Suppressors of RNA silencing encoded by tomato leaf curl betasatellites

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Virus encoded RNA-silencing suppressors (RSSs) are the key components evolved by the viruses to counter RNA-silencing defense of plants. Whitefly-transmitted begomoviruses infecting tomato crop code for five different proteins, ORF AC4, ORF AC2 and ORF AV2 in DNA-A component, ORF BV1 in DNA-B and ORF βC1 in satellite DNA β which are predicted to function as silencing suppressors. In the present study suppressor function of ORF βC1 of three betasatellites *Tomato leaf curl Bangalore betasatellite* ToLCBB-[IN:Hess:08], *Cotton leaf curl Multan betasatellite* CLCuMB–[IN:Sri:02] and *Luffa leaf distortion betasatellite* LuLDB-[IN:Lu:04] were examined. Agroinfiltration of GFP-silenced *Nicotiana tabaccum cv.* Xanthi with the cells expressing βC1 protein resulted in reversal of silenced GFP expression. GFP-siRNA level was more than 50-fold lower compared to silenced plants in plants infiltrated with βC1 gene from ToLCBB. However, in the case of 35S-βC1 CLCuMB and 35S-βC1 LuLDB construct, although GFP was expressed, siRNA level was not reduced, indicating that the step at which βC1 interfere in RNA-silencing pathway is different.

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1. Introduction

RNA silencing is an innate antiviral defense system in the plants which involves sequence-specific degradation of viral RNA. In plants double-stranded replicative intermediates of RNA viruses or overlapping structured segments of mRNA transcripts of DNA viruses are the targets, which are processed by DCL enzymes into short 21 nt dsRNA having 2-nt 3′ overhangs called viral siRNAs (Voinnet 2001). These siRNAs bind to a nuclease complex known as RNA-induced silencing complex (RISC), guiding the complex to bind and destroy homologous transcripts (Hammond *et al.* 2000). Majority of the plant viruses have evolved suppressor proteins to evade this defense mechanism of the plant (Voinnet *et al.* 1999; Roth *et al.* 2004). RNA-silencing suppressors from different plant viruses are structurally different. Many suppressors function as the pathogenicity factors that cause developmental abnormalities (Cui *et al*. 2004; Lindbo and Dougherty 2005). Viral suppressors interfere at distinct steps of silencing machinery. Begomoviruses produce three different suppressor proteins encoded by ORF AC4, ORF AC2 and ORF βC1. ORF AC2, which encodes the transcriptional activator protein (TrAP) of *African cassava mosaic virus*-[Kenya], *Tomato golden mosaic virus* and *Mungbean yellow mosaic virus*-[Vigna], C2, a positional analogue of ORF AC2 in *Tomato leaf curl virus*-[Australia] and βC1 of *Tomato yellow leaf curl China virus* (TYLCCNV) exhibit sequence non-specific DNA binding activity and are confined to nucleus (Vanitharani *et al*. 2004; Cui *et al.* 2005; Trinks *et al.* 2005). For the monopartite begomoviruses, the V2 protein of TYLCV-IL has been identified as an RNA-silencing suppressor and may exert its suppressor effect by targeting a step in the RNA-silencing pathway that occurs after siRNA production (Zrachya *et al.* 2007).

Geminiviruses (family *Geminiviridae*) are a diverse group of plant viruses with circular single-stranded DNA genomes that are composed of one or two components of 2700–3000 bp length which are encapsidated within twinned icosahedral particle of size 20×30 nm. Most of the monopartite begomoviruses are unable to induce typical symptoms and require betasatellite molecules associated with them to induce typical symptoms in plants. Betasatellites are single-stranded

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DNA satellites often required by the helper virus to induce severe disease symptom in the host (Briddon *et al.* 2003). They are approximately 1350 nt in length and share no significant sequence homology with their helper viruses, except a potential stem–loop structure which contains the ubiquitous nonanucleotide sequence TAATATT↓AC that marks the origin of virion strand DNA replication. All the betasatellites characterized so far have three highly conserved features: A sequence of approximately 100 nucleotides conserved between all DNA betasatellites (known as the satellite conserved region [SCR], a region of sequence rich in adenine (A-rich) and a positionally conserved gene, on the complementary sense strand which is known as βC1 protein. The βC1 protein is shown to up-regulate viral DNA levels *in planta*, binds DNA and is supposed to be involved in virus movement (Saeed *et al.* 2007). βC1 is a positionally conserved open reading frame which is known as major symptom modulating component and is demonstrated to function as the suppressor of RNA silencing (Cui *et al.* 2005). Nuclear localized βC1 protein appears to affect the silencing pathway similar to that of AC2 at the initiation step (Cui *et al*. 2005) We demonstrated previously that inoculation of ToLCBaV with cognate betasatellite ToLCBB and non-cognate betasatellites CLCuMB and LuLDB in *N. benthamiana* and tomato resulted in severe symptoms such as downward leaf curling, yellowing and leaf crinkling (Tiwari *et al.* 2010). In this study we use *Agrobacterium*-mediated transient expression system to establish the suppressor role of βC1 protein encoded by three different betasatellites associated with tomato leaf curl viruses.

2. Materials and methods

2.1 *Preparation of 35S-βC1 constructs*

Three different betasatellites were chosen to study the suppressor activity. They are *Tomato leaf curl Bangalore betasatellite* ToLCBB-[IN:Hess:08] (GU984046), *Cotton leaf curl Multan betasatellite,* CLCuMB–[IN:Sri:02] (AY083590), and *Luffa leaf distortion betasatellite,* LuLDB [IN:Lu:04] (AY728262), associated with *Tomato leaf curl Bangalore virus* and *Tomato leaf curl New Delhi virus* respectively. βC1 gene of these three betasatellites were amplified with specific set of primers (table 1) with a *BamH*I site at the 5′ end of forward primer and *Sac*I site at the 5′ end of reverse primer. Amplified products were gel eluted with Qiagen gel extraction kit and were restricted with *BamH*I and *Sac*I restriction enzymes and further purified with phenol:chlorophorm:isoamyl alcohol (25:24:1) and ethanol precipitated. The vector pBI121 was also restricted and purified in the same way and ligated with *BamH*I-*Sac*I-restricted βC1 PCR amplicon. Transformants were confirmed by restriction digestion with respective enzymes and all the constructs are mobilized into *Agrobacterium tumefaciens* strain EHA105. MYMIV-AC2, which has been well established as silencing suppressor, was also prepared the same way as βC1constructs and used as a positive control.

2.2 *Preparation of agrobacterium culture for infiltration*

Suppressor activity of βC1 constructs were analysed by inoculating GFP-silenced transgenic *N. tabaccum* cv. Xanthi plants. Seeds of these plants were a kind gift from Dr SK Mukherjee (ICGEB, New Delhi). Three-week-old plants of *N. tabaccum* cv. Xanthi, where the GFP gene is integrated but silent in expression due to posttranscriptional gene silencing (Karjee *et al*. 2008), were used for agroinfiltration study. All the βC1 constructs were mobilized into *Agrobacterium tumefaciens* strain EHA 105. Empty vector pBI121 without any βC1 construct was used as negative control. All the βC1 constructs and MYMIV-AC2 were inoculated into 2 mL Luria Bertani (LB) broth containing rifampicin (50 mg/L) and kanamycin (50 mg/L) and incubated at 28°C for log phase; the next day 2 mL of the primary inoculum was used to inoculate 50 mL of LB broth and grown at 28°C at 200 rpm for 2 days. Culture was pelletized by centrifugation and the pellet was suspended in MES buffer (*N*-morpholino ethane sulphonic acid), pH 7.5. The OD600 was adjusted to 0.8. βC1 constructs were infiltrated onto the underside of leaves by using an 2 mL plastic syringe. GFP expression was regularly observed by using handheld UV light or UV transilluminator. Leaves of GFP-silenced *N. tabaccum* cv. Xanthi plants fluoresce red due to the presence of chlorophyll, while the leaves infiltrated with suppressor construct will fluoresce green. The suppressor role was further investigated by siRNA analysis.

2.3 *Total RNA isolation and Northern blot analysis*

One week post-infiltration, total RNA was isolated from the treated plants. RNA from infiltrated spots was extracted by grinding leaf tissue in liquid nitrogen and suspending the frozen powder in Trizol reagent. Finally, the pellet was dissolved in DEPC-treated water. The concentration of RNA was estimated by nanodrop spectrophotometer. To analyse the transcript level, about 20–30 μg of total RNA isolated by Trizol (Invitrogen, Carlsbad, CA, USA) was resolved by electrophoresis in 1.2% agarose formaldehyde gel. RNA from the gel was transferred to Hybond N+ membrane (Millipore) and subjected to UV cross-linking (120J). Blots were prehybridized in solution containing 50% Formamide (v/v), 10× Denhardt's solution; 0.5 mg/mL sheared salmon sperm DNA, 1% SDS (w/v), 3X SSC and 50 mM phosphate buffer at 42°C for at least 4 h. The GFP gene

Table 1. Primer sequences used for 35S-βC1construct preparation

Primer name	Primer Sequence	Anticipated size
CLCuMBBF	5′CAGGATCCATGACAACGAGCGG3′	350 bp
CLCuMBBR	5′TAGAGCTCTTAAACGGTGAAC3′	
ToLCBBF	5′CAGGATCCATGACAATAAAG3′	350 bp
ToLCBBR	5′TAGAGCTCTCACACACACAC3′	
LuLDBBF	5′TAGGATCCATGGTCACTCACAACC3′	400 bp
LuLDBBR	5′ AAGAGCTCTTAAACGGTGAACTTC 3′	

Restriction sites of *BamH*I and *Sac*I are underlined.

(~750 bp) from the vector pAVA319 was released by restriction digestion with *Nco*I and *Bgl*II sites and used to prepare GFP-specific radioactive DNA probe by random primer labeling method (Feinberg and Vogelstein 1983). Hybridization of blot was performed overnight in a rotating incubator. After 19 h, washing of blots were performed four times in 2X SSC, 2% SDS for 15 min each time at room temperature and then twice with 1X SSC, 0.2% SDS at 65°C for 25 min each time. The radioactivity on the blots was quantified by phophorimaging using Cyclone Plus Storage Phosphor System (Perkin Elmer). Blots were then exposed to X-ray films.

2.4 *siRNA level of GFP*

Samples were collected 8 days post-infiltration for siRNA analysis. Total RNA was isolated by Trizol method. Further enrichment of siRNA was done by using mirVana™ miRNA isolation kit (Ambion). siRNA concentration was estimated and ~40–50 µg of RNA was resolved on 15% denaturing urea PAGE gel. Gel was pre-run before loading at 150 V for 15 min in 1× TBE buffer (45 mM Tris borate, pH 8.0, 1 mM EDTA) followed by electrophoresis at 25 V/cm. Equal volume of sample buffer (100 µL formamide, 1 µL 0.5 M EDTA, 1 µL 100X Bromo Phenol Blue (BPB)) was added and heated for 1 min at ~95°C. The mixture was further chilled on ice and spun briefly before loading onto the gel. The gel was run and stopped when BPB was 1–2 cm from the bottom of the gel frame. RNA fragments in the gel were transferred onto membrane by using BIORAD Trans-blot semi-dry transfer cell. Prehybridization of the blot was performed in a prehybridization buffer (50% formaldehyde (v/v), 50× Denhardt's solution, 0.5 mg/mL sheared salmon sperm DNA, 1% SDS (w/v), 3X SSC and 50 mM phosphate buffer) for 4 h at 42°C followed by hybridization with GFP-specific probe generated by random primer labeling method for 18–19 h. Washing of blots were performed four times in 2X SSC, 2% SDS 15 min each time at room temperature. Radioactivity on the blots was quantified by phosphorimager (Perkin Elmer). Blots were then exposed to X-ray film.

2.5 *Effect of suppressor on helper virus accumulation*

The effect of suppressors on helper viral DNA accumulation was assessed by co-inoculating βC1 constructs with partial tandem repeat constructs of *Tomato leaf curl Bangalore virus* (GU474418), *Cotton leaf curl Rajasthan virus* (NC-003199), *Mungbean yellow mosaic virus* (AJ439057) and *Mungbean yellow mosaic India virus* (AF126406). To make partial tandem repeat constructs for agroinoculation 1.1, 1.8 kb fragments of ToLCBaV, MYMIV and MYMV DNA A were restricted by *Bam*HI/*Sac*I and *Kpn*I/*Hind*III enzymes respectively and the purified fragments were cloned in the binary vector pBIN19 to give pBIN0.4mer/pBIN0.8mer. To these clones full-length 2.7 kb DNA A fragments were ligated as *Bam*HI or *Kpn*1 fragments to give pBIN1.4mer of ToLCBaV or 1.8mer of MYMV and MYMIV. The details are as given in Tiwari *et al.* (2010).

The *N. benthamiana* plants were agroinfiltrated with partial tandem repeat (PTR) and βC1 suppressors of three betasatellites (ToLCBB, CLCuMB and LuLDB) separately. Six plants were infiltrated with *Agrobacterium* cells having helper virus alone and six plants along with βC1 constructs. Plants were maintained at 28°C. The leaves were detached 3 days post-infiltration for the isolation of viral genomic DNA. Total DNA was isolated by GEMCTAB method (Rouhibakhsh *et al.* 2008). Approximately 5 µg of genomic DNA was separated in 1.2% agarose gel and DNA in the gel was transferred to a membrane by capillary method. The membrane was baked at 80°C for 2 h in vacuum. The baked nitrocellulose membrane blots were kept in hybridization cylinders. Pre-hybridization solution was added at the rate of 0.2 mL/cm^2. The cylinders were then incubated at 65°C for 4 h in a hybridization oven with gentle rotation. Full-length genomic probe of ToLCBaV was prepared with (α-^{32}P) dCTP by random priming method (Feinberg and Vogelstein 1983).

3. Results

3.1 *Reversal of GFP expression*

This assay was based particularly on suppression of RNA silencing that helps in expression of silenced reporter gene GFP. In this experiment we used GFP-silenced transgenic tobacco plants cv. Xanthi, where the GFP gene is chromosomally integrated but silent in expression due to PTGS (Karjee *et al.* 2008). GFP expression was seen in leaf lamina adjacent to the spot of agroinfiltration in all the leaves agroinfiltrated with βC1 and AC2 constructs. Intensity of fluorescence was found to be more with βC1 construct of CLCuMB and ToLCBB; it was comparable to the fluorescence shown by MYMIV-AC2 construct (figure 1). Leaves injected with vector alone exhibited only red colour. The experiment was repeated on 5 to 10 plants three times (table 2). Results clearly showed that βC1 is an efficient suppressor of established RNA silencing.

To confirm the suppressor activity of βC1, in silenced *N. tabaccum* cv. Xanthi plants, GFP transcript analysis was performed. In these experiments, leaves were inoculated with a modified pBI121 vector having GFP gene under 35S promoter to serve as positive control for detection of GFP mRNA. From figure 2 it is evident that GFP mRNA level has been restored in the plants inoculated with βC1 construct. Compared to mRNA level in un-inoculated silenced plants (figure 2, lane 5), the levels in 35S-GFP, 35S-βC1 from ToLCBB and LuLDB and MYMIV-AC2 are ~80-fold higher. Contrastingly, in mRNA level in the leaves inoculated with CLCuMB-βC1 construct, there was only a marginal increase in mRNA level. The results indicate that CLCuMB-βC1 did not restore mRNA level and may interfere with RNAi pathway at different step.

Northern blot analysis of siRNA revealed interesting results. siRNA levels in the leaves inoculated with ToLCBB-βC1 and MYMIV-AC2 showed considerable reduction. This corroborates well with higher level of GFP-mRNA. Between ToLCBB-βC1 and MYMIV-AC2, reduction in siRNA level is more pronounced in MYMIV-AC2. However, siRNA levels in CLCuMB-βC1 and LuLDB-βC1 are not affected and remain the same as in empty vector inoculated control. The size of siRNA molecules is slightly larger in CLCuMB-βC1 inoculated leaves (figure 3).

Table 2. Number of plants showing reversal of GFP expression in *Nicotiana tabacum* cv. *xanthi* plants after agroinfiltration with βC1 suppressors

	No. of plants showing GFP expression/No. of plants infiltrated				
Experiment	CLCuMB	ToLCBB	LuLDB	MYMIV-AC2	pBI121
Experiment 1	3/3	3/3	3/3	3/3	0/3
Experiment 2	5/7	6/9	7/9	8/10	0/10
Experiment 3	4/5	3/5	5/5	4/5	0/5

The effect of suppressor protein on the level of helper virus DNA was monitored by Southern blot analysis. Plants were infiltrated with suppressor construct of βC1 along with PTR of ToLCBaV (figure 4). ToLCBaV-DNA-A-alone-inoculated plants showed considerable level of replication (figure 4, lanes 4 and 5).There was ~4-fold increase in helper viral DNA level in plants inoculated with cognate betasatellites, ToLCBaV/ToLCBB. No such increase in helper viral accumulation was observed with non-cognate betasatellite combination (ToLCBaV/CLCuMB). In the case of ToLCBaV, the presence of βC1 of cognate betasatellites ToLCBB led to ~4- to 6-fold higher helper viral DNA replicative forms.

4. Discussion

Diseases caused by whitefly-transmitted begomoviruses have emerged as a challenge in tropical and subtropical countries. Increase in incidence and severity are mainly attributed to the virus genetic propensity to evolve through recombination and through mechanism of component capturing. In recent years, majority of Old World begomoviruses are associated with two types of satellites, alpha- and betasatellites, of which betasatellites are the major pathogenicity determinants. The role of betasatellites in extending host range, DNA accumulation and contributing to symptom severity is not well established in several begomoviruses–

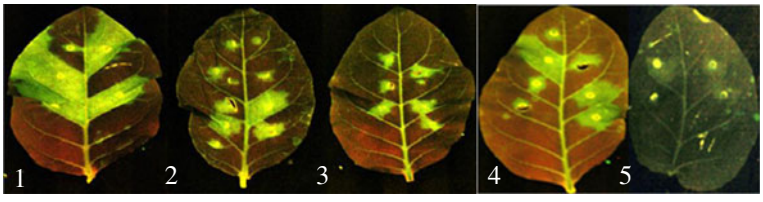

Figure 1. Reversal of GFP expression in *N.tabacum* cv. *xanthi* plants infiltrated with 35S-βC1 of (1) CLCuMB, (2) ToLCBB, (3) LuLDB, (4) ORF-AC2 of *Mungbean yellow mosaic India virus* and (5) pBI121 empty vector. Green fluorescence images of infiltrated leaves were taken 6 dpi under a long-wave UV lamp.

Figure 2. Northern blot analysis of GFP mRNA isolated from plants agroinfiltrated with 35S-βC1 constructs. Lane 1, CLCuMB; lane 2,ToLCBB; lane 3, LuLDB; lane 4, 35S-AC2 from MYMIV; lane 5, uninoculated GFP silenced *N.tabacum* cv. *xanthi* plant; lane 6, 35S-GFP in pBI121 vector; lane 7, positive control (GFP gene). Radiolabelled (α^{32}-P) GFP gene used as probe.

Figure 3. Northern blot analysis of siRNA isolated from plants agroinfiltrated with 35S-βC1 constructs 7 dpi. Lane 1, CLCuMB; lane 2, ToLCBB; lane 3, LuLDB; lane 4, empty pBI121 vector; lane 5, 35S-AC2 from MYMIV; lane 6, positive control (GFP gene). Radiolabelled (α^{32}-P) dCTP GFP gene used as probe.

betasatellites combinations. In contrast to RNA viruses, where association of satellite RNA leads to attenuation of symptoms, in begomoviruses, betasatellite is an essential component to induce typical symptoms in the primary hosts. The betasatellite molecules associated with Old World begomoviruses encode a multifunctional βC1 protein in the complementary strand. The βC1 protein induces typical symptoms, vein clearing, leaf curl, enation (Briddon *et al.* 2003; Jose and Usha 2003; Gopal *et al.* 2007) and mediates cell-to-cell movement (Saeed *et al.* 2007) and interferes with host defense pathways. Thus, it is a vital pathogenicity determinant considered as key molecule in deciding the host–virus interaction.

In plants RNA silencing is a natural defense mechanism against the virus infection and viruses encode suppressor proteins which interfere at different steps of RNAi defense pathway (Llave *et al.* 2000; Anandlakshmi *et al.* 2004; Lindbo and Dougherty 2005; Bisaro 2006). Most of the viral-encoded pathogenicity determinants are suppressors of PTGS, and mutation of viral suppressor's protein impair the viral pathogenicity (Voinnet *et al.* 1999). In the present communication the suppressor role of three betasatellites, which are associated with tomato leaf curl begomoviruses, was established by reversal of GFP silencing assay. Begomoviruses have been shown to encode three types of suppressor proteins, C2/AC2, transcription activator protein, TrAP (Moissiard and Voinnet 2004), C4/AC4 (Vanitharani

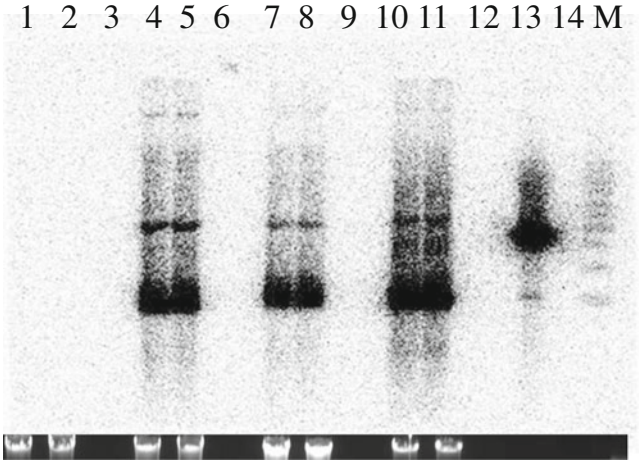

Figure 4. Southern blot analysis of DNA isolated from plants agroinfiltrated with ToLCBaV and 35S-βC1 construct. Genomic DNA was separated on a 1.2% gel and probed with full length viral genome. Lanes 1 and 2, healthy; lanes 4 and 5, ToLCBaV full-length genome; lanes 7 and 8, ToLCBaV+CLCuMB; lanes 10 and 11, ToLCBaV+ToLCBB; lane 13, 2.7 Kb linearized DNA of ToLCBaV-C[IN:Hess:08] was loaded as the positive control; blots were probed with (α^{32}-P) dCTP full-length genome of ToLCBaV; lane 14, marker.

et al. 2004; Praveen and Satendra 2006) and V2/AV2 (Zrachya *et al.* 2007). These suppressors are encoded in the helper viral genome. In the cases where the monopartite viruses are associated with betasatellites, an additional suppressor protein βC1 also is involved in viral pathogenicity. Cui *et al.* (2005) had shown that TYLCCV βC1 binds non-specifically to DNA, suppress the PTGS and is targeted to cell nucleus. Similarly, suppressor activity of βC1 protein of *Bhendi yellow vein mosaic betasatellite* betasatellite (Gopal *et al.* 2007) and *Tomato leaf curl Java betasatellite* associated with *Ageratum yellow vein virus-Indonesia* (AYVV-ID) (Kon *et al*. 2007) have been demonstrated as the suppressors of RNA silencing. In the present study we examined the suppressor activity of βC1 protein of three betasatellites associated with tomato leaf curl viruses. The three betasatellite when inoculated with ToLCBaV enhanced symptom severity and led to viral DNA accumulation (Tiwari *et al.* 2010). Reversal of GFP expression in silenced *N. tabaccum* cv. Xanthi plants clearly showed that the βC1 protein encoded by them can interfere with established (long term) silencing similar to βC1 protein of Y10β and Y35β associated with TYLCCNV-Y10 and TbCSV-Y35 (Cui *et al.*2005) respectively. There were differences in efficiency between ToLCBB, CLCuMB and LuLDB. ToLCBB-βC1 was more efficient than βC1 of other betasatellites in restoration of mRNA levels.

To understand the step at which these betasatellites may interfere with RNAi, we examined the GFP mRNA and GFP siRNA levels. In the case of βC1 gene from ToLCBB, the GFP mRNA level improved dramatically (comparable to 35S-GFP) and the siRNA level was reduced. In this way it was similar to MYMIV-AC2, although not on par with it. These two suppressors may target the step involving mRNA degradation into siRNA. On the contrary, mRNA and siRNA levels were not affected in the leaves of silenced plants inoculated by CLCuMB-βC1 and LuLDB-βC1. In this regard they may function in a way similar to TEV Hc-Pro (Burgyan and Havelda 2011) and V2 of TYLC V-IL. These suppressors may interfere with maintenance of PTGS by binding to siRNA, thus preventing siRNA–RISC assembly. Whether the βC1 protein shows siRNA binding property needs to be investigated. The diversity in βC1 sequence among the betasatellites suggests that each βC1 molecule may differ functionally targeting different step in RNAi. The step at which the suppressor interferes depends on the subcellular localization of protein. βC1 encoded by Y10β and *Tomato leaf curl Java betasatellite* have been shown to be localized in the nucleus (Cui *et al.* 2005; Sharma *et al.* 2010). A mutant version of these two βC1 proteins failed to induce disease symptoms and suppress RNA silencing, clearly indicating that nuclear localization of βC1 protein is definitely required. It will be interesting to study subcellular localization of CLCuMB-βC1 and ToLCBB-βC1 in this regard.

The RNAi-silencing pathway in due course affects viral genome replication by targeting the essential viral transcripts vital for replication of viral genome itself. In the case of begomoviruses, transcripts produced in the host cell may trigger RNA silencing affecting viral DNA genome replication (Bisaro 2006). Any potent viral suppressor by interfering with silencing shows enhanced replication. This principle of enhanced viral DNA replication in the presence of the potential suppressor was tested by co-inoculating the PTR constructs of ToLCBaV and CLCuRaV with βC1 construct of cognate/non-cognate betasatellites. Enhanced replication was seen in co-inoculation with the cognate betasatellites compared to DNA-A-alone-inoculated leaves. The exact mechanism by which the βC1 application leads to enhanced viral DNA accumulation in begomoviruses is yet to be understood. Briddon and Stanley (2006) suggested that βC1 expression may re-programme the infected cells to provide conditions more suitable for begomovirus replication. It is necessary to study the different mechanism by which βC1 interferes with RNA silencing machinery of the host. The possibility of using these suppressor proteins to enhance gene expression will be another fruitful course of investigation.

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