



Interaction between β C1 of satellite and coat protein of *Chili leaf curl virus* plays a crucial role in suppression of host RNA silencing

Anurag Kumar Sahu¹ · Neeti Sanan-Mishra¹

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Abstract

The monopartite *Chili leaf curl virus* (ChiLCV) and its β -satellite (ChiLCB) have been found to co-exist in infected plants. The ability of β C1 protein to suppress RNA silencing was investigated using an in-house developed in-planta reversal of silencing assay, using *Nicotiana tabacum* lines harboring green fluorescent protein (GFP) silenced by short hairpin GFP (_{sh}GFP). Transient expression of recombinant β C1 complemented and increased the suppressor activity of ChiLCV coat protein (CP), and this was confirmed by molecular analysis. In silico analysis followed by a yeast two-hybrid screen-identified ChiLCV-CP as the interacting partner of the ChiLCB- β C1 protein. Subcellular localization through confocal analysis revealed that when β C1 and ChiLCV-CP were co-present, the fluorescence was localized in the cytoplasm indicating that nuclear localization of both proteins was obstructed. The cytoplasmic compartmentalization of the two viral suppressors of RNA silencing may be responsible for the enhanced suppression of the host gene silencing. This study presents evidence on the interaction of ChiLCV-CP and β C1 proteins and indicates that ChiLCB may support the ChiLCV in overcoming host gene silencing to cause Chili leaf curl disease.

Key points

- CP of ChiLCV and β C1 of ChiLCB contain RNA silencing suppression activity
- The RNA silencing suppression activity of ChiLCB- β C1 complements that of ChiLCV-CP
- There is a direct interaction between ChiLCB- β C1 and ChiLCV-CP

Keywords RNA silencing suppressor · β -satellite · Yeast two-hybrid · Confocal analysis · *Chili leaf curl virus*

Introduction

Eukaryotic gene expression can be downregulated by the expression of foreign or exogenously introduced homologous sequences, as the events underlying homology-dependent gene silencing are part of the universal RNA silencing system (Rocha et al. 2005). RNA silencing is a remarkable post-transcriptional process, wherein large RNA molecules are recognized and degraded by small RNAs in a sequence-specific manner (Meins 2000; Sanan-Mishra et al. 2021; Sijen and Kooter 2000; Sinha et al. 2017). The post-transcriptional

gene silencing (PTGS) mechanism also works as a first line of defense against invading viral pathogens by recognizing and cleaving their transcripts. The characteristic feature of virus-induced gene silencing is the production of 21–24 nucleotide small interfering RNAs (siRNAs) from large viral double-stranded RNAs (Aregger et al. 2012).

To counteract RNA silencing, viruses have evolved to acquire the ability to suppress it (Csorba et al. 2015; Sanan-Mishra et al. 2017). The viral suppressors of RNA silencing (VSRs) acquired by plant viruses constitute a strong weapon in the arms race between plant and invading viruses. Most of the VSRs were originally identified as pathogenicity determinants as they are required for efficient spread of the virus in the host (Pumplin and Voinnet 2013). Therefore, the identification and functional analysis of VSRs may offer the key to comprehend the mechanism of viral infection, determination of host range and, degree of virulence. VSRs are profoundly disparate, and they can interfere with the intracellular and/or intercellular host silencing machinery

✉ Neeti Sanan-Mishra
neeti@icgeb.res.in
Anurag Kumar Sahu
anuragsahhu@gmail.com

¹ Plant RNAi Biology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India

by inhibiting the production of siRNAs or preventing their incorporation into RNA-induced silencing complex (RISC) (Samuel et al. 2016). The VSR activity has been found to vary between homologous proteins encoded by viruses of the same genus (Mangwende et al. 2009; Sundaresan et al. 2020) or isolates of the same virus species (Marques et al. 2012).

Chili leaf curl virus (ChiLCV) is a monopartite geminivirus (family *Geminiviridae*, genus *Begomovirus*), comprising a circular single-stranded DNA (ssDNA) of 2750 nt. It replicates in the nuclei of infected cells (Jeske 2009). The ssDNA genome of the geminivirus is converted into double-stranded DNA (dsDNA) by host proteins, and it is further chromatinized by association of host histones to form viral mini chromosomes (Gnanasekaran et al. 2019). ChiLCV encodes two open reading frames (ORFs), on the sense strand denoted as AV1 (CP: coat protein) and AV2 (pre-coat protein) and four ORFs, on the complementary sense strand denoted as AC1 (Rep: replication initiator protein), AC2 (TrAp: transcriptional activator protein), AC3 (REn: replication enhancer protein), and AC4 (Chattopadhyay et al. 2008; Sahu and Mishra 2021).

ChiLCV has been found to be associated with a β -satellite (ChiLCB) DNA of 1360 nt. It is approximately half the size of the begomoviral genome and requires help of ChiLCV for replication and insect transmission. It transcribes to produce two proteins, β C1, which acts as a pathogenicity determinant and β V1, which has been recently reported and may have a role in symptoms induction (Hu et al. 2020). β C1 has been shown to suppress cytoplasmic PTGS that likely targets viral transcripts as host defense (Yang et al. 2011a). A mutant

(Y10 β) associated with *Tomato yellow leaf curl China virus* (TYLCCNV), carrying a mutation in the nuclear localizing sequence (NLS) of β C1, failed to induce symptoms, suppress RNA silencing, and accumulate in the nucleus. This indicated that nuclear localization of the β C1 protein is necessary for silencing suppression (Cui et al. 2005).

In this study, we describe the generation of GFP-silenced transgenic tobacco plants by co-expressing GFP and short hairpin GFP (S_h GFP) constructs. The stably silenced lines were utilized for identification of VSR activity encoded by ChiLCV-CP and ChiLCB- β C1. The study indicated synergy of ChiLCV-CP and ChiLCB- β C1 in host silencing suppression. The interaction of the two proteins was confirmed by in silico and yeast two-hybrid (Y2H) analysis. Localization studies were performed to understand the cellular compartmentation of ChiLCV-CP and ChiLCB- β C1.

Methods

DNA isolation and cloning

Twenty chili samples showing typical leaf curl symptoms were photographed and collected from New Delhi (India) from 2016 to 2017. Total DNA was isolated from the infected leaves using the method described earlier (Xie et al. 2002). Primer pairs, β 01_{Fwd} and β 02_{Rev} and β C1_{Fwd} and β C1_{Rev} (Table 1) were used to amplify full-length β -satellite DNA and the β C1 gene, respectively. PCR master mix contained 2 μ l DNA template (100 ng/ μ l), 2 μ l of 10 \times reaction

Table 1 List of primers used in this study

Primer	Sequence (5'→3')* Target	Target	Restriction enzyme
β C1 _{Fwd}	ATACCATGGCCACACAGACACCTTCAAAG	β C1	<i>Nco</i> I
β C1 _{Rev}	TAA <u>AGATCT</u> AGACACCTTCAAACGAA		
β C1 _{Fwd}	ATACATATGCCACACAGACACCTTCAAAG	β C1	<i>Nde</i> I
β C1 _{Rev}	TAA <u>CTGCAGAGACACCTTCAAACGAA</u>		
β 01 _{Fwd}	ACCACACAGACACCTTCAAAGG	β -satellites	
β 02 _{Rev}	TCTCTGTGAACTATATCTTCT		
CP _{Fwd}	GGRTTDGARGCATGHGTACAT	Coat protein gene	Degenerate
CP _{Rev}	GCCYATRTAYAGRAAGCCMAG		
CP _{Fwd}	<u>CCATGGATGTCGAAGCGAGCTGCCGATATCG</u> <u>AGATCTTCAATT</u>	Coat protein gene	<i>Nco</i> I
CP _{Rev}	CGTTACAGAGTCATAAAAA		
CP _{Fwd}	<u>GAATTCATGTCGAAGCGAGCTGCCGATATCG</u> <u>GGATCCTCAATT</u>	Coat protein gene	<i>Eco</i> RI <i>Bam</i> HI
CP _{Rev}	CGTTACAGAGTCATAAAAA		
GFP _{Fwd}	GTGGAGAGGGTGAAGGTGAT	Green fluorescent protein	
GFP _{Rev}	TGCCATGTGTAATCCCAGCA		
35S _{Fwd}	TGACGCACAATCCCCTACTATC	Screening of transgenic plants	
Nos _{Rev}	TCATCGCAAGACCGGCAAC		
18S _{Fwd}	AACGGCTACCACATCCAAGG CCGAAGGCCAACACAATAGG	18S rRNA	
18S _{Rev}			

*Restriction sites were introduced in the 5' end of each primer and are underlined. Fwd indicates a forward primer and Rev a reverse primer
R=(A, G); D=(A, G, T); H=(A, G, T); H=(A, C, T); M=(A, C)

buffer, 1 μ l of 10 mM dNTP mix, 0.6 μ l (1.0 μ M) each of forward and reverse primers, 1.0 U of *Taq* DNA polymerase (Himedia labs, Mumbai, India), and DNase-free water to make up the volume of 20 μ l reaction mixtures. The PCR was performed in a thermocycler, with the following cycling program: denaturation at 94 °C for 3 min followed by 30 cycles, each consisting of denaturation at 94 °C for 45 s, primer annealing at 50 °C for 60 s (for full length β -satellite amplification) or 58 °C for 45 s (for β CI gene) and synthesis at 72 °C for 90 s followed by one cycle of final extension at 72 °C for 5 min. PCR products were cloned into pGEM-T Easy vector (Promega Life Science, Madison, WI, USA) as per manufacturer's protocol, and the clones were confirmed by restriction digestion (Supplemental Fig. S1).

Sequence analysis

The sequences of cloned inserts were confirmed by classical Sanger sequencing, using T7 and SP6 primers (Macrogen Service, Seoul, South Korea). The sequences of individual clones when subjected to the NCBI BLASTN using default parameters, showed homology to ChiLCV-CP (Accession number MH346125) and ChiLCB- β CI genes (Accession number MW269515), respectively. The CLUSTAL W method in SDT v.1.0 (Muhire et al. 2014) was used to determine percentage pairwise identity of the cloned and the representative sequences in the database.

Production of construct for *Agrobacterium*-mediated inoculation

Cloning strategy involved inserting specific ORF between the CaMV-35S promoter and a *NOS* terminator, using *Nco*I and *Bgl*II restriction enzyme sites. This generated the p35S- β CI and p35S-CP constructs (Supplemental Fig. S2). Each construct was used to transform *Agrobacterium tumefaciens* LBA4404 (Takara, Tokyo, Japan), and the recombinant *Agrobacterium* colonies were screened by colony PCR and finally confirmed by restriction digestion.

Generation of GFP silenced transgenic plants

GFP-silenced lines were developed in *Nicotiana tabacum* cv. *xanthi* by co-expressing GFP and short hairpin GFP (s_h GFP) using a binary vector, pCAMBIA1302 (CSIRO, Canberra, Australia). For creating the s_h GFP construct, the *GFP* gene was cloned in the sense and antisense orientations in the pHANNIBAL vector (Duan et al. 2012). The s_h GFP cassette was excised from the pHANNIBAL vector and moved into the binary vector pCAMBIA1302, which contains *GFP* under the control of the 35S promoter. To confirm the final pCAMBIA- s_h GFP constructs, sequential digestion was done using different restriction enzymes. Digestion with

*Pst*I resulted in two fragments of 4 Kb and 11 Kb, while triple digestion using *Nor*I, *Xho*I, and *Hind*III resulted in three fragments of 1.6 Kb, 3.4 Kb, and 11 Kb. Integration of the s_h GFP transgene was also checked by PCR analysis using promoter-specific 35S_{Fwd} and gene-specific GFP_{Fwd} primers (Table 1). After confirmation, it was mobilized into *Agrobacterium* (Supplemental Fig. S3).

Transgenic tobacco plants were produced by the leaf disc transformation with the *Agrobacterium* method (HORSCH et al. 1985) with minor modifications. Leaf discs were incubated with *A. tumefaciens* LBA4404 containing pCAMBIA- s_h GFP for 20 min with gentle shaking. The infected explants were blot-dried on sterile filter papers and placed on co-cultivation medium (MS medium containing 1 mg/l BAP or benzylaminopurine; 0.1 mg/l NAA or 1-naphthaleneacetic acid) at 25 °C for 2 days in the dark. After co-cultivation, the leaf discs were transferred to medium supplemented with 250 mg/l cefotaxime and 100 mg/l kanamycin or 25 mg/l hygromycin for regeneration. Regeneration of shoots from the leaf discs started after 5–6 weeks. Individual well-developed shoots were transferred to the bottle containing MS medium supplemented with 250 mg/l cefotaxime and 100 mg/l kanamycin or 30 mg/l hygromycin to induce rooting. The whole plants were transferred to vermiculite pots for hardening, before being transferred to the soil pots and grown in the green house to obtain intact transgenic plants (Supplemental Fig. S4).

To screen putative primary transgenic plants (T0), total DNA was isolated from leaves and confirmed for transgene integration using PCR primer pair 35S_{Fwd} and gene specific GFP_{Fwd}, respectively (Table 1). The amplified products were run of an agarose gel and visualized under UV illumination. Five independent T0 lines (L1, L2, L4, L5, and L6) showing expected 1.1-kb fragment for transgene integration were selected for selfing, and seeds were obtained. T1 seedlings silenced for GFP expression were selected for the further experiments.

Assay for suppression of RNA silencing

Silencing suppression activity was tested in-planta using *Agrobacterium*-mediated transient expression of the candidate viral genes. *A. tumefaciens* LBA4404 cultures harboring the desired construct were infiltrated into young (third or fourth leaf from top) leaves of transgenic GFP-silenced *N. tabacum* plants, with a 1-ml needleless syringe. The constructs used were p35S- β CI and p35S-CP individually and in combination. Infiltrations with empty vector (pCAMBIA) were used as control. The leaves were labeled for the construct used for infiltration and were plucked from the plant at different (3, 5, 7, and 11) days post infiltration (dpi) for visual observation and molecular analysis. Under UV light, leaf regions in which GFP did not express appeared red due

to chlorophyll autofluorescence whereas regions expressing GFP appeared green.

Reverse transcription quantitative real-time-PCR (qRT-PCR)

cDNA was prepared in 20 μ l reactions from total RNA isolated from the infiltrated leaf tissues, using 50 U of superscriptTM II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamers. The first strand cDNA was subjected to DNaseI treatment for 30 min and used for PCR amplification of *GFP* and viral genes (ChiLCV-*CP* or ChiLCB- β *C1*) using gene-specific primers (Table 1). The tobacco *18S rRNA* gene was used as a constitutive internal standard to evaluate cDNA content. The amplification products were analyzed on 0.8% agarose gel. The band intensities were quantified using Alpha Imager Imaging System (Alpha Innotech, CA, USA).

RNA concentration was measured by Nanodrop 2000 spectrophotometer (Thermo Scientific, CA, USA). DNase treatment and real-time qRT-PCR were performed by SYBR-green-based method in real-time PCR machine (Himedia Insta Q96, PA, USA). The relative level of transcripts was determined, after normalization with *18S rRNA* transcript using specific primers (Table 1) as previously described (Sinha et al. 2021). All samples were run in triplicate, average values were plotted, and the standard deviation is shown as error bars. Statistical comparisons were made and the level of significance was set at $p \leq 0.05$. Statistical calculations were performed using IBM SPSS 15.0 software (SPSS, Chicago, USA).

Computational analysis

ChiLCV-*CP* and ChiLCB- β *C1* protein sequences were mined from the GenBank-NCBI database (MH346125 and MW269515, respectively) for homology modeling and docking studies. Homology modeling involves several steps like template selection, target template alignment, model construction, and model assessment. As a first step, a query sequence with an unknown 3D structure was used to identify a homologous target sequence with known 3D structure. SWISSPROT (<https://swissmodel.expasy.org>) was used to search against the PDB (Protein Databank) to identify the related homologs of the query sequence. The uppermost frame had sufficiently close template in the structure database to confidently model the sequence, and this was used for generating the 3D Model.

The PDB files of ChiLCV-*CP* and ChiLCB- β *C1* were downloaded (<https://swissmodel.expasy.org>) for further analysis. The docking studies were carried out using PatchDock server (<https://bioinfo3d.cs.tau.ac.il/PatchDock/>). The results were analyzed through PDBsum (<http://www>.

[ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html](http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html)) and visualized through Rasmol software (<http://www.openrasmol.org/>).

Yeast two-hybrid analysis

To analyze the interaction of ChiLCV-*CP* and ChiLCB- β *C1*, Y2H (Clontech Laboratories, CA, USA) analysis was performed. It is a yeast-based screening system in which transcription factor, GAL4, is utilized for the detection of the protein interactions. The transcription factor is split such that its DNA-binding domain (BD) and transcription-activation domain (AD) are unable to transcribe the reporter gene independently on nutritional selection. Expression of a reporter gene can be obtained only if BD and AD are brought together by the two interacting proteins (Osman 2004).

Bait protein was expressed as GAL4 DNA-binding domain fusion of β *C1* (pGBKT7- β *C1* expressing BD- β *C1*). The prey protein was expressed as GAL4 DNA activation domain fusion with ChiLCV-*CP* (pGADT7-*CP* expressing AD-*CP*) or ChiLCV-*MP* (pGADT7-*MP* expressing AD-*MP*). To construct the vectors, the ChiLCB- β *C1* gene was cloned into the *NdeI/PstI* sites of the pGBKT7 yeast expression vector (Clontech Laboratories, CA, USA), and the *CP* and *MP* genes were cloned into the *EcoRI/BamHI* sites in the pGADT7 yeast expression vector (Clontech Laboratories, CA, USA) (Supplemental Fig. S5). The *CP* and *MP* genes were also cloned into the *NdeI/PstI* sites of the pGBKT7 vector to express as GAL4 DNA-binding domain fusion. Recombinant colonies were checked by colony PCR using gene-specific primers (Table 1) and confirmed by restriction digestion. All transformants were tested for auto-activation before mating prior to the Y2H screening. For mating, the yeast AH109 cells were co-transformed with the BD- β *C1*, AD-*CP*, or AD-*MP* constructs and plated on double dropout (Leu⁻Trp⁻) selective dropout medium. For confirmation, colonies were transferred to triple dropout (Leu⁻Trp⁻His⁻) medium supplemented with 5 mM 3-amino-1,2,4-triazole (3-AT) to reduce the background growth and allow clones showing strong interaction to grow. 3-AT is a competitive inhibitor of the *HIS3* gene product, and cells will be able to grow in presence of 3-AT only if the level of histidine (*HIS3*) is adequate to support the inhibitory impact of 3-AT.

Filter assay for β -galactosidase

The Y2H interactions were also tested by β -galactosidase activity. Yeast colonies were patched on YPD (gal) Leu⁻Trp⁻His⁻ plates and incubated at 30 °C for 2 days. A fresh Whatman 3-MM filter paper (Whatmann Co, Marlborough, USA) was placed on the yeast colonies and gently removed. The filter was placed in liquid N₂ for 5 min and kept at 37 °C for 5 min. This step was repeated thrice for

lysis of yeast cells. A filter paper was wetted with buffer (60 mM Na_2HPO_4 (dibasic), 40 mM NaH_2PO_4 (monobasic), 10 mM KCl, 1 mM MgSO_4) supplemented with X-gal (0.01 mg/ml in dimethyl sulfoxide, DMSO), and the filter containing lysed yeast cells was placed on pre-wet filter for 2–3 h at 30 °C in the dark. The colonies were observed for the appearance of blue color.

Sub-cellular localization of βC1 protein expression in tobacco cells

To examine the sub-cellular localization of ChiLCB- βC1 and ChiLCV-CP proteins in host cells, they were expressed in fusion with GFP at its C-terminus, using the pCAMBIA1302 vector (p35S- βC1 and p35S-CP). The empty pCAMBIA1302 vector backbone (p35S-GFP) was used as control. The plasmids were transformed into *A. tumefaciens* LBA4404, and positive colonies were screened by colony PCR. For infiltration leaves, 4-week-old *N. tabacum* plants growing under a 16-h light and 8-h dark photoperiod were used. A ten-min staining with the blue-fluorescent DNA stain, DAPI, or 4,6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA, USA) was used to visualize nuclei in cells during confocal microscopy (Duan et al. 2012). GFP and DAPI fluorescence images were captured with the Nikon spectral confocal microscope (Nikon A1R, Nikon Corporation, Tokyo, Japan). The captured images were processed using the Adobe Photoshop software (Technofirm Software, Guj, India).

Results

Generating GFP-silenced transgenic plants

The primary requirement for developing stably silenced *N. tabacum* lines was to perform an in-planta reversal of silencing assay. T1 transgenics, obtained on selection medium, were screened for loss of GFP fluorescence by observing under UV light. The putative positive plants were reconfirmed by genomic DNA PCR, which amplified a product of 1.1 Kb indicating positive integration of GFP (Supplemental Fig. S6). In these plants, transcription of the GFP transgene could not be detected by RT-PCR analysis, indicating silencing mediated by the s_{h} GFP construct.

Reversal of established silencing by βC1 and coat protein in planta

To analyze the VSR activity of ChiLCB- βC1 and ChiLCV-CP, the young leaves of GFP silenced lines were agro-infiltrated individually with the respective constructs and analyzed for GFP expression after 3 dpi (Fig. 1a), 5

dpi (Fig. 1b), 7 dpi (Fig. 1c), and 11 dpi (Fig. 1d). The zones infiltrated with p35S- βC1 and p35S-CP exhibited green fluorescence upon UV illumination at 365 nm indicating positive reversal of silencing. The GFP fluorescence started appearing at 3 dpi, increased up to 7 dpi and was sustained till 11 dpi. Green fluorescence was not observed in regions with mock infiltrations using empty vector. These experiments show that both ChiLCV-CP and ChiLCB- βC1 are capable of suppressing s_{h} -RNA-induced established RNA silencing.

To determine the effect on the VSR activity in co-presence of ChiLCB- βC1 and ChiLCV-CP, GFP-silenced plants were co-infiltrated with the p35S- βC1 and p35S-CP constructs (p35S- βC1 + p35S-CP). Examination of the infiltrated zones under UV light revealed that strong GFP fluorescence can be observed at 3 dpi (Fig. 1a), and the level of fluorescence was relatively higher at other time points (Fig. 1b–d) when compared to that elicited by p35S- βC1 and p35S-CP, individually. This indicated complementation of the VSR activity in the presence of the two proteins.

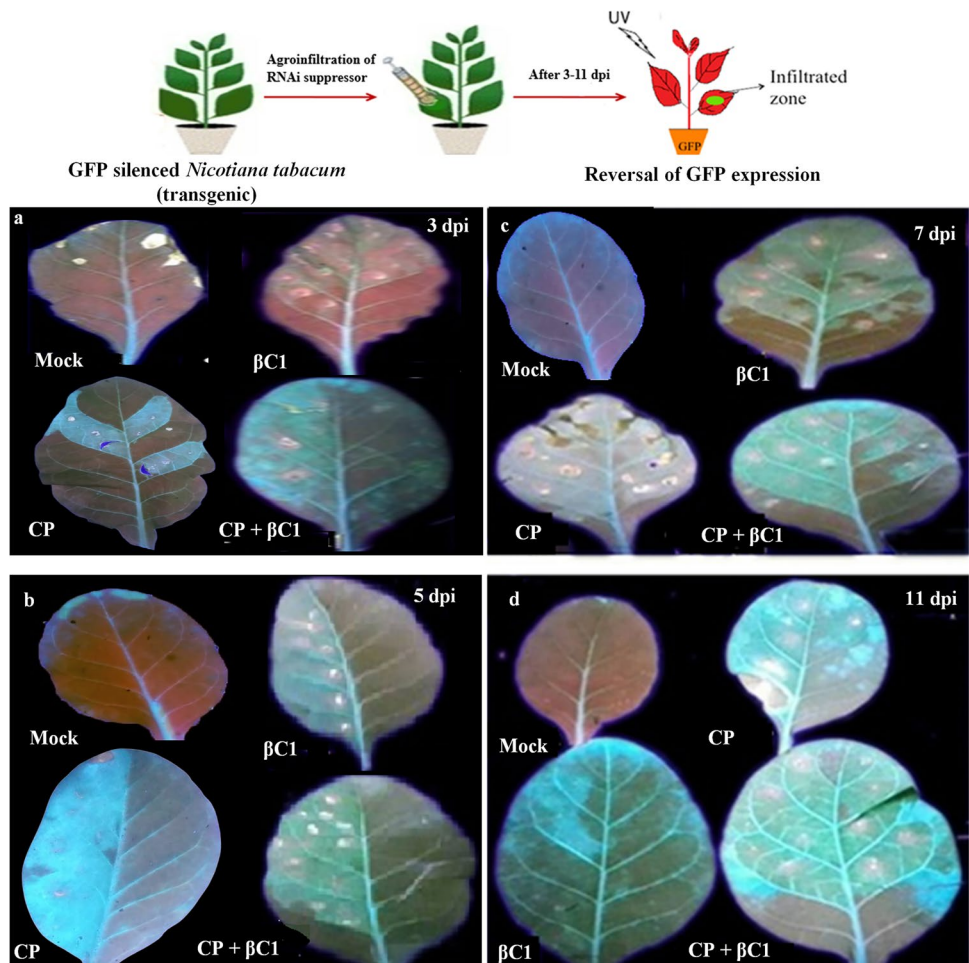
At 14 dpi, the plants which were co-infiltrated with p35S- βC1 + p35S-CP, also exhibited GFP fluorescence in the newly emerging leaves (Fig. 2). The plants infiltrated with p35S- βC1 or p35S-CP alone did not show reversal of GFP expression in the newly emerged leaves (Fig. 2). The spread of reversal of silencing beyond the region of infiltration indicates a possible interference with the spread of silencing.

Analyzing the strength of VSR

RT-PCR analysis was performed to confirm the GFP fluorescence observed upon infiltration with p35S- βC1 and p35S-CP, respectively. The detection of GFP transcripts in the infiltrated zones validated that both ChiLCV-CP and ChiLCB- βC1 contain VSR activity (Fig. 3a, b). The suppression activity of ChiLCV-CP started early and sustained to high levels till 11 dpi (Fig. 3a). In the presence of ChiLCB- βC1 , the suppression increased slowly till 11 dpi, and the values were lower than that obtained in ChiLCV-CP (Fig. 3b). When p35S- βC1 and p35S-CP were co-infiltrated, higher level of GFP transcript accumulation was observed indicating stronger reversal of suppression (Fig. 3c).

For further confirmation, GFP and VSR transcripts in the infiltrated zones were quantitated using qRT-PCR (Fig. 3d), and suppressor strength was calculated in terms of an *S* value. This was done by normalizing the GFP transcripts with respect to the VSR transcript to determine the degree of GFP reversal (Das and Sanan-Mishra 2014; Sundaresan et al. 2020). The *S* value plots showed that GFP accumulation in the presence of ChiLCV-CP was higher than that in the presence of ChiLCB- βC1 . In the presence

Fig. 1 Upper panel: Schematic representation of assay for reversal of GFP silencing. Lower panel: Representative pictures of s_{11} GFP-silenced tobacco leaves agroinfiltrated with different constructs. The constructs used are Mock: Empty vector control, CP: ChiLCV-CP, β C1: ChiLCB- β C1, and CP + β C1: ChiLCB- β C1 and ChiLCV-CP together. Leaves were observed under UV illumination after **a** 3 dpi, **b** 5 dpi, **c** 7 dpi, and **d** 11 dpi



of both ChiLCV-CP and ChiLCB- β C1, the S values show an increase at 7 and 11 dpi. This indicated synergism of ChiLCV-CP and ChiLCB- β C1 on the suppression of silencing in the host cells.

ChiLCB- β C1 and ChiLCV-CP contain interacting domains

To investigate, if there was a potential interaction between ChiLCB- β C1 and ChiLCV-CP, the two proteins were first subjected to in silico analysis. It was observed that the two proteins do not have much homology at their primary and secondary structures, so the tertiary or 3D structure of the proteins was subjected to homology modeling. SwissProt was used to predict the 3D structures of the ChiLCB- β C1 and ChiLCV-CP proteins. Ramachandran plot analysis of the predicted structures was performed using PROCHECK processing, and two homologs were identified as acceptable models (Table 2). 6bx3-pdb had 87.2% residues in the most favored region (Fig. 4a) while 6f2s.pdb had 71.82% residues in the most favored region (Fig. 4b). The selected

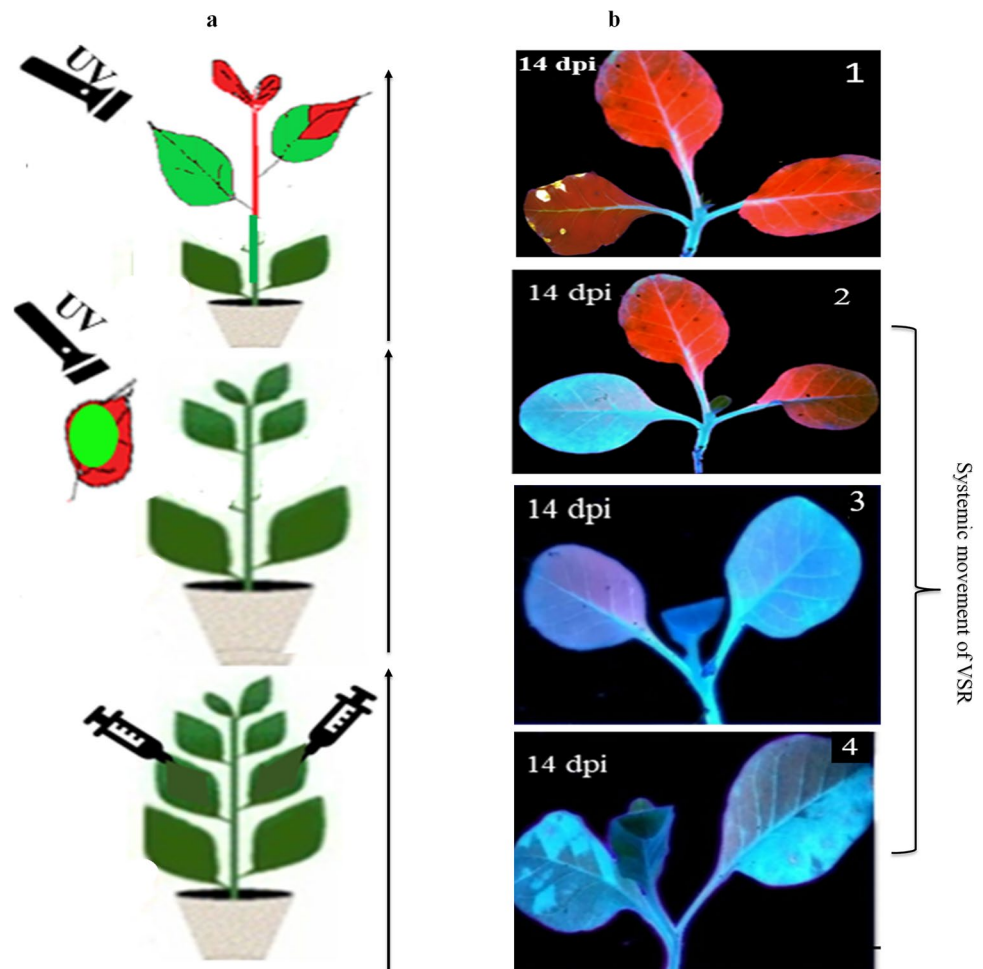
structures were subjected to docking, and the resulting clusters were ranked according to the PatchDock score. The analysis indicated that ChiLCB- β C1 could potentially interact with ChiLCV-CP through the α -helix residues (Fig. 4c).

ChiLCB- β C1 interacts with ChiLCV-CP in the yeast model

The reversal of silencing was seen to spread when ChiLCV-CP and ChiLCB- β C1 were co-infiltrated. To validate the interaction between ChiLCV-CP and ChiLCB- β C1, a Y2H assay was performed (Fig. 5a, b). For this assay, ChiLCV-MP (movement protein) was chosen as a control because MP plays an important role in the infection and spread of viruses within the host (Li et al. 2020). To eliminate the false positive results, due to self-interaction, appropriate positive and negative controls were also included in this experiment (Supplemental Fig. S7).

The interacting partners were screened by growth of transformants on double dropout (Leu⁻ Trp⁻ SD agar

Fig. 2 Representative pictures to show the reversion of GFP expression in newly emerging leaves at 14 dpi. The left panel shows schematic representation of spread of reversal of GFP silencing in newly emerging leaves. The right panel shows leaves infiltrated with ChiLCV-CP (panel 1), ChiLCB- β CI (panel 2), and ChiLCB- β CI + ChiLCV-CP (panels 3 and 4). The photographs were taken by a digital camera (Nikon, Tokyo, Japan)



minimal medium) plates. Several colonies were obtained with BD- β CI and AD-CP co-transformation (Fig. 5c) while no growth observed in case of BD- β CI and AD-MP co-transformation (Supplemental Fig. S8). The colonies co-transformed with BD- β CI and AD-CP also survived on triple dropout (Leu⁻ Trp⁻ His⁻ SD agar minimal) media (Fig. 5d). The stringency of interaction was confirmed by addition of 5 mM 3-AT (His analog) to the medium (Fig. 5e). To validate the interaction, the colonies were screened for blue color formation due to β -galactosidase (MEL reporter) on triple dropout SD agar minimal medium supplemented with X-gal (Fig. 5f). The results showed that ChiLCB- β CI does not interact with ChiLCV-MP but exhibits strong interaction with ChiLCV-CP.

ChiLCB- β CI protein localizes in the nucleus of plant cells

To determine the subcellular localization, three constructs carrying p35S- β CI:GFP, p35S-CP:GFP, and p35S-GFP (control) were introduced into *N. tabacum* leaves via agro-infiltration. Microscopic observation revealed that in 48 h

post infiltration with p35S- β CI:GFP, the GFP fluorescence could be detected in trichomes and stomata (Fig. 6a) and by 72 h post inoculation, the GFP signal became weak in this region. At the cellular level, CP:GFP fluorescence was spread in both cytoplasm and nucleus but β CI:GFP fluorescence was concentrated mostly in the nucleus (Fig. 6b). These results provide evidence that ChiLCB- β CI protein is localized primarily in the nucleus within the host cells. In leaves co-infiltrated with p35S- β CI:GFP and p35S-CP:GFP, the GFP fluorescence was localized only in the cytoplasm (Fig. 6b), and this was confirmed by merging the fluorescence image with that of DAPI-stained nuclei. This indicated that, when present together, the translocation of ChiLCV-CP and ChiLCB- β CI to the nucleus is blocked.

Discussion

ChiLCV is a ssDNA virus belonging to the *Geminiviridae* family and it has a wide host range (Sahu and Mishra 2021). Replication of the geminiviral ssDNA is performed via dsDNA intermediate, which associates with cellular histones

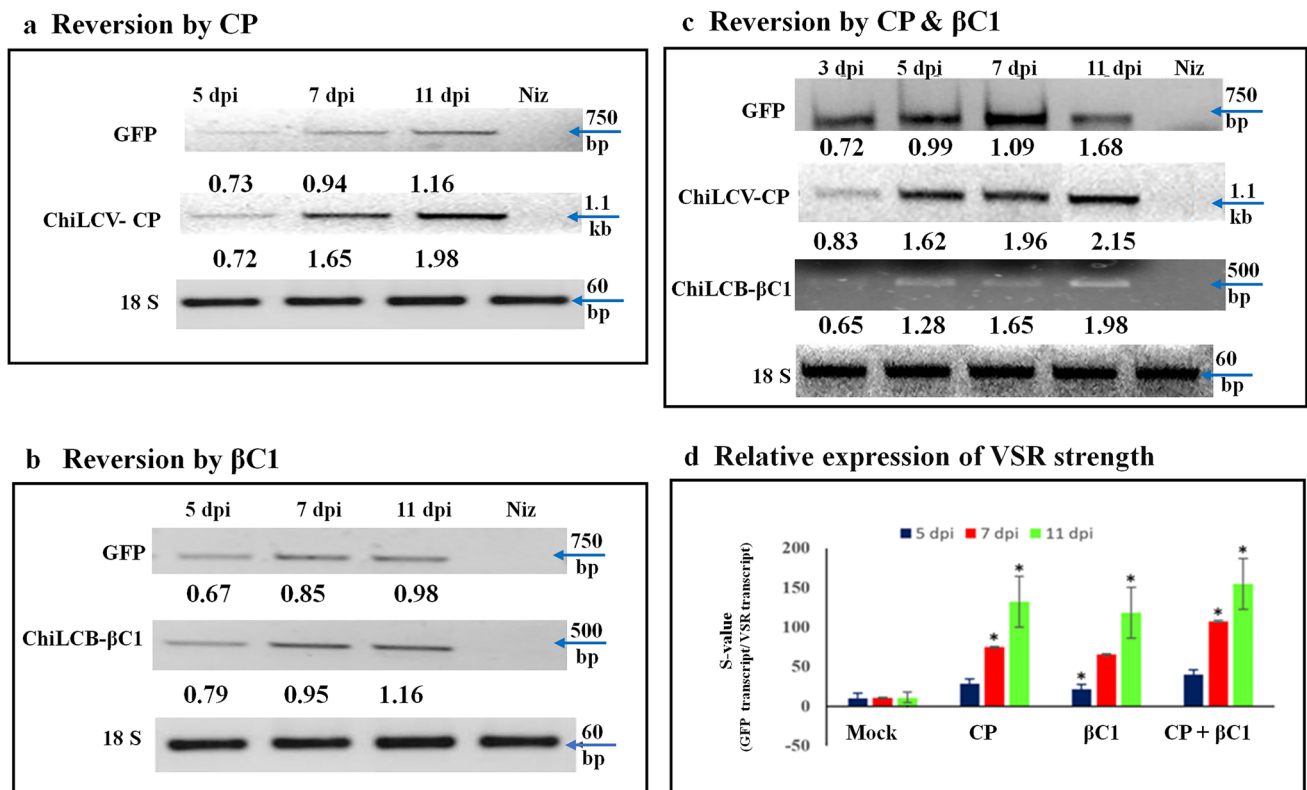


Fig. 3 Analyzing the strength of suppression. RT-PCR amplified transcripts of GFP and VSR using total RNA obtained from GFP-silenced *Nicotiana tabacum* leaves infiltrated with **a** ChiLCV-CP, **b** ChiLCB-βC1, and **c** ChiLCV-CP+ChiLCB-βC1. The samples were analyzed at 3 dpi, 5 dpi, 7 dpi, and 11 dpi. NIZ (non-infiltrated zone) served as control. The band intensities were measured by Alphamager Imaging System (Alpha Innotech, CA, USA) and normalized with respect to

18S rRNA. **d** Plot of suppressor strength calculated in terms of an S value, by quantitating the GFP transcripts with respect to the VSRs transcript. The expression levels were determined by qRT-PCR, and the individual values were normalized using 18S of tobacco, in each case. Error bars represent standard deviation of three independent experiments. Asterisks indicate significant differences calculated using ANOVA (* $p \leq 0.05$)

Table 2 Comparative analysis of Ramachandran plot statistics for the homologs of ChiLCV-CP and ChiLCB-βC1

Ramachandran plot statistics, residues (%)				
Predicted structure	Most favored region	Additional allowed region	Generously allowed region	Disallowed region
6bx3-pdb (ChiLCV-CP)	87.2	12.3	0.4	0.1
6f2s.pdb (ChiLCB-βC1)	71.8	27.5	0.6	0.0

to make minichromosomes. These minichromosomes are potential targets of epigenetic modifications and transcriptional gene silencing (TGS) involving methylation of DNA or histone leading to heterochromatinization and repression of mRNA transcription. The geminiviral DNA also triggers sequence-specific degradation of complementary mRNA transcripts through the PTGS pathways (Pandey et al. 2009). The small RNA-mediated gene-silencing pathways serves as an adaptive immune response and plays an active role in shielding plants against viral infections (Waterhouse et al. 2001; Pandey et al. 2009). It has been demonstrated that

constructs producing double-stranded RNA are the most effective method for silencing gene expression in plants. For instance, constructs expressing Plum Pox Virus (PPV)-derived intron hairpin RNA (ihpRNAs) can efficiently induce PTGS. ihpRNA originating from viral *PI* and *CP* genes were found to confer resistance against PPV in stone fruits (Wang et al. 2008), while ihpRNA from *PI* and helper component protease (*HC-Pro*) were found to confer resistance against PPV in *Nicotiana benthamiana* (Makeshkumar et al. 2021). The mechanisms have been effectively utilized by employing various sources of double-stranded RNAs,

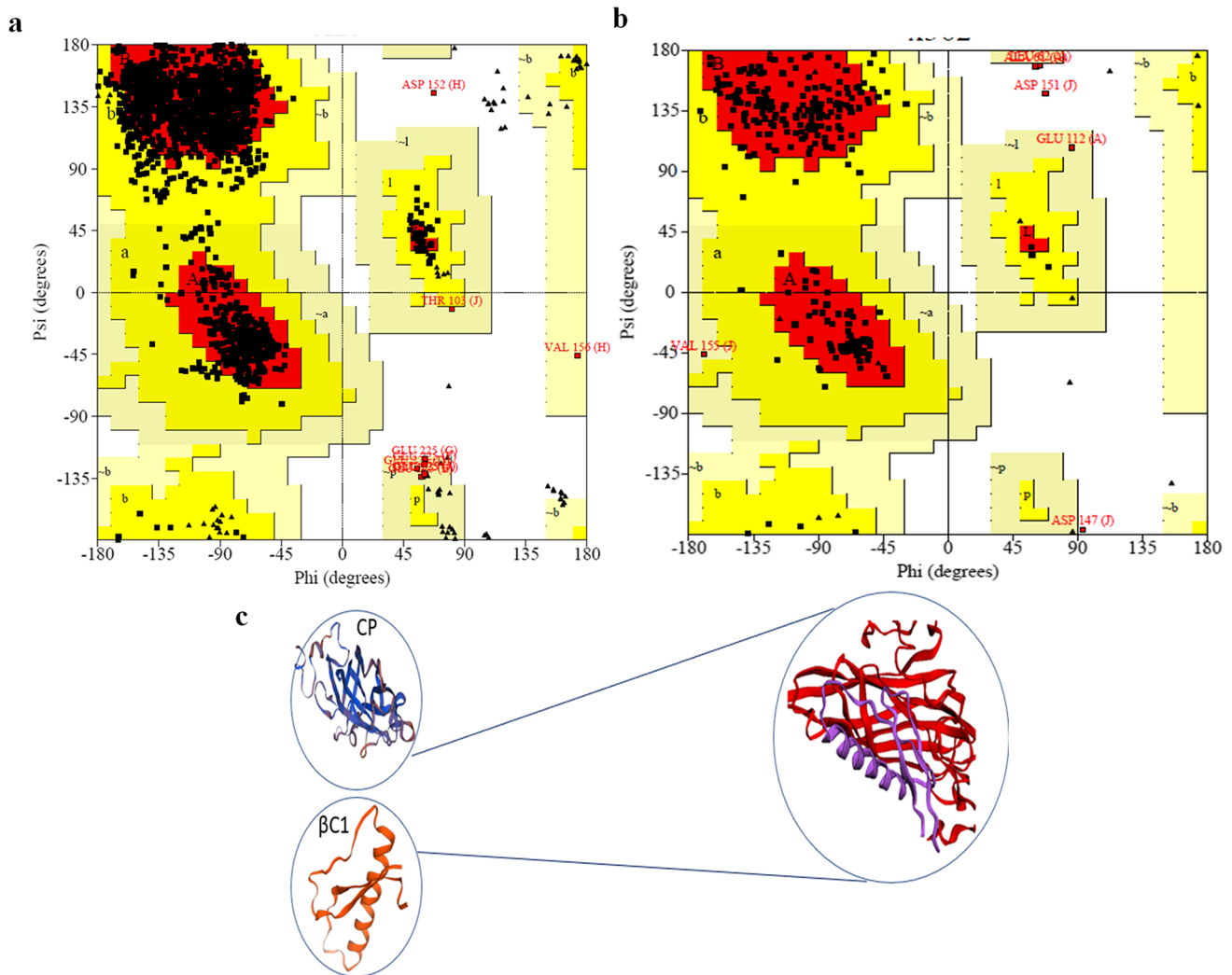


Fig. 4 Ramachandran plot of **a** 6b×3-pdb and **b** 6f2s.pdb, homologs of ChiLCV-CP and ChiLCB-βC1 proteins, respectively, as predicted using PROCHECK. **c** Ribbon representation of model of second frame protein sequence of 6f2s.pdb (upper panel) and 6b×3-pdb

(lower panel) created by using Swissprot server (<https://swissmodel.expasy.org>) and in silico docking by using PatchDock server (<https://bioinfo3d.cs.tau.ac.il/PatchDock/>) to identify potential interacting residues

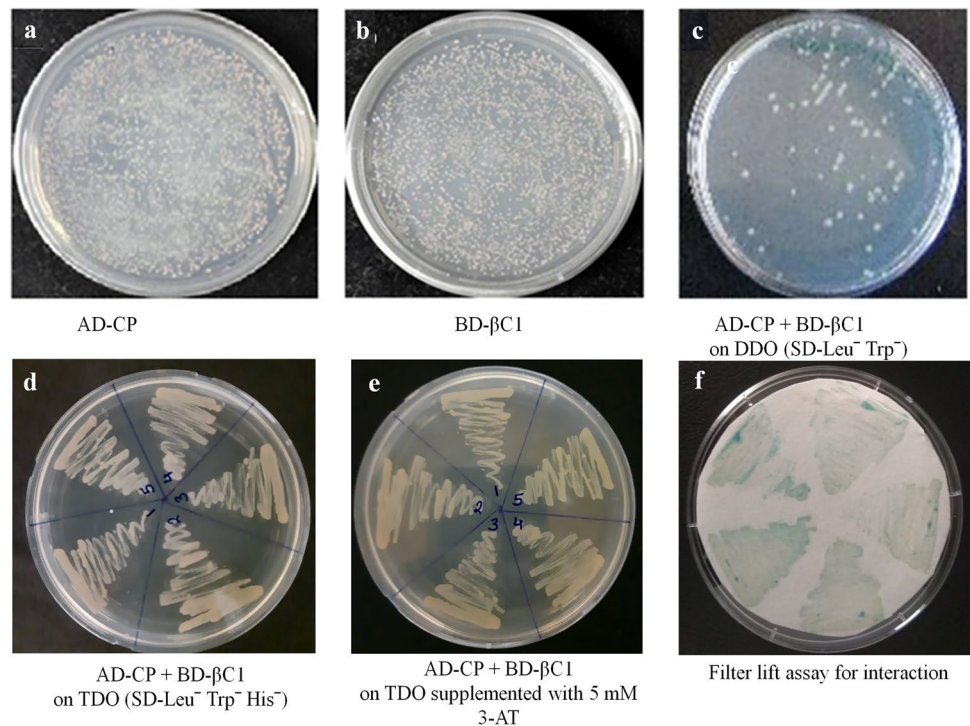
like antisense RNAs and hairpin RNAs, for preventing virus resistance (Sinha et al. 2017).

The viruses encode a limited number of proteins and some or all of them have acquired silencing suppression activity (Kumar et al. 2015b; Qu and Morris 2005; Sanan-Mishra et al. 2017). These VSR proteins act as pathogenicity determinants to dampen RNA silencing-based host antiviral defenses and play an important role in virulence (Pruss et al. 1997; Das and Sanan-Mishra 2014). These VSR activities have been characterized in virus-encoded CP (Kunik et al. 1994), MP (Duan et al. 1997), and protease (Hartitz et al. 1999) proteins of different viruses. The AC2 protein of begomovirus has been shown to possess strong VSR activity and is capable of suppressing TGS and or PTGS (Trinks et al. 2005; Vanitharani et al. 2004). To define the strength of VSR

activities, two parameters were used that were based on the degree of GFP reversal and the sustenance of GFP expression (Das and Sanan-Mishra 2014). Molecular approaches were employed to quantitate the degree of GFP reversal due to the VRS activity, and the suppressor strength was calculated in terms of an *S* value by normalizing the accumulated GFP transcripts with respect to the VRS transcripts.

The plant DNA viruses are often associated with satellite DNAs that are completely dependent on the virus for their replication and encapsidation (Hanley-Bowdoin et al. 2013). β-satellites related with chili leaf curl disease have also been identified (Khan and Khan 2017; Kumar et al. 2015a). Their DNA contains excessive nucleotide variability due to high nucleotide substitution rate in the βC1 coding regions. The βC1 protein acts as a pathogenicity

Fig. 5 Yeast two-hybrid assay for one-to-one interaction study. Yeast colonies of **a** AD-CP and **b** BD- β C1. These (AD-CP and BD- β C1) were co-transformed and selected on **c** two dropout (Leu⁻ Trp⁻), **d** triple dropout (Leu⁻ Trp⁻ His⁻), and **e** triple dropout (Leu⁻ Trp⁻ His⁻) plates supplemented with 5 mM 3-AT. **f** Filter showing blue-colored colonies upon performing galactosidase assay



determinant and plays a crucial role in pathogenesis. It has been shown that the β C1 protein works as a suppressor for both PTGS and TGS. It suppresses the jasmonic acid signaling (JA) pathway and the ubiquitin–proteasome system and defense hormones of the plants (Bhattacharyya et al. 2015; Jia et al. 2016; Yang et al. 2008; Zhou 2013).

N. benthamiana is a popular model species for studying molecular mechanisms of different aspects of plant biology. It is easy to transform and has been used in studies involving transient as well as stable gene expression (Goodin et al. 2015). The transcriptionally silenced *GFP* gene in transgenic tobacco lines has been used in many studies on the induction and maintenance of epigenetic modifications (Buchmann et al. 2009; Yang et al. 2011b). These plants have also been used to develop assays based on re-establishment of GFP expression for identifying VSRs (Karjee et al. 2008) and studying their mechanism of suppression (Sanan-Mishra et al. 2017). Using one such assay, it was shown that β C1 was able to reverse TGS of the GFP transgene in *N. benthamiana* lines, during co-infection with TYLCCNV or *Beet curly top virus* (Yang et al. 2011a). In this study, transgenic *N. tabacum* lines co-expressing GFP and s_hGFP were generated to screen for reversal of stable silencing.

ChiLCV-CP and ChiLCB- β C1 proteins play important roles in viral replication, encapsidation, and pathogenesis. Their VSR activity was confirmed by reversal of the GFP silencing, upon *Agrobacterium*-mediated ectopic expression in the leaves of the silenced tobacco plants. The suppression of silencing was not detected in regions infiltrated with the

empty vector. Time kinetics revealed an effective correlation between accumulation of GFP transcript and individual VSR transcript till 11 dpi. This indicated that ChiLCV-CP and ChiLCB- β C1 transcripts accumulated in the specific infiltrated regions and interfered with the host silencing machinery.

The assay using co-infiltration demonstrated the possible synergism between ChiLCB- β C1 and ChiLCV-CP, as the intensity of GFP fluorescence was enhanced. The reversion of expression was also monitored by molecular analyses. The strength of VSR activity, calculated in terms of an *S* value was higher when both ChiLCB- β C1 and ChiLCV-CP were present. It was observed that by 14 dpi, the plants also exhibited a bright yellow-green fluorescence in newly emerging leaves, indicating a spread of suppression of silencing outside the zone of infiltration. The mechanistic details are subject of further investigation and could be due to suppression of transitive siRNAs or systemic movement of VSRs.

In silico studies identified the possibility of interaction between ChiLCB- β C1 and ChiLCV-CP (Ainir et al. 2011). Homology modeling with predicted 3D structures of the proteins indicated direct interaction. There are earlier reports showing that the β C1 protein interacts with the CP of the helper *Bhendi yellow vein mosaic virus* (Usha et al. 2006). The Y2H experiments confirmed ChiLCV-CP as the interacting partner of ChiLCB- β C1 and this synergism is likely responsible for enhanced VSR activity seen during co-infiltration of both constructs.

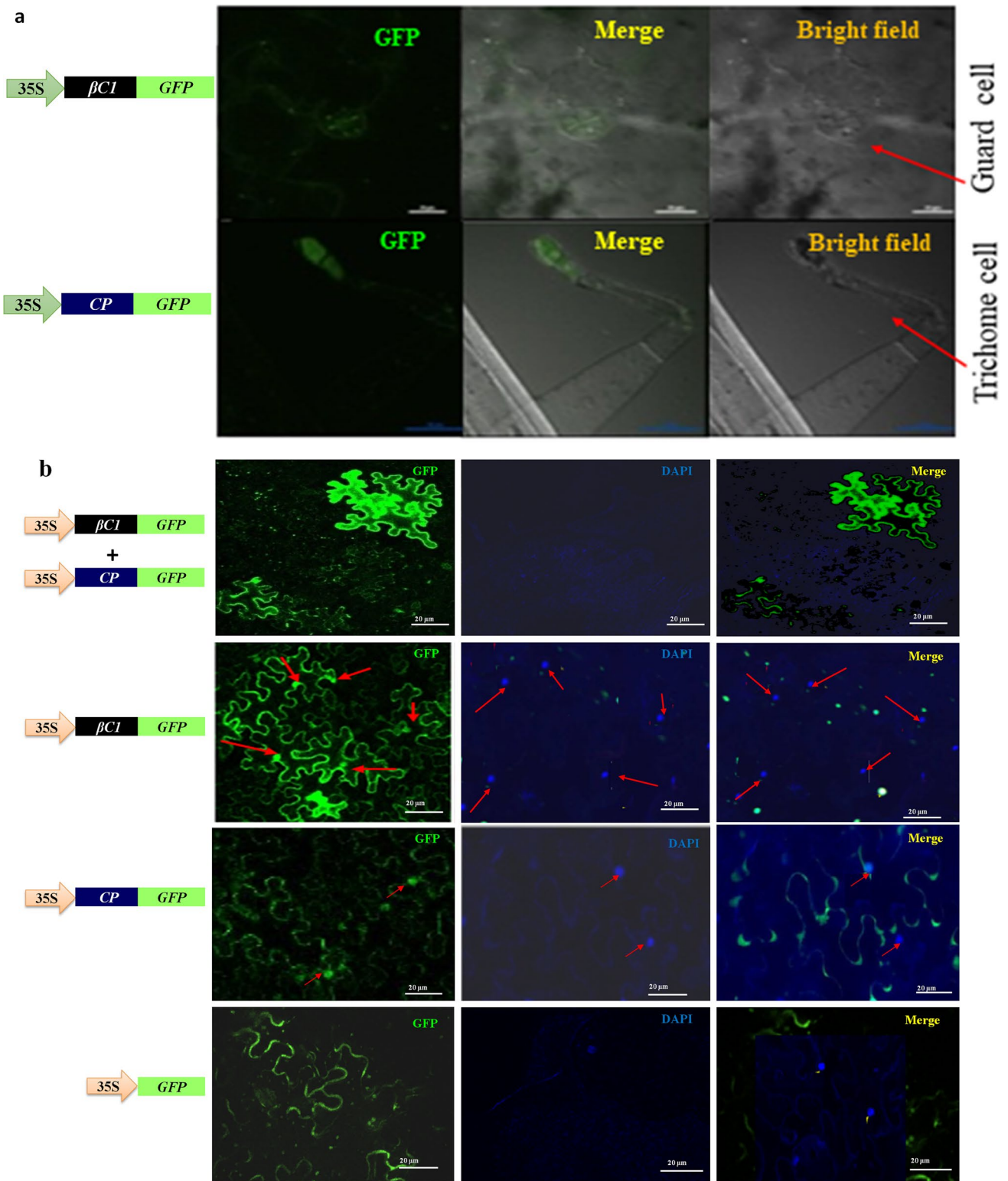


Fig. 6 Subcellular localization of ChiLCB-βC1:GFP and ChiLCV-CP:GFP fusion protein in plant cells. **a** The upper two panels show images taken 48 h post infiltration. The fluorescence was found in guard cells (upper lane) and trichomes (lower lane). **b** The lower four panels show images taken 72 h post infiltration. βC1 and CP infiltrated together were localized in the cytoplasm. βC1 protein mainly

accumulated in the nuclei of tobacco cells, CP protein was distributed in both cytoplasm and nuclei of tobacco cells. The GFP construct alone was used as control and its expression was restricted in the cytoplasm. Images were examined using confocal microscopy and arrows have been used to indicate nuclei. DAPI staining was used to visualize nuclei. Bar in these images, 20 μm

In other studies, the identified interacting partners of β C1 protein include host proteins like nuclear importin, karyopherin α (Usha et al. 2006). This protein facilitates the nuclear export and import in a process analogous to that performed by DNA-B in the bipartite virus. The DNA-B encoded NSP (BV1/BV2) and MP (BC1) are responsible for the nuclear transport and cell-to-cell movement of the viruses but their movement is limited to phloem cells (Noueiry et al. 1994; Jyothsna et al. 2013; Gafni and Epel 2002). The monopartite begomoviruses do not have a DNA-B component but are often associated with satellites. Recently, the existence of another geminivirus satellite encoded protein, β V1, was reported which may function with β C1 for a productive virus infection (Hu et al. 2020).

Sequence analysis of β C1 proteins of different β -satellites has indicated the presence of a NLS (Usha et al. 2006). β C1 encoded by *Bhendi yellow vein mosaic* betasatellite possesses a strong NLS and also physically interacts with *Bhendi yellow vein mosaic virus* encoded CP, which lacks an NLS (Usha et al. 2006). Earlier reports have correlated the increase in symptom induction and intracellular movement of β C1 with the presence of NLS (Cui et al. 2005). Mutation in the β C1 gene of *Cotton leaf curl multan* betasatellite failed to produce systemic infection (Saeed et al. 2007), although, the β C1 deletion-mutant of TYLCCN (Y10 β) was capable of systemic movement in host plants and underwent encapsidation by the helper virus (Qian and Zhou 2005).

Localization studies of ChiLCB- β C1 and ChiLCV-CP, in cells of tobacco leaf, showed that β C1 accumulated in trichomes and guard cells at early stages, but later it was found to primarily accumulate in the nuclei of cells. ChiLCV-CP was localized in both nucleus and cytoplasm. When both ChiLCV-CP and ChiLCB- β C1 were co-expressed, the GFP signal is restricted to only cytoplasm indicating that interaction limits ChiLCB- β C1 to the cytoplasm. This change in cellular localization of the two VSR proteins may play a likely role in enhancing the RNA silencing suppression activity.

Therefore, it can be concluded that ChiLCB- β C1 protein acts as a pathogenicity determinant during viral infection. In the presence of both ChiLCB- β C1 and ChiLCV-CP, the potency of silencing-suppression was increased. It was found to interact with ChiLCV-CP in host cells. ChiLCB- β C1 possibly also plays an important role in suppressing systemic spread of silencing as observed by the spread of VSR activity after 14 dpi of co-infiltration with ChiLCV-CP. The findings provide leads for further experiments for understanding the mechanism of VSR action.

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Author contribution NSM supervised the study. AKS designed and performed the experiments. AKS wrote the manuscript. NSM revised the manuscript. All authors read and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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