VIROCON, from *Indian Virological Society*, New Delhi. (February 2020)
2. Received 3rd prize in "**Oral presentation**" at International Conference of Virology, INTERVIROCON, organised by *IndianVirological Society at PGIMER*, Chandigarh. (November 2018)

8th International Geminivirus Symposium
&
6th International ssDNA Comparative Virology Workshop

November 07-10, 2016

Certificate

This is to certify that Neha Gupta, India has actively participated / made a poster / ~~oral~~ presentation in the 8th International Geminivirus Symposium & 6th International ssDNA Comparative Virology Workshop held during November 07-10, 2016 at New Delhi, India.

Anirban Roy	Basavaprabhu L. Patil	Sunil K. Mukherjee	Indranil Dasgupta
Secretary	Joint Secretary	Co-Chair	Chair

box, DEAH-box and DExD/H-box helicases, which are classified based on motif II variations. Microarray and expressed sequence tag data showed that RNA helicase protein family may play a crucial role in plant growth and development as well as in response to biotic and abiotic stresses. Though, contrasting to Arabidopsis, no in depth information about the RNA helicase family in biotic stress is currently available for tomato (*Solanum lycopersicum*) due to a restricted number of whole-genome sequences. In this study, we finda total of 103 RNA helicase genes in tomato genome. According to the structural features of the motif II region, we divided the tomato RNA helicase genes into DEAD-box (42), DEAH-box (18) and DExD/H-box (31) helicase genes. However, there were 12 RNA helicases which did not belong to either of three subfamilies, and further grouped into "other helicase". We also aimed to study the role of DEAD-box helicase in the tomato against the Tomato leaf curl New Delhi virus (ToLCNDV). Based on our previous study; we selected 3 putative candidate DEAD-box helicase genes, belonging to same clade. Their differential expression pattern was confirmed with the real time PCR. Out of three genes, DEAD-box helicase *SlDBP-12* showed upregulation in the tolerant cultivar H-88-78-1 during ToLCNDV infection. Further functional characterization of *SlDBP-12* is in progress.

Role of C-terminal of the replication initiator protein of Tomato leaf curl New Delhi virus

RajraniRuhel,PrabuGnanasekaranand Supriya Chakraborty*

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Geminiviruses are plant infecting ssDNA virusesthat cause devastating diseases in a wide range of crop plants worldwide. It codes for the conserved and multifunctional replication initiator protein (Rep, AL1 or AC1) that is indispensible for viral replication. In addition, Rep is the only member of the SFIII helicase family reported to participate in rolling circle replication (RCR) in plants. Keeping in mind the importance of Rep protein in geminivirus replication, the present study was undertaken to understand nucleotide binding and hydrolysis coupled DNA unwinding activities of this protein. Tomato leaf curl New Delhi virus (ToLCNDV) Rep belongs to AAA+ (ATPase associated with various cellular activities) family of ATPases. Rep N-terminus (1–120 residues) confers endonuclease, ligase and sequence specific DNA binding activities while the ATPase and helicase activity is associated with the C terminus (120–361 residues). Residues within 120–180 form the oligomerization domain. Role of N terminus is well documented whereas information on role of C terminus is scarce. Amino acid sequence alignment withSV40 T antigen and Human Papilloma virus E1 protein resulted in identifying the conserved motifs in the C-Terminal of the ToLCNDV Rep (RepC). Based on this model, the positively charged amino acidsresiding within the predicted beta hairpin loop and B' motif that have the potential to bind DNA were mutated to alanine by site directed mutagenesis. Circular-dichorismspectra of the mutants indicated that the mutations had not altered their overall structure. These RepC mutants were further checked for various activities such as ATPase, helicase and DNA binding. Mutational analysis revealed the importance of beta hairpin loop and B' motif present in the C terminal of Rep in the ToLCNDV replication. This study provides insight into the critical roles of amino acids involved in unwinding viral DNA during post initiation stage of the RCR of geminivirus.

Multi-facet role of 26S Proteasome subunit RPT4a, in regulating ToLCNDV in tomato

Pranav Pankaj Sahu, Namisha Sharma*, Swati Puranik, Supriya Chakraborty &Manoj Prasad

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Ubiquitin/26S proteasome (UPS) pathway has been implicated in diverse aspects of eukaryotic cell regulation as it rapidly removes intracellular proteins. In addition to these functions, involvement of 26S proteasomal subunits in plant pathogen-interactions, and the roles of each subunit in independently modulating the activity of many intra- and inter-cellular regulators controlling physiological and defense responses of a plant were well reported. In this regard, we aimed to functionally characterize a *Solanum lycopersicum* 26S proteasomal subunit RPT4a (SlRPT4) gene, which was differentially expressed after Tomato leaf curl New Delhi virus (ToLCNDV) infection in tolerant cultivar H-88-78-1. Molecular analysis revealed that SlRPT4 protein has an active ATPase activity. SlRPT4 could specifically bind to the stem-loop structure of intergenic region (IR), present in both DNA-A and DNA-B molecule of the bipartite viral genome. Lack of secondary structure in replication associated gene fragment prevented formation of DNA–protein complex suggesting that binding of SlRPT4 with DNA is secondary structure specific. Interestingly, binding of SlRPT4 to IR inhibited the function of RNA Pol-II and subsequently reduced the bi-directional transcription of ToLCNDV genome. Virus-induced gene silencing of SlRPT4 gene incited conversion of tolerant attributes of cultivar H-88-78-1 into susceptibility. Furthermore, transient over-expression of *SlRPT4* resulted in activation of programmed cell death and antioxidant enzymes system. Overall, present study highlights non-proteolytic function of SlRPT4 and their participation in defense pathway against virus infection in tomato.

Emergence of chilli-infecting begomoviruses in India and the role of associated satellites in pathogenesis

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The genus, *Begomovirus* includes a large number of viruses infecting a wide range of plant species across the continents. In the Indian subcontinent, production of chilli is constrained due to chilli leaf curl disease (ChiLCD) caused by begomoviruses. Despite the considerable economic consequences of ChiLCD on chilli cultivation in India, there have been scant studies of the genetic diversity and structure of the begomoviruses that cause this disease. Here we report a comprehensive survey across major chilli-growing regions in India. Analysis of samples collected in the survey indicates that ChiLCD-infected plants are associated with a complex of begomoviruses (including a previously unreported species) with a diverse group of alphasatellites (including a novel group) and betasatellites. The associated alphasatellite/betasatellites neither enhanced the accumulation of the begomovirus components nor reduced the incubation period in *Nicotiana benthamiana*. The ChiLCD-associated begomoviruses induced mild symptoms on *Capsicum* spp., but both the level of helper virus that

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DAY 1 ORAL

A novel role of betasatellite encoded AC1 protein in pathogenesis

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Plant virus satellites are associated with their helper viruses for their maintenance and it influences the viral pathogenesis. Geminivirus-betasatellite diseases complex causes an epidemic threat to economically important crops across the world. The betasatellite encoded pathogenicity determinant, AC1 protein plays multitasking role as a suppressor of gene silencing, attenuates plant defense response, an inducer of disease symptoms and possible involved in virus movement. Association of betasatellites with begomoviruses results in more severe symptoms in the plants and affects the yield of numerous crops leading to huge agroeconomic losses. However, the molecular function of AC1 and its mechanism of action remain unexplored due to difficulties in purification of the AC1 protein under native condition. To identify and characterize the novel molecular function of betasatellite encoded pathogenicity determinant, AC1 protein. Protein purification by affinity and ion exchange chromatograpghy, western blotting, southern hybridization, ATPase assay, leaf disc assay *Tomato leaf curl Patna betasatellite* (ToLCPaB) encoded AC1 protein was found to exhibit ATPase activity and is ubiquitously observed in AC1 proteins encoded by diverse betasatellites. Alanine scanning was carried out to identify the residues involved in ATP hydrolysis. Results revealed that residues located in the N terminal region of AC1 protein are essential for ATPase activity. Biochemical studies suggest that ATP binding interferes with the DNA binding activity of AC1. The mutant betasatellite with AC1-ATPase dominant negative mutation induces symptoms on *Nicotiana benthamiana* plants similar to that of wild-type betasatellite infection. Additionally, the ATPase function of AC1 negatively influences the geminivirus-betasatellite DNA accumulation. AC1 encoded by betsatellites can hydrolyze the ATP at different temperature and pH conditions. Further investigation on host-betasatellite interactions will explore the role of ATPase activity of AC1 proteins in viral pathogenesis.

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Virology Journal

REVIEW

Open Access

Plant responses to geminivirus infection: guardians of the plant immunity

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Abstract

Background: Geminiviruses are circular, single-stranded viruses responsible for enormous crop loss worldwide. Rapid expansion of geminivirus diversity outweighs the continuous effort to control its spread. Geminiviruses channelize the host cell machinery in their favour by manipulating the gene expression, cell signalling, protein turnover, and metabolic reprogramming of plants. As a response to viral infection, plants have evolved to deploy various strategies to subvert the virus invasion and reinstate cellular homeostasis.

Main body: Numerous reports exploring various aspects of plant-geminivirus interaction portray the subtlety and flexibility of the host–pathogen dynamics. To leverage this pool of knowledge towards raising antiviral resistance in host plants, a comprehensive account of plant's defence response against geminiviruses is required. This review discusses the current knowledge of plant's antiviral responses exerted to geminivirus in the light of resistance mechanisms and the innate genetic factors contributing to the defence. We have revisited the defence pathways involving transcriptional and post-transcriptional gene silencing, ubiquitin-proteasomal degradation pathway, protein kinase signalling cascades, autophagy, and hypersensitive responses. In addition, geminivirus-induced phytohormonal fluctuations, the subsequent alterations in primary and secondary metabolites, and their impact on pathogenesis along with the recent advancements of CRISPR-Cas9 technique in generating the geminivirus resistance in plants have been discussed.

Conclusions: Considering the rapid development in the field of plant-virus interaction, this review provides a timely and comprehensive account of molecular nuances that define the course of geminivirus infection and can be exploited in generating virus-resistant plants to control global agricultural damage.

Keywords: Autophagy, Begomovirus, Betasatellite, Defence, Geminivirus, Immunity, Pathogenesis, Resistance

Background

Geminiviruses belong to the largest family of plant viruses, *Geminiviridae*. Characterized by the circular, single-stranded DNA genome, they cause devastating diseases in plants, faring as one prominent reasons of global crop loss and compromised food security. Geminiviruses are phloem limited viruses and are transmitted by hemipterous insect vectors. Their unique virion includes a twinned icosahedral structure enclosing the circular genomic DNA. Replication of the DNA occurs through the rolling circle and recombination dependent mechanism [1]. In differentiated host cells, geminiviruses reprogram the cell cycle and transcriptional events [2], making the microenvironment suitable for its own replication. Inside the infected plant cell, host DNA polymerases convert the viral single-stranded DNA (ssDNA) into double-stranded DNA (dsDNA) and host nucleosomes pack the dsDNA forming minichromosomes that reside in host nucleus and can act as a template for virus transcription [3, 4]. Early transcription events drive the genes

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essential for virus replication and transcription, followed by late genes required for encapsidation and movement. By altering the host gene expression profile and regulating the host cell signalling pathways, geminiviruses induce severe diseases in plants which manifest as leaf curling, veinal swelling, chlorosis, growth stunting, stem bending, and smalling of leaves etc. [5, 6].

Based on their phylogenetic relationships, genome organization, host range and insect vectors, geminiviruses are categorized into nine genera- *Becurtovirus, Begomovirus, Capulavirus*, *Curtovirus*, *Eragrovirus, Grablovirus, Mastrevirus, Topocuvirus* and *Turncurtovirus* [7]. Among these, begomovirus constitutes the largest genus that are predominantly transmitted by whitefly [*Bemisia tabaci* Genn.] vector. While majority of the classified genera comprises a monopartite genome, begomoviruses can contain either monopartite or bipartite genome. Based on their geographical distributions and genetic diversities, begomoviruses are grouped into Old world (Africa, Europe, Australia, and Asia) and New World categories (America) [8]. The New World begomoviruses mostly have bipartite genome while the Old World ones contain both mono and bipartite genomes. The genome of a bipartite begomovirus contains two separately encapsidated DNA molecules, known as DNA-A and DNA-B, of sizes ranging from 2600 to 2800 nt [8]. Monopartite begomoviruses have genome of one DNA molecule which is structurally and genetically similar to DNA-A of bipartite begomoviruses. Both DNA-A and DNA-B include a common region (CR) of 200–250 nucleotides that encompasses a conserved stem-loop structure and the sequence TAATATTAC. The DNA-A component contains open reading frames (ORFs) encoding five to seven proteins while DNA-B codes for two proteins. Two of the proteins in DNA-A of bipartite begomovirus and in monopartite virus are encoded in the virion sense strand and four in the complementary sense strand. The complementary sense strand proteins are replication-associated protein (REP; AC1), transcription activator protein (TrAP; AC2), replication enhancer protein (REn; AC3), and AC4 protein. Coat protein (CP; AV1) and precoat protein (AV2) are encoded in the virion sense strand. However, the AV2 ORF is absent in new world bipartite begomoviruses [9]. DNA-B contains ORFs BC1 and BV1 encoding movement protein (MP) and nuclear shuttle protein (NSP), respectively. The geminivirus proteins work in coordination to facilitate replication, movement, and anti-defence response to establish a successful infection process [2, 10, 11].

In the infection establishment process, the subviral components of begomoviruses play important roles. Known as alphasatellite, betasatellite, deltasatellites or non-coding satellites, these satellite molecules depend on the helper virus for their replication and propagation, but some of them are adapted in modulating the biological properties of helper viruses [5, 12, 13]. While alphasatellites are self-replicating and depend on the helper virus for encapsidation, movement, and transmission, betasatellites are *trans*-replicated by helper begomovirus and mastrevirus [14–16]. With a genome of nearly 1350 nt, and two proteins βV1 and βC1 encoded in virion and complementary sense strand respectively, the betasatellites take important parts in symptom induction, host defence suppression and insect transmission [17–19]. Besides, betasatellites contain a satellite conserved region (SCR) and an adenine rich region important for betasatellite replication and maintenance [20, 21]. In natural conditions, plants can be infected by multiple viruses, and the stringency of betasatellites associated with their helper virus is very less, which increases the diversity of geminivirus-betasatellite complexes and enhances the probability of disease occurrence in new hosts. Furthermore, the high evolutionary rate of geminiviruses enables them to adapt to new hosts.

Due to the coevolution of plants and the pathogenic viruses, plants acquired multiple strategies to defend and counter viral infection and pathogenesis. However, viruses co-evolve to overcome such resistance responses [10]. Plants control the viruses by preventing the virus gene expression and inhibiting the systemic spread of viruses. Although inhibiting virus gene expression is one of the fundamental ideas of protecting the host from infection, the mainline defence against geminiviruses plays a crucial role in combating the geminivirus infections. This review deals with past and recent findings of major plant immune responses operated against the geminiviruses and a brief discussion on anti-host defence responses. Defence responses such as transcriptional gene silencing (TGS), post-transcriptional gene silencing (PTGS), autophagy, resistance genes and hypersensitive response (HR), protein kinase-mediated immunity, and ubiquitin–proteasome system are discussed in detail. In addition, regulation of phytohormones and alternations in plant primary and secondary metabolism during plant-geminivirus interactions are discussed, and host factors contribute to the pathogen resistance/tolerance are summarised.

Main text

Transcriptional gene silencing and RNA polymerase blockers

Plants deploy two major armours against geminiviruses that are based on silencing the expression of viral genes. While the methylation-mediated TGS targets viral minichromosomes, the viral mRNAs are rendered ineffective

by PTGS. In the next two sections, we will present the nuances of these two processes.

Geminiviral DNA forms a complex with the coat protein and enters the nucleus. In the nucleus, using the host cell machinery the ssDNAs replicate to the double stranded forms and by binding with host's histones exist as the minichromosomes [3]. The plant's response to this invasion is employed by the RNA-directed DNA methylation (RdDM) apparatus to suppress the viral minichromosomes, silencing the viral gene expression by transcriptional gene silencing (TGS) [22, 23]. This epigenetic silencing mechanism involves a sequence of host-virus interactions that reflect the different stages of the infection. In symptomatic tissue, during active replication/expression process, the minichromosomes exist in relaxed conformation, having a chromatin activation marker (H3K4me3) and low level of DNA methylation in comparison to the recovered tissue, where the minichromosomes bear the mark of chromatin-repression (H3K9me2) [24]. The cascade of silencing is operated by a section of small RNAs: siRNAs and miRNAs.

The canonical RdDM pathway is mediated by host DNA dependent RNA pol IV and V, which are evolved from RNA Pol II exclusively to function in plant RNA silencing pathways [25]. RNA Pol IV and V generate 24-nt siRNAs and amplify de novo methylation of target DNA [26]. Pol IV catalyses the formation of single-stranded non-coding transcripts from geminiviral chromatin which are replicated into dsRNA by RNA- DEPENDENT RNA POLYMERASE 2 (RDR2) by CLASSY 1 (CLSY1) dependent manner [26, 27]. These dsRNAs are diced by DICER-LIKE 3 (DCL-3) ribonucleases and generates 24-nt siRNAs duplexes, which are stabilised by HUA-ENHANCER 1 (HEN1), later loaded onto ARGONAUTE 4 (AGO4)/AGO-6 containing RNA-induced silencing complex (RISC). When challenged with beet curly top virus (BCTV), the Pol IV-RdDM machinery reinforced and amplified the viral DNA methylation that was performed by a pathway involving RNA Pol II and RDR6 [26].

RNA polymerase V transcription is independent of siRNA biogenesis and carried out by DDR complex, which includes DEFECTIVE IN RNA DIRECTED DNA METHYLATION 1 (DRD1), DEFECTIVE IN MERISTEM SILENCING 3 (DMS3), REQUIRED FOR DNA METHYLATION 1 (RDM1) and DMS4. The siRNA present in the AGO4-RISC complex base pairs with the transcripts processed by Polymerase V activity. This interaction is stabilized by the AGO4 association with Nuclear RNA polymerase E (NRPE1) carboxyl-terminal tail and KOW DOMAIN-CONTAINING TRANSCRIPTION FACTOR 1 (KTF1) [28]. AGO4 further binds to RDM1 protein of DDR complex and recruits cytosine methyltransferase like DOMAINS REARRANGED METHYL TRANSFERASE 2 (DRDM2) to carry out de novo methylation on the viral genome [29]. Histone modification plays a decisive role in determining the course of host-virus interaction. A histone methyltransferase SU(VAR)3-9 HOMOLOGUE 4 (SUVH4), also known as KRYPTONITE2 establishes the specific repressive epigenetic markers such as histone methylation marks K9, K27 on H3 responsible for transcription repression ultimately results in TGS [29]. Histone methyltransferase KRYPTONITE (KYP) and DNA methyltransferase CHROMOMETHYLTRANSFERASE 3 maintain the TGS and to overcome this host-mediated TGS, virus-encoded transactivator AC2 activates an EAR-motif-containing transcription repressor RELATED TO ABI3 and VP1 (RAV2) that represses KYP expression facilitating virus survival in host [30].

The complex transcriptional reprogramming that involves DNA methylation and demethylation is central in the chromatin-based systemic immune responses in plant [31]. The epigenetic studies show the role of RdDM in resistance against geminiviruses [29, 32, 33]. Arabidopsis mutants *ddm1, ago4, drm1drm2, cmt3, adk1* and, *dcl3* that shows reduced viral genome methylation, are hypersusceptible to distinct geminiviruses [32]. However, geminivirus disease complexes overcome the TGS by virus-encoded TGS suppressors. For example, AC2 encoded transactivator protein (TrAP) of bipartite begomoviruses, V2 protein of tomato yellow leaf curl virus (TYLCV), and C2/L2 encoded TrAP of curtoviruses carry out the suppression of TGS pathway [34, 35]. V2 proteins of TYLCV and cotton leaf curl Multan virus (CLCuMuV) directly interact with AGO4 and interfere with binding of AGO4 to the viral DNA, functioning as TGS suppressor and promoter of virulence [36, 37]. Tomato leaf curl Yunnan virus (TLCYnV) encoded C4 protein binds to DOMAINS REARRANGED METHYLASE2 (DRM2) and hampers its binding to viral genome followed by antiviral DNA methylation [38]. Beet severe curly top virus (BSCTV) TrAP protein inhibits the proteasomal degradation of SAMDC1 (S-adenosyl-methionine decarboxylase). This disturbs the ratio of SAM (S-Adenosyl-methionine)/dSAM (decarboxylated SAM), which leads to inhibition of geminiviral DNA methylation [39]. Furthermore, TrAP protein inhibits ADK that is involved in the production of SAM, a methyl donor [34]. TrAP and βC1 protein interact with SAHH (S-adenosyl homocysteine hydrolase), which is responsible for maintaining the methyl cycle during TGS [40] and dampens TGS. To increase the susceptibility, TYLCV pre-coat protein competes with Methytransferase 1 (MET1) and interacts with HISTONE DEACETYLASE 6 (HDA6) to repress DNA methylation [41]. On the plants' front, a

total control of transposon elements and compaction of chromatin are achieved by Pol IV-RdDM mediated TGS of viral genome involving Pol IV and Pol V [42].

Post-translational modification of histone, an inherent gene expression regulatory process of plant is used by *Arabidopsis* against viral pathogens. EMSY-LIKE 1 (EML1) is a histone reader protein binds to H3K36 modification sites on viral chromatin blocking the access of RNA pol-II to the viral genes and suppressing the expression [42]. The access of RNA pol-II to viral genes is inhibited by *Solanum lycopersicum* regulatory particle triple-a atpase 4A (RPT4a), a subunit of 26-proteasome protein, that binds to the intergenic region of tomato leaf curl New Delhi virus (ToLCNDV), inhibiting the viral transcription [43].

All these molecular dynamics are inspiring developing newer strategies against geminivirus infections. Stable or transient expression of invert repeat constructs to the homologous sequence of geminivirus promoter region inhibits the expression of downstream genes and leads to the reduced viral load as well as symptom recovery. IR region/bidirectional promoter region has been successfully employed in generating the target-specific siRNA to downregulate the virus gene expression [44]. RNA-dependent RNA polymerase 1 (RDR1) of *Nicotiana tabacum* enhances cytosine methylation of tomato leaf curl Gujarat virus (ToLCGV) promoter and represses the virus gene expression and increases virus specific siRNA accumulation eventually leads to symptom remission [45]. Moreover, *NtRDR1* overexpression in *N. benthamiana* alters the expression of host defence genes such as subunit-7 of COP9 Signalosome (CSN) complex, WRKY6 and USPA-like protein and confers reduced susceptibility to ToLCGV infection [46]. Administration of bidirectional promoter fragment from DNA-A of vigna mungo yellow mosaic virus (VMYMV) into VMYMV infected *V. mungo* plants abolished viral DNA accumulation and lead to disease recovery [47].

Post transcriptional gene silencing

The RNA transcripts produced by the viruses are targeted by the cytoplasmic siRNA-mediated silencing pathway of plant. This post-transcriptional gene silencing (PTGS) is a sequence-specific mechanism, is crucial for the host gene expression, development and defence [48]. In response to viral transcripts inside the cells, the PTGS initiates to target the dsRNA segments derived from either complementary viral transcripts (usually the products of bidirectional transcription) or viral RNA secondary structures like hairpins. DICER- LIKE protein (DCL) and dsRNA binding protein (DRB) recognise and process the dsRNAs into 21–24-nt siRNAs. HUA ENHANCER 1 (HEN1) protein methylates 3′ end of siRNAs and protects them from 3′ to 5′ exonucleolytic degradation and uridylation [49]. Alongside, these small RNAs duplexes are recruited onto AGO proteins to provide sequence specificity for targeting and forms RISC complex, resulting into mRNA degradation by cytoplasmic exonucleases or translation inhibition [50]. A second wave of amplified PTGS is generated at the systemic sites by the primary siRNAs to induce systemic resistance [25]. To counter this robust immunity response, geminiviruses have co-evolved several suppressors which interfere at multiple stages of the siRNA pathways such as sensing and activation of PTGS, siRNA biogenesis, amplification and systemic spread to mitigate the host defence [51]. Nuclear shuttle protein (NSP), encoded by the ORF BV1, induces ASYMMETRIC LEAVES2 (AS2) expression in the infected cells that enhances the decapping activity of DECAPPING 2 (DCP2), accelerating the mRNA turnover and hindering siRNA accumulation as well as host RNA silencing [52]. An endogenous RNAi suppressor calmodulin-like protein (CaM) is upregulated by βC1 protein, triggering an interaction cascade that leads to degradation of Suppressor of Gene Silencing 3 (SGS3) and suppression of RDR6 activity, eventually affecting the anti-viral RNA silencing process [53, 54]. Rep protein of wheat dwarf virus (WDV) binds to 21nt and 24nt siRNAs duplexes, inhibiting local and systemic silencing of viral RNA and spread of signals [55]. TYLCV infected and cotton leaf curl Multan betasatellite (CLCuMuB) βC1 expressing transgenic plants showing increased expression of AGO1 and DCL1 underscore the nuanced anti-PTGS process in play [56]. CLCuMuV C4 interacts with the core enzyme of methyl cycle, S-adenosyl methionine synthetase (SAMS) to inhibit TGS and PTGS and, $C4^{R13A}$ mutant fails to retain the suppressor activities [57]. SAMS utilises ATP for converting the methione to SAM [58]. Intriguingly, the arginine 13 of cotton leaf curl Kokhran Virus-Dabawali (CLCuKV-Dab) C4 protein had shown to be important for ATPase function [59]. Presumably CLCuMuV C4 exerts its ATPase action to inhibit the SAMS activity.

Exploitation of host PTGS constitutes a promising strategy in rising the potential defence strategies against geminivirus. In fact, this strategy successfully introduced three decades ago, for developing resistance against a plant RNA virus, tobacco mosaic virus (TMV), where the transgenic expression of TMV CP protein delayed the disease progression [60]. Similar result was also observed with TYLCV. However, the protection was dependent on the expression of the transgene in infected CP transgenic tomato plants [61]. Recombinant vector-mediated expression of artificial dsRNAs raised from either conserved or fusion transcripts belong to same or different virus origin triggers siRNA accumulation and potentially

triggers PTGS against broad spectrum geminiviruses [62, 63].

MicroRNAs in antiviral immunity

MicroRNAs (miRNAs) play a significant regulatory role in plant development as well as biotic and abiotic stress responses. In plants, miRNA biogenesis predominantly occurs in the following steps: (1) Transcription of primary miRNA from *MIRNA (MIR)* genes by RNA polymerase II, (2) Processing of primary miRNAs to nascent miRNA by Dicer-like proteins, (3) Methylation of nascent miRNA and assemble into RISC and, (4) Binding to target mRNA and regulation of gene expression [64]. Existing literature highlights the role of miRNAs against geminiviruses as an underexplored area with promising insights on several aspects of plant-virus interaction. Transient or transgenic expression of geminiviral proteins often exhibit phenotypic abnormalities, evidences the possible involvement of perturbations in miRNA regulatory pathways [65, 66]. AC4 protein of african cassava mosaic virus (ACMV) directly binds to the matured miRNAs and interferes with the mRNA homeostasis that results into developmental abnormalities [66]. The reports on tomato yellow leaf curl Sardinia virus (TYLCSV) and mungbean yellow mosaic India virus (MYMIV) infection in tomato and mungbean, respectively highlighted the host miRNAs that targets phytohormone pathways, resistance (R) genes, receptor-like serine/threonine-protein kinases and transcriptions factors involved in the development [67, 68]. The influence of betasatellite on induction of host miRNA has been studied in the plants co-infected with tomato yellow leaf curl China virus (TYLCCNV) and tomato yellow leaf curl China betasatellite (TYLCCNB), in the presence and absence of functional *βC1*. TYLCCNB responsive miRNAs such as miR391, miR397, and miR398 have been predicted to generate the phased secondary siRNAs (phasiRNAs) [69]. Bioinformatics analysis suggested the tendency of host miRNAs to bind to the geminiviral genome and ORFs and may negatively regulate viral transcription [70]. Prediction analysis of RNA hybrid software revealed miR/miR* sequences are capable of binding ToLCNDV ORFs includes AC1, AC2, AC3, AV1, AV2, BV1 and to betasatellite non-coding region at one or more than one site. Nonetheless, still virus dominates the host defence response by successfully deploying its silencing suppressors [70]. Transgenic plants expressing miRNAs specific to AV1 and AV2 proteins confer tolerance to tomato leaf curl virus (ToLCV), indicating the effectiveness of miRNAs against geminiviruses [71]. *Gossypium hirsutum* miR398 and miR2950 were found to bind to the genomes of both CLCuMuV and CLCuMB, and potentially augmented the CLCuD resistance in transgenic plants [72]. In silico analysis suggests the binding capability of *Glycine max* miRNAs on the genome of MYMIV and mungbean yellow mosaic virus (MYMV) but also involves in regulation of plant defence responses [73]. A stable barley transgenic line, developed with a polycistronic artificial miRNA, gains the resistance against WDV at a lower temperature ranging between 12–15 °C [74]. Recently, ToLCV resistant tomato transgenic lines have been generated by overexpressing the ATP binding domain of AC1 protein via artificial miRNA without compromising the yield [75]. Expression analysis of miRNAs sheds light on possible role of Argonaute homeostasis along with miRNA directed cleavage of virus movement protein in developing resistance against viruses along with the gene regulatory changes in hormonal signalling pathways [76]. Greater supplementary research is required to understand miRNAs as a potential tool in rising defence against geminiviruses.

Ubiquitin-proteasomal pathway and SUMOylation

Ubiquitination is a post-translational modification process, where the protein ubiquitin is conjugated to the lysine moiety of a target protein and eventually directs the protein to 26S proteasomal degradation. Ubiquitination requires the sequential action of three enzymes-ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and E3 ubiquitin ligase (E3). One of the most abundant E3 ligase families comprises Cullin Ring Ligases (CRLs) in SCF complex (SKP1-CUL1-F-box-protein) which is regulated by CSN complex. The F-box proteins contribute to hormonal regulations of plants [77]. The F-box protein CORONATIN INSENSITIVE 1 (COI1) (SCF^{COI1}), functions as one of the components of the jasmonic acid (JA) receptor, is involved in pathogenesis in plants. Upon pathogen infection, increased accumulation of jasmonoyl isoleucine (JA-Ile) facilitates the interaction of repressor protein JAZ (Jasmonate Zim domain) with SCF^{COI1} which cause degradation of JAZ proteins, and elevated the expression of JA responsive genes [78] that were earlier repressed by JAZ.

In plants, the quality control process of protein involving proteases, autophagy and proteasomal degradation systems work closely with defence pathway that requires degrading the pathogenic proteins [79, 80]. During geminivirus infection, aggregation of viral proteins in the cytosol and nucleus [81, 82] is reported often. These aggregates sequester the viral proteins and virion particles from the host immune sensors to ensure the survival, multiplication and movement of the viruses [83]. A number of reports suggest that Ubiquitin-proteasomal pathway regulates geminiviral infection by degrading either viral or cellular proteins [79, 84, 85]. NtRFP1, a tobacco RING -finger protein, which functions as a ubiquitin E3 ligase interacts with βC1 protein and mediates

βC1 ubiquitination, attenuating betasatellite mediated symptom expression [79] (Fig. 1). Ubiquitin activating enzyme (UBA1) interacts with TrAP protein and silencing of UBA1 promotes early viral infection in transgenic *N. benthamiana* [85]. TYLCV TrAP protein also regulates CSN activity to inhibit SCF^{COI1} [86]. βC1 protein interrupts SKP1 and CUL1 interaction during CLCuMuV infection disrupting the proteasomal degradation pathway and altering plant hormonal signalling cascades [87]. *S. lycopersicum* E2 enzyme UBC3 (Ubiquitin-conjugating enzyme 3) activity is also blocked by βC1, with aftermath of decreased level of total polyubiquitinylated protein and increased symptom severity [88]. Cyclin-dependent kinase/cyclins control cell cycle progression in plants and animals. CDK inhibitors (CKIs) negatively regulate CDK/Cyclins. One of the mammalian CKIs, CKI p27*kip1*, is degraded through the help of ubiquitin ligase KPC (Kip-1 ubiquitination promoting complex). Expression of C4 gene of BSCTV in *Arabidopsis* induces Ring finger protein RKP1 the protein similar to human KPC1. RKP1 acts as an ubiquitin E3 ligase and interacts with CKIs, thus lowering the protein level of CKIs during the infection with the effect of continued cell cycle progression [84]. BSCTV also couples ubiquitin-proteasomal system (UPS) to TGS defence pathways, hampering the latter [39]. Numerous molecular studies also revealed the involvement of UPS in regulating the immune responses by altering the fate of transcriptional regulators [77], virus replication [89] and, movement [90]. However, these roles need to be ascertained in the context of geminivirus infection.

SUMOylation is a transient, post-translational modification, similar to ubiquitination and is involved in ligation of a 10 kDa small ubiquitin-related modifier, SUMO to the lysine residues of target peptides to modulate protein activities and interaction as well as subcellular localization [91]. The dynamic equilibration of SUMOylation plays a crucial role in development [92], biotic and abiotic stress responses [93] of plants. Interactions of SUMO conjugating Enzyme 1A (NbSCE1), E2-SUMO conjugating enzyme of *N. benthamiana,* with N-terminal of Rep proteins of tomato golden mosaic virus, (TGMV), TYLCSV, and ACMV are vital for virus replication [94, 95]. In plant RNA virus infection, interaction of SCE1 and viral replicases has a similar positive-effects on virus replication [96]. The interaction between NbSCE1 and Rep/AL1 protein in both monopartite and bipartite begomoviruses augments virus replication, probably by altering the SUMOylation patterns of specific host factors to create a favourable environment for the viruses [95]. SUMOlyation of proliferating cell nuclear antigen (PCNA), the replication processivity factor is compromised by the Rep protein creates a similar permissive ambience for geminivirus replication [97]. Synedrella yellow vein clearing virus (SyYVCV)- βC1 undergoes via ubiquitination mediated degradation [91]. The N-terminal SUMOylation motifs of βC1 functions as stability markers whereas the C-terminal SUMO interacting motifs (SIMs) binds to the host cellular components, promoting the protein degradation. To counter the host-mediated degradation, βC1-protein interacts with *Nb*SUMO1 and recruits the host SUMOylation machinery. Both N-terminal and C-terminal SUMOylation motifs of βC1 are indispensable for the symptom expression, virus replication and systemic movement. However, chloroplast localization of βC1 solely depends on C-terminal SUMOylation motifs [91]. Further research is required to explore how the plants engage the defence response to counteract such virus induced micro-environmental modifications.

Autophagy as a viral venator

Autophagy is an evolutionary conserved process of recycling the degraded or undesirable cellular components taking place in the cell. Autophagic cargo sequestered into vesicle-like compartments and subsequently fused

(See figure on next page.)

Fig. 1 Schematic overview of plant immune strategies against geminiviruses. Geminivirus infection initiates with the release of viral ssDNA into the nucleus, subsequently leads to the replication, transcription and translation of viral genome. (**A**) Plants counteract geminivirus genetic life cycle via multiple host factors. GRAB interacts with RepA and interferes with the replication. RPT4a and EML1 hamper the geminivirus active transcription by obstructing the RNA Pol-II on virus euchromatin. Additionally, host induces RNAi via TGS and PTGS to suppress the viral gene expression. Virus-encoded VSRs potentially suppresses the RNAi. (**B**) Geminivirus induced GRIK1 autophosphorylates and activates SnRK1 which interact and phosphorylates the viral Rep, TrAP (AL2/C2) and βC1 protein. Phosphorylation of Rep and TrAP impedes Rep binding and causes a delay in the infection, respectively. βC1 phosphorylation hampers the TGS and PTGS suppressor functionalities and attenuates symptom expression via suppression of AS1-βC1 mediated downstream responses. Phosphorylated βC1 may also direct to autophagy. (**C**) Tobacco RFP1 interacts with βC1 and prompts the βC1 degradation via ubiquitin-mediated 26S proteasomal pathway and causes the symptom attenuation. (**D**) ATG8h interacts with nuclear C1 and translocate to cytosol Xpol dependent manner. The ATG8h-C1 complex is then recruited into autophagosomes with the aid of ATG5 and ATG7 for vacuolar degradation. (**E**) Defence regulated MEKK1-MKK1/MKK2-MPK4 module induced, activated by geminivirus infection and exerts the basal defence response. However, βC1 protein directly interacts with MKK2 and MPK4, thereby suppress the broad spectrum of downstream defence reactions. (**F**) NIK-1 from plasma membrane activated upon the geminivirus infection triggers dimerization and autophosphorylation. Alternatively, PTI induced DAMPs secreted from ER in response to virus attack may cause NIK-1 activation. Active NIK-1 phosphorylates and translocate L10 into the nucleus where it binds to LIMYB to block the transcription of ribosomal biosynthesis genes which affects the global translation and prevents the translation of viral genes

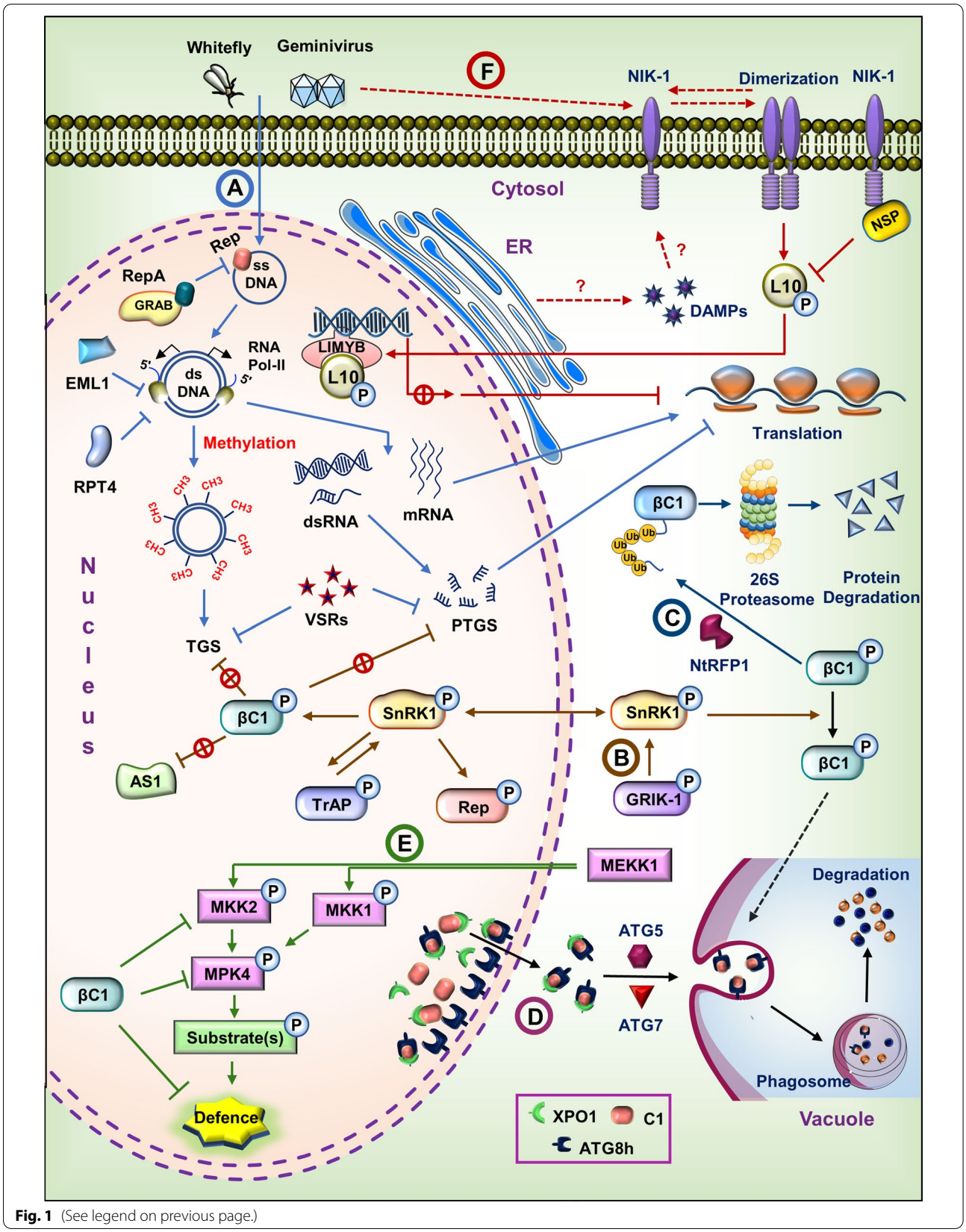

Fig. 1 (See legend on previous page.)

with lytic components such as lysosomes in animals and vesicles in the plant cells. Studies conducted on plant DNA and RNA viruses confirm that autophagy plays a potential antiviral role in host innate and adaptive immunity [98, 99]. In vivo and in vitro experiments showed that CLCuMuB βC1 protein interacts with autophagy-related protein NbATG8 through its ATG8 interacting motif (LVSTKSPSLIK) and directs it for degradation. Disruption of βC1-ATG8 interaction by a point mutation (V32A) in the ATG8 interaction motif promotes the virus replication and disease symptoms. Since ATG genes are functionally redundant, silenced ATG5 and ATG7 transgenic plants when infected with CLCuMuV and associated betasatellite showed severe and early symptoms [98]. Furthermore, interaction of ATG8h protein with Rep protein of TLCYnV leads to ATG8h mediated Rep translocation to cytoplasm and degradation [80] (Fig. 1).

Autophagy carries out both pro-viral and anti-viral roles in host cells to maintain the balance of cellular and viral proteomes. Geminiviruses have recently been reported to manipulate autophagy-mediated defence by inducing autophagy of host factors involved in other defence pathways. TYLCCNB-encoded βC1 regulates Nbrgs-CaM, which induces degradation of NbSGS3 with the help of ATG factors [54]. βC1 protein of CLCuMuB disrupts the interaction between a negative autophagic regulator and ATG3 protein to induce autophagy in *Nicotiana benthamiana* [100]. Further research is needed to reveal the mechanism behind the regulation of autophagy during viral infection and explore the potential of blocking proviral autophagic pathways as a mean to control the infection.

Kinases as transducers of defence

Protein kinases are some of the key components involved in plant growth, development and defence including pathogen sensing and defence response induction [101]. Protein kinases are accountable for setting different signalling cascades in motion for efficient plant defence against geminiviral infection [102–104]. Viral proteins modulate the signal transduction pathways via both direct and indirect interactions with different host protein kinases. This section illustrates various mechanisms by which protein kinases such as SnRK1, MAP kinases and receptor like kinases (RLKs) orchestrate the cellular responses during plant-virus interactions.

SnRK1 mediated signalling

The SUCROSE NON-FERMENTING1-related protein kinase 1 (SnRK1) is a Ser/Thr kinase that functions as an energy sensor and central regulator of energy, metabolism and stress responses. It operates multi organellar crosstalk and potentially regulates downstream transcription factors involved in diverse molecular pathways to maintain cellular homeostasis [105]. Upon cabbage leaf curl virus (CaLCuV) and BCTV infection, functionally redundant geminivirus Rep interacting kinases GRIK1 and GRIK2 expression gets enhanced, which causes SnRK1 phosphorylation and activation [106]. GRIK1 and GRIK2 also interact with Rep protein of TGMV [107]. The lower expression of SnRK1 enhances the susceptibility of plants towards geminivirus, whereas SnRK1 overexpression leads to an increased resistance in plants [103]. SnRK1 phosphorylates TYLCCNB encoded βC1 protein at serine 33 and threonine 78 residues, which negatively impacts the titres of both helper virus and betasatellite as well as disease development in *N. benthamiana* [108]. The phosphorylation of βC1 also suppresses its role in suppression of methylation mediated RNA silencing, which further explains the significant role of SnRK1 against geminiviruses (Fig. 1). SnRK1 may also induce autophagy of βC1 protein as yeast, and mammalian homologs of SnRK1 have been reported to promote autophagy by phosphorylation of different protein substrates [98, 109]. AtREM4 (*Arabidopsis thaliana* remorin group 4), which functions as a positive regulator of the cell cycle during BCTV and BSCTV infections, gets phosphorylated by SnRK1 and induce the degradation of AtREM4 by 26S proteasomal degradation pathway [110].

Geminiviral TrAP proteins AL2 from TGMV and L2 from BCTV interact with *Arabidopsis* SnRK1 and inhibit its activity to enhance pathogenesis [111]. SnRK1 maintains balance of cellular metabolic energy of host cells to defend against viral infection. Therefore, depletion of ATP or increased level of 5′-AMP activates SnRK1. As Adenosine kinase (ADK) phosphorylates adenosine to 5′-AMP, L2 and AL2 interact with ADK and disable the SNF1 kinase-related antiviral mechanism [111]. TrAP protein of CaLCuV also gets phosphorylated by SnRK1 at serine 109 position, which leads to delayed viral infection [33]. Further, SnRK1 phosphorylates TGMV-Rep at serine 97 position hindering binding of Rep onto the viral genome and inhibiting viral infection [112]. As suggested by the recent reports, being a global energy regulator of the cell and carrying out the role of metabolic modulator, SnRK1 has emerged as a pivotal player in plants antiviral defence armour.

MAP kinase cascade

Mitogen-activated protein kinase (MAPKs) are widely studied, and known to be involved in signal transduction and signal amplification processes and defence against diverse phytopathogens as well as in abiotic stresses [113]. MAPKs are activated by MAPK Kinase, which gets regulated through cross-phosphorylation.

The role of MAPK cascades in plant innate immunity against bacterial and fungal pathogens is well studied [114]. New findings are highlighting the role of MPKs in antiviral defence responses, too. *Vigna mungo* MAPK1 has been found to suppress MYMIV accumulation and upregulate salicylic acid (SA) mediated expression of pathogenesis-related (PR) genes [115]. Similarly, MAPK3 silenced tomato plants showed reduced tolerance to viral infection and attenuated expression of SA/JA regulated defence related genes [116]. Earlier, global transcriptional analysis of whitefly after TYLCCNV infection revealed the downregulation of genes involved in MAPK signalling pathways [117]. TYLCCNV infection leads to activation of MAPK signalling cascade for defence, but βC1 protein interacts with MKK2 and MPK4 inhibiting the kinase activity and limiting the anti-viral activity of MAPK [104] (Fig. 1). Recently, C4 mediated suppression of MAPK cascade activation has been discovered in *N. benthamiana* [118]. TLCYnV-encoded C4 competes with BRASSINOSTEROID INSENSITIVE 1 (BRI) to bind BRI1 KINASE INHIBITOR1 (BKI1) and stabilizes the protein complex on plasma membrane. The unavailability of free BIK1 precludes the autophosphorylation of ERECTA (ER), concomitantly leading to the inhibition of downstream MAPK cascade activation which facilitates optimal conditions for TLCYnV infection [118].

Receptor-like kinases

RLKs are transmembrane proteins that transduce extracellular signals by their specific ligand binding domains, a membrane-spanning region, and cytoplasmic serine-threonine kinase domain to regulate cell differentiation, patterning, development and innate immunity [119]. They act as pattern recognition receptors (PRRs) recognizing microbe-associated molecular patterns (MAMPs) and initiating the basal innate defence responses [119]. RLKs also recognize secondary danger signals produced in a stressed situation in the cells that boost the immune response against pathogens [120]. One of the RLKs, NSP interacting kinase (NIK) is encoded by a small multigenic family that consists of three genes NIK1, NIK2, and NIK3. Geminiviral nuclear shuttle protein (NSP) acts as a target of NIKs, implicating the existence of RLKs mediated immune response against geminivirus [121]. The plants deficient with *nik* exhibited enhanced susceptibility to begomoviruses infection [122]. Geminivirus infection triggers NIKs oligomerization and transphosphorylation of kinase domain at T474 that activates NIK1 kinase prompting the latter to phosphorylate the cytoplasmic ribosomal protein 10 (RPL10) [102]. As the phosphorylated RPL10 translocates into the nucleus with the transcription factor LIMYB (L10-interacting MYB domain-containing protein), it forms RPL10-LIMYB complex that negatively regulate the virus infection by binding to the promoters of ribosomal protein gene and represses the expression (Fig. 1). However, the host NIKs mediated resistance against geminiviruses is limited by the NSP of geminiviruses as it interacts with NIKs and suppresses its activity [122] (Table 1). Another RLK, CLAVATA 1 (CLV1) that regulates WUSCHEL gene expression and helps in maintaining the meristem, undergoes binding by the S- acylated form of BSCTV C4 protein; an interaction leading to anomalous siliques development in *Arabidopsis* [123]. As it localizes to the plasmodesmata as well as to the plasma membrane, BARELY ANY MERISTEM 1 (BAM1) and BAM2 helps in expanding systemic movement of RNAi signals and thus obstructing the spread of the virus to other cells. However, TYLCV C4 protein binds to BAM1 and inhibits the propagation of silencing signals [124]. *Arabidopsis* Shaggy like kinase protein AtSK21, also known as AtBIN2 (Brassinoids inhibitor 2), negatively regulates brassinosteroid signalling and affects male sterility [125]. During sweet potato leaf curl virus (SPLCV) infection, viral C4 protein targets AtBIN2 inducing anomalous development including male sterility in *Arabidopsis* [125]. C4 physically interacts with RLKs, FLAGELLIN SENSING 2 (FLS2) and BRI1 and affect the downstream pathways as the interaction reduces the time of apoplastic ROS burst without influencing downstream marker genes expression [126]. Shaggy-related protein kinase (SKη) also determines C4 mediated symptom induction. The affinity of the NbSKη-C4 interaction and tethering to the plasma membrane complex regulates the viral pathogenicity [127]. RLK Proline-rich extension-like receptor kinase (PERK) like protein is exploited by the viral machinery to positively regulate viral protein NSP and enhancing the infection of tomato crinkle leaf yellows virus (TCrLYV) and TGMV. PERK can be considered as a potential resource to develop viral resistance in plants as T-DNA insertional mutation in PERK attenuates infection [128]. Various other RLKs like PHLOEM INTERCALATED WITH XYLEM members (PXYs), PEP1 RECEPTOR members (PEPRs) are some potential targets of viral C4 proteins. Manipulation of such RLKs which mediate defence and developmental processes by geminivirus points towards the possible roles of these RLKs in antiviral activity [129]. RLKs, being critically important in the perception of pathogens, need a broader exploration to reveal the molecular and genetic pathway against geminiviruses.

Table 1 Host factors involved in the antiviral defence against geminiviruses

Host	Host factor	Function	Viral factor	Geminivirus	Precise role in defence response	References
Host defence responses						
A. thaliana	SnRK1	It is a global regulator of energy metabolism during growth and stress conditions	Rep	TGMV	SnRK1 phosphorylates Ser 97 of Rep protein and interferes replication and infection	[98]
			TrAP	CaLCuV	SnRK1 phosphorylates AL2 at ser 109 that delays infection process	[33]
N. benthamiana	PsbP	It is a core protein of oxygen-evolving complex that stabilizes Photosystem II	βC1	ToLCNDV/RaLCB	PsbP binds to the viral genome and reduces virus replication	[191]
N. benthamiana	ATG8	It is involved in autophagy, mediates protein degradation	βC1	CLCuMuV/CLCuMuB	ATG8 binds to βC1 and degrades via autophagy thus causes reduction in viral titre	[84]
S. lycopersicum	ATG8h	It is an autophagy factor, mediates protein degradation	Rep	TLCYnV	C1 gets exported to the cytoplasm by ATG8h and degraded which restricts viral infection	[66]
N. tabacum	RFP1	It is an E3 ligase that mediates protein ubiquitination	βC1	TYLCCNV/TYLCCNB	RFP1 interaction with βC1 leads to βC1 degradation thus reduces viral infection	[65]
S. lycopersicum	UBA1	A ubiquitin-activating enzyme, catalyses the first step in 26S proteasomal degradation pathway	TrAP	TYLCSV	Silencing of UBA1 increases viral infection. TrAP protein interacts with UBA1, thus inhibit its defensive activity	[71]
A. thaliana	EML1	A histone reader protein, represses active transcription by binding on H3K36 modification	Viral minichromosome	CaLCuV	EML1 binds to viral minichromosome and blocks transcription, therefore attenuates infection	[39]
S. lycopersicum	RPT4a	A subunit of 26S proteasome, aid in unfolding target proteins	Intergenic region	ToLCNDV	RPT4a binds to the viral intergenic region and blocks transcription	[40]
V. mungo	MAPK1	A protein kinase involved in SA mediated defence	Not known	MYMIV	It mediates defence by inducing SA responsive genes	[101]
Triticum monococcum	GRAB	A Geminivirus Rep A-Binding protein. It may have role in plant development	RepA	WDV	GRAB1 and GRAB2 bind to C-terminus of RepA and inhibit replication	[193]
N. benthamiana	*Nb*SUMO1	A component of SUMOylation system	βC1	SyYVCV/ SyYVCB	It promotes ubiquitin mediated degradation of βC1 protein	[77]
Host	**Host factor**	**Function**	**Viral factor**	**Geminivirus**	**Remarks**	**References**
Geminivirus adaptations to host defence						
N. benthamiana	HDA6	It interacts with MET1 and maintains chromatin silencing	Pre-coat protein	TYLCV	Pre-coat protein interfere with MET1 interaction to HDA6 and inhibit TGS	[38]
A. thaliana	ADK	It converts adenosine to AMP which is essential to maintain the methyl cycle during TGS and aid in the activation of SnRK1	TrAP	TGMV, BCTV	TrAP inactivates ADK and causes TGS suppression and inactivation of SnRK1 defence pathway	[34]
A. thaliana	SAHH	It catalyses the generation of methyl donor during TGS mediated methylation of viral genome	βC1	TYLCCNV/TYLCCNB	βC1 inhibits SAHH enzyme and suppress TGS	[37]
A. thaliana	BAM1	It facilitates cell to cell movement of RNAi signals	C4	TYLCV	C4 interacts with BAM1 domain and inhibits spread of silencing signals	[109]
N. benthamiana	MKK2 MPK4	Regulates defence responses by via phytohormones, ROS and defence-related gene expression during biotic stress	βC1	TYLCCNV/TYLCCNB	βC1 inhibits the kinase activity of both MKK2 and MPK4 and increases host susceptibility	[90]

Table 1 (continued)

Host	Host factor	Function	Viral factor	Geminivirus	Remarks	References
A. thaliana	SnRK1	It is a global regulator of energy metabolism during growth and stress conditions	TrAP	TGMV, CaLCuV	TrAP inactivates SnRK1 and leads to enhanced susceptibility	[97]
				BCTV		[98]
A. thaliana	NIKs	It undergoes reversible phosphorylation within its activation loop domain to carry out the defensive function	NSP	CaLCuV	NSP inhibits autophosphorylation of NIK and thus breaks suppression of NIK mediated global protein synthesis	[88]
A. thaliana	CSN5	It regulates SCF complex activity during ubiquitination	TrAP	TYLCV, TYLCSV	TrAP interacts with CSN5 and impairs SCF complex function thereby inhibits jasmonic acid signalling and enhanced infection	[72]
N. benthamiana	*Nb*SUMO1	A component of SUMOylation system	βC1	SyYVCV/SyYVCB	It stabilises the βC1 protein	[77]

Phytohormone modulations

Phytohormones not only regulate various physiological activities related to development, metabolism, reproduction but they are also essential in management of abiotic and biotic stresses [130, 131]. Various phytohormones like salicylic acid (SA), jasmonic acid (JA), and ethylene have been known to work either synergistically or antagonistically to generate diverse host defence responses against pathogens. Crucial roles of JA, SA against geminiviruses have been elucidated and the involvement of auxin, cytokinin, gibberellic acid, brassinosteroids and abscisic acid in the anti-virus activity are being explored [132].

SA is synthesized in plants during biotic stress and establishes both local and systemic acquired resistance (SAR) via synthesis of PR proteins. CaLCuV infected *A. thaliana* transcriptome analysis revealed activation of the SA pathway during infection as *Arabidopsis cpr1* plants that exhibited high endogenous SA level and increased PR proteins expression were less susceptible to CaLCuV infection [133]. Similarly, overexpression of GLUTAMINE DUMPER 3 (LSB1/GDU3), a gene important in amino acid transport, activates the SA pathway and weakens DNA replication of BSCTV [134]. However, during TYLCSV infection in *S. lycopersicum*, biosynthesis of SA has been reported to be reduced. SA minimises the egg hatchability of vector whitefly putting pressure on viral propagation [135]. Recent report suggests induction of expression of SA responsive PR genes (*SlPR1, SlPR2, and SlPR5*) and ROS scavenging enzymes (*SlSOD, SlPOD, SlCAT2*) following TYLCV infection in *S. lycopersicum* which contributes to increased resistance against the virus [136].

Methyl jasmonate (MeJA) treated plants also developed milder symptoms and low viral titre compared to the control plants when infected with BCTV [86]. Tomato plants infected with TYLCSV had several JA responsive genes upregulated including JA signaling pathway gene *COI1* but had the lower level of *JASMONATE INSENSITIVE1* (JAI1), a transcription factor activated by the JA [67]. *N. tabacum* transgenic plants expressing TrAP protein had increased expression of JA biosynthetic genes as well. Although betasatellite encoded βC1 protein did not have a direct impact on JA biosynthetic genes, it represses JA downstream marker genes such as *PLANT DEFENSIN1.2* (*PDF1.2*), *PATHOGENESIS RELATED4* (*PR4)* and *CORONATINE INSENSITIVE13* (*CORI3*), thus hampering the hormonal defence suppression mechanisms [137]. As βC1 interacts with MYC2, the MYC2-mediated JA responses gets suppressed [138]. Activation of JA leads to reduced development of whitefly *B. tabaci* nymphaea; however, the adult population can suppress JA related defence [139].

Gibberellic acid (GA) signalling is mediated via the degradation of DELLA proteins, which are negative growth regulators. GA biosynthesis and GA receptors genes were found to be upregulated in tomato plants infected with TYLCSV while the repressor protein of GA, Gibberellic-Acid Insensitive (GAI) downregulates, suggesting the fine tuning of GA homeostasis during the geminiviral infection [67]. CLCuMuB βC1, when interacts with the DELLA protein, represses its degradation, affecting the GA response pathway.

Role of classic growth hormones like auxins and cytokinins against geminiviruses are yet to be explored conclusively. Auxin is a pivotal regulator of growth and development of stem and roots as responsiveness to light and temperature. Through its precursor Tryptophan, auxin signalling is related to chemical defence pathways involving camalexins and glucosinolates that selectively inhibits the growth of necrotrophic and biotrophic pathogens [140]. ToLCNDV-encoded AC4 disrupts endogenous auxin content and downstream signaling cascade in tomato by interacting with auxin biosynthetic enzymes such as TAR1 (tryptophan amino transferase 1)-like protein, CYP450 monooxygenase. It also upregulates the expressions of miRNAs that target auxin signaling components, reprogramming the virus replication and altering the symptom manifestation [141]. RNA sequence analysis of WDV infected samples showed downregulation of auxin-induced protein 15A and auxin-responsive protein SAUR72 compared to the control plants. Small auxin upregulated RNA (SAUR) family proteins are involved in the Auxin/Indole-3-acetic acid (AUX) signalling pathway. The downregulation of SAUR72 suggested the possible roles of auxins in antiviral response. Similarly, two-component response regulator encoding ORR22 and ORR4 genes that are involved in cytokinin signalling pathways were upregulated and downregulated, respectively [142]. TGMV TrAP protein and spinach curly top virus (SCTV) C2-encoded TrAP protein expression resulted in inhibition of activity of ADK, a regulator of primary cytokinin responsive genes. Inhibition of ADK prevents cytokinin nucleosides phosphorylation leading to the accumulation of more bioactive cytokinins [143]. This increases the cell division rate and promotes severe infection. Regulation of level of cytokinins may decisively control the susceptibility of plants towards geminiviruses.

Abscisic acid is a widely studied phytohormone in plant abiotic stress tolerance. However, the correlation between geminivirus infection and abscisic acid has largely remained unexplored. Exogenous application of abscisic acid and auxin induces expression of *A. thaliana* homeobox ATHB7 and ATHB12 genes that encode homeodomain-leucine zipper family transcription

factors. Similar genes were found to be induced during BSCTV infection [67]. Since expression of ATHB12 in BSCTV infected plants leads to several abnormalities like stunting, curling of leaves, abnormal floral and root structure, callous like outgrowths in plants, this indicates a regulation of geminiviral response by abscisic acid [144]. ABA is known to enhance the survival capability of plants in drought conditions. Recently, an interesting finding described enhanced drought tolerance capacity of transgenic *A. thaliana* plants expressing TYLCV C4 protein [131]. However, this alteration of the physiological aspect of infected plants is through ABA independent mechanism.

Ethylene (ET) is another plant hormone that is involved in defence mechanism. The level of 1-aminocyclopropane-1-carboxylate oxidase (ACCO), a vital molecule of ethylene biosynthesis pathway, is increased during TYLCSV and CaLCV infection. However, 1-aminocyclopropane-1-carboxylate synthase 8 (ACS8) that catalyses the rate-limiting step in the biosynthetic pathway of ethylene is downregulated during TYLCSV infection [67]. The level of ACS8 targeting miR159 increases along with the disease progression, reducing the level of 1-aminocyclopropane-1 carboxylic acid (ACC), another ethylene precursor, which is probably compensated by the upregulation of ACCO. A dynamic equilibrium involving ET signalling plays at the interface of host and virus interaction [67]. Ethylene responsive factor 1 (ERF1) gene, the regulator of ethylene-responsive genes was also upregulated when ACMV TrAP protein was overexpressed in *N. tabacum* [145]. Likewise, the systemic silencing of CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), a negative regulator of ET signaling enhances upregulation of defence marker genes during tomato leaf curl Joydebpur virus (ToLCJoV) infection [146]. Suppressed expression of essential ET responsive EIN2 in case of WDV infecting a monocot plant might underline the evolutionary diversification of the plants [142].

BCTV C4 protein induces severe development abnormalities like hyperplasia of phloem tissue and tumour-like outgrowths in infected plants, and conversely, a mutation in C4 causes reduction in disease symptoms [147]. When brassinosteroids and abscisic acid were applied exogenously, the C4 transgene-induced phenotype of seedlings was partially rescued. However, seedlings became more sensitive to gibberellic acid and kinetin [148]. An earlier report revealed that *Arabidopsis* Shaggy-like kinase proteins (AtSKs), which targets transcription factors that regulate brassinosteroid signalling also interact with the C4 protein of BCTV and TGMV [149]. Current transcriptomic studies in response to the *WDV* in *Triticum aestivum* showed differential expression of BR signalling genes [142]. Different classic and stress-responsive phytohormones act in concert in the plants' immunity and it is important to decipher the roles of these hormones in this complex network.

Metabolite interplay

Plants varied responses against biotic stresses are often associated with the production of a variety of metabolites. Beet mild curly top virus (BMCTV) infection on chilli pepper induces a high level of glucose and fructose, galactose, and myoinositol compared to asymptomatic samples [150]. Glucose and fructose act as energy sources for running viral machinery, while galactose may be required for the synthesis of the glycoprotein required for capsid formation [151]. As an osmoregulator, myoinositol is also involved in tissue deformation during ageratum enation virus (AEV) infection [152]. In chilli, geminivirus infection induces prominent symptoms of leaf curling, yellowing, etc., and reduces the total chlorophyll a and b content affecting the CO_2 fixation rate and total soluble sugars, proteins and starch content [6, 153–155]. TYLCV infection increases total phenolics, tannins, and the related gene expression but reduces the soluble sugars and free amino acids that impacts the growth and fecundity of whitefly [156]. Alteration of nutritional changes brought about by geminiviruses favour the abundance, fecundity, and transmission ability of vector whitefly to promote the spread of the virus. Various volatile organic substances released from secretory organs such as glandular trichomes, secretory cavities, and resin ducts, specifically acts as an attractant or repellent to specific herbivores and insect vectors. A fatty acid derivative undecanone, sesquiterpene zingiberene, and its transformed form, curcumene produced from tomato plants are reported to be toxic to whitefly [157]. Resistance towards one of the whitefly species was observed when zingiberene containing ginger oil was applied on the leaves of the tomato plant [158]. P-cymene, one of the active and toxic volatile substances may also play a role in repelling whitefly [157]. The infestation of whitefly leads to the upregulation of terpenoid biosynthesis genes. This secondary metabolite mediated defence is compromised by viral infection as the virus attenuates the terpenoid release. The number of whiteflies in different development stages were also higher in plants with silenced 5-epi-aristolochene synthase (EAS) gene, a terpenoid synthesis gene in tobacco [159]. βC1 protein encoded from TYLCCNB associated with TYLCCNV inhibits terpene synthesis by interacting with MYC2 transcription factor [138]. Utilizing the chemistry of secondary metabolites in controlling the herbivory can be an easy and time-efficient approach in managing the whitefly populations in field conditions.

Innate genetic factors and hypersensitive response mediated responses

Disease resistance to phytopathogens is classified into nonhost resistance and host resistance. In the context of viral pathogenesis, nonhost resistance is a species-dependent phenomenon where the genotypes belonging to particular plant species might exhibit resistance or susceptibility to a specific virus [160]. While in the case of host resistance, it is typically limited to specific genotypes or cultivars of same or different species and renders the rest of them to be susceptible to the virus infection. There are multiple genetic studies describing the disease resistance phenotype is explicitly associated with occurrence of gene loci encode for resistance (R) genes. In the dynamic population, R-genes segregate into dominant and recessive. Often it is found that the former exerts defence responses mostly by induction of HR response [161] and the latter inhibits the virus life cycle by impeding the protein translation [162]. Identification of promising resistant genetic sources from domestic and wild varieties has been a longstanding successful approach in managing the geminiviruses against several crops such as tomato, bhendi, cassava, cotton and mung bean [163].

Frequent occurrence of TYLCV epidemics in tomato cultivating regions has posited the tomato infecting geminivirus such as TYLCV as a potential threat for the production. Genetic approaches to gain tolerance/resistance to TYLCV resulted in the mapping of six resistance Ty- loci, i.e. *Ty* 1–6 from different wild tomato species and, except for *Ty-4* and *Ty-6*, rest of the *Ty* genes have been characterized (Table 1). *Ty* genes confer phenotypic disease tolerance to begomoviruses, but unlike the reported R-genes, doesn't induce HR. Tomato varieties possessing Ty-1/Ty-3 alleles upon infection with TYLCV produced mild or no symptoms with low virus titre, but an increased accumulation of siRNAs. Ty-1 and Ty-3 are allelic forms of ϒ type of RNA dependent RNA polymerase gene (RDRϒ) [164]. As increased siRNAs production derived from V1 and C3 genes, enhanced TGS conferred Ty-1 mediated resistance in TYLCV infected plants where hypermethylation of cytosine residues in the V1 and C3 promoters of tomato severe rugose virus (ToSRV) were observed [165]. However, the resistance mediated by *Ty-1* against TYLCV was compromised by mixed infection with cucumber mosaic virus (CMV). CMV encodes silencing suppressor proteins which counteract the host RNAi machinery and trade-off TYLCV resistance in plants and enhances the viral titre and infection severity [165]. Ty-1/Ty-3 have been found to be essential for achieving broad-range resistance against geminiviruses as they provide a high degree of resistance to both mono and bipartite begomoviruses [166]. *Ty-2* is a functional R gene that encodes for nucleotide-binding-leucine rich repeat protein. The insertion of *Ty-2* gene into the domestic susceptible tomato plants conferred resistance to TYLCV [167]. Quantitative trait locus, *Ty-5* majorly has also been implicated in recessive resistance against TYLCV in TY172 line of tomato [168]. At this locus, *Pelo* gene which encodes for mRNA surveillance factor pelota (pelo) homolog in tomato, is involved in ribosome recycling phase of protein biosynthesis and controls the disease resistance [162]. Experimental evidence suggests that *Pelo* silenced susceptible transgenic plants infected with TYLCV failed to produce disease symptoms, and viral titre was decreased by 20–60-fold. The possible mechanism might involve affected ribosome dissociation, leading to low availability of ribosomal subunits for translation initiation of viral proteins [162]. Ty-4 and Ty-6 also provide resistance against TYLCV and tomato mottle virus (ToMV) [169, 170]. Apart from exploiting *Ty* loci in breeding, devising the molecular biology tools may provide an opportunity for developing broad resistance against plant viruses. However, the resistance conferred by Ty genes can be compromised by the presence of betasatellite during the infection [171]. Breeding approaches also have identified inter simple sequence repeat (ISSR), a key diagnostic marker, in ToLCNDV tolerant cultivar *Solanum habrochaites* LA1777 which can be exploited for marker-assisted breeding in rising defence against ToLCNDV. Two genetic markers $SSR18_{170\text{-}145}$ and $SSR304_{158\text{-}186}$ have been identified from the F2 population of susceptible variety Punjab Chhuhara (PBC), 'H-24', and *S. habrochaites* accession 'EC-520061' with possible implications in TYLCV resistance [172].

Three resistance genes CMD1, CMD2 and CMD3 have been identified against cassava mosaic geminiviruses that are prevalent in South Africa and India. CMD1 is a polygenic resistance gene originally from *Manihot glaziovii* [173]. Several CMD resistant varieties were obtained utilizing CMD1 through breeding that exhibited lower viral titre than the susceptible ones and had reduced systemic movement of virus enabling to develop virus-free plants from infected cuttings [174]. Molecular genetic mapping and analysis have led to the identification of CMD2, a monogenic dominant locus from *M. esculenta*. Crossing CMD1 and CMD2 carrying parents, that together produce complementary resistant effect, have generated CMD3, another quantitative trait loci responsible for resistance in cassava [175]. Although several breeding programs successfully obtained CMD resistant varieties, molecular characterization of these genes has not yet progressed much. As geminiviruses can evolve into more virulent strains and break the natural resistance provided by marker-assisted selection and breeding, it is important to develop resistance against virus by additional genetic engineering methods.

Because of its high-quality fibre and superior lint *G. hirsutum* accounts for more than 90% of total cotton production all over the world. But it is highly susceptible to cotton leaf curl disease (CuLCD) caused by Cotton leaf curl virus [176]. *G. arboreum*, one of the wild progenitors of *G. hirsutum,* has been highly tolerant to various biotic and abiotic stresses and a major source of genes for natural resistance. Through introgression and conventional hybridization programs, single genes with dominant effect for resistance were transferred from *G. arboreum* to *G. hirsutum* [177]. Massive screening of 22 cotton varieties revealed two genes $R_{1CLCuDhir}$ and $R_{2CLCuDhir}$ that were involved in *G. hirsutum* resistance and one gene $S_{CLCuDhir}$ as suppressor of resistance [178]. However, introgression of multiple genes for resistance with minor effects can provide plants with durable resistance [179].

Bhendi (*Abelmoschus esculentus)* is an important vegetable crop in the tropical and subtropical countries of Indian subcontinent is greatly challenged by bhendi yellow vein mosaic virus (BYVMV) and okra enation leaf curl virus (OELCV). Various bhendi resistant varieties have been developed through conventional breeding experiments in the past 50 years. The responsible factors of natural resistance transferred during the breeding were either two recessive genes or two complementary dominant genes [180–184]. Presence of a single dominant gene or two dominant genes may also provide resistance against the virus [185–187] (Table 2). Various molecular markers RAPD, SSR, AFLP, have been seemingly associated with the resistant genes identified which may assist in characterizing resistance genes.

Responsible for a yield loss of up to 100%, *Bean golden mosaic virus* is one of the major concerns for common bean (*Phaseolus vulgaris*) production. Resistant recessive genes, *bgm-1* and *bgm-2* were identified from a highly disease-resistant variety of bean from Mexico [188, 189]. Later on, several durable breeding lines were developed utilizing these resistance genes. Another R gene from *Phaseolus vulgaris* cultivar Othello, named *PvVTT1* (*Phaseolus vulgaris* VIRUS response TIR-TIR GENE 1) found to be responsible for resistance against bean dwarf mosaic virus (BDMV) through HR mediated defence response [161]. The dominant resistance gene *Bgp-1* has also been reported to account for normal pod development during viral infection and involve in providing resistance against BGYMV [190]. Similarly, bean leaf crumple disease of *P. vulgaris* is associated with TYLCV and is controlled by the dominant gene *PvBlc* [191]. *Phaseolus* also has resistance gene *Bct-1*, linked to the RAPD marker against BCTV [192]. In addition, the expansion of genetic studies on cucurbits against ToLCNDV infection revealed the occurrence of a single dominant resistance gene in *Luffa cylindrica* (Roem.) [193, 194]. Three candidate genes [195] and recently, one major quantitative trait locus (QTL) on chromosome 8 conferring resistance to ToLCNDV have been found in melon [196].

Natural hybridization and conventional breeding programs have utilized the natural resistance resources of plant genomes to combat virus infection via introgression for successful management of plant viruses. However, to attain stable resistance, it is important to further analyse and characterize these genetic sources and implementation of advanced speed breeding techniques for advanced phenotyping and quick transformation outputs. Although findings are promising in controlling the geminiviruses, resistance breakdown occurs frequently requiring continuous exploration of new resistance sources among geminivirus infecting crops. As geminiviruses have been observed in association with nanovirus, potyvirus, and other plant RNA viruses, investigation of commonly conserved genetic factors among the phytopathogens is crucial for tackling the mixed infections in regular filed conditions [197]. For instance**,** the phenomenon of single R-gene controls the unrelated pathogen clusters. Wheat *Bdv1* locus shows resistance to barley yellow dwarf virus (BYDV) also interfere R-genes of fungal pathogens, causing leaf rust [198].

Plant immune system has evolved multi-layer receptor systems to sense and induce the pathogen defence responses. The first layer of defence employs the cell surface radars known as pathogen recognition receptors (PRR) that recognise extracellular immune targets called pathogen associated molecular patterns (PAMPs). However, to circumvent this basal defence, pathogens employ sophisticated intracellular immune suppressors called effectors (Avr) which in turn are intercepted by the host resistance (R) genes, that encode for NBS-LRR type receptors, leading to rapid immune responses called effector-triggered immunity (ETI) [199]. These R-genes sense the effectors via direct or indirect interactions to initiate physiological and biochemical defence responses such as reactive oxygen species (ROS) generation, cell wall fortification, defence gene expression and HR at the infection site followed by induced SAR, at the distant leaves to restrict the pathogen growth and systemic movement [199]. Bean dwarf mosaic virus (BDMV)-NSP elicits HR response in BDMV resistant bean cultivar Pinto bean cvs. Othello [200]. Similarly, Rep protein of ACMV and TYLCV, pre-coat protein of tomato leaf curl Java virus (ToLCJV), and TrAP of TYLCSV also induce HR, so also does the recently identified a novel TYLCCNB βV1 gene [19, 201–203]. Often, overexpression of individual geminiviral ORFs induces visible HR or necrotic symptoms, which do not appear during virus infection, suggesting the geminiviruses also encode for proteins that mediate HR suppression. AC4

Table 2 Sources of resistance genes and exploitation of wild varieties for developing resistance to geminiviruses

Host	Genetic factor	Encoded protein	Source	Target geminivirus	References
Tomato	*Ty-1*	RDRγ	Chromosome 6 of *S. chilense*	Tomato yellow leaf curl virus (TYLCV), honey suckle yellow vein mosaic virus (HYVMV) and tobacco leaf curl Japan virus (TbLCJV)	[148]
	Ty-2	NBS-LRR	Chromosome 11 of *S. habrochaites*	TYLCV	[151]
	Ty-3	RDRγ	Chromosome 6 of *S. chilense*	TYLCV	[148, 150]
	Ty-4	Uncharacterized	Chromosome 3 of *S. chilense*	TYLCV	[154]
	Ty-5	Pelota	Chromosome 4 of *S. chilense*	TYLCV	[146]
	Ty-6	Uncharacterized	Chromosome 10 of *S. chilense*	TYLCV	[153]
	Inter simple sequence repeat (ISSR) Marker	–	*S. habrochaites*	Tomato leaf curl New Delhi virus (ToLCNDV)	[203]
	$SSR304_{158–186}$ and $SSR18_{170–145}$	–	Chromosome 7 and 10 of *Solanum habrochaites* accession 'EC-520061	Tomato leaf curl virus (ToLCV)	[156]
Cassava	CMD1, recessive gene and polygenic	Uncharacterized	*Manihot glaziovii* (TMS)	African cassava mosaic virus (ACMV), East African cassava mosaic virus (EAMCV)	[157]
	CMD2, a major dominant gene	Uncharacterized	Chromosome 12 of *M. esculenta* (TME)	ACMV, EAMCV	[159]
	CMD3, a QTL	Uncharacterized	TMS 97/2205	ACMV, EAMCV	[204]
Okra	Two duplicate dominant genes and two complementary genes	Uncharacterized	BCO-1 and VNR Green	Bhendi yellow vein mosaic virus (BYVMV)	[164]
	Two dominant genes	Uncharacterized	*A. manihot* ssp. *Manihot*	BYVMV	[170]
	Two recessive genes	Uncharacterized	Pusa Sawani (IC-1542 X Pusa Makhmali)	BYVMV	[165]
	Two complementary dominant genes	Uncharacterized	*A. manihot* (L.) Medikus ssp. manihot	BYVMV	[167]
	Two complementary dominant genes and two duplicate dominant genes	Uncharacterized	Arka Anamika, Punjab Padmini and Arka Abhay	BYVMV	[168]
	Single dominant gene	Uncharacterized	*A. manihot*	BYVMV	[171]
	Two dominant complementary genes	Uncharacterized	*A. manihot* ssp. *Manihot*	BYVMV	[166]
	A single dominant gene (Not Identified)	Uncharacterized	*A. manihot* (L.) *Medik* and *A. manihot* (L.) *Medik* ssp. *Manihot*	BYVMV	[169]
Black gram	*CYR1*, a dominant gene	Non-TIR-NBS-LRR class R-gene	*V. mungo* line VM1 (MYMIV resistant)	Mung bean yellow mosaic virus (MYMV)	[205]
Common bean	*PvVTT1*, a dominant gene	Protein with a double TIR motif	*Phaseolus vulgaris* cv. Othello	Bean dwarf mosaic virus (BDMV)	[145]
	bgm-1, a recessive gene	Uncharacterized	Chromosome 5 of DOR476 or Dry bean landrace cultivar Garrapato (Mexico)	Bean golden mosaic virus (BGMV)	[172]
	bgm-2, a recessive gene	Uncharacterized	DOR303	BGMV	[173]
	Bct-1, a dominant gene	Uncharacterized	Chromosome 7 of *P.* coccineus Burtner	Beet curly top virus (BCTV)	[176]
	Bgp-1, a dominant gene	Uncharacterized	PR9556-171	BGMV	[174]
	PvBlc, a dominant gene	Uncharacterized	*P. vulgaris* line GG12	TYLCV	[175]
Cotton	*R1CLCuDhir* and *R2CLCuDhir*	Uncharacterized	CP-15/2, LRA-5166, CIM-443, Ravi, Cedex	Cotton leaf curl Multan virus (CLCuMV), cotton leaf curl Kokhran virus	[162]

Table 2 (continued)

Host	Genetic factor	Encoded protein	Source	Target geminivirus	References
Melon	A major QTL located in chromosome 11, and two other locus on chromosome 2 and 12	–	*Cucumis melo* subsp. *Agrestis* (WM-7)	ToLCNDV	[179]
	A single recessive gene	Uncharacterized	Chromosome 8 of *Cucurbita moschata*	ToLCNDV	[180]
Sponge gourd	A single dominant gene	Uncharacterized	DSG-6 and DSG-7	ToLCNDV	[177]
	A single dominant gene and two sequence-related amplified polymorphism (SRAP) markers	Uncharacterized	DSG-6	ToLCNDV	[178]

protein of ACMV may negatively affect the Rep mediated HR responses to promote virulence [201] while TLCYnV encoded C4 physically interacts with HIR1 (hypersensitive induced reaction 1) and perturbs the HIR1 homodimerisation to counteract HIR mediated HR response [204]. Due to limited coding capacity, unlike other plant pathogens, viruses do not strictly fit into the Gene-for-Gene theory of host–pathogen interactions. However, to overcome the limitation, viruses have evolved multifunctional proteins that maintain a coevolutionary relationship with the host to invade the host defence machinery.

Other cellular factors

The chloroplast is a hub of plant's immune arsenal, not only by producing immune signals such as ROS and SA but also to initiate the biosynthesis pathways of the phytohormones GA, ABA and JA that are crucial for biotic and abiotic stress tolerance [205]. Hence, to suppress the active host defence, viruses primarily target the chloroplast machinery to attain successful infection [206]. Radish leaf curl betasatellite (RaLCB) encoded βC1 protein disturbs chloroplast organization, photosynthetic efficiency and causes veinal chlorosis [6]. A recent finding suggests PsbP (photosystem II subunit P), an extrinsic protein of oxygen-evolving complex (OEC), plays a defensive role against geminiviruses [207]. *PsbP*-silenced plants, had higher virus titre than the control plants and *PsbP* overexpression lines showed reduced disease symptoms evidenced by lower virus replication at early stages of infection. However, during later phases of infection, RaLCB-βC1 interacts with PsbP and permits successful virus replication [207]. Host regulatory proteins such as GRAB (Geminivirus Rep-A binding) proteins of NAC transcription factor families, that are involved in leaf development and senescence, can be modulated to interacting with viral proteins such as RepA to facilitate viral replication or suppress host immunity [208]. Interaction with GRAB protein has been shown to inhibit WDV infection, but enhance TYLCSV infection [209]. Overexpression of *Arabidopsis* TIFY4B, a plant DNA binding protein, responsible for cell cycle arrest, causes a reduction in viral titre and increase the latent period for symptom production. Increased expression of TIFY4B after geminiviral infection also suggests its crucial role in defence against the viruses. Furthermore, to hinder the plant defence response, TrAP protein encoded by TGMV and CaLCuV interacts with TIFY4B to counteract the cell cycle arrest leading to increased viral load [210].

CRISPR-Cas9 based resistance against geminivirus

The application of advanced genetic engineering methods has helped to overcome the limitations of labour-intensive traditional approaches for developing resistant plants. CRISPR-Cas (Clustered, regularly interspaced short palindromic repeats-CRISPR associated protein) is a bacterial adaptive immune strategy against invasive foreign nucleic acids, that has been exploited to target plant viruses. In this technique, single guide RNA (sgRNA) directs an endonuclease Cas9 to modify specified viral DNA targets by inducing double-stranded breaks which eventually leads to viral genome degradation. This technique has already been proved successful in reducing the viral titer and symptom expression against the monopartite and bipartite geminiviruses [211]. Transient expression of sgRNA-Cas9 directed to dsDNA intermediate forms of BSCTV has reduced the viral accumulation by 80% and abolished the disease symptoms in *N. benthamiana* plants [211]. The findings of Baltes et al. [212] corroborated the CRISPR system's efficiency against bean yellow dwarf virus (BeYDV)-eGFP by estimating the fluorescence intensity relative to the control plants. Systemic targeting of BeYDV key regulatory elements such as conserved nonanucleotide hairpin, rep binding sites (RBS), and Rep protein motifs that are crucial for replication has reduced the viral DNA accumulation [212]. Considering the sequence-specificity of the technique, targeting the conserved geminiviral IR region with a suitable a sgRNA (IR-sgRNA) can provide a universal approach to control geminivirus infections, even for the commonly occurring mixed infections in the field conditions. Systemic delivery of IR-sgRNA in *N. benthamiana* Cas9OE plants infected separately with TYLCV, BCTV, and merremia mosaic virus (MeMV) displayed symptom attenuation and suppression of viral DNA against infections [213]. Notwithstanding the promising CRISPR applications, CRISPR-induced virus evolution must be critically examined to monitor the emergence of resistant viruses [214]. Recently the breakdown of CRISPR resistance has been reported, where ACMV-AC2 evolved a conserved single "T" insertion that can affect the Cas9 target-cleavage activity [215]. Additionally, non-homologous end joining (NHEJ) repair of plant system is a posing threat that could repair the Cas9 targeted viral dsDNA intermediates [213, 215]. Importantly recombination, a driving force of geminivirus evolution, which allows swapping of genomic sequences during mixed infections, also enables the geminiviruses to escape the CRISPR induced cleavage, subsequently leading to CRISPR resistance.

Conclusions

Plants have evolved to develop very complex defence strategies against geminiviral infection. RNA silencing machinery remains to be one of the prominent mechanisms. While TGS carries out viral genome methylation, consequently, leads to the repression of viral pathogenicity proteins, PTGS mediates the degradation of the viral mRNAs, thereby inhibit the viral infection. Several chromatin remodelers have evolved in plants that carry out repressive modifications on host genome, diverts targeting viral genome. miRNAs have emerged as effective tools for achieving broad spectrum resistance against geminiviruses. Another level of defence against geminiviruses is mediated through R-genes that are well studied in case of fungi and bacteria and in this context geminiviruses are highlighted recently. Several other host defence regulatory mechanisms like autophagy, ubiquitination, hormonal signalling, protein kinases also play a significant role in guarding and shielding the host from geminivirus by providing the ammunition to the host to act against geminivirus. Against these wide array of defence mechanisms, various suppressor proteins and evolved sophisticated strategies are deployed by the geminiviruses that emphasize the dynamic relationship between the host and the pathogens and unique role of geminiviruses in driving the co-evolution of both plants along with their own. Molecular studies carried out to elucidate the antiviral responses involve the characterization of potential targets in cellular transcriptome, proteome, metabolome in the background of geminivirus interaction. In the vast array of cellular pathways, identifying the mechanisms which does not influence the plant growth remains the principal task. Current efforts focus on the use of precise gene-editing tool for providing a broad range of resistance against viruses. Various laboratories worldwide are standardizing the CRISPR-cas9 system for providing broad range of adaptive immunity and resistance against geminiviruses. Selection of targets within viral genome is crucial factor in achieving the durable resistance. In this context, non-coding targets are more efficient over coding regions as they embed the crucial elements essential for virus replication and pathogenicity maintenance [216]. Incidences of geminiviral diseases are increasing at an accelerated pace due to high evolution rate expanding their geographical barrier and host range. Although, various techniques ranging from conventional methods to molecular approaches have been adopted to control the geminiviral infections, due to mixed virus infections the success is limited. Identifying the suitable host factors involved in the resistance during plant-geminivirus interaction, the introduction of multiplexed genetic engineering tools targeting multiple targets, and targeted deletion of large sequences from the viral genomes can aid in the development of disease-free plants and preventing the emergence of CRISPR resistant geminiviruses [217, 218].

Abbreviations

CR: Common region; REP: Replication-associated protein; TrAP: Transcription activator protein; REn: Replication enhancer protein; CP: Coat protein; MP: Movement protein; NSP: Nuclear shuttle protein; TGS: Transcriptional gene silencing; PTGS: Post-transcriptional gene silencing; HR: Hypersensitivity response; RDR2: RNA- DEPENDENT RNA POLYMERASE 2; CLSY1: CLASSY 1; DCL-3: DICER-LIKE 3; HEN1: HUA- ENHANCER 1; AGO4: ARGONAUTE 4; RISC: RNA-induced silencing complex; BCTV: Beet curly top virus; DDR complex: DEFECTIVE IN RNA DIRECTED DNA METHYLATION 1 (DRD1), DEFECTIVE IN MERISTEM SILENCING 3 (DMS3), REQUIRED FOR DNA METHYLATION 1 (RDM1) complex; NPRE1: Nuclear RNA polymerase E; KTF1: KOW DOMAIN-CONTAINING TRANSCRIPTION FACTOR 1; DRDM2: DOMAINS REARRANGED METHYL TRANSFERASE 2; SUVH4: SU(VAR)3–9 HOMOLOGUE 4; KYP: KRYPTONITE; CMT3: CHROMOMETHYLTRANSFERASE 3; RAV: RELATED TO ABI3 and VP1; DDM1: Decrease in DNA methylation 1; Adk1: ADENOSINE KINASE 1; TYLCV: Tomato yellow leaf curl virus; BSCTV: Beet severe curly top virus; SAMDC1: S-adenosylmethionine decarboxylase; SAM: S-Adenosyl-methionine; dSAM: Decarboxylated SAM; SAHH: S-adenosyl homocysteine hydrolase; MET1: Methyltransferase 1; HDA6: Histone deacetylase 6; EML1: EMSY-LIKE 1; RPT4a: REGULATORY PARTICLE TRIPLE-A ATPASE 4A; ToLCNDV: Tomato leaf curl New Delhi virus; RDR1: RNA-dependent RNA polymerase 1; ToLCGV: Tomato leaf curl Gujarat virus; VMYMV: Vigna mungo yellow mosaic virus; DCL: DICER- LIKE protein; DRB: DsRNA binding proteins; AS2: ASYMMETRIC LEAVES 2; DCP2: DECAPPING 2; CaM: Calmodulin-like protein; SGS3: SUPPRESSOR OF GENE SILENCING 3; WDV: Wheat dwarf virus; CLCuMuB: Cotton leaf curl Multan betasatellite; TMV: Tobacco mosaic virus; ToLCV: Tomato leaf curl virus; MYMIV: Mung bean yellow mosaic India virus; MYMV: Mungbean yellow mosaic virus; CRLs: Cullin ring ligases; SCF complex: SKP1 (S phase kinase-associated protein 1)-CUL1(CULLIN 1)-F-box-protein; CSN: COP9 Signalosome; COI1: CORONATIN INSENSITIVE 1; JA: Jasmonic acid; JA-Ile: Jasmonoyl isoleucine; JAZ: Jasmonate Zim domain; NtRFP1: Nicotiana tabacum RING FINGER PROTEIN 1; UBA1: Ubiquitin activating enzyme; UBC3: Ubiquitin-conjugating enzyme 3; CKIs: Cyclin-dependent kinase inhibitors; KPC: Kip-1 ubiquitination promoting complex; RKP1: Ring finger protein; UPS: Ubiquitin-proteasomal system; SCE1: SUMO CONJUGATING ENZYME 1A; TGMV: Tomato golden mosaic virus; TYLCSV: Tomato yellow leaf curl Sardinia virus; ACMV: African cassava mosaic virus; PCNA: Proliferating cell nuclear antigen; ATG8: AUTOPHAGY-RELATED 8; CLCuMuV: Cotton leaf curl Multan virus; TLCYnV: Tomato leaf curl Yunnan virus; TYLCCNB: Tomato yellow leaf curl China betasatellite; RLKs: Receptor like kinases; SnRK1: SUCROSE NON-FERMENTING1 related protein kinase 1; CaLCuV: Cabbage leaf curl virus; GRIK1: Geminivirus Rep interacting kinase 1; AtREM4: Arabidopsis thaliana remorin group 4; ATP: Adenosine triphosphate; AMP: Adenosine monophosphate; MAPKs: Mitogen-activated protein kinase; SA: Salicylic acid; PR: Pathogenesis-related; MKK2: Map kinase kinase 2; PRRs: Pattern recognition receptors; MAMPs: Microbe-associated molecular patterns; NIK: NSP interacting kinase; RPL10: Ribosomal protein 10; LIMYB: L10-interacting MYB domain-containing protein; CLV1: CLAVATA 1; BAM1: BARELY ANY MERISTEM 1; AtBIN2: Arabidopsis thaliana Brassinosteroid insensitive 2; SPLCV: Sweet potato leaf curl virus; FLS2: FLAGELLIN SENSING 2; BRI1: BRASSINOSTEROID INSENSITIVE 1; PERK: Proline-rich extension-like receptor kinase; TCrLYV: Tomato crinkle leaf yellows virus; PXYs: PHLOEM INTERCALATED WITH XYLEM members; PEPRs: PEP1 RECEPTOR members; GDU3: GLUTAMINE DUMPER 3; SOD: Superoxide dismutase; POD: Peroxidase; CAT: Catalase; MeJA: Methyl jasmonate; JAI1: JASMONATE INSENSITIVE 1; PDF1.2: PLANT DEFENSIN 1.2; CORI3: CORONATINE INSENSITIVE 13; GA: Gibberellic acid; GAI: Gibberellic-acid insensitive; SAUR: Small auxin upregulated RNA; AUX: Auxin/Indole-3-acetic acid; ORR22: Oryzae sativa response regulator; SCTV: Spinach curly top virus; ATHB7: Arabisopsis thaliana homeobox 7; ET: Ethylene; ACCO: 1-Aminocyclopropane-1-carboxylate oxidase; ACS8: 1-Aminocyclopropane-1-carboxylate synthase 8; ACC

: 1-Aminocyclopropane-1 carboxylic acid; ERF1: Ethylene responsive factor 1; CTR1: CONSTITUTIVE TRIPLE RESPONSE 1; ToLCJoV: Tomato leaf curl Joydebpur virus; BMCTV: Beet mild curly top virus; AEV: Ageratum enation virus; EAS: 5-Epi-aristolochene synthase; RDR: RNA dependent RNA polymerase; ToSRV: Tomato severe rugose virus; CMV: Cucumber mosaic virus; ToMV: Tomato mottle virus; ISSR: Inter simple sequence repeat; CMD: Cassava mosaic disease; CuLCD: Cotton leaf curl disease; CuLCV: Cotton leaf curl virus; BYVMV: Bhendi yellow vein mosaic virus; OELCV: Okra enation leaf curl virus; RAPD: Random amplified polymorphic DNA; AFLP: Amplified fragment length polymorphism; BGMV: Bean golden mosaic virus; BDMV: Bean dwarf mosaic virus; BYDV: Barley yellow dwarf virus; NBS-LRR: Nucleotide-binding site and leucine-rich repeat; ETI: Effector-triggered immunity; SAR: Systemic acquired resistance; ToLCJV: Tomato leaf curl Java virus; HIR1: Hypersensitive induced reaction 1; RaLCB: Radish leaf curl betasatellite; PsbP: Photosystem II subunit P; OEC: Oxygen-evolving complex; GRAB: Geminivirus Rep-A binding; CRISPR-cas9: Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9; NHEJ: Non-homologous end joining.

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GENOME REPLICATION AND REGULATION OF VIRAL GENE EXPRESSION

Geminivirus Betasatellite-Encoded βC1 Protein Exhibits Novel ATP Hydrolysis Activity That Influences Its DNA-Binding Activity and Viral Pathogenesis

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ABSTRACT Plant virus satellites are maintained by their associated helper viruses, and satellites influence viral pathogenesis. Diseases caused by geminivirus-betasatellite complexes can become epidemics and therefore have become a threat to economically important crops across the world. Here, we identified a novel molecular function of the betasatellite-encoded pathogenicity determinant βC1. The tomato leaf curl Patna betasatellite (ToLCPaB)-encoded βC1 protein was found to exhibit novel ATPase activity in the presence of the divalent metal ion cofactor $MgCl_2$. Moreover, ATPase activity was confirmed to be ubiquitously displayed by βC1 proteins encoded by diverse betasatellites. Mutational and sequence analysis showed that conserved lysine/arginine residues at positions 49/50 and 91 of βC1 proteins are essential for their ATPase activity. Biochemical studies revealed that the DNA-binding activity of the βC1 protein was interfered with by the binding of ATP to the protein. Mutating arginine 91 of βC1 to alanine reduced its DNA-binding activity. The results of docking studies provided evidence for an overlap of the ATP-binding and DNA-binding regions of βC1 and for the importance of arginine 91 for both ATP-binding and DNA-binding activities. A mutant betasatellite with a specifically βC1-ATPase dominant negative mutation was found to induce symptoms on *Nicotiana benthamiana* plants similar to those induced by wild-type betasatellite infection. The ATPase function of βC1 was found to be negatively associated with geminivirus-betasatellite DNA accumulation, despite the positive influence of this ATPase function on the accumulation of replication-associated protein (Rep) and βC1 transcripts.

IMPORTANCE Most satellites influence the pathogenesis of their helper viruses. Here, we characterized the novel molecular function of βC1, a nonstructural pathogenicity determinant protein encoded by a betasatellite. We demonstrated the display of ATPase activity by this βC1 protein. Additionally, we confirmed the ubiquitous display of ATPase activity by βC1 proteins encoded by diverse betasatellites. The lysine/arginine residues conserved at positions 49 and 91 of βC1 were found to be crucial for its ATPase function. DNA-binding activity of βC1 was found to be reduced in the presence of ATP. Inhibition of ATPase activity of βC1 in the presence of an excess concentration of cold ATP, GTP, CTP, or UTP suggested that the purified βC1 can also hydrolyze other cellular nucleoside triphosphates (NTPs) besides ATP *in vitro*. These results established the importance of the ATPase and DNA-binding activities of the βC1 protein in regulating geminivirus-betasatellite DNA accumulation in the infected plant cell.

KEYWORDS betasatellite, βC1 protein, ATP hydrolysis, DNA binding, geminivirus, viral pathogenesis

Satellites are extraviral particles shown to be pathogenic to a wide range of plant, animal, and yeast organisms. The ability of satellites to associate with their helper viruses is a key feature required for the maintenance of the satellites. Satellites associate with

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various helper viruses to carry out the essential functions of their infection cycle, such as replication, movement, transmission, and/or encapsidation (1). Satellites are categorized into satellite viruses, which are encapsidated by the capsid protein encoded by their own genome, and into satellite nucleic acids, which are encapsidated by the capsid protein encoded by their helper viruses, as satellite nucleic acids do not encode any structural protein (1). Although most satellites influence the pathogenesis of their helper viruses either synergistically or antagonistically, the molecular details of the interactions between helper viruses and satellites are largely unknown.

Satellites of geminiviruses namely, alphasatellites, betasatellites, and deltasatellites, have been studied. Geminiviruses are nonenveloped, twinned, icosahedral particles that each possesses a circular, single-stranded DNA genome of 2.5 to 5.4 kb. *Geminiviridae* is the largest family of plant viruses, comprising more than 360 species, and these pathogens infect numerous economically important dicot and monocot plants in the tropical and subtropical regions of the world (2). Considering host range, type of transmission vector, genome organization, and phylogenetic relationship as criteria for classification, the family *Geminiviridae* is subdivided into nine genera, namely, *Becurtovirus*, *Begomovirus*, *Capulavirus*, *Curtovirus*, *Eragrovirus*, *Grablovirus*, *Mastrevirus*, *Topocuvirus*, and *Turncurtovirus* (2). Of these, the whitefly (*Bemisia tabaci*)-transmitted begomoviruses constitute the largest group of plant viruses and are the most numerous and geographically widespread (2, 3). Begomoviruses possess genomes that are either bipartite, comprising two molecules of circular single-stranded DNA (ssDNA) termed DNA-A and DNA-B, or monopartite, comprising a single DNA-A-like genomic component (4). Begomoviruses are associated with single-stranded satellite DNAs, namely, alphasatellites, betasatellites, and deltasatellites (3, 5–8). The majority of the begomoviruses of the Old World are monopartite, and each requires a betasatellite for its efficient infection and pathogenesis. Moreover, the association of a betasatellite with bipartite begomoviruses, such as mung bean yellow mosaic India virus (9) and tomato leaf curl New Delhi virus (10), has also been reported.

Betasatellites are small, circular, single-stranded DNA molecules of approximately 1.3 kb, associated primarily with monopartite begomoviruses (5, 8, 11). This molecule has also been shown to exacerbate the symptoms induced by its helper virus and to cause enhanced accumulation of helper virus DNA in the infected host plants. The typical genome of a betasatellite contains a satellite conserved region (SCR), an adenine-rich region, and a region that encodes the βC1 protein (12, 13). Despite lacking substantial sequence similarities with their respective associated helper viruses, betasatellites rely on their helper viruses for replication, encapsidation, insect transmissibility, and cell-to-cell and long-distance movement (12–16). The origin of replication of a betasatellite encompasses a stem-loop structure, the SCR region, and part of the intergenic region immediately upstream from the SCR (14). Earlier studies demonstrated that betasatellite *trans*-replication can be achieved by diverse begomoviruses, indicating a more relaxed specificity of its origin of replication than helper begomoviruses (12, 15). Both cognate and noncognate begomovirus-encoded replication-associated proteins (Reps) can bind to the betasatellite Rep-binding motif (RBM), although with different affinities (16). However, the promiscuous *trans*-replication of betasatellites by diverse begomoviruses remains largely unexplored.

Tomato yellow leaf curl China betasatellite (TYLCCNB)-encoded βC1 protein interacts with the Asymmetric leaves 1 (AS1) protein in the molecular disguise of Asymmetric leaves 2 (AS2) and in this way alters leaf polarity and suppresses the jasmonic acid response (17). The βC1 protein also interacts with the MYC2 transcription factor to suppress the jasmonic acid-mediated resistance of the plant to whiteflies (18). Also, βC1 encoded by *Cotton leaf curl Multan betasatellite* elicits betasatellite-specific symptoms by interacting with the ubiquitin-conjugating enzyme SlUBC3 (19). The tobacco RING E3 ligase NtRFP1, however, attenuates the tomato yellow leaf curl China virus (TYLCCNV)-βC1-induced symptoms by catalyzing the ubiquitination and 26S proteasome-mediated degradation of the βC1 protein (20). The βC1 protein functions as

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a suppressor of gene silencing, which is required for symptom induction and effective virus infection (21, 22). It suppresses the host antiviral RNA silencing defense by upregulating the expression of the host calmodulin-like protein Nbrgs-CaM (22). The tomato Sucrose-nonfermenting1-related kinase (SlSnRK1), however, attenuates this symptom expression and geminivirus infection by interacting with and phosphorylating the TYLCCNB-βC1 protein (23). TYLCCNB-βC1 also regulates the mitogen-activated protein kinase cascade to suppress host defense responses and promote infection (24). Also, radish leaf curl betasatellite (RaLCB)-encoded βC1 interacts with chloroplast oxygen-evolving enhancer protein 2 (PsbP), and interferes with PsbP-mediated antiviral defense in plants (25). Multifaceted biological functions of betasatellite-encoded βC1 proteins have been demonstrated to influence the helper virus and betasatellite DNA accumulation in the infected plants. However, the mechanism by which betasatellites regulate the accumulation of helper begomoviruses is largely undefined. Similarly, the biochemical function of the βC1 protein and its role in regulating viral pathogenesis have not yet been determined.

Quite a few viral proteins have shown the ability to hydrolyze ATP, which is an extremely important reaction in biology. ATP is an important high-energy molecule that drives many metabolic reactions in living cells. In addition, ATP acts as a substrate for kinases, whose activity provides signals for several biological events, and the hydrolysis of ATP provides energy for driving motor proteins. Enzymes of several biological pathways commonly display ATPase (ATP-hydrolyzing) activity (26). ATPases constitute a class of enzymes that catalyze the hydrolysis of ATP to ADP and free inorganic phosphate (Pi). The catalysis of the ATP hydrolysis is the most common enzymatic activity of proteins and is associated with several functions, such as ion transport, protein degradation, protein folding, DNA helicases, and gene regulation (27, 28). Almost all biochemical pathways and transport in cells are driven by energy released during the hydrolysis of ATP and other abundant cellular nucleoside triphosphates (NTPs), such as GTP, CTP, and TTP. The NTPase domains of several viral proteins include a consensus phosphate binding loop (Walker A motif, G/AXXXXGKT/S), required for ATP binding, the Walker B motif (XXXXD), required for ATP hydrolysis, and motif C (conserved arginine residue), required for binding to the γ-phosphate of ATP (29–31).

Geminivirus-encoded Rep uses the consensus Walker A motif, Walker B motif, and motif C to exhibit NTPase activity needed for its DNA helicase motor function (29). However, several noncanonical ATPases do not have the consensus sequences for binding and hydrolyzing ATP. ATP-grasp fold enzymes constitute a superfamily of proteins that bind ATP using the characteristic ATP-grasp fold domain (26). In addition to the involvement of canonical and noncanonical motifs/domains in ATPase activity, a few viral proteins have been reported to exhibit ATPase activity in their natively unfolded forms. The natively unfolded C4 protein of *Cotton leaf curl Kokhran virus-Dabawali* (CLCuKV-Dab) and the *Sesbania mosaic virus* (SeMV)-encoded polyprotein 2a domain P10 each display ATPase activity despite lacking a canonical ATPase domain (32, 33).

Renatured/refolded recombinant βC1 protein has been demonstrated to bind single-stranded as well as double-stranded DNA in a size- and sequence-independent manner (21). However, the biochemical properties of the βC1 protein remain to be elucidated, probably due to difficulties in purifying βC1 protein under native conditions. In this study, we set out to identify molecular functions of βC1 by electing to study *Tomato leaf curl Patna virus* (ToLCPaV) and its associated betasatellite ToLCPaB, which has been observed to cause serious tomato leaf curl diseases in central India (34). We were able to characterize intrinsic properties of the ToLCPaB-encoded βC1 protein.

To the best of our knowledge, this study demonstrated for the first time the display of a novel ATPase activity by a protein encoded by a plant DNA-virus-associated satellite nucleic acid. Mutational studies and conserved sequence analyses revealed that a Lys/Arg residue at either position 49 or 50 and Arg/Lys-91 are the crucial residues for the noncanonical ATPase activity of the βC1 protein. Additionally, ATPase function

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was found to be ubiquitous in different βC1 proteins encoded by diverse betasatellites. The βC1 protein, in addition to being able to hydrolyze ATP, was also found to be able to hydrolyze other abundant cellular NTPs, such as GTP, CTP, and UTP. Interestingly, the ATPase activity of the βC1 protein was also shown to interfere with its ability to bind DNA. Further, infectivity studies with wild-type and dominant negative ATPase mutant betasatellite indicated a negative correlation between the ATPase activity of βC1 and the DNA accumulation of helper begomovirus and betasatellite. An observed increased accumulation of helper virus *Rep* transcripts and betasatellite *βC1* transcripts at 21 days postinoculation (dpi) in wild-type betasatellite infection compared to that in dominant negative ATPase mutant betasatellite infection suggested a positive correlation between the ATPase activity of βC1 and helper begomovirus and betasatellite transcription. Although the biological implications of the ATPase activity of the βC1 protein are not clear at present, the results of biochemical and infectivity studies suggest that it is involved in the regulation of viral transcription.

RESULTS

The ToLCPaB-encoded βC1 protein displays ATPase activity. To explore the molecular function of the ToLCPaB-encoded βC1 protein, *in silico* analysis was carried out using the Protein Function Prediction (FFPred) program (http://bioinf.cs.ucl.ac.uk/psipred/). Functions of the βC1 protein predicted with high reliability by the FFPred tool were categorized as nucleic acid binding, NTP/NDP binding, and functions related to hydrolase/transferase activities (data not shown). Additionally, a motif prediction using the Motif search tool indicated that the βC1 protein might include the ATP-grasp motif (http://www.genome.jp/tools/motif/; data not shown). Together, the *in silico* analysis prediction results indicated that βC1 protein might have an ATPase function, in addition to its known nucleic acid binding (21) function. To study the ATPase function of ToLCPaB-βC1 *in vitro*, the glutathione *S*-transferase (GST)-tagged βC1 (GST-βC1) fusion protein was expressed in a bacterial system, purified under native, nondenaturing conditions using affinity chromatography, and further purified using anion-exchange chromatography (Fig. 1A). An ATPase assay was performed using specified amounts of purified recombinant GST-βC1 protein and the desired amount of [γ-^{32}P]ATP. Here, the reaction products were run on thin-layer chromatography (TLC) plates and autoradiographed to detect the released free inorganic phosphate (Pi) as well as nonhydrolyzed [γ-^{32}P]ATP. The ATPase assay showed that GST-βC1 hydrolyzed ATP, and the amount of released free Pi was found to increase with increasing protein concentration (Fig. 1B). Purified GST protein used as a negative control did not hydrolyze ATP (Fig. 1B). These results confirmed that the ToLCPaB-βC1 protein functions as an ATPase.

The βC1 protein exhibited ATPase activity despite not including in its sequence any of the canonical motifs previously reported to be involved in ATP binding and hydrolysis, such as the Walker A and Walker B motifs present in geminivirus replication-associated proteins (Reps) and several other viral NTPases (29). Interestingly, a geminivirus-encoded C4 protein was reported to display noncanonical ATPase activity in its native unfolded condition (32). Thus, we were interested in analyzing the noncanonical ATPase function of βC1 in terms of protein folding. The overall ordered structure formation by the βC1 protein was predicted using the PonDR tool. With this tool, the absolute mean charge and mean scaled hydropathy of the βC1 protein were predicted to be similar to those of ordered proteins (Fig. 1C), indicating that βC1 probably at least starts out as an ordered folded protein. Also, the prediction, made using the PSIPRED server, of the presence of secondary structure elements in the βC1 structure (Fig. 1D) indicated that the βC1 protein undergoes an ordered folding.

To determine the optimum conditions required for the ToLCPaB-βC1 protein to hydrolyze ATP, we tested its activity with different cofactors and at various pH levels, temperatures, and time points. ATPase activity of purified GST-βC1 was tested in the

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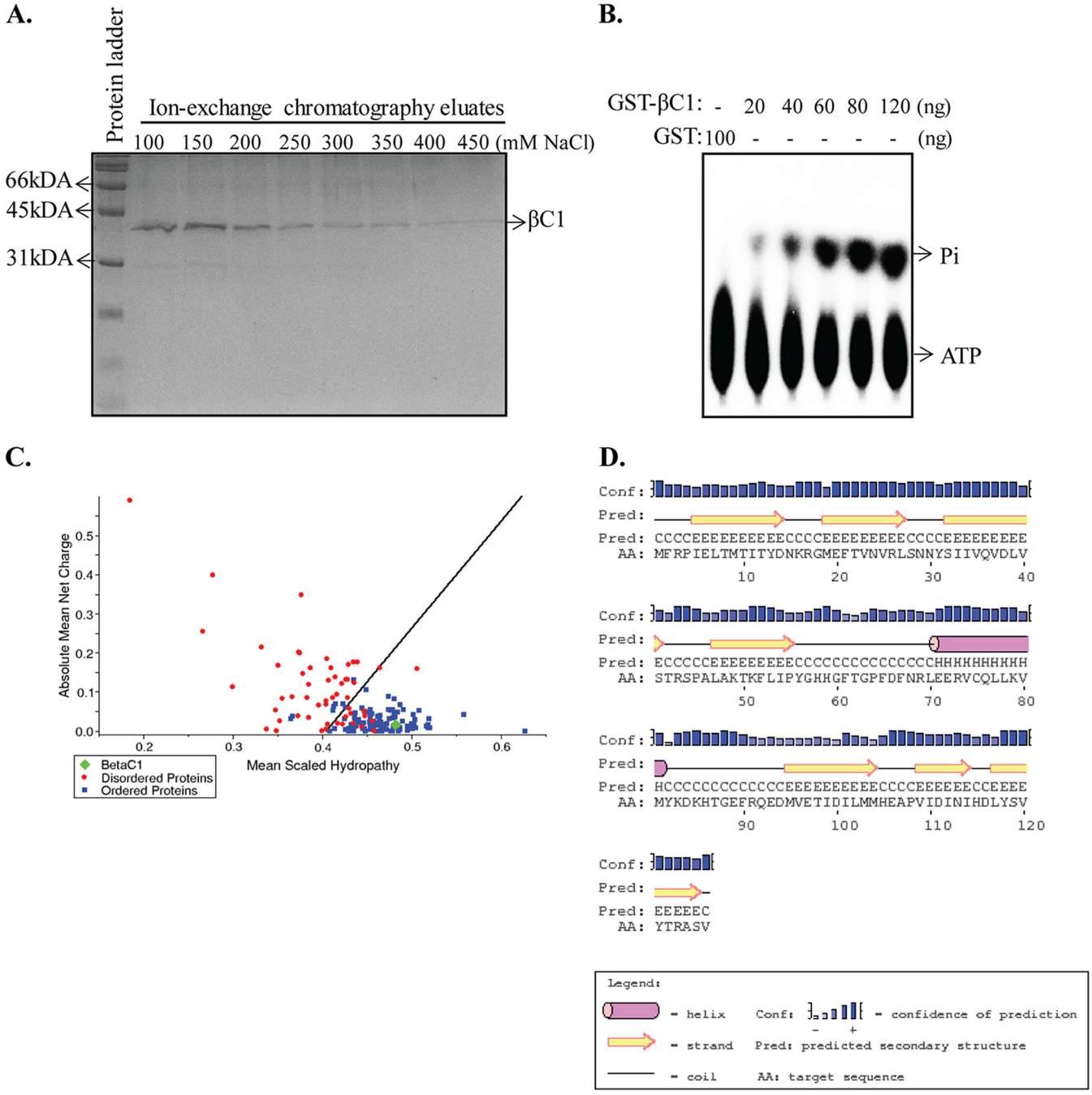

FIG 1 Betasatellite-encoded βC1 exhibits ATPase activity. (A) Gel of purification of ToLCPaB-encoded βC1 using ion-exchange chromatography with DEAE-Sepharose beads and buffer containing increasing salt concentrations, as indicated. (B) ATPase assay performed using GST-βC1 fusion protein purified under native conditions and [γ-^{32}P]ATP. The hydrolytic cleavage of γ-phosphate from ATP was analyzed by performing autoradiography on samples run in polyethyleneimine thin-layer chromatography plates. Purified GST protein was used as a negative control. Pi indicates the released free inorganic phosphate, and ATP indicates the nonhydrolyzed [γ-^{32}P]ATP. (C) Prediction of the overall disorder or order of ToLCPaB-encoded βC1 using the PonDR server (http://www.pondr.com/). (D) Secondary structure prediction of ToLCPaB-encoded βC1 using the PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred/).

absence of any divalent cation cofactor as well as in the presence of Mg^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+}, or Mn^{2+}. In these experiments, the βC1 protein did not exhibit ATPase activity in the absence of cofactor ions, and the activity was found to be higher in the presence of the Mg^{2+} ion cofactor (Fig. 2A) than in the presence of any of the other cofactors tested. Similarly, ATPase activity of the βC1 protein was tested at each of various pH levels, from 3.0 to 6.0 (with 20 mM sodium citrate buffer) and from 7.0 to 9.0 (with

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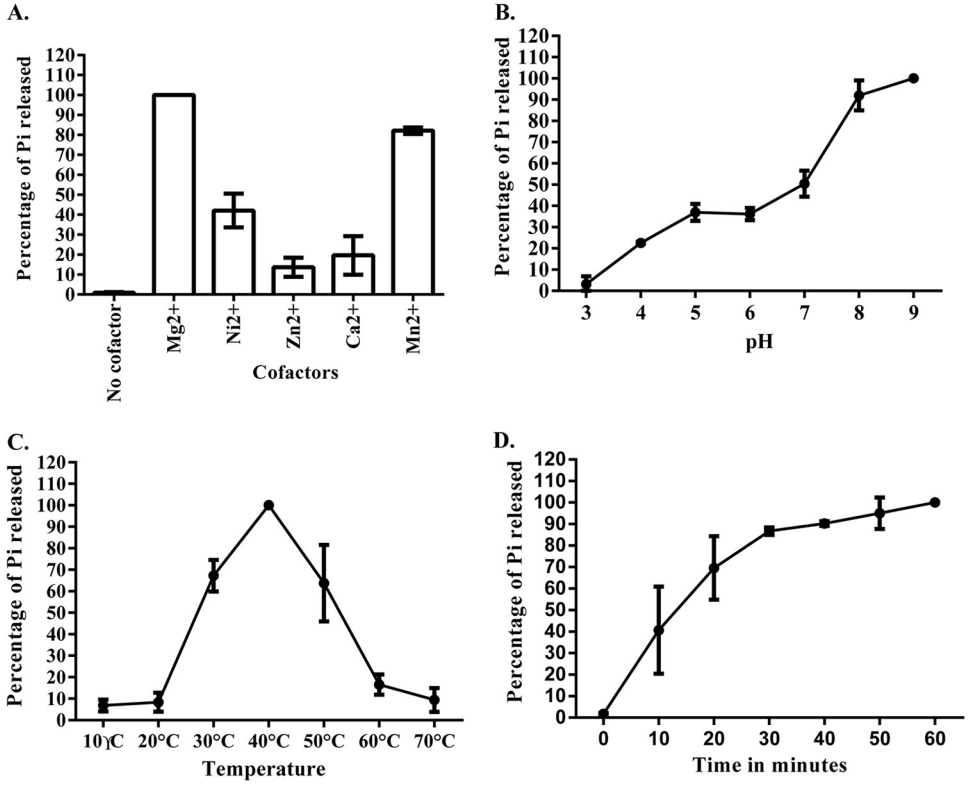

FIG 2 Characterization of the novel ATPase activity of the βC1 protein. (A) ATPase assays performed with the purified GST-βC1 protein in the absence of any introduced divalent metal cation as well as in the presence of Mg^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+}, or Mn^{2+}. The amount of Pi released in the presence of Mg^{2+} was set as 100%. (B) ATPase assays performed with GST-βC1 at various pH levels, from 3.0 to 9.0. The amount of Pi released at pH 9.0 was set as 100%. (C) ATPase assays of GST-βC1 performed at various temperatures, from 10°C to 70°C. The amount of Pi released at 40°C was set as 100%. (D) ATPase assay of the GST-βC1 protein as a function of assay time, from 0 to 60 min. The amount of Pi released at 60 min incubation was set as 100%. The amount of Pi released was quantified using ImageJ software and plotted using GraphPad Prism 6 software. Data are means and standard deviations (SD) from three independent experiments.

20 mM Tris buffer); here, maximum ATPase activity was observed at pH 9.0 (Fig. 2B). Also, of the various temperatures tested, ATPase activity was found to be at a maximum at 40°C (Fig. 2C). The time point experiment showed a linear increase in the activity until 20 min of incubation (Fig. 2D). Autoradiographs of ATPase assays performed with different cofactors (Fig. 2A), pH levels (Fig. 2B), temperatures (Fig. 2C), and times (Fig. 2D) were quantified using ImageJ software and plotted. Taken together, these results showed the ToLCPaB-βC1 protein to be a functional ATPase capable of efficiently hydrolyzing ATP using Mg^{2+} ions as cofactor ions and displaying optimal activity at approximately the physiological temperature of the host.

Identification of residues associated with ATPase activity of βC1. Earlier studies demonstrated that geminivirus-encoded Rep (2) acts as an ATPase and includes canonical ATPase motifs, namely, Walker A (required for ATP binding), Walker B (required for ATP hydrolysis), and motif C (involved in binding γ-phosphate of ATP) (29). However, sequence analysis of the βC1 protein encoded by the geminivirus-associated betasatellite revealed that it lacks any known canonical ATPase motif. To identify the motif/domain of βC1 that endows it with ATPase activity, we carried out ATPase assays with C-terminally truncated proteins, namely, $\beta C1_{1-85aa}$ and $\beta C1_{1-55aa}$, which include only amino acid residues 1 to 85 and 1 to 55, respectively, of the βC1 protein. In these experiments, none of the C-terminally truncated proteins was found to display ATPase activity, while an equal amount of full-length βC1 protein used as a positive control did show ATPase activity (Fig. 3A). This observation suggested that amino acid residues

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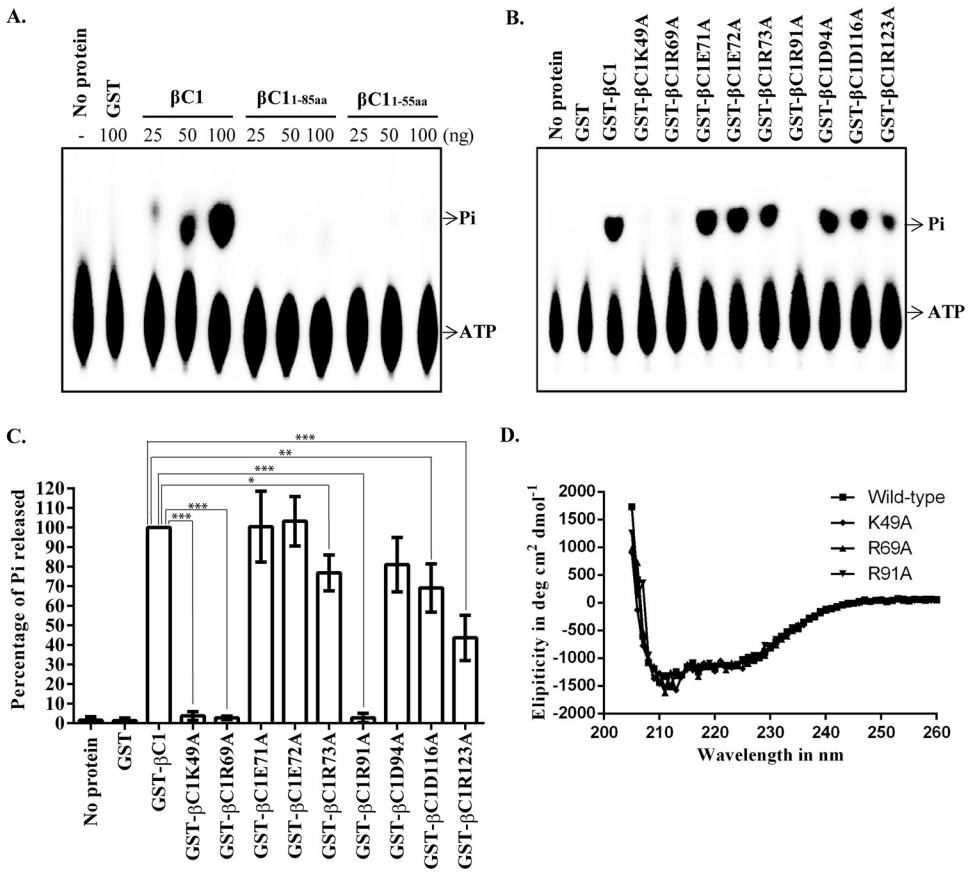

FIG 3 Identification of the motif associated with ATPase activity of the βC1 protein. (A) Autoradiograph from ATPase assays showing the ATP hydrolysis levels seen with purified full-length GST-βC1 and the truncated GST-$\beta C1_{1-85aa}$ and GST-$\beta C1_{1-55aa}$ forms of the protein. The purified GST protein was used as a negative control. Pi indicates the released free inorganic phosphate, and ATP indicates the nonhydrolyzed [γ-^{32}P]ATP. (B) Autoradiograph from ATPase assays performed with equal amounts of purified GST-βC1 wild-type and mutant proteins. Purified GST protein was used as a negative control. Pi indicates the released free inorganic phosphate, and ATP indicates the nonhydrolyzed [γ-^{32}P]ATP. (C) Comparative ability of purified GST-βC1 wild-type and mutant proteins to hydrolyze ATP. The amount of Pi released with the GST-βC1 wild-type protein was set as 100%. The amount of Pi released was quantified using ImageJ software and plotted using GraphPad Prism 6 software. Asterisks indicate values of Pi released as a result of the activity of the indicated protein that were significantly different from the amount released as a result of the activity of the GST-βC1 wild-type protein (considered 100%). (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$), as determined using Dunnett's multiple-comparison test in an analysis of variance (ANOVA). Values are means from three independent experiments. (D) CD spectra of the wild-type and mutant βC1 proteins.

associated with the ATPase activity of the βC1 protein either are distributed over the full length of the protein or lie in its C-terminal region.

We were further interested in identifying the amino acid residues of the βC1 protein that are associated with its noncanonical ATPase activity. Since the βC1 protein forms a large multimeric complex *in vivo* and *in vitro* (35), we postulated that the charged residues at the βC1 homodimeric interface may be involved in the ATPase activity of the protein. Thus, we simulated the structure of the βC1 protein by using PHYRE (http://www.sbg.bio.ic.ac.uk/phyre2) and then selected the charged residues K49, R69, E71, E72, R73, R91, D94, D116, and R123 at its homodimeric interface and made nine different single-residue-substitution mutant proteins by replacing these residues with alanine. These mutant proteins, designated GST-βC1K49A, GST-βC1R69A, GST-βC1E71A, GST-βC1E72A, GST-βC1R73A, GST-βC1R91A, GST-βC1D94A, GST-βC1D116A, and GST-βC1R123A, were expressed and purified using corresponding protein expression constructs generated using site-directed mutagenesis. Ten ATPase assays were carried out with equal amounts of GST-βC1 wild-type and mutant

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proteins, and the results showed that each of the mutations K49A, R69A, and R91A abolished the ATP hydrolysis activity of the βC1 protein (Fig. 3B and C). In addition, the ATPase activity levels of the GST-βC1R73A, GST-βC1D116A, and GST-βC1R123A mutant proteins were found to be significantly decreased relative to that of the wild type (Fig. 3B and C). Together, these results suggested that K49, R69, and R91 are the crucial residues associated with ATPase activity of the ToLCPaB-βC1 protein.

In order to test the structural integrity of each of the identified strongly ATPase-altering βC1 mutants, circular dichroism (CD) was carried out. The CD spectra of the purified GST-βC1 mutant proteins K49A, R69A, and R91A were found to be similar to that of wild-type GST-βC1 (Fig. 3D). This result confirmed that the K49A, R69A, and R91A mutations did not affect the structural integrity and global conformation of the GST-βC1 protein.

ATP influences the DNA-binding activity of βC1. Previous studies have demonstrated that the βC1 protein can bind to both double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA) *in vitro* in a sequence-independent manner (21, 25). In order to test the influence of ATP binding/hydrolysis on the DNA-binding activity of the βC1 protein, we deployed an electrophoretic mobility shift assay (EMSA) to test the dsDNA- and ssDNA-binding abilities of the protein in the presence of ATP.

According to the results of these experiments, the purified GST-βC1 protein did bind dsDNA in the absence of ATP, while the GST protein used as a negative control did not bind dsDNA (Fig. 4A). In addition, the dsDNA-binding activity of βC1 was found to decrease with increasing concentration of ATP (Fig. 4A). Due to this result of ATP interfering with the DNA-binding activity of βC1, we tested the dsDNA-binding activities of wild-type and mutant βC1 proteins in the absence or presence of ATP and specifically did so using 5 mM ATP. Here, all of the wild-type and the mutant βC1 proteins, except for the βC1R91A mutant, bound dsDNA in the absence of ATP (Fig. 4B). Similarly, GST-βC1 was shown to bind ssDNA in the absence of ATP, and increased ATP concentration interfered with its ssDNA-binding activity (Fig. 4C). Also, except for βC1R91A, the wild-type and other mutant proteins bound ssDNA without ATP (Fig. 4D). These results indicated the crucial nature of Arg-91 of the βC1 protein for its ability to bind DNA.

EMSA performed with wild-type and all of the mutant βC1 proteins in the presence of ATP showed reduced dsDNA/ssDNA-binding activity. Intriguingly, the βC1R91A mutant protein showed slight DNA-binding activity in the presence of ATP (Fig. 5A to D). The results of our previous experiments showed the βC1K49A, βC1R69A, and βC1R91A mutant proteins to be ATPase deficient. The EMSA results taken together revealed that, similar to the case for wild-type βC1, the DNA-binding abilities of the ATPase-deficient mutants were also affected by the presence of ATP. Specifically, upon introduction of ATP, βC1K49A, and βC1R69A showed reduced DNA-binding activities, while βC1R91A gained a small amount of DNA-binding activity. These results suggested that the ATPase-deficient βC1K49A, βC1R69A, and βC1R91A mutants can bind ATP and such binding would modulate the DNA-binding activity of the protein. Taken together, these results further showed ATP interfering with both dsDNA- and ssDNA-binding activities of βC1 *in vitro*. Furthermore, these findings indicated that binding of ATP rather than ATP hydrolysis interferes with the DNA-binding activity of βC1.

To understand the functional dynamics of ATP-binding and DNA-binding activities of wild-type and mutant βC1 proteins, we used computational approaches. The 3D structures of wild-type, K49A, R69A, and R91A βC1 proteins were modeled using Phyre2, and the resulting molecular dynamics (MD)-simulated structures each showed a stable conformation. Since the βC1 protein has been shown to form a multimeric complex both *in vitro* and *in vivo* (35), we further modeled the 3D structure of the βC1 homodimer using two identical subunits of βC1, subunits A and B. The complexes of each of the wild-type, K49A, R69A, and R91A βC1 homodimers with ATP were generated using the AutoDock Vina program. Inspection of the generated ATP-βC1 homodimer complex structures suggested the involvement of Lys-49, Thr-50, and Lys-51 of

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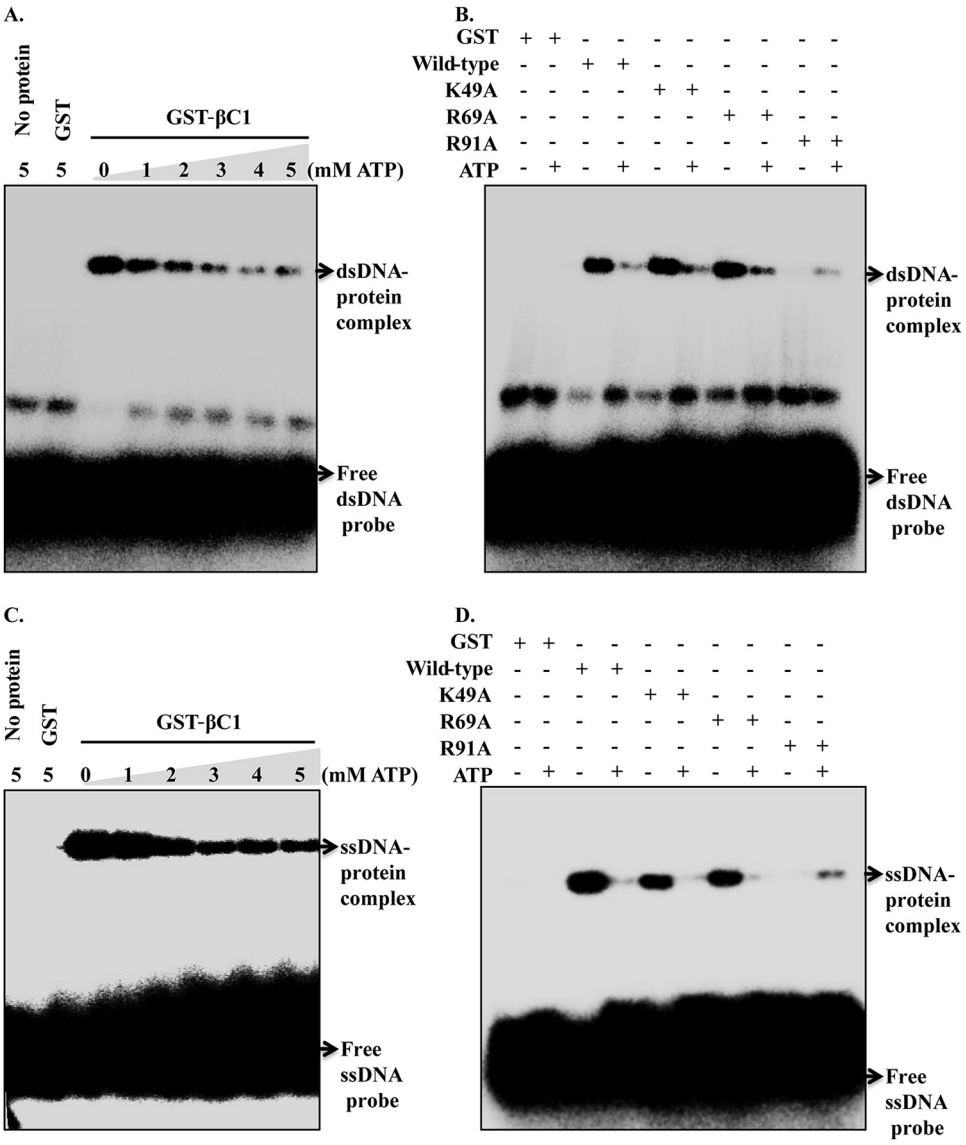

FIG 4 Effect of ATP on the dsDNA/ssDNA-binding properties of the ToLCPaB-βC1 protein. (A) Autoradiograph of the EMSA performed using purified GST-βC1 protein, radiolabeled dsDNA probe, and various concentrations of ATP. (B) Effects of ATP on the dsDNA-binding properties of the purified wild-type and K49A, R61A, and R91A mutant GST-βC1 proteins. (C) Autoradiograph of the EMSA performed using purified GST-βC1 protein, ssDNA probe, and various concentrations of ATP. (D) Effect of ATP on the ssDNA-binding properties of purified wild-type and K49A, R61A, and R91A mutant GST-βC1 proteins. The purified GST protein was used as a negative control.

subunit A and Arg-91 and Glu-93 of subunit B in ATP binding (Fig. 6A). Inspection of the modeled ATP-βC1 homodimer complex structures generated using the K49A (Fig. 6B), R69A (Fig. 6C), and R91A (Fig. 6D) βC1 proteins suggested that there is no direct effect of these mutations on the ATP-binding pocket region of subunit A but that these mutations would locally alter the positions of the residues of the ATP-binding pocket region of subunit B and hinder the direct interaction of Arg-91 with ATP. An *in vitro* ATPase assay indicated that the K49A, R69A, and R91A mutations each abolished the ATP hydrolysis activity of the βC1 protein (Fig. 3B and C). Thus, the K49A, R69A, and R91A mutations were concluded to have apparently abolished the ATP hydrolysis activity of the βC1 protein but not its ability to bind ATP.

Similarly, the structures of dsDNA-wild-type-ATP (Fig. 7A), dsDNA-K49A-ATP (Fig. 7B), dsDNA-R69A-ATP (Fig. 7C), and dsDNA-R91A-ATP (Fig. 7D) βC1 homodimeric

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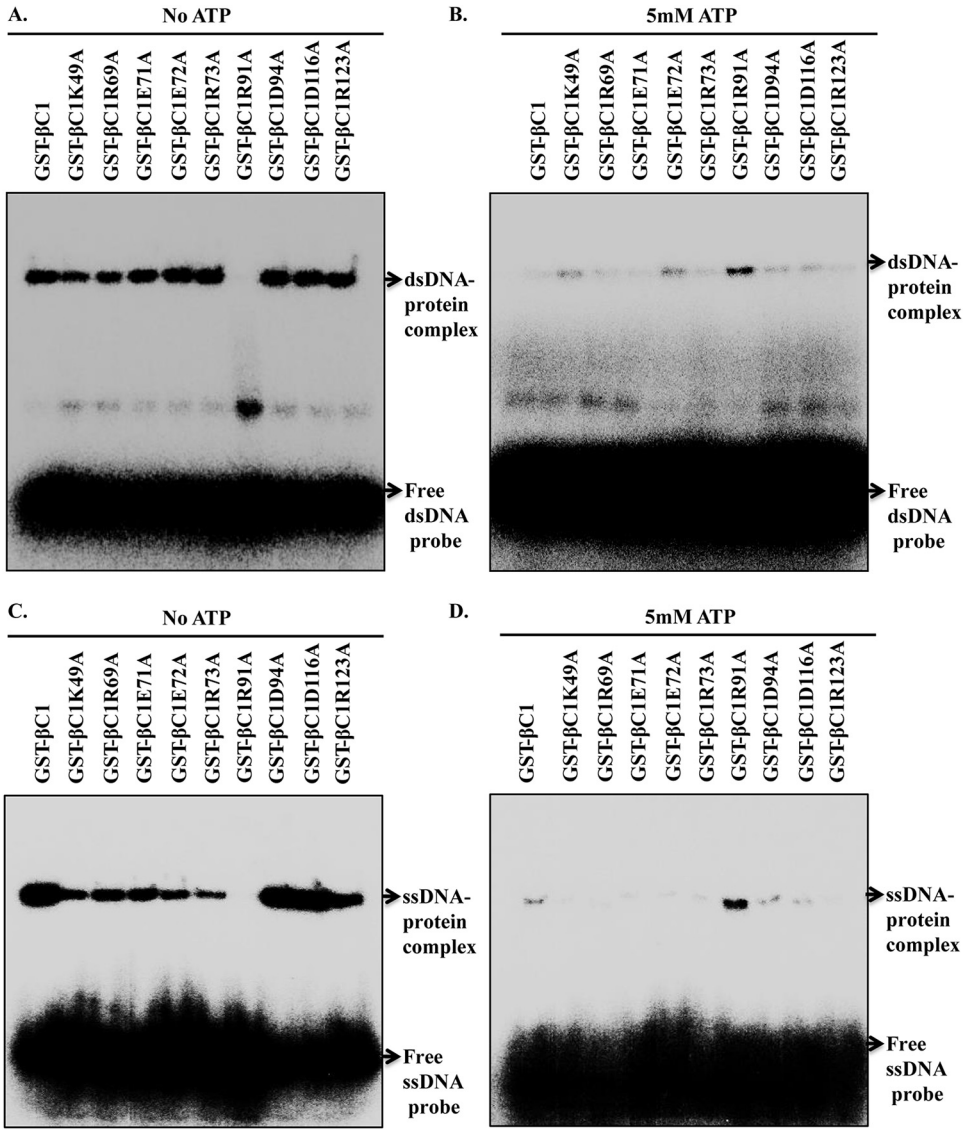

FIG 5 Effects of ATP on the dsDNA/ssDNA-binding properties of the mutated βC1 proteins. Autoradiographs showing the dsDNA (A and B) and ssDNA (C and D) binding properties of the purified wild-type and mutant GST-βC1 proteins in the absence (A and C) and presence (B and D) of 5 mM ATP.

complexes were generated using HADDOCK. The Lys-49, Arg-69, and Arg-91 residues of the wild-type βC1 protein are highlighted in Fig. 7A in orange, red, and blue, respectively. The alanine residues located at the positions of the respective mutations in the K49A (Fig. 7B), R69A (Fig. 7C), and R91A (Fig. 7D) mutant βC1 proteins are also highlighted. Interestingly, inspection of the generated structures indicated an overlap of the DNA-binding and ATP-binding pockets of βC1. Similarly, based on inspection of the structures of ssDNA-wild-type-ATP, ssDNA-K49A-ATP, ssDNA-R69A-ATP, and ssDNA-R91A-ATP βC1 homodimer complexes, one could also predict an overlap of the DNA-binding and ATP-binding pockets of the βC1 protein (data not shown). We predicted, based on these dockings, that there is a strong interaction between dsDNA/ssDNA and the βC1 protein and that ATP binding would interfere with this interaction (Fig. 7E and F). The K49A and R69A mutant βC1 proteins were also predicted to strongly bind dsDNA/ssDNA, at binding levels as high as those of the wild-type protein for dsDNA/ssDNA (Fig. 7E and F), with the presence of ATP affecting these interactions. However, our prediction showed a weak interaction between dsDNA/ssDNA and the

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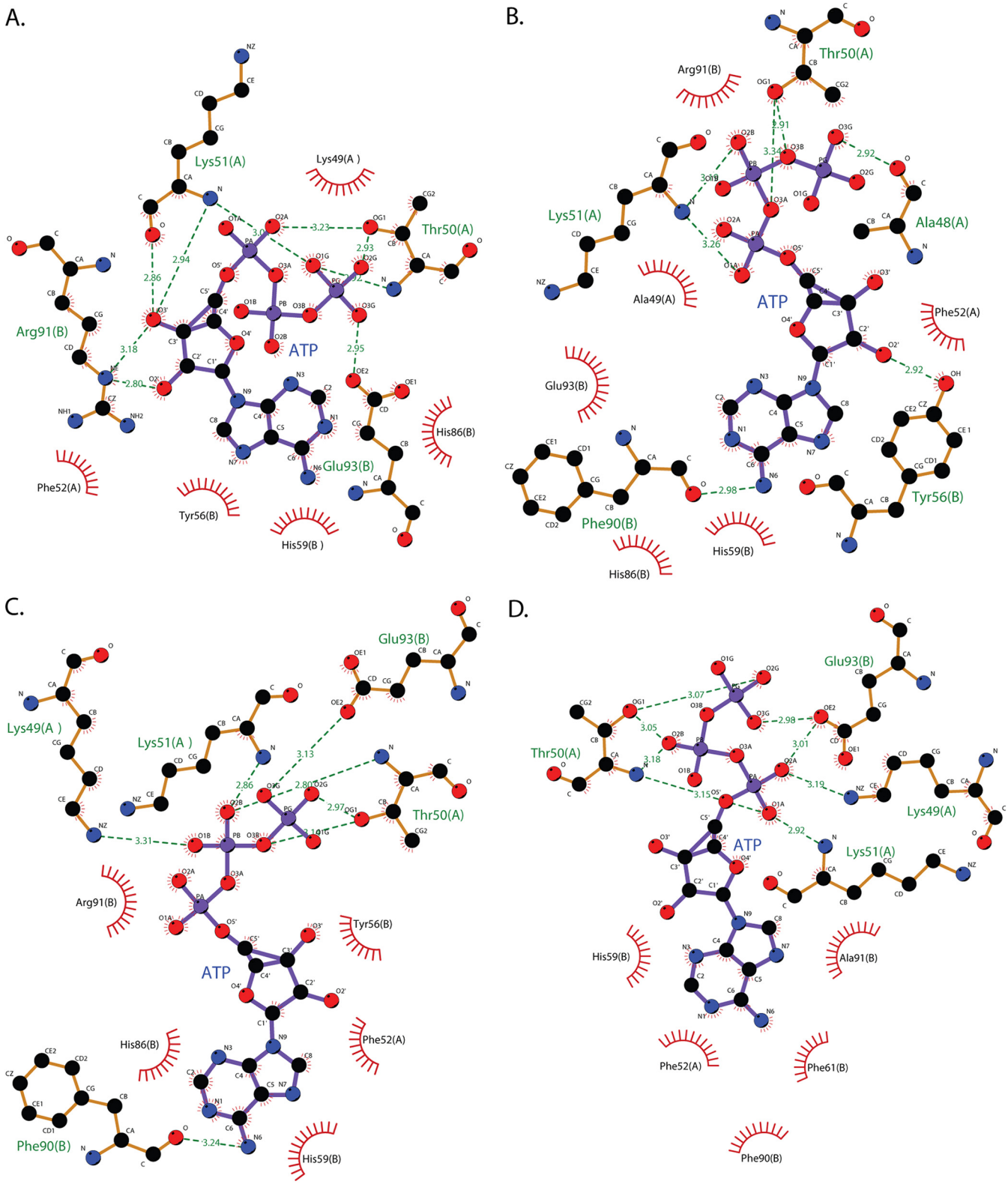

FIG 6 *In silico* docking of ATP into ToLCPaB-βC1 wild-type and mutant proteins. Structures of ATP docked into the wild-type (A), K49A (B), R69A (C), and R91A (D) βC1 homodimers, done to identify the ATP-binding pocket.

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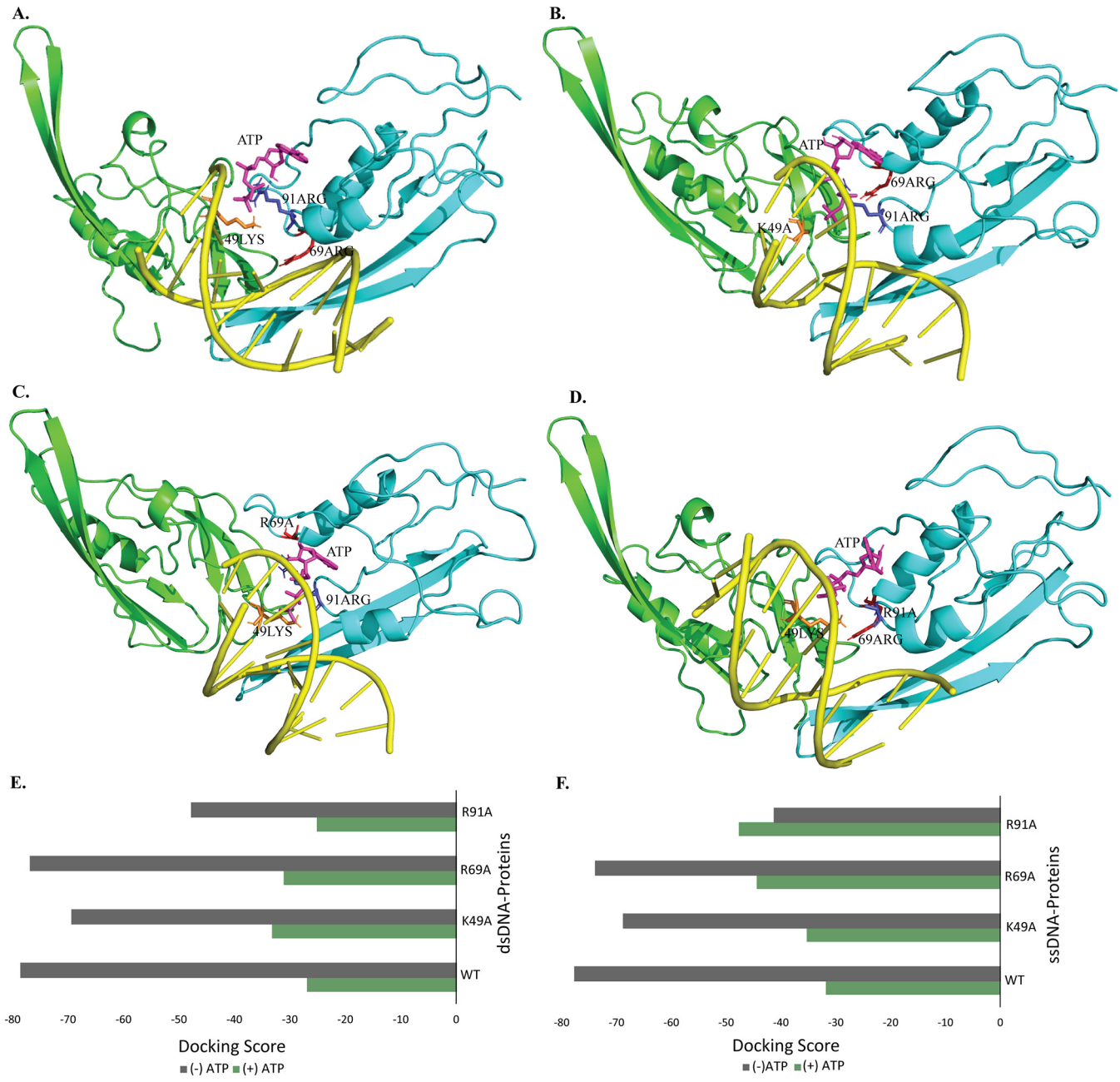

FIG 7 *In silico* docking of dsDNA with and without ATP into wild-type and mutant ToLCPaB-βC1 proteins. (A to D) Structures of dsDNA with ATP docked into the wild-type (A), K49A (B), R69A (C), and R91A (D) βC1 homodimers, done to identify the residues associated with ATP and dsDNA binding. (E) Docking scores of dsDNA with wild-type, K49A, R69A, and R91A proteins in the presence or absence of ATP. (F) Docking scores of ssDNA with wild-type, K49A, R69A, and R91A proteins in the presence or absence of ATP.

R91A mutant βC1 protein, irrespective of the presence or absence of ATP in the complexes (Fig. 7E and F). Taken together, our prediction results suggested an overlap of the ATP-binding and DNA-binding regions of βC1 protein and suggested that residue Arg-91 of the βC1 protein is crucial for both its ATP-hydrolysis and DNA-binding activities.

ATPase activity is ubiquitously displayed by diverse βC1 proteins. The conservation of amino acid residues among βC1 proteins encoded by 66 distinct betasatellites (8) was assessed using Weblogo (https://weblogo.berkeley.edu/). This analysis revealed the presence of conserved Lys/Arg residues at positions 49/50 and 91 of βC1 proteins encoded by distinct betasatellites (Fig. 8A). Arg-69 of ToLCPaB-βC1, although

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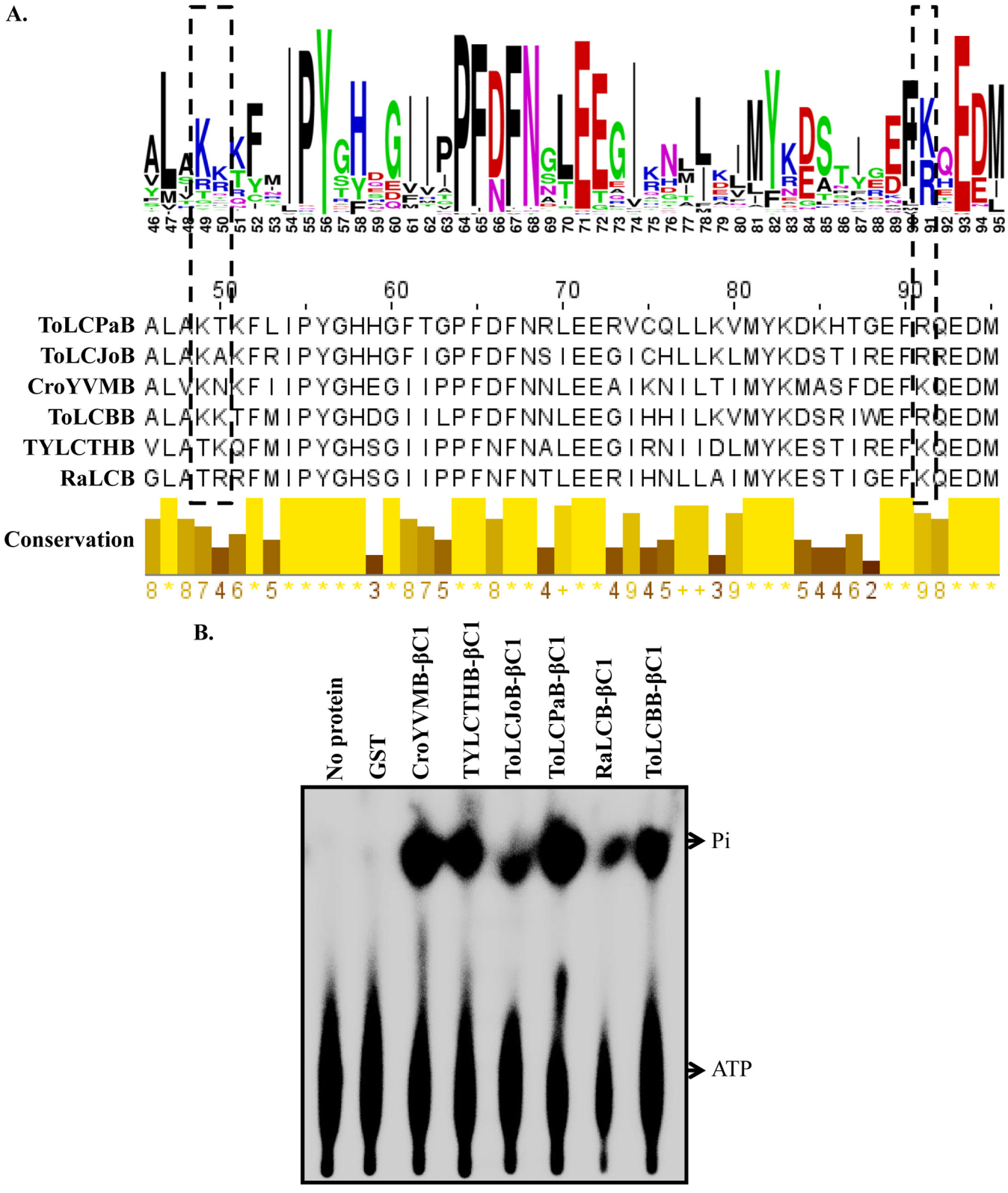

FIG 8 Sequence analysis and ATPase assays of βC1 proteins obtained from diverse betasatellites. (A) Weblogo-generated depiction of the degree of conservation of amino acid residues of βC1 proteins encoded by 66 distinct betasatellites (top) and multiple-sequence alignment of βC1 proteins encoded by *Tomato leaf curl Patna betasatellite* (ToLCPaB-βC1), *Tomato leaf curl Joydebpur betasatellite* (ToLCJoB-βC1), *Croton yellow vein mosaic betasatellite* (CroYVMB-βC1), *Tomato leaf curl Bangladesh betasatellite* (ToLCBB-βC1), *Tomato yellow leaf curl Thailand betasatellite* (TYLCTHB-βC1), and *Radish leaf curl betasatellite* (RaLCB-βC1) (bottom). (B) ATPase assay performed with equal amounts of purified βC1 protein encoded by different betasatellites. The purified GST protein was used as a negative control. Pi indicates the released free inorganic phosphate, and ATP indicates the nonhydrolyzed [γ-^{32}P]ATP.

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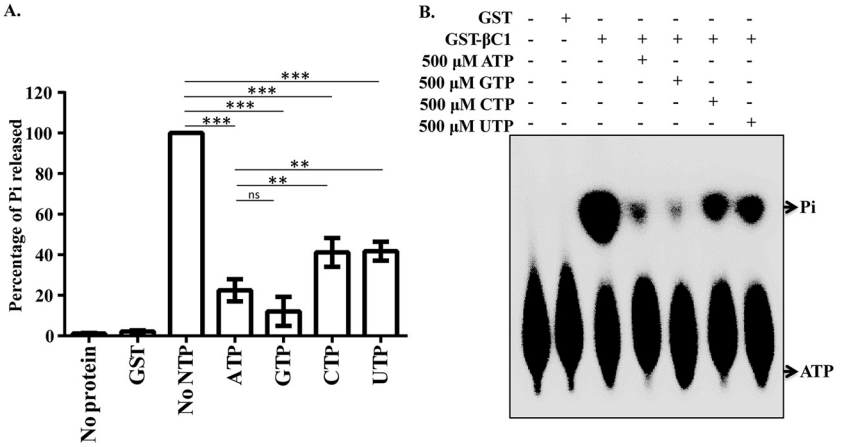

FIG 9 Effects of various NTPs on the ATPase activity of the βC1 protein. (A) ATPase activities were specifically measured for the purified GST-βC1 protein in the absence of any introduced NTP as well as in the presence of 500 μM cold NTP, specifically, ATP, GTP, CTP, or UTP. The amount of Pi released in the absence of any NTP was set as 100%. The amount of Pi released was quantified using ImageJ software and plotted using GraphPad Prism 6 software. Asterisks indicate reactions in which the amount of Pi released was significantly different from the amount of Pi released in the reaction without cold NTP (ns, nonsignificant; **, $P < 0.01$; ***, $P < 0.001$) as determined using Dunnett's multiple-comparison test in an ANOVA. Values are means from three independent experiments. (B). Autoradiograph of ATPase assays performed with the purified GST-βC1 protein in the absence of any introduced cold NTP as well as in the presence of 500 μM cold NTP, specifically, ATP, GTP, CTP, or UTP. The purified GST protein was used as a negative control. Pi indicates the released free inorganic phosphate, and ATP indicates the nonhydrolyzed [γ-^{32}P]ATP.

important for its ATPase activity, was found to be nonconserved between distinct βC1 proteins (Fig. 8A). We were interested in determining whether ATPase activity is ubiquitous in βC1 proteins encoded by diverse betasatellites. To test this hypothesis, and considering the variations in the conservation of lysine/arginine residue at positions 49 and 91, we cloned, expressed and purified the βC1 proteins encoded by croton yellow vein mosaic betasatellite (CroYVMB-βC1), tomato leaf curl Bangladesh betasatellite (ToLCBB-βC1), tomato leaf curl Joydebpur betasatellite (ToLCJoB-βC1), tomato yellow leaf curl Thailand betasatellite (TYLCTHB-βC1), and radish leaf curl betasatellite (RaLCB-βC1) under the conditions used for the expression and purification of ToLCPaB-βC1.

ATPase assays were carried out using equal amounts of purified selected βC1 proteins, and ToLCPaB-βC1 protein was used as the positive control. All of the tested βC1 proteins were found to be able to hydrolyze ATP, whereas purified GST protein, used as a negative control, did not. The CroYVMB-βC1, ToLCBB-βC1, and ToLCJoB-βC1 proteins, each of which was found to have the conserved Lys/Arg residues at positions 49 and 91 similar to ToLCJoB-βC1, also showed ATPase activity (Fig. 8A and B). Surprisingly, TYLCTHB-βC1 and RaLCB-βC1, each lacking the Lys/Arg residue at position 49, nevertheless also showed ATPase activity. These proteins, however, were observed to have the Lys/Arg residue at position 50 (Fig. 8A and B). These results taken together suggested the presence of ATPase activity for all of the tested βC1 proteins. In addition, from these results, it was concluded that conserved Lys/Arg residues at positions 49/50 and 91 are associated with the ATPase activity of the βC1 proteins.

Different NTPs modulate the ATPase activity of the βC1 protein. In order to further characterize the biochemical activities of the βC1 protein, we were interested in determining whether it can hydrolyze other cellularly abundant ribonucleotides. Thus, we carried out ATPase assays using radiolabeled [γ-^{32}P]ATP and purified ToLCPaB-βC1 protein in the absence of any introduced cold NTP as well as in the presence of a cold NTP, namely, cold ATP, GTP, CTP, or UTP. ATPase activity of the ToLCPaB-βC1 protein was found to be significantly reduced in the presence of cold ATP, GTP, CTP, or UTP (Fig. 9A and B). Specifically, the inhibition of ATPase activity of ToLCPaB-βC1 achieved by the addition of either 500 μM ATP or 500 μM GTP was found to be greater than that

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achieved by the addition of 500 μM CTP or 500 μM UTP (Fig. 9A and B). These results taken together suggest that, in addition to being able to hydrolyze ATP, the ToLCPaB-βC1 protein can also hydrolyze other ribonucleotides, namely, GTP, CTP, and UTP, albeit at lower rates for the last two.

ATPase activity of βC1 negatively regulates the accumulation of helper begomovirus and betasatellite. In order to determine the biological significance of the ATPase activity of βC1 from the perspective of viral pathogenesis, we were interested in determining the effects of the ATPase-deficient mutants on viral pathogenesis. For this purpose, we generated infectious clones of betasatellite mutants encoding βC1 proteins with alanine mutations at either K49, R69, E71, E72, R73, R91, D94, D116, or R123 using the corresponding mutant monomeric clone of ToLCPaB (referred to as β).

Nicotiana benthamiana plants were inoculated with either mock virus, ToLCPaV (referred to as A) alone, A+β, A+βΔβC1, A+βK49A, A+βR69A, A+βE71A, A+βE72A, A+βR73A, A+βR91A, A+βD94A, A+βD116A, or A+βR123A. *N. benthamiana* plants inoculated with A alone showed typical symptoms, such as upward leaf curling, vein thickening, and stunted plant growth (Fig. 10A). The plants inoculated with the wild type, as well as all those with the mutant betasatellites coinoculated with A, showed downward leaf curling, vein thickening and stunted plant growth except for A+βΔβC1, which consistently induced typical symptoms of A infection (Fig. 10A). These observations suggested that the ATPase activity of βC1 is not associated with its ability to induce betasatellite symptoms. The accumulation of the viral and betasatellite DNA from the uppermost systemically symptomatic leaves of infected plants was assessed using Southern blotting. Here, the accumulation of helper virus and betasatellite DNA in plants inoculated with A+βE71A, A+βE72A, A+βR73A, A+βD94A, A+βD116A, or A+βR123A was found to be comparable to that in wild-type betasatellite (A+β)-infected *N. benthamiana* plants at 14 dpi (Fig. 10B and C). However, the accumulation of helper virus and betasatellite DNA in plants inoculated with A+βΔβC1 was found to be decreased compared to that in A+β-infected plants. Interestingly, A+βK49A-, A+βR69A-, and A+βE91A-infected *N. benthamiana* plants showed increased accumulation of both helper virus and betasatellite DNA compared to A+β-infected plants (Fig. 10B and C). These findings indicated that the ATPase activity of βC1 negatively regulates the accumulation of helper begomovirus and betasatellite.

ATPase activity of the βC1 protein is positively correlated with viral transcription. Since the ATPase activity of βC1 was found to negatively regulate the accumulation of betasatellite and helper begomovirus in the *N. benthamiana* plants, the expression of *Rep* and *βC1* transcripts at 14 dpi and 21 dpi was analyzed using Northern blotting. Compared to the level of accumulation of *Rep* transcripts at 21 dpi in *N. benthamiana* plants inoculated with A+β, markedly reduced levels were found in the plants inoculated with A+βΔβC1, A+βK49A, and A+βR91A, and slightly reduced and comparable levels, respectively, were found in the plants inoculated with A+βR69A and A+βR123A (Fig. 11A). Similarly, at 21 dpi, βC1 transcript accumulation in plants inoculated with A+βK49A, A+βR69A, and A+βR91A, but not in the plants inoculated with A+βR123A, showed a marked reduction compared to that in the plants inoculated with A+β (Fig. 11B). Conversely, in plants inoculated with A+β as well as those with A+βR123A, the levels of *Rep* and *βC1* transcripts were found to be increased at 21 dpi in comparison to their levels at 14 dpi. These results indicated a decrease in the accumulation of *Rep* and *βC1* transcripts in the plants inoculated with mutant betasatellite encoding an ATPase-deficient βC1 protein but an increase in the levels of these transcripts in plants inoculated with wild-type betasatellite. The *βC1* transcript was found to be increased at 21 dpi in comparison to its level at 14 dpi in plants inoculated with A+βR69A (Fig. 11B). Taken together, these results suggested that the ATPase activity of the βC1 protein positively regulates the viral transcription during betasatellite infection.

DISCUSSION

Geminivirus disease complexes exert harmful effects on numerous economically important food and fiber crops throughout the world. The endemic threat posed by the

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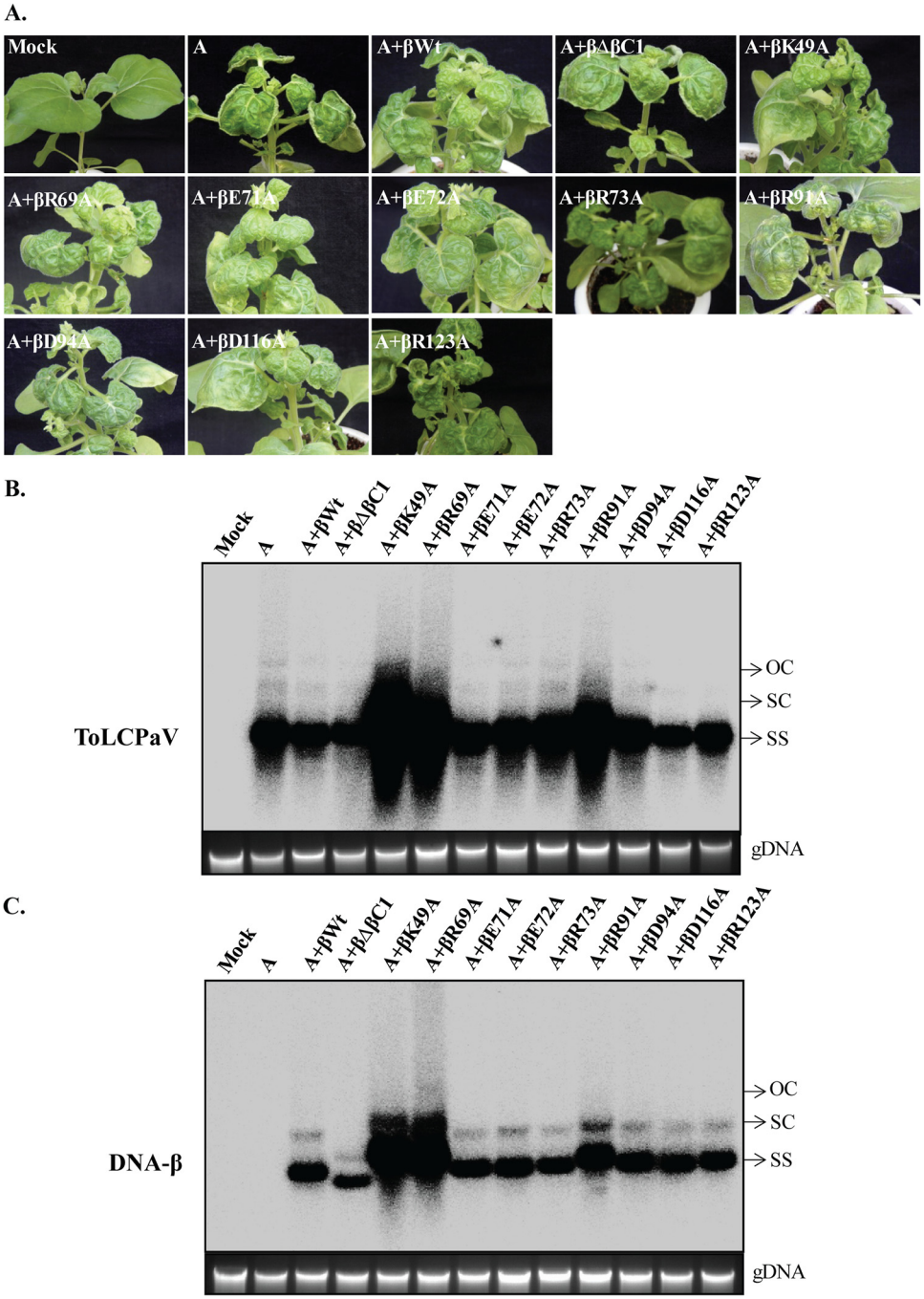

FIG 10 ATPase activity of βC1 negatively regulates helper begomovirus and betasatellite DNA accumulation. (A) Photographs of representative wild-type- or mutant-betasatellite-infected *N. benthamiana* plants at 21 dpi, showing betasatellite-induced symptoms. (B and C) Southern blotting analyses showing the comparative levels of the DNA of helper virus (ToLCPaV) (B) and betasatellites (DNA-β) (C) in the indicated infected *N. benthamiana* plants at 14 dpi. Viral DNA forms are indicated as OC (open circular), SC (supercoiled), and SS (single stranded). An ethidium bromide-stained gel showing plant genomic DNA (gDNA) served as a loading control.

geminivirus disease complex on economically important crops is aggravated by most monopartite begomoviruses being associated with betasatellites (2, 6, 36). Betasatellites, in addition to their association with begomoviruses, were also reported to be naturally associated with a mastrevirus-infected wheat sample from the field (37). Betasatellite associations with viruses that infect both monocot and dicot plants may have implications for the

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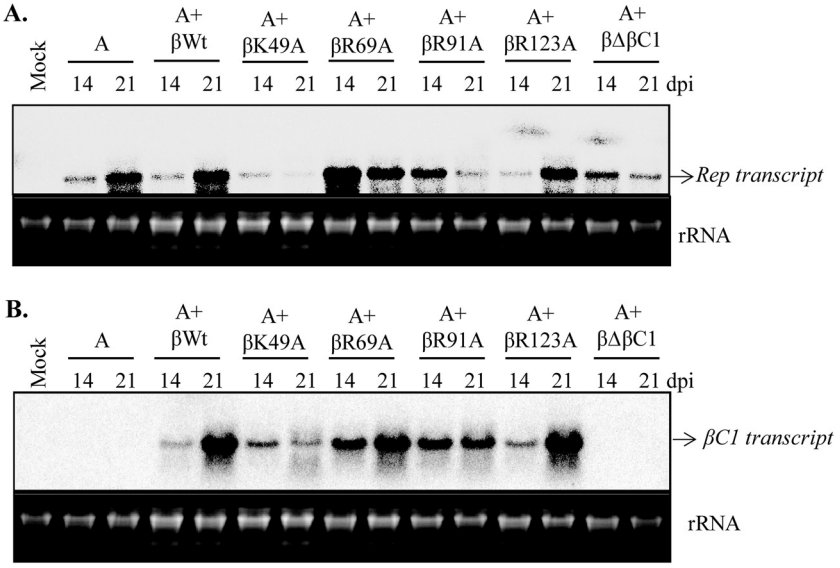

FIG 11 βC1 ATPase activity influences accumulation of Rep and βC1 transcripts. (A and B) Northern blotting analyses showing the comparative amounts of Rep transcripts (A) and βC1 transcripts (B) at 14 dpi and 21 dpi in *N. benthamiana* plants infected with wild-type or mutant betasatellites. An ethidium bromide-stained gel showing rRNA served as a loading control.

economic impact of betasatellites on crop yield (37). The pathogenicity determinant βC1 plays a pivotal role in viral pathogenesis by contributing to geminivirus virulence and by mitigating the plant defense response.

Earlier studies investigating the associations of the biological functions of βC1 as a pathogenicity factor revealed that βC1 interacts with various host proteins to reinforce geminivirus virulence and to suppress the host defense response. The binding of βC1 to ssDNA and dsDNA has been found to correlate with its suppression of gene-silencing activity (21). The current study demonstrates for the first time that βC1 protein exhibits ATPase activity in the presence of divalent cation cofactors (Mg^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+}, and Mn^{2+}). Even though the βC1 protein sequence does not include the canonical ATPase motif, the natively purified protein was shown to bind ATP and hydrolyze γ-phosphate of the substrate. Binding of ATP is achieved possibly through the ATP-grasp like motif in its sequence. ATP-grasp enzymes constitute a superfamily of proteins that each comprise a unique ATP-binding site named ATP-grasp fold. An ATP-grasp enzyme binds to an ATP molecule by grasping it in the typical 2 $\alpha+\beta$ domain of the ATP-grasp fold (26). ATP-grasp superfamily enzymes contribute to various metabolic pathways of prokaryotes, eukaryotes, and viruses. The ATP-grasp fold enzymes are involved in diverse functions associated with purine biosynthesis, pyrimidine biosynthesis, fatty acid biosynthesis, glutathione biosynthesis, lipid biosynthesis, urea hydrolysis, and dipeptide synthesis (26). Mutational analysis in the current work revealed the critical roles of βC1 residues Lys-49, Arg-69, and Arg-91 for βC1-mediated hydrolysis of ATP. In addition, the purified βC1 protein was shown in the current work to be able to hydrolyze other NTPs, such as GTP, CTP, and UTP.

Building on earlier findings showing the ability of the βC1 protein to bind DNA (21), our current study showed that ToLCPaB-βC1 also binds to both ssDNA and dsDNA. Several studies have indicated that ATP binding to and hydrolysis of DNA-binding proteins lead to changes in the conformations of these proteins that in turn either positively or negatively affect their abilities to bind DNA. The T7 gene 4-encoded protein is a well-studied helicase, with its nucleotide-binding and DNA-binding region spanning between the subunits. The connection between NTP binding and DNA binding activities of T7 helicase is complex, but NTP binding and oligomerization are prerequisites for DNA binding (38). The ToLCV-Rep protein has been shown to undergo an

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ATP-induced change in conformation to expose the DNA-binding hairpin loop and thereby facilitate DNA-binding activity of the protein (29). Adeno-associated virus 2 Rep40 helicase packages its genomic DNA into virions, in which case Rep40 protein requires stimulation of its ATPase activity to bind DNA (39). Inhibition of DNA binding activity of viral helicase by addition of ATP and $MgCl_2$ is unusual but has been reported in baculovirus-p143 and hepatitis C virus (HCV) helicase NS3 protein (40, 41). HCV-NS3 protein functions as a helicase and displaces the complementary strands of DNA or RNA and the bound proteins. It is driven by ATP hydrolysis. The DNA binding activity of wild-type HCV-NS3 protein has been demonstrated to be inhibited by the addition of 2 mM ATP, while the mutant HCV-NS3, which is defective in ATP hydrolysis, remains unaffected. However, no working model is available for HCV-NS3 helicase to explain all the available experimental data (41, 42).

A previous study showed that the baculovirus-p143 protein binds to both single-stranded DNA and double-stranded DNA more efficiently in the absence of ATP and $MgCl_2$ and that this protein undergoes conformational changes as a result of binding to ATP and $MgCl_2$, which subsequently lead to dissociation of the DNA-protein complex (41). The inchworm model of the rolling mechanism proposes that viral enzymes undergo conformational changes upon ATP binding which advance the viral protein on the DNA even before ATP hydrolysis. However, for translocation on the DNA, the enzyme must release NTP from the DNA binding pocket (40, 41). Similarly, the addition of ATP was shown in the current work to hamper the DNA-binding ability of wild-type and mutant βC1 proteins. Since an ATPase-deficient mutant protein was shown to be able to affect the DNA binding, the inhibition of DNA binding was concluded to be associated with its ATP-binding activity and not with its ATP hydrolysis activity. Our *in silico* docking analyses substantiated the results of our biochemical assays. Together, the results of our biochemical and *in silico* analyses demonstrated an overlap of the ATP-binding and DNA-binding regions of the βC1 protein. Although the three βC1 proteins containing the K49A, R69A, and R91A mutations could not hydrolyze ATP, they all showed altered ssDNA/dsDNA binding in the presence of ATP. These results suggested that ATP binding controls the ability of the βC1 protein to bind DNA.

Transient expression of the βC1 protein has been found to induce leaf curling symptoms similar to those of betasatellite-infected plants (16, 43). A previous deletion mutation analysis provided evidence that the myristoylation-like motif of the βC1 protein is required to exhibit typical betasatellite symptoms (19). In addition, betasatellite-mediated symptom development has been demonstrated to occur through the interactions of βC1 with various host proteins. Interactions of βC1 with host factors such as AS1 transcription factor (17), UBC3 (19), RING finger protein (20), SnRK1 (23), and autophagy protein (ATG8) (44) have each been demonstrated to facilitate betasatellite-mediated symptom induction. The βC1 protein localizes into the chloroplast, eventually affecting the ultrastructure and photosynthetic function of the organelle (45). A betasatellite mutant lacking the βC1 open reading frame (ORF) exhibited a typical symptom of DNA-A infection, and this observation corroborated the indispensable role of the βC1 protein in betasatellite symptom development. Nevertheless, the mutational analysis revealed that ATPase activity of βC1 is not required for betasatellite-mediated induction of symptoms such as downward leaf curling, vein thickening, and stunted plant growth. Note that viral ATPase activity is mostly known to be associated with biological motor functions, such as DNA packaging and DNA translocation.

In 2004, Cui et al. reported that a betasatellite associated with TYLCCNV enhances the DNA accumulation of helper begomovirus (16). Although the βC1 protein is dispensable for its own replication, the replication/accumulation of betasatellite is highly compromised (70%) in the absence of the βC1 protein (46). However, mutual regulation of helper begomovirus and betasatellite replication in the infected plant remains unexplored. Our study also showed that the complete deletion of the βC1 ORF markedly reduces the levels of betasatellite replication. Plants inoculated with betasatellite-encoding ATPase-defective βC1 mutants such as βK49A, βR69A, or βR91A showed

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increased accumulation of betasatellite DNA. It is important to note that increased accumulation of helper begomovirus was observed upon inoculation with each betasatellite encoding ATPase-deficient mutant βC1. This finding suggested that ATPase activity of the βC1 protein perhaps negatively regulates the replication of helper begomovirus and betasatellite in order to maintain an optimum level of viral titer. The transcription regulation patterns of Rep and βC1 transcripts were found to be altered in plants inoculated with betasatellite harboring ATPase-deficient βC1-encoding betasatellite. Taken together, these observations suggest that the ATPase activity of βC1 is associated with altered regulation of viral transcription and subsequently leads to a greater accumulation of helper begomovirus and betasatellite DNA.

ATP binding and hydrolysis alter the conformations of proteins and subsequently affect DNA binding affinity either positively or negatively depending on functional obligations. For example, ATPase activity has been shown to induce conformational changes in motor proteins to power the packing of viral DNA into the procapsid (47). Also, an ATP-hydrolysis-induced conformational rearrangement of amino acid residues of the baculovirus-encoded P143 protein has been shown to lead to dissociation of the DNA-protein complex (41).

In conclusion, βC1 protein encoded by the geminivirus-associated betasatellite has been shown, for the first time in the current work, to display novel ATPase activity in the presence of $MgCl_2$. The βC1 protein binds and hydrolyzes ATP despite lacking the Walker A and Walker B motifs and instead does so by using novel noncanonical motifs. Amino acid residues Lys-49, Arg-69, and Arg-91 of βC1 were found to be crucial for endowing the protein with ATPase activity. The ATPase activity of βC1 probably induces a conformational rearrangement that reduces its DNA binding affinity. The biological function of the βC1 protein as a symptom determinant is independent of its ATPase activity. The altered regulation of viral and betasatellite transcription is concomitant with the ATPase activity of the βC1 protein. The ATPase activity of βC1 negatively correlates with the accumulation of helper begomovirus and betasatellite. In addition to its showing ATPase activity, βC1 protein can hydrolyze other NTPs, such as GTP, CTP, and UTP.

MATERIALS AND METHODS

Construction of wild-type and mutant protein expression clones. The βC1 ORF of tomato leaf curl Patna betasatellite (ToLCPaB) (34) was PCR amplified from an infectious clone of ToLCPaB with a specific primer pair (ToLCPaBFP/ToLCPaBRP) and cloned into a pJET1.2 cloning vector. Furthermore, the pGEX-6p2-βC1 expression construct was generated by cloning the βC1 ORF in frame with GST of the pGEX-6p2 vector (GE Healthcare, Piscataway, NJ, USA) at BamHI and SalI restriction sites. The βC1 mutant protein expression constructs were generated by carrying out site-directed mutagenesis (SDM) of the pGEX-6p2-βC1 construct. GST-βC1K49A, GST-βC1R69A, GST-βC1E71A, GST-βC1E72A, GST-βC1R73A, GST-βC1R91A, GST-βC1D94A, GST-βC1D116A, and GST-βC1R123A mutant protein expression constructs were generated using K49AFP/K49ARP, R69AFP/R69ARP, E71AFP/E71ARP, E72AFP/E72ARP, R73AFP/R73ARP, R91AFP/R91ARP, D94AFP/D94ARP, D116AFP/D116 ARP, and R123AFP/R123ARP primer pairs, respectively (primer sequences will be made available upon request). All of the above-mentioned βC1 mutant protein expression constructs were ascertained by having their DNA sequenced. All of the protein expression constructs were transformed into the *Escherichia coli* ArcticExpress (DE3) strain (Agilent Technologies).

βC1 ORFs of *Croton yellow vein mosaic betasatellite* (CroYVMB; GenBank accession number FJ593630), *Tomato leaf curl Joydebpur betasatellite* (ToLCJoB; GenBank accession number JN663863), *Radish leaf curl betasatellite* (RaLCB; GenBank accession number EF175734), *Tomato leaf curl Patna betasatellite* (ToLCPaB; GenBank accession number EU862324), *Tomato leaf curl Bangladesh betasatellite* (ToLCBB; GenBank accession number JN663876), and *Tomato yellow leaf curl Thailand betasatellite* (TYLCTHB; GenBank accession number EU573713.1) were each cloned into the pGEX-6p2 vector using the appropriate primer pairs.

Expression and purification of wild-type and mutant βC1 proteins. Each pGEX-6p2-βC1 expression construct was transformed into the ArcticExpress (DE3) strain (Agilent Technologies, New York, USA). The expression conditions of GST-βC1 recombinant fusion protein were standardized as induction with 0.2 mM isopropyl β-D-thiogalactoside (IPTG) and followed by incubation at 12°C and 200 rpm for 24 h. The protein expression strain, ArcticExpress (DE3) expressing GST-βC1, was harvested by centrifugation at 4°C and 5,000 rpm for 5 min. The pelletized cells were suspended in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.05% Triton X-100. The cell lysate was sonicated and pelletized at 4°C and 13,000 rpm for 30 min. The supernatant obtained after centrifugation was used for purifying the GST-βC1 fusion protein.

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The purification of the GST-βC1 protein was achieved by performing affinity chromatography using glutathione resin (G-Biosciences, St. Louis, MO, USA). The protein-bound glutathione beads were washed with wash buffer containing 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol, and 0.05% Triton X-100. After equilibration, the GST-βC1 fusion protein was eluted using buffer containing 25 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol, 0.05% Triton X-100, and 50 mM reduced glutathione. GST protein was purified following the same procedure from *E. coli* ArcticExpress (DE3) strain transformed with the pGEX-6p2 vector. The purified GST-βC1 was further purified by performing anion-exchange chromatography using DEAE-Sepharose beads (Sigma-Aldrich, St. Louis, MO, USA). The GST-βC1 bound to DEAE-Sepharose beads was eluted with Tris buffers containing 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 350 mM, 400 mM, and 450 mM NaCl as final concentrations. The expression and purification of GST-βC1K49A, GST-βC1R69A, GST-βC1E71A, GST-βC1E72A, GST-βC1R73A, GST-βC1R91A, GST-βC1D94A, GST-βC1D116A, and GST-βC1R123A mutant proteins were carried out using the protocol used for purification of the GST-βC1 wild-type protein. Similarly, CroYVMB-βC1, ToLCJoB-βC1, RaLCB-βC1, ToLCBB-βC1, and TYLCTHB-βC1 proteins were expressed and purified using conditions similar to those used for the expression and purification of the ToLCPaB-βC1 protein.

ATPase assay. ATPase assays were performed as previously described (28, 29). The desired amounts of proteins and 0.2 μCi of [γ-^{32}P]ATP (6,000 Ci mmol^{-1}) were used in this study. The indicated protein was incubated in a buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 100 mM KCl, 8 mM dithiothreitol (DTT), 80 μg/ml bovine serum albumin, 100 μM cold ATP and [γ-^{32}P]ATP in a total reaction volume of 10 μl at 37°C. After incubation, 1 μl of the reaction mixture was spotted on a polyethyleneimine thin-layer chromatography (TLC) plate (Sigma-Aldrich, St. Louis, MO, USA) and air dried. Thin-layer chromatography was carried out with running solvent containing 0.5 M LiCl and 1 M HCOOH. Subsequently, the TLC plates were autoradiographed to detect the [γ-^{32}P]ATP and cleaved γ-^{32}P-labeled inorganic phosphate. An equal amount of purified GST protein was used as a negative control.

To identify the residues of βC1 crucial for its ATPase activity, a mass of 100 ng of purified wild-type or mutant βC1 protein was used. Similarly, to study the effect of different NTPs on the ATPase activity of βC1, the ATPase assay was performed with 100 ng of purified GST-βC1 protein in either the absence or presence of 500 μM ATP, 500 μM GTP, 500 μM CTP or 500 μM UTP. In order to study the ubiquity of βC1 ATPase activity, masses of 100 ng of different βC1 proteins were used in the ATPase assay. For each different type of ATPase assay, the autoradiographs obtained from three independent repeated experiments were quantified using ImageJ software and plotted using the program GraphPad Prism 6.

Electrophoretic mobility shift assay. The electrophoretic mobility shift assay was carried out as previously described by Gnanasekaran et al. with slight modification (25). The affinity-purified wild-type and various mutant βC1 proteins were each further purified by anion-exchange chromatography using DEAE-Sepharose resin (Sigma, St. Louis, MO, USA). To test the DNA-binding ability of the βC1 protein, either an [α-^{32}P]dCTP-radiolabeled dsDNA fragment (corresponding to bp 1103 to 50 of RaLCB) or [γ-^{32}P]ATP-radiolabeled M13-17 mer ssDNA (5′-GTTTTCCCAGTCACGAC-3′) was used as the DNA probe. GST or GST-βC1 protein (0.5 μg) was incubated in reaction buffer containing 20 mM Tris-HCl (pH 8.0), 2 mM DTT, 5 mM $MgCl_2$, 12% glycerol, 25 mM KCl, and 20 nM radiolabeled dsDNA/ssDNA for 10 min at room temperature. The reaction product in each case was run on a 3.7% native polyacrylamide gel for resolving dsDNA/ssDNA-protein complexes from free dsDNA/ssDNA probe. Subsequently, the vacuum-dried polyacrylamide gels were autoradiographed using a phosphorimager. The purified GST protein was used as a negative control.

The effect of ATP on the DNA-binding properties of the βC1 protein was studied by carrying out various experiments each using 0.5 μg of a different form of the protein, namely, GST-βC1, GST-βC1K49A, GST-βC1R69A, GST-βC1E71A, GST-βC1E72A, GST-βC1R73A, GST-βC1R91A, GST-βC1D94A, GST-βC1D116A, or GST-βC1R123A, and in each case in either the presence or absence of 5 mM ATP. For each case, the autoradiographs of three independent experiments were quantified using ImageJ software and plotted using the program Graph Pad Prism 6.

Construction of wild-type and mutant partial-tandem-repeat infectious clones. An infectious partial tandem dimer of *Tomato leaf curl Patna virus* (ToLCPaV; referred to as A) and that of *Tomato leaf curl Patna betasatellite* (ToLCPaB; referred to as β) were already available in our laboratory (3). To construct ToLCPaB$\Delta\beta$C1, the βC1-coding region was removed from ToLCPaB; this removal was accomplished by using SDM to introduce the NotI restriction on both the 5′ and 3′ ends of the βC1 ORF. Using the monomeric clone of ToLCPaB$\Delta\beta$C1, the infectious tandem repeat of ToLCPaB$\Delta\beta$C1 (referred to as $\beta\Delta\beta$C1) was constructed at the KpnI restriction site of pCAMBIA 2300. The βC1K49A, βC1R69A, βC1E71A, βC1E72A, βC1R73A, βC1R91A, βC1D94A, βC1D116A, and βC1R123A mutations were each generated by performing SDM using the monomeric clone of ToLCPaB and corresponding sets of primers (primer sequences will be made available upon request). The introduction of the above-mentioned mutations into the monomeric clones of ToLCPaB was confirmed by sequencing the DNA of these clones. An infectious partial tandem dimer of each of βK49A, βR69A, βE71A, βE72A, βR73A, βR91A, βD94A, βD116A, and βR123A was constructed using the corresponding mutant monomeric clone of ToLCPaB.

Virus inoculation and DNA and RNA blotting. The *Agrobacterium tumefaciens* strain EHA105 carrying each of the infectious partial tandem dimers of ToLCPaV along with either ToLCPaB or a ToLCPaB mutant ($\beta\Delta\beta$C1, βK49A, βR69A, βE71A, βE72A, βR73A, βR91A, βD94A, βD116A, or βR123A) was inoculated into *N. benthamiana* plants as described previously (34, 48). For each experiment, total genomic DNA was extracted from the uppermost symptomatic leaves as described earlier by Singh et al. (49), detection of viral DNA from the isolated total DNA was carried out by performing Southern blotting

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following the standard procedure, and total genomic DNA was run on a 0.8% agarose gel and transferred to a Hybond-N$^+$ membrane. The membrane was then probed with an [α-^{32}P]dCTP-labeled DNA probe specific for ToLCPaV-Rep and ToLCPaB-SCR, and the membrane was autoradiographed using the phosphorimager. Total RNA was extracted from tissue of symptomatic uppermost leaves using the standard RNA isolation protocol with TRIzol (25). Northern hybridization was performed after running total RNA on denaturing agarose gel containing 1% formaldehyde, and total RNA was transferred to a Hybond-N$^+$ membrane. The membrane was then probed with [α-^{32}P]dCTP-labeled DNA probe specific for ToLCPaV-Rep, and ToLCPaB-βC1 and autoradiographed using the phosphorimager.

Circular dichroism spectroscopy studies of wild-type and mutant proteins of βC1. Circular dichroism spectra of wild-type and mutant GST-βC1 proteins at 25°C were each obtained in a quartz cell with a 1-mm light path using an Applied Photosystem Chirascan model CD spectrophotometer (50). Three scans in each case were averaged to correct for buffer contributions. The secondary structure content of each protein (wild-type, K49A, R69A, and R91A proteins) was predicted by using K2D and CDNN software.

Computational analysis. The Protein Function Prediction (FFPred) program (http://bioinf.cs.ucl.ac.uk/psipred/) was used for the prediction of the molecular function of the βC1 protein. To find a sequence motif that would explain the molecular function of βC1, the prediction of motifs in the βC1 protein was performed using the Motif search tool (http://www.genome.jp/tools/motif/). The prediction of whether the protein is natively ordered or disordered was carried out using the PONDR server (http://www.pondr.com/). The secondary structure prediction was performed using the PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred/). The standard default threshold values were used for all prediction tools used in this study.

BLASTp analysis of the ToLCPaB-βC1 amino acid sequence against the Protein Data Bank (PDB) did not give any significant hit (https://www.rcsb.org/). Thus, the protein structure of ToLCPaB-βC1 was modeled using Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/) as described by Gnanasekaran et al. (8). The obtained 3D structure of the βC1 protein was subjected to 20 ns of MD simulation using the program GROMACS. The docking studies of the βC1 homodimer were carried out using the HADDOCK server (http://milou.science.uu.nl/services/HADDOCK2.2/haddock.php). The residues conserved in βC1 proteins encoded by 66 distinct betasatellites were identified by using Weblogo (https://weblogo.berkeley.edu/).

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