Published in ***The Plant Journal*** **(2011) 65, 156-168**

Virus-mediated efficient induction of epigenetic modifications of endogenous genes with phenotypic changes in plants

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Running title: Epigenetic modifications of endogenous genes

Keywords: transcriptional gene silencing, epigenetic changes, RNA-directed DNA methylation, histone modification, *Cucumber mosaic virus*, short interfering RNA

Summary

Gene silencing through transcriptional repression can be induced by targeting double-stranded RNA (dsRNA) to a gene promoter. It has been reported that a transgene was silenced by targeting dsRNA to the promoter, and the silenced state was inherited to the progeny plant even after removal of the silencing inducer from cells. In contrast, no plant has been produced that harbors silenced endogenous gene after removal of promoter-targeting dsRNA. Here, we show that heritable gene silencing can be induced by targeting dsRNA to the endogenous gene promoters in petunia and tomato plants, using the *Cucumber mosaic virus* (CMV)-based vector. We found that efficient silencing of endogenous genes depends on the function of the 2b protein encoded in the vector virus, which has the ability to facilitate epigenetic modifications through the transport of short interfering RNA to nucleus. Bisulfite sequencing analyses on the targeted promoter in the virus-infected and its progeny plants revealed that cytosine methylation was found not only at CG or CNG but also at CNN sites. The observed inheritance of asymmetric DNA methylation is quite unique, suggesting that plants have a mechanism to maintain even asymmetric methylation. This CMV-based gene silencing system provides a useful tool to artificially modify DNA methylation in plant genomes and elucidate the mechanism for epigenetic controls.

Introduction

Nucleotide sequence-specific interactions mediated by double-stranded RNA (dsRNA) have been known to induce gene silencing, either through mRNA degradation or transcriptional repression (Baulcombe, 2004; Matzke and Birchler, 2005). The dsRNAs are processed into 21- to 26-nulceotide (nt) short interfering RNAs (siRNAs) by dsRNA-specific ribonuclease, Dicer or Dicer-like (DCL) (Baulcombe, 2004; Carmell and Hannon, 2004). In Arabidopsis, DCL2, DCL3 and DCL4 produce 22-, 24- and 21-nt siRNAs, respectively (Fusaro et al., 2006). DCL3-generated 24-nt siRNAs have been recently found to be mobile signals and direct epigenetic modification in plants (Molnar et al., 2010). The siRNAs are incorporated into AGO proteins and serve as a guide for sequence-specific cleavage of a target RNA (Brodersen and Voinnet, 2006; Vaucheret, 2008). Transcriptional repression can also be induced by dsRNA, which contain a sequence homologous to a gene promoter and can trigger cytosine methylation on the promoter sequence in the nuclear DNA resulting in transcriptional gene silencing (TGS) (Mette et al., 2000; Jones et al., 2001; Sijen et al., 2001). Such RNA-guided epigenetic modification of the genome is referred to as RNA-directed DNA methylation (RdDM), and the RdDM is also correlated with histone modifications involving histone H3 lysine 9 (H3K9) dimethylation on the target sequence, which is the initial step of heterochromatin formation (Matzke and Birchler, 2005).

Gene silencing through transcriptional repression can be induced by dsRNA targeted to a gene promoter, and this phenomenon, termed RNA-mediated TGS, was first discovered in plants using a transgene that transcribes an inverted repeat of promoter sequence and later reported in cultured human cells and in *Schizosaccharomyces pombe* (Mette et al., 2000; Volpe et al., 2002; Schramke et al., 2003; Morris et al., 2004; Ting et al., 2005). Plant RNA viruses such as the *Potato*

virus X (PVX), *Tobacco rattle virus* (TRV) and *Cucumber mosaic virus* (CMV) vectors have also been used as a tool to induce TGS (Jones et al., 1999; Jones et al., 2001; Otagaki et al., 2006) because replication of the RNA virus generates dsRNA intermediates that are processed into siRNAs by the host RNA silencing pathway. When viruses are designed to carry a portion of host gene sequence, the processed siRNAs can become inducer of gene silencing targeting to the corresponding homologous mRNA or promoter DNA.

In plants, there is a marked difference between transgenes and endogenous genes in the feasibility of the TGS induction by targeting dsRNA to a promoter (Okano et al., 2008). Transgenes in plant genome can be easily silenced and the silenced state was heritable in the presence or absence of the silencing inducer (Jones et al., 2001). On the other hand, endogenous genes can be silenced only in the presence of the silencing inducer (Sijen et al., 2001; Cigan et al., 2005; Heilersig et al., 2006). The PVX and TRV vectors have been shown to induce heritable RNA-mediated TGS against transgenes such as the green fluorescent protein (*GFP*) and β-glucuronidase (*GUS*) genes (Jones et al., 1999; Jones et al., 2001), but no success has been reported in RdDM and RNA-mediated TGS against endogenous genes such as the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*rbcS*) gene (Jones et al., 1999). Thus, plants that retain a silenced endogenous gene after removal of promoter-targeting dsRNA have not been reported so far.

In our previous work, we developed the RNA virus vector based on CMV, which is able to rapidly induce sequence-specific gene silencing through targeting the coding sequence or the promoter of transgene (Otagaki et al., 2006; Nagamatsu et al., 2007). In the present report, we tested whether heritable RdDM and RNA-mediated TGS can be induced using the CMV-based vector and found that the CMV vector carrying the endogenous gene promoter efficiently induced RdDM and heritable gene silencing, and

that the 2b protein of CMV was involved in the efficient RNA-mediated TGS. Because the virus is not normally transmitted to seeds in many plant species, the epigenetically modified plants are inducer-free in the next generation. The great advantage of the absence of any transgenes is discussed for practical use of such a class of modified plants.

Results

CMV carrying endogenous gene promoter induces phenotypic changes in petunia flower color

In this study, we targeted the promoter of the *CHS-A* gene for chalcone synthase in petunia (*Petunia hybrida*) because silencing of this gene can be manifested as an altered visible phenotype. When transcriptional silencing of the *CHS-A* gene is induced in petunia, pigment in flower petal is expected to decrease, and white sectors can appear because chalcone synthase is essential for the synthesis of anthocyanins as shown by *CHS-A* co-suppression (Napoli et al., 1990; van der Krol et al., 1990). Silencing of the *CHS-A* gene also reduces flavonol content resulting in decrease of fertile pollen (Mo et al., 1992). To induce epigenetic modification to the *CHS-A* gene, the region spanning -244 to -2 relative to the transcription start site of the *CHS-A* promoter was inserted into the cloning site of the CMV-A1 vector (Figure 1a). To avoid excessive symptom induction that may mask effects of specific silencing, we used a pseudorecombinant virus that consists of RNA components derived from different CMV strains; CMV-L RNA1 and CMV-L RNA3 were mixed with the RNA2 transcribed from CMV-A1 (derived from CMV-Y), and found that the pseudorecombinant virus systemically infected petunia plants and caused very mild symptoms. We used petunia variety Red Star that produces flowers with a red and white bicolor pattern (Figure 1b, Mock), and this phenotype has been shown to be the

result of naturally occurring sequence-specific degradation of *CHS-A* transcripts in the white sector of petal (Koseki et al., 2005). Red Star plants grown in a growth chamber produced flowers having 18-45% (an average of 30%) of white area in petal (Figure 1b, Mock). When Red Star plants were infected with the pseudorecombinant virus that lacked the *CHS-A* promoter insert, the plants produced almost entirely red flowers (Figure 1b, CMV-A1) because sequence-specific RNA degradation was suppressed by the silencing suppressor protein 2b, encoded by the vector virus, as previously reported (Koseki et al., 2005). In contrast, when Red Star plants were infected with the virus carrying the *CHS-A* promoter sequence (referred to hereafter as CMV-A1:CHSpro), the plants exhibited visible change in their flower color. About 80% of CMV-A1:CHSpro-infected plants had a similar phenotype to that of the CMV-A1-infected plants, but at about 20%, plants having petals with wider white area (69-89%; an average of 79%) were generated. At a low frequency, plants with almost completely reduced pigmentation in petal were generated (Figure 1b, CMV-A1:CHSpro). Furthermore, plants having petals with wider white area produced less pollen. In an in vitro pollen germination assay (Jahnen et al., 1989), the frequency of pollen germination was significantly low in the CMV-A1:CHSpro-infected plants (Figure 1c). RT-PCR was performed to confirm viral accumulation in the flowers inoculated with CMV-A1:CHSpro and no deletion of the insert from the vector virus (Figure 1d).

We then obtained 60 seeds from four flowers after self-fertilization of the plant with the altered phenotype, although these plants had only limited pollen grains. Most of the plants of the next generation did not survive after germination, but three plants did survive. The all progeny of the CMV-A1:CHSpro-infected plant had a unique flower phenotype with wider (61-75%; an average of 69%) and/or distorted distribution of white area, e.g., white stripes within red portions of the petals (Figure

1b, CMV-A1:CHSpro progeny). In addition, the male-sterile phenotype, identified by severe inhibition of pollen production, was also maintained in the progeny plants (Figure 1b and 1c). Seed production was normal when stigmas of the CMV-A1-infected and CMV-A1:CHSpro-infected plants were pollinated with pollen from virus-uninfected plants, indicating that these plants are female fertile. None of more than 200 mock-inoculated plants or CMV-A1-infected plants or plants obtained by self-fertilization of these plants, had such altered flower pigmentation and pollen production.

CMV-induced phenotypic changes are associated with DNA methylation and histone modification on the targeted CHS-A promoter in petunia

A quantitative RT-PCR revealed that the mRNA level of the *CHS-A* gene in flower (anther and petal) tissues markedly decreased in the CMV-A1:CHSpro-infected plant and its progeny, indicating that flower pigmentation and altered pollen production were induced as a consequence of downregulation of the *CHS-A* gene. A representative result of RT-PCR in anther tissue was shown in Figure 2a, which was consistent with the previous report that *CHS-A* gene silencing causes male sterility (Mo et al., 1992). Because siRNAs homologous to the *CHS-A* promoter are required for inducing sequence-specific DNA methylation and/or changes in histone modification, we examined the production of siRNAs as a consequence of CMV-A1:CHSpro infection. Hybridization of low-molecular weight RNA with a probe specific to the *CHS-A* promoter indicated that siRNAs homologous to the *CHS-A* promoter were actually produced in the CMV-A1:CHSpro-infected plant (Figure 2b). On the other hand, the siRNAs homologous to the *CHS-A* coding region were not detected both in the CMV-A1:CHSpro-infected plant and its progeny (Figure S1). Recently, 24-nt small RNAs have been proved to be essential for epigenetic modification (Molnar et al.,

2010), although small RNAs of the other size classes can also have the role (Brodersen and Voinnet, 2006; Vazquez 2006). We thus conducted deep sequencing analysis of small RNAs and found that there were indeed 24-nt siRNAs of the target sequence generated in CMV-A1:CHSpro-infected tissues (Figure 2c). We also found that 21-nt small RNAs were most abundant among small RNAs homologous to the *CHS-A* promoter (Figure S2). In addition, we also analyzed cytosine methylation and histone modification to detect epigenetic marks in the CMV-A1:CHSpro-infected plant and in its progeny. Bisulfite sequencing analysis indicated that de novo methylation of cytosine was induced in the *CHS-A* promoter in the CMV-A1:CHSpro-infected plant (Figure 2d). Cytosine methylation was also detected in the progeny of the CMV-A1:CHSpro-infected plant, although the frequency was lower than in the parent plants (Figure 2d), indicating that cytosine methylation of the *CHS-A* promoter is inherited but the extent of its inheritance is limited. The data also indicated that in addition to symmetric DNA methylation, asymmetric DNA methylation could also be transmitted to the progeny. Furthermore, a chromatin immunoprecipitation (ChIP) assay on the *CHS-A* promoter region revealed a marked difference between the CMV-A1-infected plants and CMV-A1:CHSpro-infected plant: the presence of dimethylation at Lys9 of histone H3 (H3K9me2) and the absence of acetylation of histone H3 (H3Ac) in the *CHS-A* promoter region in the CMV-A1:CHSpro-infected plant (Figure 2e). More noteworthy was that histone modification was also altered in the progeny plants (Figure 2e). These results demonstrated that infection of Red Star plants with CMV-A1:CHSpro resulted in a heritable and downregulated state of the *CHS-A* gene, which is associated with epigenetic changes in the *CHS-A* promoter.

Transmission of CMV through seeds has been reported in a limited number of plant species, and normally CMV is not transmitted to seeds in *Solanaceae* plants including petunia and tomato. We confirmed the absence of virus in the progeny

petunia plants by both an enzyme-linked immunosorbent assay (ELISA) and RT-PCR; a representative result is shown in Figure 2f. Thus, the altered phenotype induced by targeting dsRNA to the *CHS-A* promoter was inherited to the next generation in the absence of any silencing trigger. To further confirm the phenomenon observed in Red Star, we also tested the *CHS-A* promoter-targeted silencing using another petunia variety. When petunia line V26 were infected with CMV-A1:CHSpro, 20 of 230 plants produced flowers with patchy or striped color patterns and showed severe inhibition of pollen production (Figure S3). No changes were detected in the plants infected with an empty vector. As observed in Red Star, the mRNA levels of the *CHS-A* gene in the petal tissues of these plants and their progeny were remarkably reduced, and furthermore, epigenetic modifications in CMV-A1:CHSpro-infected plants and its progeny were also observed; histone modification was well maintained, although DNA methylation was mostly canceled in the progeny plants (Figure S3).

CMV-induced epigenetic changes by targeting the LeSPL-CNR promoter in tomato

We also tested the ability of the vector to induce promoter-targeted heritable gene silencing for another gene and another host plant by targeting the *LeSPL-CNR* (CNR, colorless non-ripening) gene, which is essential for fruit ripening in tomato. The 286-bp contiguous region 2.4 kb upstream from the *LeSPL-CNR* coding sequence has been demonstrated to be the site for a naturally occurring epigenetic mutation (Manning et al., 2006). We therefore tested the possibility that we could accelerate the natural epigenetic process by targeting the cytosines reported for the epigenetic allele of the *LeSPL-CNR* gene. Infection of the tomato plant with the virus carrying the target gene promoter resulted in phenotypic changes: inhibition of fruit ripening by silencing the *LeSPL-CNR* gene (Figure 3a). Although the fruit color was almost recovered, the progeny of the CMV-A1:LeSPL-CNRpro-infected plant produced a fruit

with mottled phenotype (Figure 3a). These changes were accompanied by heritable reduction in the mRNA level of the gene and an increase in the frequency of methylcytosine in the promoter (Figure 3b and 3c). Methylated promoter region in the progeny of the CMV-A1:LeSPL-CNRpro-infected tomato seems to spread out compared to the parent plants. The *LeSPL-CNR* promoter is always highly and widely methylated in plants containing an epigenetic allele of *LeSPL-CNR* (*Cnr* mutation) (Manning et al., 2006). Manning et al. (2006) suggested that the *Cnr* mutation resulted from methylation of normally unmethylated cytosines in the promoter with unknown mechanism and the changes were stable and inheritable to the next generation. We therefore speculate that targeting this region by the virus vector may have activated the potential mechanism of the *Cnr* mutation, resulting in the spread of DNA methylation in the progeny plants. In ChIP assay, the altered state in histone modification was maintained in the progeny of CMV-A1:LeSPL-CNRpro-infected tomato (Figure 3d). Phenotypic changes were observed in 3 of 12 plants inoculated with the virus containing the *LeSPL-CNR* promoter. No phenotypic changes other than those expected from the gene silencing were observed, indicating that the promoter-targeted silencing caused no side effect. Microarray analysis of RNA isolated from the progeny of CMV-A1:LeSPL-CNRpro-infected tomato plants and its control plants also indicated that the *LeSPL-CNR* silencing was substantially accompanied by no profound change in global gene expression in leaf tissues (Figure S4).

The 2b protein binds to siRNA and promotes siRNA accumulation in nucleus

In CMV-infected cells, the 2b protein (2b) has been shown to accumulate predominantly in or on the host cell nucleus (Mayers et al., 2000; Wang et al., 2004). On the other hand, 2b of *Cucumovirus* has been shown to have the ability to bind to

dsRNAs (Goto et al., 2007; Rashid et al., 2008). We thus hypothesized that 2b could promote siRNA accumulation in nucleus, probably by transporting siRNA to the nuclei, and facilitate CMV-induced epigenetic modification. To test this idea, we first confirmed the accumulation in nucleus and the ability of siRNA-binding of the 2b encoded by our CMV vector CMV-A1 [referred to hereafter as 2b(2/3)], because the 2b(2/3) lacks the C-terminal one-third of the intact 2b protein as a consequence of introducing restriction sites for cloning a foreign fragment (Otagaki et al., 2006). As shown in Figure 4a and 4b, 2b(2/3) could be localized in nuclei and bind to chemically synthesized 24-nt siRNAs in vitro. In our previous work, we have demonstrated that intact 2b could bind to siRNAs in CMV-infected plants (Goto et al., 2007). Here, a sequence analysis of siRNAs bound to 2b(2/3) was conducted to identify their sizes. Because both 2b and 2b(2/3) tend to become insoluble in infected tissues, it is quite difficult to isolate these proteins as soluble form and thus immunoprecipitation of 2b [2b(2/3)] did not work. We therefore added a tag peptide to the C-terminal of 2b(2/3) and expressed the modified 2b(2/3) in vivo from a recombinant virus for affinity purification (Figure S5). cDNA cloning and the subsequent sequencing analysis revealed that the isolated 2b(2/3) actually recruited siRNAs including not only 21-nt and 22-nt siRNAs but also 23-nt and 24-nt siRNAs (Figure 4c and 4d).

We next examined whether siRNA accumulation in nuclei is facilitated in the presence of the 2b protein using tobacco cultured BY2 cells transformed with the 2b(2/3) gene (the cell line is referred as BY2-2b). When 6-carboxyfluorescein (FAM)-labeled siRNAs were introduced into the BY2-2b and wild-type BY2 protoplasts with lipofectamine, distinct, bright fluorescence from the siRNAs was detected in the nuclei of the BY2-2b protoplasts, whereas the fluorescence was faint and dispersed inside the wild-type BY2 protoplasts (Figure 4e), suggesting that the accumulation of siRNAs in nuclei was indeed facilitated in the presence of 2b.

The 2b protein promotes efficient epigenetic modification induced by the CMV vector

To further confirm our hypothesis that 2b transports siRNA to nucleus, resulting in efficient induction of the promoter-targeted silencing, we next compared the ability to induce silencing between CMV-A1 and CMV-H1, the latter of which was a plant expression vector and lacks the entire 2b gene (Matsuo et al., 2007). Considering that TGS of endogenous genes is not efficiently induced, we here used a transgene promoter to examine the involvement of 2b in TGS. The 345-bp sequence of the *Cauliflower mosaic virus* (CaMV) 35S promoter was inserted into the cloning site of CMV-A1 and CMV-H1 to create CMV-A1:35Spro and CMV-H1:35Spro, respectively (Figure 5a). These recombinant viruses were used to inoculate *Nicotiana benthamiana* line 16c plants that contain a single-copy *GFP* gene expressed under the control of the CaMV 35S promoter (Ruiz et al., 1998). As a consequence, the upper leaves of plants infected with CMV-A1:35Spro started to lose GFP fluorescence at 12 days post inoculation (dpi), while all the CMV-H1:35Spro-infected plants retained *GFP* fluorescence at this stage (Figure 5b). Comparable results were obtained by Northern blot analysis (Figure 5b, lower panel). Similar changes were reproduced in all 12 plants independently infected with either CMV-A1:35Spro or CMV-H1:35Spro. CMV-H1:35Spro-infected plants subsequently lost GFP fluorescence much later at 53 dpi (data not shown). These results indicate that the 2b protein affects the efficient promoter-targeted silencing. In addition, the efficient induction of silencing by CMV-A1:35Spro was associated with a high frequency of cytosine methylation on the promoter region (Figure 5c). By a ChIP assay, we confirmed the alterations in histone modification; the increase of H3K9me2 and the decrease of H3Ac in the CMV-A1:35Spro-infected plants (Figure 5d). In contrast, we were not able to detect any siRNAs derived from the *GFP* coding region (Figure S6a). Furthermore, in a

ChIP assay using RNA Polymerase II (Pol II) antibodies (Swiezewski et al., 2009), the level of Pol II binding to the CaMV 35S promoter decreased (Figure S6b). These results suggest that *GFP* silencing induced by CMV occurred at transcriptional repression. To deny the possibility that the disability of CMV-H1 to induce efficient TGS is due to its low infectivity, we compared its infectivity with that of CMV-A1. Actually, the two constructs, CMV-A1:35Spro and CMV-H1:35Spro did not differ in viral accumulation and systemic spread at 14 dpi and in the stability of the insert (Figure S7) as previously reported for 2b-expressing and 2b-deficient CMVs in other *Nicotiana* plants (Ji and Ding, 2001), although 2b is essential for systemic infection of CMVs in cucumber and *Arabidopsis* plants (Diaz-Pendon et al., 2007).

To verify the hypothesis further, we analyzed the levels of siRNAs corresponding to the CaMV 35S promoter in nuclei. Northern blot analysis of low-molecular-weight RNAs extracted from isolated nuclei indicated that the siRNAs were more abundant in the nuclei of CMV-A1:35Spro-infected plants than those in the CMV-H1:35Spro-infected plants, while siRNAs were detected at considerable levels in total cell extracts of both plants (Figure 5e). We also tested CMV-G1, which lacks the two nuclear localization signals in 2b, and found that this vector did not induce complete loss of *GFP* fluorescence, even at 30 dpi (Figure S8). Therefore, the nuclear localizing nature of the 2b protein is important for its ability to promote silencing. Overall, these results suggest that 2b enhances transport of siRNA into the nucleus, resulting in efficient induction of cytosine methylation and silencing of a target gene.

The 2b protein alone is responsible for the efficient epigenetic modification

We now have the evidence that 2b is associated with efficient siRNA transport to the nucleus and hypothesized that 2b could facilitate RdDM and histone modifications

eventually leading to RNA-mediated TGS. To test this idea, we developed a protoplast assay coupled with a ChIP analysis and examined whether 2b can alone enhance silencing of the *CHS-A* gene and subsequent histone modifications on the *CHS-A* promoter (Figure 6a). Because the petunia *CHS-A* gene is preferentially expressed in flowers, we here used petal protoplasts. The result of quantitative RT-PCR following introduction of dsRNA of the *CHS-A* promoter into protoplasts showed that the mRNA levels of the *CHS-A* gene were significantly reduced when 2b was expressed simultaneously in protoplasts (Figure 6b). We then conducted sequence analysis of and the *CHS-A* promoter-specific siRNAs (Figure 6c) and Northern hybridization (Figure S9a), and confirmed that the siRNAs generated in protoplasts contained 23-nt and 24-nt siRNAs as well as 21-nt and 22-nt siRNAs. Furthermore, the ChIP analysis revealed that H3K9me2 increased whereas H3Ac decreased on the *CHS-A* promoter in protoplasts that were transfected with dsRNA of the *CHS-A* promoter in the presence of 2b (Figure 6d), suggesting that histone modifications were indeed induced by the 2b expression. As to DNA methylation status on the *CHS-A* promoter, bisulfite sequencing revealed that DNA methylation was induced to some extent, although the frequency was low (Figure S9b). Taken together, these results thus strongly suggest that 2b is the major determinant for induction of epigenetic changes in our vector system.

Discussion

It has been little understood whether virus-induced silencing can induce heritable chromatin inactivation particularly when the endogenous gene was targeted for TGS. In a previous study using the PVX vector (Jones et al. 1999), targeting dsRNA to a transcribed region induced sequence-specific degradation of the *rbcS* RNA in *N. benthamiana* but it did not induce de novo methylation of the gene. In contrast, our

CMV vector induced cytosine methylation at a high frequency on endogenous gene promoter. It seems likely that this difference may be due to a difference in the vector system used, i.e., PVX versus CMV. Through the CMV-based efficient induction of gene silencing, we consider that induction of epigenetic changes on the endogenous gene promoter depends on activity of the viral protein 2b encoded by the vector itself; the entire scheme of epigenetic induction by the CMV vector is shown in Figure S10. In brief, virus-derived siRNAs including those corresponding to an endogenous gene promoter are transported to nucleus, a process facilitated by 2b. The target gene then undergoes epigenetic changes and consequently, heritable silencing.

As CMV-H1:35Spro could sometimes (not always) induce silencing more than one month after CMV-A1:35Spro induced TGS, 2b may not be absolutely necessary for TGS induction. We assume that the CMV-H1:35Spro-induced silencing would also be the consequence of promoter-targeted silencing. When 2b is not supplied, the siRNAs derived from the viral vector may not efficiently target the CaMV 35S promoter but diffuse into nucleus and cause silencing at a low frequency.

In the CMV vector system used here, virus-derived dsRNA efficiently induced DNA methylation on the *CHS-A* promoter in the inoculated petunia plants but the frequency of DNA methylation was significantly reduced in the progeny plants, although DNA methylation was still maintained to some extent. In contrast, histone H3 modification was well maintained even in the progeny plants, suggesting that DNA methylation is not always associated with histone modification. On the other hand, DNA methylation on the *LeSPL-CNR* promoter in tomato was found to be high even in the progeny plants, but the frequency of CNN methylation was greatly decreased. This is perhaps due to the fact that this region is naturally methylated with fruit ripening and thus has different epigenetic features compared to the other host genes (Manning et al., 2006). At this stage, we cannot answer whether the alteration in

histone modification was induced through the initial DNA methylation triggered by the viral vector, and which event is more important for epigenetic changes in phenotypes. Considering that we could not induce some drastic phenotypic changes, maintenance of DNA methylation including CNN methylation as well as histone modification may be important for induction of stable TGS. Interestingly, we could induce not only symmetric DNA methylation at a CG or CNG site but also asymmetric methylation at a CNN site at a relatively high frequency. In animal cells, DNA methylation is mostly found at CG sites and that at a CNN site is very rare (Lister et al., 2009); the asymmetric DNA methylation is quite unique in plants (Chan et al. 2006; Suzuki and Bird, 2008). Our data indicate that asymmetric DNA methylation is transmitted to the progeny of virus-infected plants, suggesting that asymmetric DNA methylation is meiotically maintained in the absence of RNA trigger for RdDM. Thus, there must be some mechanism(s) to maintain CNN methylation in plants. Indeed, MET1 has been considered to be a candidate for an enzyme contributing to siRNA-independent non-CG methylation in plants (Henderson et al., 2006).

Distribution of CMV and the accumulation of CMV-derived siRNA have been detected even in shoot meristem tissues in tobacco (Mochizuki and Ohki, 2004), indicating that siRNAs can be transmitted to germ cells; these features may account for the induction of RNA-mediated, heritable epigenetic changes in our CMV vector system. In human cells, Morris et al. (2004) successfully demonstrated promoter-targeted silencing of the EF1A (elongation factor 1 alpha) gene using MPG, which is the short peptide vector (27 residues) consisting of the peptide domains derived from HIV gp41 and SV40 T-antigen (Morris et al., 1997) and efficiently promotes delivery of siRNAs to the nucleus by its nuclear localization sequence. Therefore, the features of MPG most closely resemble those of 2b. Likewise, our results indicate that the 2b protein plays a crucial role in the nuclear transport of

siRNAs; hence, CMV vector has intrinsic features that facilitate the induction of promoter-targeted silencing of endogenous genes.

The present results also provide direct evidence that acquired epigenetic changes resulting from the targeting of dsRNA to endogenous gene promoters can be heritable in plants. The virus-induced TGS is thus useful in that chromatin inactivation keeps on the progeny without TGS inducer because virus itself is not transmitted to seeds. This approach is the technique for the production of a class of modified plants that do not carry a transgene while still having an altered level of gene expression and the resultant altered phenotype. To advance this technique to a practical level, our trials to target endogenous genes in several plants are well under way. Such efforts revealed that the success to obtain epigenetically modified plants and its progeny depended on the host genes targeted. For example, we could successfully induce RNA-mediated TGS of genes involved in flower morphology; such epigenetically modified progeny accounted for 20-30% of the harvested seeds. We are currently investigating the detailed molecular mechanism for the involvement of the 2b protein in RNA-mediated TGS by using Arabidopsis, which provides many mutant lines in the pathway for RdDM.

Experimental procedures

Plant materials

Petunia hybrida variety Red Star (Takii Seed Co., Japan) and *Lycopersicon esculentum* cv. Ailsa Craig was used as host plants to induce transcriptional repression of endogenous genes. *Nicotiana benthamiana* line 16c having a single copy of the GFP transgene (Ruiz et al., 1998) was obtained from Dr. D. Baulcombe (The Sainsbury Laboratory, UK) and was also used for the analysis. Plants were grown in growth chambers under a 16 h light and 8 h dark regime at 24°C.

Primers

All the primers used in this study have been listed in Table S1.

Construction of viral vectors and inoculation of created recombinant virus

The −244 to −2 region (positions are relative to the transcription start site) of the *CHS-A* promoter (van der Meer et al., 1990) was amplified by PCR using primers (StuI-CHS5-244F and MluI-CHS3-2R) and genomic DNA from *P. hybrida* line V26. The −2555 to −2268 region (positions are relative to the translation start site) of the *LeSPL-CNR* promoter (Manning et al., 2006; DDBJ/EMBL/GenBank accession DQ672601) was amplified by PCR using primers (CNR-5-St-286 and CNR-3-Ml-286) and genomic DNA from *L. esculentum*. These fragments were cloned between the *Stu*I and *Mlu*I sites of the CMV-A1 vector (Otagaki et al., 2006). A 346-bp fragment of the CaMV 35S promoter sequence (−345 to +1) was amplified by PCR using primers (35S-StuI-345F and 35S-MluI+1R) and genomic DNA from *N. benthamiana* line 16c, and then the fragment was cloned in CMV-A1 and CMV-H1 (Matsuo et al., 2007).

Each plasmid containing full length cDNA of RNA1 or RNA3 (derived from CMV-L) and the CMV-A1 vector (derived from RNA2 of CMV-Y) were transcribed in vitro after linearization with a restriction enzyme (Otagaki et al., 2006). Infectious viruses were created by mixing transcripts of RNAs 1 to 3. For virus propagation, leaves of 4-weeks-old plants of *N. benthamiana* were dusted with carborundum and rub-inoculated with the RNA transcripts. For inoculation of *P. hybrida* and tomato plants, leaves of young plants were rub-inoculated with sap from infected leaf tissue from *N. benthamiana* plant. Successful infection of plants without deletion of the inserted sequences was confirmed by conventional ELISA (Masuta et al., 1995) and

RT-PCR of the viral RNA using primers 2b-5up and RNA2-2814R.

Analysis of flower color pattern

The proportion of the petal area occupied by white area was analyzed by Multi Gauge software (Fujifilm, Japan) using digital images of petunia flowers.

In vitro germination of pollen

Petunia pollen grains were suspended in germination medium (Jahnen et al., 1989) and incubated at 25°C. After overnight incubation, the percentage of germinating pollen grains was determined with a light microscope.

Analysis of RNA

Total RNA was isolated from petunia and tomato plants to use for quantitative RT-PCR as described previously (Koseki et al., 2005). Primers for quantitative RT-PCR were as follows (primer sequences are shown in Table S1): 4246 and 5003 for the petunia *CHS-A* gene, tub-1110F and tub-201R for the petunia *α-tubulin* gene, CNR-5M-200 and CNR-3S-200 for the *L. esculentum LeSPL-CNR* gene, LeAct-F and LeAct-R for the *L. esculentum actin* gene. For northern hybridization, low molecular weight RNA was isolated as described previously (Goto et al., 2003). For probes, DNA fragments were amplified by PCR using primers Stu1-CHS5-244F and Mlu1-CHS3-2R for the petunia *CHS-A* promoter and primers 35S-Stu1-345F and 35S-Mlu1+1R for the CaMV 35S promoter. The amplified fragments were cloned into the downstream of T7 promoter of pGEM-T easy vector (Promega, USA) in antisense orientation. The sense RNA probes specific for these promoters were prepared using DIG RNA Labeling Mix (Roche, Switzerland).

Cloning of siRNAs

Purified low molecular weight RNA including siRNAs was cloned using the small RNA cloning kit (Takara, Japan) according to the manufacturer's protocol. The cDNAs were then cloned into pGEM-T easy vector (Promega, USA) and the recombinant clones were randomly sequenced. Thirty and 34 clones of 2b-bound siRNAs in CMV-infected plants and siRNAs in dsCHSpro-transfected protoplasts were sequenced, respectively.

Deep sequencing of small RNAs

Total RNA was extracted from CMV-infected petunia Red Star plants. Small RNAs were isolated essentially as described by Goto et al. (2007), and submitted to Hokkaido System Science (Sapporo, Japan), where deep sequencing analysis was performed on Illumina Genome Analyzer using the standard protocol of manufacturer. A total of 13,849,330 raw sequence tags were generated from a single run of the analysis. The 18-45 nt small RNA reads were extracted from raw reads and aligned with the *CHS-A* promoter sequence using SOAP (Li et al. 2008) to search for perfectly matched sequences. The small RNA reads mapped on the *CHS-A* promoter sequence are available in DNA Data Bank of Japan (DDBJ) under the accession numbers AOAAA0000001-AOAAA0001053.

Bisulfite sequencing analysis

For analysis of DNA methylation by bisulfite sequencing, DNA was isolated from plant tissues using a Nucleon PhytoPure DNA extraction kit (Amersham Biosciences, USA). Bisulfite treatment of DNA was performed as described previously (Kanazawa et al., 2007a). In two rounds of PCR to amplify the target sequences, primers CHS-336FbisulfiteT and CHS+107RbisulfiteA were used for the first round of

PCR, and primers CHS-298FbisulfiteT and CHS+34RbisulfiteA were used for the second for the petunia *CHS-A* promoter. For the *LeSPL-CNR* promoter in *L. esculentum*, primers CNR-2681FbisulfiteT and CNR-2131RbisulfiteA were used for the first round, and primers CNR-2611FbisulfiteT and CNR-2228RbisulfiteA were used for the second round. For the CaMV 35S promoter in *N. benthamiana* 16c, primers 35S-346FbisulfiteT and 35S+1RbisulfiteA were used for the first round, and primers 35S-323FbisulfiteT and 35S-21RbisulfiteA were used for the second round. The PCR cycling conditions were: 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min. This cycle was repeated 40 times, and the reaction mixture was then further incubated at 72°C for 10 min. The PCR products were cloned in the pGEM-T Easy vector (Promega, USA), and then subjected to sequence analysis. For each product, 10-25 clones were sequenced. As a control to ensure that the bisulfite treatment was complete, we isolated DNA from *Arabidopsis thaliana* leaves and amplified a region of *ASA1* gene, which is not methylated as previously reported (Kanazawa et al., 2007a). All five cloned sequences of the *ASA1* PCR products showed complete conversion of cytosines to thymidines.

Chromatin immunoprecipitation (ChIP) analysis

Cross-linking of histones to DNA and sonication of chromatin were performed as described (Johnson et al., 2002). Immunoprecipitation, elution, and reverse cross-linking of chromatin were performed by using a ChIP Kit (Upstate, USA). The following antibodies were used for ChIP assays: anti-acetyl-histone H3 (No. 06-599) and anti-dimethyl-histone H3-K9 (No. 07-441) (Upstate, USA). For the petunia, primers CHS-273F and CHS-2R were used to amplify the *CHS-A* promoter region from DNA purified after the ChIP reaction. For the tomato, primers CNR-5-286 and CNR-3-286 were used to amplify the *LeSPL-CNR* promoter region. For the CaMV

35S promoter in *N. benthamiana* 16c, primers 35S-345F and 35S+1R were used to amplify the 35S promoter region. For each material, the ChIP experiment was repeated three times, and the reproducibility of the results was confirmed.

Western blot analysis

Western blot analysis was performed using anti-2b polyclonal antibodies essentially as described previously (Masuta et al., 1995).

In vitro binding assay between 2b and siRNAs

The 2b(2/3) gene in CMV-A1 was PCR-amplified using a forward primer containing the T7 promoter sequence at the 5′ end and a reverse primer containing the FLAG-tag sequence at the 3′ end. The PCR product was used as a template for *in vitro* transcription, and the RNA transcripts were translated into proteins using the wheat germ in vitro translation system (Proteios; Toyobo, Japan). The in vitro synthesized protein [2b(2/3)-FLAG] was mixed with the chemically synthesized 24-nt siRNA that had been designed to target the *CHS-A* promoter. The siRNAs bound to 2b(2/3)-FLAG were extracted with phenol/chloroform, and siRNAs were then precipitated with ethanol. The recovered siRNAs were detected by Northern blot analysis using the *CHS-A* promoter-specific probes as described above.

Purification of in vivo-synthesized 2b from virus-infected tissues

The 2b(2/3) protein with the C-terminal strep-tagII peptide [2b(2/3)-strepII] is expressed from the CMV-A1-strepII, which was created from the CMV-A1 vector. Purification steps in vivo-synthesized 2b(2/3) are summarized in Figure S5b. Briefly, proteins were isolated from CMV-A1-strepII-infected *N. benthamiana* plants by using the P-PER Plant Protein Extraction kit (Thermo scientific, Rockford, IL, USA). After

soluble protein fraction containing the 2b(2/3) complex was incubated with Dynabeads M-280 Streptavidin beads (Invitrogen, USA), 2b(2/3) was purified essentially as described before (Sueda et al., 2010).

Isolation of nuclei and nuclear RNA

Nuclei were isolated as described previously (Kanazawa et al., 2007b). Nuclei were suspended in buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0), and nuclear RNA was purified from the suspension as described (Otagaki et al., 2006). For Northern blot analysis, 0.4 μg of nuclear low-molecular weight RNA was used.

Protoplast experiments in BY2 cells

BY2 suspension culture cells were prepared and transfected as previously described (Shimura et al., 2008a). The *DsRed* gene was cloned into CMV-A1 and *in vitro* transcripts from the fusion protein gene was transfected into BY2 protoplasts. To visualize siRNA accumulation in nucleus, fluorescent labeling of siRNA was performed by annealing the synthetic 21-nt 5′-6-FAM-labeled RNA oligonucleotide (5′-UGAUUGAGCCGCGCCAAUAUC-3′) and its complementary RNA molecule. Lipofectamine 2000 (Invitrogen) was suspended in 50 μl of 10 mM Tris-HCl (pH7.5) and left for 5 min at room temperature. The suspension was then mixed with 100 pmol of FAM-labeled siRNAs in 50 μl of 10 mM Tris-HCl (pH7.5) and left for 20 min at room temperature. The liposome-siRNA solution was mixed with 500 μl of protoplast suspension and incubated for 30 min at room temperature. After washing the protoplasts with 0.4 M mannitol twice, protoplasts were resuspended in 1 ml growth medium (Shimura et al., 2008b). The protoplasts were then observed with a fluorescence microscope (model AF600; Leica, Germany).

Protoplast experiments in petunia tissues

Petunia protoplasts were prepared from petal tissues of petunia and transfection was performed essentially as described (Yoo et al., 2007; Shimura et al., 2008b). The dsRNA of *CHS-A* promoter (dsCHSpro) was prepared from in vitro transcription of the PCR products that were amplified by the primer pair, T7-CHS-P5-700 and T7-CHS-P3-700. The control dsRNA of the firefly luciferase gene (Fluc) (dsFluc) was prepared as described previously (Shimura et al., 2008b). The cDNA clone of 2b was inserted into the cloning site in pBI121 (Clontech, Japan) to create pBI121-2b. The prepared protoplasts were transfected with dsCHSpro (3 μg) or dsFluc (3 μg) with/without pBI121-2b (3 μg). When necessary, an irrelevant plasmid (pBI121) was included to adjust the total amount of nucleic acids for transfection. After incubation for 48 h, RNA was extracted from the harvested protoplasts by Trizol reagent (Invitrogen) and then mRNA levels of the *CHS-A* gene were measured by quantitative RT-PCR as described above. The ChIP assay was also performed using dsRNA-introduced protoplasts to analyze histone modifications on the *CHS-A* promoter region as described above.

Acknowledgements

We are grateful to Dr. David Baulcombe for 16c *N. benthamiana* plants. We also thank Ms. Kae Sueda, Ms. Kozue Kamiya and Dr. Ryusuke Fujita for technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Nos. 21024001, 21380203, 21658014, 22380002 and 22658001), and Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

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Supporting Information

The following information is available on line.

Figure S1. Accumulation of siRNAs derived from the *CHS-A* coding region.

Figure S2. Size distribution of the *CHS-A* promoter small RNA populations.

Figure S3. Changes in the phenotype of *Petunia hybrida* V26 infected with the CMV-A1 vector carrying the *CHS-A* promoter insert.

Figure S4. Microarray analysis of RNA transcripts from the progeny of CMV-A1:LeSPL-CNRpro-infected tomato plants.

Figure S5. Detection and purification of the 2b protein containing a tag peptide in CMV-infected plants.

Figure S6. GFP silencing induced by the CMV vector occurs at the transcriptional level.

Figure S7. Comparison of viral accumulation and systemic spread in *Nicotiana benthamiana* plants between CMV-A1 and CMV-H1.

Figure S8. The ability of CMV-G1 to induce TGS of *Cauliflower mosaic virus* (CaMV) 35S promoter in *GFP*-transgenic 16c plants.

Figure S9. Generation of siRNAs and DNA methylation on the *CHS-A* promoter in petunia protoplasts.

Figure S10. Scheme of induction of promoter-targeted silencing and epigenetic changes by the CMV vector.

Figure legends

Figure 1. Changes in the phenotype of petunia Red Star infected with the virus that carries the *CHS-A* promoter. (a) Schematic representation of the CMV vector construct that contains the *CHS-A* promoter sequence (position -244 to -2). (b) Changes in flower color and anther phenotype in Red Star. Mock-inoculated, CMV-A1-infected, CMV-A1:CHSpro-infected plants and self-pollinated progeny of the CMV-A1:CHSpro-infected plants were shown. (c) Percentage of in vitro germination of pollen derived from plants shown in (b). Results are given as means with standard error obtained from three replicates. (d) Detection of the insert from CMV-A1:CHSpro-infected petunia plants (five individuals) by RT-PCR. RNA was extracted from flower tissues 2 weeks after inoculation with CMV-A1:CHSpro. The *α-tubulin* sequence was amplified as an internal control. The insert sequence intervenes between the forward and reverse primers; if the vector has the intact insert, a 616-bp fragment is amplified. CMV-A1 and CMV-A1:CHSpro lanes indicate fragments amplified from each plasmid.

Figure 2. Characterization of the changes induced by CMV-A1:CHSpro infection in petunia Red Star. (a) A representative result of the mRNA levels of the *CHS-A* gene in anther tissues determined by quantitative RT-PCR (mean ± SE; n = 3). Similar results were obtained when petal tissues were used. (b) Accumulation of siRNAs corresponding to the *CHS-A* promoter in CMV-A1-infected, CMV-A1:CHSpro-infected plants and its progeny plants. Ethidium bromide-stained 5S RNA and tRNA bands in bottom panel demonstrate that an equal amount of the small RNA fraction was loaded. (c) The *CHS-A* promoter-derived 24-nt siRNAs determined by deep sequencing. Small RNAs were isolated from

CMV-A1:CHSpro-infected petunia plants and submitted to deep sequencing. 24-nt siRNAs were mapped to the *CHS-A* promoter sequence in either sense (above the X-axis) or antisense (below the X-axis) orientation. The region inserted in the viral vector was indicated by arrows. (d) Cytosine methylation status of the *CHS-A* promoter assayed by bisulfite sequencing. The percentages of methylcytosine are shown. Red, green, and blue lines indicate frequencies of methylcytosine at CG, CNG, and CNN sites, respectively. (e) Histone modifications at the *CHS-A* promoter. ChIP analysis was performed on anther tissues from the CMV-A1-infected, CMV-A1:CHSpro-infected plants and its progeny plants using antibodies against histone H3 dimethyl K9 (H3K9me2) and acetylated histone H3 (H3Ac). Input, amplification from the input DNA in chromatin before immunoprecipitation; −, no antibody control for precipitation. (f) Confirmation of the absence of virus in the progeny petunia plants by RT-PCR. RNA was extracted from the CMV-A1:CHSpro-infected plants or its progeny plants. The CMV-A1-infected plant was used as a control.

Figure 3. Induction of promoter-targeted heritable silencing of *LeSPL-CNR* gene in *Lycopersicon esculentum* cv. Ailsa Craig. (a) Changes in phenotype as a consequence of the infection of the virus carrying the *LeSPL-CNR* promoter. CMV-A1, CMV-A1-infected plants; CMV-A1:LeSPL-CNRpro, CMV-A1:LeSPL-CNRpro -infected plants, CMV-A1:LeSPL-CNRpro progeny, the progeny of the CMV-A1:LeSPL-CNRpro-infected plants. (b) The mRNA levels of the *LeSPL-CNR* gene in fruit tissues determined by quantitative RT-PCR (mean ± SE; n = 3). The *LeSPL-CNR* mRNA level relative to the actin mRNA level in the CMV-A1-infected plants was assigned a value of 1. (c) Cytosine methylation status of the *LeSPL-CNR* promoter assayed by bisulfite sequencing. The percentages of methylcytosine are

shown. For colored bars, see the legend to Figure 2d. Uninfected plants had a methylation profile similar to that of CMV-A1-infected plants (data not shown). (d) Histone modifications at the *LeSPL-CNR* promoter. ChIP analysis was performed on the CMV-A1-infected, CMV-A1:LeSPL-CNRpro-infected plants and the progeny plant using antibodies against histone H3 dimethyl K9 (H3K9me2) and acetylated histone H3 (H3Ac). Input, amplification from the input DNA in chromatin before immunoprecipitation; −, no antibody control for precipitation.

Figure 4. The 2b protein binds to siRNAs in vivo and facilitates the accumulation of siRNAs in nuclei. (a) Schematic representation of the CMV vector construct containing the *DsRed* sequence. Images indicate localization of the 2b-DsRed fusion protein in BY2 cells transfected with CMV-A1:DsRed. Light micrographs (BF, left) and DsRed-derived fluorescence micrographs (DsRed, right) are shown. Images were taken 24 h after transfection. Bars, 10 μm. (b) Ability of 2b(2/3) bind to 24-nt siRNAs. 2b(2/3) is encoded by CMV-A1 and lacks the C-terminal one-third of the intact 2b protein. The chemically synthesized 24-nt siRNA was incubated with the products of the in vitro translation mixture containing no transcript (N.C.) and with the products of the in vitro translation mixture containing transcripts of 2b(2/3). B, blank lane. (c) Electrophoresis of RT-PCR products from the siRNAs bound to 2b(2/3). The 50-bp band is the ligation product of 5′ and 3′ adaptors. The cDNAs of siRNAs appear between 60-nt and 80-nt positions. (d) Size distribution of the siRNAs bound to 2b(2/3) in CMV-infected tissues. The CMV-synthesized 2bs bound to siRNAs were isolated by affinity purification. siRNAs were extracted from the 2b complex, then cloned and sequenced randomly. Most of the clones were virus-specific siRNAs. Numbers for the X-axis indicate the sizes of siRNAs. Numbers above the columns are the numbers of cloned siRNAs. (e) Transport of siRNAs to the nuclei in the

presence of the 2b protein. BY2 and BY2-2b protoplasts were transfected with 6-FAM-labeled 21-nt siRNAs using lipofectamine. Images of FAM-derived fluorescence and light micrographs (upper left of each panel) at 30 min after transfection are shown. Bars, 10 μm. The number of protoplasts with fluorescing nuclei vs. the number of total protoplasts in four observations is shown at the bottom. The data indicates that almost half of the BY2-2b protoplasts transfected with FAM-labeled siRNAs had fluorescence in nuclei because the transfection efficiency by lipofectamine was at most 20%.

Figure 5. Comparison of CMV-A1 and CMV-H1 to induce TGS of the 35S promoter in *GFP*-transgenic *Nicotiana benthamiana*. (a) Schematic representations of the CMV vector constructs containing the 35S promoter sequence. (b) *GFP* fluorescence in the *N. benthamiana* 16c plants at 12 days post-inoculation (dpi) with CMV-A1:35Spro or CMV-H1:35Spro (upper panel) and northern blot analysis of *GFP* mRNAs at 12 dpi (lower panel). Ethidium bromide-stained 25S rRNA is shown as a loading control. (c) Cytosine methylation status of the 35S promoter in plants infected with CMV-A1:35Spro or CMV-H1:35Spro assayed by bisulfite sequencing. The percentages of methylcytosine are shown. For colored bars, see the legend to Figure 2d. (d) Histone modifications at the 35S promoter. ChIP analysis was performed on the CMV-A1-infected and CMV-A1:35Spro-infected plants using antibodies against histone H3 dimethyl K9 (H3K9me2) and acetylated histone H3 (H3Ac). Input, amplification from the input DNA in chromatin before immunoprecipitation; −, no antibody control for precipitation. (e) Northern blots analysis of 35S promoter-derived siRNAs in isolated nuclei and total cell extracts from the plants infected with either CMV-A1:35Spro or CMV-H1:35Spro at 25 dpi. Relative intensity of hybridization signal was described below the panel. Ethidium

bromide-stained small nuclear RNAs (snRNAs) (for nucleus) and 5S RNA and tRNA (for total cell) bands were shown as a loading control.

Fig. 6. Effect of 2b on promoter-targeted silencing in the protoplast assay. (a) Schematic flow of the developed protoplast assay. Protoplasts were isolated from petals of petunia line V26. Isolated protoplasts were transfected either with dsRNA of the *CHS-A* promoter sequence (700 bp, dsCHSpro) or with dsCHSpro together with the plant expression plasmid containing the 2b gene (pBI121-2b). As a control, dsRNA of the firefly luciferase gene (dsFluc) was used instead of dsCHSpro. The transfected protoplasts were harvested at 48 h after transfection, and used for the subsequent analyses. (b) The mRNA level of the *CHS-A* gene after introduction of dsRNA into protoplasts. Isolated protoplasts were treated with dsCHSpro with or without the plant expression plasmid containing the 2b gene. As a control, dsRNA of the firefly luciferase gene (dsFluc) was used instead of dsCHSpro. Protoplasts were harvested at 48 h after transfection, and the mRNA levels of the *CHS-A* gene were measured by quantitative RT-PCR (mean ± SE; n = 3). The *β-tubulin* mRNA levels were used for data normalization. (c) Size distribution of sequenced siRNAs that were derived from dsCHSpro-transfected protoplasts. Numbers for the X-axis indicate the sizes of siRNAs. Numbers above the columns are the numbers of cloned siRNAs. (d) ChIP assay with antibodies against histone H3K9 dimethylation (H3K9Me2) and H3 acetylation (H3Ac). Input, amplification from the input DNA in chromatin before immunoprecipitation; −, no antibody control for precipitation.

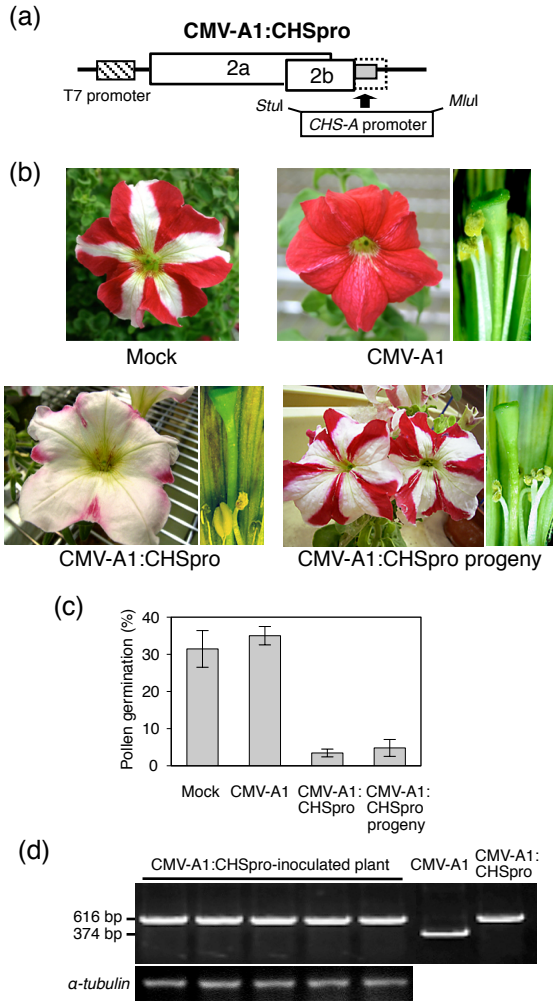
(a)
CMV-A1:CHSpro
2a
2b
T7 promoter
StuI
MluI
CHS-A promoter
(b)
Mock
CMV-A1
CMV-A1:CHSpro
CMV-A1:CHSpro progeny
(c)
Pollen germination (%)
40
30
20
10
0
Mock
CMV-A1
CMV-A1: CHSpro
CMV-A1: CHSpro progeny
(d)
CMV-A1:CHSpro-inoculated plant
CMV-A1
CMV-A1: CHSpro
616 bp
374 bp
α-tubulin

Fig. 1

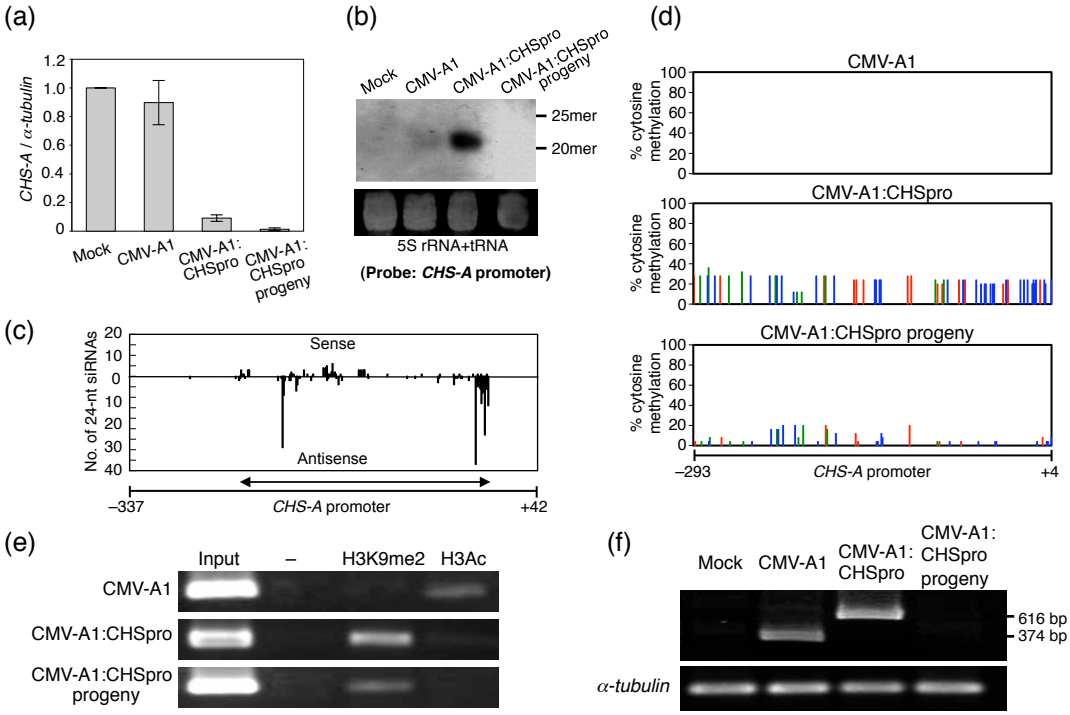
(a)
CHS-A / α-tubulin
1.2
1.0
0.8
0.6
0.4
0.2
0
Mock
CMV-A1
CMV-A1: CHSpro
CMV-A1: CHSpro progeny
(b)
Mock
CMV-A1
CMV-A1:CHSpro
CMV-A1:CHSpro progeny
25mer
20mer
5S rRNA+tRNA
(Probe: CHS-A promoter)
(c)
No. of 24-nt siRNAs
20
10
0
10
20
30
40
Sense
Antisense
–337
CHS-A promoter
+42
(d)
CMV-A1
CMV-A1:CHSpro
CMV-A1:CHSpro progeny
% cytosine methylation
100
80
60
40
20
0
–293
CHS-A promoter
+4
(e)
Input
–
H3K9me2
H3Ac
CMV-A1
CMV-A1:CHSpro
CMV-A1:CHSpro progeny
(f)
Mock
CMV-A1
CMV-A1: CHSpro
CMV-A1: CHSpro progeny
616 bp
374 bp
α-tubulin

Fig. 2

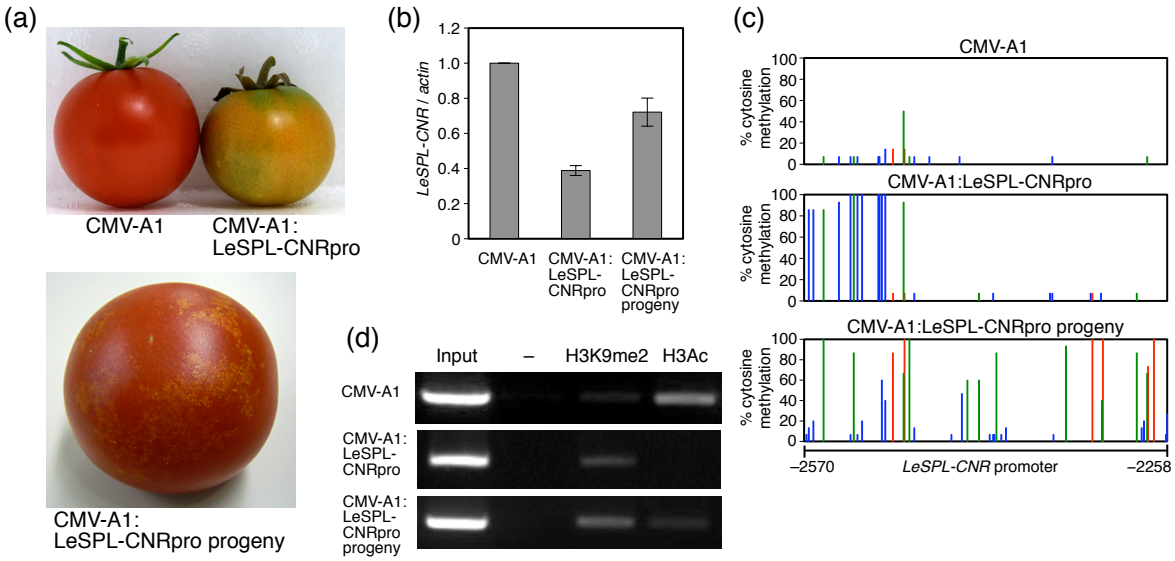
(a)
CMV-A1
CMV-A1:
LeSPL-CNRpro
CMV-A1:
LeSPL-CNRpro progeny
(b)
LeSPL-CNR / actin
1.2
1.0
0.8
0.6
0.4
0.2
0
CMV-A1
CMV-A1:
LeSPL-
CNRpro
CMV-A1:
LeSPL-
CNRpro
progeny
(c)
CMV-A1
CMV-A1:LeSPL-CNRpro
CMV-A1:LeSPL-CNRpro progeny
% cytosine
methylation
100
80
60
40
20
0
–2570
LeSPL-CNR promoter
–2258
(d)
Input
–
H3K9me2
H3Ac
CMV-A1
CMV-A1:
LeSPL-
CNRpro
CMV-A1:
LeSPL-
CNRpro
progeny

Fig. 3

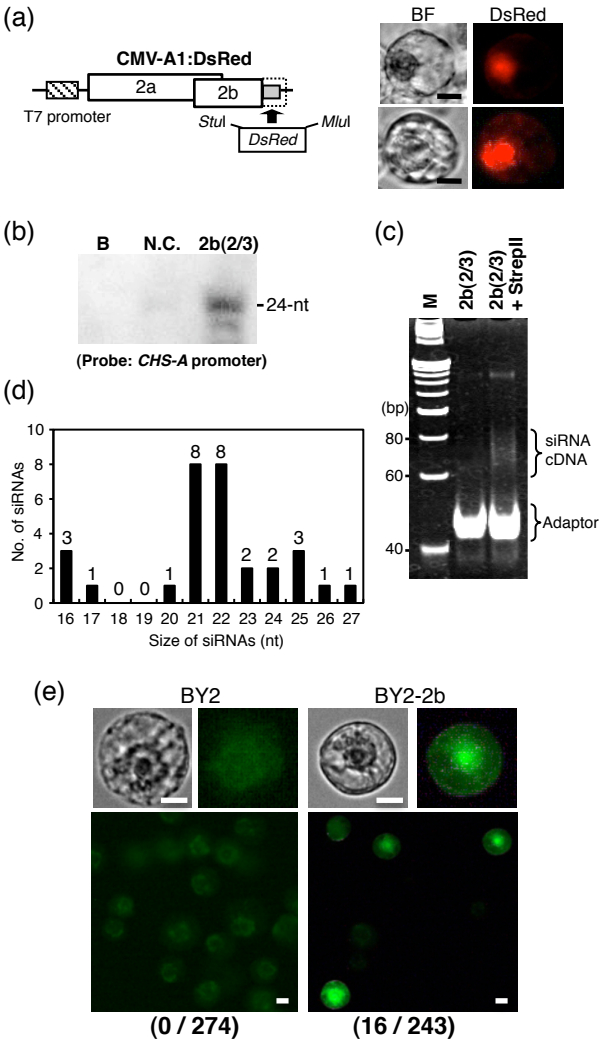
(a)
CMV-A1:DsRed
2a
2b
T7 promoter
StuI
MluI
DsRed
BF
DsRed
(b)
B
N.C.
2b(2/3)
24-nt
(Probe: CHS-A promoter)
(c)
M
2b(2/3)
2b(2/3) + StrepII
(bp)
80
60
40
siRNA
cDNA
Adaptor
(d)
No. of siRNAs
Size of siRNAs (nt)
3 1 0 0 1 8 8 2 2 3 1 1
16 17 18 19 20 21 22 23 24 25 26 27
(e)
BY2
BY2-2b
(0 / 274)
(16 / 243)

Fig. 4

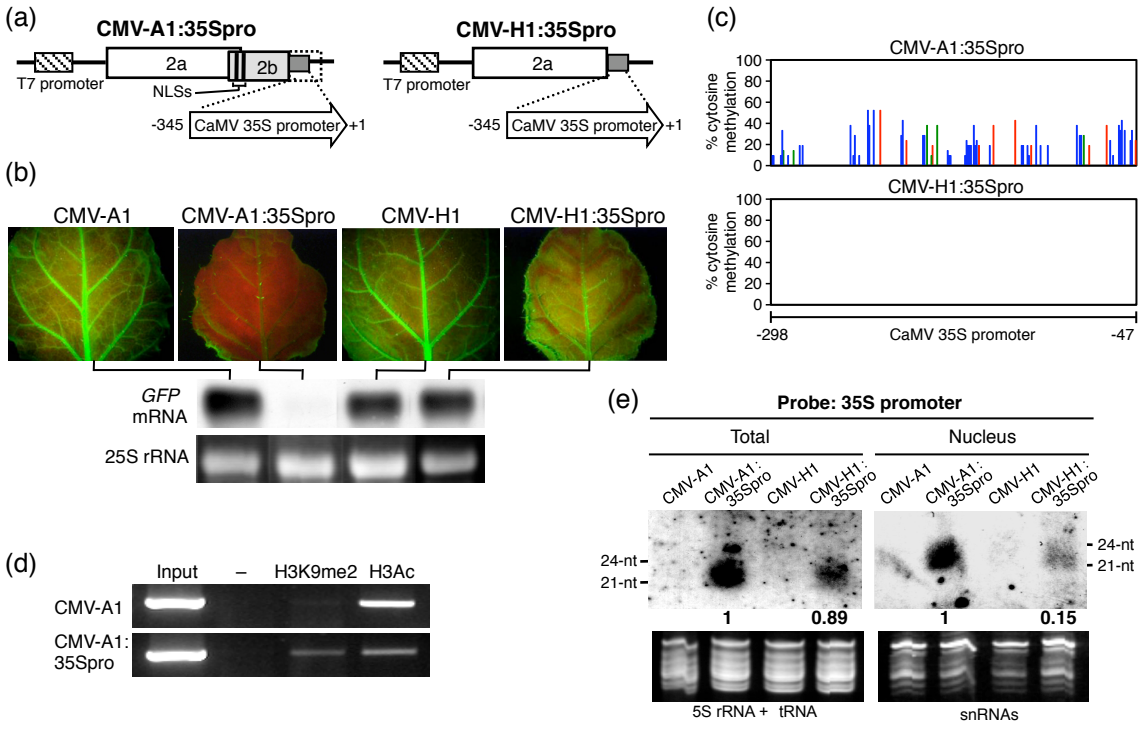
(a)
CMV-A1:35Spro
T7 promoter
2a
2b
NLSs
-345
CaMV 35S promoter
+1
CMV-H1:35Spro
T7 promoter
2a
-345
CaMV 35S promoter
+1
(b)
CMV-A1
CMV-A1:35Spro
CMV-H1
CMV-H1:35Spro
GFP mRNA
25S rRNA
(c)
CMV-A1:35Spro
% cytosine methylation
100
80
60
40
20
0
CMV-H1:35Spro
% cytosine methylation
100
80
60
40
20
0
-298
CaMV 35S promoter
-47
(d)
Input
–
H3K9me2
H3Ac
CMV-A1
CMV-A1:35Spro
(e)
Probe: 35S promoter
Total
Nucleus
CMV-A1
CMV-A1::35Spro
CMV-H1
CMV-H1::35Spro
24-nt
21-nt
1
0.89
1
0.15
5S rRNA + tRNA
snRNAs

Fig. 5

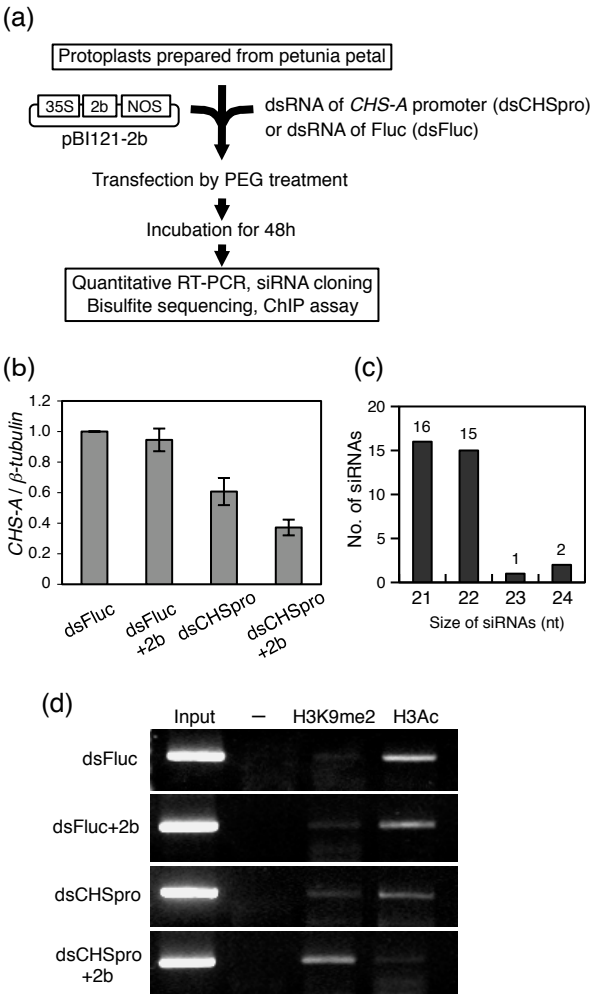
(a)
Protoplasts prepared from petunia petal
35S
2b
NOS
pBI121-2b
dsRNA of CHS-A promoter (dsCHSpro)
or dsRNA of Fluc (dsFluc)
Transfection by PEG treatment
Incubation for 48h
Quantitative RT-PCR, siRNA cloning
Bisulfite sequencing, ChIP assay
(b)
CHS-A / β-tubulin
1.2
1.0
0.8
0.6
0.4
0.2
0
dsFluc
dsFluc +2b
dsCHSpro
dsCHSpro +2b
(c)
No. of siRNAs
20
15
10
5
0
16
15
1
2
21
22
23
24
Size of siRNAs (nt)
(d)
Input
–
H3K9me2
H3Ac
dsFluc
dsFluc+2b
dsCHSpro
dsCHSpro +2b

Fig. 6