BSCTV C2 Attenuates the Degradation of SAMDC1 to Suppress DNA Methylation-Mediated Gene Silencing in Arabidopsis

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INTRODUCTION

Geminiviruses are a group of single-stranded plant DNA viruses that can infect a wide range of plant species and cause considerable losses of agricultural products. Plants have evolved various mechanisms to respond to viral invasion, such as pathways mediated by gene silencing, salicylic acid, and metabolism regulation (Herbers et al., 1996; Vanitharani et al., 2005; Bisaro, 2006; Ascencio-Ibáñez et al., 2008; Chen et al., 2010). Among them, gene silencing-mediated antiviral defense is a well-conserved and effective pathway. All the geminiviruses, which are classified into four groups, Mastrevirus, Curtovirus, Begomovirus, and Topocuvirus, amplify themselves in the nucleus of plant host cells using double-stranded DNA (dsDNA) intermediates as replication and transcription templates. The dsDNA intermediates can form minichromosomes that are associated with plant host histone proteins (Pilartz and Jeske, 1992). Viral mRNAs can be transcribed bidirectionally from the common region that is conserved in geminivirus genomes, and the resulting two divergent transcripts overlapping in the 3’ termini likely form dsRNAs, which can trigger plant host RNA silencing-mediated defense mechanisms (Voinnet, 2005). Therefore, both the dsDNA intermediates and their transcripts can become the targets of plant gene silencing-mediated antiviral defense (Voinnet et al., 1999; Seemanpillai et al., 2003; Bian et al., 2006; Blevins et al., 2006). However, geminiviruses also encode proteins to counteract these defense mechanisms, such as C2/L2/AC2/AL2 proteins, a series of complementary positional homologs that have been reported to function during pathogenesis (Voinnet et al., 1999; van et al., 2002; Trinks et al., 2005; Wang et al., 2005; Kon et al., 2007).

Each of the C2/L2 proteins in curtoviruses and their positional homologs AC2/AL2 in begomoviruses has a conserved zinc-finger motif, despite having only limited homology in the overall amino acid sequence. Begomovirus AC2/AL2, also known as TrAP, is a transcriptional activator for viral genes and some plant host genes (Sunter and Bisaro, 1992, 1997; Trinks et al., 2005), while curtovirus C2/L2 proteins lack an obvious transcriptional activation domain and transcriptional activation activity (Sunter et al., 1994; Baliji et al., 2007). Furthermore, AC2 encoded by the Begomovirus African cassava mosaic virus was first reported to suppress established RNA silencing in a transient infiltration
system, and later a series of begomovirus AC2/AL2 proteins and the curtovirus L2 protein were identified as RNA silencing suppressors (Voinnet et al., 1999; van et al., 2002; Trinks et al., 2005; Wang et al., 2005). Meanwhile, the functional role of geminivirus AC2/AL2/C2/L2 proteins became better understood through studies that screened for their interacting proteins in plant hosts (Hao et al., 2003; Wang et al., 2003). Tomato golden mosaic virus (TGMV) AL2 and Beet curly top virus (BCTV) L2 were found to interact with and inactivate adenosine kinase (ADK) (Wang et al., 2003), which is a key enzyme for converting adenosine to 5’AMP and essential for sustaining the methyl cycle and S-adenosylmethionine (SAM)–dependent methylation (Lecoq et al., 2001; Moffatt et al., 2002).

Further studies indicated that AL2 and L2 suppress both posttranscriptional gene silencing and transcriptional gene silencing (TGS) by inactivating ADK (Raja et al., 2008; Buchmann et al., 2009). Meanwhile, the balance of SAM and its metabolic analogs or products have become more notable in the methylation-related process. In addition, TGMV AL2 and BCTV L2 were also found to interact with and inactivate SNF1-related kinase (also known as Arabidopsis protein kinase 11 [AKIN11]), which is a global regulator of metabolism. However, this inactivation of SNF1 does not appear to be involved in RNA silencing suppression, suggesting that AL2 and L2 may be involved in the SNF1-mediated antiviral defense (Hao et al., 2003; Wang et al., 2005). All these findings suggest that geminivirus AC2/AL2/C2/L2 proteins play diverse roles in the plant–virus interaction.

S-Adenosyl-methionine decarboxylase (SAMDC) is a key enzyme in polyamine biosynthesis, which catalyzes the conversion of SAM to decarboxylated S-adenosyl-methionine (dcSAM). The product dcSAM acts as an aminopropyl donor for biosynthesis of spermidine and spermine, while the substrate SAM is also used in three other key metabolic pathways: transmethylation, ethylation biosynthesis, and trans-sulfuration. However, as dcSAM shares similar radicals with SAM, dcSAM also may act as a competitive inhibitor against SAM for methyltransferases (Heby et al., 1988). Therefore, SAMDC is also relevant for SAM/dcSAM balance and transmethylation. SAMDC is synthesized as an inactive proenzyme that generates two subunits, termed α and β, by an autocatalytic cleavage reaction. SAMDCs in many organisms are highly conserved, sharing a defined autocleavage recognition site and a PEST region enriched in Pro (P), Glu (E), Ser (S), and Thr (T) that is correlated with the protein’s rapid turnover (Salama et al., 1994; Rechsteiner and Rogers, 1996; Berset et al., 2002; Blondel et al., 2005; Pal et al., 2007). SAMDC is expressed throughout the cell, and its expression is regulated at multiple levels, such as the transcriptional, translational, and posttranslational levels (Shantz et al., 1992; Stanley et al., 1994; Hanfrey et al., 2003; Yerlikaya and Stanley, 2004). Previous studies indicated that SAMDC is involved in diverse growth and developmental processes, including cell division, cell proliferation and differentiation, morphological development, and response to abiotic stress (Kumar et al., 1996; Frostesjö et al., 1997; Shibata et al., 1998; Wi et al., 2006). However, whether SAMDC is involved in the response to viral invasion remains unclear.

In this study, to explore the role of BSCTV C2 in the plant–virus interaction, yeast two-hybrid screening was performed and a BSCTV C2-interacting protein, SAMDC1, was identified. Infection by C2-deficient BSCTV caused an increase in DNA methylation of the BSCTV genome in virus-infected plants. DNA analysis further showed that BSCTV C2 interfered with de novo methylation of the FWA transgene in BSCTV C2 transgenic Arabidopsis thaliana. Analysis of transient induction of RNA silencing revealed that both BSCTV C2 and SAMDC1 possess silencing suppressor activity. Biochemical and genetic analysis indicated that BSCTV C2 may interfere with the gene silencing-mediated antiviral defense in planta via positive regulation of the stability of SAMDC1 protein, which is ubiquitinated and degraded by the 26S proteasome in plant cells.

RESULTS

BSCTV C2 Interacts with Arabidopsis SAMDC1

Plant viruses are excellent tools for studying mechanisms of plant development and microbial–host interactions. Geminivirus proteins were found to interact with host proteins to regulate the host environment to facilitate virus replication and transmission (Xie et al., 1995, 1999; Castillo et al., 2003; Hao et al., 2003; McGarry et al., 2003; Wang et al., 2003; Fontes et al., 2004). To explore the functional role of BSCTV C2 in plant–virus interactions, we searched for plant cellular proteins that interact with BSCTV C2 using a yeast two-hybrid system based on the yeast strain HF7C, which harbors both HIS3 and lacZ reporter genes. Using GBD-C2 (a fusion protein consisting of the GAL4 DNA binding domain and BSCTV C2) as bait, a cDNA corresponding to the C-terminal residues 181 to 363 of SAMDC was recovered from an Arabidopsis GAL4 activation fusion library, and the interaction was confirmed on selective plates (Figure 1A). To rule out potential false positive interactions between BSCTV C2 and SAMDC1, an in vitro pull-down assay was also performed. The SAMDC1 cDNA clone recovered from the yeast two-hybrid screening was fused to gluthione S-transferase (GST). This fusion protein was expressed in Escherichia coli and purified with gluthione sepharose beads. 35S-Met–labeled BSCTV C2 generated by in vitro translation in wheat germ extracts was incubated with the fusion protein captured by gluthione sepharose beads, and the GST protein alone was included in a parallel binding assay as a negative control. Incubation of wheat germ extracts containing 35S-Met–labeled BSCTV C2 with GST fusion protein GST-SAMDC1181-363 resulted in pull down of BSCTV C2, but not the GST control, suggesting a specific interaction between BSCTV C2 and SAMDC1 in vitro (Figure 1B). The complete α-chain open reading frame (ORF) of SAMDC1 was also demonstrated to interact with BSCTV C2 in the in vitro pull-down assay (Figure 1C). All these data suggest that BSCTV C2 directly interacts with SAMDC1.

To investigate whether BSCTV C2 interacts with SAMDC1 in planta, we performed a well-established Agrobacterium tumefaciens–based firefly luciferase complementation imaging (LCI) assay for the detection of protein–protein interaction in planta (Chen et al., 2008) in Nicotiana benthamiana. C2 protein and SAMDC1α were fused with N-terminal luciferase (NLuc) and C-terminal luciferase (CLuc) regions, respectively. A. tumefaciens strains harboring CLuc and NLuc constructs (or their derivative
constructs C2-NLuc and CLuc-S1α were mixed and infiltrated into different positions at the same leaf of *N. benthamiana*. Leaves coexpressing different combination of constructs were then examined for LUC activity. Figure 1D showed that, in the same leaf, the combination of the empty vector NLuc and CLuc, the combination of NLuc and CLuc-S1α, or the combination of C2-NLuc and CLuc did not show LUC complementation. By contrast, the combination of C2-NLuc and CLuc-S1α did show LUC complementation, suggesting that C2 interacts with SAMDC1 in planta.

Furthermore, to locate the approximate domains of SAMDC1 involved in interactions with C2, yeast two-hybrid assays using C2 and defined regions of SAMDC1 were performed. The results
showed that SAMDC1-1-83 and SAMDC1-292-366 were not able to interact with the C2 protein, while SAMDC1-182-292 and SAMDC1-226-366 were able to interact with C2 protein (Figure 1E). These experiments suggested that the region of amino acids 226 to 292 of SAMDC1 is essential for its interaction with C2.

BSCTV C2 Inhibits 26S Proteasome-Mediated Degradation of SAMDC1

Interestingly, a highly conserved region known as the PEST sequence, enriched in Pro, Glu, Ser, and Thr, is located in the region essential for interaction between SAMDC1 and BSCTV C2 (Figure 1D). The PEST sequence has been reported to be located in some proteins with fast turnover rates and be essential for the interaction between SCF complex and target proteins, which consequently leads to ubiquitination/26S proteasome-mediated degradation (Kiernan et al., 2001; Berset et al., 2002; Blondel et al., 2005; Pal et al., 2007). Human SAMDC was reported to have a short half-life and is regulated at multiple levels, such as the transcriptional, translational, and posttranslational levels (Shantz et al., 1992; Stanley et al., 1994; Hanfrey et al., 2003; Yerlikaya and Stanley, 2004). Taking all the information above together, we wanted to ask whether the interaction with BSCTV C2 can affect with the stability of SAMDC1. Therefore, we designed two different cell-free degradation assays to address this question.

First, SAMDC1 with a 6× myc tag in the C terminus was generated by in vitro translation in a wheat germ extract. Notably, the SAMDC1-myc protein remained stable in the proenzyme form, possibly due to lack of some important factors essential for its autocleavage or due to containing inhibitors of its autocleavage in the wheat germ extract (Figures 2A and 2E). Equal volumes of wheat germ extracts containing SAMDC1-myc were added into the wheat germ extract-based protein degradation system with or without 50 μM of the 26S proteasome inhibitor MG132 (see Methods). After incubation at 30°C, equal volumes of the protein degradation mix were sampled at different time points and subjected to immunoblot analysis using the anti-myc antibody. As shown in Figure 2A, SAMDC1-myc depletion was observed in the absence of MG132, whereas SAMDC-myc was much more stable in the presence of MG132. The results indicated that the degradation of SAMDC1-myc was inhibited by MG132, suggesting that the degradation of SAMDC1 is mediated by the 26S proteasome. Next, we explored whether BSCTV C2 affects the proteasome-mediated degradation of SAMDC1. We added BSCTV C2 protein generated by in vitro translation into the protein degradation system. A luciferase protein also generated by in vitro translation was used as a control in a parallel assay. The results showed that the SAMDC1-myc protein was more stable in the presence of the BSCTV C2 protein than with that control luciferase protein, suggesting that BSCTV C2 could attenuate the proteasome-mediated degradation of SAMDC1 (Figures 2B and 2F).

Considering that the degradation of SAMDC1 in Arabidopsis might be different from the degradation in wheat germ extract, we prepared fresh total protein extracts from 4-week-old wild-type Arabidopsis rosette leaves to replace the wheat germ extract in an in vitro cell-free degradation assay (see Methods for details). During the incubation in Arabidopsis total protein extract, SAMDC1-myc proteins were cleaved into a smaller form that is close in size to the SAMDC1 α-subunit (~43 kD) (Figure 2C). However, the sum of SAMDC1-myc and SAMDC1 α-myc was still depleted faster in the degradation system without MG132 than that with MG132 (Figures 2C and 2G), similar to results in the wheat germ extract assay (Figures 2A and 2E). When MG132 was replaced by in vitro–translated C2 protein in the in vitro protein degradation system, the depletion of SAMDC1-myc was retarded compared with coinubcation with in vitro–translated luciferase protein (Figures 2D and 2H), suggesting further the BSCTV C2 plays a role in attenuating the proteasome-mediated degradation of SAMDC1. To determine whether the suppressive activity of the protein degradation is specific for the interaction between BSCTV C2 and SAMDC1, we also performed a similar cell-free assay for ELONGATED HYPOCOTYL5 (HY5), another rapidly turned-over protein, whose degradation is mediated by the COP1-dependent ubiquitin/26S proteasome degradation pathway (Ma et al., 2002; Sajio et al., 2003). Similar assays of coinubcation of HY5 protein with C2 or luciferase in Arabidopsis cell extract were performed. There was no alteration of degradation of HY5 with C2 compared with that with luciferase (Figures 2I and 2J), suggesting that the protein degradation suppression activity of C2 may have specificity on SAMDC1.

We also noticed that the input of the wheat germ extract, due to adding in vitro–translated C2 or luciferase protein, in the cell-free system would prevent the autocleavage of SAMDC1-myc; as a consequence, the degradation rate of the SAMDC1-myc was affected to a certain extent in both coinubcation with C2 and luciferase control assays (Figure 2D).

Furthermore, we also used an A. tumefaciens infiltration-based transient expression system in N. benthamiana to confirm the effect of C2 on the stability of SAMDC1 in vivo. Agrobacteria harboring 35S-SAMDC1-myc and 35S-red fluorescent protein (RFP) constructs were mixed with A. tumefaciens harboring either 35S-HA-GFP for green fluorescent protein) or 35S-GFP-C2 construct and coinfublated into the leaves of N. benthamiana, in which 35S-RFP was used as an internal control for all the infiltration samples and 35S-HA-GFP was used as a negative control for 35S-GFP-C2. Total proteins were extracted for each samples 3 d after coinubilation and subjected to immunoblot assay for the detection of RFP and SAMDC1-myc. Results showed that, with equal RFP protein levels, SAMDC1-myc coexpressed with GFP-C2 protein accumulated to a higher level than when coexpressed with HA-GFP protein (Figure 2K). However, when the RNA levels of RFP were equal in the two samples, the RNA level of SAMDC1-myc in the sample coexpressed with GFP-C2 didn’t show any obvious difference compared with the sample coexpressed with HA-GFP. These data indicate that the GFP-C2 protein facilitates the accumulation of SAMDC1-myc, and this effect is not due to the impact of C2 on the transcription level, also indicating that C2 probably attenuate the protein degradation of SAMDC1.

The BSCTV C2 Transgene Interferes with Plant Host de Novo Methylation

SAMDC1 belongs to a family of SAMDCs that is involved in catalyzing the decarboxylation of SAM to dcSAM. The substrate
of SAMDC1, SAM, is also the methyl donor, and it is known that changing the content and ratio of SAM/dcSAM and SAM/SAH may lead to alteration of host transmethylation (Frostesjö et al., 1997; Rocha et al., 2005). Thus, we asked whether BSCTV C2 could affect the host transmethylation changes by stabilizing the SAMDC1 protein, resulting in an altered SAM/dcSAM ratio in plants. Because we could not measure the content of SAM and dcSAM in vivo, we designed experiments to address whether BSCTV C2 affects methylation of the host DNA. Wild-type Arabidopsis plants were transformed with vectors expressing SAMDC1-myc. Figure 2. Degradation Assays for SAMDC1.

(A) Wheat germ extract degradation assay of SAMDC1-myc in wheat germ extract with or without MG132. +MG132, final concentration 50 μM; –MG132, equal volume of DMSO (MG132 solvent) as a negative control. Samples were collected at 0, 0.5, 1, and 2 h after incubation at 30°C.

(B) Wheat germ extract degradation assay of SAMDC1-myc in wheat germ extract with BSCTV C2 or luciferase. +C2, incubated with C2 protein; +LUC, incubated with luciferase as a control.

(C) and (D) Degradation assays of SAMDC1-myc performed in an Arabidopsis cell-free system with or without MG132 (C) and with BSCTV C2 or luciferase (D) as described above in (A) and (B).

(E) to (H) Normalized plots for the degradation of SAMDC1-myc in (A) to (D), respectively. The details for quantification and normalization are described in Methods. Three independent repeats were analyzed for each experiment. Error bars represent SD.

(I) Cell-free degradation assay of myc-HY5 performed in an Arabidopsis cell extract as described in (D).

(J) Normalized plots for the degradation of HY5 in (I). Three independent experiments were analyzed. Error bars represent SD.

(K) In vivo A. tumefaciens infiltration assay in N. benthamiana. The top two panels show the accumulation of SAMDC1-myc and RFP. The bottom two panels show the SAMDC1-myc and RFP RNA levels. RFP protein level and RFP RNA level were used as internal controls for infiltration.
BSCTV C2 under the control of the 35S promoter, and the expression of C2 mRNA was detected by an RNA gel blot (see Supplemental Figure 1A online). The C2 transgenic plants showed no apparent morphologically or developmentally abnormal phenotypes under the standard growth conditions and displayed a similar flowering time as the wild-type plants (see Supplemental Figures 1B and 1C online), whereas some mutants defective in host methylation genes, such as *met1*, *cmmt3*, and *kyp*, display developmental defects and the late flowering phenotype. However, in a study of the phenotypic characterization of a de novo methyltransferase mutant *dmr1 dmr2*, it was found that the double mutant showed no apparent morphologically and developmentally abnormal phenotype under standard growth condition (Cao and Jacobsen, 2002). When *dmr1 dmr2* was transformed with an *FWA* genomic fragment containing tandem repeats in its promoter region, in which certain methylation sites remained unmethylated, the resulting transgenic plants displayed a late flowering phenotype with the control, suggesting that DRM proteins are required for de novo methylation of *FWA* transgene (Cao and Jacobsen, 2002). Therefore, we used the same assay in our study to evaluate whether the overexpression of BSCTV C2 in plants would affect de novo methylation of the promoter region of the *FWA* transgene and alter the flowering time of plants. Several C2 transgenic lines, together with the Columbia wild-type control, were transformed with a copy of the *FWA* genomic fragment, and several resulting double transgenic lines were generated. Three independent *FWA* transgenic lines in the C2 transgene background were selected for detailed analysis, and all three resulting transgenic lines displayed a late flowering phenotype compared with the wild type as determined by flowering bolting times (Figures 3A and 3B) and counting of rosette leaves (Figure 3C). To confirm the late flowering phenotype of C2 overexpression in the *FWA* transgene lines, DNA gel blot analysis was performed to evaluate the DNA methylation status of the *FWA* transgene region. Analysis by digestion with CfoI, a cytosine methylation-sensitive restriction enzyme, showed that a larger portion of the *FWA* transgene (Figure 3D) remained unmethylated in plants of all three C2-overexpressing lines, compared with that of the *FWA* transgene in a wild-type background (Figure 3D). These results indicate that C2 may interfere with plant host de novo methylation, even though C2 stabilized the activity of SAMDC1, resulting in changes in the content and the ratio of SAM/dcSAM.

### C2 Deficiency Causes Enhanced DNA Methylation of the BSCTV Genome in Plants

The results of the biochemical and genetic analyses above led us to hypothesize that C2 might interact with SAMDC1 to attenuate its degradation and to maintain its high enzyme activity in transforming its substrate SAM to dcSAM. Previous studies in mammalian stem cells indicated that positive feedback regulation of SAMDC caused a dramatic increase of dcSAM and was associated with genomic DNA hypomethylation (Frostesjö et al., 1997). Geminivirus replication is host dependent; thus, BSCTV C2 may also affect the DNA methylation of the virus genome itself. To test this hypothesis, we constructed a C2 minus BSCTV strain (*c2* mutant) by introducing a stop codon in the C2 ORF (see Supplemental Figure 2 online) and infected plants with the resulting mutant virus together with the intact BSCTV as a control. *Arabidopsis* plants were inoculated before bolting, and only the bolted inflorescences were sampled for total DNA isolation, avoiding the contamination of *A. tumefaciens* inocula in the following PCR reactions. We then performed bisulfite sequencing of the wild-type and *c2* mutant BSCTV genomes isolated from infected plants, mainly focusing on the intergenic region (IR), which contains divergent viral promoters flanking the origin of replication. As any cytosine could be a potential methylation site in plants, the region containing no or rare methylation sites was selected for PCR primer design. Based on these principles, PCR primers were designed for the intergenic region to amplify a fragment covering 57 potential methylation sites, including eight CG methylation sites, nine CNG methylation sites, and 43 CHH methylation sites, where N represents any nucleotide and H represents A, T, or C. Total DNA samples were treated with bisulfite reagent to convert unmethylated cytosines to uracil and then purified for PCR amplification and sequencing. Cytosine methylation levels in the IR of the wild-type and *c2* mutant BSCTV genomes are summarized in Figure 4. The cytosine methylation levels in the wild-type BSCTV genome is relatively low, and only one methylated CHH-type cytosine was detected in three clones (see Supplemental Figure 3 online). By contrast, almost all of the potential methylation sites in the *c2* mutant BSCTV genome were detected with various frequencies in different sequenced clones, and the total cytosine methylation level was 18.8%, in which 17.5% of potential CG-type methylation sites, 16.30% of potential CNG-type methylation sites, and 19.2% of potential CHH-type methylation sites were methylated (Figure 4). These data suggested that C2 deficiency caused the enhanced DNA methylation of the BSCTV genome in plants.

### Loss of Function of SAMDC1 Has a Similar Effect on Virus Infectivity and Viral DNA Accumulation as with C2 Deficiency

The plant host de novo methylation system plays an important role in the defense against exogenous DNA invasion. In geminivirus infection, it is known that the replication efficiency affects viral infectivity and pathogenesis. Thus, we hypothesized that changes affecting host DNA methylation may also affect host antiviral defenses and geminivirus infectivity. To test this idea, we first compared the infectivity of *c2* mutant BSCTV to that of intact BSCTV using an *A. tumefaciens* spraying method described previously (Chen et al., 2010). The *c2* mutant BSCTV showed milder and delayed symptoms, such as longer inflorescence, mild curling inflorescence, and more production of lateral branches (Figure 5A). Reduced infectivity (92% by *c2* versus 99% by wild-type BSCTV) was also observed (Figure 5B). Total DNA from whole plants at different time points after the inoculation was extracted and evaluated by DNA gel blot to detect virus accumulation in infected plants. A lower level of viral DNA (about twofold reduction) was detected in the *c2* mutant BSCTV infection compared with that of wild-type BSCTV (Figure 5C). Our methods were designed to exclude the possibility that the lower viral DNA level in *c2* BSCTV-infected plants at early time points was due to sampling of noninfected plants. The *Arabidopsis* plants inoculated with either wild-type or *c2* mutant BSCTV
began to develop symptoms ~7 d after inoculation (DAI), and the final infection rate reached a steady level by ~15 DAI. One inoculated rosette leaf from each of the infected Arabidopsis plants (40 plants each) was collected at each time point and kept separately. Only the leaves of plants that developed symptoms in each time point were mixed, while the final infection rate was determined. Total DNA at different time points after infection was extracted and analyzed by DNA gel blot. Consistent with results of the previous DNA gel blot, viral DNA accumulation in the local leaves inoculated with c2– mutant BSCTV was less than that with the wild-type BSCTV (Figure 5D), suggesting that C2 indeed resulted in decreased symptom severity and less viral DNA accumulation in host plants. The C2 null mutant of BCTV, a close relative of BSCTV, with no significant direct effects on replication (Hormuzdi and Bisaro, 1995) also showed the weak infection phenotype. This also supports our hypothesis that C2 is important for the suppression of the host methylation pathway to reduce host de novo methylation and to favor geminivirus DNA replication.

Next, we wanted to investigate the biological relevance of SAMDC1 to BSCTV infection. A T-DNA insertion null mutant of SAMDC1, Salk_020185, which was first identified as samdc1-1 (Ge et al., 2006), was obtained from the ABRC. Meanwhile, transgenic plants overexpressing SAMDC1 were generated by fusing the SAMDC1 ORF with 6×myc tag downstream of the cauliflower mosaic virus (CaMV) 35S promoter. Three different lines, including 4-week-old samdc1-1, 35S-SAMDC1-myc, and Columbia wild-type plants were agroinoculated with BSCTV. Repeated experiments showed that the final infection rate of samdc1-1 was only 75%, while almost 100% of the 35S-SAMDC1-myc transgenic plants inoculated with BSCTV were infected and exhibited similar phenotypes with wild-type plants, suggesting that the samdc1-1 plants are less susceptible to BSCTV infection than the wild-type plants (Figure 5E). DNA gel blotting was also used to detect the accumulation of BSCTV DNA in infected plants at 3 weeks after inoculation. The viral DNA accumulation in inoculated samdc1-1 was much less than that in wild-type plants, while the inoculated 35S-SAMDC1-myc transgenic plants accumulated viral DNA levels comparable to that of the inoculated wild-type plants (Figure 5F). Therefore, less susceptibility to BSCTV infection also correlated with less viral DNA accumulation in samdc1-1 transgenic plants, suggesting that loss of SAMDC1 function was responsible for these observations. Therefore, loss of the C2-interacting SAMDC1 has a similar

Figure 3. The FWA Transgenic Assay in the BSCTV C2 Transgene Background.

(A) Bolting phenotype of FWA transgenic plants in the wild-type (FW-2) or BSCTV C2 transgene (FC14-3, FC14-6, and FC18-2) background while growing in 12-h-daylight/12-h-dark cycles. Bar = 2 cm.

(B) The histogram represents bolting days in 12-h-daylight/12-h-dark cycle growth conditions. A total of 35, 29, 38, and 28 independent transformants for FW-2, FC14-3, FC14-6, and FC18-2, respectively, were analyzed. Error bars represent ±d. Asterisks indicate P < 0.01.

(C) The histogram represents number of rosette leaves while bolting in 12-h-daylight/12-h-dark cycles. A total of 35, 29, 38, and 28 independent transformants for FW-2, FC14-3, FC14-6, and FC18-2, respectively, were analyzed. Error bars represent ±d. Asterisks indicate P < 0.01.

(D) A DNA gel blot of CfoI-digested genomic DNAs probed with a 1.74-kb fragment corresponding to the DNA shown as solid line in the diagram at the top. The arrows indicate the CfoI digestion sites in the FWA genomic fragment. The positions and sizes in (kilobases) of the methylated and unmethylated bands are shown in the blot below the diagram. DNAs from T2 plants of three independent transformants with the BSCTV C2 transgene background and of one transformant with the Columbia wild-type background are shown. The probe fragment was completely digested by CfoI and loaded onto the first lane as a marker.
Figure 4. Analysis of DNA Methylation of Wild-Type and c2− Mutant BSCTV Genomes.

The histogram shows the percentage of total cytosine sites methylated in different sequence contexts in the intergenic region of BSCTV wild-type and c2− mutant DNA isolated from infected *Arabidopsis* plants. N represents any nucleotide, and H represents A, T, or C.

...effect on virus infectivity and viral DNA accumulation as with C2 deficiency, suggesting that the C2 and SAMDC1 interaction may play a key role in regulation of host de novo methylation during BSCTV infection.

To further investigate the relationship between BSCTV C2 and SAMDC1 during the viral infection, we also inoculated the transgenic plants of 35S-SAMDC1-myc with wild-type BSCTV, c2− mutant BSCTV, and empty vector. The inoculated plants at 14 DAI were sampled for immunoblot with anti-myc antibody. Figure 5G shows that SAMDC1 protein level in the wild-type virus-infected plants are higher than both the one in the c2− mutant virus-infected plants and the one in the mock-infected plants. This result indicates that a native BSCTV C2 protein was essential for SAMDC1 protein accumulation during viral accumulation, supporting that BSCTV C2 may attenuate the degradation of SAMDC1 protein.

Both BSCTV C2 and SAMDC1 Proteins Possess Silencing Suppression Activity

Key cellular and viral factors affecting host methylation have been shown to regulate host gene silencing processes (Buchmann et al., 2009). Here, we asked whether BSCTV C2 and its interacting protein SAMDC1 could also affect, that is, suppress, the gene silencing mechanism. First, the suppression activity of BSCTV C2 and SAMDC1 on host gene silencing was tested in a two-component infiltration system using *N. benthamiana* line 16c plants, which constitutively express the GFP transgene (Ruiz et al., 1998). Leaves were co-infiltrated with the A. *tumefaciens* culture harboring 35S-GFP (the silencing trigger) and 35S-C2 or 35S-SAMDC1. The construct expressing the p19 protein, an efficient silencing suppressor from the *Cymbidium ringspot virus*, was used as a positive control, while an empty vector and 35S-GUS (for β-glucuronidase) were used as negative controls. At 4 DAI, the induction of GFP silencing was observed under red fluorescence in the areas co-infected with 35S-GFP and empty vector or 35S-GUS (Figure 6A). However, the zones co-infected with 35S-GFP and 35S-C2 maintained GFP fluorescence (Figure 6A), suggesting that BSCTV C2 can suppress the silencing activity. Leaves co-infected with 35S-GFP and 35S-SAMDC1 also showed GFP signals, which suggested that SAMDC1 also possesses silencing suppression activity. However, both SAMDC1 and BSCTV C2 displayed weaker silencing suppression activities compared with the strong gene silencing activity of suppressor p19 (Figure 6A). Additionally, the silencing suppression activity was also tested using the three-component transient system (Johansen and Carrington, 2001). The wild-type *N. benthamiana* leaves were co-infiltred with *A. tumefaciens* culture harboring 35S-GFP, 35S-dsGFP, and a test or control. Total RNA were extracted from these co-infiltred leaves 4 DAI and analyzed by RNA gel blotting. The results showed that both BSCTV C2 and SAMDC1 could suppress the dsGFP-directed GFP silencing in the infiltrated leaves, as judged by the greater accumulation of GFP mRNA than that in the negative control, which was consistent with the observations in the two-component system (Figure 6B). These results indicate that both BSCTV C2 and SAMDC1 possess silencing suppression activities.

In other words, overexpression of SAMDC1 could at least partially mimic the silencing suppression function of BSCTV C2, which supports the hypothesis that BSCTV C2 positively regulates SAMDC1 to suppress DNA methylation-mediated gene silencing.

**DISCUSSION**

The replication and transmission of viruses are dependent on host mechanisms. A number of studies have demonstrated that viral proteins can regulate host mechanisms at different levels, such as host DNA replication, gene transcription, and host protein modification and stability, in order to facilitate the viral presence and replication in the host (Xie et al., 1995; Fontes et al., 2004; Trinks et al., 2005; Baumberger et al., 2007). Plant viruses usually encode suppressors that interfere with the host defense machinery directly by inhibiting the function of the host defensive components and/or indirectly by exploiting the host cellular components and finally facilitating viral replication (Wang et al., 2003; Lakatos et al., 2004, 2006; Mérai et al., 2006; Zhang et al., 2006; Baumberger et al., 2007; Bortolamiol et al., 2007). However, the mechanisms underlying the interaction between viral suppressors and plant host cellular components still remain poorly understood, especially for plant DNA viruses. In a yeast two-hybrid screening, we successfully identified a BSCTV C2-interacting cellular protein, SAMDC1. Our biochemical and genetic data suggest that BSCTV C2 can attenuate the degradation of SAMDC1 to suppress host DNA methylation-mediated gene silencing, further facilitating viral replication.

**BSCTV C2 Positively Regulates SAMDC1**

To counteract the plant host antiviral gene silencing defense, small interfering RNA sequestration is the most common strategy...
for plant virus-encoded silencing suppressors (Lakatos et al., 2004, 2006; Mérai et al., 2006). Up to now, only a few viral suppressors have been reported to suppress gene silencing through protein–protein interactions (Wang et al., 2003; Zhang et al., 2006; Baumberger et al., 2007; Bortolamiol et al., 2007). Cucumber mosaic virus–encoded silencing suppressor 2b inhibits RNA-induced silencing complex activity through direct interaction with the PAZ domain of the ARGOANAUTE1 (AGO1) protein (Zhang et al., 2006). Geminivirus-encoded AL2 and L2 suppress gene silencing by inactivation of ADK2 (Wang et al., 2003; Raja et al., 2008; Buchmann et al., 2009). Meanwhile, polerovirus encodes the F-box–like silencing suppressor P0, which has been suggested to interact with Arabidopsis ASK1 and directly target AGO1 for 26S proteasome-independent degradation (Pazhouhandeh et al., 2006; Baumberger et al., 2007; Bortolamiol et al., 2007; Csorba et al., 2010). Here, we identified a protein–protein interaction between a viral suppressor and plant cellular component, SAMDC1, which is involved in catalyzing the decarboxylation from SAM to dcsSAM. Research in both humans and animals indicated that SAMDC is a fast turnover protein degraded by the ubiquitination/26S proteasome pathway (Shirahata and Pegg, 1985; Kramer et al., 1988; Stjernborg and...
may lead to changes in SAM and dcSAM contents and the ratio in cells. Overexpression of SAMDC in *Xenopus laevis* early embryos resulted in abnormal development at the early gastrula stage and was associated with a dramatic decrease in both the putrescine/spermidine ratio and SAM levels (Shibata et al., 1998). However, it was not the restoration of the putrescine/spermidine ratio but that of the SAM levels that could rescue the abnormal development, indicating that overexpression of SAMDC led to a SAM-deficient state in vivo (Shibata et al., 1998). On the other hand, both overexpression of SAMDC in Chinese hamster ovary cells and *Arabidopsis* resulted in a dramatic increase in dcSAM content, while the SAM content remained relatively unaffected (Kramer et al., 1995; Hanfrey et al., 2002). However, it is not disputed that positive regulation of SAMDC may lead to an increase of dcSAM/SAM ratios. Previous studies indicate that BCTV L2 interacts with and inactivates ADK2, and this inactivation may interfere with the metabolism of methyl donor SAM and alter the SAH/SAM ratio, leading to inhibition of host cellular transmethylation (Hao et al., 2003; Cui et al., 2005; Yang et al., 2007; Raja et al., 2008; Buchmann et al., 2009). Considering that the cellular SAH/SAM ratio has a great impact on the activity of cellular methyltransferases and on the genomic DNA methylation level (Chiari et al., 1996; Tanaka et al., 1997), it is conceivable that cellular regulation of the SAMDC-to-dcSAM/SAM ratio also may be essential for the regulation of cellular DNA methylation. Alternatively, the product of SAMDC, dcSAM, with a structure similar to SAM, does not act as a methyl donor but instead acts as a competitive inhibitor of DNA methyltransferase when present at a high level (Heby et al., 1988). In the study of F9 teratocarcinoma cells, the inhibition of ornithine decarboxylase, another key enzyme of polyamine biosynthesis, often led to a feedback positive regulation of the SAMDC enzyme activity, which was associated with a dramatic increase of dcSAM levels and decrease of cytosine methylation in the genome (Frostesjö et al., 1997). All this evidence suggests that SAMDC plays an important role in the regulation of cellular methylation processes. Combined with the observation that SAMDC1 had a similar effect on gene silencing as that of BSCTV C2, these results suggest that SAMDC1 may be also involved in DNA methylation-mediated gene silencing.

**De Novo Methylation-Mediated Gene Silencing Targets the Viral Genome to Suppress Viral Replication**

De novo methylation usually occurs as a defense against foreign DNA in organisms with well-established methylation mechanisms. Foreign DNA, such as adenovirus, plastids, and phage, become the target of de novo methylation when integrated into the mammalian genome (Doerfler, 2008). Similarly, plant viruses and transgenes also become methylated de novo (Morel et al., 2000; Vaucheret and Fagard, 2001). Geminiviruses, like typical DNA viruses that display a double-strand viral genomic DNA during some point in their life cycle (Jeske et al., 2001; Alberter et al., 2005), are challenged by the host DNA methylation-mediated gene silencing defense and must develop strategies to counter it (Seemannpillai et al., 2003; Raja et al., 2008; Buchmann et al., 2009). Bisulfite sequencing of the IR of wild-type and c2⁻ mutant BSCTV genomes in infected plants in our

**SAMDC, dcSAM/SAM, and DNA Methylation**

As SAMDC functions in catalyzing the conversion of SAM to dcSAM, it is not surprising that positive regulation of SAMDC
study showed that C2 deficiency caused the enhanced DNA methylation of viral genomes in virus-infected plants, including CG-, CNG-, and CHH-type methylation, suggesting that BSCTV C2 can suppress methylation-mediated antiviral defense against viral genome in the host plant. Meanwhile, plants with the FWA transgene in the BSCTV C2 transgenic background were found to be insensitive to host de novo methylation, suggesting that BSCTV C2 effects were not restricted to the viral genome and interfered with the host cellular de novo DNA methylation process. Recently, several studies also strongly supported that the methylation-related TGS is a very important defense mechanism against geminiviruses in plant hosts (Raja et al., 2008; Rodríguez-Negrete et al., 2009), and BCTV L2 can suppress the TGS defense and cause the reduction of methylation in both the viral and plant host genomes (Raja et al., 2008; Buchmann et al., 2009). Clearly, during invasion of the plant host by geminiviruses, viral suppressors such as BSCTV C2 can perturb the de novo methylation machinery by different mechanisms, resulting in a hypomethylation of the host cell and facilitation of viral replication. Similar functions were also observed with other microbes. *Tobacco mosaic virus* infection, for example, led to hypomethylation of some host genomic regions, such as the pathogen-responsive tobacco (*Nicotiana tabacum*) gene *Nt Alix1* (Wada et al., 2004). Several centromeric loci, including the 180-bp retrotransposon *Athila* and some of the nuclear insertions of mitochondrial DNA, were shown to be hypomethylated in *Arabidopsis* infected by *Pseudomonas syringae*. All these examples showed that microbes develop different mechanisms to counteract the plant host de novo methylation-mediated defense during their coevolution.

Host de novo methylation-related defenses often lead to the epigenetic modification of the geminivirus genomes and repression of viral gene expression, ultimately interfering with viral DNA accumulation and pathogenesis. The integrated adenovirus type 12 (Ad12) genome was shown to be de novo methylated in hamster cells, and early Ad12 genes were only transcribed to a limited level, while late Ad12 genes were almost completely silenced (Doerfler, 2008). Upon infection by the *Tomato leaf curl virus* (TLCV), the TLCV C4 transgenic plants lost the phenotype conferred by overexpression of the transgene and the C4 transcript disappeared, and this disappearance was associated with the hypermethylation of both the 3SS-C4 and C4 loci in intact TLCV (Bian et al., 2006). Therefore, it is reasonable that BSCTV replication decreased and that pathogenesis was attenuated after C2 was depleted and its ability to counteract the host methylation-related defense was abolished. Based on the known abilities of SAMDC1 and BSCTV C2 to suppress host gene silencing activity, it is highly likely that BSCTV C2 may interfere with gene silencing-mediated antiviral defense in plant hosts by attenuating the degradation of SAMDC1. Based on our work and the results by others, we propose a working model (Figure 7).

Figure 7. Proposed Model for C2 and SAMDC1 Interaction in the Regulation of Transmethylation-Mediated Gene Silencing and Geminivirus Infection.

The machinery of host DNA methylation may modify the geminivirus genome and inhibit viral DNA accumulation. This model proposes that BSCTV C2 attenuates the 26S proteasome-mediated degradation of SAMDC1 to positively regulate cellular SAMDC activity, thereby leading to an altered SAM/dcSAM ratio. The change in the SAM/dcSAM ratio further inhibits the DNA methylation of geminivirus DNA and promotes viral replication. This process is associated with DNA hypomethylation and the release of gene silencing in some loci of the host genome.

In conclusion, our research suggests that BSCTV C2 attenuates the degradation of SAMDC1 to establish a hypomethylated environment to facilitate viral accumulation, providing us new insight into the mechanisms developed by viruses to counteract with the plant antiviral system. Additionally, it is notable that SAM not only is the methyl donor in the transmethylation reactions, but also is a substrate for polyamine biosynthesis, ethylene biosynthesis, and transsulfuration. SAMDC may also be subjected to regulation by other pathways. Previous data showed that the infection of *Herpes simplex virus type 1* (HSV-1), a DNA virus, suppressed synthesis of most host proteins but induced SAMDC mRNA and protein synthesis, perhaps because spermidine and spermine, two of the final products of polyamine metabolism, are components of the HSV-1 virion (Greco et al., 2005). Inhibition of SAMDC was further shown to prevent HSV-1 infection (Greco et al., 2005). Therefore, we cannot exclude the possibility that the interaction between BSCTV C2 and SAMDC1 may have other effects. Considering that SAMDC functions as a key regulator for SAM–related pathways and it is conserved in diverse organisms, the mechanism for its regulation during virus infection is worthy of further investigation.

METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* ecotype Columbia was used for this study. Seeds were surface sterilized with 10% bleach and washed three times with sterile water. Sterile seeds were suspended in 0.15% agarose and plated on Murashige and Skoog (Duchefa Biochemie) medium plus 1.5% sucrose. Plates were stratified in darkness for 2 to 4 d at 4°C and then
transferred to a tissue culture room at 22°C under a 16-h-light/8-h-dark photoperiod. After 2 to 3 weeks, seedlings were potted in soil and placed in a growth chamber at 22°C and 70% relative humidity under a 16-h-light/8-h-dark photoperiod.

**Transformation Vectors and Construction of Transgenic Plants**

To produce 35S-C2 plants, a 522-bp fragment containing the BSCVT C2 full-length ORF (GenBank accession no. U02311.1, position 1550 to 2071) was cloned into the vector pCambia1300-221 (Liu et al., 2010) in which transgene expression is under the control of the CaMV 35S promoter. Transformation of Arabidopsis was performed by the vacuum infiltration method (Bechtold and Pelletier, 1998) using Agrobacterium tumefaciens strain EHA105.

To produce 35S-SAMDC1-myc plants, an 1101-bp fragment containing Arabidopsis SAMDC1 full-length ORF was PCR cloned and inserted into the vector pVIPmyc (Xie et al., 2002) in which transgene expression is under the control of the CaMV 35S promoter (SAMDC1-Forward, 5’-GGGCGGCCGCGCTTGGGTCTTCGATGG-3’; SAMDC1-Reverse, 5’-GGGCGTGCCCGCTTGCCTGCTCTCGTCTC-3’, primers contain the Apal restriction site, which is underlined). A 6× myc tag was fused to the C terminus of SAMDC1 lacking a stop codon. Transformation of Arabidopsis was performed by the vacuum infiltration method (Bechtold and Pelletier, 1998), but using the A. tumefaciens strain ABI.

**Yeast Two-Hybrid Screen**

Two-hybrid screens and experiments to assess the interaction of BSCVT C2 and defined regions of SAMDC1 were performed using the system described previously (Xie et al., 1999). Bait constructs were expressed as GAL4 DNA binding domain fusion proteins from the yeast expression plasmid pGBKT7 (Clontech). Prey constructs were expressed as GAL4 activation domain fusion proteins from pGADT7 (Clontech). Initial screens were performed with BSCVT C2 as the bait and an Arabidopsis cDNA library constructed by our lab. Positive interaction was indicated by the ability of HY7C cells cotransformed with bait and prey constructs to grow on SD medium minus His and containing 5 mM 3-aminoazonole. SD medium minus Leu and Trp ensure the maintenance of both expression plasmids. The interactions were confirmed by assessing β-galactosidase activity using a filter-lift assay (Breeden and Nasmyth, 1988).

The construct containing full-length BSCVT C2 was generated by PCR using pCambia1300-BSCVT (1.8 copy) (Chen et al., 2010). Constructs containing defined regions of SAMDC1 were generated by different restriction endonuclease digestions of full-length SAMDC1 cDNA: truncation from amino acids 1 to 83 was produced by KpnI; truncation from amino acids 182 to 292 was produced by EcoRI and XhoI; truncation from amino acids 292 to 366 was produced by XhoI; and truncation from amino acids 226 to 366 was produced by EcoRI. All truncations were cloned into the yeast expression plasmid pGADT7 (Clontech) in the correct frame fused to downstream of the GAL4 activation domain.

**In Vitro Pull-Down Assay**

35S-Met-labeled BSCVT C2 was generated by in vitro transcription and translation with the use of a T7/SP6-coupled Tnt kit (Promega). ORFs of SAMDC1 truncated proteins were cloned into the pGEX-6P-1 vector (Amersham Biosciences) and expressed in *Escherichia coli*. Expression of GST fusion proteins and in vitro binding experiments were performed as described by Xie et al. (1999).

**LCI Assay**

LCI assays were performed as described by Chen et al. (2008). C2 and SAMDC1 were amplified by PCR using primers (both C2LUC-UP, 5’-CGGATCCATGAAACAACCGTGTCATT-3’, and C2LUC-DP, 5’-CAG-GTCGACTCCAGATATCCTCTAGTCTC-3’, for C2; StLuc-UP, 5’-CGG-GATCCAGCGTTCTTTGTCTACCTC-3’, and StLuc-DP, 5’-CAGGTCT-GACCTAAGTTCCCTCTGCTTTCTC-3’, for SAMDC1; primers contained BamHI or Sall, which are underlined) and inserted into pCAMBIA-NLuc and pCAMBIA-CLuc (Chen et al., 2008) following the digestion with BamHI and Sall. All the constructs were transformed into A. tumefaciens strain EHA105. An equal volume of A. tumefaciens harboring pCAMBIA-NLuc and pCAMBIA-CLuc (or their derivative constructs) was mixed to a final concentration of OD600 = 1.5. Four different combinations of A. tumefaciens were infiltrated into four different positions at the same leaves of Nicotiana benthamiana. Plants were placed in 23°C and allowed to recover for 60 h. A low-light cooled CCD imaging apparatus (NightOWL II LB983 with indiGO software) was used to capture the LUC image.

**Immunoblotting**

Immunoblotting was performed as described previously (Liu et al., 2010) with primary anti-c-myc (9E10; Santa Cruz Biotechnology) and anti-RFP antibodies (AbM59004-25-PU; Beijing Protein Innovation), followed by secondary goat anti-mouse antibody conjugated to horseradish peroxidase and visualized using chemiluminescence as instructed by the manufacturer (ECL; Amersham Pharmacia).

**Protein Degradation Assay in Wheat Germ Extracts**

Fresh wheat germ extract (6.5 μL) was added to 15 μL of the protein degradation system (40 mM Tris-HCl, pH 7.4, 5 mM ATP, 5 mM MgCl2, 2 mM DTT, 0.2 μg/μL His-Ubiquitin, 1 mM PMSF, 10 mM creatine phosphate, 10 μg/μL creatine phosphate kinase, and 1× protease inhibitor cocktail from Roche). For the protein degradation assay with or without the proteasome inhibitor MG132 (Sigma-Aldrich), MG132 (dissolved in DMSO or DMSO was added to the test system or control system, respectively (final concentration 50 μM). For the protein degradation assay with C2 or luciferase, 3 μL wheat germ extract containing C2 or luciferase protein translated and labeled with 35S-Met by the TNT Coupled Wheat Germ Extract System was added to the test system or to the control system, respectively. Then, 2 μL wheat germ extract containing SAMDC1-myc translated by the TNT Coupled Wheat Germ Extract System was added to each of the systems, and the final volumes were adjusted to 15 μL. All the steps for adding components to the mixtures were conducted on ice (4°C), and then all of the degradation systems were incubated at 30°C for up to 2 h. The samples were collected at 0, 0.5, 1, and 2 h and analyzed by immunoblot assays described above.

**Cell-Free Protein Degradation Assay in Arabidopsis Total Protein Extracts**

Fresh total protein extracts were extracted from 4-week-old wild-type Arabidopsis plants by rosette leaves in protein extraction buffer DE (40 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 5 mM ATP, 1 mM PMSF, and 1× protease inhibitor cocktail from Roche) at 4°C. For the assay without MG132, MG132 (dissolved in DMSO) or DMSO was added to the test system or control system, respectively (final concentration 50 μM). For the assay with C2 or luciferase, 3 μL wheat germ extract containing C2 or luciferase protein translated in vitro was added to the test system and control system, respectively. Then, 2 μL wheat germ extract containing SAMDC1-myc translated in vitro was added to the respective system to make the final volume of the system equal to 50 μL. All the steps were performed in an ice-water mixture (4°C). The degradation systems were incubated at 30°C for 3 h, and samples...
were collected at 0, 1, 2, and 3 h and subjected to standard immunoblot assay as described above. HYS fused with 6× myc tag was also translated by the TNT Coupled Wheat Germ Extract System and was used for a similar degradation assay. Degradation systems were incubated at 30°C for 2 h, and samples were collected at 0, 0.5, 1, and 2 h and subjected to standard immunoblot assay.

Quantification and Normalization for the Protein Degradation Assay

The results of the protein degradation assay were quantified by the software ImageJ (National Institutes of Health). The percentage of SAMDC1-myc remaining in the degradation assay using wheat germ extract and the percentage of myc-HYS in cell-free degradation assay were normalized by the following formula: \( P_t = \frac{C_t}{C_0} \times \frac{L_t}{L_0} \times 100\% \); \( P_t \) the percentage of the test protein remaining in each sampling time point; \( C_t \), the quantified content of the test protein in each sampling point; \( C_0 \), the quantified content of the test protein in 0 h; \( L_t \), the loading content in each sampling time point; \( L_0 \), the loading content in 0 h. Notably, considering that certain portions of SAMDC1-myc proenzyme were autocleaved to SAMDC1-myc subunit and β-subunit without the myc tag in a 26S proteasome-independent manner during the degradation process, the percentages of SAMDC1-myc remaining for SAMDC1-myc in the cell-free degradation assay were calculated differently by the following formula: \( P_{SAMDC1\text{-}myc} = \frac{C_{SAMDC1\text{-}myc}}{C_0} \times \frac{L_t}{L_0} \times 100\% \); \( P_{SAMDC1\text{-}myc\text{-}} \), the percentage of SAMDC1-myc remaining; \( C_{SAMDC1\text{-}myc} \), and \( C_{SAMDC1\text{-}myc\text{-}} \), the content of SAMDC1-myc and SAMDC1-myc, respectively, in each sampling time point; \( W_{SAMDC1\text{-}myc} \), the molecular weight of SAMDC1-myc, ~51 kD; \( W_{SAMDC1\text{-}myc\text{-}} \), the molecular weight of SAMDC1-myc, ~43 kD; \( C_0 \), the content of SAMDC1-myc at 0 h; \( L_0 \), the loading content in each sampling time point; \( L_0 \), the loading content at 0 h.

BSCTV Agroinoculation

Rosette leaves of 4-week-old plants were agroinoculated with BSCTV using an established A. tumefaciens spraying method described previously (Chen et al., 2010). The concentration of the A. tumefaciens strain EHA105 (Hood et al., 1993) carrying pCambia1300-BSCTV (1.8 copy) or the C2 mutant was at a dose of OD600 of 2.0.

DNA Gel Blotting

Total DNA was extracted using a CTA buffer as described (Chen et al., 2010). Genomic DNA was stained using ethidium bromide as a loading control. After depurination and neutralization, total DNA was transferred to Hybond N+ nylon membranes (Amersham Pharmacia Biotech) by upward capillary transfer in a 0.4 M NaOH solution. The membranes were hybridized at 65°C with the whole genome of BSCTV as a probe labeled with [α-32P]dCTP.

FWA Transformation Assay

As described by Cao and Jacobsen (2002) with some modifications, 6.1 kb of the FWA gene was inserted into the pCambia2300 vector and mobilized into the A. tumefaciens strain EHA105. Plants were transformed using vacuum infiltration, and transformed seedlings were selected on Murashige and Skoog plates containing 50 μg/mL kanamycin and 150 μg/mL carbenicillin. Individual T2 plants were transferred to soil and scored for flowering time in 12-h-daylight/12-h-dark cycles. Genomic DNA was extracted from whole plants of T2 plants and digested with methylation-sensitive restriction enzyme CfoI. DNA gel blots were probed with a 1.7-kb PCR-generated DNA fragment corresponding to positions 498 to 2281 (GenBank accession no. AF178668).

Bisulfite Sequencing

Two micrograms of genomic DNA isolated from the symptomatic plants infected by intact or C2 mutant BSCTV were digested overnight using BamHI and EcoRI. Sodium bisulfite treatment was performed as described (Jacobsen et al., 2000). Primers for the BSCTV intergenic region of 2866 to 275 (GenBank accession no. U02311.1) are as follows: forward, 5’-TTGGTTATTGGAATTTGAGGTTATGAAAGG-3’, and reverse, 5’-ACCGAATGAATCTTATTTAATTCTT-3’.

A. tumefaciens–Mediated Co-infiltration Assay

The A. tumefaciens–mediated co-infiltration assay in N. benthamiana plants or N. benthamiana line 16c, constitutively expressing the GFP gene, was based on the transient expression system described by Johansen and Carrington (2001). Equal volumes of A. tumefaciens cultures harboring Ti plasmids expressing GFP, double-stranded GFP RNA (dsGFP, silencing trigger) and a test, or a control were mixed and co-infiltrated into leaves of N. benthamiana. The optimal concentration was at OD600 of 1.0. Total RNA was isolated from infiltration zones and subjected to RNA gel blot hybridization. The Ti plasmids expressing GFP, dsGFP, and p19 (pCAMBIA-GFP, pCAMBIA-GFP, and pCAMBIA-p19) were kindly provided by Guo Huishan. The binary vector expressing GUS was pCAMBIA1300-221 from our lab (Liu et al., 2010). Then, the GUS gene was replaced by the C2 gene and the SAMDC1-myc fusion gene to generate pCAMBIA-C2 and pCAMBIA-SAMDC1-myc, respectively.

Similarly, equal volumes of A. tumefaciens cultures harboring Ti plasmids expressing double-stranded GFP RNA (dsGFP, silencing trigger) and a test or a control were mixed and co-infiltrated into leaves of N. benthamiana line 16c. Photographs of representative leaves were taken under UV light 4 DAI and uniformly processed using Adobe Photoshop. For the in vivo assay of SAMDC1-myc protein stability in N. benthamiana, A. tumefaciens cultures harboring Ti plasmids expressing SAMDC1-myc, GFP, and GFP-C2/GFP were mixed together (the final concentrations are OD600 of 0.6, 0.3, and 0.6, respectively) and co-infiltrated into leaves of N. benthamiana. Here, GFP was used as an internal control for SAMDC1-myc. Total protein and RNA were isolated from infiltration zones and subjected to immunoblot and RT-PCR, respectively. The details for the co-infiltration were described previously (Liu et al., 2010). The Ti plasmid expressing GFP (pGDR) was kindly provided by Wang Guoliang. The construct expressing HA-GFP and pCanG-HA-GFP was described previously (Liu et al., 2010). C2 was fused to the N terminus of GFP and replaced the HA-GFP and generated construct expressing GFP-C2.

RNA Gel Blotting

Total RNA was isolated using Trizol reagent (Tiangen). Total RNA was stained with methylene blue as a loading control. Total RNA was transferred to Hybond N+ nylon membranes (Amersham Pharmacia Biotech) by upward capillary transfer in a 10× SSC solution. The membranes were hybridized at 65°C as described (Chen et al., 2010) with the full-length GFP gene as a probe labeled with [α-32P]dCTP.

RT-PCR Amplification

To examine the expression of SAMDC1-myc mRNAs by RT-PCR, DNase I–treated total RNA was denatured and subjected to reverse transcription reaction using SuperScript II (200 units per reaction; Invitrogen) at 37°C for 1 h followed by heat inactivation of the reverse transcriptase at 70°C for 15 min. PCR amplification was performed using AIGSAMDC1 forward (SIRT-Fw, 5’-TCCATACCTGCTGTTCTTC-3’) and reverse (SIRT-Rev, 5’-CTGATCTGGGCTTCTTGCCTC-3’) primers for 25 cycles. Expression levels of GFP, which was co-infiltrated with SAMDC1-Myc, were
monitored with forward (DsR-UP, 5'-ATGGCCCTCCTCCGAAGAAGC-3') and reverse (DsR-DP, 5'-CAGGAAAACTGGTGGCCG-3') primers to serve as an internal control.

Accession Numbers

The Arabidopsis Genome Initiative locus mentioned in this article is as follows: SAMDC1 (At3G02470). A T-DNA insertion null mutant of SAMDC1, Salk_020185, which was initially identified as samdc1-1 (Ge et al., 2006), was obtained from the ABRC.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. RNA Gel Blotting and Phenotype of BCTV C2 Transgenic Plants.

Supplemental Figure 2. Construction of the c2'-Mutant of BCTV.

Supplemental Figure 3. Distribution of Methylated Cytosine Sites Detected by Bisulfite Sequencing Assay.

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BSCTV C2 Attenuates the Degradation of SAMDC1 to Suppress DNA Methylation-Mediated Gene Silencing in *Arabidopsis*
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