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Identification and subcellular location of an RNA silencing suppressor encoded by mulberry crinkle leaf virus

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ABSTRACT

Mulberry crinkle leaf virus (MCLV) is a novel geminivirus recently identified from the woody plant mulberry (*Morus alba* L.). Little is known about the functions of the proteins encoded by the MCLV genome. Here, all the MCLV-encoded proteins were examined for the ability to suppress gene silencing by an agroinfiltration assay in combination with northern blot analysis of green fluorescent protein (GFP) mRNA and western blot analysis. Of the six proteins, only one protein, V3, which has been predicted to play a role in viral movement, was found to suppress the gene silencing induced by a sense GFP gene in *Nicotiana benthamiana* 16c. The minimal amino acid sequence of V3 that maintains suppressor activity was also determined by constructing truncated mutants lacking different lengths of the amino acid sequences at the N- or C-terminus of the V3 protein. The results showed that the 94 N-terminal amino acid residues of V3 are sufficient to maintain V3 suppressor activity. In addition, the subcellular location of the V3 protein was investigated by confocal laser scanning microscopy after the expression of a V3-RFP fused protein in leaf epidermal cells of *N. benthamiana*. The results indicated that the V3 protein localized not only to the cytoplasm but also to the nucleus of *N. benthamiana*, implying that V3 can shuttle between the nucleus and the cytoplasm. Deletion mutant analysis indicated that a putative nuclear localization signal (NLS) between aa 118–134 might be responsible for the nuclear distribution of the V3 protein. Given the importance of RNA silencing in plant-virus interactions, the identification of a silencing suppressor of MCLV should be valuable in understanding the pathogenicity and molecular biology of this virus.

1. Introduction

Gene silencing in plants occurs either through miRNA- or siRNA-mediated mRNA degradation and translational suppression, generally referred to as posttranscriptional gene silencing (PTGS), or through siRNA-directed DNA methylation (RdDM) and transcriptional suppression, referred to as transcriptional gene silencing (TGS) (Vaucheret and Fagard, 2001; Baulcombe, 2004). Gene silencing, which was first discovered in a transgenic plant (Napoli et al., 1990), has since been found in most eukaryotes, including fungi, plants, and animals. Key components in the gene silencing pathways include Dicer-like (DCL) ribonucleases, RNA-dependent RNA polymerase (RDR), and Argonaute (AGO) proteins (Raja et al., 2010). Gene silencing is triggered by double-stranded RNAs (dsRNAs), which are recognized and diced by DCL ribonucleases into small interfering RNAs (siRNAs) (Hammond et al., 2000; Vaucheret, 2006). In PTGS, these duplex 21–25 nt siRNAs are unwound, and one strand is incorporated into the RNA-induced silencing complex (RISC) and then guides RISC by base pairing to degrade or translationally suppress cellular mRNAs, viral mRNAs, or transcripts produced by DNA viruses such as geminiviruses (Anandalakshmi et al., 1998; Hamilton and Baulcombe, 1999; Ding and Voinnet, 2007;). In TGS, RdDM in plants is largely guided by Pol IV-dependent 25–50 nt P4 RNA, a previously unappreciated class of Dicer-independent noncoding RNAs, rather than the 24 nt RNA previously thought to be involved (Yang et al., 2016). Upon infection with DNA viruses such as geminiviruses, plants employ RdDM to suppress replication and/or transcription of the DNA virus genome, leading to TGS. Gene silencing has emerged as an important host defense mechanism against plant viruses (Waterhouse et al., 2001; Ruiz-Ferrer and Voinnet, 2009; Ding, 2010; Llave, 2010). To counter PTGS and/or TGS and achieve infection, the majority of plant viruses have evolved to encode suppressors of gene silencing that specifically counteract the host antiviral defense by

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interfering with silencing pathways at different steps (Vance and Vaucheret, 2001; Li and Ding, 2001). Viral suppressors of PTGS have been identified in almost all plant virus genera (Burgyán and Havelda, 2011). The helper component-proteinase (HC-Pro) protein encoded by members of *Potyviridae* was the first PTGS suppressor identified (Kasschau and Carrington, 1998; Anandalakshmi et al., 1998). Subsequently, the 2b protein of *Cucumber mosaic virus* (Brigneti et al., 1998), the NSs protein of *Tomato spotted wilt virus* (Takeda et al., 2002), the P6 protein of *Strawberry vein banding virus* (Feng et al., 2018), the P20, P23 and capsid proteins of *Citrus tristeza virus* (Lu et al., 2004), the P6 protein of *Cauliflower mosaic virus* (CaMV) (Haas et al., 2008), the C2, C4 and V2 proteins of geminiviruses (Voinnet et al., 1999; Bisaro, 2006; Wang et al., 2005; Trinks et al., 2005; Vanitharani et al., 2004; Zrachya et al., 2007; Zhang et al., 2012), and several other viral proteins (Lakatos et al., 2004; Thomas et al., 2003; Wu et al., 2011) have been identified as suppressors of PTGS. The molecular mechanism of PTGS varies greatly with the viral suppressors of RNA silencing (VSRS) encoded by viruses from different families (Bisaro, 2006; Danielson and Pezacki, 2013). Geminivirus-encoded suppressors of TGS were first reported in 2009. These two suppressors of TGS, C2 or transcriptional activator protein (TrAP) encoded by *Begomovirus* and the L2 protein found in the *Curtovirus* genus, reverse TGS by nonspecifically inhibiting cellular transmethylation reactions (Buchmann et al., 2009). Thereafter, at least two geminivirus-encoded proteins, Rep (Rodriguez-Negrete et al., 2013) and V2 (Wang et al., 2014), a Cotton leaf curl Multan alphasatellite (CLCuMuA)-encoded replication-associated protein (Rep) (Abbas et al., 2018), and one Tomato yellow leaf curl China betasatellite (TYLCCNB)-encoded βC1 protein (Yang et al., 2011) were identified as suppressors of TGS and were shown to revert TGS by suppressing plant methylation reaction (Zhang et al., 2011).

Mulberry crinkle leaf virus (MCLV), which was recently identified from the woody plant mulberry (*Morus alba* L.), is a novel species of the family *Geminiviridae* but does not fit into the nine established genera of this family according to phylogenetic analysis of the complete nucleotide sequence of the genome or of the amino acid sequences of the putative replication-associated protein (RepA) and the putative capsid protein (Lu et al., 2015; Varsani et al., 2014, 2017). The genome of MCLV consists of six open reading frames (ORFs), four in the viral sense strand and two in the complementary sense strand. By BLASTp, the V1 protein (encoded by ORF3), V3 protein (encoded by ORF2), C1 protein (encoded by ORF5), and C2 protein (encoded by ORF6) were predicted to be a coat protein (CP), a movement protein (MP), a RepA protein, and a Rep protein, respectively, while the functions of V2 (encoded by ORF1) and V4 (encoded by ORF4) are not known because of their lack of detectable homology to proteins of known functions. With the exception of these features mentioned above, we know very little about this novel geminivirus.

To obtain insight into MCLV, we examined all proteins encoded by MCLV using an Agrobacterium-mediated transient expression assay in *Nicotiana benthamiana* 16c plants. Our results demonstrate that the V3 protein functions as a suppressor of PTGS. Furthermore, the results of subcellular localization experiments showed that the V3 protein is localized to the cytoplasm and nucleus of *N. benthamiana*.

2. Materials and methods

2.1. DNA extraction

The genomic DNA of MCLV and total DNA of *N. benthamiana* line 16c were extracted from MCLV-infected mulberry leaves and *N. benthamiana* line 16c leaves, respectively, using the Ezup Column Plant Genomic DNA Purification Kit (Sangon Biotech, Shanghai, China), following the manufacturer's instructions.

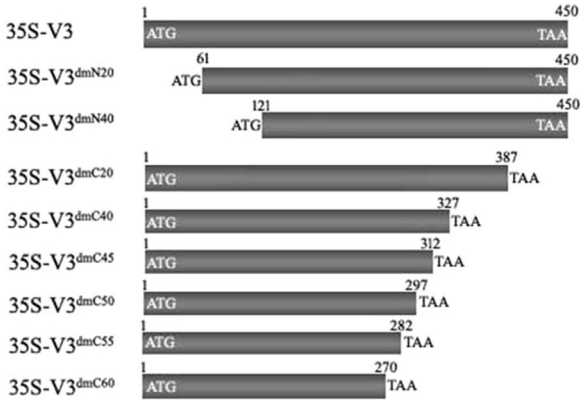

Fig. 1. Schematic representation of the expression cassettes of the mulberry crinkle leaf virus (MCLV) V3 and deletion mutants of V3. Here, 35S represents the 35S promoter of CaMV, and 'dm' indicates a deletion mutant. The numbers are the positions of the nucleotides. The white 'ATG' or 'TAA' in the black solid column is the original start or stop codon of the *V3* gene. The black "ATG' or 'TAA' outside the black solid column is the additional start or stop codon.

2.2. Plasmid construction

The sequences of the primers used in this study are listed in Table S1. Primers for the identification of viral RNA silencing suppressor and subcellular localization studies were designed according to the genomic sequence of MCLV deposited in GenBank (accession number: KR131749). The forward primers contained an *att*B1 adaptor, and the reverse primers contained an *att*B2 adaptor (italic lowercase sequence before each primer). The MCLV genes and the deletion mutants of the *V3* gene were amplified using the primers detailed in Table S1, and the resulting deletion mutants of the *V3* gene are shown in Fig. 1. Additionally, the green fluorescent protein (*gfp*) gene, 738 nt in size (Kon et al., 2007), was amplified from the total DNA extracted from *N. benthamiana* line 16c plants using GFP F/GFP R. The sequences of all primers used in this paper are shown in Table S1. In the primers for amplification of the deletion mutants of V3, a start codon (ATG) and the complementary sequence (TTA) to a stop codon (TAA) were added at the 5′-terminus following the *att*B1 adaptor and *att*B2 adaptor sequences for the N-terminal and C-terminal deletion mutants, respectively. All primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Sangon, Shanghai, China).

The vector pEarleyGate 101 was used for studies of subcellular localization. pEarleyGate 101 contains an enhanced CaMV 35S promoter to drive expression and allows the rapid recombinational cloning of cDNAs from Gateway entry vectors to produce in-frame C-terminal fusions of encoded proteins with yellow fluorescence protein (YFP)-human influenza hemagglutinin epitope (HA). To obtain V3-YFP fused proteins for transient expression in *N. benthamiana*, the *V3* gene without the stop codon was amplified from DNA extracted from MCLV-infected mulberry leaves using the primer pairs LoORF2F/LoORF2R.

PCR products were separated by 1% agarose gel electrophoresis, and the target fragments were recovered using the SanPrep Column DNA Gel Extraction Kit (Sangon Biotech, Shanghai, China). The recovered fragments were recombined with the pDONR221 vector (Invitrogen) using Gateway® BP Clonase® II enzyme mix (Invitrogen) following the manufacturer's instructions. The fidelity of the positive entry clones identified by colony PCR with M13 primer pairs was verified by DNA sequencing. The plasmid DNA of the recombinational entry clones was extracted using the EZ-10 Spin Column Plasmid Mini-Preps Kit (Sangon Biotech, Shanghai China) and linearized by the restriction enzyme *Mlu* I (TaKaRa). To identify the viral RNA silencing suppressor, the linearized plasmid DNAs were then individually recombined into the destination vector pEarleyGate 100, which contains a CaMV 35S promoter, with the help of the LR Clonase® II Plus enzyme

mix (Invitrogen). For simplicity, the resultant plasmids were named 35S-V2, 35S-V3, 35S-V1, 35S-V4, 35S-C1, 35S-C2, and 35S-ΔV3. For subcellular localization studies, the linearized plasmid DNAs were individually recombined into the destination vector pEarleyGate 101 with LR Clonase® II Plus enzyme mix (Invitrogen). For simplicity, the resultant plasmids were tentatively named 35S-V3-YFP.

2.3. Agrobacterium infiltration (agroinfiltration) assay and GFP observation

Binary vector constructs were introduced into *Agrobacterium tumefaciens* strain EHA105 by the freeze-thaw method (Chen et al., 1994). A single colony of *A. tumefaciens* containing the resultant plasmids was cultured overnight at 28 °C in Luria-Bertani (LB) medium supplemented with 50 μg/ml kanamycin and 20 μg/ml rifampin. Cells were collected by centrifugation for 2 min at 8000*g* and resuspended to a final concentration of OD_{600} = 1.0 in a buffer containing 0.5% (W/V) D-glucose, 50 mM 2-(N-morpholino)-ethane sulfonic acid (MES, pH 5.6), 2 mM Na_3PO_4, and 100 μM acetosyringone. The resuspended cells were incubated for 3 h at room temperature before infiltration.

For viral RNA silencing suppressor assays, equal volumes of an *Agrobacterium* suspension carrying one of the MCLV constructs and an *Agrobacterium* suspension harboring 35S-GFP binary plasmid were mixed. The mixtures were pressure-coinfiltrated into young leaves of 4-week-old chamber-grown transgenic *N. benthamiana* line 16c using a 1-ml syringe without a needle as described previously (Hamilton et al., 2002). Mixtures (1:1, v/v) of *Agrobacterium* suspension harboring 35S-GFP binary plasmid and of *Agrobacterium* suspension carrying the empty vector pEarleygate 100 or 35S-HC-Pro were coinfiltrated into different sites of the same leaf and used as negative and positive controls, respectively. The infiltrated *N. benthamiana* line 16c plants were placed in a dark growth chamber for 20 h at 22 °C and then cultured at 25 °C with 16-h daylengths. GFP fluorescence in the infiltrated plant leaves was monitored using a handheld longwave UV lamp (Black Ray Model B-100AP, UVP Corporation, USA). Each coinfiltration experiment was performed three times, and five *N. benthamiana* line 16c plants were coinfiltrated in each coinfiltration experiment.

For the MCLV-V3 subcellular localization study, equal volumes of an *Agrobacterium* suspension carrying 35S-V3-YFP constructs and an *Agrobacterium* suspension harboring 35S-SV40-RFP (nuclear localization marker fused to red fluorescent protein) were mixed and coinfiltrated into the young leaves of 4-week-old chamber-grown *N. benthamiana* plants using a 1-ml syringe without a needle as described previously (Hamilton et al., 2002). The infiltrated *N. benthamiana* plants were incubated in a dark growth chamber for 20 h at 25 °C and then cultured at 25 °C with 16-h daylengths. The subcellular localization of MCLV-V3 genes was directly visualized by a Leica TCS SP8 laser scanning confocal microscope (Leica Microsystems, Germany) at 48 h post inoculation.

2.4. RNA analysis

Total RNA was extracted from the *Agrobacterium*-infiltrated leaf patches using the RNAiso Plus Kit (TAKARA) following the manufacturer's instructions. The amplified products of the *gfp* gene were used to prepare a digoxigenin (DIG)-labeled DNA probe of the *gfp* gene using a DIG High Prime DNA Labeling and Detection Starter Kit (Roche, Switzerland). For the northern blot analysis of GFP mRNA, 30 μg of total RNA was separated on a 1.5% agarose-formaldehyde gel, transferred to a Hybond-N^+ membrane (Amersham, GE Healthcare Life Sciences) by capillary transfer, and hybridized and detected using a DIG High Prime DNA Labeling and Detection Starter Kit (Roche, Switzerland) according to the manufacturer's instructions. As a control, 5S rRNA bands visualized by ethidium bromide staining were used to monitor the equal loading of total RNA per lane.

2.5. Western blot analysis

The extraction of total protein from the infiltrated leaf zones and analysis of western blotting were carried out as described previously (Zrachya et al., 2007) with some modifications. In brief, 1 g of the infiltrated leaf zones was homogenized in 3 ml of the extraction buffer (75 mM Tris-HCL pH 6.5, 2% SDS, 8 M urea, and 2.0% β-mercaptoethanol). The resulting homogenate was centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was a protein sample used for western blotting analysis. For western blotting, protein samples were separated by 12% SDS-PAGE followed by semidry transfer to a polyvinylidene fluoride (PVDF) membrane using a Hoefer™ TE70X semidry transfer unit (Thermo Fisher Scientific, USA). The membrane was blocked with 5% nonfat milk in TBST buffer (10 mM Tris-Cl, pH 7.5, 150 mM NaCl and 0.05% Tween-20) for 2 h at room temperature and incubated with an anti-GFP primary antibody for 2 h at room temperature. After three 10-min washes in TBST buffer, the membrane was incubated with goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (HRP) (Beyotime, China) for 1 h at room temperature. Finally, the membrane was washed thoroughly with TBST buffer. Immunoreactive bands were visualized using the ECL Western Blotting Detection Kit (GE Healthcare) according to the manufacturer's instructions. Images were acquired using a Tanon 5200 Multi imaging system (Tanon, Shanghai, China) following the manufacturer's specifications.

3. Results

3.1. Screening for RNA silencing suppressors encoded by MCLV

To identify the potential suppressor of PTGS encoded by MCLV, the six proteins predicted to be encoded by the virus were assessed for their ability to suppress the silencing of a GFP reporter gene in the GFP-transgenic *N. benthamiana* line 16c. All leaf patches coinfiltrated with empty vector/35S-GFP, 35S-HC-Pro/35S-GFP, or 35S-driven constructs for the expression of the MCLV genes/35S-GFP showed the same intense GFP fluorescence at 2 days post infiltration (dpi) (data not shown). In the leaf patches coinfiltrated with a negative control (empty vector/35S-GFP), 35S-V2/35S-GFP, 35S-V1/35S-GFP, 35S-V4/35S-GFP, 35S-C1/35S-GFP, or 35S-C2/35S-GFP, the GFP fluorescence intensity started to decline at 3 dpi and had almost completely disappeared at 6 dpi (Fig. 2A). In contrast, in leaf patches coinfiltrated with 35S-V3/35S-GFP or 35S-HC-Pro/35S-GFP which were used as positive controls, the GFP fluorescence remained strong at 6 dpi (Fig. 2A). These findings indicate that only the product of the *V3* gene has the ability to suppress PTGS.

To confirm whether GFP mRNA levels in the infiltrated leaves were correlated with the variable GFP fluorescence strength, the levels of accumulation of GFP transcripts were analyzed by northern blotting. The accumulation levels of GFP mRNA in the leaf patches coinfiltrated with 35S-HC-Pro/35S-GFP or 35S-V3/35S-GFP were significantly higher at 6 dpi than those in the negative control (Fig. 3E). These results were further confirmed by western blot analysis of the GFP protein using anti-GFP antibodies, showing that changes in the levels of GFP transcripts generally lead to changes in the total amounts of GFP proteins accumulated in the GFP-transgenic *N. benthamiana* line 16c. The results of the western blot analysis indicated that, as expected, the amounts of GFP protein in the leaf patches coinfiltrated with 35S-HC-Pro/35S-GFP or 35S-V3/35S-GFP were high, which was consistent with the levels of GFP transcripts. These results implied that the expression of the *V3* gene suppressed transgene-induced RNA silencing and that the GFP mRNA in the GFP-transgenic *N. benthamiana* line 16c was expressed at high levels. Correspondingly, the amount of GFP protein was elevated.

To exclude the possibility that RNA transcribed from the *V3* gene was responsible for the RNA silencing suppressor activity described

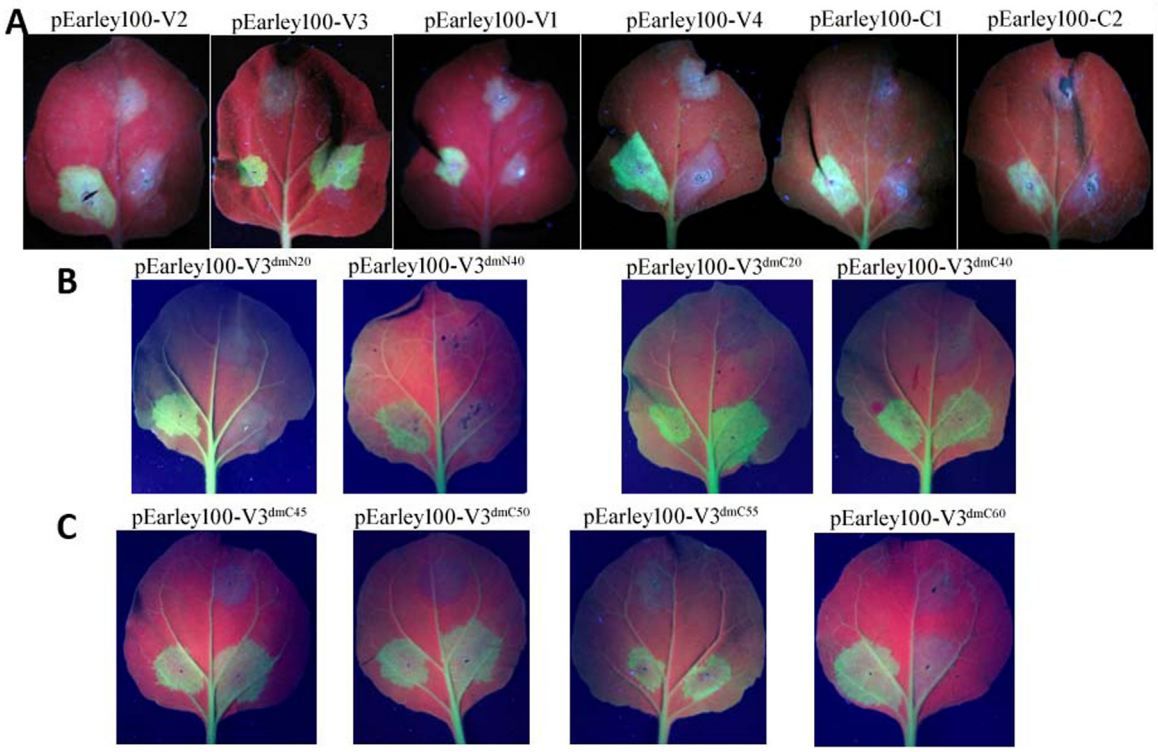

Fig. 2. Leaves of the *N. benthamiana* line 16c coinfiltrated with *Agrobacterium* cultures carrying 35S-GFP and *Agrobacterium* cultures harboring different constructs. The top, left and right of the leaves were coinfiltrated with negative control/35S-GFP, positive control/35S-GFP, and the genes of MCLV (panel A) and the deletion mutants (panel B, and C) of V3/35S-GFP, respectively.

above, a *V3* gene mutant, 35S-ΔV3, was constructed. ΔV3 lacked the protein-coding function because the first nucleotide (A) of the *V3* gene start codon was eliminated during amplification. In comparison to that in the positive control, the GFP fluorescence intensity in leaf patches coinfiltrated with empty vector pEarleyGate 100/35S-GFP or 35S-ΔV3/35S-GFP started to decline at 3 dpi and was hardly detectable at 6 dpi (Fig. 3A, D). The results showed that the V3 protein, but not the RNA transcribed from the *V3* gene, has RNA silencing suppression activity.

3.2. The N-terminal 94 amino acid residues of V3 are sufficient to maintain the gene silencing suppression activity of V3

Four deletion mutants were constructed and coinfiltrated with 35S-GFP into leaves of *N. benthamiana* 16c plants to determine whether the N-terminus region or the C-terminus region is required for the silencing suppressor activity of V3. $V3^{dmN20}$ and $V3^{dmN40}$ lacked the first 20 and 40 amino acids of the N-terminus of V3, respectively, and $V3^{dmC20}$ and $V3^{dmC40}$ lacked the last 20 and 40 amino acids from the C-terminus of V3. GFP fluorescence was hardly detectable in the leaf patches coinfiltrated with $V3^{dmN20}$/ 35S-GFP or $V3^{dmN40}$/35S-GFP at 6 dpi, similar to the negative control. In contrast, strong GFP fluorescence was readily detectable in leaf patches coinfiltrated with $V3^{dmC20}$/35S-GFP, $V3^{dmC40}$/35S-GFP, or 35S-V3/35S-GFP (Fig. 2B). These results indicate that the amino acids at the N-terminus of V3 are required for its silencing suppression activity. The suppression activity was completely lost if the first 20 aa of the N-terminus of V3 were deleted. In contrast, the amino acids at the C-terminus of V3 are nonessential, and the gene silencing suppression remained unchanged when the last 40 aa of the C-

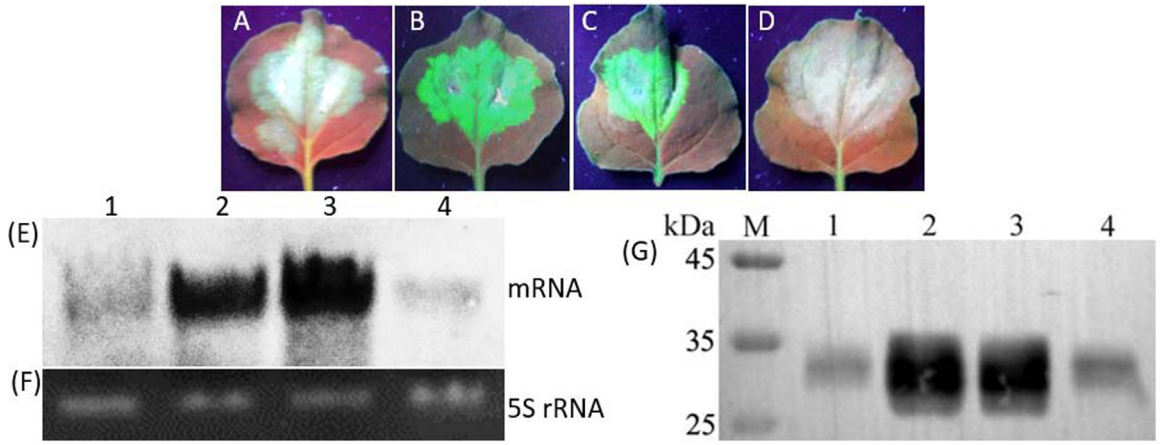

Fig. 3. Northern blot analysis of GFP mRNA (E) and western blot analysis of GFP (G) in the leaf patches of the *N. benthamiana* line 16c coinfiltrated with *Agrobacterium* cultures carrying 35S-GFP and *Agrobacterium* harboring empty pEarleyGate 100 vector (A, lane 1), 35S-Hc-Pro (B, lane 2), 35S-V3 (C, lane 3), or 35S-ΔV3 (D, lane 4). (F): The 5S rRNA band on the agarose gel stained with ethidium bromide indicates equal loading of the samples in E. M: Protein marker.

terminus of V3 were deleted.

To define the minimal V3 amino acid sequence required for suppressor activity, four additional deletion mutants, $V3^{dmC45}$, $V3^{dmC50}$, $V3^{dmC55}$, and $V3^{dmC60}$, which lacked 45, 50, 55, and 60 amino acids from the C-terminus of V3, respectively, were constructed and coinfiltrated with 35S-GFP into leaves of *N. benthamiana* 16c plants. Leaf patches coinfiltrated with $V3^{dmC45}$/35S-GFP, $V3^{dmC50}$/35S-GFP, and $V3^{dmC55}$/35S-GFP, similar to those with intact V3, exhibited strong GFP fluorescence intensity at 6 dpi, while leaves with $V3^{dmC60}$/35S-GFP, similar to the negative control, did not exhibit GFP fluorescence at 6 dpi (Fig. 2C). These results indicated that the gene silencing suppression activity of V3 remained if 55 aa at the C-terminus were deleted and was abolished if 60 aa were deleted.

These results indicate that only the 94 N-terminal amino acids of V3 are required for suppressor activity.

3.3. Subcellular localization of V3 protein encoded by MCLV

The *V3* gene without a stop codon was fused in-frame to the 5′ end of the YFP gene in pEarleyGate 101 and coexpressed with 35S-SV40-RFP in *N. benthamiana* leaves using an *Agrobacterium*-mediated transient expression system to determine the subcellular localization of V3 encoded by MCLV. As shown in Fig. 4, some V3-YFP fusion proteins were dispersed throughout the cell cytoplasm in the form of irregular bodies, and others were colocalized with the nucleus-localization marker RFP-fused SV40 protein in the form of aggregated bodies (Fig. 4). These results indicated that the V3 protein is sublocalized to both the cytoplasm and the nuclei of the host plant cells.

Online prediction was performed using WoLF PSORT software (http://psort.hgc.jp), cNLS Mapper software for the prediction of importin α-dependent nuclear localization signals (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) and Virus-mPLoc: Predicting the subcellular localization of viral proteins within host and virus-infected cells (http://www.csbio.sjtu.edu.cn/bioinf/virus-multi/). The results showed that V3 contains a monopartite nuclear localization signal (NLS) consisting of seventeen amino acid residues (aa 118–134) in the C-terminal region. To examine the importance of the putative NLS to the nuclear localization of V3, a V3 deletion mutant expression vector (35S-$V3^{dmNLS}$) in which the putative NLS sequence was deleted was constructed by artificially synthesizing the full-length *V3* gene without the NLS sequence and stop codon ($V3^{dmNLS}$), amplifying $V3^{dmNLS}$ by PCR using the specific primers LoORF2F/LoORF2R (Table S1), and finally recombining $V3^{dmNLS}$ into the destination vector pEarleyGate 101. For simplicity, the deletion mutant expression vector was tentatively named 35S-$V3^{dmNLS}$-YFP (Fig. 4 A). The transient coexpression of 35S-$V3^{dmNLS}$-YFP and 35S-SV40-RFP in *N. benthamiana* leaves showed that the $V3^{dmNLS}$-YFP fusion proteins were observed only in the cytoplasm, and none of them colocalized with the nucleus-localization marker RFP-fused SV40 protein (Fig. 4), suggesting that aa 118–134 are responsible for the nuclear localization of the V3 protein.

4. Discussion

PTGS plays a key role in defending plants against viral pathogens. To counteract the antiviral properties of PTGS, plant viruses have evolved to encode one or more PTGS suppressors to counteract host defense (Vance and Vaucheret, 2001). MCLV is a novel geminivirus recently identified from mulberry and is a natural pathogen of mulberry trees. The functions of gene products encoded by MCLV have not been confirmed experimentally. We speculate that to counteract host small RNA-mediated antiviral defense, MCLV must encode at least one suppressor of PTGS. In this paper, we examined the silencing suppressor

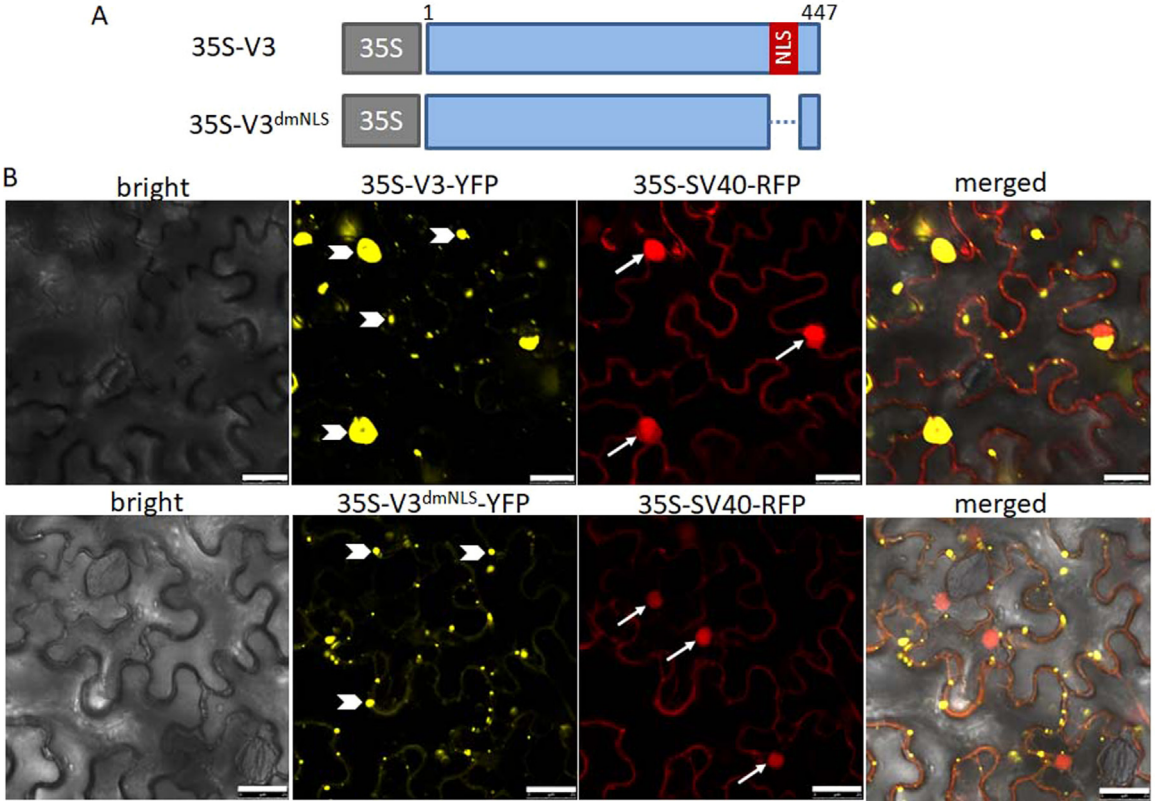

Fig. 4. Schematic representation of the expression cassettes (A) and cellular and subcellular distribution in *N. benthamiana* plants (B) of MCLV V3 (upper panel) and its deletion mutant (lower panel). Each coinfiltration experiment was performed on five leaves of *N. benthamiana* plants, three of which were selected randomly to observe the cellular and subcellular distribution of V3 and its deletion mutant. Here, 'dm' indicates a deletion mutant, and 35S represents the 35S promoter of CaMV. NLS, nuclear location signal. Dashed lines indicate the deleted regions in the *V3* gene. The bar in these images indicates 25 μm.

activity of all six proteins encoded by MCLV and confirmed for the first time that V3 acts as a suppressor of PTGS. The minimal amino acid sequence that is necessary and sufficient for the silencing suppressor activity of V3 was also defined by a combination of experiments using a series of N- or C-terminally truncated V3 mutants. This minimal sequence is 94 aa at the N-terminus of V3. In other words, 94 aa at the N-terminus of V3 is sufficient to maintain the PTGS suppressor activity of V3, and 55 aa at the C-terminal did not contribute to this function.

Geminiviruses have circular, single-stranded DNA (ssDNA) genomes. These viruses do not encode polymerases and are therefore dependent on the host machinery for replication and transcription. Their replication occurs in the nucleus of an infected cell by a rolling circle replication (RCR) mechanism, and their transcription occurs on double-stranded (ds) DNA replicative intermediate templates (Pilartz and Jeske, 2003). The viral DNA genome and viral mRNA exist simultaneously in the infected plant cell during geminivirus infection. Geminivirus infection triggers the host PTGS and/or TGS mechanism, which suppresses transcription of the viral DNA genome and translation of the viral mRNA, probably via the Dicer-catalyzed processing of structured regions in the viral mRNA, the action of host RDR on aberrant or overexpressed virus transcripts, overlapping read-through transcription from the divergent promoters in the intergenic region (IR), or some combination of these mechanisms (Bisaro, 2006). Generally, geminiviruses encode more than one silencing suppressor to counter TGS and PTGS (Bisaro, 2006). For example, the Cotton leaf curl Multan virus/Cotton leaf curl Multan betasatellite (CLCuMuV/CLCuMuB) complex encodes five suppressors, and the alphasatellite of the complex encodes an additional suppressor (Abbas et al., 2018; Nawaz-ul-Rehman et al., 2010; Amin et al., 2011; Cui et al., 2005). Among the identified geminivirus-encoded suppressors, some show the ability to suppress PTGS, TGS, or both. The molecular mechanism used by geminivirus-encoded suppressors of RNA silencing is complex and varies depending on the geminivirus. The suppression activity varies depending on the suppressors. For example, L2/C2 proteins exhibit relatively weak suppression of PTGS but strong suppression of TGS mediated by the siRNA-directed methylation of DNA and histone proteins (Buchmann et al., 2009). The V3 protein was found to be the only PTGS suppressor encoded by MCLV. There are two probable explanations for this result. One is that the viruses in distinct genera of the family *Geminiviridae* have evolved to use different numbers and types of functional proteins encoded by the virus to suppress PTGS. The other explanation is that MCLV also encodes other suppressors of PTGS that were not identified by the method used in this paper. Additionally, whether MCLV encodes a TGS suppressor(s) needs to be studied further.

In general, the physiological functions of a protein are closely related to its localization in cells. The subcellular localization studies provide important insights into the functions of the protein encoded by MCLV. The results in this paper showed that V3 was localized not only in the cytoplasm but also in the nucleus. A putative NLS between aa 118–134 in the C-terminal region plays an important role in the nuclear localization of V3; deleting this region caused cytoplasmic redistribution and abolished the nuclear localization of V3. The subcellular localization pattern of the V3 protein implies that V3 may possess the property of nucleocytoplasmic shuttling, which might play an important role in successful MCLV infection. During the life cycle of geminiviruses, viral DNA enters the nucleus of the infected cell for the replication, transcription, and encapsidation of viral particles. For systemic infection, the progeny viruses must be transported out of the nucleus, through plasmodesmata, and then to other plant tissues via the phloem. In geminiviruses with bipartite genomes, the heavy responsibility of shuttling the progeny viruses out of the nuclei is carried out by the nuclear shuttle protein (NSP) encoded by DNA B (Sanderfoot et al., 1996). In monopartite viruses, this shuttle process is mediated by CP or MP (Liu et al., 1999; Kotlizky et al., 2000; Unseld et al., 2001). We thus speculate that in MCLV, this shuttle process is most likely mediated by V3.

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Ethics approval and consent to participate

All authors have read the manuscript and approved its submission to Virology. This article does not contain any studies with human participants or animals performed by any of the authors.

Competing interests

The authors declare that they have no competing interests.

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