Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors

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RNA silencing is an evolutionarily conserved system that functions as an antiviral mechanism in higher plants and insects. To counteract RNA silencing, viruses express silencing suppressors that interfere with both siRNA- and microRNA-guided silencing pathways. We used comparative *in vitro* and *in vivo* approaches to analyse the molecular mechanism of suppression by three well-studied silencing suppressors. We found that silencing suppressors p19, p21 and HC-Pro each inhibit the intermediate step of RNA silencing via binding to siRNAs, although the molecular features required for duplex siRNA binding differ among the three proteins. None of the suppressors affected the activity of preassembled RISC complexes. In contrast, each suppressor uniformly inhibited the siRNA-initiated RISC assembly pathway by preventing RNA silencing initiator complex formation.

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Introduction

In eukaryotes, RNA silencing serves as a sequence-specific gene inactivation system. Although RNA silencing operates through diverse pathways in plants and animals, production of effector small RNAs always relies on a set of core reactions triggered by double-stranded (ds) or self-complementary foldback RNAs that are processed into 21–24-nt short interfering RNA (siRNA) or microRNA (miRNA) duplexes by the RNase III-type DICER enzymes. Small RNAs are then incor-

porated into a ribonucleoprotein complex termed RNA induced silencing complex (RISC) (Tomari and Zamore, 2005). Assembly of RISC involves guide strand incorporation and passenger strand elimination to program active RISC (Schwarz *et al*, 2003; Matranga *et al*, 2005). In extracts from *Drosophila* embryos, DICER2-R2D2 (DCR2-R2D2) complexed with duplex siRNA serves as an initiator of RISC assembly. This complex then interacts with an AGO2-containing multiprotein complex and cleaves the passenger strand of the siRNA duplex to form the single-stranded (ss) siRNA-containing 80S holo-RISC, which catalyses sequence-specific cleavage of target RNA (Pham *et al*, 2004; Tomari *et al*, 2004; Matranga *et al*, 2005).

Natural roles of RNA silencing include genome defence and specification of heterochromatin formation, post-transcriptional inhibition of gene expression by miRNAs and trans-acting siRNAs, and antiviral defence (Matzke and Matzke, 2004; Mello and Conte, 2004). In higher plants, these functions are mediated by several RNA interference (RNAi) pathways. In the nucleus, DCL3-, RDR2-, AGO4- and NRPD1-dependent RNAi is associated with DNA cytosine methylation, heterochromatin-associated modifications of histone H3 tails and transcriptional silencing (Zilberman *et al*, 2003; Lippman and Martienssen, 2004; Xie *et al*, 2004; Herr *et al*, 2005; Kanno *et al*, 2005; Onodera *et al*, 2005). Post-transcriptional RNAi-related pathways for endogenous gene regulation involve miRNAs (DCL1-dependent) or ta-siRNAs (DCL4- and RDR6-dependent) that interact with mRNA targets, although a few miRNAs interact with primary transcripts for ta-siRNAs (Allen *et al*, 2005; Gascioli *et al*, 2005; Yoshikawa *et al*, 2005). Virus-induced RNA silencing is triggered by dsRNA intermediates of cytoplasmically replicating viruses, RDR1- or RDR6-dependent formation of dsRNA, or structured regions of viral RNAs (Molnar *et al*, 2005; Voinnet, 2005). Virus-induced silencing likely leads to the sequence-specific degradation of viral RNA and generation of a mobile silencing signal that activates or potentiates RNA silencing in noninfected cells (Baulcombe, 2004; Voinnet, 2005). Therefore, systemic virus infection of plants requires effective mechanisms to suppress RNA silencing.

To counteract antiviral RNA silencing, most plant and many animal viruses have evolved silencing suppressor proteins (Silhavy and Burgyán, 2004; Voinnet, 2005). The molecular bases for suppressor activity have been proposed for several viruses, including p21 of closteroviruses, HC-Pro of potyviruses, p19 of tombusviruses and B2 protein of Flock House virus (FHV). Detailed studies demonstrated that the molecular basis of silencing suppression of p19 protein of tombusviruses and p21 of *Beet yellows virus* (BYV) is siRNA-sequestration (Silhavy *et al*, 2002; Vargason *et al*, 2003; Chapman *et al*, 2004; Dunoyer *et al*, 2004; Lakatos *et al*, 2004), while B2 binds to dsRNAs and inhibits siRNA formation (Chao *et al*, 2005; Lu *et al*, 2005).

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HC-Pro was one of the first viral proteins identified as a suppressor of transgene- and virus-induced RNA silencing. Analyses of data from variant experimental systems led to the development of several different models for the mechanism of HC-Pro silencing suppression. In one model, HC-Pro was proposed to reverse established RNA silencing (Anandalakshmi *et al*, 1998; Brigneti *et al*, 1998; Voinnet *et al*, 1999; Llave *et al*, 2000). Another model involved the enlistment of a cellular negative regulator of RNA silencing, such as rgs-CaM, a calmodulin-related protein (Anandalakshmi *et al*, 2000). A third model proposed that HC-Pro acts downstream of an RNA-dependent RNA polymerase but inhibits accumulation of siRNAs, suggesting that DICER activity was impaired (Mallory *et al*, 2001; Dunoyer *et al*, 2004). A fourth model predicted that RISC activation was suppressed through interaction between HC-Pro and a protein or complex required for siRNA duplex unwinding (Chapman *et al*, 2004). Importantly, most comparative studies concluded that the possible mechanism by which HC-Pro suppresses RNA silencing differs from the mechanism of other suppressor proteins, including p19 of tombusviruses and p21 of BYV (Chapman *et al*, 2004; Dunoyer *et al*, 2004; Voinnet, 2005).

To establish a more detailed model for the molecular basis of RNA silencing suppression by these suppressor proteins, we developed a multipronged approach for their comparative characterization. This approach included *in vitro* and *in vivo* approaches to explore the molecular mechanisms by which p19, p21 and HC-Pro interfere with RNA silencing machinery. We present evidence that all three silencing suppressors are dsRNA-binding proteins that interact physically with siRNA duplexes *in vivo* as well as *in vitro*. We also demonstrate that, similar to p19, HC-Pro and p21 inhibit siRNA-directed target RNA cleavage in the *Drosophila in vitro* RNA silencing system. Moreover, p19, HC-Pro and p21 uniformly inhibit the siRNA-initiated RISC assembly pathway by preventing RNA silencing initiator complex formation through siRNA sequestration. We further show that none of these silencing suppressors inhibit preassembled RISC activity *in vitro* or *in vivo*.

Results

Plant viral RNA silencing suppressors inhibit siRNA-directed RNA cleavage in *Drosophila* embryo extracts

The *Drosophila* embryo extract-based *in vitro* RNA silencing system allows analysis of RISC assembly (Pham *et al*, 2004; Tomari *et al*, 2004) and was used successfully to analyse silencing suppression (Lakatos *et al*, 2004). In this study, we analysed viral RNA silencing suppressors p19 of *Carnation Italian ringspot virus* (CIRV), p21 of BYV and HC-Pro of TEV. P19 and a GST-p21 fusion protein were expressed and purified from bacteria, while HC-Pro was purified from virus-infected plants as described in Supplementary Experimental Procedures. To better understand the actions of these silencing suppressors, we analysed their effects on intermediate steps of RISC assembly and RISC activity. We tested the effects on siRNA-guided RNA cleavage in direct competition assays in which inducer siRNA, target RNA containing sequences complementary to the inducer siRNA, and purified suppressor proteins were added simultaneously to *Drosophila* embryo extracts. RISC activity was measured by quantifica-

tion of the 5'-end product of siRNA-directed cleavage of target RNA generated over a dilution series of the suppressor protein. To analyse the effect of silencing suppressors on preassembled RISC activity, siRNA was preincubated with extracts to allow RISC formation, then target RNA and suppressor proteins were added and RNA cleavage was measured as described above.

CIRV p19 was a potent inhibitor of target RNA cleavage in direct competition assays, resulting in complete inhibition of cleavage with a half-maximal inhibition concentration (IC_{50}) of 15.24 ± 2.3 nM (Figure 1A and C). However, CIRV p19 had no effect on preassembled RISC activity, since target RNA cleavage occurred with similar efficiency regardless of the concentration of p19 added to the reactions containing preassembled RISC (Figure 1C). Consistent with our previous observations using *Cymbidium ringspot virus* (CymRSV) p19, these results support the direct siRNA-binding model for p19 suppressor function, whereby p19 inhibits RISC programming by siRNA duplex sequestration, but does not inhibit the activity of preassembled (ss siRNA-containing) RISC (Lakatos *et al*, 2004) (Figure 1B and C). To further support the direct siRNA binding model for p19, we analysed target cleavage inhibition the p19 W39/42R mutant (Vargason *et al*, 2003). P19 W39/42R does not effectively suppress transgene silencing, and introduction of this allele in a CIRV mutant results in reduced symptom severity during infection (Vargason *et al*, 2003). Due to participation of Trp39 and Trp42 in end-capping interactions with small RNA duplexes, the W39/42R mutant is likely compromised in siRNA binding. In target cleavage assays, p19 W39/42R was a less effective suppressor ($IC_{50} = 75.9 \pm 3.4$ nM) than wild-type (wt) p19 (Supplementary Figure S1A and C)). These results confirmed that the inhibition of target cleavage depends on the siRNA binding activity of p19.

In the direct competition assays, p21 also inhibited siRNA-mediated target RNA cleavage, although complete suppression of cleavage was not achieved with the GST-p21 preparation (Figure 1D and F). Activity of preassembled RISC was refractory to this suppressor at all concentrations tested (Figure 1E and F). P21 mutant 8A-21, which fails to suppress RNA silencing *in vivo* (Chiba *et al*, 2006), did not show any siRNA binding activity in electrophoretic mobility shift assays while the apparent dissociation constant (K_d) of wt p21 is 22 nM (Supplementary Figure S1F). These results are consistent with previous data in which p21 was shown to bind siRNA and miRNA duplexes *in vitro* and *in vivo* (Chapman *et al*, 2004).

Purified HC-Pro also inhibited target RNA cleavage in direct competition assays, and this effect was dose dependent (Figure 1G and I) ($IC_{50} = 118.22 \pm 5.36$ nM). Surprisingly, HC-Pro did not inhibit the activity of preassembled RISC at any concentration tested (Figure 1H and I). This indicates that the behaviour of HC-Pro in *in vitro* RNA silencing suppression assays is very similar to that of p19 and p21, suggesting that HC-Pro may possess siRNA duplex binding activity.

Silencing suppressors impair RISC assembly via inhibiting RNA silencing initiator complex formation *in vitro*

Ordered pathways for stepwise RISC formation and assembly have been described using *Drosophila* embryo extracts

Figure 1 Silencing suppressors inhibit siRNA-guided target RNA cleavage *in vitro*. Direct competition target cleavage assays used embryo extracts, target RNA (0.5 nM), 32 P-labelled siRNA (5 nM), and 0.125–256.2 nM p19 (A), 93.75–6000 nM GST-p21 (D), or 89–570 nM HC-Pro (G). Note that HC-Pro was considered as a monomer in all experiments. Preassembled RISC target cleavage assays used embryo extract preincubated with 32 P-labelled siRNA (5 nM) prior to addition of target RNA (0.5 nM) and 0.125–256.2 nM p19 (B), 93.75–6000 nM GST-p21 (E), or 89–570 nM HC-Pro (H). In panels A, B, D, E, G and H, lane 1 contains target RNA only. Lane 2 contains inducer siRNA and target RNA. Effect of p19 (C), GST-p21 (F) and HC-Pro (I) on target RNA cleavage by RISC (black squares) and preassembled RISC (red triangles), plotted as a function of the concentration of suppressor used.

(Pham *et al*, 2004; Tomari *et al*, 2004). Intermediate and fully assembled RNA silencing complexes can be analysed quantitatively by electrophoretic mobility shift assays. To assess the putative step(s) of RISC assembly inhibited by the three silencing suppressors, we analysed the effects of purified suppressor proteins on the formation of RISC and intermediate complexes in *Drosophila* embryo extracts. A method developed previously (Pham *et al*, 2004) was modified by omitting heparin, a potent competitor for siRNA duplex binding, from our experiments. In the modified system, the molecular weight of the first complex formed with siRNA is similar to that of the R1/R2D2-DCR2 initiator complex (data not shown); thus, this complex corresponds to siRNA-DCR2-R2D2 (Pham *et al*, 2004). Formation of the second complex requires ATP (Supplementary Figure S2), and is referred to as RISC loading complex (RLC), as suggested previously (Tomari and Zamore, 2005). The highest molecular weight

complex is RISC, as shown by binding of a 2'-O-methyl mRNA analogue (Supplementary Figure S3). We analysed suppressor effects on RISC assembly in direct competition assays in which labelled inducer siRNAs and a dilution series of purified suppressor proteins were added simultaneously to embryo extracts, and RISC and intermediate siRNA-containing complexes were quantified. To analyse suppressor effects on preassembled RISC accumulation, labelled inducer siRNA was preincubated with extracts, then a dilution series of suppressor proteins was added and silencing complexes were quantified as above.

As the concentration of p19 was increased in direct competition experiments, formation of silencing complexes siRNA-DCR2-R2D2, RLC, and RISC was compromised and a p19-siRNA complex accumulated (Figure 2A). Complete inhibition of RISC formation occurred at p19 concentrations sufficient to shift the majority of siRNA into a p19-siRNA

The EMBO Journal VOL 25 | NO 12 | 2006 **2771**

complex. Next, we examined the effect of p19 when added following RISC assembly. In these experiments, siRNA-DCR2-R2D2 complex formation gradually diminished as the p19 concentration increased; however, formation of RLC and RISC did not decrease below 80% of the control (Figure 2B). This is consistent with results of p19 target cleavage suppression assays, since addition of p19 after RISC formation had only slight effects on target cleavage (Figure 1B). Consistent with the results of cleavage assays and the weak *in vivo* suppressor activity of the p19 W39/42R mutant (Vargason *et al*, 2003), p19 W39/42 showed only a slight effect on the formation of RISC complexes (Supplementary Figure 1D). Accumulation of preassembled RISC was not compromised, even at the highest p19 W39/42R concentration (Supplementary Figure 1E).

Similar to p19, p21 effects on RISC formation were observed in direct competition assays (Figure 2C, lanes 1–11). At high concentrations of p21, accumulation of RISC decreased to 30% of the control. However, these concentrations of p21 did not affect accumulation of preassembled RISC (Figure 2C, lanes 13–20). The p21 mutant 8A-21 affected neither RISC assembly nor the amount of preassembled RISC (not shown).

In direct competition assays with HC-Pro, as the concentration of suppressor was increased, RISC formation was eliminated (Figure 2D). The IC_{50} (117.04 ± 1.05 nM) of HC-Pro for inhibition of RISC formation was very similar to the IC_{50} (118.22 ± 5.36 nM) measured in the target cleavage experiments. These values, and the IC_{50} values necessary for p19 inhibition of RISC formation and target cleavage (19.97 ± 1.5 and 15.24 ± 2.3 nM), suggest that both RISC formation and target cleavage experiments reflected the absolute amount of RISC. When HC-Pro was applied following RISC assembly, only the siRNA-DCR2-R2D2 complex was reduced (Figure 2E). To determine whether the silencing suppressor activity of HC-Pro might be based on siRNA duplex binding, the binding properties of purified HC-Pro were analysed with gel mobility shift assays. HC-Pro bound siRNA duplexes *in vitro*, although only at the two highest concentrations of HC-Pro tested (Figure 2D).

Strikingly, HC-Pro inhibited RISC assembly in embryo extracts at a concentration (142.5 nM) that did not support siRNA binding by purified HC-Pro alone (Figure 2D). These results suggest that, although purified HC-Pro is sufficient for siRNA duplex binding, addition of *Drosophila* embryo extract significantly increased the affinity of HC-Pro for RNA. We

also observed formation of a new complex migrating close to RLC and higher than the HC-Pro-siRNA complex in the absence of embryo extracts (Figure 2D). Accumulation of the new complex increased with HC-Pro concentration and accompanied reduction of DCR2-R2D2-siRNA and RISC. We hypothesized that this new complex corresponds to an HC-Pro-siRNA duplex complex containing a cellular factor that increases both the affinity of HC-Pro to siRNA and the molecular weight of the complex. Alternatively, this new complex may be an HC-Pro-induced silencing-related complex, although this is unlikely as the higher molecular weight complex is independent of the RNA silencing complexes (Supplementary Figure S4).

HC-Pro and p21 recognize the 3' end overhangs of 21-nt siRNA duplexes *in vitro*

siRNAs are produced by RNase III-like enzymes in the DICER family, which process dsRNA or foldback precursors to generate 21–25-nt siRNA duplexes with 2-nucleotide (nt) 3' overhangs and 5' monophosphates (Nykanen *et al*, 2001). These structural features are required to route siRNAs into RNAi pathways. We hypothesized that these features may be important for siRNA binding by some silencing suppressors, although it has been demonstrated that 2-nt 3' overhangs are not required for duplex small RNA binding by p19 (Vargason *et al*, 2003). To this end, we determined the structural features of siRNA required for HC-Pro and p21 binding.

Synthetic 21-nt or 24-nt siRNA duplexes and 19-nt or 21-nt blunt-ended RNA duplexes were used to test the small RNA binding specificity of HC-Pro and GST-p21 (Figure 3A–D). HC-Pro bound 21-nt siRNA duplexes with the highest affinity among the RNAs tested (Figure 3A). Complexes of HC-Pro with 24-nt siRNA duplexes were detected, although in much lower quantities than with 21-nt siRNA duplexes (Figure 3C). Complexes of HC-Pro with a blunt-ended 21-nt RNA duplex were not detected (Figure 3D). Distinct complexes of HC-Pro with a blunt 19-nt RNA duplex were not detected; however, reduction of the unbound RNA at high HC-Pro concentrations (Figure 3B) might indicate that a complex forms but dissociates during electrophoresis. Single-stranded 21-nt RNAs were used to confirm the specificity of HC-Pro for RNA duplexes. At a range of concentrations (17.8–570 nM), HC-Pro failed to bind 21-nt ss RNAs (not shown). These data demonstrate that HC-Pro binds with size specificity to 21-nt siRNA duplexes, and with higher binding affinity for duplexes with 2-nt overhangs than for single-stranded small RNAs or blunt-ended small RNA duplexes.

Since the results of RISC formation assays suggested that a cellular factor enhanced the affinity of HC-Pro for siRNA, HC-Pro-small RNA binding assays were repeated in the presence of *Arabidopsis thaliana* plant extracts. Strikingly, an unidentified plant factor significantly enhanced the affinity of HC-Pro for all duplexes tested (Figure 3A–D). Plant extracts did not similarly affect the binding of p19 or p21 to siRNA duplexes (not shown).

Comparison of the apparent dissociation constant (K_a) of GST-p21 to small RNA duplexes revealed that GST-p21 binds synthetic 21-nt siRNA duplexes with high affinity ($K_a = 22$ nM) (Figure 3E). Significantly lower affinity was measured for a blunt-ended 19-nt RNA duplex ($K_a = 165$ nM). Similarly, the affinity of GST-p21 to a 24-nt siRNA duplex ($K_a = 565$ nM) was higher than the affinity of

GST-p21 to a blunt-ended 21-nt RNA duplex ($K_a = 647$ nM). These results indicate that p21 binds siRNA duplexes in a size-selective manner, and suggest a preference for 21-nt siRNA duplexes containing 2-nt 3' end overhangs over blunt-ended RNA duplexes (Figure 3E).

Silencing suppressors bind silencing-generated 21-nt siRNA duplexes and miRNA/miRNA* intermediates *in vivo*

Previously, it was shown that CymRSV-infected plants accumulate high levels of siRNA, suggesting that p19 does not prevent siRNA production *in vivo* (Szittyá *et al*, 2002). It was subsequently shown that p19 sequesters siRNA duplexes *in vivo* (Lakatos *et al*, 2004). Based on our findings that p21 and HC-Pro also sequester siRNA duplexes, the effect of these suppressors on siRNA accumulation *in vivo* was analysed. In addition, the *in vivo* interactions between these two suppressors and siRNAs were analysed. To determine whether HC-Pro or p21 inhibit siRNA production *in vivo*, virus-specific siRNA accumulation and genomic RNA accumulation were measured in TEV- and BYV-infected *Nicotiana benthamiana* plants. In systemic leaves, viral genomic RNA and virus-derived 21-nt siRNA increased during an infection time course, suggesting that siRNA production is not inhibited *in vivo* (Figure 4A and B). The effects of p21, HC-Pro and p19 on siRNA biogenesis in *N. benthamiana* plants that were coinfiltrated with *Agrobacterium* strains carrying constructs encoding GFP, GFP-IR (Inverted Repeat) and the silencing suppressors were analysed. To avoid secondary siRNA generation, we used *N. benthamiana* line GFP16c/RDR6i, in which NbRDR6 is constitutively silenced by an RNAi hairpin construct (Schwach *et al*, 2005). At 3 days postinfiltration, plants were analysed for siRNA, GFP mRNA (Figure 5) and suppressor protein expression (Supplementary Figure S5). None of the silencing suppressors, nor the corresponding suppression-defective mutants, inhibited the generation of siRNA from GFP-IR. In contrast, control Reovirus sigma3 protein (Lichner *et al*, 2003), which is known to bind long dsRNA, inhibited GFP-IR RNA processing to siRNA and diminished mRNA cleavage. wt p19, HC-Pro and p21 each prevented the degradation of GFP mRNA, while the suppression-defective mutants p19 W39/42 (Vargason *et al*, 2003), HC-Pro AS3 (Kasschau and Carrington, 2001) and p21 8A-21 (Chiba *et al*, 2006) did not inhibit degradation of GFP mRNA (Figure 5). These results strongly suggest that p19, HC-Pro and p21 do not prevent siRNA formation from a long dsRNA precursor.

To demonstrate directly that p21 binds virus-derived siRNA *in vivo*, BYV-derived siRNAs were analysed in α -HA immunoprecipitates (IP eluates) from BYV-infected plants expressing HA-tagged p21. BYV-derived siRNAs and p21 were detected in total extracts (inputs) and α -HA IP eluates from virus-infected plants (Figure 4D). Neither p21 nor BYV-derived siRNAs were detected in the eluate of the α -His control IP (Figure 4D).

Previously, p21 and p19 were shown to interact with miRNA/miRNA* duplexes *in vivo* (Chapman *et al*, 2004). Although HC-Pro did not interact directly with miRNA/miRNA* in those experiments, miRNA* intermediates and two of three cognate miRNAs accumulated to higher levels in *Arabidopsis* plants expressing an HC-Pro transgene than in wt plants (Chapman *et al*, 2004). To re-investigate potential

Figure 3 Affinity of HC-Pro and p21 to RNA duplexes. 21-nt siRNA duplex (A), blunt-ended 19-nt RNA duplexes (B), 24-nt siRNA duplex (C), and blunt-ended 21-nt RNA duplex (D) at 1 nM final concentration were incubated with a dilution series of HC-Pro without (lanes 2–9) or with 4 μ g of *Arabidopsis thaliana* cell culture extract (lanes 10–17) for 30 min, then loaded onto a 3.9% native gel. (E) Determination of K_a for GST-p21 to different short RNA molecules. Binding reactions contained 7 pM of siRNA and 0.24–1000 nM of GST-p21 protein (black squares), 7 pM of 19-nt blunt siRNA and 3.9–1000 nM of GST-p21 protein (red circles), 10 pM of 24-nt siRNA and 7.81–2000 nM of GST-p21 protein (green triangles), or 10 pM of 21-nt blunt siRNA and 60–2000 nM of GST-p21 protein (blue triangles). Bound fractions are plotted as a function of the concentration of GST-p21.

interactions between HC-Pro and small RNA duplexes, HC-Pro containing an N-terminal 6xHis tag was expressed from TEV (Ruiz-Ferrer *et al*, 2005) in infected *N. benthamiana* plants and immunoprecipitated using conditions less stringent than those used by Chapman *et al* (2004). miR171, miR171* and virus-derived siRNAs were analysed in total extracts and α -His IP eluates from mock-inoculated plants and TEV-infected plants. miR171 and miR171* were both detected in total extracts from infected plants (IP inputs) and in α -His IP eluates (Figure 4C). miR171 was detected in mock-inoculated plant extracts, but at lower levels than in extracts from TEV-infected plants (Figure 4C). miR171* was not detected in total extracts from mock-inoculated plants,

consistent with previous findings (Mallory *et al*, 2002; Chapman *et al*, 2004; Dunoyer *et al*, 2004). Neither miR171 nor miR171* was detected in α -His IP eluates from mock-inoculated plants. TEV-derived siRNAs also co-immunoprecipitated with tagged HC-Pro. The immunoprecipitated TEV-derived siRNAs co-migrated during electrophoresis with a size standard corresponding to 21-nt duplex siRNA, but not a single-stranded 21-nt siRNA (Figure 4E).

The findings that p19, p21 and HC-Pro bind 21-nt siRNAs and duplex miRNAs *in vivo* support the idea that the mechanism by which these structurally divergent silencing suppressors inhibit RNA silencing is sequestration of small RNA duplexes.

Figure 4 HC-Pro and p21 bind silencing-generated small RNA duplexes *in vivo*. (A) Accumulation of viral genomic RNA and virus-derived siRNA during a TEV infection timescourse. (B) Accumulation of viral genomic RNA, p21 protein and virus-derived siRNA during a BYV infection timescourse. (C) Analysis of HC-Pro, TEV-derived siRNAs, and miRNA171 and miRNA171* in IP inputs and eluates from TEV-infected and mock-inoculated plants. (D) Analysis of p21 and BYV-derived siRNAs in IP inputs and eluates from BYV-infected plants. (E) Nondenaturing RNA gel blot analysis of TEV-derived siRNAs in α -His IP eluates from TEV-infected plants expressing 6xHis-tagged HC-Pro. 21-nt siRNA duplexes and 21-nt ss RNAs were used as markers.

SiRNA duplex-binding silencing suppressors do not inhibit siRNA and miRNA-programmed RISC in planta

SiRNA duplex-binding silencing suppressors impair target cleavage activity by inhibiting RISC assembly, but do not inhibit the activity of programmed RISC. To determine if this applies *in planta*, a new sensor-based assay was developed. Plants were infected with the CymRSV mutant Cym19stop, which triggers silencing in upper leaves. These leaves are resistant to challenge inoculation by viruses with sequences homologous to the primary virus, indicating that they contain active RISC complexes (Szittya *et al*, 2002). To analyse the activity of CymRSV-activated RISC and suppressor effects on this activity, GFP-Cym sensor RNAs and suppressor proteins were analysed in Cym19stop-infected *N. benthamiana* plants. SiRNA sensor constructs contained a GFP-encoding ORF fused to a sequence of 194 nt of CymRSV (GFP-Cym) or 204 nt of PoLV (GFP-PoLV) (Figure 6A). The PoLV sensor construct was used as a negative control, as it does not

Figure 5 Duplex siRNA-binding silencing suppressors inhibit target RNA cleavage *in planta*. Analysis of GFP mRNA and siRNA in leaves of *N. benthamiana* GFP16c/RDR6i expressing 35S-GFP (lane 2), 35S-GFP and 35S-GFP-IR (lane 3), 35S-GFP, 35S-GFP-IR and p19 wt (lane 4), 35S-GFP, 35S-GFP-IR and p19 W39/42R (lane 5), 35S-GFP, 35S-GFP-IR and HC-Pro (lane 6), 35S-GFP, 35S-GFP-IR and HC-Pro-AS3 mutant (lane 7), 35S-GFP, 35S-GFP-IR and p21 wt (lane 8), 35S-GFP, 35S-GFP-IR and p21 8A-21 mutant (lane 9), 35S-GFP, 35S-GFP-IR and sigma3 dsRNA binding protein (lane 10), or mock-infiltrated (lane 1). For suppressor constructs see Supplementary Experimental Procedures.

contain sequence similarity to CymRSV. Both sensor constructs were expressed when they were infiltrated into control, noninfected plants (Figure 6B and D).

Nonsymptomatic leaves emerging 14–18 days after inoculation with Cym19stop were coinfiltrated with *Agrobacterium* strains carrying the GFP-Cym or GFP-PoLV sensor and suppressor cDNA constructs. Expression of the suppressor proteins in infiltrated leaves was confirmed by immunoblotting (Figure 6C). At 3 days after infiltration, GFP fluorescence in patches expressing the GFP-Cym sensor was compared to fluorescence in patches expressing the GFP-PoLV sensor, and GFP mRNA and protein accumulation was analysed by RNA gel blot hybridization and immunoblotting. Loss of GFP-Cym sensor activity was detected in the presence of each suppressor in infected plants (Figure 6B). Consistently, the amount of GFP protein was significantly lower from the GFP-Cym sensor than from the GFP-PoLV sensor (Figure 6D). Moreover, a GFP-specific RNA shorter than the predicted sensor mRNA transcript was detected in RNA samples recovered from GFP-Cym-, but not GFP-PoLV-infiltrated patches (Figure 6D). These GFP-specific RNAs were identified by cloning and sequencing as 5' cleavage products of GFP-Cym sensor RNA (Pantaleo *et al*, unpublished data) and the observed reduction of GFP expression correlated with accumulation of these RNA cleavage products. Sequence data (not shown) indicated that RNA cleavage occurred only in the CymRSV-containing region of the GFP-Cym sensor RNA and not in the GFP sequence. In addition, no siRNA corresponding to GFP was detected by RNA blot analyses of RNA derived from patches infiltrated with the GFP-Cym sensor (data not shown).

To examine suppressor effects on miRNA-programmed RISC activity, we designed similar *Agrobacterium* coinfiltration experiments using a GFP-171.1 sensor to detect miR171-programmed RISC, and GFP-171.2 as a noncleavable sensor

Figure 6 Duplex siRNA-binding silencing suppressors do not inhibit activity of siRNA-programmed RISC. (A) Graphical representation of GFP-Cym and GFP-PoLV constructs. (B) GFP fluorescence in leaves of Cym19stop-infected *N. benthamiana* expressing GFP-Cym or GFP-PoLV, compared to leaves expressing each suppressor and the GFP-Cym or GFP-PoLV sensor. (C) Immunoblot analyses of the expression of silencing suppressors in infiltrated patches. (D) RNA gel blot and immunoblot analyses of GFP sensor mRNAs and GFP protein isolated from infiltrated patches.

control (Parizotto *et al*, 2004) (Figure 7A). Immunoblot analyses confirmed expression of each suppressor protein in the infiltrated patches (Figure 7C). GFP fluorescence and protein accumulation was reduced in patches expressing the GFP-171.1 sensor compared to patches expressing the GFP-171.2 sensor construct (Figure 7B and D). Similarly, accumulation of GFP-171.1 sensor transcripts was reduced, while the mutant target sequence-containing sensor RNA arising from GFP-171.2 remained intact (Figure 7D). The expression levels of the two miRNA sensors were the same regardless of the presence or the absence of suppressor, further demonstrating that miR171-programmed RISC was active in the presence of each suppressor.

These data are consistent with results from the RISC assembly experiments *in vitro*, and indicate that the three suppressors do not compromise assembled RISC activity.

Discussion

p19, p21 and HC-Pro suppress RNA silencing by inhibiting RNA silencing initiator complex formation *in vitro*

A multipronged approach was used to compare the activities of three distinct RNA silencing suppressors. Results obtained

in the *Drosophila* embryo extracts revealed that p19, p21 and HC-Pro each inhibited siRNA programming of RISC and target cleavage. This occurred when the suppressors were introduced into embryo extracts simultaneously with effector siRNA duplexes, but not when suppressors were added after siRNA duplexes. Analysis of RISC assembly indicated that each suppressor acted primarily to block formation of initial siRNA-DICER2-R2D2 intermediate complexes (Pham *et al*, 2004). These data support the hypothesis that suppressor-mediated reduction of target cleavage was primarily due to inhibition of RISC programming rather than inhibition of activity of assembled RISC.

The basis for inhibition of RISC assembly and target cleavage by p19, p21 and HC-Pro was clearly sequestration of siRNA duplexes. p19 and p21 bound siRNA with high affinity in the presence or absence of *Drosophila* extract, as predicted from the known properties of both proteins. However, effective suppression of silencing by HC-Pro required a cellular factor that increased the affinity of HC-Pro to siRNA duplexes. The cellular factor was detected in *Arabidopsis* extracts and increased the size of the HC-Pro-siRNA complex. However, the question of whether HC-Pro requires a cellular factor for efficient silencing suppression *in planta* requires further study. The finding that

Figure 7 Duplex siRNA-binding silencing suppressors do not inhibit activity of microRNA-programmed RISC. (A) Representation of microRNA sensor constructs used in this experiment (Parizotto *et al*, 2004). (B) GFP fluorescence in leaves of *N. benthamiana* expressing GFP-171.1 or GFP-171.2 sensors, compared to leaves expressing each suppressor and the GFP-171.1 or GFP-171.2 sensor. (C) Immunoblot analyses of the expression of silencing suppressors in infiltrated patches. (D) RNA gel blot and immunoblot analyses of microRNA sensor RNAs and GFP protein isolated from infiltrated patches.

p19, p21 and HC-Pro each bind siRNA duplexes in a dose-dependent manner extends the sequestration model to three evolutionarily distinct virus-encoded suppressors (Silhavy *et al*, 2002; Lakatos *et al*, 2004).

The inhibition of siRNA-DCR2-R2D2 intermediate complex formation is likely due to the silencing suppressors possessing higher affinities than DCR2-R2D2 complexes for siRNA duplexes. The sequestration model for suppressor function is essentially a competitive inhibition model. Although the suppressors functioned primarily to inhibit siRNA-DCR2-R2D2 complex formation, slight reductions in RISC and RLC abundance and in target cleavage activity were detected when high levels of suppressor were added to extracts after siRNA duplexes. Although this could be due to nonspecific effects, suppression of ATP-dependent complexes that contain siRNA might reflect a low proportion of complexes with siRNA duplexes. Although such complexes are difficult to explain mechanistically, RLC and RISC may contain some siRNAs in a ds form (Tomari *et al*, 2004).

Mechanisms for suppression of RNA silencing by HC-Pro, p19 and p21 in vivo

Both structural properties and biochemical activities reveal clearly that p19 binds siRNA duplexes in a size-selective

(~19 bp) manner regardless of the presence of 3' 2-nt overhangs (Vargason *et al*, 2003). Here, p21 is reported to bind small RNAs *in vitro* and *in vivo*, possess a higher affinity for 21-nt siRNA duplexes relative to 24-nt siRNA duplexes, bind preferentially to duplexes with 3' 2-nt overhangs, and to lack ss siRNA-binding activity. These data are fully consistent with our previous *in vitro* and *in vivo* analyses of p21-small RNA interaction (Chapman *et al*, 2004). However, these data are not easy to reconcile with a recent study suggesting that p21 is a general nucleic acid binding protein (Ye and Patel, 2005). One possible explanation for the disparate results is the concentration of protein, and protein/RNA ratios, used in different experiments. p21-siRNA duplex complexes form specifically at protein/siRNA ratios between 10:1 and 10 000:1 and protein concentrations up to 1 μ M (Chapman *et al*, 2004; Figure 3E). At high protein concentrations (1–10 μ M) that exceed nucleic acid concentrations by 10 000-fold (Ye and Patel, 2005), we detected primarily non-specific complexes with very slow mobility in gel shift assays (EJC and JCC, unpublished data). It is possible that these slow mobility complexes correspond to the p21 octameric structures analysed at atomic resolution by Ye and Patel (2005). We suggest that the octameric complexes may represent a structural form of p21 with functions that are distinct from RNA silencing suppressor functions.

Quantitative data showed that HC-Pro binds to 21-nt siRNA duplexes containing 3' 2-nt overhangs with higher affinity than to 19-nt duplexes lacking overhangs or to 24-nt siRNA duplexes. Thus, both p21 and HC-Pro differ from p19 by a requirement for 2-nt 3' end overhangs within the 21-nt siRNA duplex. TEV HC-Pro was also shown here to co-immunoprecipitate with virus-derived siRNAs and miR171/miR171* duplexes in extracts from infected plants. Infection by TEV promoted accumulation of miR171*, which normally accumulates to very low levels in noninfected plants. Previously, HC-Pro from *Turnip mosaic virus*, as well as p19 and p21, expressed in transgenic *Arabidopsis* were each shown promote accumulation of miR171*, miR167b* and miR160c*, leading to the suggestion that the three suppressors inhibit miRNA/miRNA* duplex unwinding (Chapman *et al*, 2004). However, unlike p19 and p21, which co-immunoprecipitated with siRNAs from long dsRNA and miRNA/miRNA* duplexes, no direct interaction between siRNAs or duplexes were detected with HC-Pro (Chapman *et al*, 2004). The failure to detect direct interactions between small RNAs and HC-Pro in the previous study may have been due to several reasons. First, the immunoprecipitation conditions used in the previous study were more stringent than that used in the current experiments. And second, the N-terminal 6xHis epitope tag used here differed from the C-terminal HA tag used previously. And third, the tagged HC-Pro used here was recovered from virus-infected plants rather than transgenic plants, the latter of which may have resulted in protein that was less accessible for immunoprecipitation.

None of the three suppressors affected si- and miRNA programmed RISC activity *in planta* as analysed using sensor constructs in transient assays. This is consistent with experiments using the *Drosophila* extract system. Previous reports suggested that HC-Pro could reverse established RNA silencing in a transgene-based system (Anandalakshmi *et al*, 1998; Brigneti *et al*, 1998; Voinnet *et al*, 1999). It was also reported that in HC-Pro-expressing transgenic plants, transgene-derived siRNAs are downregulated, suggesting that HC-Pro interferes with siRNA production (Llave *et al*, 2000; Mallory *et al*, 2001; Dunoyer *et al*, 2004). However, our results (Figure 5) clearly showed that co-expression of HC-Pro, p19, or p21 with GFP-IR in *N. benthamiana* GFP16c/RDR6i did not compromise dsRNA processing to siRNAs, which agrees with previous data obtained with HC-Pro using a different GFP inverted repeat construct (Johansen and Carrington, 2001). In plants, initiator-dependent maintenance of RNA silencing requires the RNA-dependent RNA polymerase, RDR6 (Dalmay *et al*, 2000; Mourrain *et al*, 2000). Sequestration of siRNAs by suppressors in RDR6-dependent systems would inhibit the maintenance/amplification step and lower the levels of siRNAs produced. This would have the same effect as losing DICER activity. Therefore, the proposed sequestration mechanism for HC-Pro provides a reasonable explanation for many observations derived from different systems. Of course, HC-Pro may also possess other activities or mechanisms related to RNA silencing. The association of HC-Pro with one or more cellular factors, as indicated by the *in vitro* assays (Figure 2) and other analyses (Anandalakshmi *et al*, 2000), means this remains an open possibility.

Is siRNA duplex sequestration a widely used strategy to suppress RNA silencing?

Inhibition of antiviral RNA silencing is critical prerequisite for the successful systemic invasion by many or most plant viruses. Silencing inhibition through siRNA sequestration seems advantageous, as production of siRNAs is a conserved element of the antiviral silencing in any host. p19, p21 and HC-Pro are structurally and evolutionarily unrelated proteins, each representing a small protein family specific to a respective viral taxon (Koonin *et al*, 1991; Reed *et al*, 2003; Vargason *et al*, 2003; Ye *et al*, 2003; Dolja *et al*, 2006). Although only a limited number of silencing suppressors have been shown experimentally to bind small RNA duplexes (Chapman *et al*, 2004; Dunoyer *et al*, 2004; Lakatos *et al*, 2004), there are several additional suppressors for which a similar mechanism is predicted. For example, p14 of PoLV is an siRNA-interacting and long dsRNA binding protein (Merai *et al*, 2005), and there are several other viruses that trigger production of siRNA-binding proteins during infection (Merai *et al*, 2006). However, there are also other known or suggested mechanisms of silencing suppression, including F-box-like activity that leads to destruction of silencing components (Pazhouhandeh *et al*, 2006) direct inhibition of one or more DICER activities (Qu *et al*, 2003), or sequestering of mature small RNAs after duplex unwinding (Chellappan *et al*, 2005). The siRNA duplex-binding mechanism, however, represents a recurring mechanism that has evolved independently in several families (*Tombusviridae*, *Potyviridae*, and *Closteroviridae*) within the positive-strand RNA viruses.

Materials and methods

Target cleavage assays

Drosophila embryo extract preparation, target RNA labelling, and siRNA annealing were described previously (Haley *et al*, 2003). Reactions used 2 µl of embryo extract, 5 nM siRNA (final concentration) and 1 × lysis buffer containing 10% v/v of glycerol in a total volume of 10 µl. Cap-labelled GFP target RNA was used at 0.5 nM final concentration. In direct competition assays, reactions were incubated for 1 h. In preassembled RISC assays, siRNA and embryo extracts were preincubated for 30 min to allow RISC assembly prior to addition of target RNA and suppressor proteins. Samples were deproteinized and RNA was analysed on an 8% denaturing gel.

RISC formation assays

Reaction conditions were as described for target cleavage assays. In direct competition assays, embryo extracts were incubated for 30 min with 5 nM ³²P-labelled siRNA duplexes and suppressor protein, diluted with 10 µl of loading buffer (1 × lysis buffer, 6% ficoll 400) and analysed on a 3.9% (39:1 acrylamide:bisacrylamide) native acrylamide gel. In preassembled RISC assays, ³²P-labelled siRNA duplexes and embryo extracts were preincubated for 30 min to allow RISC assembly prior to addition of target RNA and suppressor proteins. Native gel electrophoresis for separation of silencing complexes was essentially as described in Pham *et al* (2004) with modifications. Gels were dried and exposed to a storage phosphor screen, and bands were quantified using a Genius Image Analyzer (Syngene).

Statistical analysis

All *in vitro* target cleavage and RISC formation experiments were performed three times. The curves were best fitted to the indicated sets of data with the computer program Microcal Origin 5.00. The average of three trials ± standard deviation is shown.

Electrophoretic mobility shift assays

Labelling and annealing of RNA duplexes was carried out as described previously (Lakatos *et al*, 2004). Purified proteins and

labelled RNAs were incubated for 30 min at room temperature in lysis buffer (Zamore *et al*, 2000) supplemented with 0.02% Tween-20. Complexes were resolved on 6% polyacrylamide 0.5 × TBE gels. Gels were dried and exposed to a storage phosphor screen (Molecular Dynamics Typhoon Phosphorimager, Amersham Biosciences), and bands were quantified with a Genius Image Analyser (Syngene).

Agrobacterium tumefaciens infiltration

A. tumefaciens infiltration was performed according to Silhavy *et al* (2002). For coinfiltration of *N. benthamiana* leaves, mixtures of strains carrying sensor constructs (OD₆₀₀ = 0.15) and strains carrying suppressor constructs (OD₆₀₀ = 0.3) were used. *N. benthamiana* GFP16c/RDR6i line was co-infiltrated with a mixture of strains carrying constructs encoding 35S-GFP (OD₆₀₀ = 0.1), 35S-GFP-IR (OD₆₀₀ = 0.4) and the indicated suppressors (OD₆₀₀ = 1.0).

RNA isolation and hybridization analyses

Total RNA from *Agrobacterium*-infiltrated *N. benthamiana* leaves and leaves of TEV-infected and mock-inoculated *N. benthamiana* plants was isolated in 2 × PK buffer with Proteinase K as described previously (Lakatos *et al*, 2004). Total RNA from leaves of BYV-infected and mock-inoculated *N. benthamiana* plants was isolated using Trizol reagent (Johansen and Carrington, 2001). Denaturing RNA gel blot hybridization and analyses were performed as described in Silhavy *et al* (2002). Nondenaturing RNA gel blot hybridization was performed as described previously (Lakatos *et al*, 2004).

Immunoprecipitation and immunoblotting

Extracts for immunoprecipitation (IP inputs) were prepared in IP buffer containing 40 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM MgCl₂, 1 mM DTT. For immunoprecipitation of HA-tagged p21, leaves of BYV-infected *N. benthamiana* plants were infiltrated with *Agrobacterium* carrying a construct for expression of HA-tagged p21 (OD₆₀₀ = 1.0). Tissue was collected 48 h postinfiltration for preparation of extracts in IP buffer, and immunoprecipitations using α-HA or α-His were performed as described in Chapman *et al*

(2004). For immunoprecipitation of 6xHis-tagged HC-Pro, α-His antibody-conjugated beads were added to total extracts, incubated for 1 h at 4°C, and washed with IP buffer. Immunoprecipitated complexes were eluted as described previously (Lakatos *et al*, 2004), and IP eluates were divided for protein extraction and RNA isolation.

Protein extracts from *Agrobacterium*-infiltrated *N. benthamiana* leaves were normalized for SDS-PAGE using the Bradford assay (BioRad). Protein loading was visualized by staining with Ponceau S. Immunoblotting to TEV HC-Pro was performed with the polyclonal TEV HC-Pro antibody (Blanc *et al*, 1999). To detect CIRV p19, the polyclonal antibody against CIRV p19 was used (Vargason *et al*, 2003). Commercially available antibodies were used for detection of eGFP, 6xHis- and HA-tagged proteins. Immunoblots used for detection of suppressor proteins were probed with a mixture of antibodies.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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