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Original Paper

Rapid induction of transcriptional and post-transcriptional gene silencing using a novel *Cucumber mosaic virus* vector

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Abstract We developed a novel RNA virus vector based on the *Cucumber mosaic virus* (CMV), which is able to efficiently induce gene silencing in plants. We manipulated the RNA 2 of the CMV Y strain, whose genome consists of tripartite components, and introduced restriction sites for cloning a foreign sequence into the vector. To evaluate the vector (designated CMV2-A1) in terms of the ability to induce gene silencing, we cloned portions of the green fluorescent protein (*GFP*) cDNA or *Cauliflower mosaic virus* (CaMV) 35S promoter sequences into the vector and inoculated the infectious transcripts into *Nicotiana benthamiana* plants that express the *GFP* gene under the control of the CaMV 35S promoter. In both cases, a loss of GFP fluorescence accompanying a reduction in the level of *GFP* mRNA was induced. The short interfering RNAs (siRNAs) harboring the sequences inserted in the CMV2-A1 vector were detected in the silenced plants. When plants were infected with the virus containing the CaMV 35S promoter sequence, the CaMV 35S promoter sequence in the genomic DNA was heavily methylated. A reduction in the mRNA level of the *GFP* gene and loss of GFP fluorescence were induced as early as 6 and 12 days post-inoculation, respectively, earlier than the 20 days previously achieved with a *Potato virus X* vector. These results suggest that the CMV2-A1 vector is suitable for the rapid induction of both transcriptional and post-transcriptional gene silencing.

Key words: *Cucumber mosaic virus*, green fluorescent protein, transcriptional gene silencing, virus-induced gene silencing, virus vector.

Infection by a virus causes activation of a defense mechanism that is specifically targeted to the viral RNA in the host plant. When viruses are designed to carry a portion of the host gene sequence, the process can also be targeted against the corresponding mRNA. This phenomenon is called virus-induced gene silencing (VIGS; for a review, see Lu et al. 2003). Replication of the recombinant virus and generation of double-stranded RNA (dsRNA) intermediates trigger the RNA-mediated antiviral defense mechanism, and dsRNA intermediates are processed into short interfering RNAs (siRNAs). The siRNAs then target the RNase complex to the corresponding RNA, and the phenotypic changes in the infected plant reflect the loss of function of the encoded protein (Lu et al. 2003). Over the past 10 years, several RNA virus vectors have been developed especially for VIGS, such as those based on *Tobacco mosaic virus* (TMV; Kumagai et al. 1995), *Potato virus X* (PVX; Ruiz et al. 1998), *Tobacco rattle virus* (TRV; Ratcliff et al. 1999), *Barley stripe mosaic virus* (BSMV; Holzberg et al. 2002), *Satellite tobacco mosaic virus* (STMV; Gossele et al. 2002), *Pea early browning virus* (PEBV; Constantin et al. 2004), *Tomato mosaic tobamovirus* (ToMV; Hori et al. 2004), and *Bean pod mottle virus* (BPMV; Zhang and Ghabrial 2005).

Plant viral vectors are also used for inducing transcriptional gene silencing (TGS), which represses transcription. DsRNAs that contain a sequence homologous to a promoter sequence can induce cytosine methylation on the promoter sequence in the nuclear DNA and subsequently induce TGS (for a review, see Matzke and Birchler 2005). Thus far, induction of TGS has been reported only by the PVX vector (Jones et al. 1999) and TRV vector (Jones et al. 2001) harboring a portion of the transgene promoter sequence.

Cucumber mosaic virus (CMV) is one of the best characterized positive-sense single-stranded RNA viruses and has the widest host range among the plant viruses (Palukaitis et al. 1992). The CMV genome consists of the tripartite components RNAs 1, 2, and 3

Abbreviations: BSMV, *Barley stripe mosaic virus*; BPMV, *Bean pod mottle virus*; CaMV, *Cauliflower mosaic virus*; CMV, *Cucumber mosaic virus*; CMV-Y, CMV Y strain; DIG, digoxigenin; dpi, days post-inoculation; dsRNA, double-stranded RNA; GFP, green fluorescent protein; PEBV, *Pea early browning virus*; PTGS, post-transcriptional gene silencing; PVX, *Potato virus X*; STMV, *Satellite tobacco mosaic virus*; siRNA, short interfering RNA; TMV, *Tobacco mosaic virus;* TRV, *Tobacco rattle virus*; ToMV, *Tomato mosaic tobamovirus*; TGS, transcriptional gene silencing; VIGS, virus-induced gene silencing.

(Peden and Symons 1973). RNA 1 and RNA 2 encode the 1a and 2a proteins, respectively, that are involved in virus replication. RNA 2 also encodes the silencing suppressor protein (2b). RNA 3 encodes the movement protein (3a) and coat protein (Palukaitis and Garcia-Arenal 2003). In this study, we manipulated the RNA 2 of the CMV Y strain (CMV-Y) to construct a viral vector and examined whether this vector could induce both transcriptional and post-transcriptional gene silencing (PTGS) of a transgene in *Nicotiana benthamiana* plants.

Materials and methods

Plant materials

N. benthamiana line 16c containing a single-copy green fluorescent protein (*GFP*) transgene (Ruiz et al. 1998) was used. This plant expresses the *GFP* gene under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter (Ruiz et al. 1998). The plants were grown under a 16-h light : 8-h dark cycle at 24°C.

Plasmid construction

Modifications were introduced into the pCY2 plasmid DNA containing the full-length cDNA of CMV-Y RNA 2 (Suzuki et al. 1991) as follows. The fragment nt 2753-3051 of CMV-Y RNA 2 was amplified by PCR with the primers 5′-CGAGGCCTGACGCGTGTACGTAAACCT-CCCCTTCCGCATC-3′ and 5′-CCATCGATTGGTCT-CCTTTTGGAGGCC-3′. The former contains *Stu*I, *Mlu*I, and *Sna*BI recognition sites and a stop codon. The amplified fragment was treated with *Stu*I and *Bln*I and then inserted into compatibly digested pCY2 to give CMV2-A1.

Cloning of the GFP *gene and CaMV 35S promoter sequences into the virus vector*

Portions of the *GFP* cDNA sequence from positions +24 to +240 and +328 to +430 (position numbers are relative to the ATG codon) were amplified by PCR using plasmid DNA containing a *GFP* gene cloned from *N. benthamiana* line 16c as a template. The PCR primers were: 5′-AGGCCTCACTGGAGTTGTCCCAATTC-3′ and 5′-ACGCGTCCGCTTCATATGATCTGGGT-3′ for amplifying the region +24 to +240; and 5′-AGGCC-TGCTGAAGTCAAGTTTGAGGG-3′ and 5′-ACGCGT-TGTATTCCAACTTGTGGCCG-3′ for amplifying the region +328 to +430. Similarly, portions of the CaMV 35S promoter sequence from positions −345 to −89 and −208 to −89 (position numbers are relative to the transcription start site) were amplified by PCR using a plasmid DNA template and the following primers: 5′-AGGCCTCATCGTTGAAGATGCCTCTG-3′ and 5′-ACGCGTATATCACATCAATCCACTTGC-3′ for amplifying the region −345 to −89; and 5′-AGGCCTCAT-CGTTGAAGATGCCTCTG-3′ and 5′-ACGCGTATAT-CACATCAATCCACTTGC-3′ for amplifying the region −208 to −89. The first six nucleotides of the forward primers provided a *Stu*I site, and those of the reverse primer provided a *Mlu*I site, which were used in the subsequent plasmid construction. After cloning the PCR products into the pGEM-T Easy vector (Takara Bio Inc., Otsu, Japan), the *Stu*I–*Mlu*I fragment of the plasmid containing the portions of the *GFP* gene or CaMV 35S promoter sequences were cloned into the *Stu*I and *Mlu*I sites of the CMV2-A1 vector. The CMV2-A1 vectors containing the +24 to +240 and +328 to +430 regions of the *GFP* cDNA sequence were designated A1 : GFP-5′-long and A1 : GFP-3′-short, respectively, and those containing the −345 to −89 and −208 to −89 regions of the CaMV 35S promoter sequence were designated A1 : 35S-long and A1 : 35S-short, respectively.

In vitro transcription

The plasmids pCY1 (Suzuki et al. 1991) and CMV2-A1 (this study) were linearized with *Not*I, and the plasmid pCY3 (Suzuki et al. 1991) was linearized with *Eco*RI prior to *in vitro* transcription. The *in vitro* transcription reaction was performed using 1 μg of linearized vector DNA, 25 units of T7 RNA polymerase (Takara), 20 units of ribonuclease inhibitor, 5 mM DTT, 1 mM ATP, 1 mM CTP, 1 mM UTP, 0.1 mM GTP, and 1 mM m7G(5′)PPP(5′)G (Invitrogen, Carlsbad, CA, USA) as a cap analog in 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, and 2 mM spermidine-HCl at 37°C for 60 min.

Inoculation of transcripts to plants

The leaves of 4-week-old plants of *N. benthamiana* line 16c were dusted with carborundum and rub-inoculated with the *in vitro* transcripts. Successful infection of the virus in the *N. benthamiana* plants without deletion of inserted sequences was confirmed by RT-PCR of the viral RNA.

Imaging of GFP fluorescence

GFP fluorescence was examined under an epifluorescence microscope (SZX-12; Olympus, Tokyo, Japan) equipped with a GFP cube (SZX-MGFPA; Olympus).

Northern blot analysis

Total RNA was isolated as previously described (Napoli et al. 1990), except that we removed genomic DNA from the RNA fraction using DNase I (Takara). Two micrograms of the total RNA were treated with 50% formamide, 17.5% formaldehyde, and 0.5% SDS in MOPS buffer (20 mM 4-morpholinepropanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) and separated on a 1% agarose gel containing 20 mM MOPS buffer. After electrophoresis, RNA was transferred onto nylon membranes (Hybond N^+; Amersham Biosciences,

Piscataway, NJ, USA) and allowed to hybridize with labeled probes. A digoxigenin (DIG)-labeled *GFP* probe was prepared by PCR amplification of a portion of the *GFP*-coding sequence using plasmid DNA (see above) as a template. The reaction was performed using a PCR-DIG labeling kit (Roche, Basel, Switzerland). To amplify the target sequence, the primers 5′-ATGAGTAAAGG-AGAACTTTTC-3′ and 5′-TTATTTGTATAGTTTCAT-CCATGC-3′ were used. The labeled probe was hybridized with membranes at 58°C overnight. The membranes were washed twice with washing solution containing 2x SSC and 0.1% SDS for 15 min each at room temperature and then twice with washing solution containing 0.2x SSC and 0.1% SDS for 15 min each at 68°C. The hybridized probe was detected using anti-DIG-AP Fab fragments (Roche) and CDP-Star (Roche).

Detection of siRNA

A fraction of low-molecular-weight RNA was extracted from the upper leaves of *N. benthamiana* plants at 18 days post-inoculation (dpi) and used for detection of siRNAs according to the method of Goto et al. (2003). To obtain a probe specific for the CaMV 35S promoter sequence, the −345 to +1 region of the promoter sequence was amplified by PCR with the primers 5′-ATTGAGACTTTTCAACAAAGGG-3′ (35S −345F) and 5′-TCCTCTCCAAATGAAATGAAC-3′ (35S +1R) and labeled with DIG as described above. The *GFP* gene-specific probe was prepared as described above.

Southern blot analysis

Total DNA was extracted from the upper leaves as described by Kanazawa and Tsutsumi (1992) at 2 months post-inoculation. Total DNA (25 μg) was digested with the methylation-sensitive restriction enzyme *Alu*I and separated by electrophoresis on a 1.5% agarose gel in 1x TBE buffer. The DNA was transferred onto a Hybond N^+ membrane and hybridized with probes labeled using an AlkPhos Direct nucleic acid labeling and detection system (Amersham). The hybridization, membrane wash, and signal detection were performed according to the manufacturer's instructions. A DNA fragment amplified by PCR using the primers 35S −345F and 35S +1R was labeled and used as a probe for the CaMV 35S promoter. To obtain an *actin* gene probe, a 396-bp portion of the *actin*-coding sequence (GenBank accession no. AY179605) was amplified from *N. benthamiana* genomic DNA using the primers 5′-GAAGATACTCACAGAA-AGAGG-3′ and 5′-GGAGCTAATGCAGTAATTTCC-3′. The amplified fragment was cloned into the pGEM-T Easy vector (Takara) and used as a probe.

Results

Construction of the CMV2-A1 vector

The full-length cDNAs of RNAs 1, 2, and 3 of CMV-Y have been previously cloned into plasmids and named pCY1, pCY2, and pCY3, respectively (Suzuki et al. 1991). In this study, we modified pCY2 by adding restriction sites for cloning a foreign sequence. RNA 2 contains two ORFs, ORF 2a and 2b; two-thirds of ORF 2b overlaps ORF 2a (Figure 1A). We deleted the 3′ portion of ORF 2b and introduced new *Mlu*I and *Sna*BI recognition sites as well as an in-frame stop codon for ORF 2b between the *Stu*I and *Mlu*I recognition sites (Figure 1A). The resulting vector was designated CMV2-A1.

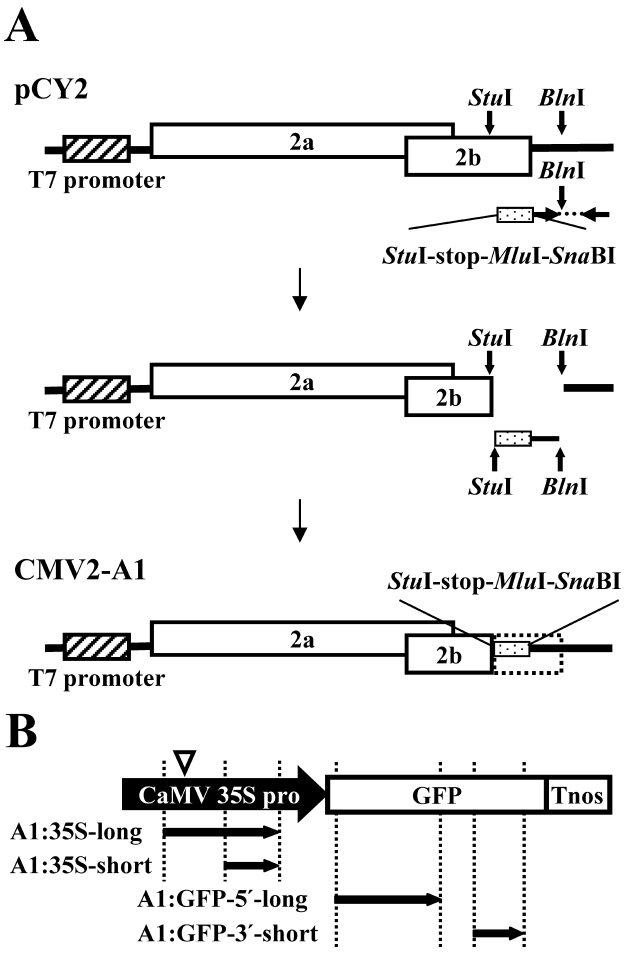

Figure 1. Construction of the CMV2-A1 vector and portions of the *GFP* transgene used for virus-induced gene silencing. (A) Schematic representation of the construction of the CMV2-A1 vector. A *Stu*I–*Bln*I fragment located in the 3′ region of CMV-Y RNA 2 was deleted from the plasmid pCY2. The deleted portion was refilled with a PCR-amplified DNA fragment containing cloning sites and an in-frame stop codon for the 2b protein. The hatched box indicates the T7 RNA polymerase promoter sequence. (B) Portions of the *GFP* transgene used for induction of gene silencing. The fragments that were cloned into the vector are shown as arrows. Tnos, nopaline synthase terminator. The open triangle indicates the recognition site of the methylation-sensitive enzyme *Alu*I.

Induction of gene silencing by the CMV2-A1 vector

We examined whether the CMV2-A1 vector mediates post-transcriptional silencing of a *GFP* reporter gene. The 217-bp sequence of the 5′ region (A1 : GFP-5′-long) or 103-bp sequence of the 3′ region (A1 : GFP-3′-short) of the *GFP* cDNA sequence was cloned into the vector (see Figure 1B), and *in vitro* transcripts of these constructs were co-inoculated with the CMV-Y RNAs 1 and 3 into 4-week-old *N. benthamiana* line 16c plants containing a single-copy *GFP* gene located downstream of the CaMV 35S promoter (Ruiz et al. 1998).

The upper leaves of the plants infected with a control empty vector exhibited strong fluorescence continuously even after 28 dpi (Figure 2). In contrast, the upper leaves of the plants infected with the CMV2-A1 vector containing the *GFP* cDNA sequence lost GFP fluorescence and exhibited red fluorescence from chlorophyll at 12 dpi (Figure 2A). The GFP fluorescence was not restored at 24 dpi. Both A1 : GFP-5′-long and A1 : GFP-3′-short efficiently induced the silencing. The loss of GFP fluorescence was observed in the upper leaves of all 12 plants independently infected with A1 : GFP-5′-long or A1 : GFP-3′-short (data not shown). Northern blot analysis of RNA from the same leaves showed that the loss of GFP fluorescence accompanied a reduction in the level of *GFP* mRNA (Figure 2A). Furthermore, this analysis indicated that the reduction in the mRNA level of *GFP* had already started at 6 dpi, which preceded the loss of GFP fluorescence (Figure 2A).

We also inoculated the *N. benthamiana* plants with viruses containing a sequence of a transgene promoter, which was designed to target the promoter sequence in the nuclear DNA and induce transcriptional gene silencing. The 257-bp sequence (A1 : 35S-long) or 120-bp sequence (A1 : 35S-short) of the CaMV 35S promoter was cloned into the CMV2-A1 vector (see Figure 1B). When *N. benthamiana* plants were infected with the viruses containing these sequences, the loss of GFP fluorescence as well as a reduction in the mRNA level of the *GFP* gene was observed in the upper leaves (Figure 2B). Again, this phenomenon was observed in all 12 plants independently infected with A1 : 35S-long or A1 : 35S-short (data not shown). The results indicate that the promoter-targeted silencing and transcribed sequence-targeted silencing were induced with similar time courses.

Silencing induced by the virus infection accompanied accumulation of siRNA

The production of siRNA is a hallmark of the occurrence of both the sequence-specific degradation of a target mRNA and sequence-specific inactivation of a target promoter in nuclei, and thus we next looked for the

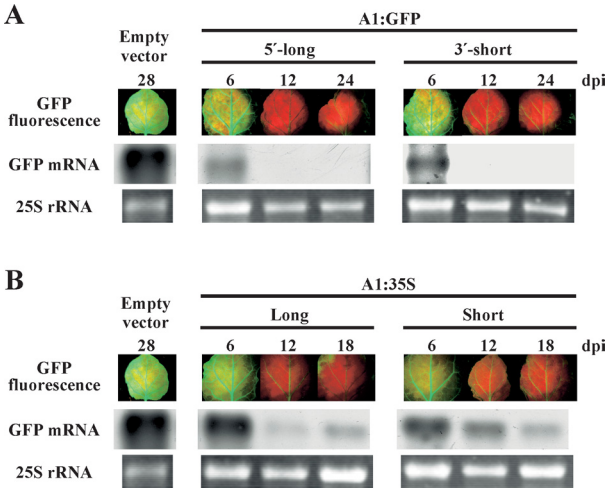

Figure 2. Changes in GFP fluorescence and mRNA level of the *GFP* gene by virus-induced gene silencing. GFP fluorescence of the upper leaves was photographed under UV illumination after viral inoculation. Northern blot analysis of RNAs from the respective leaves was performed using a *GFP* gene-specific probe. (A) Induction of silencing targeted to the transcribed sequence of the *GFP* gene. The upper leaf samples were taken from plants at 6, 12, and 24 days after inoculation with either A1 : GFP-5′-long or A1 : GFP-3′-short. (B) Induction of silencing targeted to the CaMV 35S promoter sequence. The upper leaf samples were taken from plants at 6, 12, and 18 days after inoculation with either A1 : 35S-long or A1 : 35S-short. The systemic leaf of the empty vector-inoculated plant and the mRNA level of the *GFP* gene in the leaf are shown as a control. Ethidium bromide staining of 25S rRNA is shown as a loading control.

presence or absence of siRNA homologous to the sequence inserted in the viral genome. A Northern blot analysis of the small RNA fraction revealed that siRNAs with homology to the *GFP* gene (Figure 3A) or CaMV 35S promoter (Figure 3B) sequences accumulated in the upper leaves when plants were infected with the viruses containing these sequences.

Targeting of the promoter sequence caused DNA methylation in nuclei

As the presence of siRNAs homologous to a transgene promoter sequence is associated with the induction of methylation on the promoter sequence in nuclear DNA, we analyzed the methylation status of the CaMV 35S promoter sequence. Total DNAs extracted from the upper leaves were digested with the methylation-sensitive enzyme *Alu*I and analyzed by Southern hybridization using a probe specific to the CaMV 35S promoter sequence. A hybridization signal of 1.3 kb was detected in the DNAs from plants infected with A1 : 35S-long or A1 : 35S-short, whereas only 0.7-kb and 0.6-kb signals were detected in DNAs from mock-inoculated or empty vector-inoculated plants (Figure 4A and B). To eliminate the possibility that insufficient digestion of *Alu*I caused the generation of the higher molecular weight signals, the same DNA samples were re-hybridized with the *actin* gene probe (Figure 4C). An

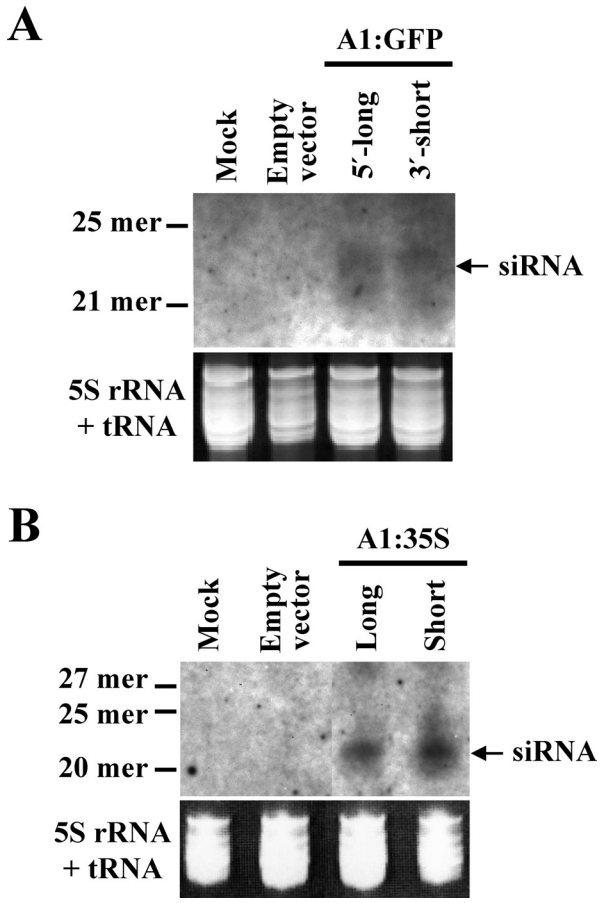

Figure 3. Accumulation of siRNAs in the *GFP*-silenced plants. (A) Northern blot analysis of low-molecular-weight RNAs from a mock-inoculated plant or from plants inoculated with the empty vector, A1 : GFP-5′-long, or A1 : GFP-3′-short, probed for the *GFP* gene. (B) Northern blot analysis of low-molecular-weight RNAs from a mock-inoculated plant or from plants inoculated with the empty vector, A1 : 35S-long, or A1 : 35S-short, probed for the CaMV 35S promoter. Ten micrograms of low-molecular-weight RNAs were loaded. DNA oligonucleotides were also loaded for a size control; their sizes are indicated to the left. Ethidium bromide staining of 5S rRNA and tRNAs are shown as a loading control.

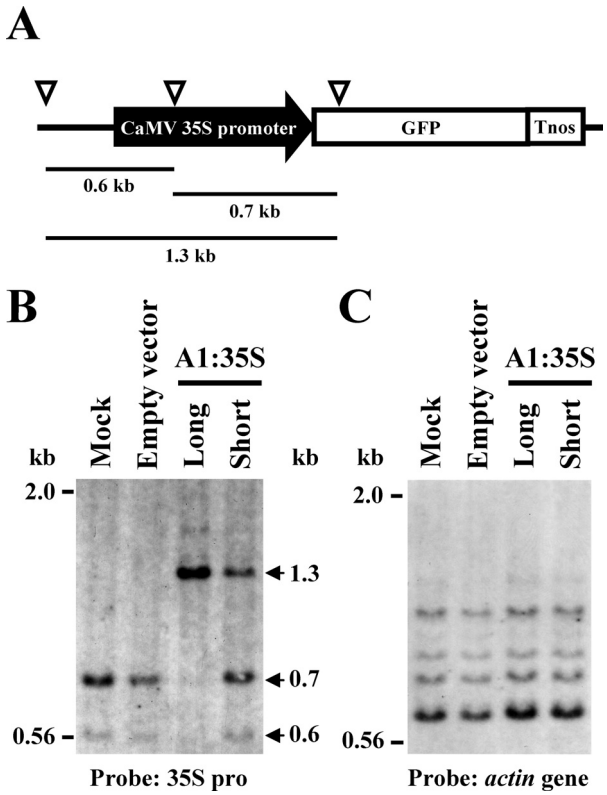

Figure 4. Methylation status of the CaMV 35S promoter. Genomic DNA was isolated from mock-inoculated plants or from plants inoculated with the empty vector, A1 : 35S-long, or A1 : 35S-short. The DNA was digested with the methylation-sensitive enzyme *Alu*I and used for a gel-blot analysis. (A) Restriction map of the transgene showing the expected sizes of *Alu*I-digested fragments. Open triangles indicate the *Alu*I recognition sites. (B) Hybridization with a probe specific for the CaMV 35S promoter sequence. Note that the 1.3-kb signal corresponds to a DNA fragment that was generated by non-digestion of the *Alu*I site in the CaMV 35S promoter as a result of cytosine methylation in the restriction site, while the 0.7-kb and 0.6-kb signals correspond to DNA fragments generated by digestion at the *Alu*I site. (C) Re-hybridization of the membrane with a probe specific for the *actin* gene confirming that the digestion with the enzyme was complete in all DNA samples.

identical hybridization pattern was detected in the four DNA samples, indicating that all samples were digested to the same extent and that the differences detected by the CaMV 35S promoter probe were attributable to the methylation of the *Alu*I recognition site in the promoter. The *Alu*I site methylation was induced even by A1 : 35S-short, whose insert sequence did not cover the site. We speculate that this may have resulted from the spread of methylation along the promoter sequence. We have also found that methylation on the promoter sequence as well as the silenced state of the *GFP* gene is transmitted to the next generation at a high frequency (S. Otagaki et al. unpublished data). These results suggest that the viral vector containing the promoter sequence can induce TGS.

Discussion

In the present study, we developed a CMV-based vector that can induce sequence-specific silencing of a transgene by targeting a transcribed sequence and a promoter sequence, which cause mRNA degradation and transcriptional repression, respectively. Previously, Zhao et al. (2000) made a complementation-dependent CMV vector. They modified the RNA 3 of the CMV lx strain and split the RNA 3 into two subcomponents, RNAs 3A and 3B. In RNA 3A, the movement protein gene was replaced by a reporter gene encoding GFP; in RNA 3B, the ORF of the coat protein was eliminated, and a multiple cloning site was created for foreign gene insertion. This engineered virus could move from cell to cell in the inoculated leaf and enter the minor veins of the inoculated leaf. However, intermolecular

recombination between RNAs 3A and 3B occurred frequently, resulting in a loss of the foreign gene insert in the systemic leaves. In this study, we modified RNA 2, instead of RNA 3, and did not adapt the complementation-dependent strategy in order to avoid possible intermolecular recombination.

Although many plant viral vectors have been reported, only the PVX vector (Jones et al. 1999) and TRV vector (Jones et al. 2001) have thus far been reported to induce TGS in a transgene. The *N. benthamiana* line 16c plants infected with a PVX vector containing the CaMV 35S promoter sequence showed a loss of GFP fluorescence at 20 dpi (Jones et al. 1999), whereas 16c plants infected with A1 : 35S-long or A1 : 35S-short showed a loss of GFP fluorescence at 12 dpi as well as a reduction in the mRNA level of the *GFP* gene at 6 dpi (Figure 2B). These results imply that the CMV vector induces TGS more efficiently than the reported PVX vector.

One advantage of applying the CMV vector is its wide host range. Different strains of CMV preferentially infect different plant species. For example, the *Glycine max* strain of CMV can infect soybean (Senda et al. 2004), while ordinary strains such as CMV-Y and CMV-O can infect *Petunia hybrida* (Koseki et al. 2005). Viable pseudorecombinant CMV strains can be created by mixing the three viral RNAs from different viral strains. Accordingly, the use of pseudorecombinant CMV consisting of the CMV2-A1 vector and RNA 1 and/or RNA 3 of different strains of CMV allows optimizing its infectious ability and the viral host range.

When the CMV2-A1 vector was inoculated into *N. benthamiana* plants, a mild mosaic symptom appeared on systemically infected leaves (Figure 5). The symptom had no influence on the GFP fluorescence. However, viral infection without outward symptoms may be preferable depending on the phenotype of the target gene. One method that may eliminate symptoms without affecting systemic infection by a virus is to use an attenuated viral strain. Hori et al. (2004) demonstrated that the attenuated strain of tomato mosaic tobamoviruses-based vectors induced silencing of the *phytoene desaturase* gene without exhibiting any visible symptoms after infection. As the cucumoviral 2b protein is involved in the control of symptom expression (Ding et al. 1995; Soards et al. 2002), replacing the 2b sequence of the CMV2-A1 vector with that of an attenuated CMV such as CM95 (Kosaka and Fukunishi 1997) would reduce the viral symptom. Also, it may be effective to use a pseudorecombinant virus consisting of CMV2-A1 and RNA 1 and/or RNA 3 of other strains of CMV, because it is known that symptom expression is also affected by RNA 1 or RNA 3 in addition to the activity of the 2b protein (Shintaku et al. 1992; Suzuki et al. 1995). We have found that a pseudorecombinant virus consisting of CMV2-A1 and RNA 1 and RNA 3 of the leguminous strain of CMV (CMV-L; Karasawa et al. 1997) infects *N. benthaminana* plants without exhibiting visible symptoms (Figure 5). Overall, these approaches may improve the vector in terms of controlling viral symptoms, in addition to extending its host range, to avoid viral influence on the phenotypic changes after VIGS.

Figure 5. Elimination of viral symptom by the use of pseudorecombinant virus. Left, a mock-inoculated *N. benthamiana* plant; center, a *N. benthamiana* plant infected with a virus consisting of CMV2-A1 and RNA 1 and RNA 3 of CMV-Y (Y1/CMV2-A1/Y3); right, a *N. benthamiana* plant infected with a pseudorecombinant virus consisting of CMV2-A1 and RNA 1 and RNA 3 of CMV-L (L1/CMV2-A1/L3). Plants were photographed at 7 days after viral inoculation.

In the present study, we demonstrated that the CMV2-A1 vector is suitable for the rapid induction of gene silencing targeted to both transcribed and promoter sequences. The targeting of these sequences by the vector is potentially useful as a tool for functional genomics: this system is especially useful for silencing endogenous genes in plants that are difficult to transform. In addition, the rapid induction of methylation on a promoter sequence by the vector has proved to be useful in our ongoing study, in which epigenetic effects on transcriptional regulation are being analyzed using the CaMV 35S promoter as a model.

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