Chapter 9

Mutational Analysis of Effectors Encoded by Monopartite Begomoviruses and Their Satellites

Muhammad Shafiq Shahid, Pradeep Sharma and Masato Ikegami

Additional information is available at the end of the chapter

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1. Introduction

The geminiviruses are plant-infecting viruses with genomes consisting of circular, single-stranded DNA (ssDNA) geminate particles [86]. Members of the family *Geminiviridae* have been grouped into four genera (*Begomovirus, Curtovirus, Mastrevirus and Topocuvirus*) based on genome organization, host range and insect vector [29, 87]. The majority of geminiviruses belong to the genus Begomovirus, are transmitted by whiteflies (*Bemisia tabaci*: Gennadius), and infect dicotyledonous plant species [85]. The monopartite begomovirus genome is ~2.8 kb nucleotides in length and encode genes both in complementary and virion sense from a non-coding intergenic region that contains promoter sequences and the origin (*ori*) of virion-strand DNA replication. The *ori* consists of a predicted hairpin structure that contains the absolutely conserved (for geminiviruses) nonanucleotide (TAATATTAC) loop sequence and repeated motifs upstream known as iterons.

2. Functions of effectors encoded by monopartite begomoviruses

2.1. Complementary-sense

The complementary-sense strand encodes the Rep proteins, also known as C1, AC1 and AL1, is a multifunctional protein and the only viral protein absolutely required for virus replication. Rep is encoded on the complementary sense strand (Fig. 1 DNA A). This protein is involved in several biological processes: initiation and termination of rolling circle replication (RCR) by nicking and religating the replication origin of viral DNA [45] and repression of its own gene transcription [19]. The Rep proteins of geminiviruses are closely related and show substantial sequence conservation. Four functional domains have been delineated for begomovirus Rep : the N-terminal domain (amino acids 1 to 120), which is involved in initiation by geminiviruses [63], AC1 protein initiates rolling circle replication

by a site-specific cleavage within the loop of the conserved nonamer sequence, TAATATTAC [30]. The AC1 protein binding site is located between the TATA box and the transcription start site for the *Rep* gene and acts as the origin recognition sequence and as a negatively regulatory element for *AC1* gene transcription [19], the oligomerization domain (121 to 180 aa), leading to interactions with itself and with host factors [28]. The AC1 protein alone can initiate RCR without requiring other accessory viral factors [34]. AC1 protein also has DNA helicase activity which depends upon the oligomeric state of the protein [14].

The transcriptional activator protein (TrAP); is also known as AC2, C2 an AL2. AC2 is a ~15-KD a transcriptional activator protein unique to begomoviruses because it is absent in mastreviruses and a related protein in curtoviruses, AC2 protein, seems to play a different role. In mastreviruses, AC1 protein provide the functions of AC2 [51]. TrAP is necessary for transactivation of late genes [90]. Recently, several researchers have shown that the AC2 gene of *Cabbage leaf curl virus* (CaLCuV) activates the CP promoter in mesophyll and acts to derepress the promoter in vascular tissue, similar to that observed for TGMV [44]. Further, since AC2 1-100 is as effective a suppressor as the full-length AC2 protein, activation and silencing suppression appear to be independent activities. For example Gopal et al. [26] showed that AC2 of *Bhindi yellow vein mosaic virus* (BYVMV) is involved in transactivation and only mildly in suppression of gene silencing of monopartite begomoviruses viruses and not in transmission.

The replication enhancer protein (REn); also named as AC3/AL3. AC3 is a ~16 KD a protein in curtoviruses and in begomoviruses. The AC3 protein greatly enhances viral DNA accumulation of curtoviruses and begomoviruses [22, 92] by interacting with Rep [81]. Experimental observations suggested that AC3 protein might increase the affinity of Rep for the origin. Complementation studies revealed that AC3 could act on heterologous viruses [93].

The C4 protein, for which the function remains unclear but for some viruses is a pathogenicity determinant and a suppressor of PTGS [73]. AC4 is highly variable among begomoviruses, which is expressed from an open reading frame (ORF) embedded in the Rep ORF.

2.2. Virion-sense

The virion-sense strand encodes the genes required for insect transmission and movement in plants, coat protein (CP) and V2 protein. Monopartite begomovirus capsids are composed of a single CP, encoded by the *V1* gene or (also known as AV1), depending on the geminivirus [107]. For monopartite geminiviruses, CP is essential for systemic spread through the plant [12]. Besides the encapsidation function, CP is also required for transmission of the virus between the plants. The CP of the monopartite begomoviruses facilitates the transfer of infecting viral DNA into the host cell nucleus and is essential for systemic virus movement [5, 46, 50, 104]. The CP also determines the vector specificity [10, 32, 33] and protects the viral ssDNA from degradation during transmission by the insect vector [3], or mechanical inoculation [24].

In contrast to New World (NW) begomoviruses, Old World (OW) begomoviruses have AV2/V2 and this is involved in the movement of monopartite viruses. A recent report shown that the V2 (a homolog of AV2) of a monopartite begomovirus is involved in overcoming host defenses mediated by post-transcriptional gene silencing as well as in movement [114, 115]. V2 targets a step in the RNA silencing pathway which is subsequent to the Dicer-mediated cleavage of dsRNA [109, 70].

3. Role of effectors encoded by satellites

3.1. Betasatellite

Recently, the majority of the begomoviruses originating from the OW have been shown to be monopartite and to associate with a class of ssDNA satellites known as betasatellites (earlier known as DNAβ) [9]. Betasatellites are approximately half the size of their helper begomoviruses (approx.1.4 kb) and are required by the helper virus to induce typical disease symptoms in their original hosts. The success of begomovirus-betasatellite disease complexes appears to be due to the promiscuous nature of betasatellites that allows them to be *trans*-replicated by several distinct begomoviruses [53, 59]. These begomovirus-betasatellite disease complexes are widespread throughout OW and outnumber bipartite begomoviruses whereas in the NW only bipartite begomoviruses are native. There have been recent reports which showed that betasatellite can complement the function of DNA B, suggesting that the betasatellite may provide movement functions to its helper begomovirus [74]. Betasatellite can be associated with distinct begomoviruses and it can interact and make new complex with diverse monopartite begomoviruses [110-112].

Tomato leaf curl virus (ToLCV), originating from Australia, was shown to be associated with a single-stranded DNA satellite molecule [18]. The ToLCV satellite (ToLCV-sat) is approximately 682 nt in length and sequence unrelated to ToLCV and it depends on ToLCV for replication and encapsidation. It has no discernable effects on viral replication or symptoms caused by ToLCV. Betasatellites have three structural features: a approx.115 bp highly conserved region, *βC1* gene and a region rich in adenine, [76, 108] (Fig. 1 betasatellite). This gene has the capacity to encode a 13-14-kDa protein comprising 118 amino acids, although some betasatellites have additional N-terminal amino acids [79, 108]. Recently it has been shown that betasatellite to be pathogenicity determinant and suppressor of RNA silencing [16, 66]. It also induced abnormal cell division in *N. benthamiana* [17]. Betasatellites do not contain the iterons of their helper begomoviruses, although betasatellite clearly must possess sequences that are recognized by the begomovirus-encoded Rep in order to allow transreplication of the betasatellite [76].

All the reported betasatellites [54] or defective betasatellites (half size of wild type betasatellite) [7] contain the A-rich region, the A-rich region may play biological role in betasatellites [95]. A-rich region is not required for *trans*-replication of betasatellite and not related with encapsidation also. However, the A-rich region deleted mutant caused milder symptom [95]. The begomovirus accumulates to normal levels in Ageratum in the presence

of betasatellite suggesting that the satellite functions either by facilitating the replication or movement of the begomovirus or by suppressing a host defense mechanism such as gene silencing. Recently it has been shown that a betasatellite can override the AC4 pathogenicity phenotype of TLCV and it can complement the function of DNA B [73]. Despite its importance to the disease phenotype, there is still no information available concerning even the most fundamental properties of the satellite.

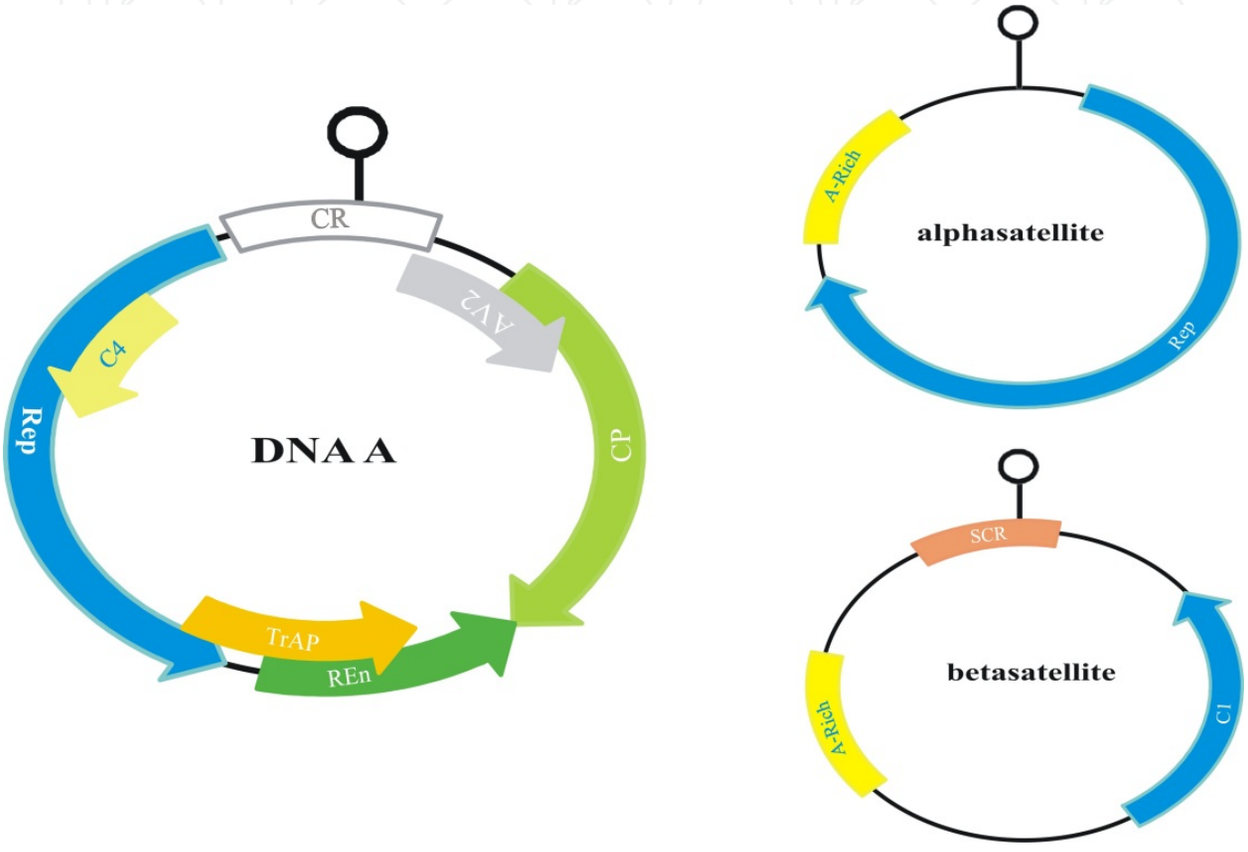

Figure 1. Genome organization of monopartite begomoviruses-satellite complex. DNA-A (encoding replication-associated protein [Rep], coat protein [CP], replication enhancer protein [REn], transcriptional activator protein [TrAP] and proteins possibly involved in virus movement [AV2], pathogenicity determinant and a suppressor of RNA silencing [AC4], viral genome replication [AC5]) Alphasatellites are self-replicating molecules encoding their own Rep. Betasatellites are dependent on their helper viruses for their replication and encode a single protein, βC1, which upregulate replication of helper virus and suppress host defense. Both satellites have an A-rich region and in addition to this betasatellites have a region of sequence conserved between all examples known as the satellite conserved region (SCR).

In Arabidopsis, these pathways are affected by the DICER- like proteins (DCL1, DCL2, and DCL3) that are nuclear localized and are required for miRNA and siRNA biogenesis. Thus, βC1 protein may affect the activity of the DICER-like proteins in plants during nuclear activities that function in silencing suppression. The other possibilities are that βC1 protein could down-regulate transcription of a host protein that acts in the PTGS pathway in the cytoplasm or that βC1 protein could activate transcription of a host PTGS inhibitor [15].

4. Alphasatellites

Many begomovirus betasatellite complexes are also associated with a third ssDNA component for which the collective term alphasatellite (earlier known as DNA 1; R.W. Briddon, manuscript in preparation). However, alphasatellites are dispensable for virus infection and appear to play no significant role in the etiology of the diseases with which they are associated [56]. Alphasatellite components are satellite-like, circular ssDNA molecules approx.1375 nucleotides in length (Fig.1 alphasatellite). They encode a single gene, a rolling circle replication initiator protein (Rep), and are capable of autonomous replication in plant cells. Closely related to the replication associated protein encoding components of nanoviruses (a second family of plant infecting ssDNA viruses), from which they are believed to have evolved, they require a helper begomovirus for movement within and between plants [56, 80].

Several alphasatellites are capable of replicating and systemically infecting their plant host in the presence of a helper begomovirus without a visible effect on symptom development or virulence [6, 40]. However AYVSGA a different type of 'DNA-2'-class alphasatellite that ameliorates symptom severity in an infected host and also capable of reducing virulence and the relative accumulation of its associated Tomato leaf curl betasatellite (ToLCB) [1]. Alphasatellites have been acquired by helper begomoviruses to restrain virulence to achieve increased viral fitness [76, 105].

Recently, two 'DNA-1-type' alphasatellites Gossypium mustelinium symptomless alphasatellite (GMusSLA) and Gossypium darwinii symptomless alphasatellite (GDarSLA) phylogenetically divergent from the DNA-2-type alphasatellite have each been shown to attenuate symptoms caused by their helper begomovirus [60]. However [35] hypothesize that symptom attenuation and a relative reduction in betasatellite accumulation might result from DNA-2-mediated modulation of betasatellite activity. Possibly alphasatellite modulates begomovirus-betasatellite pathogenicity by interfering with βC1, a key virulence factor [8]. Also alphasatellite rep can interact with C4 of CLCuRaV that might be providing an additional possible mechanism for symptom amelioration by alphasatellites. Furthermore alpha-Rep down regulate betasatellite replication (In the field), and thus down-regulation of the manifestation of the pathogenicity determinant βC1 [60], moreover alpha-Rep proteins GMusSLA and GDarSLA can act as a strong suppression of posttranscriptional gene silencing (PTGS) [60].

5. Post-transcriptional gene silencing (PTGS)

Post-transcriptional gene silencing (PTGS) which is initiated by double stranded RNA (dsRNA) is common in plant–virus interactions and is an evolutionarily conserved mechanism that protects host cells against invasive nucleic acids, such as viruses, transposons and transgenes [100]. As a counter to this host defense, most plant viruses encode proteins which act as suppressors of PTGS [71]. Viral suppressors of PTGS interfere with various steps of this pathway including initiation, maintenance or systemic silencing which are mainly downstream of dsRNA production [52, 57].

RNA silencing in plants operates as an antiviral defense response; to establish infection, viruses must suppress RNA silencing by the host [100]. Begomoviruses have been shown to induce PTGS in infected plants by producing virus specific siRNAs (21, 22 and 24 nt) [97]. To counteract this host defence, geminiviruses encode RNA silencing suppressors [4]. However, depending on each intrinsic virus and its interaction with plant host factors, the efficacy of virus-induced PTGS may vary [99]. At least three RNA-silencing suppressors have been reported in TYLCD-associated or related begomoviruses. Thus, the V2 protein of TYLCV functions as an RNA-silencing suppressor; it counteracts the innate immune response of the host plant by interacting with SISGS3, the tomato homolog of the Arabidopsis SGS3 protein involved in the RNA-silencing pathway. The TrAP protein of the related monopartite begomovirus. *Tomato yellow leaf curl China virus* (TYLCCNV) is also involved in suppression of RNA silencing [98], probably by activating transcription of host genes that control silencing [97]. The C4 protein of the monopartite begomoviruses ToLCV, *Ageratum yellow vein virus* (AYVV), and *Bhendi yellow vein mosaic virus* (BiYVMV) also have the ability to suppress RNA silencing [26, 84].

6. Mutagenesis of effectors encoded by monopartite begomoviruses

Little is known about gene function in monopartite begomoviruses. However, gene function has been studied extensively in other types of geminiviruses which share organization and nucleotide sequence similarities with TYLCV. Mutational analysis of few monopartite begomoviruses like TYLCV define similarities and differences between this single component geminivirus and bipartite geminiviruses in functions essential for systemic spread and infectivity [103]. The CP appeared to be required for systemic movement of TYLCV in *N. benthamiana* and tomato, consistent with those of mutation analyses obtained with other monopartite geminiviruses such as MSV, BCTV, and TLCV [5, 12, 69, 61] have shown that *Tomato yellow leaf curl virus*-Sardinia (TYLCV-Sar) C2 can form stable complexes with ssDNA (and less preferably with dsDNA) and that the binding is sequence nonspecific. AC2 of TYLCV have also been involved in the activation of other viral genes and was considered as a transcriptional activator [91]. However, transcription factors usually show high sequence specificity.

TYLCV ORF V1 truncated either 133 nt upstream or 19 nt downstream of the initiation codon of ORF V2 would altered the viral DNA forms, it suggested that the V1 protein may participate in the switch from dsDNA to ssDNA synthesis. Indeed, interaction between V1 and the CP has already been proposed, in view of the concerted evolution of these two protein sequences following a geo-graphical gradient of similarity [39], and the synergistic reduction in ssDNA levels of a TYLCV V1-V2 double mutant compared to single mutants [69]. Although TYLCV V1 mutants did not greatly overproduce dsDNA, the similarity of phenotype between BCTV V2 and TYLCV V1 mutants may indicated that the two corresponding gene products serve a related function. It has shown [69] (Table 1) has shown that disruption of the V1 gene in the monopartite Australian isolate of TLCV did not affect its ability to spread in tomato, although the infection was asymptomatic and the DNA levels reduced.

Virus	Accession number	Mutated ORF	Mutation Position	Type of mutation	References
TLCV-[AU]	AF084006	V1	N-terminal/ *Bam*HI	frameshift	Rigden et al., 1993
		V2	N-terminal/ *Bgl*II	frameshift	
		V/V2	N-terminal/*Bgl*II/ *Bam*HI	frameshift	
		V/V2	C-terminal/*Bgl*II/ *Bam*HI	deletion	
		V/V2	C-terminal/*Bam*HI/ *Bgl*II	inversion	
TLCV-[AU]	AF084006	C4	N-terminal (at 2457&2463)	deletion	Rigden et al., 1993
		C4	N-terminal (at 2457&2463)	revertion	
TYLCV-Sar[ES:Psp95:93]	Z25751	C4	C>T at 2432	point	Jupin et al., 1994
		C4	C>T at 2423		
TYLCV-Sar[ES:Psp95:93]	Z25751	C2	ΔCC2>31	deletion	Noris et al., 1996
		C2	ΔNC2>33	deletion	
		C2	ΔC2>33-104	deletion	
TYLCV-Sar[FR:98]	X61153	C2	1523+GATC	frameshift	Wartig et al.,1997
		V1	156+GATC	frameshift	
		V1	324C>T	stop	
		V2	748>CTAG	stop	
TYLCV-Sic[IT:pSic36:95]	Z28390	CP	H134	substitution	Noris et al., 1998
		RepC1	L198	substitution	
TYLCV-Sar[ES:Psp95:93]	Z25751	CP	Q134H	site-directed	Noris et al., 1998
TYLCV-DO[DO:99]	AF024715	CP	ΔNCP>180	deletion	Rojas et al., 2001
		CP	ΔCCP>150	deletion	
		V1	ΔNV1>63	deletion	
		V1	ΔCV1>84	deletion	
		C4	ΔNC4>54	deletion	
		C4	ΔCC4>60	deletion	
AYVV-[SG:pHN419:97]	X74516	C4	A>T at 2419 C4mut	substitution	Saunders et al., 2004

Virus	Accession number	Mutated ORF	Mutation Position	Type of mutation	References
PaLCuV-[PK:02]	AJ436992	V2	N-terminal (1-32)	deletion	Mubin et al., 2010
		V2	N-terminal (1-60)	deletion	
		V2	C-terminal (130-149)	deletion	
		V2	C-terminal (111-149)	deletion	
		V2	C-terminal (101-149)	deletion	
		V2	C-terminal (91-149)	deletion	
		V2	C-terminal (71-149)	deletion	
		V2	C-terminal (30-149)	deletion	
TYLCV-IL[IL:89]	X15656	CP	Lys-Thr CPmut3	substitution	Yaakov et al., 2011
		CP	Arg-Pro CPmut4	substitution	
		CP	Arg-Leu CPmut19	substitution	
TYLCV-Mld[ES:72:97]	AF071228	C4	C>G at 9 C4mut	substitution	Tomas et al., 2011
ToLCJV-A[ID]	AB100304	CP	CPΔ191-257	deletion	Sharma et al., 2009
		CP	CPΔ1-190	deletion	
		CP	CPΔ31-257	deletion	
		CP	CPΔ1-30/Δ50-257	deletion	
		CP	CPΔ1-30/Δ191-257	deletion	
		CP	CPΔ1-31	deletion	
		CP	CPΔ16-20	deletion	
		CP	CPΔ1-190/Δ245-250	deletion	
		CP	CPΔ1-30/Δ62-257	deletion	
ToLCJV-A[ID]	AB100304	V2	N-terminal (58aa)		Sharma et al., 2010
		V2	C-terminal (58aa)		
ToLCJV-A[ID]	AB100304	V2	pGEMV2ΔC		Sharma et al., 2011

Table 1. List of published studies reporting deletion mutants of monopartite begomoviruses

For example Noris et al. [62] suggested that the region of the CP between amino acids 129 and 134 is essential for both the correct assembly of virions and transmission by the insect vector. The genome of the SicRcv (infectious) had the same size as the original Sic DNA 9 (non-infectious) differed by only 2 nt. One change was at nt 2025 (A instead of T in the plus strand), determining a CAC-to-CUC codon change in the RepC1 mRNA and an H198L

replacement in the RepC1 protein. The other mutation located at nt 708 (C instead of G), determining a CAG-to-CAC codon change in the CP mRNA and a Q134H replacement in the CP. This indicated that the Q134H mutation changed a viral DNA, only capable of replicating in single cells (Sic), into one that was systemically infectious, but not insect transmissible (SicRcv). Comparative analysis of Sic, SicRcv, and the hybrid genomes and showed that the mutation in the CP gene, not in the Rep gene, was responsible for restoring infectivity in SicRcv; however, it still did not result in a whitefly-transmissible TYLCV. In TYLCV-Sar, the two capsid protein alterations resulted in the same either non-infectious or non-transmissible phenotype. Mutants containing the combinations QQ, QH, and PH at positions 129 and 134 were infectious in plants, whereas those with PQ are not. The PQ mutants can replicate and accumulate CP and V2 protein in leaf discs, but appear unable to produce virus particles. Mutants having the PH combination at positions 129 and 134 infect plants and form apparently normal virions, but are not transmissible by whiteflies. Changing the amino acid at position 152 (D or E) does not influence the phenotype. Requirement of the CP for infection has been demonstrated previously [62] suggested that accurate particle assembly is also necessary. In fact, the PQ mutants, which are unable to assemble virions, accumulate CP in leaf discs, showing that its expression and stability were not altered. Another TYLCV protein, V2, for which a role in virus assembly has recently been, suggested [103].

For example Rojas et al. [70] has shown that C4, V1, and CP gene may function in TYLCV-DO movement. The CP localized to nuclei and nucleoli and was found to act as a nuclear shuttle, mediating the import and export of DNA [70]. It was consistent with results obtained for the TYLCV CP in heterologous experimental systems [43, 68]. Recently, Liu et al [49] also showed the same behavior for the CP of the monopartite mastrevirus, MSV. TYLCV CP was found to accumulate in the nucleolus and the absence of the N-and C-terminal CP mutants from the nucleolus implicates CP motifs in this localization. As the nucleolus is the site of rRNA synthesis and packaging of ribosomal proteins, it may also serve as the site of geminiviral replication/gene expression [70]. The TYLCV C4 targeted to the cell periphery and/or cell wall, consistent with a role in cell-to-cell movement of viral DNA [65, 75, 101].

Disruption of the AYVV C4 ORF (A>T at position 2419nt) alters the phenotype in agroinoculated *N. benthamiana* from upward leaf roll and vein swelling to downward leaf curl [79] (Table 2). Previously,[88] also found the identical functions of BCTV C4 ORF mutant in this host. The AYVV C4 proteins may perform partially redundant functions involving convergent pathways and the behavior of ACMV AC4 and TYLCCNV C4 is consistent with such a function [98].

For example Stanley and Latham [58] have shown that V2 protein of *Papaya leaf curl virus* (PaLCuV) is potentially involved in the elicitation of cell death response. The deletion mutants (having deletions of 32 and 60 amino acids, respectively, at the N-terminal end of V2) exhibited a systemic HR in *Nicotiana benthamiana* plants. While C-terminal end deletions of 60 , 80 and 119 amino acids abolished the induction of HR, however 50 amino acids deletion

induced local necrosis, but not systemic. The mutants with 20 and 40 amino acids deletion produce HR both at the inoculated and in newly emerged leaves, although the systemic symptoms for the 40-amino-acid deletion mutant were delayed and were milder. The amino acid sequences between positions 92 and 101 are essential for the elicitation of HR, whereas those between 102 and 115 affect the timing and severity of the response. V2 of PaLCuV at amino acid positions 116 and 118 contains a conserved CxC. Mutations of this motif have been shown to abolish both the pathogenicity and suppressor of RNA silencing activities of the protein [64, 109]. Phosphorylation of MPs may also play a role in controlling the switch from viral replication to translation [36, 37]. Few earlier studies showed that for PaLCuV V2, deletion of sequences encompassing this motif abrogates the ability to induce HR [58].

The first 30 N-terminal amino acids of the TYLCV-IL CP are needed for nuclear import of the protein into the plant cell, suggesting the CP's involvement in nuclear shuttling of the virus genome [43]. This was confirmed by the finding of a strong interaction between the CP and the plant nuclear import receptor karyopherin α1 (Kap α1) [94]. The TYLCV CP has been found to inter act with itself (CP–CP or homotypic interaction) which may be important for capsule assembly as it is made up solely of CP units serving as building blocks. Mutations in the TYLCV-IL V1 gene coding for the TYLCV-IL CP by replacing Lys with Thr, Arg with Pro, and Arg with Leu, according to the positions of amino acids mutated [31]. TYLCV CP mutated failed to interact with the w.t. CP, while the w.t. protein showed strong homo typic interaction. As the CP has been suggested to be a shuttle protein for the viral genome into the plant cell nucleus [43, 70], its interaction with the nuclear-transport mediator Kap α1 is an important step and has been shown to occur at high affinity [94]. A mutation in the NLS domain, in particular at Arg19, disrupts the CP's interaction with proteins that are known to interact with the w.t. CP [106.]. Earlier Sharma et al. [113-115] demonstrated by the constructed a series of single and double deletions into the coding sequence of *Tomato leaf curl Java virus* ToLCJAV-A[ID] CP and found that, amino acids (aa)16KVRRR20 in the N-terminal region of CP functioned as nuclear/nucleolar localization signals (NLSs). Further, the region from aa 52RKPR55 contained basic amino acid cluster was capable to redirect the CP to the nucleus. Deletion mutant analysis revealed that this property was attributed to a nuclear export signal (NES) sequence consisted of aa (245LKIRIY250) reside at C-terminal part of CP. Additionally ToLCJV V2 is a target of host defense responses. Deletion of 58 amino acids (aa) from the N-terminus did not affect the HR, suggesting that this region has no role in the HR, while deletion of 58 aa from the C-terminus of V2 abolished both the HR response and V2 silencing suppressor activity, suggesting that these sequences are required for the HR-like response and suppression of PTGS. He also demonstrated that ToLCJV V2 is a pathogenicity determinant that elicits an HR-like response. Further deletion analysis that fusion of Nterminal part of the V2, containing the nuclear export signals (NES), directed the accumulation of fluorescence towards the cell cytoplasm. Also V2 enhances the coat protein-mediated nuclear export of ToLCJV and is consistent with the model in which V2 mediates viral DNA export from the nucleus to the plasmodesmata.

Satellite	Accession number	region (nt.)	Type of mutation	References
AYVB-[SG:pBS-beta:99]	AJ252072	AT>TA at 547/ 548 βC1mut1		Saunders et al., 2004
		G>T at 486 βC1mut2		
TYLCCNB-[CN:Y10:01]	AJ421621	ATG>ATC (CIM-F)	site-derected	Cui et al., 2004
		ACT>TGA (ACIM-S)	site-derected	
		ATG (2) >ATC (2) (CIM-B)	site-derected	
		GAA>TAG (CIM-T)	site-derected	
TYLCCNB-[CN:Y10:01]	AJ421621	742-952	deletation	Xiaorong et al., 2004
TYLCCNB-[CN:Y10:01]	AJ421621	ΔC1β	truncation	Qian and Zhou 2005
CLCuVβ-[PK:00]	AJ298903	195-484	site-derected	Saeed et al., 2005
		504-596	stop	
		586	frame-shift	
		ATG>ATA	stop	
BYVMB-[IN:Muth:01]	AJ308425	51-140 ΔNβC1	deletation	Kumar et al., 2006
		1-80 ΔCβC1	deletation	
TYLCV-satDNA-[AU:96]	U74627	Δnt 35-146 (112nt)	deletation	Li et al., 2007
		Δnt 146-296 (151nt)	deletation	
		Δnt 35-296 (262nt)	deletation	
		Δnt 296-420 (1251nt)	deletation	
		Δnt 296-492 (197nt)	deletation	
		Δnt 492-540 (49nt)	deletation	
		Δnt 540-641 (105nt)	deletation	
AYVJB-[ID:04]	AB162142	4>ATGtga	stop	Kont et al., 2007
AYVB-[SG:pBS-beta:99]	AJ252072	794-795	deletation	Saunders et al., 2008
		118-119	deletation	
		804-806	deletation	
		801-1047	deletation	
		1048-1051	deletation	
		1146-1147	deletation	
		1146-1150	deletation	
		1269-1271	deletation	
		1229-1234	deletation	
TbCSVB-[CN:Y35:01]	AJ420318	DNAΔC1β		Qian et al., 2008

Satellite	Accession number	region (nt.)	Type of mutation	References
CLCuMA-[PK:2:99]	AJ132345	915-1117	deletation	Shahid et al., 2009
CLCuMuB-[PK:09]	FJ607041	Δ150-840	deletation	Nawaz-ul-Rehman et al., 2009
		Δ1130-116	deletation	
		Δ995-1095	deletation	
CLCuMB-[PK:00]	AJ298903	ΔC1β	deletation	Kharazmi et al., 2012
TYLCCNB-[CN:Y10:01]	AJ421621	N-terminal (NTG)	deletation	Cheng et al., 2011
		C-terminal (CTG)	deletation	
		βC1-ΔCTD	deletation	

Table 2. List of published studies reporting deletion mutants of DNA Satellites

7. Mutational analysis of effectors encoded by satellites

Betasatellite molecules have been associated with numerous monopartite begomoviruses in China, including *Tobacco curly shoot virus* (TbCSV) and TYLCCNV that infect tomato and tobacco field plants [108, 47]. TbCSB is not essential for infection but increases symptoms in some hosts [17, 47]. However in case of TYLCCNB which is essential for symptom induction, The βC1 gene of TYLCCNB is required for symptom induction but not for the replication of betasatellite. Also a mutated βC1 deleted is stably maintained in few hosts by TYLCCNV [17, 67]. TbCSB with the complete βC1 deleted (ΔβC1) returns to a size comparable to that of the intact betasatellite in few systemically infected *N. glutinosa*, *N. tabacum Samsun* and *P. bybrida* plants plants. The levels of accumulation of the size revertant betasatellite were similar to those of ΔβC1 in same hosts (*N. benthamiana* and *N. glutinosa*) plants showing size reversion of the betasatellite developed viral symptoms similar to those induced by TbCSV and DNAΔβC1 [67]. A βC1 gene frame-shift mutant of TYLCCNVB was unable to induce disease symptoms and consequently, did not play a role in silencing suppression [67]. The complete coding region of Y10βC1 (TYLCCNB), followed by N- and C-terminal deletion mutants showed multimerization mediated by amino acids between positions 60 and 100 [13]. Karyopherin-α, a transport receptor involved in nuclear import were reported to interact with the C-terminal sequences of BYVMB- βC1 [42]. A myristoylation-like motif (GMDVNE) positioned at the C-terminal of CLCuMB-βC1 (103 to 108aa) interacted with a ubiquitin-conjugating enzyme involved in targeting proteins for degradation by the 26S proteasome [20, 78] identified sequences on AYVB by deletion mutagenesis required for trans-replication by AYVV. βC1 of Cotton leaf curl Multan betasatellite (CLCuMuB) has been shown to have possible virus movement function [74]. Generally, sequences between the *βC1* gene and the A-rich region are not essential for trans-replication by begomoviruses. Nevertheless, deletion of these sequences abolish the ability of the betasatellite to upregulate virus levels in plants and the symptoms expression [59]. For geminiviruses hairpin structure that contains the nonanucleotide sequence is an essential part of the virion sense origin of replication that is recognized and nicked (within

the nonanucleotide sequence) by Rep to initiate rolling-circle replication of the virion strand. Similarly, deletion of betasatellite sequences from 1130 to 116 that is conserved (between all betasatellites) stopped the betasatellite's ability to be trans-replicated and maintained by helper viruses both from OW (CLCuRaV) and New world and *Cabbage leaf curl virus* (CbLCuV). Trans-replication of CLCuMuB remained unaffected by deletion of the sequence between coordinates 995 and 1095 by CLCuRaV [59].

ToLCJAV alone can cause infection and displayed leaf curl symptoms. But, symptom expression of ToLCJAV in the presence of ToLCJAB is enhanced. In contrast, ToLCJAV and AYVB (mutated βC1) restored mild symptoms. It suggested that the βC1 protein was required for symptom induction and is a determinant of pathogenicity, βC1 protein expression in *N. benthamiana* plants and as a suppressor of PTGS [41].

For example Li et al. [47] have shown the deletion mutant of TYLCV sat-DNA (from 296-641nt) lacked the ability to replicate or replicated poorly by deleting of (region nt 35-296). Also sequence from nt 296-35 is to be essential for sat-DNA replication. The deletion of a 112 nt region downstream of the stem-loop from nt 35-146 and 151nt from 146-296 cannot effect on the replication of sat-DNA but reduced significantly. However, the deletion from nt 35-296 regions diminished sat-DNA replication these deletions loss of genomic sequences required for replication or due to changes in genome size. Heterologous non-viral DNA fragments can restore the wild-type 682 nt sat-DNA size and of replication when the replacement occurred in the region between nt 35 and 296. However, the sequence replacements in the region nt 35 to 296 of the sat-DNA improved the accumulation of sat-DNA considerably relative to the deleted constructs in this region. The sequence elements distributed within the entire sat-DNA molecule contribute to replication activity, but that sequence elements within the region from nt 35 to 296 are dispensable for replication.

For example Saeed et al. [72] used mutagenesis study of CLCuMB and tobacco was used as the host plant rather that cotton, the natural host of CLCuB. Few studies showed that it was symptomless when inoculated with *Cotton leaf cur Multan virus* (CLCuMV) alone but showed drastic symptoms when coinoculated with CLCuMB [9]. *Nicotiana benthamiana* showed a severe symptom on inoculated with CLCuMV with or without CLCuMB. Evidence for the involvement of the βC1 ORF in modulation of symptom expression also provided by [108] demonstrated few DNA β species associated with tomato and tobacco infecting begomoviruses and found that in-frame mutation of the βC1 initiation codon resulted in loss of symptom severity in *N. benthamiana*.

In recent studies Saunders et al. [79] have proved that disruption of the βC1 ORF prevented infection of the AYVB complex in ageratum and altered their phenotype in *N. benthamiana* to that produced by AYVV alone. For example Kumar et al. [42] tested the infectivity of two βC1 mutant constructs, first carrying a stop codon at amino acid position 41 and second with two stop codons at positions 9 and 41, and both resulted in loss of pathogenicity in tobacco plants on coinoculated with TLCV as helper virus. These mutation studies indicated that the βC1 ORF is involved in pathogenicity and that the expression of its N terminal 40 amino acids is not sufficient for its function.

Disruption of the βC1 ORF of AYVB by introducing an internal in-frame nonsense codon (G>T) did not prevent transreplication and systemic movement of the βC1 mutant by AYVV in lab host (*N.benthamiana*). The mutated βC1 removed the influence of the satellite on symptom development in this host and prevented symptomatic infection of ageratum. That suggested the βC1 protein is an important pathogenicity factor that plays an essential role in the proliferation of the AYVV-betasatellite complex in its real host. For example Saunders et al. [79] also shown that βC1 ORF initiation codon (AT) to a nonsense codon (TA) did not completely eliminate betasatellite activity. A similar mutation in the βC1 ORF of a satellite associated with TYLCCNB was shown previously [108]. The βC1 ORF encodes a pathogenicity determinant that suppressed a host defense mechanism [76].

For example [78] have demonstrated that the region of AYVB between the introduced nt 114 and 1047 sites is not required for betasatellite replication. This region includes the βC1 open reading frame (ORF), which encodes a gene essential for pathogenicity [79] and an A-rich region that may serve to maintain the size integrity of the satellite [76]. For example [78] found that the entire ORF is dispensable and is consistent with the findings of [67] for the betasatellite associated with TYLCCNV. In addition, removal of the A-rich region from TYLCCNB was tolerated, although the deletion mutant was associated with milder infection than those produced by the wild-type satellite [95]. In contrast, deletion of this region in AYVB did not affect the phenotype, at least in *N. benthamiana*. Maximum deletions within non coding regions of the begomovirus genome were not tolerated and the deletion mutants revert to wild type size by both intra-and intermolecular recombination during systemic movement [23, 25,]. For example Saunders et al. [78] also demonstrated removal of 361 nt of betasatellite representing 27% of the satellite and the region between nt 1047 and 1146 is important for betasatellite replication. It contained an inverted repeat flanking a sequence that is identical to the ToLCV iteron ToLCV sat-DNA [18]. Protein binding assays followed by mutagenesis have demonstrated that this motif in both ToLCV and sat-DNA represents a high affinity Rep binding site, although it is not required for replication of either the begomovirus or its satellite [48]. Saunders and associates [80] found that the region between nt 1146-1229 and sequences across the nt 1268 of AYVB are also required for replication.

SCR is highly conserved nature between distinct satellites [typically above 65% sequence identity with blocks of absolutely conserved sequence [7] strongly suggests that it also plays an important role in the virus replication cycle. In addition, the adjacent stem-loop and conserved nonanucleotide sequence would be expected to participate in replication. Approximately the 386 nt upstream of the stem–loop structure in ToLCV sat-DNA, as well as the stem–loop structure itself, are essential for replication [47].

βC1 is a multi-functional protein encoded by betasatellites that are associated with the majority of monopartite begomoviruses [11]. For example Cheng et al. [13] proved by deletion mutants of Y10βC1 that multimerization was mediated by amino acids between positions 60 and 100. Previous studies say that the C-terminal sequences of BYVMB-βC1 were interact with karyopherin α, a transport receptor involved in nuclear import [42]. A myristoylation-like motif (GMDVNE) located at the C-terminal of CLCuMB-βC1 (103-108aa)

interacted with a ubiquitin-conjugating enzyme involved in targeting proteins for degradation by the 26S proteasome [21]. It also seems to indicate interference with a functionality associated with the C terminus of Y10βC1. βC1 protein of AYVB, CLCuMB or BYVMB with GFP fused at the N-terminus also presented as granular spots in the cytoplasm and around the nucleus [42, 84].

TYLCCNB presumably has one or more *cis*-acting elements needed for replication and binds to TYLCCNV replication protein (Rep) for replication, and these elements are most probably located in the 115-nucleotide highly conserved region of betasatellite upstream of its stem-loop structure. Recently, Astroga [2] showed that a 5-bp core sequence (GGN1N2N3) is a typical constituent of Rep-binding iterons. Conserved GG motifs occur upstream of the115-nucleotide highly conserved region of betasatellite. One or more of these GG motifs, combined with the 115 nucleotide highly conserved region, possibley responsible for Rep binding to betasatellite. However, the Rep binding activity of the TLCV-sat from Australia seems much less specific: TLCV-sat contains an A-rich region but lacks a βC1 gene and is believed to be a defective betasatellite molecule [48]. The effect of mutation of the conserved βC1 gene of TYLCCNVB indicated that the βC1 protein plays a key role in symptom induction.

The position and size of the βC1 gene of the betasatellite molecules are conserved in all betasatellite molecules, and the mutation of the start codon of C1 gene in TYLCCNB showed that it's a pathogenicity determinant [108, 6]. Few studies has been also shown that the βC1 protein of betasatellite associated with TYLCCNV or AYVV is an essential pathogenicity determinant [17, 79], it may act as suppressors of post-transcriptional gene silencing that interfering the host defense system, thus, the presence of C1 protein facilitates efficient infection of the virus in hosts [102]. For example Tau and Zhou [96] showed that *βC1* gene was not required for the TYLCCNV and betasatellite replication and truncated betasatellite molecules with the deletion of the entire *βC1* gene were stable in infected plants. Defective DNAs, betasatellite and alphasatellite associated with begomoviruses are maintained at approximately half the size of the genomic components [83, 89, 55, 80, 77]. Some proofs have been displayed that geminiviruses CP can encapsidate circular ssDNA molecules of about half or quarter the size of the genomic DNA [18, 51]. Immunocapture PCR indicated that the truncated TYLCCNB of about 1 kb in length may be encapsidated with TYLCCNV coat protein in vivo [67].

βC1of BYVMB have a nuclear export or peripheral localization function and βC1 interacts with itself, also with CP and the tomato protein karyopherin α. Mutagenesis of βC1 protein showed that the domain of βC1 interacting with CP is at the N terminal half whereas the domain(s) of βC1interacting with itself and karyopherin α are at the C terminal half and the role of BYVMD βC1 as a suppressor of posttranscriptional gene silencing was explored [42]. Karyopherins are soluble transport receptors that interact with basic NLS sequences and help in nuclear import [27]. Full length betasatellite of CLCuMB can substitute for the movement function of the DNA B of a bipartite begomovirus *Tomato leaf cur New Dehli virus DNA-B* (ToLCNDV DNA-B). However, the betasatellite containing a disrupted βC1 ORF did not mobilize the DNA A for systemic infection, suggested that the βC1 protein was required for movement [74].

8. Potential of mutated satellites using a virus induced gene silencing vectors

8.1. Betasatellite

Betasatellites have about 200nt sequences (known as a-Rich region) conserved among all that indicating may be these sequences have some biological roles in satellites. The role of A-Rich sequence may be to increase the required size of the molecule that is essential for encapsidation or systemic movement by the coat protein or movement protein encoded by begomovirus. TYLCCNB-Y10 could be infectious and mutant betasatellite (deleted a-rich region) could be encapsidated in the coat protein encoded by DNA-A that suggested may be A-Rich region is not required for trans-replication of TYLCCNB but only for size maintaining [95]. For example [20] reported that only a small region of the nucleotide sequence of CLCuMB upstream of the start codon of βC1 (a 68-nt fragment), which contains a G-box, was important for βC1 promoter activity. In addition to βC1 ORF of CLCuMB delete a larger region (complete βC1) to make it a gene delivery vector for plants. It can potentially tolerate the insertion of larger foreign sequences without affecting promoter activity [38]. Putative promoter and TATA box are located upstream of the *βC1* gene. Thus, the *βC1* gene of betasatellite could be replaced by a foreign gene and be modified to convert it into an expression vector [17]. The modified betasatellite might be an candidate gene silencing vector to study functional genomics in plants [54]. Also leaf curl symptoms in Nicotiana species can be brought by transgenic expression of the *βC1* gene of TYLCCNB that the severity of the symptoms parallels the level of βC1 transcript in the transgenic plants and their ability to induce symptoms is abolished by mutation of the *βC1* gene. Possibly *βC1* gene of betasatellite may be replaced with a foreign gene and used as an expression vector for gene function analysis in plants [20].

Evidence has been shown that TYLCCNB modified by deletion of its *βC1* gene but retaining the βC1 promoter and terminator, can be turned into a gene silencing vector. Also, insertion in the vector with fragments of endogenous plant genes or a transgene, in either the sense or antisense direction, can result in effective silencing of the cognate gene in plants [95]. The size of the mutated satellite DNA molecule significantly influences replication efficiency. TLCV sat-DNA can be used as a potential gene expression/silencing vector [47].

8.2. Alphasatellite

Also alphasatellite is a small molecule and easy to manipulate and have a wide host range and can apparently be maintained by a large number of distinct Begomovirus species. It has some sequences (A-rich approx.200 nt.), similar to betasatellite which can, potentially, be removed and still it can replicate autonomous. The A-rich deleted sequences of CLCuMA can not affect its ability to replicate autonomously and move, in trans, by a helper begomovirus that provide a space suitable for insertion of foreign sequences to increase its capacity to accept and maintain foreign gene sequences [82] (Table 2). This ability to amplify itself is useful for construction of VIGS vectors it will increase the copy number (and thus

also expression) of inserted sequences [82]. Rolling-circle replication initiator protein of GmusSA and GDarSLA act as a strong suppressor of PTGS.

9. Conclusions

The monopartite begomovirus associated with DNA-satellites (Betasatellite and Alphasatellite) complex is in the norm throughout the Old World, particularly in South Asian countries. The epidemiology and evolution of this complex has been extensively analyzed since its first description. Monopartite begomovirus encoded all the genes needed to cause a successful infection. Many of these genes are coding for multifunctional proteins, adding another level of complexity in their interaction with host proteins, and their de novo creation. This shows the ability of begomoviruses and their associated satellites to rapidly evolve in response to selection pressures such as host plant resistance.

Author details

Muhammad Shafiq Shahid and Masato Ikegami
NODAI Research Institute, Tokyo University of Agriculture, Tokyo, Japan

Pradeep Sharma *
Division of Crop Improvement, Directorate of Wheat Research, Karnal, India

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* Corresponding Author

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