Plant Cell Physiol. 48(7): 1050–1060 (2007)
doi:10.1093/pcp/pcm074, available online at www.pcp.oxfordjournals.org

Characterization of Silencing Suppressor 2b of Cucumber Mosaic Virus Based on Examination of its Small RNA-Binding Abilities

Kazunori Goto [1], Takashi Kobori [2], Yoshitaka Kosaka [2], Tomohide Natsuaki [3] and Chikara Masuta [1,*]

[1] *Cell Biology and Manipulation Laboratory, Graduate School of Agriculture, Hokkaido University, Sapporo, 060-8589 Japan*
[2] *Kyoto Prefectural Institute of Agricultural Biotechnology, Kyoto, 619-0244 Japan*
[3] *Laboratory of Plant Pathology, Faculty of Agriculture, Utsunomiya University, Utsunomiya, 321-8505 Japan*

Double-stranded (ds) RNAs and imperfect hairpin RNAs of endogenous genes trigger post-transcriptional gene silencing (PTGS) and are cleaved by a Dicer-like nuclease into small interfering RNAs (siRNAs) and microRNs (miRNAs), respectively. Such small RNAs (siRNAs and miRNAs) then guide an RNA-induced silencing complex (RISC) for sequence-specific RNA degradation. While PTGS serves as an antiviral defense in plants, many plant viruses encode suppressors as a counter defense. Here we demonstrate that the PTGS suppressor (2b) of a severe strain (CM95R) of cucumber mosaic virus (CMV) can bind to in vitro synthesized siRNAs and even to long dsRNAs to a lesser extent. However, the 2b suppressor weakly bound to a miRNA (miR171) duplex in contrast to another small RNA-binding suppressor, p19 of tombusvirus that can effectively bind miRNAs. Because the 2b suppressor of an attenuated strain of CMV (CM95), which differs in a single amino acid from the 2b of CM95R, could barely bind siRNAs, we hypothesized that the weak suppressor activity of the attenuated strain resulted from a loss of the siRNA-binding property of 2b via a single amino acid change. Here we consider that 2b interferes with the PTGS pathway by directly binding siRNAs (or long dsRNA).

Keywords: Cucumber mosaic virus — PTGS — siRNA — Viral suppressor.

Abbreviations: AGO1, Argonaute; ARR, arginine-rich region; CHS, chalcone synthase; CMV, cucumber mosaic virus; dpi, days post-infiltration; GFP, green fluorescent protein; GUS, β-glucuronidase; IR, inverted repeat; LMW, low molecular weight; LUC, luciferase; miRNA, microRNA; NLS, nuclear localization signal; PTGS, post-transcriptional gene silencing; RISC, RNA-induced silencing complex; siRNA, small interfering RNA; TBSV, tomato bushy stunt virus.

Introduction

Post-transcriptional gene silencing (PTGS) is a sequence-specific RNA degradation that is triggered by double-stranded RNAs (dsRNAs). Based upon the current model, a dsRNA-specific enzyme, Dicer, digests long dsRNAs into short interfering RNA (siRNAs) of 21–25 nucleotides (nt). Dicer also digests imperfect hairpin-forming RNA precursors transcribed from endogenous genes into 21 nt microRNAs (miRNAs). Both siRNAs and miRNAs are then incorporated into RNA-induced silencing complexes (RISCs) and guide the RISC to homologous single-stranded RNAs (ssRNAs) for either sequence-specific cleavage or translational repression of ssRNA targets (Meister et al. 2004, Vance and Vaucheret 2001, Baulcombe 2004). Because viral dsRNAs (PTGS inducers) are generated as replicative forms during virus replication or by a host RNA-dependent RNA polymerase, PTGS is therefore considered to be a natural antiviral defense mechanism (Voinnet 2001). However, plant viruses have evolved a counterdefense strategy, producing silencing suppressors that interfere with PTGS (Vance and Vaucheret 2001). Viral PTGS suppressors target different points in the PTGS pathway. Among the known suppressors, potyvirus HC-Pro, cucumber mosaic virus (CMV) 2b and tombusvirus p19 have been studied most extensively (Roth et al. 2004). Although HC-Pro has been reported to reduce siRNA accumulation in local silencing (Llave et al. 2000, Mallory et al. 2001), it increases the level of endogenous miRNAs (Mallory et al. 2002, Kasschau et al. 2003). In addition, HC-Pro does not inhibit the systemic silencing signal, a phenomenon that suggests that HC-Pro works downstream from production of the systemic signal. Chapman et al. (2004) suggested that HC-Pro works at the point of RISC assembly and most probably unwinds miRNA duplexes. On the other hand, p19 binds both siRNAs and miRNAs directly and blocks local and systemic silencing (Silhavy et al. 2002, Chapman et al. 2004). In the three-dimensional X-ray crystal structure of a p19–siRNA complex, a p19 dimer held one molecule of siRNA (Ye et al. 2003). At the present time, several viral suppressors have been reported to have siRNA- and/or miRNA-binding properties: p21 of beet yellows virus (Closterovirus) (Chapman et al. 2004), p14 of pothos latent virus (Aureusvirus) (Mérai et al. 2005), NS1 of influenza A virus (Bucher et al. 2004), AC4 of african cassava mosaic virus (Geminivirus) (Chellappan et al. 2005) and B2 of flock house virus (Nodavirus) (Lu et al. 2005). Recently many other suppressors including

*Corresponding author: E-mail, masuta@res.agr.hokudai.ac.jp; Fax, +81-11-706-2483.

HC-Pro were also found to bind small RNA, suggesting that small RNA binding is a general strategy to suppress RNA silencing (Mérai et al. 2005, Lakatos et al. 2006).

CMV has three single-stranded, genomic RNAs (RNAs 1–3). RNA 4A, a subgenomic RNA is derived from the 3′ half of RNA 2 and encodes the 2b protein (2b) (Ding et al. 1994). Early studies focusing on the function of 2b demonstrated that 2b was dispensable for replication of the CMV genome and a satellite RNA (Ding et al. 1995). In addition, 2b can affect viral movement (Soards et al. 2002), functions as a viral virulence determinant (Shi et al. 2002) and enhances viral pathogenicity in *Nicotiana benthamiana* when expressed through potato virus X vector (Brigneti et al. 1998, Lucy et al. 2000).

In an *Agrobacterium* co-infiltration assay with a stable transgenic plant, 2b acted as a PTGS suppressor that inhibited both local and systemic silencing (Brigneti et al. 1998, Guo et al. 2002). In a recent report, 2b could suppress PTGS even at the single cell level (Qi et al. 2004). The suppressor ability of 2b has also been shown to depend on the presence of a nuclear localization signal (NLS) and was localized in the nucleus (Lucy et al. 2000, Mayers et al. 2000). Motif analysis of 2b raised the possibility that the arginine-rich region (ARR) near the NLS might function to bind dsRNA (Mayers et al. 2000). Although 2b has been extensively characterized from a mechanistic standpoint, the point of operation for 2b in the PTGS pathway is not fully understood. For example, Argonaute1 (AGO1) was reported to be the target of 2b (Zhang et al. 2006). Another possibility is that 2b acts by binding to siRNAs. Considering that 2b is in the nucleus, 2b may act indirectly in the PTGS pathway by affecting transcription factors.

In this study, we used an attenuated CMV strain (CM95) that induces very mild symptoms and is used for cross-protection against cucumber CMV diseases in Japan (Kosaka and Fukunishi 1997). In addition, we isolated a spontaneous mutant of CM95 (CM95R) that induces severe symptoms on the inoculated plants in the field. We also recently showed that the 2b of CM95 is weakly suppressive in comparison with the 2b of CM95R (T. Kobori et al. unpublished data). Interestingly, the 2b proteins of CM95 and CM95R only differ in a single amino acid. We took advantage of having 2bs with different suppressor activities and used them as our model system to analyze the mode of 2b suppression in the PTGS pathway.

We could produce 2b by using a wheat germ in vitro translation system because it was difficult to produce 2b in *Escherichia coli*. In this article, we correlate the weak suppressor activity of CM95 2b with poor binding to siRNAs. Our finding that CM95R can strongly bind to siRNA supports the hypothesis that 2b interferes with the PTGS pathway by directly binding to siRNAs.

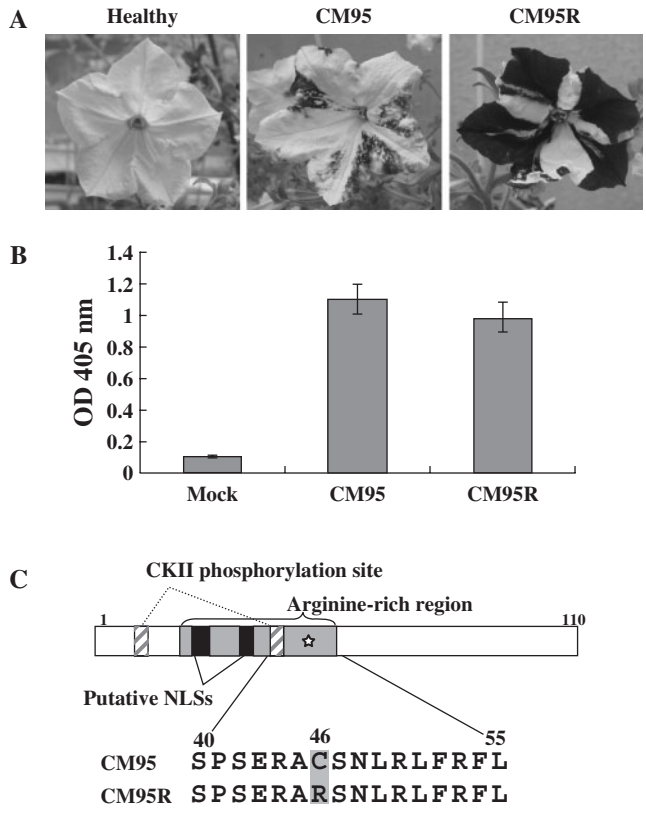

Fig. 1 Interference of 2b with PTGS of the CHS gene in transgenic petunia line C001. (A) Flower color before and after CMV infection. Left, transgenic petunia line C001 with typical white flowers. Middle, infection with CM95 induced purple mottles. Right, infection with CM95R induced a broad purple area on the flowers. (B) Mean viral concentrations (±SE) in four infected flowers as measured by enzyme-linked immunosorbent assay. (C) Comparison of the 2b amino acid sequences between CM95 and CM95R. The differing amino acid is shaded. The CKII phosphorylation site (Lucy et al. 2000), putative NLSs (Wang et al. 2004) and arginine-rich region (Mayers et al. 2000) were taken from previous reports.

Results

2bs with different PTGS suppressor activities

When the white-petaled transgenic petunia line C001 (Fig. 1A, left panel) was infected with the attenuated strain (CM95), the flowers were mottled with purple (Fig. 1A, middle panel). If C001 was inoculated with the revertant (CM95R), most of the petals developed a deep purple color (Fig. 1A, right panel). Importantly, the viral concentrations measured in the flowers confirmed that there was little difference in the viral levels between the two isolates (Fig. 1B). The purple areas in the flowers infected with CM95R were much broader than those in the flowers infected with CM95. As expected, the level of mRNA of the chalcone synthase (CHS) gene was higher in the

CM95R-infected tissues than in the CM95-infected tissues (Supplementary Fig. 1A), while the level of CHS siRNAs was decreased in the CM95R-infected tissues (Supplementary Fig. 1B). The accumulation of the 2b proteins of CM95R and CM95 was found to be almost equivalent (Supplementary Fig. 1C). These data are consistent with the phenotypic changes and clearly suggest that the CHS PTGS was more strongly suppressed by CM95R than CM95. It was therefore concluded that PTGS suppressive activity differed between the two isolates. Because 2b is the PTGS suppressor of CMV, we attributed the variable color recovery to the suppressive abilities of 2b. The 2b proteins of CM95 (A2b) and CM95R (R2b) only differ from one another by a single amino acid at position 46 (C and R, respectively) (Fig. 1C; T. Kobori et al., unpublished data). The A2b and R2b genes contain a U and a C at nucleotide position 136 in the 2b open reading frame, respectively. This nucleotide change was a silent mutation in the overlapped 2a protein.

Localization of R2b and A2b in plant cell

To investigate whether the weak suppression by A2b might be the result of intracellular localization, R2b or A2b fused with green fluorescent protein (GFP) was transiently expressed in onion epidermal cell. As a result, the free GFP was diffused through the cytoplasm and nucleus (Fig. 2). The distributions of R2b and A2b clearly differed from that of the free GFP; R2b and A2b levels were apparently equally enhanced in the nucleus.

Comparison of R2b and A2b in local silencing

To confirm directly that R2b is a stronger suppressor than A2b, we evaluated the suppressor activities directed against local gene silencing in transient expression assays using *Agrobacterium tumefaciens* (Brigneti et al. 1998, Voinnet et al. 1998). Although 2b has been reported to have little effect on local silencing (Guo et al. 2002), our preliminary assays revealed that 2b from subgroup I CMV could affect local silencing. The cDNA clones of R2b and A2b were inserted into the pBE2113 plant expression vector. Tomato bushy stunt virus (TBSV) p19, which is a well-characterized suppressor, was also included as a control. GFP silencing was induced in *Nicotiana benthamiana* plants by infiltrating a mixture of *Agrobacterium*, each expressing a sense or an inverted repeat construct of GFP. *Agrobacterium* expressing viral suppressors were co-infiltrated to interfere with the induced GFP silencing. As expected, in the leaf patch co-infiltrated with the β-glucuronidase (GUS) gene (control), the GFP gene was not expressed at 4 days post-infiltration (dpi), but when the p19 gene was co-infiltrated, strong GFP fluorescence was observed. The patch co-infiltrated with R2b exhibited GFP fluorescence, whereas A2b resulted in notably weak

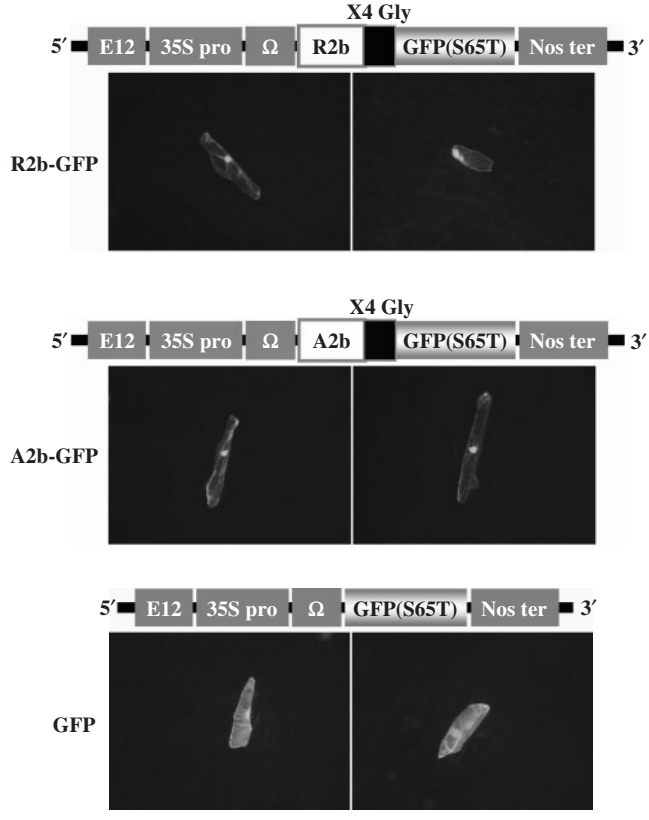

Fig. 2 Localization of A2b and R2b in onion epidermal cells. To localize A2b and R2b in plant cells, the cDNA clones of A2b and R2b were fused to the GFP gene (S65T) and inserted in the plant expression vector pE2113. The schematic representations of the expression cassettes are shown above each picture. Onion epidermal cells were then bombarded with the recombinant constructs. GFP fluorescence was observed 36 h after bombardment, using an epifluorescence microscope. As a control bombardment (bottom), the GFP gene alone was used. XGly, four glycine residues.

fluorescence, suggesting that A2b cannot suppress the induced GFP silencing as efficiently as R2b (Fig. 3A, upper panel). Western blot analysis confirmed that similar amounts of 2b were produced in the infiltrated patches (Fig. 3A, lower panel). These observations were consistent with the result of Northern blot analysis (Fig. 3B); the level of GFP mRNA in the A2b lane was under the detection limit. We then analyzed the accumulation of GFP siRNAs in the patches. The levels of siRNAs of R2b and A2b were similar to that in GUS, while p19 markedly reduced GFP siRNAs of both orientations (Fig. 3C).

Comparison of R2b and A2b in systemic silencing

Because 2b is known to target systemic silencing (Guo et al. 2002, Hamilton et al. 2002) by blocking

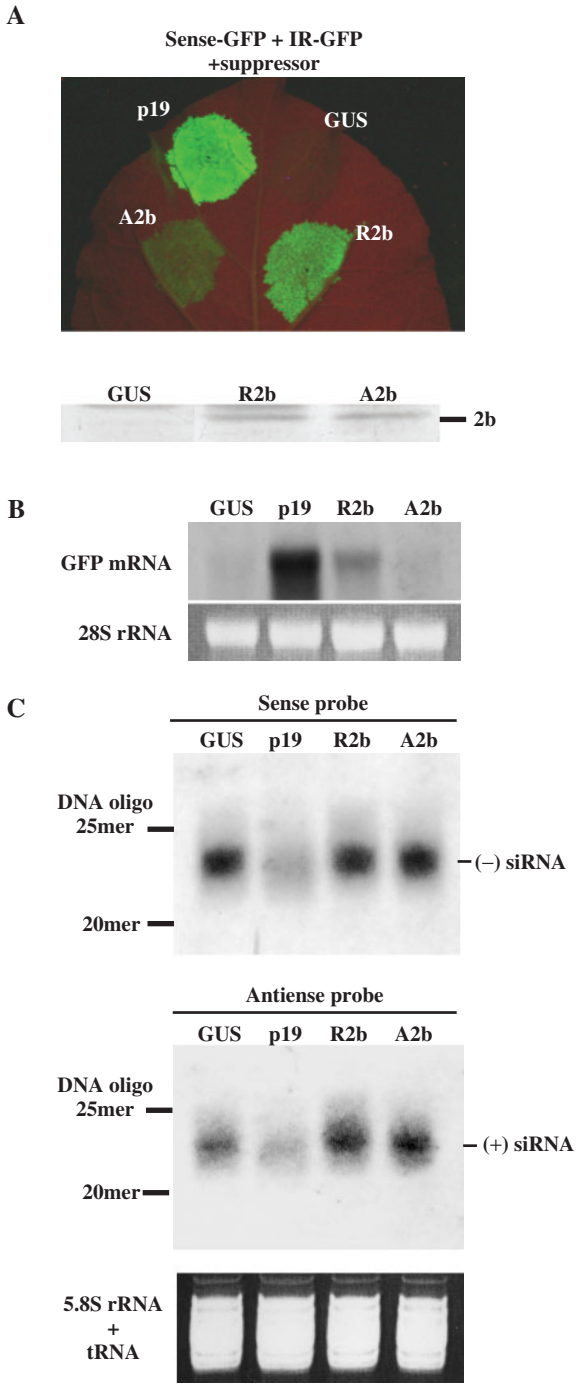

Fig. 3 Effect of 2b on local gene silencing. (A) Transient expression assay using *Agrobacterium* (upper panel). GFP silencing was induced by an inverted repeat construct. The photograph was taken 4 days post-infiltration (dpi). The inoculum was a combination of *Agrobacterium* cultures carrying the various constructs of GFP-sense, GFP-inverted repeat and a viral suppressor in the ratio 5 : 1 : 5, respectively. *Agrobacterium* suspensions were prepared to an optical density at 600 nm of 1.0. The GUS and p19 constructs were used as a negative and a positive control, respectively. The lower panel is the result of Western blot analysis of the 2b protein produced in the infiltrated patches. Equal amounts of protein (50 μg) extracted from the patches were separated by SDS–PAGE, and 2b was detected with anti-2b antibodies. (B) Accumulation of the GFP mRNA in the infiltrated patches. The levels of mRNA were analyzed by a Northern blot hybridization using an antisense RNA probe for GFP. Ethidium bromide-stained 26S rRNAs are shown as a loading control. (C) Accumulation of the GFP siRNAs in the infiltrated patches. Low molecular weight (LMW) RNAs were separated in a 15% polyacrylamide gel, blotted to a nylon membrane and the blots were hybridized with sense and antisense RNA probes for GFP. Ethidium bromide-stained 5.8S rRNAs + tRNAs are shown as a loading control.

movement of the unidentified systemic signal (possibly siRNAs), we assayed its function in transient expression experiments. GFP transgenic plants (16c) were infiltrated with *Agrobacterium* co-expressing the inverted repeat (IR) and a viral suppressor. The treated plants were observed until the top leaf of the GFP + IR plant turned red, an indicator of GFP silencing. Most of the plants induced by GFP + IR had turned red by 16 dpi. For each suppressor, 20 plants were used. As shown in Fig. 4A, all plants that were infiltrated with GFP + IR turned red. Eighty-five percent and 77% of the plants that were infiltrated with the 2b protein of CMV-Y (Y2b) and with R2b, respectively, did not have GFP silencing. On the other hand, only 18% of the plants infiltrated with A2b continued to fluoresce green at 16 dpi. As shown in Fig. 4B, the plants that were treated with either Y2b or R2b continued to fluoresce green; however, the plants treated with GFP-IR and the A2b-infiltrated plants turned red. Taken together, these results suggest that R2b can interfere with the systemic silencing, but A2b cannot. The results of Northern blot analyses agreed well with our observations of GFP fluorescence (Fig. 4C, D). We also confirmed systemic GFP silencing by detecting siRNAs from the top leaves of the plants that were treated with IR in the presence of A2b (Fig. 4D).

Direct interaction between 2b and siRNAs

We have demonstrated here that 2b from subgroup I CMV can suppress both local and systemic silencing. It is known that two molecules of p19 bind directly to one molecule of siRNA (Ye et al. 2003). If 2b is capable of binding to siRNAs in a similar fashion to p19, it certainly affects both local and systemic silencing. We were therefore interested to investigate the ability of 2b to bind to siRNAs and/or dsRNAs. Using the wheat germ in vitro translation system (Proteios), the FLAG peptide was fused to the C-terminus of the viral suppressors as a tag to facilitate their purification and detection by Western analysis. The siRNAs were prepared from the dsRNAs of the luciferase (LUC) gene using a human Dicer according to the manufacturer's instructions. The viral suppressors were

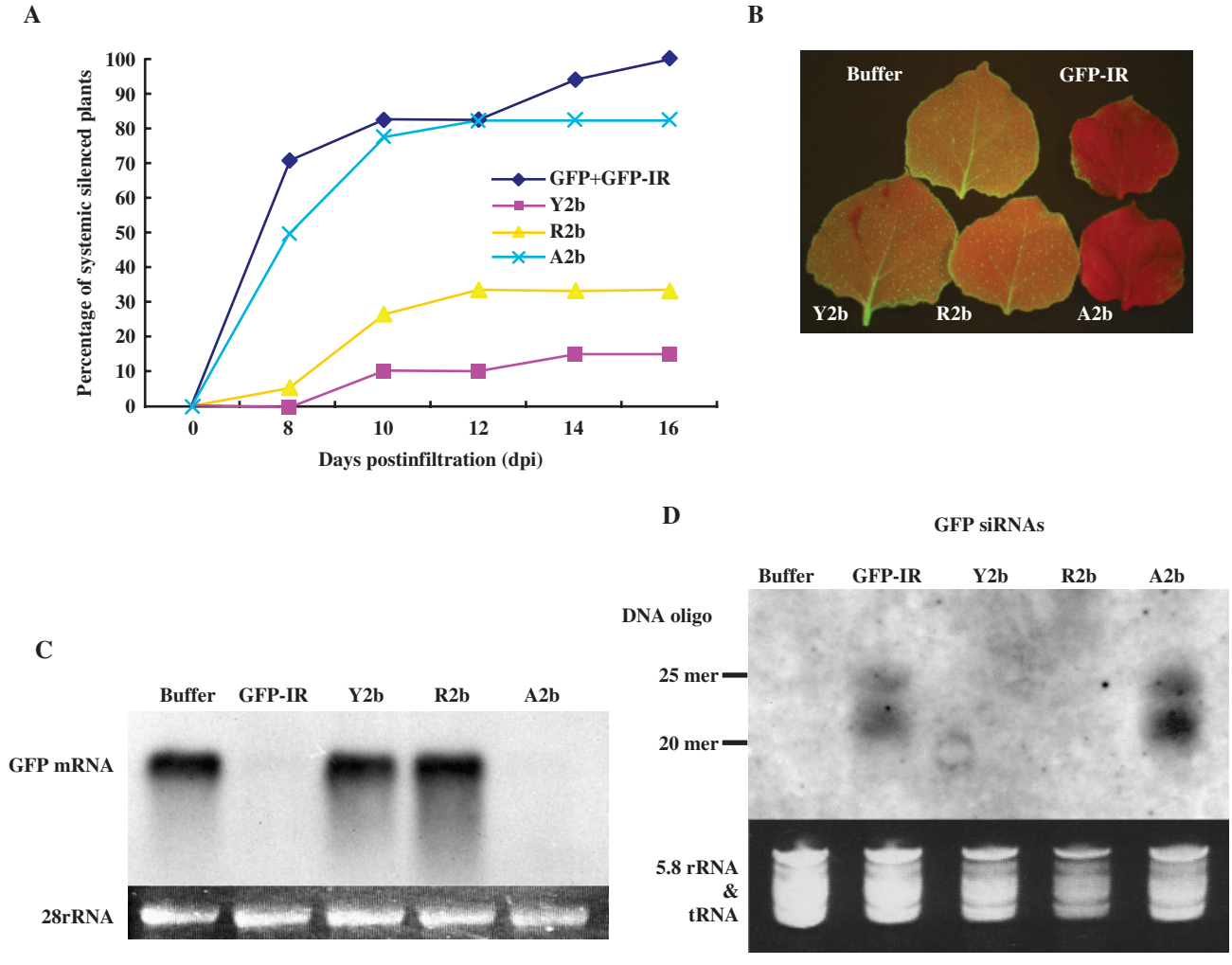

Fig. 4 Effect of CM95 and CM95R 2b on *A. tumefaciens*-mediated systemic gene silencing in GFP transgenic *N. benthamiana*. (A) GFP transgenic *N. benthamiana* plants were infiltrated with *Agrobacterium* combinations of a sense GFP construct (S), an inverted repeat GFP construct (IR) and a viral suppressor in the ratio 2 : 1 : 2, respectively. The plants were monitored for the initiation of systemic silencing in the top leaf at 8, 10, 12, 14 and 16 dpi. For each test, 20 plants were used. (B) A GFP photograph of the systemic leaf was taken 13 d post-infiltration. Infiltrated constructs are indicated at the top or bottom of each leaf. (C) Accumulations of GFP mRNA in the systemic leaf. Total RNAs were separated in a 1.5% denaturing gel and hybridized with DNA probes. Ethidum bromide-stained 26S rRNAs are shown as a loading control. (D) Accumulation of GFP siRNAs in the systemically silenced leaf. LMW RNAs were separated in a 15% denaturing polyacrylamide gel and hybridized with DNA probes after transfer to the membrane. Ethidium bromide-stained 5.8S rRNAs and tRNAs are shown as a loading control.

then incubated with siRNAs and purified through an anti-FLAG affinity column. The charged RNAs were extracted from the eluted proteins and subjected to Northern blot analysis using LUC sense or antisense RNA probes. As a result of the purification, we detected siRNAs that bound to p19 and R2b, while it was apparent that A2b bound siRNAs poorly (Fig. 5A, B). These data support the supposition that R2b has the ability to bind to siRNAs. In another binding assay using the partially digested LUC dsRNAs, we used two kinds of epitope tags (His and FLAG) to purify 2b (or 2b–siRNA complex) after incubation with siRNAs. Neither epitope prevented 2b from binding siRNAs, regardless of any difference in sequence. In a control experiment, we further confirmed that GFP with a C-terminal His tag (six residues) did not bind siRNAs, showing that the His tag itself does not bind to siRNAs. These data indicated that the C-terminus of 2b was not involved in the siRNA-binding activity (Fig. 6). In addition, we found that R2b could also bind the chopped dsRNAs that are longer than siRNAs (Fig. 6). These data support the notion that R2b might bind dsRNAs.

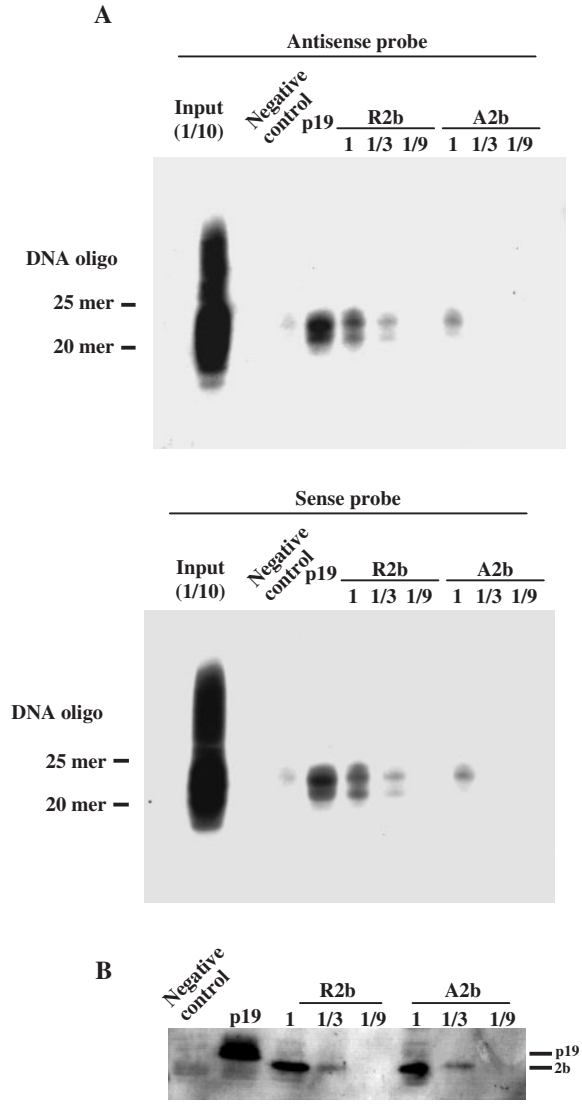

Fig. 5 Ability of R2b to bind siRNAs in vitro. (A) Northern blot analysis of LUC siRNAs bound to the suppressors. After incubation of the viral suppressors and in vitro synthesized LUC siRNAs, the charged proteins were purified through a FLAG affinity column, and siRNAs were extracted and subjected to Northern blot analysis. Signals were visualized with either a LUC sense or antisense RNA probe. LUC siRNAs were incubated with a 3-fold dilution series of in vitro translation mixture including suppressors. p19 was included as a control. One-tenth of the amount of the LUC dsRNAs including siRNAs used for the incubation was loaded in the input lane. For the negative control, LUC siRNAs were incubated with the in vitro translation cocktail containing no transcripts. (B) The in vitro translated suppressors used for the assay were detected with anti-FLAG antibodies to confirm the yields.

To investigate whether NLS of 2b is involved in its siRNA-binding ability, we created another mutant that lacks the two NLSs in 2b. The mutant 2b was found not to bind siRNAs, suggesting that NLS of 2b is also important for siRNA binding (data not shown).

Direct interaction between 2b and miRNA duplex

Because some viral suppressors having siRNA-binding ability (p19 and p21) can also bind miRNAs, we considered the notion that 2b could also have this dual binding capacity. To test this hypothesis, a chemically synthesized miR171 was used for the binding assay. miR171 interacts with the mRNA targets encoding several members of the Scarecrow-like (SCL) family, which are putative transcriptional factors. miR171 is complementary to the internal region of *SCL* mRNAs and leads to digestion of the mRNA target with RISC (Llave et al. 2002, Parizotto et al. 2004). As shown in Fig. 7A, siR171 was also created with the selected strand; siR171 is a complete dsRNA mimicking an siRNA. In our in vitro binding assays, we always detected a stronger band in the siR171 lane than in the miR171 lane, although the same amount of R2b was incubated with the RNA duplexes (Fig. 7B). To confirm the observation further, miR171 and siR171 were incubated with the in vitro synthesized suppressor at a series of dilutions of the translation mixture. When p19 was tested, it did not show preference for binding to small RNAs (miR171 and siR171). In contrast, R2b could not bind to miR171 at dilutions greater than 1/9 but bound to siR171 even at 1/12, confirming that R2b preferentially binds to siR171 over miR171.

Discussion

In this study, we found that an attenuated isolate (CM95) and its revertant (CM95R) had different suppressor activities. The difference in viral suppression of silencing was mapped to a single amino acid residue (position 46) in the 2b protein. Previously, Lucy et al. (2000) showed that mutations in NLS of 2b abolished suppressor activity, suggesting that the nuclear location of 2b may be important for its function. Although the altered amino acid residue does not reside within the NLS, we used a GFP fusion approach to confirm if A2b and R2b differed in their location within the cells. When onion epidermis was bombarded with the 2b–GFP fusion construct, GFP fluorescence was observed with an epifluorescence microscope. As expected, both A2b and R2b were localized in the nucleus, indicating that the single amino acid alteration did not affect the subcellular localization of 2b.

Can 2b bind siRNAs in vitro?

In our next line of investigation, we determined whether 2b could bind dsRNAs in vitro and whether the two 2b proteins differ in their ability to bind to dsRNAs, including siRNAs. Many viral suppressors have been identified, and the mechanisms of suppression by those proteins have been extensively investigated. Viral suppressors have low sequence similarities and are believed to

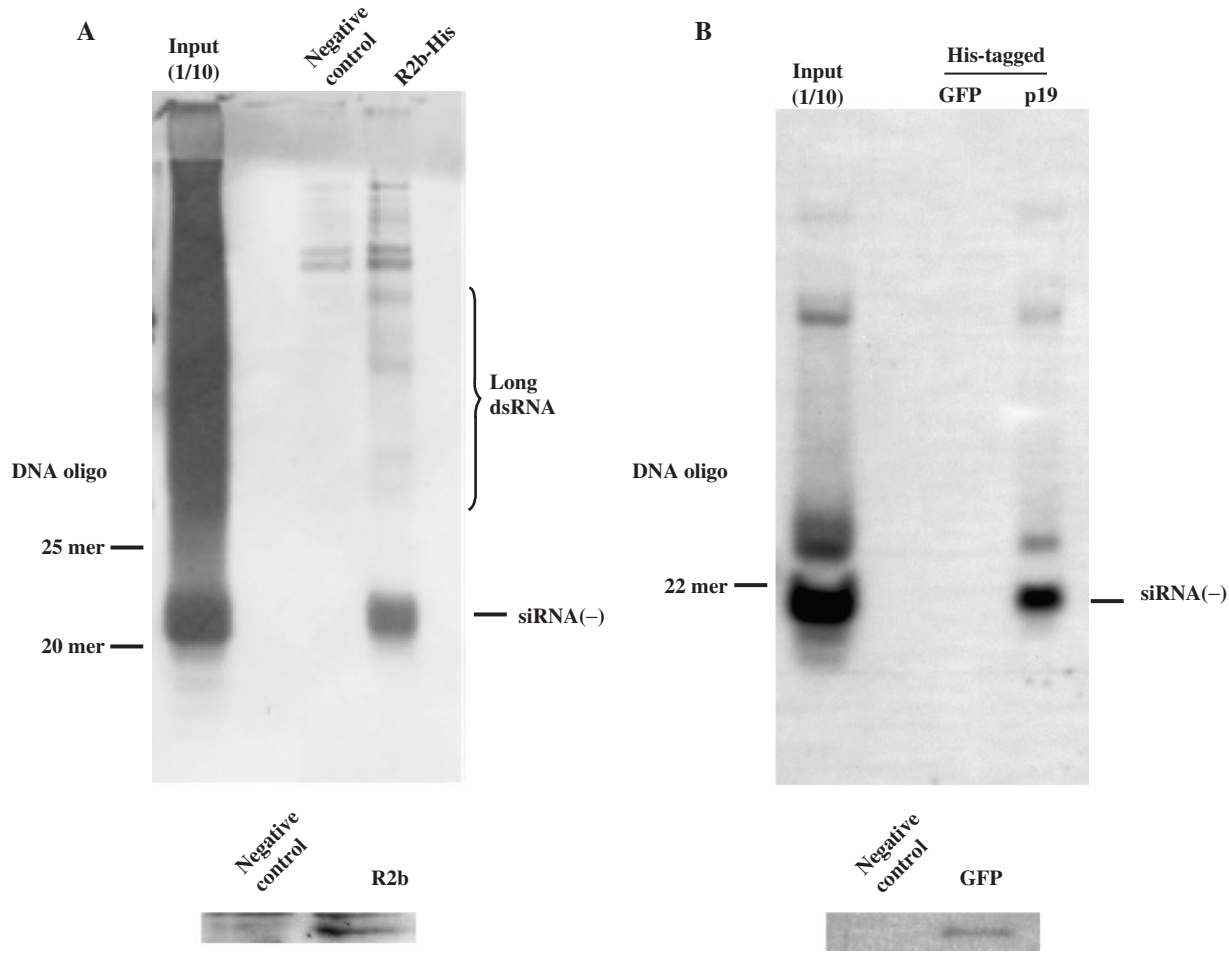

Fig. 6 Ability of R2b fused to the His tag to bind to siRNAs. (A) Northern blot analysis of LUC siRNAs bound to CM95R 2b containing the C-terminal His-tag (R2b-His). After incubation of R2b-His and the in vitro synthesized LUC siRNAs, the charged proteins were purified through a His-tag affinity column, and siRNAs were extracted and subjected to Northern blot analysis. One-tenth of the amount of the partially digested LUC dsRNAs including siRNAs used for the incubation was loaded in the input lane. Signals were visualized with a LUC antisense RNA probe. For the negative control, siRNAs were incubated with the in vitro translation cocktail containing no transcripts. The in vitro translated R2b-His was detected with anti-His-tag antibodies. The Western blot of the in vitro translated His-tagged 2b is shown below the Northern blot. (B) His-tagged GFP that does not bind to Luc siRNAs. Proteins and Luc siRNAs were prepared as described above. The in vitro translated GFP was detected with anti-His-tag antibodies and is shown below the Northern blot. Note that the His-tag fusion with 2b did not affect the binding between 2b and siRNAs. His-tagged p19 was included as a positive control.

operate at different steps in different ways in the silencing pathway(s). The only suppressor protein whose mechanism is well understood is p19 from tombusvirus. Based upon some biochemical studies and the X-ray crystallography, we now know that the p19 homodimer binds to a siRNA (Silhavy et al. 2002, Baulcombe and Molnar 2004). On the other hand, Mayers et al. (2000) suggested that the arginine-rich N-terminal domain of 2b might bind dsRNAs, a prediction that has not yet been tested. It is possible that this lack of confirmation may be partly due to the difficulty of recombinant 2b production in *E. coli* (Mayers et al. 2000; our unpublished data). Here we overcame this problem using the wheat germ in vitro translation system. We were thus able to initiate the first investigation to determine whether 2b can bind siRNAs or dsRNAs in vitro. Our in vitro binding assays confirmed that 2b could bind to siRNAs that were generated by a human Dicer.

After many attempts to isolate plant-expressed 2b, we found that in planta isolation of soluble 2b was very difficult, whereas the p19–siRNA complex was relatively easily isolated by the same system. We believe that this complication results from the fact that when 2b is expressed in planta, it forms inclusions after synthesis and becomes localized in the nucleus of the plant cell, as shown by Mayers et al. (2000). It is thus technically difficult to purify 2b (or a 2b–siRNA complex) in a soluble and active form

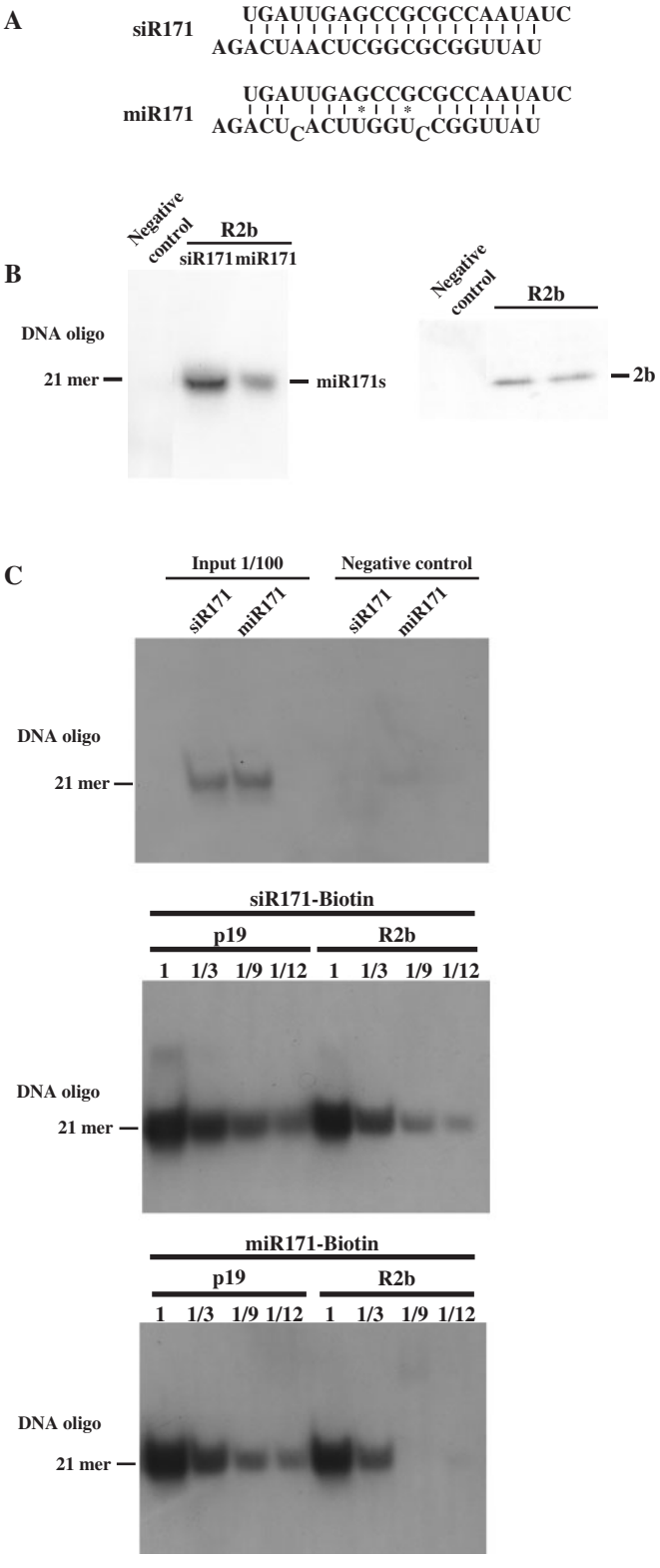

Fig. 7 In vitro assay of 2b binding to different RNA duplexes. (A) The structures of miR171 and siR171 perfectly match the selected strand (miR171). (B) Northern blot analysis of miR171 bound to R2b. After incubation of the viral suppressors with the synthesized RNA duplexes (1 μg each), the charged proteins were purified through a FLAG affinity column, and the bound RNAs were then isolated and subjected to Northern blot analysis. Signals were visualized by the sense RNA probe, which was complementary to miR171. For the negative control, the miR171 duplex was incubated with the in vitro translation cocktail containing no transcripts. In the right panel, the in vitro translated R2b used for the binding assay was detected with the anti-FLAG antibodies to confirm the yields. (C) Comparison of the abilities of 2b to bind miR171 and siR171. 5′-Biotinylated miR171 and siR171 were purified from a polyacrylamide gel to remove ssRNAs and then incubated with a series of dilutions of the in vitro translation mixture containing suppressors. The original translation mixture (used for lane 1) contained 50–75 ng of suppressors. The upper panel shows the inputs of siR171 and miR171 (1/100 of RNA used for the incubation). For the negative control, miR171 or siR171 was incubated with the in vitro translation cocktail containing no transcripts. The RNA-charged proteins were purified through a FLAG affinity column. RNA was then extracted by phenol/chloroform followed by ethanol precipitation, separated on a denaturing polyacrlyamide gel and transferred to a nylon membrane. Signals were detected by using the LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL, USA). p19 was used as a positive control.

that can be released from the inclusions. This technical problem might implicate a functional role based on the nuclear localization of 2b. It is reasonable to consider that 2b-bound siRNAs would be transported to the nucleus where the complex forms inclusions so that siRNAs are not released to the cytoplasm again or are targeted by RNases in the nucleus. Wang and Metzlaff (2005) recently suggested that siRNAs released from viral suppressors might be important for cross-protection, which is mediated by PTGS (Kurihara and Watanabe 2003, Qi et al. 2004). This idea is consistent with practical cross-protection by an attenuated CMV strain, CM95. Because 2b of CM95 binds siRNAs weakly, it is conceivable that most siRNAs remain active in the cytoplasm and always available for PTGS against an invader (severe strain challenger).

2b binds miRNA duplex weakly

miRNAs are chemically similar to siRNAs, but miRNA duplexes contain some mismatched base pairs because they are generated from imperfect hairpin-forming RNA precursors by Dicer. Our results revealed that 2b could bind siRNAs but had bound weakly to the miR171–miR171* duplex. In contrast, p19 could bind both siRNAs and miRNAs efficiently. This observation is in good agreement with the report that *Arabidopsis* expressing 2b had minor phenotypic changes (Chapman et al. 2004) and that transgenic plants expressing either p19 or p21 had abnormal phenotypes most probably because of a disorder in miRNA biogenesis and its target mRNA (Chapman et al. 2004).

Recently Zhang et al. (2006) reported that *E. coli*-synthesized 2b did not bind to siRNAs but instead bound to

AGO1, a component of the RISC. Another attempt also failed to detect the siRNA-binding ability of 2b by using protein extract from CMV-infected leaf (Merai et al., 2006). However, as we discussed earlier, 2b tends to form inclusions not only in *E. coli* but also in plant cells; 2b isolated from cells may not be fully functional. Here, we clearly demonstrated that in vitro synthesized 2b had the ability to bind to small RNAs. This inconsistency may be explained by some structural difference in the 2b proteins synthesized by different methods. In addition, Guo and Ding (2002) previously provided evidence that 2b binds and sequesters the RNA silencing signal (possibly siRNAs) by grafting experiments, in which a 2b-expressing interstock was sufficient to suppress intercellular signaling. To test further whether 2b actually bind to siRNAs in vivo, we isolated siRNA-charged 2b from CMV-infected tissues. Although the yields were not good, perhaps due to the inclusion formation of 2b, we could detect endogenous siRNAs (Supplementary Fig. 2). Taken together, we consider that 2b can inhibit RNA silencing by targeting small RNAs as well as AGO1 reported by Zhang et al. (2006).

Nuclear localization signal and siRNA-binding ability

As we noted earlier, Mayers et al. (2000) previously found that the ARR in the N-terminal half of 2b is probably a dsRNA-binding site based on a motif analysis. In accordance with this suggestion, the mutated amino acid in A2b located at position 46, which is actually in the ARR, abolished siRNA-binding ability of 2b. As for the NLS, Lucy et al. (2000) previously found by substituting NLS with alanine residues that the NLS was important for the suppressor ability of 2b of CMV-Q (subgroup II). Because the putative NLSs are also in the ARR, the alanine substitutions may have affected not only nuclear targeting but also siRNA-binding ability.

To determine whether the suppressor ability of 2b actually requires both siRNA-binding ability and an NLS, we attempted to separate the two functions by manipulating the two NLSs in R2b (subgroup I). Wang et al. (2004) reported that unlike subgroup II CMV, alanine substitutions at the two NLSs did not affect nuclear targeting of 2b of subgroup I CMV, but the NLS deletion prevented nuclear localization. As a result, we created a mutant R2b lacking the NLSs to prevent the nuclear localization of R2b. The NLS deletion resulted in complete loss of siRNA-binding ability, suggesting that it is difficult to separate the two functions of 2b of subgroup I CMV. Therefore, at the present time we cannot conclude that nuclear targeting is absolutely necessary for the suppressor ability of 2b. However, considering that A2b, which can be localized in the nucleus but bound siRNAs poorly, has poor suppressor ability, the ability to bind siRNA may be important for 2b suppressor ability.

Materials and Methods

Plants

The V26 line of petunia (*Petunia hybrida*) and transgenic line C001 of V26 in which the CHS-A gene was silenced (Metzlaff et al. 1997, O'Dell et al. 1999, Kanazawa et al. 2000, Metzlaff et al. 2000, Goto et al. 2004, Koseki et al. 2005) were used to assay CMV suppressor activity. Transgenic C001 has white petals due to PTGS of the CHS-A gene, while the wild-type plant bears purple flowers (Fig. 1; Metzlaff et al. 1997, Metzlaff et al. 2000). The GFP-expressing *N. benthamiana* (16c) was obtained from Dr. D. Baulcombe (Sainsbury Laboratory, John Innes Centre, Norwich, UK).

Inoculation with virus

CMV strains (CM95, CM95R and CMV-Y) were propagated in *N. benthamina*. CM95 is an attenuated CMV isolate that has been used to control CMV diseases in Japan (Kosaka and Fukunishi 1997). CM95R is a spontaneous mutant generated from CM95 in the field and induces severe symptoms on inoculated plants. CMV-Y is a typical strain that causes severe symptoms in inoculated plants (Suzuki et al. 1991). CMV isolates are divided into two groups (subgroups I and II), based on serological relationships and nucleic acid similarities. All three isolates used in this study belong to subgroup I. Inoculated plants were kept in a greenhouse at 25°C in natural light. Inoculum was prepared by grinding infected tissues in 0.1 M phosphate buffer (pH 7.1, 10 mM sodium *N*, *N*-diethyldithiocarbomate trihydrate) for mechanical inoculation of plants with carborundum.

Transient expression of 2b in onion cells

The 2b genes (R2b and A2b) were fused to the GFP gene (S65T) by recombinant PCR and cloned into the pE2113 vector (Mitsuhara et al. 1996), resulting in construction of R2b–GFP and A2b–GFP. Onion epidermal cells were then bombarded with the plasmids using a PDS-1000/He Particle Delivery System (Bio-Rad, Hercules, CA, USA) as described before (Sato et al. 2003). GFP fluorescence was observed, 36 h after bombardment, with an epifluorescence microscope (SZX-12, Olympus, Tokyo, Japan) with a filter block containing a 460–490 nm excitation filter, a 505 nm dichroic mirror and a 510–550 nm barrier filter (SZX-MGFPA, Olympus).

Agrobacterium tumefaciens infiltration

cDNA clones of the 2b genes of CMV-Y, CM95 and CM95R were inserted between the *Xba*I and *Sac*I sites in pre-digested pBE 2113 vector. The p19 gene of TBSV was a kind gift of Dr. D. Baulcombe. The constructs of GFP and the GFP IR in pIG-121-HG were generously provided by Dr. I. Uyeda (Hokkaido University, Japan). Each suppressor construct was then introduced into *A. tumefaciens* KYRT1 strain, which was kindly supplied by Dr. G. B. Collins (University of Kentucky, USA). *Agrobacterium tumefaciens*-mediated gene silencing was induced according to methods used in Dr. Baulcombe's laboratory. In brief, pre-cultured cells of *Agrobacterium* were grown overnight in LB medium at 28°C. After centrifugation, the bacterial pellet was dissolved in buffer (10 mM MES, 10 mM $MgCl_2$, 100 μM acetosyringone, pH 5.2) to an optical density (OD) at 600 of 1.0, and the inoculum was left on the bench for 3–4 h. The inoculum

was a combination of the *Agrobacterium* cultures containing the GFP sense construct, the GFP-IR construct and the viral suppressor in the ratio 5 : 1 : 5, respectively. The bacterial culture was then injected into the plants with a 1 ml syringe. For local silencing, RNA was extracted for Northern blot analysis from each infiltrated area (patch) at 4 dpi. For systemic silencing, the plants were observed for at least 3 weeks.

RNA isolation and Northern hybridization

RNA extraction was performed according to the method of Senda et al. (2004). Leaves were ground in buffer (100 mM Tris–HCl, pH 8.8, 20 mM EDTA, 200 mM NaCl, 4% *N*-lauryl sarcosine) using liquid nitrogen, and a conventional phenol/chloroform extraction was used to isolate total nucleic acids. After lithium chloride precipitation of nucleic acids, the pellet was used as the total RNA fraction and the supernatant was kept for low molecular weight (LMW) RNAs. Genomic DNAs were eliminated with the addition of polyethylene glycol precipitation (final 10% PEG 8000/1 M NaCl). Total RNA was dissolved in RNA pre-mix (20× MOPS, 50% formamide, 17.5% formaldehyde, 0.5% SDS), heated at 65°C for 5 min, separated in a 1.5% denaturing gel and then blotted to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech). The membrane was hybridized with a specific cDNA probe in hybridization buffer (50% formamide, 5× SSC, 7% SDS, 0.1% *N*-lauryl sarcosine, 2% blocking regent) at 40°C for 1 h. The signals were detected by exposing the membrane to X-ray film with the chemical fluorescent substrate. LMW RNA was dissolved in formamide and electrophoresed in a 15% polyacrylamide gel with 8 M urea. Separated RNAs were transferred to the membrane by capillary blotting, and the blots were hybridized as mentioned.

DNA probe and RNA probe preparation

DNA probes were labeled with digoxigenin by PCR. The reaction mixture contained 1 mM dATP, dGTP, dCTP, 0.65 mM dTTP, 0.35 mM Dig-11-dUTP (Roche, Basel, Switzerland), 1 × EX-Taq buffer, 5 U of EX-Taq (TAKARA SHUZO CO. LTD, Otsu, Japan), template DNA and primers. RNA probes were labeled with digoxigenin by in vitro transcription according to the manufacturer's instructions (Roche, Basel, Switerland). RNA probes for the LUC gene were prepared from a partial LUC cDNA that was provided in the TaKaRa siRNA Cocktail Kit (TAKARA SHUZO CO. LTD).

Preparation of LUC siRNAs for in vitro binding assay

The LUC dsRNAs were prepared by the TaKaRa siRNA cocktail kit. To eliminate any residual ssRNAs, the dsRNA (25 μg) was then treated at 37°C for 30 min with RNase A (2.5 μg ml^{-1}) in incubation buffer (10 mM Tris–HCl, pH 7.5, 200 mM NaCl, 100 mM LiCl and 1 mM EDTA). After phenol/chloroform extraction followed by ethanol precipitation, dsRNA was digested by a human Dicer to generate siRNAs. The small RNA fraction (20–30 nt) was eventually separated in a 3% agarose gel, recovered from the gel and used as LUC siRNA.

siRNA and miR171 binding assay

PCR-amplified genes of viral suppressor (p19 and 2b) containing the T7 promoter sequence at the 5′ end and the FLAG peptide sequence at the 3′ end were used for in vitro transcription with T7 RNA polymerase. Likewise, PCR-amplified genes of 2b and GFP containing the His-tag sequence at the 3′ end (six His residues) were prepared. The transcripts were then in vitro translated with the wheat germ extract system (Proteios) (TOYOBO, Osaka, Japan) at 26°C for 20 h according to the manufacturer's instructions. A part of the translation mixture containing p19 (100–150 ng) or 2b (50–75 ng) was mixed with the LUC dsRNAs that were digested by a human Dicer, and incubated overnight at 25°C. The reaction mixtures were then purified through an anti-FLAG M2 affinity column. The bound RNAs were then extracted with phenol and subsequently analyzed by Northern blot hybridization. For the miRNA binding assay, we used miR171 (Llave et al. 2002, Parizotto et al. 2004). The selected and non-selected strands (miR171 and miR171*, respectively) were chemically synthesized, and the duplex was formed in annealing buffer (100 mM acetic potassium, 30 mM HEPES-KOH, pH 7.4, 2 mM acetic magnesium). Instead of miR171*, another RNA strand was prepared to make siR171, a complete dsRNA with miR171S. The annealed duplexes were confirmed by PAGE, and excised from the gel by electroelution for the subsequent binding assay.

Detection of 5′-biotinylated miR171 and siR171

Synthetic 5′-biotinylated miR171 was used to create miR171 and siR171, and the annealed RNA duplexes were purified as described above. A series of dilutions of the in vitro translation mixture containing a suppressor were incubated with the prepared RNA duplexes. The suppressor-bound RNA duplexes were isolated through a FLAG affinity column. RNA was then extracted, analyzed in a 15% denaturing polyacrylamide gel and transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech, Piscataway, NJ, USA). The biotinylated miR171 and siR171 were detected by using the LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

Acknowledgments

We would like to thank Ichiro Uyeda and Kenji Nakahara for their helpful discussions. We also thank David Baulcombe for kindly providing us with the transgenic plant (16c) and the p19 gene from TBSV. We are grateful to Akira Kanazawa for help with the experiments using the petunia plants. We also thank Kaien Fujino and Ayano Meguro for their technical help.

References

Baulcombe, D.C. (2004) RNA silencing in plants. *Nature* 431: 356–363.

Baulcombe, D.C. and Molnar, A. (2004) Crystal structure of p19—a universal suppressor of RNA silencing. *Trends Biochem. Sci.* 29: 279–281.

Brigneti, G., Voinnet, O., Li, W.X., Ji, L.X., Ding, S.W. and Baulcombe, D.C. (1998) Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO J.* 17: 6739–6746.

Bucher, E., Hemmes, H., Haan, P.D., Goldbach, R. and Prins, M. (2004) The influenza A virus NS1 protein binds small interfering RNAs and suppresses RNA silencing in plants. *J. Gen. Virol.* 85: 983–991.

Chapman, E.J., Prokhnevsky, A.I., Gopinath, K., Dolja, V.V. and Carrington, J.C. (2004) Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. *Genes Dev.* 18: 1179–1186.

Chellappan, P., Vanitharani, R. and Fauquet, C.M. (2005) MicroRNA-binding viral protein interferes with *Arabidopsis* development. *Proc. Natl Acad. Sci. USA* 102: 10381–10386.

Ding, S.W., Anderson, B.J., Haase, H.R. and Symons, R.H. (1994) New overlapping gene encoded by the cucumber mosaic virus genome. *Virology* 198: 593–601.

Ding, S.W., Rathjen, J.P., Li, W.X., Swanson, R., Healy, H. and Symons, R.H. (1995) Efficient infection from cDNA clones of cucumber mosaic cucumovirus RNAs in a new plasmid vector. *J. Gen. Virol.* 76: 459–464.

Goto, K., Kanazawa, A., Kusaba, M. and Masuta, C. (2004) A simple and rapid method to detect plant siRNAs using nonradioactive probes. *Plant Mol. Biol. Rep.* 21: 1–8.

Guo, H.S. and Ding, S.W. (2002) A viral protein inhibits the long range signaling activity of the gene silencing signal. *EMBO J.* 21: 398–407.

Hamilton, A., Voinnet, O., Chappell, L. and Baulcombe, D. (2002) Two classes of short interfering RNA in RNA silencing. *EMBO J.* 21: 4671–4679.

Kanazawa, A., O'Dell, M., Hellens, R., Hitchin, E. and Metzlaff, M. (2000) Mini-scale method for nuclear run-on transcriptional assay in plant. *Plant Mol. Biol. Rep.* 18: 377–383.

Kasschau, K.D., Xie, Z., Allen, E., Llave, C., Chapman, E.J., Krizan, K A. and Carrington, J.C. (2003) P1/HC-Pro, a viral suppressor of RNA silencing, interferes with Arabidopsis development and miRNA function. *Dev. Cell* 4: 205–217.

Kosaka, Y. and Fukunishi, T. (1997) Multiple inoculation with three attenuated viruses for the control of cucumber virus disease. *Plant Dis.* 81: 733–738.

Koseki, M., Goto, K., Masuta, C. and Kanazawa, A. (2005) The star-type color pattern in *Petunia hybrida* 'red star' flowers is induced by the sequence-specific degradation of the chalcone synthase RNA. *Plant Cell Physiol.* 46: 1879–1883.

Kurihara, Y. and Watanabe, Y. (2003) Cross-protection in *Arabidopsis* against crucifer tobamovirus Cg by an attenuated strain of the virus. *Mol. Plant Pathol.* 4: 259–269.

Lakatos, L., Csorba, T., Pantoaleo, V., Champman, E.J., Carrington, J.C., Liu, Y.-P., Dolja, V.V., Calvino, L.F., López-Moya, J.J. and Burgyán, J. (2006) Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. *EMBO J.* 80: 5747–5756.

Llave, C., Kasschau, K.D. and Carrington, J.C. (2000) Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. *Proc. Natl Acad. Sci. USA* 97: 13401–13406.

Llave, C., Xie, Z., Kasschau, K.D. and Carrington, J.C. (2002) Cleavage of scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. *Science* 297: 2053–2056.

Lu, R., Maduro, M., Li, F., Broitman-Maduro, G., Li, W.X. and Ding, S.W. (2005) Animal virus replication and RNAi-mediated antiviral silencing in *Caenorhabditis elegans*. *Nature* 436: 1040–1043.

Lucy, A.P., Guo, H.S., Li, W.X. and Ding, S.W. (2000) Suppression of post-transcriptional gene silencing by a plant viral protein localized in the nucleus. *EMBO J.* 19: 1672–1680.

Mallory, A.C., Ely, L., Smith, T.H., Marathe, R., Anandalakshmi, R., Fagard, M., Vaucheret, H., Pruss, G., Bowman, L. and Vance, V.B. (2001) HC-Pro suppression of transgene silencing eliminates the small RNAs but not transgene methylation or the mobile signal. *Plant Cell* 13: 571–583.

Mallory, A.C., Reinhart, B.J., David, B., Vance, V.B. and Bowman, L.H. (2002) A viral suppressor of RNA silencing differentially regulates the accumulation of short interfering RNAs and micro-RNAs in tobacco. *Proc. Natl Acad. Sci. USA* 99: 15228–15233.

Mayers, C.N., Palukaitis, P. and Carr, J.P. (2000) Subcellular distribution analysis of the cucumber mosaic virus 2b protein. *J. Gen. Virol.* 81: 219–226.

Meister, G. and Tuschl, T. (2004) Mechanisms of gene silencing by double-stranded RNA. *Nature* 431: 343–349.

Mérai, Z., Kerényi, Z., Kertész, S., Magna, M., Lakatos, L. and Shihavy, D. (2006) Double-stranded RNA binding may be a general plant RNA viral strategy to suppress RNA silencing. *J. Virol.* 80: 5747–5756.

Mérai, Z., Kerényi, Z., Molnár, A., Barta, E., Válóczi, A., Bisztray, G., Havelda, Z., Burgyán, J. and Shihavy, D. (2005) Aureusvirus P14 is an efficient RNA silencing suppressor that binds double-stranded RNAs without size specificity. *J. Virol.* 79: 7217–7226.

Metzlaff, M., O'Dell, M., Cluster, P.D. and Flavell, R.B. (1997) RNA-mediated RNA degradation and chalcone synthase A silencing in petunia. *Cell* 88: 845–854.

Metzlaff, M., O'Dell, M., Hellens, R. and Flavell, R.B. (2000) Developmentally and transgene regulated nuclear processing of primary transcripts of chalcone synthase A in petunia. *Plant J.* 23: 63–72.

Mitsuhara, I., Ugaki, M., Hirochika, H., Ohshima, M., Murakami, T., et al. (1996) Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. *Plant Cell Physiol.* 37: 49–59.

O'Dell, M., Metzlaff, M. and Flavell, R.B. (1999) Post-transcriptional gene silencing of chalcone synthase in transgenic petunia, cytosine methylation and epigenetic variation. *Plant J.* 18: 33–42.

Parizotto, E.A., Dunoyer, P., Rahm, N., Himber, C. and Voinnet, O. (2004) In vivo investigation of the transcription, processing, endonucleolytic activity and functional relevance of the spatial distribution of a plant miRNA. *Genes Dev.* 18: 2237–2242.

Qi, Y., Zhong, X., Itaya, A. and Ding, B. (2004) Dissecting RNA silencing in protoplasts uncovers novel effects of viral suppressors on the silencing pathway at the cellular level. *Nucleic Acids Res.* 32: e179.

Roth, B.M., Pruss, G.J. and Vance, V.B. (2004) Plant viral suppressors of RNA silencing. *Virus Res.* 102: 97–108.

Sato, M., Masuta, C. and Uyeda, I. (2003) Natural resistance to *Clover yellow vein virus* in beans controlled by a single recessive locus. *Mol. Plant-Microbe Interact.* 16: 994–1002.

Senda, M., Masuta, C., Ohnishi, S., Goto, K., Kasai, A., Sano, T., Hong, J.-S. and MacFarlane, S. (2004) Patterning of virus-infected *Glycine max* seed coat is associated with suppression of endogenous silencing of chalcone synthase genes. *Plant Cell* 16: 807–818.

Shi, B.J., Palukaitis, P. and Symons, R. (2002) Differential virulence by strains of *Cucumber mosaic virus* is mediated by the 2b gene. *Mol. Plant-Microbe Interact.* 15: 947–955.

Silhavy, D., Molnár, A., Lucioli, A., Szittya, G., Hornyik, C., Tavazza, M. and Burgyán, J. (2002) A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. *EMBO J.* 21: 3070–3080.

Soards, A.J., Murphy, A.M., Palukaitis, P. and Carr, J.P. (2002) Virulence and differential local and systemic spread of *Cucumber mosaic virus* in tobacco are affected by the CMV 2b protein. *Mol. Plant-Microbe Interact.* 15: 647–653.

Suzuki, M., Kuwata, S., Kataoka, J., Masuta, C., Nitta, N. and Takanami, Y. (1991) Functional analysis of deletion mutants of Cucumber mosaic virus RNA3 using an in vitro transcription system. *Virology* 183: 106–113.

Vance, V. and Vaucheret, H. (2001) RNA silencing in plants—defense and counterdefense. *Science* 292: 2277–2280.

Voinnet, O. (2001) RNA silencing as a plant immune system against viruses. *Trends Genet.* 17: 449–459.

Voinnet, O., Vain, P., Angell, S. and Baulcombe, D.C. (1998) Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell* 95: 177–187.

Wang, M.B. and Metzlaff, M. (2005) RNA silencing and antiviral defense in plants. *Curr. Opin. Plant Biol.* 8: 216–222.

Wang, Y., Tzfira, T., Gaba, V., Citovsky, V., Palukaitis, P. and Gal-On, A. (2004) Functional analysis of the Cucumber mosaic virus 2b protein: pathogenicity and nuclear localization. *J. Gen. Virol.* 85: 3135–3147.

Ye, K., Malinina, L. and Patel, D.J. (2003) Recognition of small interfering RNA by a viral suppressor of RNA silencing. *Nature* 426: 874–878.

Zhang, X., Yuan, Y.R., Pei, Y., Lin, S.S., Tuschl, T., Patel, D.J. and Chua, N.H. (2006) Cucumber mosaic virus-encoded 2b suppressor inhibits Arabidopsis Argonaute1 cleavage activity to counter plant defense. *Genes Dev.* 20: 3255–3268.

(Received February 12, 2007; Accepted June 7, 2007)