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RNA-mediated epigenetic modifications of an endogenous gene targeted by a viral vector—a potent gene silencing system to produce a plant that does not carry a transgene but has altered traits

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Nucleotide-sequence-specific interactions mediated by double-stranded RNA (dsRNA) can induce gene silencing. Gene silencing through transcriptional repression can be induced by dsRNA targeted to a gene promoter. However, until recently, no plant has been produced that harbors an endogenous gene that remains silenced in the absence of promoter-targeting dsRNA. We have reported for the first time that transcriptional gene silencing can be induced by targeting dsRNA to the endogenous gene promoters in petunia and tomato plants, using a Cucumber mosaic virus (CMV)-based vector and that the induced gene silencing is heritable. Efficient silencing depended on the function of the 2b protein encoded in the vector, which facilitates epigenetic modifications through the transport of short interfering RNA (siRNA) to the nucleus. Here we show that gene silencing that is mediated by targeting dsRNA to a gene promoter via the CMV vector can be as strong as co-suppression in terms of both the extent of mRNA decrease and phenotypic changes. We also show that the expression of genes involved in RNA-directed DNA methylation and in demethylation are upregulated and downregulated, respectively, in Arabidopsis plants infected with CMV. Thus, along with the function of the 2b protein, that transports siRNA to the nucleus, the promoter-targeted silencing system using the CMV vector has some property that facilitates heritable epigenetic changes on endogenous genes, enabling the production of a novel class of modified plants that do not have a transgene.

Nucleotide-sequence-specific inhibition of gene expression can be induced by diverse processes that are mediated by RNA.[1] Gene silencing through transcriptional repression can be induced by dsRNA targeted to a gene promoter,[2] a process known as RNA-mediated transcriptional gene silencing (TGS). This phenomenon was first discovered in plants using a transgene that transcribes an inverted repeat of a promoter sequence and was later reported in cultured human cells and in *Schizosaccharomyces pombe*.[3-7] Plant RNA viruses such as the Potato virus X (PVX), Tobacco rattle virus (TRV) and Cucumber mosaic virus (CMV) have also been used as a tool to induce TGS.[8-11]

There is a marked difference between transgenes and endogenous genes in plants in the feasibility of the induction of silencing by targeting RNA to a promoter.[12] Transgenes can be easily silenced and the silenced state is heritable in the presence or absence of the silencing inducer.[9] On the other hand, endogenous genes can be silenced only in the presence of the silencing inducer,[13-15] and no plant has been produced that harbors a silenced endogenous gene after the promoter-targeting dsRNA has been removed.

We previously developed a CMV-based vector, CMV-A1, which is suitable for inducing RNA silencing.[10,16,17] We found that the CMV-A1 vector, which carries the endogenous gene promoter, efficiently induced heritable gene silencing that was accompanied by epigenetic changes and that the 2b protein of CMV was involved in this efficient RNA-mediated TGS.[11] Here we describe the potency and other features of this silencing system.

The Effect of RNA-Mediated TGS Is as Strong as that of Co-suppression

We previously targeted the promoter of the *CHS-A* gene for chalcone synthase in petunia (*Petunia hybrida*) because silencing of this gene is manifested as an altered visible phenotype on flower organs. A prominent, visible change is a reduction in pigmented

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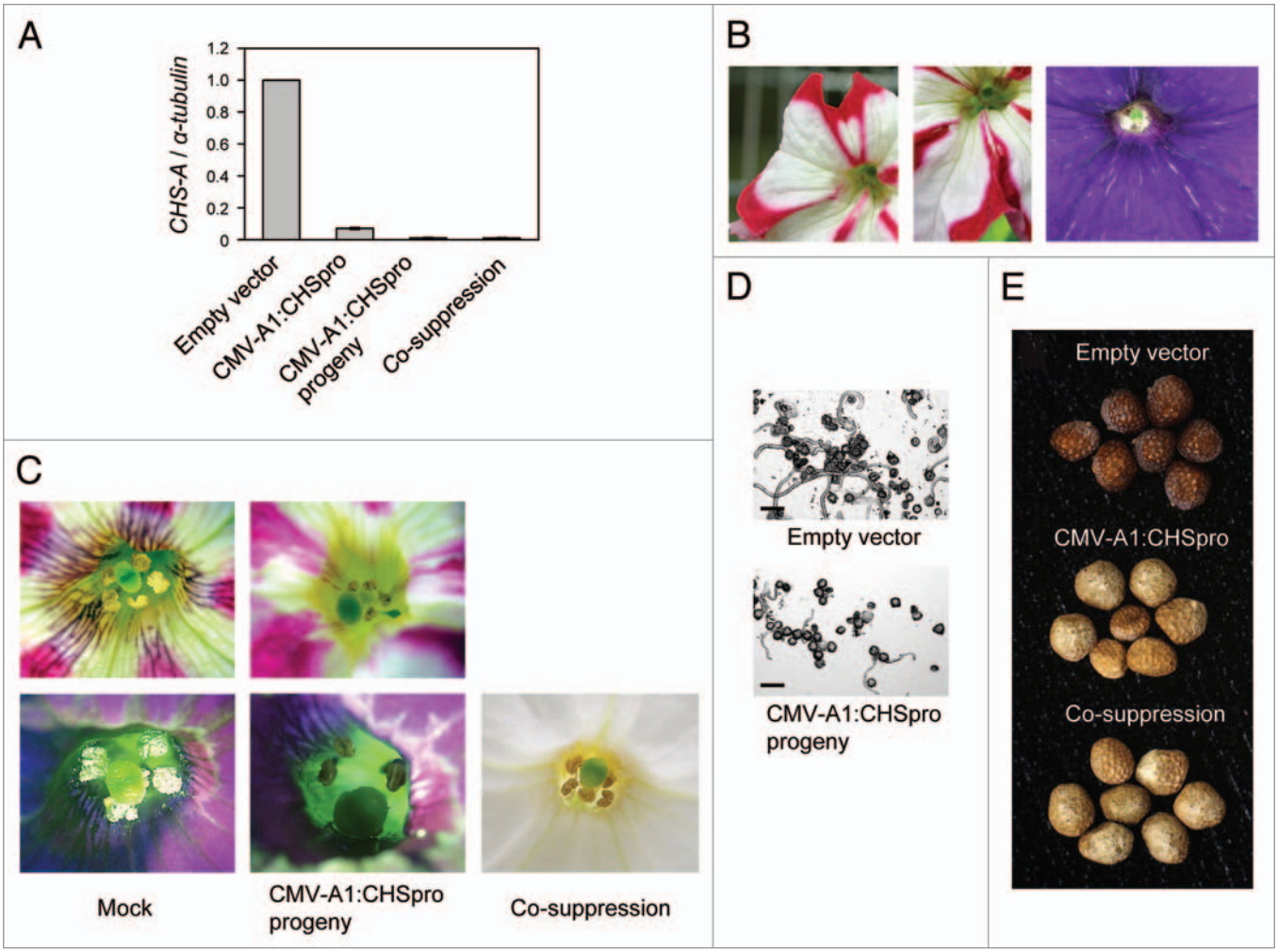

Figure 1. Characterization of the changes induced by CMV-A1:CHSpro infection in petunia plants. (A) The mRNA level of the *CHS-A* gene (mean ± SE; $n = 3$) in the petal tissues of V26 plants infected with the empty vector (negative control) or CMV-A1:CHSpro, the progeny of virus-infected plants, and plants that had *CHS-A* co-suppression. The *CHS-A* mRNA level relative to the α-*tubulin* mRNA level in empty vector-inoculated plants was assigned a value of 1. (B) Nonpigmented sectors generated in the pigmented portions of the petals in the self-pollinated progeny of the CMV-A1:CHSpro-infected plant. Left and center, var. Red Star; right, V26 line. (C) Changes in anther phenotype maintained in the progeny of CMV-A1:CHSpro-infected plant. Upper row, var. Red Star; lower row, V26 line. The phenotype of male-sterile transgenic line C001 that exhibits *CHS-A* co-suppression is also shown. (D) In vitro germination of pollen. Bars indicate 50 μm. (E) Changes in pigmentation of seed coat tissues in V26: upper, seeds produced by empty-vector infected plants; center, seeds produced by CMV-A1:CHSpro-infected plants; lower, seeds produced by male-sterile transgenic line C001.

area in the petal tissues of Red Star,[11] a petunia variety that produces flowers with a red and white bicolor pattern as a result of naturally occurring sequence-specific degradation of *CHS-A* transcripts in the white sector of the petal.[18] To evaluate the effect of RNA-mediated TGS, we compared the mRNA level of the *CHS-A* gene between plants infected with the virus carrying the *CHS-A* promoter (referred to as CMV-A1:CHSpro) and a transgenic line C001 that exhibits *CHS-A* co-suppression (**Fig. 1A**). We previously demonstrated that *CHS-A* silencing in this line is induced by degradation of RNA transcribed from both an endogenous gene and its homologous transgene.[19] This co-suppressed line produces entirely white petals. Quantitative RT-PCR analysis indicated that the mRNA level for the *CHS-A* gene in the CMV-A1:CHSpro-infected plants was as low as that in the C001 line (**Fig. 1A**).

In the progeny of CMV-A1:CHSpro-infected petunia plants, white sectors were often generated inside the pigmented portions of the petal (**Fig. 1B**). This phenomenon suggests that strong de novo *CHS-A* silencing occurred in the lineage of pigmented cells during development. This observation is consistent with the fact that *CHS-A* mRNA level was very low even though the petal tissues were pigmented; the mRNA level was presumably only slightly higher than the threshold mRNA level that allows pigmentation in the petal tissues. Our previous data indicate that the estimated threshold level of transcripts of a gene involved in flavonoid synthesis associated with pigmentation in a plant tissue is about 3% of the steady-state mRNA level of the gene.[20]

Plants having petals with a greater white area produced less pollen compared with the control plants.[11] The outward phenotype of anthers in CMV-A1:CHSpro-infected plants and their

progeny was very similar to that of a plant line that exhibits *CHS-A* co-suppression; pollen grains remained clumped within the anther case, and anthers degenerated without exposing a large amount of pollen grains on the surface of the anther at anthesis (**Fig. 1C**). This male-sterile phenotype is ascribed to a reduction in flavonol content brought about by *CHS-A* silencing.[21] In vitro pollen germination assay according to the method of Jahnen et al. showed that the frequency of pollen germination was lower in the progeny of CMV-A1:CHSpro-infected plants than in the control plants (**Fig. 1D**).

In addition to the changes in the anther and petal tissues, the seed coat of CMV-A1:CHSpro-infected plants did not have typical brown pigmentation, and the color was very similar to that of the *CHS-A* co-suppressed line (**Fig. 1E**). Taken together, these observations suggest that the effect of promoter-targeted silencing was equivalent to that of co-suppression in terms of both the extent of mRNA decrease and phenotypic changes.

Promotive Function of Viral Infection in the Induction of Epigenetic Changes

Previously, we found that the phenotypic changes were accompanied by epigenetic changes including cytosine methylation and histone modification.[11] CMV encodes the 2b protein, which binds to siRNA and is localized in the nucleus.[23] We found that these features are associated with efficient siRNA transport to the nucleus and that they facilitate RNA-directed DNA methylation (RdDM) and histone modifications, which eventually lead to RNA-mediated TGS.[11]

To identify additional feature(s) that may contribute to the efficient induction of epigenetic changes, here we analyzed changes in gene expression of the host plant after viral infection. Arabidopsis plants that were infected with CMV were examined for changes in the levels of genes known to be involved in RNA-mediated TGS. The mRNA levels of *NRPD1*, *NRPE1*, *NRPD2* and *AGO4* all increased after CMV infection. On the other hand, the mRNA level of *ROS1*, which is known to be involved in cytosine demethylation, decreased (**Fig. 2**). These results suggest that CMV infection upregulates the genes that drive RNA-mediated TGS and downregulates the gene that antagonizes the pathway. We thus believe that these changes presumably facilitate RNA-mediated TGS.

A previous study indicated that the mRNA levels of numerous genes of the host plant are either upregulated or downregulated after CMV infection,[24] whereas the regulatory mechanisms of many of these genes are still unknown. Similarly, why the expression of genes involved in RdDM changed after viral infection remains unsolved. In these circumstances, it is conceivable that CMV has acquired a mechanism to fine-tune the expression of host genes to levels suitable for infection via RdDM during the arms race between plants and virus.

Another feature that may promote heritable epigenetic changes is the distribution of CMV within meristematic tissues, where heritable changes can be made on the genome; the accumulation of CMV-derived siRNAs has actually been detected in shoot meristems of tobacco.[25] A combinatory effect of these features may account for the efficient induction of RNA-mediated, heritable epigenetic changes in the CMV vector system.

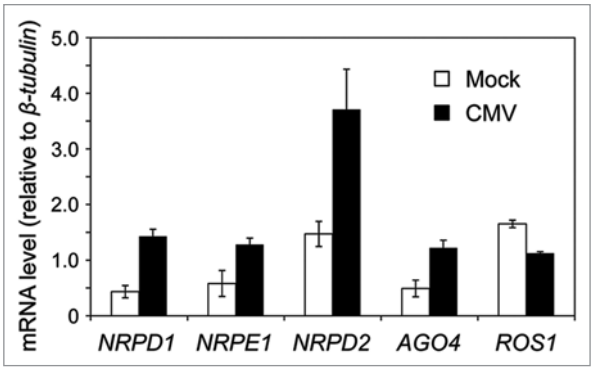

Figure 2. Changes in the mRNA level of the *NRPD1*, *NRPE1*, *NRPD2*, *AGO4* and *ROS1* genes in the *Arabidopsis thaliana* (Columbia ecotype) plants infected with CMV. Total RNA was isolated from mock-inoculated plants or plants infected with CMV-Y at 14 d postinoculation. The expression levels of these genes were analyzed with an eXpress profiling multiplex RT-PCR assay (Beckman, USA). The mRNA level of the β-*tubulin* gene was used as a control. The data are means and standard errors obtained from three replicates. Gene-specific sequences of the primers were as follows: for the *NRPD1* gene, 5'-TAC GCT GCT TAT GAT GGC AC-3' and 5'-GCT GCC TCA GAT AAT GCA CA-3'; for the *NRPE1* gene, 5'-ACA GGA ACA ACC AAG ATG CC-3' and 5'-CCT GAG CCT GAG ATG GAG AC-3'; for the *NRPD2* gene, 5'-TCA AGA TGG GGA AAC GAA AG-3' and 5'-TGT GGA CAA GTC GCT GGT AG-3'; for the *AGO4* gene, 5'-TGA GGC ATT ACC ACC TCC TC-3' and 5'-CCC CTT GTT CCA AAT CCT TT-3'; for the *ROS1* gene, 5'-CTA ATT GCA ATG CAT GTC CG-3' and 5'-CCT TGC TCT CTC TGG AAT GG-3'; for the β-*tubulin* gene, 5'-GTC AAT ACG TCG GCG ATT CT-3' and 5'-CAT GGT ACC AGG CTC CAG AT-3'.

Final Remarks

Recent reports indicate that induction of epigenetic changes is potentially useful for plant breeding.[26-29] However, just as mutagenesis causes mutations randomly in a genome, treatment of plants with an agent that induces epigenetic changes (e.g., a demethylating agent) also causes random changes. In contrast, our method allows induction of epigenetic changes on a target gene. Another advantage of the RNA-mediated TGS using the CMV vector is that the progeny plants do not have any transgene because the virus is eliminated during meiosis. Plants that are produced by this system have altered traits but do not carry a transgene, thus constituting a novel class of modified plants. The scheme of our silencing system is shown in **Figure 3**. The changes in the expression of host genes involved in RdDM or demethylation as a consequence of CMV infection suggest that CMV may have sophisticated epigenetic control of host genes and that it uses this control to infect the host. Thus, aside from using CMV for practical applications, we can also use CMV to provide new insights into the interactions between the virus and its host plant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

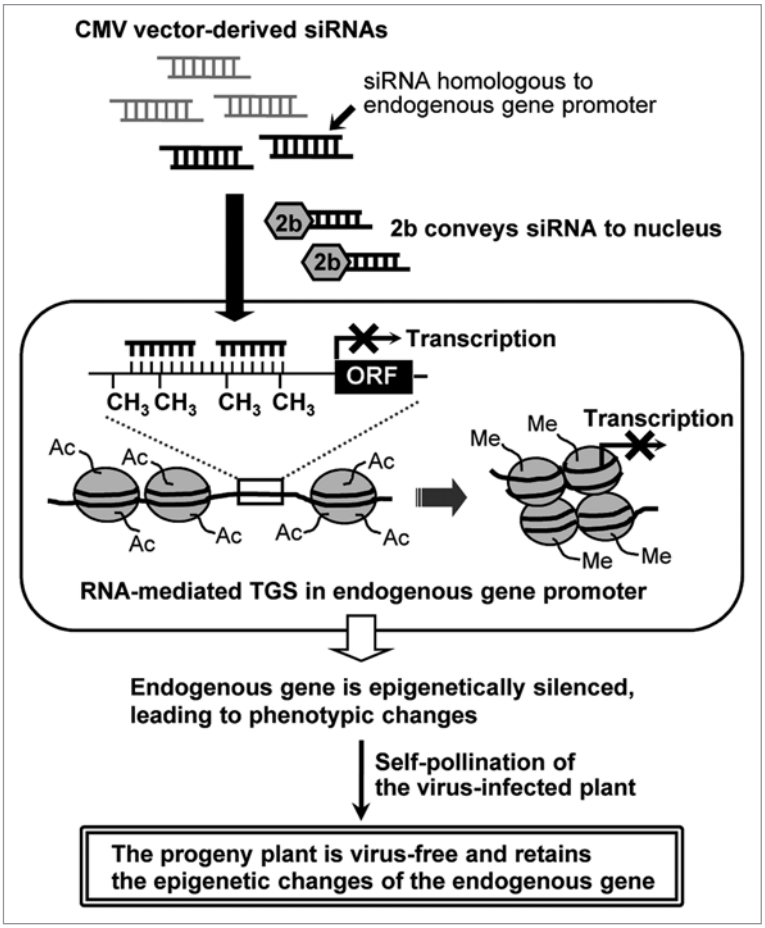

Figure 3. Scheme of induction of promoter-targeted silencing and epigenetic changes by the CMV vector. The CMV vector containing an endogenous gene promoter sequence produces siRNAs in the infected plants via the RNA silencing machinery. The 2b protein (2b) of CMV functions as an RNA silencing suppressor that inhibits posttranscriptional gene silencing (PTGS), but meanwhile it enhances nuclear transport of siRNAs. The siRNAs homologous to the endogenous gene promoter are conveyed by 2b and possibly by other mechanisms such as diffusion. The siRNAs are targeted to the promoter sequence of the endogenous gene in the nucleus, resulting in RNA-directed DNA methylation and histone modification. These epigenetic modifications eventually induce TGS of the endogenous gene and consequent phenotypic changes. In addition, the epigenetic modifications are maintained in the self-pollinated progeny, and thus the progeny plants have an altered phenotype but do not carry promoter-targeting dsRNAs. Ac and Me indicate acetylation and methylation of histone tails, respectively. ORF, open reading frame.

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