A Single Amino Acid Substitution in the Intervening Region of 129K Protein of Cucumber Green Mottle Mosaic Virus Resulted in Attenuated Symptoms

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ABSTRACT

Cucumber green mottle mosaic virus (CGMMV), a member of the genus *Tobamovirus*, is a major threat to economically important cucurbit crops worldwide. An attenuated strain (SH33b) derived from a severe strain (SH) of CGMMV caused a reduction in the viral RNA accumulation and the attenuation of symptoms, and it has been successfully used to protect muskmelon plants against severe strains in Japan. In this study, we compared GFP-induced silencing suppression by the 129K protein and the methyltransferase domain plus intervening region (MTIR) of the 129K protein between the SH and SH33b strains, respectively. As a result, silencing suppression activity (SSA) in the GFP-silenced plants was inhibited efficiently by the MTIR and 129K protein of SH strain, and it coincided with drastically reduced accumulation of GFP-specific small

interfering RNAs (siRNAs) but not by that of SH33b strain. Furthermore, analyses of siRNA binding capability (SBC) by the MTIR of 129K protein and 129K protein using electrophoretic mobility shift assay revealed that SBC was found with the MTIR and 129K protein of SH but not with that of SH33b, suggesting that a single amino acid mutation (E to G) in the MTIR is responsible for impaired SSA and SBC of SH33b. These data suggest that a single amino acid substitution in the intervening region of 129K protein of CGMMV resulted in attenuated symptoms by affecting RNA silencing suppression.

Keywords: attenuated virus, biological control, cucumber green mottle mosaic virus, RNA silencing suppression, tobamovirus

Cucumber green mottle mosaic virus (CGMMV) is a member of the genus *Tobamovirus* in the family Virgaviridae with a positive-sense, single-stranded RNA of 6.4 kb and encodes 186 kDa (K), 129K, 29K, and 17.3K proteins (Ugaki et al. 1991); 186K is the read-through product of 129K, and both are RNA replication proteins. The 29K and 17.3K proteins are movement and coat proteins (CPs), respectively. CGMMV is prevalent particularly in cucurbitaceous crops, such as watermelon, muskmelon, and cucumber (Ainsworth 1935; Dombrovsky et al. 2017; Fletcher et al. 1969; Inoue et al. 1967; Rajamony et al. 1987). An attenuated strain (SH33b) of CGMMV was isolated through nitrous acid, ultraviolet (UV) irradiation, and high-temperature treatment, and it has been used for cross-protection of muskmelon in greenhouses in Japan (Motoyoshi and Nishiguchi 1988; Nishiguchi and Kobayashi 2011; Oosawa 1990).

Plants have developed a sophisticated system termed RNA silencing against the invading viruses (Baulcombe 1999; Boshier and Labouesse 2000; Burguán 2008; Catalanotto et al. 2000; Matzke et al. 2001; Waterhouse et al. 2001). However, many viruses have evolved counterdefensive strategies to counteract RNA silencing by encoding a viral suppressor of RNA silencing (VSR), a protein that inhibits RNA silencing (Burguán 2008;

Csorba et al. 2015). A number of VSRs have been reported, including HC-Pro protein of tobacco etch virus (Anandalakshmi et al. 1998; Kasschau and Carrington 1998), P19 protein of tomato bushy stunt virus (Silhavy et al. 2002), 2b protein of cucumber mosaic virus (CMV) (Guo and Ding 2002), p38 protein of turnip crinkle virus (Thomas et al. 2003), and p21 protein of beet yellows virus (Reed et al. 2003). *Tobamovirus* replication proteins are reported to be responsible for RNA silencing suppression (Csorba et al. 2007; Ding et al. 2004; Kubota et al. 2003; Várallyay and Havelda 2013; Vogler et al. 2007; Wang et al. 2012). These proteins suppress RNA silencing by preventing small interfering RNA (siRNA) generation, inhibiting the incorporation of siRNAs into effector complexes, or interfering with one of the effector complexes in different RNA silencing pathways (Burguán 2008).

Previously, we reported that RNA silencing was suppressed by a CGMMV severe strain SH but partially by strain SH33b, and we found that the 129K/186K protein region of the genome was responsible for RNA silencing suppression activity (SSA) (Ali et al. 2016). Mutant plasmid construct from an attenuated strain of tomato mosaic virus (ToMV) showed much lower extent of RNA silencing suppression compared with severe strain (Kubota et al. 2003). Furthermore, two types of amino acid substitutions at amino acid position 284 (R284A or R284S) in the methyltransferase domain (MT) of CGMMV isolate responsible for stable attenuated symptoms have been implicated as potential candidates for cross-protection (Liu et al. 2017). However, it is not known whether these two mutants affect RNA SSA. In this study, as a step toward better understanding the underlying mechanism of symptom attenuation in the SH33b strain in relation to silencing suppression, we further investigated the SSA of the 129K protein as well as methyltransferase domain plus intervening regions (MTIR) from SH33b and SH strains of CGMMV. The small interfering RNA binding capability (SBC) of the MTIR and 129K proteins in the two strains was also analyzed using electrophoretic mobility shift assay (EMSA), and

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the three-dimensional (3D) structures of the MTIR of 129K proteins from the two strains were predicted.

MATERIALS AND METHODS

Plants and viruses. Muskmelon (*Cucumis melo* 'Earls Favorite') and *GFP*-overexpressed (no. 11), and *GFP*-silenced (no. 24) *Nicotiana benthamiana* (Shimono et al. 2005) were used for virus inoculation and/or agroinfiltration. Onion (*Allium cepa*) and rice (*Oryza sativa*) were used for particle bombardment. The complementary DNA (cDNA) clones of pCG3A (severe strain) and pCG22 (attenuated strain) were described previously (Tan et al. 1997; Ugaki et al. 1991).

Plasmid constructs. DNA fragments of the MTIR (1,899 base pairs) and 129K protein (3,435 base pairs) from cDNA plasmids of pCG3A and pCG22 were amplified by PCR using forward primer 5'-CGGGATCCATGGCAAACATTAATGAACAAATC-3' containing the *Bam*HI site (underlined) and reverse primer 5'-CGGAGCTCCTACATCAACACTATATCCTCCATATC-3' (MT) or 5'-CCGAGCTCCTATTTGGTAGGCACAGTGGTAGC-3' (129K) containing the *Sac*I site (underlined), respectively. The fragments were digested with *Bam*HI and *Sac*I restriction enzymes and ligated into similarly digested pBI121 vector. The resulting plasmids (pCGSH-MTIR, pCGSH-129K, pCG33b-MTIR, and pCG33b-129K) were used for particle bombardment. The plasmid of p35S-dsGFP (Miki and Shimamoto 2004) was digested with *Bam*HI and *Sac*II restriction enzymes to remove dsGFP fragment and blunt-end religated; the resulting plasmid of empty vector (EV) was used for particle bombardment as a control. For the *Agrobacterium*-mediated transient gene expression, the fragments of the MTIR and 129K protein from both strains were amplified by PCR using a forward primer 5'-CGGGATCCATGGCAAACATTAATGAACAAATC-3' (*Bam*HI; underlined) and a reverse primer 5'-CGGAATTCCTACATCAACACTATATCCTCCATATC-3' (MTIR) or 5'-CCGAATTCCTATTTGGTAGGCACAGTGGTAGC-3' (129K; *Eco*RI; underlined), and the fragments were digested with *Bam*HI and *Eco*RI restriction enzymes and inserted into similarly digested pBICP35 (Takeda et al. 2005), which was digested by the same enzymes. The resulting plasmids were named pSH-MTIR, pSH-129K, pSH33b-MTIR, and pSH33b-129K. For siRNA binding analysis, the fragments of the MTIR and 129K protein from SH and SH33b strains were amplified by PCR using gateway attB forward primer 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTC + MTIR or 129K-3' (without start codon) and reverse primer 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTC + MTIR or 129K-3' and recombined into the entry vector pDONR221 using Gateway BP Clonase II Enzyme mix (Invitrogen). The target fragments in pDONR221 vector were further recombined into the Gateway-compatible pEarleyGate-201 using Gateway LR Clonase Enzyme mix (Invitrogen), resulting in pCGSHt-MTIR, pCGSHt-129K, pCG33bt-MTIR, and pCG33bt-129K that expressed the hemagglutinin (HA)-tagged MTIR and 129K proteins. All plasmids were verified by DNA sequencing.

Transient suppression assay by particle bombardment. Particle bombardment was performed as described previously (Chen et al. 2008, 2010). Fluorescence images of *GFP*- and DsRed-expressing cells in all treatments were captured by a C7070 digital camera (Olympus; <https://olympus-imaging.jp>) using the same illumination intensity and exposure time under an IX70 fluorescence microscope (Olympus; <https://www.olympus-lifescience.com/en/microscope-resource/>).

Transient suppression assay by agroinfiltration. The *Agrobacterium*-mediated transient suppression assay using *Agrobacterium tumefaciens* strain C58C1 was performed as described previously (Kubota et al. 2003). The concentration of the *Agrobacterium* carrying respective plasmid constructs in all infiltration experiments was normalized to 1.0 optical density (OD) at 600 nm. Infiltrated plants were grown at 22°C, and GFP

fluorescence was observed at 3 days postinoculation (dpi) with a 100-W handheld UV lamp (UV Products). Photographs were taken using a digital camera (Fujifilm; FinePix S1 Pro).

RNA isolation and analysis. Total RNA extraction and northern blot analysis for *GFP* messenger RNA (mRNA) and *GFP*-specific siRNAs were performed as described previously (Chen et al. 2008; Shimono et al. 2005). The band intensity of *GFP* mRNA and *GFP*-specific siRNAs in the captured images was measured using ImageJ software (<https://imagej.nih.gov/ij/index.html>). Total RNA was used for reverse transcription PCR (RT-PCR) and reverse transcription quantitative PCR (RT-qPCR) for *GFP* mRNA and CGMMV RNA. Synthesis of cDNA was performed using 1 µg of total RNA and M-MLV Reverse transcription (Nippon Gene) with a reverse primer SH-CP-3' (5'-AGCGTTAAGCGACTCAGC-3'), and the CP fragment was amplified with the CP-specific primers, forward primer SH-CP-5' (5'-CCGGATATCATGGCTTACAATCCGATCA-3') and SH-CP-3'. The PCR amplifications were performed at 94°C for 2 min followed by 30 cycles of amplification (94°C for 30 s, 56°C for 20 s, and 72°C for 40 s). *GFP* was amplified as reported previously (Chen et al. 2008). *Actin* and *L23* genes were used as internal standard for RT-PCR and RT-qPCR, respectively (Liu et al. 2012).

siRNA binding analysis. Crude HA-tagged protein was extracted from *N. benthamiana* leaves at 2 days after agroinfiltration with respective plasmids expressing MTIR or 129K protein of SH (pCGSHt-MTIR and pCGSHt-129K) and SH33b (pCGSH33bt-MTIR and pCGSH33bt-129K) using a protocol described previously by Kurihara et al. (2007); 21-nucleotide double-stranded small interfering RNAs (ds-siRNAs) were in vitro transcribed and digoxigenin (DIG) labeled as described (Chen et al. 2015, 2016). Western blot and EMSAs were performed using previously described protocols (Kurihara et al. 2007; Valli et al. 2008, 2011).

3D structure modeling analysis. The 3D structures of the MTIR from the SH and SH33b strains were modeled using the I-TASSER web server (Zhang 2008) based on multiple threading alignments by LOMETS and iterative TASSER simulations (Roy et al. 2010). The protein secondary structure and the protein with high structure similarity in the Protein Data Bank (<https://www.rcsb.org/>) were obtained using PyMol (<https://pymol.org/2/>).

RESULTS

CGMMV SH infection suppressed RNA silencing. To investigate whether RNA silencing was suppressed by CGMMV infection, crude sap from CGMMV SH-infected leaves was used to inoculate *GFP*-silenced *N. benthamiana* plants (no. 24) (Shimono et al. 2005). At 16 dpi, severe mosaic symptoms were observed in coincidence with high *GFP* expression and viral accumulation on systemically infected leaves, whereas the *GFP* expression and disease symptoms did not appear in the mock-inoculated leaves (Fig. 1). Accumulation of *GFP* mRNA and CP in the high-fluorescence area in the infected leaves was higher than that in the low-fluorescence area (Fig. 1). Northern blot, RT-PCR, and RT-qPCR analyses showed a higher level of *GFP* mRNA and viral accumulation in systemically infected leaves than the mock-inoculated leaves of plant no. 24. These results demonstrate that CGMMV infection suppressed *GFP* silencing in plant no. 24, and they lead to the next step to examine differential suppression activities between the two strains, SH and SH33b.

Differential RNA SSAs between the SH and SH33b strains of CGMMV using particle bombardment. To compare the RNA SSAs between the two CGMMV strains, a transient suppression assay was conducted by particle bombardment of the plasmids of severe and attenuated strains that carry either the fragments of the MTIR (1,899 base pairs) or 129K protein (3,435 base pairs) (Fig. 2A). p35S-GFP and p35S-dsGFP were used as reporter and inducer of RNA silencing, respectively, and *Discosoma* red fluorescent

protein gene (*DsRed*; pUbq-DsRed) was used as an internal control (Miki and Shimamoto 2004). The cobombardment mixtures with different combinations of plasmids (5 µg of each plasmid and 10 µg of each silencing inducer) include (i) pUbq-DsRed (expressing *DsRed*), p35S-GFP (expressing *GFP*), EV, and pBI221 (control vector); (ii) pUbq-DsRed, p35S-GFP, p35S-dsGFP (silencing inducer), and pBI221; (iii) pUbq-DsRed, p35S-GFP, p35S-dsGFP, and pCGSH-MTIR; (iv) pUbq-DsRed, p35S-GFP, p35S-dsGFP, and pCG33b-MTIR; (v) pUbq-DsRed, p35S-GFP, p35S-dsGFP, and pCGSH-129K; and (vi) pUbq-DsRed, p35S-GFP, p35S-dsGFP, and pCG33b-129K. When mixture (i) was cobombarded into the epidermal cells of onion, both GFP and *DsRed* fluorescent expressions reached near the maximum levels at 36 h postbombardment (Fig. 2B and C). For consistency, samples were taken at 36 h postbombardment for analysis in subsequent experiments. Similarly, fluorescent images were obtained from the same GFP-expressing and *DsRed*-expressing cells using their respective fluorescence filters. The number of cells with GFP or *DsRed* fluoresce was counted from six independent experiments

($n = 6$) for quantitative analysis. When mixture (ii) that contains a silencing inducer (p35S-dsGFP) to trigger specific silencing against *GFP* expression was cobombarded, the number of GFP fluoresced cells was drastically reduced (Fig. 2B and C). When mixtures (iii) and (v), which carry a silencing suppressor from the SH strain, were cobombarded, the number of GFP fluoresced cells increased. However, when mixtures (iv) and (vi), which include a silencing suppressor from the SH33b strain, were cobombarded, the number of GFP fluoresced cells did not increase. In contrast, the number of cells with *DsRed* fluorescence remained high in all of the treatments. These results suggest that CGMMV strain SH but not SH33b suppressed *GFP* silencing, likely by encoding the strong silencing suppressor proteins 129K/MTIR. The similar results were obtained using rice shoot tissues (data not shown).

Comparison of RNA SSAs and SBCs between the strains of SH and SH33b by the *Agrobacterium*-mediated transient expression. To further investigate RNA SSAs of CGMMV strains, the *Agrobacterium*-mediated transient suppression assay

Fig. 1. Symptoms associated with suppression of RNA silencing in a transgenic *GFP*-silenced *Nicotiana benthamiana* line (no. 24) infected by cucumber green mottle mosaic virus (CGMMV) SH strain. **A**, Viral symptoms and GFP fluorescence in the upper leaves were monitored in **i** and **ii**, a CGMMV SH-infected plant and **iii** and **iv**, a mock-inoculated plant at 16 days postinoculation. Images were taken under **i** and **iii**, white light and **ii** and **iv**, ultraviolet light. **i** and **ii**, Arrows indicate low (L) and high (H) intensity of fluorescence of GFP. The white scale bar represents 2 cm. **B**, Level of *GFP* messenger RNA accumulation detected by northern blot analysis. H and L indicate high and low intensity of GFP fluorescence in CGMMV SH-infected leaves, respectively. Mock, inoculation with buffer; no. 11, *GFP*-overexpressed plant; no. 24, *GFP*-silenced plant; NT, nontransformed plant; rRNA, ribosomal RNA. **C**, *GFP* expression and viral accumulation (coat protein [CP]) were detected by reverse transcription PCR analysis. The *L23* gene was used as an internal control. **D**, *GFP* expression and **E**, viral accumulation were also detected by reverse transcription quantitative PCR analysis. The *actin* gene was used as an internal control. Data are mean $\pm$ standard error ($n = 3$). Values with different letters differ significantly among inoculated leaves in Tukey's test at $P < 0.05$.

was performed. As described above, two fragments with only one-amino acid difference in the MTIR (1,899 base pairs) and 129K protein (3,435 base pairs) derived from the two CGMMV strains were cloned into a pBICP35 expression vector, resulting in the plasmids pSH-MTIR, pSH-129K, pSH33b-MTIR, and pSH33b-129K. GFP fluorescence in infiltrated leaves from *GFP*-silenced *N. benthamiana* plants (no. 24) was observed under a UV light at 3 dpi (Fig. 3A). GFP fluorescence was much stronger in the area expressing the 129K protein from strain SH (pSH-129K) than in the area expressing the same protein from strain SH33b (pSH33b-129K) (Fig. 3A). *GFP* silencing was suppressed by expressing MTIR from pSH-MTIR (SH) but not by pSH33b-MTIR (SH33b) in *GFP*-silenced *N. benthamiana* plants (no. 24) (Fig. 3A). Northern blot analysis showed that the level of *GFP* mRNA in leaves expressing the MTIR or 129K protein from strain SH was higher than that in leaves expressing the MTIR or 129K protein from strain SH33b (Fig. 3B). The level of *GFP* siRNAs in leaves expressing the MTIR or 129K protein from strain SH was much lower than that for the MTIR or 129K protein from strain SH33b (Fig. 3C). These results indicate that the MTIR or 129K protein from CGMMV SH was a stronger suppressor of RNA silencing compared with CGMMV strain SH33b. These data also suggest that the MTIR and 129K proteins of CGMMV strain SH independently suppressed *GFP*-induced RNA silencing in *N. benthamiana* plants.

MTIR and 129K proteins of SH and SH33b differentially bound to siRNA in EMSA. To further examine whether 129K of CGMMV can bind siRNA and if this capacity is correlated with its SSA, we performed EMSA using HA-tagged MTIR or 129K protein from *N. benthamiana* leaves agroinfiltrated with the plasmids expressing the MTIR and 129K protein of SH (pCGSHt-MTIR and pCGSHt-129K) or SH33b (pCGSH33bt-MTIR and pCGSH33bt-129K), respectively. The MTIR and 129K proteins of SH caused a shift of DIG-labeled 21-nucleotide ds-siRNA and produced a protein:siRNA complex (Fig. 3D, upper left and lower left panels). In contrast, the MTIR and 129K protein of SH33b did not affect the 21-nucleotide ds-siRNA mobility and were unable to generate a protein:siRNA complex at detectable levels (Fig. 3D, upper right and lower right panels). These results indicated that a single amino acid substitution from E to G at 480 amino acid position in the intervening region (IR) of SH33b impaired SBC.

Structural difference in the MTIR of 129K protein between SH and SH33b strains. As previously discussed, a single nucleotide change (A to G) in the MTIR of 129K protein of SH33b causes an amino acid substitution E to G at amino acid position 480 (Tan et al. 1997). To further investigate whether impairing SSA and SBC in the SH33b is owing to any structural changes in the MTIR of 129K protein, the 3D structures of the MTIR 129K protein from both SH and SH33b strains were predicted using web server I-TASSER (Zhang 2008). An appropriate template was identified by LOMETS by searching for homologous proteins in a representative Protein Data Bank structure library (Roy et al. 2010). The protein's secondary structures as well as proteins with high structural similarity in the Protein Data Bank were then obtained. Template 3ungC (Cmr2) (Cocozaki et al. 2012), the largest and essential subunit of a CRISPR (clusters of regularly interspaced short palindromic repeats) RNA-Cas (CRISPR-associated) RNA silencing complex, was generated and ranked as the best template (Wu and Zhang 2007). The MTIR of 129K protein in SH had a high coverage (81.5%) of template 3ungC. The 3D structure of the MTIR of 129K protein was triangular, containing many α -helices and loops as well as several β -sheets (Fig. 4A and B). The SH's MTIR domain had amino acid E at position 480 in the α -helix (Fig. 4C). However, the MTIR from SH33b had G at this position, which was between a coil structure and an α -helix structure (Fig. 4D). The amino acid substitution resulted in a shorter α -helix but longer random coil. Comparing the exposed surface

area of the MTIR of 129K protein between SH and SH33b showed that E at amino acid position 480 in SH was completely exposed (Fig. 4E), but a glycine residue at amino acid position 480 was hidden entirely in SH33b (Fig. 4F). The structural divergence of the MTIR of 129K protein between SH and SH33b is consistent with their functional roles in SSA. Therefore, a single amino acid substitution at amino acid position 480 in the IR in SH33b resulted in a structural change in the suppressor, which is most likely responsible for impairing SBC and SSA.

DISCUSSION

In this study, we investigated SSA of CGMMV 129K protein by using a *GFP*-silenced transgenic *N. benthamiana* line, particle bombardment, and transient agroinfiltration assays. The CGMMV SH strain infection resulted in suppression of *GFP* silencing in the no. 24 line accompanied by severe mosaic symptoms in the systemically infected leaves. The levels of suppression activity positively correlated with the intensity of symptoms (Fig. 1). The effects of suppressor activity between the SH and SH33b strains were compared in a transient suppression assay by particle bombardment and agroinfiltration (Figs. 2B and 3A). Both results demonstrated that 129K protein encoded by CGMMV SH is a strong suppressor of RNA silencing, even if the MTIR of 129K protein can also suppress local silencing in the absence of other CGMMV-encoded proteins (Figs. 2B and 3A). This

Fig. 2. A, Comparison of genome organization between cucumber green mottle mosaic virus (CGMMV) SH severe and SH33b attenuate strains. CP, coat protein. **B,** *GFP* silencing suppressed by expressing the methyltransferase domain plus intervening region (MTIR) and 129K protein encoded by CGMMV SH in the epidermal cells of onion bulb through particle bombardment. Fluorescent images of *GFP* and *DsRed* (control) expression in the different mixtures of plasmids at 36 h postcobombardment. Scale bar = 25 μ m. **C,** Quantitative analysis of fluorescence-expressing cells per shot in the different mixtures of plasmids cobombarded. Data are mean $\pm$ standard error ($n = 6$). * $P < 0.05$ (Fisher's test) versus value of *GFP*-expressing cells in i.

MTIR result shows an important role of a single amino acid substitution at amino acid position 480 in the IR of SH33b (Tan et al. 1997) (Fig. 2A) for SSA. The MTIR of 126K protein by tobacco mosaic virus (TMV) is involved in both cell-to-cell movement and long-distance movement in *N. benthamiana* (Knapp et al. 2005, 2007). Mutation in the IR of 126K protein is one of the major pathogenicity determinants of Pepper mild mottle virus (PMMoV), which is responsible for the attenuated symptoms and decreased accumulation of the CP in infected pepper plants (Hagiwara et al. 2002; Ichiki et al. 2005; Yoon et al. 2006). It was reported that a single amino acid substitution in the MTIR of Paprika mild mottle virus replicase proteins led to a change in disease symptoms in inoculated *Capsicum* plants carrying the *Hk* resistance gene at 24°C (Matsumoto et al. 2009). The IR of ToMV 130K protein is also responsible for symptom attenuation and SSA (Kubota et al. 2003). RNA silencing suppression by ToMV 130K protein was thought to occur via 130K protein in soluble fraction (Hagiwara-Komoda et al. 2008). They showed larger quantity of ToMV 130K protein in soluble fraction than that of TLJ (a ToMV

mutant and named TLJ), an attenuated strain, indicating that quantitative difference of 130K protein caused different levels of silencing suppression.

Specific amino acids in the nonconserved region II (NONII) in the C-terminal half of the IR in TMV 126K have been reported to be crucial for silencing suppression (Wang et al. 2012), which coincides with our result that one amino acid substitution occurred at amino acid position 480 located in NONII. In PMMoV, the mutations for attenuation were also located in NONII (Ichiki et al. 2005). The attenuated strain, CGMMV-VIROG-43, had the amino acid substitution at 534 and 698 amino acid positions in NONII, although another amino acid substitution occurred at 86 amino acid position in MT domain (Slavokhotova et al. 2016). Liu et al. (2017) showed that amino acid substitution at 284 (in NONI) amino acid positions in hn-CGMMV isolate (a CGMMV isolate obtained from Henan province, China) were involved in symptom attenuation. In the case of ToMV, the amino acid substitution in NONI affected silencing suppression (Kubota et al. 2003). The same amino acid substitution by in vitro mutagenesis in PMMoV was also found to

Fig. 3. A, Suppression of local GFP silencing by the *Agrobacterium*-mediated transient expression of the cucumber green mottle mosaic virus SH methyltransferase domain plus intervening region (MTIR) and 129K protein in a transgenic GFP-silenced *Nicotiana benthamiana* line (no. 24). The white scale bar indicates 2 cm. PVY HC-Pro, positive control; vector, negative control. B, Detection of GFP messenger RNA by northern blot analysis. rRNA, ribosomal RNA. C, Detection of GFP-specific small interfering RNAs (siRNAs) by northern blot analysis. Total RNA was extracted from the leaves of line no. 24 infiltrated with the *Agrobacterium* carrying the respective plasmids indicated in the panels. tRNA, transfer RNA. D, siRNA binding analysis by electrophoretic mobility shift assay. Increasing amounts of the purified hemagglutinin (HA)-tagged MTIR and 129K (100, 200, and 400 nM) and bovine serum albumin (BSA; 300 nM) were incubated with the digoxigenin (DIG) 21-nucleotide double-stranded small interfering RNAs (ds-siRNAs). The amount of HA-tagged proteins present in the crude extracts was estimated by western blot analysis with HA antibody (lower panel). The upper and lower arrows indicate 129K protein:siRNA complex and free DIG-labeled 21-nucleotide ds-siRNAs, respectively.

be involved in virus attenuation (Tsuda et al. 2007). Thus, NONI and/or NONII are responsible for symptom attenuation. Analysis of MTIR and 129K protein SBC by EMSA revealed that SH showed SBC, whereas SH33b did not (Fig. 3D), suggesting that the single amino acid substitution in the IR of SH33b strain was sufficient for impairing SBC and SSA and eventually, led to the attenuated symptoms. This finding is also consistent with previous work that the cr-TMV (now belonging to the virus species turnip vein-clearing virus) 122K and TMV-cg (now belonging to the virus species youcai mosaic virus) 126K proteins acted as a silencing suppressor and had binding activity to small RNA duplexes (Csorba et al. 2007; Kurihara et al. 2007).

Here, we showed that SBC of CGMMV 129K protein of an attenuated strain was impaired, leading to its attenuation. SBC of viral silencing suppressors has been reported in many viruses so far (Burguán 2008; Csorba et al. 2015). This is the first report of impaired SBC of silencing suppressor protein in a tobamovirus. So far, lower or impaired SBC has been reported for different attenuated strains/isolates in a tobamovirus (this study), a cucumovirus, CMV, (Goto et al. 2007), and a potyvirus, tobacco etch virus, (Torres-Barceló et al. 2010). Thus, it may be common among different viruses that lower/impaired SBC leads to attenuated symptom phenotypes. CP of CGMMV affects virus infectivity using several mutations, including single amino acid substitution (Zhang 2008). In SH33b, there is one amino acid substitution A to V at 156 amino acid position of CP (Tan et al. 1997). We cannot exclude the possibility that this substitution affects symptom attenuation. Generally, it is thought that infectivity/symptoms reflect a total sum of mutations all over the viral genome. Thus, it is reasonable to understand that not only RNA silencing but also, other mechanisms could affect symptoms in hosts as a total. Further investigation is needed for elucidation

of any effects of each mutation in SH33b. In conclusion, here we show that a single amino acid substitution from E to G at amino acid position 480 in the IR of CGMMV caused SSA and SBC to be repressed, contributing to symptom attenuation.

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Fig. 4. Impairment of the suppression activity of RNA silencing probably resulting from a structural change of suppressor caused by a single amino acid substitution in the methyltransferase domain plus intervening region (MTIR) of 129K protein of the SH33b strain. Modeling of the MTIR of 129K protein in the cucumber green mottle mosaic virus SH and SH33b strains using the I-TASSER server with 3ungC (Cmr2) as a template. The predicted cartoon structure is for **A**, SH-methyltransferase domain (MT) and **B**, SH33b-MT. Close views in the region from 475 to 500 are for **C**, SH and **D**, SH33b. Exposed surface structures are for **E**, SH and **F**, SH33b. For clarity, the position of amino acid substitution at 480 amino acid is shown in red outlined with a yellow rectangle.

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