Effects of the Crinivirus Coat Protein–Interacting Plant Protein SAHH on Post-Transcriptional RNA Silencing and Its Suppression

M. Carmen Cañizares,¹ Rosa Lozano-Durán,² Tomás Canto,³ Eduardo R. Bejarano,² David M. Bisaro,⁴ Jesús Navas-Castillo,¹ and Enrique Moriones¹

¹Instituto de Hortofruticultura Subtropical y Mediterránea ‘La Mayora’, Universidad de Málaga-Consejo Superior de Investigaciones Científicas (IHSM-UMA-CSIC), Estación Experimental ‘La Mayora’, 29750 Algarrobo-Costa, Málaga, Spain;

²IHSM-UMA-CSIC, Departamento de Biología Celular, Genética y Fisiología, Universidad de Málaga, Campus Teatinos, 29071 Málaga, Spain; ³Centro de Investigaciones Biológicas, Departamento de Biología de Plantas, Ramiro de Maeztu 9, 28040 Madrid, Spain; ⁴Department of Molecular Genetics, Center for Applied Plant Sciences, and Center for RNA Biology, The Ohio State University, Columbus 43210, U.S.A.

Submitted 1 February 2013. Accepted 14 May 2013.

In plants, post-transcriptional gene silencing (PTGS) is a sequence-specific mechanism of RNA degradation induced by double-stranded RNA (dsRNA), which is processed into small interfering RNAs (siRNAs). siRNAs are methylated and, thereby, stabilized by the activity of the S-adenosyl-methionine-dependent RNA methyltransferase HEN1. PTGS is amplified by host-encoded RNA-dependent RNA polymerases (RDR), which generate dsRNA that is processed into secondary siRNAs. To counteract this RNA silencing-mediated response of the host, plant viruses express proteins with silencing suppression activity. Here, we report that the coat protein (CP) of crinivirus (family *Closteroviridae*, genus *Crinivirus*) *Tomato chlorosis virus*, a known suppressor of silencing, interacts with S-adenosyl-homocysteine hydrolase (SAHH), a plant protein essential for sustaining the methyl cycle and S-adenosylmethionine-dependent methyltransferase activity. Our results show that, by contributing to an increased accumulation of secondary siRNAs generated by the action of RDR6, SAHH enhances local RNA silencing. Although downregulation of SAHH prevents local silencing, it enhances the spread of systemic silencing. Our results also show that SAHH is important in the suppression of local RNA silencing not only by the crinivirus *Tomato chlorosis virus* CP but also by the multifunctional helper component-proteinase of the potyvirus *Potato virus Y*.

(siRNA) (Hamilton and Baulcombe 1999). The methylation of the 3′ terminal nucleotides of the siRNAs is carried out by HEN1, a class of S-adenosylmethionine-dependent RNA methyltransferase (Yang et al. 2006; Yu et al. 2005), and protects them from degradation and oligouridylation (Li et al. 2005). The subsequent incorporation of the siRNAs into an RNA-induced silencing complex leads to the sequence-specific degradation of target RNAs. The amplification of this process is directed by host-encoded RNA-dependent RNA polymerases (RDR), which generate dsRNA using primed or unprimed single-stranded RNA (ssRNA) (Voinnet 2008) that is processed by RNase III-type Dicer enzymes, producing transitive secondary siRNAs (Himber et al. 2003). The siRNAs generated are able to spread from cell to cell, presumably via plasmodesmata, and over long distances through the vasculature, leading to a systemic propagation of silencing (Brosnan and Voinnet 2011; Dunoyer et al. 2010; Molnar et al. 2010). In addition to the importance in developmental regulation and maintenance of genome integrity (Meins et al. 2005), RNA silencing is a potent defense mechanism against viruses (Ding and Voinnet 2007).

To counteract the RNA silencing-mediated defense response of the host and to thereby ensure successful infection, plant viruses express proteins with silencing-suppression activity (Voinnet 2005; Voinnet et al. 1999). The great diversity in sequence, structure, and mechanism of action of these proteins reinforces the importance of the studies directed to know their modes of action (Burguán and Havelda 2011; Díaz-Pendón and Ding 2008; Voinnet 2005). Although most silencing suppressors can act by binding small RNA duplexes or long dsRNA (Lakatos et al. 2006; Merai et al. 2006; Silhavy and Burguán 2004), some act by interacting with host proteins that are either important components of the silencing machinery (Giner et al. 2010; Glick et al. 2008; Pazhouhandeh et al. 2006; Zhang et al. 2006) or proposed regulators of the silencing pathway (Anandalakshmi et al. 2000; Endres et al. 2010).

Multiple-component RNA silencing-suppression strategies have been reported for *Tomato chlorosis virus* (ToCV; genus *Crinivirus*) (Cañizares et al. 2008; Kreuze et al. 2005; Lu et al. 2004). ToCV, which is a member of the complex plant virus family *Closteroviridae* (Dolja et al. 2006), has a large bipartite, single-stranded, positive-sense RNA genome (Lozano et al. 2006, 2007; Wintermantel et al. 2005; Wisler et al. 1998). Al-

Post-transcriptional RNA silencing is a sequence-specific process of RNA degradation in plants. This mechanism is generally induced by double-stranded RNA (dsRNA), which is processed by RNase III-type Dicer enzymes into small RNA species of 21 to 24 nucleotides, termed small interfering RNA

Current address for R. Lozano-Durán: The Sainsbury Laboratory, Norwich Research Park, Norwich NR4 7UH, U.K.

Corresponding author: E. Moriones; Telephone: (+34) 952548990; Fax: (+34) 952552677; E-mail: moriones@eelm.csic.es

*The e-Xtra logo stands for “electronic extra” and indicates that a supplementary figure is published online.

though RNA-1 encodes the suppressor protein p22, the suppressor function in RNA-2 is performed by the structural proteins CP (coat protein) and CPm (minor coat protein) (Cañizares et al. 2008).

In the present work with ToCV and its experimental host *Nicotiana benthamiana* Domin, we have identified a host protein, *S*-adenosylhomocysteine hydrolase (SAHH), that interacts with the viral CP. We have analyzed the effect of *SAHH* downregulation on both RNA silencing and the ability of CP to block silencing. Our results show that, although downregulation of *SAHH* can suppress local silencing, it can enhance systemic silencing. In addition, we show that the presence of SAHH is important for suppression of local silencing not only by the crinivirus CP but also by the multifunctional helper component-proteinase (HC-Pro) of the potyvirus (genus *Potyvirus*, family *Potyviridae*) *Potato virus Y* (PVY).

RESULTS

ToCV CP interacts with SAHH.

A yeast two-hybrid screen was carried out to identify cellular proteins that interact with ToCV CP suppressor protein. The assay used a cDNA library from *N. benthamiana* leaves as prey and the ToCV CP fused to the GAL4 DNA-binding domain (BD) as bait. Yeast cells harboring the bait plasmid were transformed with the *N. benthamiana* cDNA library and plated onto selection medium. Screening of approximately 7×10^5 transformants led to the identification and isolation of four independent cDNA clones that interacted with ToCV CP, which represented partial clones of two independent cDNAs. Cotransformation of CP and either of these interactors activated the reporter genes and enabled yeast transformants to grow on a histidine and adenine dropout medium. One of the clones was selected for further analysis. The selected interaction was specific because cells with the identified plant interactor-expressing construct, when cotransformed with the unrelated protein lamin C (Bartel et al. 1993), were unable to survive in the dropout medium (Fig. 1A).

Nucleotide sequence analysis showed that the identified CP interactor was closely related to the SAHH from *N. tabacum* L. SAHH hydrolyses *S*-adenosylhomocysteine, a product of the methyl donor *S*-adenosyl methionine (SAM), and, thus, plays a key role in sustaining the methylation cycle and *S*-adenosylmethionine-dependent methyltransferase activity in plants (Pereira et al. 2007; Weretilnyk et al. 2001).

The interaction between ToCV CP and SAHH was confirmed with an in vitro pull-down assay. For this, the complete open reading frame of SAHH from *N. benthamiana* (*NbSAHH*) was obtained by reverse-transcriptase polymerase chain reaction (RT-PCR) with primers specific to the SAHH nucleotide sequence from *N. tabacum* (GenBank accession number AY639864) and cloned. The *NbSAHH* gene nucleotide sequence was deduced from the full-length cDNA clone and deposited in GenBank under the accession number JQ890096. An *NbSAHH* protein of 485 amino acids with an N-terminal His-tag and a C-terminal S-tag (His-SAHH-S), and an N-terminal His-tagged ToCV CP (His-CP), were expressed in *Escherichia coli* and purified as described below (Fig. 1B, lanes 4 and 5). His-SAHH-S was captured on S-protein agarose and incubated with purified His-CP. After extensive washing, the bound proteins were analyzed by Western blotting with an anti-His antibody. His-CP efficiently bound to His-SAHH-S adsorbed to S-protein agarose, whereas His-CP did not bind to S-protein agarose in the absence of His-SAHH-S (Fig. 1B, compare lanes 1 and 3). These results confirmed that ToCV CP interacts with SAHH in vitro.

Specific interaction of *NbSAHH* with ToCV CP in two-hybrid screen and in vitro assays suggested a biological signifi-

cance. To further verify the interaction between *NbSAHH* and ToCV CP in planta, a bimolecular fluorescence complementation (BiFC) assay (Hu and Kerppola 2003) was employed. To perform this assay, *NbSAHH* was fused to the C-terminal yellow fluorescent protein (YFP) fragment (CYFP) and ToCV CP was fused to the N-terminal YFP fragment (NYFP). The PVY HC-Pro suppressor of silencing that we used as a positive control in our assays was also fused to the NYFP. Co-expression of specific pairs of proteins by agroinfiltration in *N. benthamiana* leaves showed reconstruction of YFP fluorescence with the combination of plasmid constructs expressing NYFP-CP or NYFP-HC-Pro plus CYFP-SAHH. No fluorescence was detected in co-infiltrations with NYFP plus CYFP or CYFP-SAHH and in co-infiltrations with NYFP-CP or NYFP-HC-Pro plus CYFP (Fig. 1C). Although the distribution of the cytoplasmic fluorescence observed varied, being uniform for the interaction between CP and SAHH proteins and with inclusions in the HC-Pro-SAHH interaction, these results demonstrated that ToCV CP and PVY HC-Pro interact with SAHH in planta.

The role of SAHH in local RNA silencing is connected to RDR6 activity.

Previous studies have shown that inhibiting the expression of methyl cycle enzymes such as adenosine kinase results in post-transcriptional gene silencing (PTGS) suppression (Wang et al. 2005). To confirm whether knock-down of *NbSAHH* also results in suppression of PTGS directed against GFP, we carried out a local GFP-silencing assay (Johansen and Carrington 2001; Voinnet et al. 2000). A construct designed to express *NbSAHH* dsRNA (35S-dsSAHH) was used to trigger silencing of *NbSAHH*. *N. benthamiana* leaves were examined after they were co-infiltrated with *Agrobacterium tumefaciens* cultures harboring 35S-GFP plus 35S-GF-IR (an inverted repeat GFP construct as a strong silencing inducer) with or without 35S-dsSAHH (the latter, empty vector control). At 6 days postinfiltration (dpi), tissues infiltrated with 35S-GFP + 35S-GF-IR + empty vector exhibited a low level of green fluorescence under UV light (Fig. 2A, upper panel, left side of left leaf) as a consequence of RNA silencing activation against the GFP reporter mRNA (Brigneti et al. 1998; Voinnet et al. 2000). Consistent with this reduced fluorescence, Northern blot analysis revealed that the steady-state levels of GFP mRNA were very low and were associated with increased levels of GFP-specific siRNAs (Fig. 2A, lane 1). In contrast, agroinfiltration of wild-type *N. benthamiana* plants with a mixture of 35S-GFP + 35S-GF-IR + 35S-dsSAHH resulted in suppression of silencing directed against GFP as described (Buchmann 2008; Raja et al. 2010). The strong green fluorescence observed correlated with high steady-state levels of GFP mRNA and a remarkable reduction of GFP siRNAs relative to the level of GFP siRNAs in leaves in which the expression of *SAHH* was not silenced (Fig. 2A, first leaf from the left in upper panel, and lane 2 versus lane 1). These results indicate that SAHH is required for local silencing.

We tested whether the GFP-specific siRNAs included transitive siRNAs dependent on RDR6 (Moissiard et al. 2007) by agroinfiltrating *N. benthamiana* RDR6i plants in which RDR6 is silenced (Schwach et al. 2005). At 6 dpi, RDR6i plants co-infiltrated with 35S-GFP + 35S-GF-IR + empty vector did not exhibit local RNA silencing (Fig. 2B, lane 1). In this latter case, the GFP fluorescence observed was consistent with the presence of high levels of GFP mRNAs and reduced accumulation of siRNAs. Therefore, the secondary siRNAs generated by RDR6 seemed to play an important role in local silencing of wild-type *N. benthamiana* plants. Taken together, these results indicate that, at 6 dpi, expression of *SAHH* is required for local silencing of GFP and that SAHH and RDR6 activities seem to be linked.

By 9 dpi, GFP fluorescence had disappeared, and reduced levels of GFP mRNA were detected in wild-type *N. benthamiana* leaves co-infiltrated with either the empty vector or 35S-dsSAHH (Fig. 2C, lanes 1 and 2, respectively). By this time,

These results indicate that, at 9 dpi, local downregulation of *SAHH* causes a drastic reduction of GFP siRNAs.

Downregulation of *SAHH* enhances the spread of systemic silencing.

We asked whether downregulation of *SAHH* interferes with cell-to-cell or long-distance spread of RNA silencing. To do this, we agroinfiltrated GFP-expressing transgenic *N. benthamiana* line 16c (Brigneti et al. 1998) with either 35S-GFP + 35S-IR-GFP + empty vector or 35S-GFP + 35S-IR-GFP + 35S-dsSAHH constructs. As was the case with nontransgenic *N. benthamiana* plants, local RNA silencing did not occur

when leaves were co-infiltrated with 35S-dsSAHH at 6 dpi (Fig. 3A). In contrast, no GFP fluorescence was observed in patches co-infiltrated with the empty vector, indicating that the infiltrated GFP and the endogenous *GFP* transgene had been locally silenced. Accumulation of GFP mRNA and siRNAs was consistent with the fluorescence assay results (Fig. 3B). We monitored whether downregulation of *SAHH*, which is linked to reduced accumulation of local GFP siRNAs, interfered with cell-to-cell spread of RNA silencing by observing *GFP* expression in the cells neighboring the agroinfiltrated patches. If the silencing signal exits from the agroinfiltrated area, the cell-to-cell movement should inhibit GFP expression,

Fig. 2. Effect of the downregulation of *S-adenosyl-homocysteine hydrolase* (*SAHH*) on local RNA silencing and suppression of silencing mediated by *Tomato chlorosis virus* (ToCV) coat protein (CP) and *Potato virus Y* helper component-proteinase (HC-Pro) protein. Green fluorescent protein (GFP) fluorescence in leaves from **A** and **C**, wild-type *Nicotiana benthamiana* plants (Nb) and **B**, RNA-dependent RNA polymerase 6i *N. benthamiana* plants agroinfiltrated to coexpress 35S GFP + 35S GF-IR with or without (+ or -) 35S dsSAHH alone or in combination with *Potato virus X* (PVX), PVX expressing ToCV CP (PVX-CP), or a construct expressing HC-Pro. Leaves were photographed under UV light at **A** and **B**, 6 and **C**, 9 days postinfiltration (dpi). Northern blot analyses are shown for GFP mRNA and small interfering RNA (siRNA) extracted from the zones infiltrated with *Agrobacterium tumefaciens* harboring the constructs indicated above. Ethidium bromide staining of rRNA and tRNA was used as the loading control for mRNA and siRNA, respectively. These results were reproduced in three independent assays.

which is indicated by a narrow red ring around the infiltrated patch (Himber et al. 2003). The narrow red ring observed at 6 dpi around patches in leaves co-infiltrated with 35S-dsSAHH (Fig. 3A) indicated that this was the case. Interestingly, although the patches co-infiltrated with 35S-dsSAHH still exhibited some fluorescence at 7 to 8 dpi, systemic RNA silencing of GFP expression was evident in upper noninfiltrated leaves. However, under the experimental conditions used, this rapid spread of the systemic silencing was never observed in plants co-infiltrated with the empty vector at 7 to 8 dpi. Moreover, at 15 dpi, the percentage of systemically silenced plants was consistently higher after infiltration with 35S-dsSAHH than with the empty vector (Table 1; Fig. 3C). Therefore, these results indicate that local downregulation of *SAHH* is linked to an enhanced spread of systemic silencing.

SAHH is required for suppression of gene silencing.

To determine whether SAHH plays a role in CP-mediated suppression of PTGS, we used the same local GFP-silencing assays described above. Like other suppressors of RNA silencing (Jing et al. 2011), ToCV CP efficiently suppresses local silencing in plant cells when expressed from the heterologous *Potato virus X* (PVX) vector (Cañizares et al. 2008). A plasmid (35S-HC-Pro) expressing the HC-Pro suppressor of silencing from PVY (Brigneti et al. 1998) was used as a positive control.

At 6 dpi, *N. benthamiana* leaves co-infiltrated with a mixture of 35S-GFP + 35S-GF-IR + PVX + empty vector exhibited low GFP fluorescence as a consequence of the RNA silencing of the reporter (Brigneti et al. 1998; Voinnet et al. 2000) (Fig. 2A, lane 3). However, when the dsSAHH construct was co-infiltrated, increased fluorescence was evident in the agroinfiltrated patches (Fig. 2A, lane 4). High levels of GFP fluorescence were also observed in co-infiltrations that included the ToCV CP or PVY HC-Pro suppressors and either the empty vector or the 35S-dsSAHH construct (Fig. 2A, lanes 5 to 6 and 7 to 8, respectively). Northern blot analysis showed that the GFP mRNA and siRNA levels correlated directly and inversely, respectively, with the intensity of green fluorescence. As expected, tissues infiltrated with PVX plus the empty vector contained a low level of GFP mRNA and high levels of GFP siRNAs (Fig. 2A, lane 3); GFP siRNAs, however, were barely detectable in co-infiltrations with 35S-dsSAHH (Fig. 2A, lane 4). At this time point (6 dpi) and in the presence of CP or HC-Pro suppressors (PVX-CP or 35S-HC-Pro), high levels of GFP mRNA and very low levels of GFP siRNAs were detected regardless of whether leaves were infiltrated with the empty vector or 35S-dsSAHH (Fig. 2A, lanes 5 to 6 and 7 to 8, respectively).

By 9 dpi, efficient suppression by CP and HC-Pro depended on whether or not 35S-dsSAHH had been co-infiltrated. Fluorescence was absent in patches co-infiltrated with PVX and

Fig. 3. Effect of downregulation of *S-adenosyl-homocysteine hydrolase* (SAHH) on the spread of systemic silencing of green fluorescent protein (GFP) in *Nicotiana benthamiana* line 16c plants. **A**, GFP transgenic *N. benthamiana* leaves at 6 days postinfiltration (dpi) with *Agrobacterium tumefaciens* harboring 35S GFP + 35S GF-IR with empty vector (vector) or with the construct 35S dsSAHH. Leaves were photographed under UV light. **B**, Northern blot analyses of GFP mRNA and small interfering RNA (siRNA) extracted from the zones infiltrated with *A. tumefaciens* harboring the constructs indicated above each lane at 6 dpi. Ethidium bromide staining of rRNA and tRNA was used as the loading control for mRNA and siRNA, respectively. **C**, GFP fluorescence of plants agroinfiltrated with the same mixtures. Plants were photographed under UV light at 15 dpi.

either the empty vector or 35S-dsSAHH (Fig. 2C, lanes 3 and 4, respectively). In the presence of suppressors (expressed from PVX-CP or from the construct 35S-HC-Pro), strong fluorescence was maintained in patches infiltrated with the empty vector, and the strong fluorescence was associated with high accumulation of GFP mRNA (Fig. 2C, lanes 5 and 7). In contrast, lower fluorescence and low accumulation of GFP mRNA were evident in patches in which 35S-dsSAHH was co-infiltrated (Fig. 2C, lanes 6 and 8). The analysis of GFP-specific siRNAs, however, showed a reduced accumulation in all cases in which suppressors were co-infiltrated, and levels of GFP mRNAs were unrelated to the levels of siRNAs. GFP siRNA levels were lower in patches co-infiltrated with PVX plus 35S-dsSAHH than with PVX plus the empty vector but the changes in GFP siRNA levels were not correlated with changes in GFP mRNA levels or fluorescence (Fig. 2C, lanes 3 and 4).

To confirm that the results obtained at 9 dpi with the 35S-dsSAHH construct were associated with reduced accumulation of *SAHH* transcripts, even in the presence of a suppressor of silencing, we performed semiquantitative RT-PCR with gene-specific primers. *SAHH* transcript levels were consistently decreased following 35S-dsSAHH co-agroinfiltration (Fig. 4). In all the cases, levels of cytochrome c oxidase (*CYT C*) and ribulose biphosphate carboxylase (*RBCL*) mRNAs were unaffected by 35S-dsSAHH co-agroinfiltration. *CYT C* and *RBCL*, in addition to serving as internal controls for RNA quality and RT-PCR amplifications, indicated that the reduced levels of GFP mRNA following co-agroinfiltrations with 35S-dsSAHH were not the result of cytotoxic effects.

Taken together, these results indicate that, although expression of *SAHH* has no impact on silencing suppressor activity at 6 dpi, it is required for suppression of silencing by both ToCV CP and PVY HC-Pro by 9 dpi.

Downregulation of *SAHH* does not affect mRNA cap methylation.

The downregulation of *SAHH* might reduce GFP siRNA levels (Fig. 2A, lane 2) because of a lack of methyl groups required for the siRNA stabilization. As noted earlier, siRNA stabilization is mediated by HEN1 and depends on the methyl cycle that requires SAHH activity (Yang et al. 2007; Yu et al. 2005). However, SAHH is also involved in mRNA cap methylation, a process that regulates mRNA stability and is essential for efficient gene expression (Cowling 2010; Fernández-Sánchez et al. 2009; Masuta et al. 1995). Therefore, we investigated whether downregulation of *SAHH* affects GFP mRNA capping and whether changes in mRNA capping explain some of the differences in accumulation of GFP mRNA. To answer these questions, we used an assay based on 5' rapid amplification of cDNA ends (RACE)-PCR, which was performed after an RNA adaptor was ligated to either untreated (Gazzani et al. 2004; Llave et al. 2002) or de-capped RNA samples. Only RNAs lacking a 5' cap have the phosphate group required for the ligation step. The same amount of total RNA extracted at 9 dpi from leaves co-infiltrated with the empty vector or 35S-dsSAHH was used to PCR amplify GFP mRNA either before or after 5' cap removal. In the case of samples that had not been enzymatically de-capped, no amplification occurred re-

Table 1. Percentage of *Nicotiana benthamiana* 16c plants with systemic green fluorescent protein (GFP) silencing at 15 days postinfiltration with *Agrobacterium tumefaciens* harboring 35S GFP + 35S GF-IR + empty vector (vector) or 35S GFP + 35S GF-IR + 35S double-stranded S-adenosylhomocysteine hydrolase (dsSAHH)

Infiltrated construct	Plants with GFP silencing (%)		
	Experiment 1 ^a	Experiment 2 ^b	Experiment 3 ^a
35S GFP + 35S GF-IR + vector	17.5	10	5
35S GFP + 35S GF-IR + 35 dsSAHH	43.5	40	25

^a In total, 40 plants were co-infiltrated for each of the different combinations indicated in the first column.

^b In total, 20 plants were co-infiltrated for each of the different combinations indicated in the first column.

Fig. 4. Semiquantitative reverse-transcriptase polymerase chain reaction analysis of *Nicotiana benthamiana* S-adenosyl-homocysteine hydrolase (*NbSAHH*) transcripts. RNAs extracted at 9 days postinfiltration from patches coexpressing 35S green fluorescent protein (GFP) + 35S GF-IR with 35S dsSAHH or the empty vector control alone or in combination with *Potato virus X* (PVX), PVX expressing a *Tomato chlorosis virus* coat protein (PVX-CP), or a construct expressing *Potato virus Y* helper component-proteinase (HC-Pro) protein were analyzed using appropriate primers. Aliquots were removed at the indicated cycles. Ribulose biphosphate carboxylase (*RBCL*) and cytochrome c oxidase (*CYT C*) transcript levels were used as internal controls. Results were independently reproduced two times.

gardless of whether the plants were agroinfiltrated with 35S-dsSAHH or with the empty vector (Fig. 5). Therefore, the apparent absence of uncapped GFP mRNA indicated that downregulation of *SAHH* did not affect the capping process under the conditions tested. Amplification occurred only when de-capped RNAs were used as the 5'-RACE PCR substrate, and levels of GFP mRNAs were greater when agroinfiltration included the construct expressing CP or HC-Pro. In the latter case, levels of GFP mRNA seemed similar whether or not *SAHH* was downregulated, probably because the PCR was close to saturation (Fig. 5). Therefore, we conclude that, although mRNA cap methylation requires *SAHH*, mRNA cap methylation was unaffected by the downregulation of *SAHH* under our experimental conditions.

Downregulation of *SAHH* does not affect accumulation of PVX or PVX-CP.

SAHH is involved in a number of *S*-adenosylmethionine-dependent transmethylation reactions, including the 5' capping of mRNA during animal virus replication (Wolfe and Borchardt 1991) and, possibly, during plant virus replication (Masuta et al. 1995). Therefore, we tested whether the downregulation of *SAHH* might have affected the accumulation of PVX or PVX-CP and thereby account for some of the differences in GFP mRNA accumulation observed above. The levels of PVX and PVX-CP were analyzed in *N. benthamiana* leaves co-infiltrated with either the empty vector or 35S-dsSAHH, in which the downregulation of *SAHH* had been confirmed by semiquantita-

tive RT-PCR. At 9 dpi, levels of PVX or PVX-CP genomic RNA in ethidium-bromide-stained gels were similar to the empty vector or 35S-dsSAHH co-infiltrations (Fig. 6, left panel). Northern blot analysis confirmed that the bands observed corresponded to the viral genomic RNA and that accumulation levels were similar whether or not *SAHH* was downregulated (Fig. 6, right panel).

These findings demonstrated that downregulation of *SAHH* did not affect the accumulation of PVX or PVX-CP under the experimental conditions. Also, the high quality of the total RNA extractions (Fig. 6, left panel) even when *SAHH* had been downregulated indicated that the downregulation in the analyzed patches was not associated with cytotoxic effects.

DISCUSSION

In the current study, we used a yeast two-hybrid assay with an *N. benthamiana* cDNA library to identify a protein, NbSAHH, that interacts with ToCV CP. We also determined that *SAHH* specifically interacts with ToCV CP in vitro and with ToCV CP and PVY HC-Pro suppressor in planta by BiFC. *SAHH* is a key enzyme in maintaining the methyl cycle that generates SAM as a methyl group donor (Lecoq et al. 2001; Moffatt et al. 2002; Weretilnyk et al. 2001). Because, as for PVY HC-Pro, ToCV CP has been reported to be a viral suppressor of gene silencing (Cañizares et al. 2008), we suspected that *SAHH* could be involved in gene silencing. In this context, it is interesting to note that *SAHH* encoded by the

Fig. 5. Rapid amplification of cDNA ends detection of cDNA corresponding to de-capped or untreated green fluorescent protein (GFP) mRNAs. RNA extracts were obtained at 9 days postinfiltration from patches coexpressing 35S GFP + 35S GF-IR with 35S ds *S*-adenosylhomocysteine hydrolase (*SAHH*) or the empty vector control (–) alone or in combination with *Potato virus X* (PVX), PVX expressing *Tomato chlorosis virus* coat protein (PVX-CP), or a construct expressing *Potato virus Y* helper component-proteinase (HC-Pro) protein. For the “mRNA de-capped” treatment, extracted RNA was digested with tobacco acid pyrophosphatase to remove the caps from all mRNAs in the population. Size standards are shown to the left.

Fig. 6. Accumulation of genomic RNAs of *Potato virus X* (PVX) and PVX expressing *Tomato chlorosis virus* coat protein (PVX-CP). RNA extracts were prepared at 9 days postinfiltration from *Nicotiana benthamiana* patches agroinfiltrated to coexpress 35S green fluorescent protein (GFP) + 35S GF-IR with or without (+ or –) 35S double-stranded *S*-adenosylhomocysteine hydrolase (dsSAHH) alone or in combination with PVX or PVX CP. Shown are the results of ethidium-bromide-stained RNA gel electrophoresis (left panel) and Northern blot analysis with a PVX probe (right panel) of total RNA extracts.

Arabidopsis thaliana *HOMOLOGY-DEPENDENT GENE SILENCING1* gene is required for DNA methylation-dependent transcriptional gene silencing (TGS) (Rocha et al. 2005). Methylation-mediated TGS is an important epigenetic defense mechanism against geminiviruses (Bisaro 2006; Raja et al. 2008, 2010), and the pathogenicity factor β CI1, which is encoded by the DNA β of the DNA monopartite geminivirus *Tomato yellow leaf curl China virus*, interacts with SAHH and inhibits its activity (Yang et al. 2011). Although we cannot rule out the possibility that the identified interaction affects TGS, in this report we examined the role of SAHH in PTGS.

In plants, siRNA duplexes generated by Dicer-processed dsRNA molecules are methylated by the RNA-modifying enzyme HEN1 that catalyzes SAM-dependent 2'-O-methylation of the 3'-terminal nucleotides, protecting them from degradation (Yang et al. 2007; Yu et al. 2005). The transfer of the methyl group from SAM generates S-adenosylhomocysteine (SAH), a strong inhibitor of the SAM-dependent methyltransferases, that is rapidly hydrolyzed by SAHH (Pereira et al. 2007). Therefore, SAM-dependent methyltransferases such as HEN1 depend on SAHH for a correct functioning of the process. We have demonstrated here that SAHH is essential for local RNA silencing. The decreased level of a transient GFP reporter mRNA at 6 dpi was linked to the presence of high levels of GFP siRNAs (Fig. 2A, lane 1) that were fully resistant to a β -elimination reaction (Supplementary Fig. S1) (Akbergenov et al. 2006), which suggested that they were all methylated. This result supported the importance of SAHH to maintain this type of methylation reaction that resulted in stabilization of the GFP siRNAs. In agreement with this, downregulation of *SAHH* expression resulted in high levels of GFP mRNAs and an associated decrease in the GFP siRNAs (Fig. 2A, lane 2) which could indicate that they were unstable. It is interesting to note that downregulation of SAHH would lead to the increase of the substrate, SAH, which has been shown to inhibit the SAM-dependent methyltransferase HEN1 (Horwich et al. 2007). As a consequence, the modification of siRNAs may be inhibited. The unstable siRNAs would then be subjected to rapid degradation via polyuridylation at their 3'-unmodified end (Li et al. 2005; Shen and Goodman 2004), thus impairing RNA silencing as observed (Fig. 2A, lane 2). Although it can be argued that downregulation of *SAHH* could also lead to a lack of suppression of SAHH, the results of semiquantitative RT-PCR clearly show that the downregulation of *SAHH* is maintained. It is possible that the expression of SAHH might cycle between these extremes (expression and suppression) over the time.

We have also shown that the accumulation of siRNAs is important during the initial steps of RNA silencing (Fig. 2A, first leaf from the left in the upper panel; compare lanes 1 and 2). This pool of siRNAs contains primary siRNAs and secondary siRNAs generated by the transitivity-silencing amplification process (Himber et al. 2003). The presence of secondary siRNAs is important for the modulation of local RNA silencing (Voinnet 2008), and our results suggest a possible link between SAHH and RDR6 activities. Secondary siRNA accumulation requires an amplification step in which dsRNAs are generated. Production of these dsRNAs is directed by host-encoded RDR (Chapman and Carrington 2007; Voinnet 2008) that convert ssRNA molecules into dsRNAs that are subsequently processed into secondary siRNAs by a Dicer nuclease. Thus, RDR catalyze both primer-dependent and primer-independent synthesis of complementary RNAs using ssRNA molecules as templates (Makeyev and Bamford 2002; Schiebel et al. 1993; Voinnet 2008). Among the different plant RDR, the roles of RDR1 and RDR6 in this process have been described in detail (Chapman and Carrington 2007; Qu 2010; Voinnet 2008). Because *N.*

benthamiana contains a natural RDR1 mutation (Yang et al. 2004), we determined whether the presence of secondary siRNAs is important for SAHH modulation of local RNA silencing by using *N. benthamiana* RDR6i plants in which RDR6 had been silenced (Schwach et al. 2005). Our results showed that the presence of RDR6 and, therefore, of secondary siRNAs is essential for SAHH to support local RNA silencing (Fig. 2A, lane 1 versus Fig. 2B, lane 1), indicating that SAHH might act after the action of RDR6 (i.e., on products generated by the action of RDR6). Thus, we conclude that SAHH is essential for the triggering of silencing in wild-type *N. benthamiana* plants and that its involvement in silencing is related to the presence of RDR6.

Although downregulation of *SAHH* resulted in substantial GFP fluorescence in *N. benthamiana* at 6 dpi, the fluorescence was greatly reduced at 9 dpi. By 9 dpi, GFP mRNA levels were essentially undetectable, although this was not correlated with high levels of GFP siRNAs. Our results show that, although downregulation of *SAHH* was linked to the presence of low levels of GFP siRNAs at 6 dpi, these were sufficient to trigger and amplify the silencing process. As has been previously suggested (Li et al. 2005), unmodified siRNAs may act as efficient primers, enabling RDR to amplify the silencing process. Accumulation of GFP siRNAs, however, might not be detected at 9 dpi because of the subsequent degradation of unprotected siRNAs caused by the downregulation of the methyl cycle enzyme SAHH. This inference is supported by the results of the agroinfiltration of *N. benthamiana* 16c plants, which showed that, although co-infiltration with 35S-dsSAHH suppressed silencing by 6 dpi, probably because of the low levels of GFP siRNAs (Fig. 3A and B), these low levels of siRNAs were very efficient at spreading silencing throughout the plant (Fig. 3C; Table 1). Because RDR6 is involved in this process by amplifying the secondary siRNAs that move from the original site of silencing via cell-to-cell and systemic transport (Himber et al. 2003; Qin et al. 2012; Voinnet 2005; Voinnet et al. 2000), the enhanced spreading observed when *SAHH* is downregulated could be explained by these low levels of unmodified siRNAs being used in a very efficient way as primers by this enzyme. That RDR6 is involved in this fast spreading of systemic silencing when *SAHH* is downregulated is clear because, by using *N. benthamiana* line 16c/RDR6i, in which NbRDR6 is silenced (Schwach et al. 2005), we have never seen systemic silencing (*data not shown*).

In addition to being essential for gene silencing, SAHH is also essential for the suppression of gene silencing. We showed that downregulation of *SAHH* affects the ability of the ToCV CP or PVY HC-Pro silencing suppressors to suppress GFP RNA silencing by 9 dpi. By 9 dpi, the level of *SAHH* downregulation did not seem to have affected important SAHH roles such as the mRNA capping process (Cowling 2010; Fernández-Sánchez et al. 2009; Masuta et al. 1995), which is essential for gene expression, or PVX accumulation, that could complicate the interpretation of the results. By using a BiFC assay, we found that both ToCV CP and PVY HC-Pro suppressors interact with SAHH in planta. This result could support the functional significance of the interaction identified for efficient suppression of silencing. Thus, the limitation to interact when *SAHH* is downregulated could preclude an efficient suppression of silencing activity. In addition to this, the efficient suppressor activity of CP and HC-Pro when SAHH is present might suggest that these suppressors may have their action over some products generated by the action of SAHH. Although further work is needed, it is interesting to note that the fact of finding that HC-Pro interacts in vivo with the plant factor SAHH is in agreement with previous results that showed that effective suppression of silencing by *Tobacco etch virus* HC-Pro seems

to require a plant cellular factor that increases the affinity of HC-Pro to siRNA duplexes (Lakatos et al. 2006).

MATERIALS AND METHODS

Plasmid constructs.

To obtain pGBKT7-CP, we first PCR amplified the *CP* gene from total RNA extracts of tomato (*Solanum lycopersicum* L.) plants infected with isolate PI-1-2 of ToCV (García-Cano et al. 2010); the primers were 5'-AGGAATTCATGGAGAACGATGCTGTTACAAACACTG-3' and 5'-CATACGGATCCCTTAGCACCAGTTATCGATGC-3'. The amplified *CP* gene was then cloned into the *EcoRI*-*Bam*HI sites of pGBKT7 (Clontech, Palo Alto, CA, U.S.A.). Expression of the CP-GAL4 BD from the bait plasmid was confirmed by Western blotting, as described by Castillo and associates (2003).

A similar approach was followed to obtain constructs for protein expression in *E. coli*. CP from ToCV-infected plants was amplified by RT-PCR with primers 5'-ACCGAATTCA TGGAGAACGATGCTGTTACA-3' and 5'-TAACTCGAGTT AGCAACCAGTTATCGATGC-3' and cloned into the *EcoRI*-*Xho*I sites of pET-28a(+) vector (Novagen, Madison, WI, U.S.A.) to obtain pET28.CP. For expression of the plant protein, the *NbSAHH* gene was amplified by RT-PCR with primers 5'-AATGTCGACATGGCTCTATTAGTCGAGAA-3' and 5'-ACCAGATCTGTACCTGTAGTGAGCAGGCT-3' from total RNA extracts of *N. benthamiana* plants; the amplified product was then cloned into the *Sal*I-*Bgl*II sites of the pETDuet-1 vector (Novagen) to obtain pETDuet.SAHH. The constructs pET28.CP and pETDuet.SAHH harbored the *CP* and *SAHH* genes fused to a sequence coding for a hexa-His tag; SAHH was also fused to an S-tag.

To generate pROK-based vectors for BiFC, the fragment corresponding to the *N. benthamiana* SAHH coding sequence was amplified by PCR from pETDuet.SAHH plasmid with appropriate oligonucleotides that added a *Bam*HI restriction enzyme site at its 5' end and a stop codon plus a *Sac*I site at its 3' end. The fragment was *Bam*HI/*Sac*I digested and cloned into binary construct pROK-CYFP (González et al. 2010) linearized with the same enzymes, creating construct pROK-CYFP-SAHH. In a similar way, the fragments of ToCV CP and PVY HC-Pro coding sequences were amplified from plasmids harboring full-length cDNAs, digested, and cloned into binary construct pROK-NYFP (González et al. 2010), creating constructs pROK-NYFP-CP and pROK-NYFP-HC-Pro, respectively.

The PVX and PVX derivative containing the ToCV CP constructs were described by Cañizares and associates (2008). The 35S-dsSAHH construct was described by Buchmann (2008).

Plasmids 35S-GFP (Voinnet and Baulcombe 1997), 35S-GF-IR (Bayne et al. 2005), 35S-HC-Pro (Hamilton et al. 2002), and pGR107 (Jones et al. 1999) were provided by D. C. Baulcombe. Each of the constructs was transformed by electroporation into *Agrobacterium tumefaciens* GV3101 containing the helper plasmid pJIC SA_Rep.

Yeast two-hybrid screen.

The *N. benthamiana* cDNA library was kindly provided by L. Jongejan (Leiden University, Leiden, The Netherlands). This library was constructed in the *EcoRI*-*Xho*I sites of ACTII as described by J. Memelink on the Technical Tips Online site (T01111) and contains 1.2×10^6 primary transformants. Automatic subcloning conversion of the cDNA library from lambda to plasmid vectors was done as described by Elledge and associates (1991).

The yeast two-hybrid screen and subsequent confirmation of interactions in yeast were performed as described by Castillo and associates (2004). The yeast strain PJ696, which contains

the reporter genes *lacZ*, *HIS3*, and *ADE2*, was used in two-hybrid assays (Fields and Song 1989). Yeasts were co-transformed and selected for bait and prey plasmids as described (Yeast Protocols Handbook, Clontech Laboratories, Inc. web-site).

Protein expression.

The expression constructs pET28.CP and pETDuet.SAHH were used to transform *E. coli* Rosetta (DE3)pLysS (Novagen). Expression of His-tagged/S-tagged proteins was induced from the transformed bacteria with 0.4 mM isopropyl- β -D-thiogalactopyranoside for 3 h at 28°C; the proteins were purified using Ni-NTA columns according to the manufacturer's recommendations (Qiagen). The purified recombinant proteins were analyzed by 15% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis after Coomassie brilliant blue staining.

In vitro pull-down assay.

S-protein agarose (Novagen) was equilibrated in binding buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.6% Triton X-100). The agarose was pelleted and washed with 1 ml of binding buffer. Purified proteins produced as described above were incubated for 1 h at room temperature in 25 mM Tris-HCl (pH 7.5), 25% glycerol, 0.05 mM dithiothreitol, and 125 mM NaCl (in rotation) and then added to the agarose. After incubation for 1 h at room temperature, the agarose was pelleted and the supernatant was removed. After the S-protein agarose was washed three times with 1 ml of binding buffer, 35 μ l of 0.2 M sodium citrate (pH 2) was added, and the preparation was incubated for 5 min before it was centrifuged. The supernatant was run on a 10% SDS gel, and proteins were subjected to immunoblot analysis by using an anti-His antibody.

Plant material, agroinfiltration, and GFP imaging.

Wild-type *N. benthamiana* plants, GFP-expressing transgenic *N. benthamiana* line 16c plants (Brigneti et al. 1998), and transgenic *N. benthamiana* RDR6i plants in which RDR6 is silenced (Schwach et al. 2005) were grown in an insect-free growth chamber at 25°C with a photoperiod of 16 h of light and 8 h of darkness. *N. benthamiana* line RDR6i and 16c were kindly provided by D. C. Baulcombe, The Sainsbury Laboratory, Norwich, U.K. Plants at the four- to six-leaf growth stage were agroinfiltrated with the *A. tumefaciens* GV3101 strain carrying the plasmids indicated above and as described by Voinnet and associates (1998) to coexpress 35S GFP plus 35S GF-IR with or without 35S dsSAHH (empty vector control) alone or in combination with PVX, PVX-CP, or the construct expressing HC-Pro. For co-infiltration, equal volumes of individual *A. tumefaciens* cultures (optical density = 1 at 600 nm) were mixed before infiltration. GFP fluorescence was observed with long-wavelength UV light (Black Ray model B 1000AP; UV Products, Upland, CA, U.S.A.) and photographed with a Coolpix 8700 Nikon digital camera.

In planta BiFC.

Epidermal cells in *N. benthamiana* tissue agroinfiltrated with *A. tumefaciens* cultures at optical densities of 0.2 harboring binary constructs were monitored for fluorescence derived from reconstituted YFP fluorophores of interacting tagged proteins at 4 dpi. Imaging was conducted with a Leica SP2 (Leica Microsystems GmbH, Heidelberg, Germany) confocal laser-scanning microscope using fresh, nontreated leaf tissue, and water immersion or water dipping objectives.

RNA analysis.

RNA was extracted from leaf tissue as described by Noris and associates (1996). For Northern blot analysis of GFP and

PVX mRNAs, total RNA aliquots (5 µg) from each sample were separated on 1% formaldehyde agarose gels, transferred to nylon membranes, and probed with digoxigenin-labeled probes specific for GFP or PVX, as described previously (Liu et al. 2004).

For Northern blot analysis of GFP siRNAs, low molecular weight RNAs were enriched from total RNAs by eliminating high molecular weight RNA using 5% polyethylene glycol (PEG 8000) plus 0.5 M NaCl; the low molecular weight RNAs were then separated on a 15% polyacrylamide 7 M urea gel, transferred to nylon membranes, and probed with a digoxigenin-labeled probe specific for GFP, as described previously (Cañizares et al. 2004).

Semi-quantitative RT-PCR.

Total RNA was isolated from agroinfiltrated patches as described above and further treated with DNase I to remove genomic DNA. RNA pellets were resuspended in water and quantified in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, U.S.A.). A 500-ng quantity of total RNA was reverse transcribed with oligo-dT primers using the AMV RT (Promega, Madison, WI, U.S.A.). The resulting single-stranded cDNA was used for PCR to detect *SAHH* transcripts by using the following primers: NbSAHH forward (5'-AACTTTGCAGGAGTAC TGGTGG-3') and NbSAHH reverse (5'-TGTTTCAAGGC AGCAGCACATC-3'). PCR with primers specific for the *CYT C* transcripts (5'-TATCTAGGCATGGTTTATGCC-3' and 5'-CTGCTAAGGGCATTCCATCC-3') and RBCL transcripts (5'-ATGTCACCACAAACAGAGACTAAAGC-3' and 5'-GTAAATCAAGTCCACRCG-3') were used as internal standards. Aliquots were withdrawn from the PCR reaction after 20, 25, 30, and 35 cycles and analyzed by agarose gel electrophoresis.

RNA ligase-mediated RACE assay.

RNA 5' ends were detected using the GeneRacer kit (Invitrogen Co., Carlsbad, CA, U.S.A.), with or without the initial de-capping reaction (Gazzani et al. 2004; Llave et al. 2002). After isolation of RNA, samples were de-capped or directly used. An RNA oligonucleotide (5'-GCACGAGGACACUGAC AUGGACUGAAGGAGUAGAA-3') was ligated to exposed 5' ends, and the reverse-transcription reaction was carried out using an oligo (dT) primer. In total, 200 ng of RNA was ligated to the RNA adaptor using 5 units of T4 RNA ligase at 37°C for 1 h; the protein was then removed from the solution by phenol/chloroform extraction and ethanol precipitation. The RNA thus obtained was used for cDNA synthesis with 15 units of AMV reverse-transcriptase enzyme, and the cDNA was subjected to PCR amplification using a *Taq* polymerase enzyme (Bioline, London). PCR was performed using a primer specific for the RNA oligonucleotide (5'-CACTGACATGGAC TGAAGGAGTAGAA-3') and a primer specific for GFP (5'-CAGCAGCTGTTACAACTCAAGAAGGAC-3'). The PCR products were separated on a 1% agarose gel.

ACKNOWLEDGMENTS

This work was supported, in part, by grants AGL2007-66062-C02 and AGL2010-22287-C02 (Ministerio de Ciencia, e Innovación, Spain, co-financed by FEDER). M. C. Cañizares was the recipient of an I3P contract from the Consejo Superior de Investigaciones Científicas, Spain (co-financed by Fondo Social Europeo, FSE), and R. Lozano-Durán was awarded an Introducción a la Investigación Fellowship from the Consejo Superior de Investigaciones Científicas, Spain. We thank D. C. Baulcombe and F. Tenllado for sharing materials, F. Aparicio for providing the pET vectors and helpful discussions, B. Jaffee for scientific English editing of the manuscript and M. V. Martín and R. Tovar for technical assistance.

LITERATURE CITED

- Akbergenov, R., Si-Ammour, A., Blevins, T., Amin, I., Kutter, C., Vanderschuren, H., Zhang, P., Gruissem, W., Meins, F., Jr., Hohn, T., and Pooggin, M. M. 2006. Molecular characterization of geminivirus-derived small RNAs in different plant species. *Nucleic Acids Res.* 34:462-471.
- Anandalakshmi, R., Marathe, R., Ge, X., Herr, J. M., Jr., Mau, C., Mallory, A., Pruss, G., Bowman, L., and Vance, V. B. 2000. A calmodulin-related protein that suppresses posttranscriptional gene silencing in plants. *Science* 290:142-144.
- Bartel, P., Chien, C. T., Sternglanz, R., and Fields, S. 1993. Elimination of false positives that arise in using the two-hybrid system. *BioTechniques* 14:920-924.
- Bayne, E. H., Rakitina, D. M., Morozov, S. Y., and Baulcombe, D. C. 2005. Cell-to-cell movement of Potato potexvirus X is dependent on suppression of RNA silencing. *Plant J.* 44:471-482.
- Bisaro, D. M. 2006. Silencing suppression by geminivirus proteins. *Virology* 344:158-168.
- Brigneti, G., Voinnet, O., Li, W. X., Ji, L. H., Ding, S. W., and Baulcombe, D. C. 1998. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:6739-6746.
- Brosnan, C. A., and Voinnet, O. 2011. Cell-to-cell and long-distance siRNA movement in plants: Mechanisms and biological implications. *Curr. Opin. Plant Biol.* 14:580-587.
- Buchmann, R. C. 2008. Reversal of RNA mediated gene silencing pathways by geminivirus AL2 and L2 proteins. Ph.D. thesis, The Ohio State University, Columbus, OH, U.S.A.
- Burguán, J., and Havelda, Z. 2011. Viral suppressors of RNA silencing. *Trends Plant. Sci.* 16:265-272.
- Castillo, A. G., Collinet, D., Deret, S., Kashoggi, A., and Bejarano, E. R. 2003. Dual interaction of plant PCNA with geminivirus replication accessory protein (Rep) and viral replication protein (Rep). *Virology* 312:381-394.
- Castillo, A. G., Kong, L. J., Hanley-Bowdoin, L., and Bejarano, E. R. 2004. Interaction between a geminivirus replication protein and the plant sumoylation system. *J. Virol.* 78:2758-2769.
- Cañizares, M. C., Taylor, K. M., and Lomonosoff, G. P. 2004. Surface exposed C-terminal amino acids of the small coat protein of *Cowpea mosaic virus* are required for suppression of silencing. *J. Gen. Virol.* 85:3431-3435.
- Cañizares, M. C., Navas-Castillo, J., and Moriones, E. 2008. Multiple suppressors of RNA silencing encoded by both genomic RNAs of the crinivirus, *Tomato chlorosis virus*. *Virology* 379:168-174.
- Chapman, E. J., and Carrington, J. C. 2007. Specialization and evolution of endogenous small RNA pathways. *Nat. Rev. Genet.* 8:884-896.
- Cowling, V. H. 2010. Regulation of mRNA cap methylation. *Biochem. J.* 425:295-302.
- Díaz-Pendón, J. A., and Ding, S. W. 2008. Direct and indirect roles of viral suppressors of RNA silencing in pathogenesis. *Annu. Rev. Phytopathol.* 46:303-326.
- Ding, S. W., and Voinnet, O. 2007. Antiviral immunity directed by small RNAs. *Cell* 130:413-426.
- Dolja, V. V., Kreuze, J. F., and Valkonen, J. P. T. 2006. Comparative and functional genomics of closteroviruses. *Virus Res.* 117:38-51.
- Dunoyer, P., Schott, G., Himber, C., Meyer, K., Takeda, A., Carrington, J. C., and Voinnet, O. 2010. Small RNA duplexes function as mobile silencing signals between plant cells. *Science* 328:912-916.
- Elledge, S. J., Mulligan, J. T., Ramer, S. W., Spottswood, M., and Davis, R. W. 1991. Lambda YES: A multifunctional cDNA expression vector for the isolation of genes by complementation of yeast and *Escherichia coli* mutations. *Proc. Natl. Acad. Sci. U.S.A.* 88:1731-1735.
- Endres, M. W., Gregory, B. D., Gao, Z., Foreman, A. W., Mlotshwa, S., Ge, X., Pruss, G. J., Ecker, J. R., Bowman, L. H., and Vance, V. 2010. Two plant viral suppressors of silencing require the ethylene-inducible host transcription factor RAV2 to block RNA silencing. *PLoS Pathog.* 6:e1000729.
- Fernández-Sánchez, M. E., Gonatopoulos-Pournatzis, T., Preston, G., Lawlor, M. A., and Cowling, V. H. 2009. S-adenosyl homocysteine hydrolase is required for Myc-induced mRNA Cap methylation, protein synthesis, and cell proliferation. *Mol. Cell Biol.* 29:6182-6191.
- Fields, S., and Song, O. 1989. A novel genetic system to detect protein-protein interactions. *Nature* 340:245-246.
- García-Cano, E., Navas-Castillo, J., Moriones, E., and Fernández-Muñoz, R. 2010. Resistance to *Tomato chlorosis virus* in wild tomato species that impair virus accumulation and disease symptom expression. *Phytopathology* 100:582-592.
- Gazzani, S., Lawrenson, T., Woodward, C., Headon, D., and Sablowski, R. 2004. A link between mRNA turnover and RNA interference in *Arabidopsis*. *Science* 306:1046-1048.

- Giner, A., Lakatos, L., García-Chapa, M., López-Moya J. J., and Burgyan, J. 2010. Viral protein inhibits RISC activity by argonaute binding through conserved WG/GW motifs. *PLoS Pathog.* 6:e1000996.
- Glick, E., Zrachya, A., Levy, Y., Mett, A., Gidoni, D., Belausov, E., Citovsky, V., and Gafni, Y. 2008. Interaction with host SGS3 is required for suppression of RNA silencing by *Tomato yellow leaf curl virus* V2 protein. *Proc. Natl. Acad. Sci. U.S.A.* 105:157-161.
- González, I., Martínez, L., Rakitina, D. V., Lewsey, M. G., Atencio, F. A., Llave, C., Kalinina, N.O., Carr, J. P., Palukaitis, P., and Canto, T. 2010. *Cucumber mosaic virus* 2b protein subcellular targets and interactions: Their significance to RNA silencing suppressor activity. *Mol. Plant-Microbe Interact.* 23:294-303.
- Hamilton, A. J., and Baulcombe, D. C. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 286:950-952.
- Hamilton, A. J., Voinnet, O., Chapell, L., and Baulcombe, D. C. 2002. Two classes of short interfering RNA in RNA silencing. *EMBO (Eur. Mol. Biol. Organ.) J.* 21:4671-4679.
- Himber, C., Dunoyer, P., Moissiard, G., Ritzenthaler, C., and Voinnet, O. 2003. Transitivity-dependent and independent cell-to-cell movement of RNA silencing. *EMBO (Eur. Mol. Biol. Organ.) J.* 22:4523-4533.
- Horwich, M. D., Li, C., Matrangola, C., Vagin, V., Farley, G., Wang, P., and Zamore, P. D. 2007. The *Drosophila* RNA methyltransferase, DmHen1, modifies germline piRNAs and single-stranded siRNAs in RISC. *Curr. Biol.* 17:1265-1272.
- Hu, C., and Kerppola, T. 2003. Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis. *Nat. Biotechnol.* 21:539-545.
- Jing, X., Fan, M., Jia, G., Liu, L., Ma, L., Zheng, C., Zhu, X., Liu, H., and Wang, X. 2011. A multifunctional protein encoded by Turkey herpesvirus suppresses RNA silencing in *Nicotiana benthamiana*. *J. Virol.* 85:12792-12803.
- Johansen, L. K., and Carrington, J. C. 2001. Silencing on the spot. Induction and suppression of RNA silencing in the *Agrobacterium*-mediated transient expression system. *Plant Physiol.* 126:930-938.
- Jones, L., Hamilton, A. J., Voinnet, O., Thomas, C. L., Maule, A. J., and Baulcombe, D. C. 1999. RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. *Plant Cell* 11:2291-2301.
- Kreuzer, J. F., Savenkov, E. I., Cuellar, W., Li, X., and Valkonen, J. P. T. 2005. Viral class I RNase III involved in suppression of RNA silencing. *J. Virol.* 79:7227-7238.
- Lakatos, L., Csorba, T., Pantaleo, V., Chapman, E. J., Carrington, J. C., Liu, Y. P., Dolja, V. V., Calvino, L. F., López-Moya, J. J., and Burgyan, J. 2006. Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. *EMBO (Eur. Mol. Biol. Organ.) J.* 25:2768-2780.
- Lecoq, K., Belloc, I., Desgranges, C., and Daignan-Fornier, B. 2001. Role of adenosine kinase in *Saccharomyces cerevisiae*: Identification of the ADO1 gene and study of the mutant phenotypes. *Yeast* 18:335-342.
- Li, J., Yang, Z., Yu, B., Liu, J., and Chen, X. 2005. Methylation protects miRNAs and siRNAs from a 3'-end uridylation activity in *Arabidopsis*. *Curr. Biol.* 15:1501-1507.
- Liu, L., Grainger, J., Cañizares, M. C., Angell, S. M., and Lomonosoff, G. P. 2004. *Cowpea mosaic virus* RNA-1 acts as an amplicon whose effects can be counteracted by a RNA-2-encoded suppressor of silencing. *Virology* 323:37-48.
- Lozano, G., Moriones, E., and Navas-Castillo, J. 2006. Complete nucleotide sequence of the RNA2 of the crinivirus *Tomato chlorosis virus*. *Arch. Virol.* 151:581-587.
- Lozano, G., Moriones, E., and Navas-Castillo, J. 2007. Complete sequence of the RNA1 of a European isolate of *Tomato chlorosis virus*. *Arch. Virol.* 152:839-841.
- Lu, R., Folimonov, A., Shintaku, M., Li, W. X., Falk, V. W., Dawson, W. O., and Ding, S. W. 2004. Three distinct suppressors of RNA silencing encoded by a 20-kb viral RNA genome. *Proc. Natl. Acad. Sci. U.S.A.* 101:15742-15747.
- Llave, C., Xie, Z., Kasschau, K., and Carrington, J. C. 2002. Cleavage of Scarecrow-like mRNA targets directed by a class of *Arabidopsis* miRNA. *Science* 297:2053-2056.
- Makeyev, E. V., and Bamford, D. H. 2002. Cellular RNA-dependent RNA polymerase involved in posttranscriptional gene silencing has two distinct activity modes. *Mol. Cell* 10:1417-1427.
- Masuta, C., Tanaka, H., Uehara, K., Kuwata, S., Koiwai, A., and Noma, M. 1995. Broad resistance to plant viruses in transgenic plants conferred by antisense inhibition of a host gene essential in S-adenosylmethionine-dependent transmethylation reactions. *Proc. Natl. Acad. Sci. U.S.A.* 92:6117-6121.
- Meins, F., Si-Ammour, A., and Blevins, T. 2005. RNA silencing systems and their relevance to plant development. *Annu. Rev. Cell Dev. Biol.* 21:297-318.
- Merai, Z., Kerenyi, Z., Kertesz, S., Magna, M., Lakatos, L., and Silhavy, D. 2006. Double-stranded RNA binding may be a general plant RNA viral strategy to suppress RNA silencing. *J. Virol.* 80:5747-5756.
- Moffatt, B. A., Stevens, Y. Y., Allen, M. S., Snider, J. D., Pereira, L. A., Todorova, M. I., Summers, P. S., Weretilnyk, E. A., Martin-Caffrey, L., and Wagner, C. 2002. Adenosine kinase deficiency is associated with developmental abnormalities and reduced transmethylation. *Plant Physiol.* 128:812-821.
- Moissiard, G., Parizotto, E. A., Himber, C., and Voinnet, O. 2007. Transitivity in *Arabidopsis* can be primed, requires the redundant action of the antiviral Dicer-like4 and Dicer-like2, and is compromised by viral-encoded suppressor proteins. *RNA* 13:1268-1278.
- Molnar, A., Melnyk, C. W., Bassett, A., Hardcastle, T. J., Dunn, R. D., and Baulcombe, D. C. 2010. Small silencing RNAs in plants are mobile and direct epigenetic modification in recipient cells. *Science* 328:872-875.
- Noris, E., Accotto, G. P., Tavazza, T., Brunetti, A., Crespi, S., and Tavazza, M. 1996. Resistance to tomato yellow leaf curl geminivirus in *Nicotiana benthamiana* plants transformed with a truncated viral C1 gene. *Virology* 224:130-138.
- Pazhouhandeh, M., Dieterle, M., Marrocco, K., Lechner, E., Berry, B., Brault, V., Hemmer, O., Kretsch, T., Richards, K. E., Genschik, P., and Ziegler-Graff, V. 2006. F-box-like domain in the poliovirus protein P0 required for silencing suppressor function. *Proc. Natl. Acad. Sci. U.S.A.* 193:1994-1999.
- Pereira, L. A. R., Todorova, M., Cai, X., Makaroff, C. A., Emery, R. J. N., and Moffatt, B. A. 2007. Methyl recycling activities are co-ordinately regulated during plant development. *J. Exp. Bot.* 58:1083-1098.
- Qin, C., Shi, N., Gu, M., Zhang, H., Li, B., Shen, J., Mohammed, A., Ryabov, E., Li, C., Wang, H., Liu, Y., Osman, T., Vatis, M., and Hong, Y. 2012. Involvement of RDR6 in short range intercellular RNA silencing in *Nicotiana benthamiana*. *Sci. Rep.* 2:467.
- Qu, F. 2010. Antiviral role of plant encoded RNA dependent RNA polymerases revisited with deep sequencing of small interfering RNAs of virus origin. *Mol. Plant Microbe Interact.* 23:1248-1252.
- Raja, P., Sanville, B. C., Buchmann, R. C., and Bisaro, D. M. 2008. Viral genome methylation as an epigenetic defense against geminiviruses. *J. Virol.* 82:8997-9007.
- Raja, P., Wolf, J. N., and Bisaro, D. M. 2010. RNA silencing directed against geminiviruses: Post-transcriptional and epigenetic components. *Biochim. Biophys. Acta* 1799:337-351.
- Rocha, P. S. C. F., Sheikh, M., Melchiorre, R., Fagard, M., Boutet, S., Loach, R., Moffatt, B., Wagner, C., Vaucheret, H., and Fumier, I. 2005. The *Arabidopsis* HOMOLOG-DEPENDENT GENE SILENCING1 gene codes for an S-adenosyl-L-homocysteine hydrolase required for DNA methylation-dependent gene silencing. *Plant Cell* 17:404-417.
- Schiebel, W., Haas, B., Marinkovic, S., Klanner, A., and Sanger, H. L. 1993. RNA-directed RNA polymerase from tomato leaves. Catalytic in vitro properties. *J. Biol. Chem.* 268:11858-11867.
- Schwach, F., Vaistij, F. E., Jones, L., and Baulcombe, D. C. 2005. An RNA-dependent RNA polymerase prevents meristem invasion by *Potato virus X* and is required for the activity but not the production of a systemic silencing signal. *Plant Physiol.* 138:1842-1852.
- Shen, B., and Goodman, H. M. 2004. Uridine addition after microRNA-directed cleavage. *Science* 306:997.
- Silhavy, D., and Burgyan, J. 2004. Effects and side-effects of viral RNA silencing suppressors on short RNAs. *Trends Plant Sci.* 9:76-83.
- Voinnet, O. 2005. Induction and suppression of RNA silencing: Insights from viral infections. *Nat. Rev. Genet.* 6:206-220.
- Voinnet, O. 2008. Use, tolerance and avoidance of amplified RNA silencing by plants. *Trends Plant Sci.* 13:317-328.
- Voinnet, O., and Baulcombe, D. C. 1997. Systemic signalling in gene silencing. *Nature* 389:553.
- Voinnet, O., Vain, P., Angell, S., and Baulcombe, D. C. 1998. Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell* 95:177-187.
- Voinnet, O., Pinto, Y. M., and Baulcombe, D. C. 1999. Suppression of gene silencing: A general strategy used by diverse DNA and RNA viruses. *Proc. Natl. Acad. Sci. U.S.A.* 96:14147-14152.
- Voinnet, O., Lederer, C., and Baulcombe, D. C. 2000. A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. *Cell* 103:157-167.
- Wang, H., Hao, L., Shung, C.-Y., Sunter, G., and Bisaro, D. M. 2005. Adenosine kinase is inactivated by geminivirus AL2 and L2 proteins. *Plant Cell* 15:3020-3032.
- Weretilnyk, E. A., Alexander, K. J., Drebenstedt, M., Snider, J. D., Summers, P. S., and Moffatt, B. A. 2001. Maintaining methylation activities during salt stress. The involvement of adenosine kinase. *Plant Physiol.* 125:856-865.
- Wintermantel, W. M., Wisler, G. C., Ancheta, A. G., Liu, H. Y., Karasev,

- A. V., and Tzanetakis, I. E. 2005. The complete nucleotide sequence and genome organization of *Tomato chlorosis virus*. Arch. Virol. 150:2287-2298.
- Wisler, G. C., Li, R. H., Liu, H. Y., Lowry, D. S., and Duffus, J. E. 1998. Tomato chlorosis virus: A new whitefly-transmitted, phloem-limited, bipartite closterovirus of tomato. Phytopathology 88:402-409.
- Wolfe, M. S., and Borchardt, R. T. 1991 *S*-adenosyl-L-homocysteine hydrolase as a target for antiviral chemotherapy. J. Med. Chem. 34:1521-1530.
- Yang, S. J., Carter, S. A., Cole, A. B., Cheng, N. H., and Nelson, R. S. 2004. A natural variant of a host RNA-dependent RNA polymerase is associated with increased susceptibility to viruses by *Nicotiana benthamiana*. Proc. Natl. Acad. Sci. U.S.A. 101:6297-6302.
- Yang, X., Xie, Y., Raja, P., Li, X., Wolf, J. N., Shen, Q., and Bisaro, D. M. 2011. Suppression of methylation-mediated transcriptional gene silencing by β Cs-SAHH protein interaction during geminivirus-betasatellite infection. PloS Pathog. e1002329.
- Yang, Z., Ebright, Y. W., Yu, B., and Chen, X. 2006. HEN1 recognizes 21-24 nt small RNA duplexes and deposits a methyl group onto the 2'OH of the 3' terminal nucleotide. Nucleic Acids Res. 34:667-675.
- Yang, Z., Vilkaitis, G., Yu, B., Klimasauskas, S., and Chen, X. 2007. Approaches for studying microRNA and small interfering RNA methylation in vitro and in vivo. Methods Enzymol. 427:139-154.
- Yu, B., Yang, Z., Li, J., Minakhina, S., Yang, M., Padgett, R. W., Steward, R., and Chen, X. 2005. Methylation as a crucial step in plant microRNA biogenesis. Science 307:932-935.
- Zhang, X., Yuan, Y. R., Pei, Y., Lin, S. S., Tuschl, T., Patel, D. J., and Chua, N. H. 2006. *Cucumber mosaic virus*-encoded 2b suppressor inhibits *Arabidopsis* Argonaute1 cleavage activity to counter plant defense. Genes Dev. 20:3255-3268.

AUTHOR-RECOMMENDED INTERNET RESOURCE

Clontech Laboratories, Inc. website: www.clontech.com