Double-Stranded RNA Binding May Be a General Plant RNA Viral Strategy To Suppress RNA Silencing

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In plants, RNA silencing (RNA interference) is an efficient antiviral system, and therefore successful virus infection requires suppression of silencing. Although many viral silencing suppressors have been identified, the molecular basis of silencing suppression is poorly understood. It is proposed that various suppressors inhibit RNA silencing by targeting different steps. However, as double-stranded RNAs (dsRNAs) play key roles in silencing, it was speculated that dsRNA binding might be a general silencing suppression strategy. Indeed, it was shown that the related aureusvirus P14 and tombusvirus P19 suppressors are dsRNA-binding proteins. Interestingly, P14 is a size-independent dsRNA-binding protein, while P19 binds only 21-nucleotide ds-sRNAs (small dsRNAs having two-nucleotide 3′ overhangs), the specificity determinant of the silencing system. Much evidence supports the idea that P19 inhibits silencing by sequestering silencing-generated viral ds-sRNAs. In this study we wanted to test the hypothesis that dsRNA binding is a general silencing suppression strategy. Here we show that many plant viral silencing suppressors bind dsRNAs. Beet yellows virus Peanut P21, clump virus P15, Barley stripe mosaic virus γB, and Tobacco etch virus HC-Pro, like P19, bind ds-sRNAs size-selectively, while Turnip crinkle virus CP is a size-independent dsRNA-binding protein, which binds long dsRNAs as well as ds-sRNAs. We propose that size-selective ds-sRNA-binding suppressors inhibit silencing by sequestering viral ds-sRNAs, whereas size-independent ds-sRNA-binding suppressors inactivate silencing by sequestering long dsRNA precursors of viral sRNAs and/or by binding ds-sRNAs. The findings that many unrelated silencing suppressors bind dsRNA suggest that dsRNA binding is a general silencing suppression strategy which has evolved independently many times.

RNA silencing (termed RNA interference [RNAi] in animals) is an RNA-based eukaryotic gene regulatory system that plays essential roles in many biological processes. RNA silencing is induced by accumulation of double-stranded RNAs (dsRNAs). dsRNAs are first processed by an RNase III-like nuclease called DICER (in plants it is termed DICER-LIKE [DCL]) into (21- to 25-nucleotide [nt]) small RNAs (sRNAs), which guide these sRNAs into different silencing effector complexes. In the active effector complexes sRNAs are present as single-stranded molecules, which guide these complexes to the complementary nucleic acids for suppression (2, 3, 22, 61).

In plants, different dsRNA precursors are processed by distinct DCLs into functionally different short (21- to 22-nt) and long (23- to 25-nt) sRNAs (24, 25, 67). Short sRNAs guide a multicomponent nuclease (RNA-induced silencing complex [RISC]) to homologous mRNAs for suppression. RISC cleaves targeted mRNA in the case of (near) perfect base pairing between mRNA and guide RNA. When the guide RNA is only partially complementary to the mRNA, RISC mediates translational repression. Short sRNAs could also provide (directly or indirectly) sequence specificity for plant-encoded RNA-dependent RNA polymerase (RdRP) which transforms homologous mRNAs into dsRNAs, thus amplifying silencing (3, 42, 65). Long sRNAs play a role in transcriptional silencing by directing the histone and DNA methylation of homologous DNA (66, 72).

RNA silencing plays important antiviral roles in plants and animals (15). In plants, double-stranded replicative intermediates of RNA viruses or strongly structured segments of viral mRNAs (40) are processed by DCL2 and perhaps by other DCL enzymes into viral sRNAs (18, 24, 67), short 21-nt dsRNAs having 2-nt 3′ overhangs (also called viral short interfering RNAs [siRNAs]). Viral sRNAs incorporate and target RISC to viral mRNAs for suppression, and thereby silencing could reduce pathogen levels in the infected cells. Virus-induced silencing can also act as a systemic defense system. Viral sRNAs are supposed to spread 10 to 15 cell layers and activate silencing in still noninvaded neighboring cells, limiting the extent of virus invasion (27).

To counteract RNA silencing most plant viruses express silencing suppressor proteins (44, 48, 53, 63, 65). Since silencing suppressors operate differently in widely used silencing inhibition assays it is believed that viral suppressors target different steps of the silencing response (64). However, as dsRNAs play key roles in silencing, it was speculated that binding of a dsRNA component of the silencing machinery could be a frequently used viral suppression strategy (53). In theory, dsRNA-binding silencing suppressors could target silencing response at two different steps, by binding and sequestering the silencing inducer long viral dsRNAs and/or by binding and sequestering viral ds-sRNAs.

Recent results suggest that two unrelated viral suppressors (tombusvirus P19 and clasterovirus P21) inhibit silencing by...
binding viral ds-sRNAs (11, 19, 31, 54). It has been shown that P19 sequesters viral sRNAs in infected plants, and thus it depletes the specificity determinant of antiviral silencing effectors (31). As P21 also binds ds-sRNAs in plant cells (11), it is likely that sequestering of ds-sRNAs is a silencing suppression strategy that has evolved at least twice independently. Moreover, the P19-related aureusvirus P14 silencing suppressor is also a dsRNA-binding protein (39).

Interestingly, P19 and P14 bind dsRNAs differently. P19 binds dsRNAs size-selectively (62, 68) and it binds efficiently only short, 21-nt ds-sRNAs, whereas P14 efficiently forms complexes with long dsRNAs and with 21-nt ds-sRNAs (39). These differences could be functionally relevant. P14 might suppress silencing by sequestering both long viral dsRNAs and viral ds-sRNAs, whereas P19 sequesters only viral ds-sRNAs. Moreover, flock house virus B2 is also a dsRNA-binding protein that could suppress antiviral silencing in Caenorhabditis elegans by targeting the dsRNA precursor of viral sRNAs (38).

In this study we wanted to test the hypothesis that dsRNA binding is a general silencing suppression strategy of plant RNA viruses. Here we show that many viral suppressors can bind dsRNAs. CP of Turnip crinkle virus (TCV), a carmovirus, like P14, binds dsRNA without obvious size selection. By contrast, P15 of Peanut clump virus (PCV), a pecluvirus, γB of Barley stripe mosaic virus (BSMV), a hordeivirus, P21 of Beet yellow mosaic virus (BYV), a closterovirus, and HC-Pro of Tobacco etch virus (TEV), a potyvirus, like P14, are size-specific ds-sRNA-binding silencing suppressors. These proteins efficiently form complexes with 21-nt ds-sRNA but fail to bind long dsRNA. We have also demonstrated that the structural basis of size-selective ds-sRNA binding is different for P19 and for PCV P15, BSMV γB, and TEV HC-Pro size-specific dsRNA-binding suppressors.

Our data strongly suggest that dsRNA binding is a general plant viral silencing suppression strategy which has evolved independently many times.

MATERIALS AND METHODS

Plasmid constructs. Suppressors (except BYV P21) were cloned into BIN61S for agroinfiltration assays. The BYV P21 binary construct was previously described (47). The P19, P14, and recombinant Sigma3 (Rec) BIN61S constructs were also described (34, 39, 54). PVX HC-Pro was amplified by reverse transcription (RT)-PCR (Stratagene, ProSTAR ULTRA HF RT-PCR system) from infected plants with a forward primer containing a start codon (italics) (5’-ATGGGGAATTCAATGGT) and with a reverse primer defining the stop codon (5’-ATGCTTTTCTTCCTTGTT) and then this fragment was cloned into SmaI site of BIN61S (BIN61S CP). Note that the underlined restriction sites (BamH I and SalI) were not used in this cloning. The orientation was determined by orientation-dependent PCR. BSMV γB was PCR amplified with forward (5’-CATTTGGAACATGGTCACATCTTTCTGTT) and reverse (5’-TCATCTAGATCTCACATCTGTGACCAGAAGTT) primers carrying a BamH I and XbaI site, respectively. The PCR fragment was cleaved with BamH I and XbaI and cloned into BIN61S.

Plasmid pTAS TV2 (K. Salanki, unpublished) was partially digested with KpnI and XbaI, and then the digested TV2 2b fragment was purified and cloned into BIN61S. Binary suppressor constructs were sequenced and used for agroinfiltration assays. PVXP14 and PVXP19 were previously described (39, 57). To create N-terminally c-Myc-tagged binary constructs, P15, TCV CP, and BSMV γB suppressors were cloned into BInMyC, a derivative of BIN61S in which the multiple cloning site was replaced with the c-Myc fusion cloning sites from pMESH (21). To create Myc-P15, the BInMyC-digested P15 fragment which was amplified from KS-P15 with P15BamHI forward (5’-ATGGGGAATTCAATGGT) and reverse (5’-ATGCTTTTCTTCCTTGTT) and then this fragment was cloned into BamH I and SalI sites of BInMyC. Myc-CP was obtained by cloning the BamH I fragment from BIN61S CP into BInMyC.

To obtain Myc-γB, the BIN61S γB construct was cleaved with XbaI, filled in, and then digested with BamH I. The γB fragment was cloned into BamH I- and SalI-digested vector GEX2T (GEX γB). The BInMyC-EcoR I fragment was isolated from GEX γB and cloned into BInMyC. C-terminally hemagglutinin (HA)-tagged P21 was described previously (11). His-HC-TEV was created by PCR amplifying the His-tagged TEV HC-Pro fragment with forward (5’-AAATGATCACCACATGCTACGCCG) and reverse (5’-TCATCCAAATG CTAAGTTTCTTAC) primers from His-HC-Pro TEV-infected plants (6), and then it was cloned into SalI-cleaved pBluescript KS. The proper orientation was determined, and then the BInMyC-Sall fragment was moved into Bin61S.

Preparation of viral and suppressor extracts. To prepare protein extract, 0.25 g leaf tissue was ground in 1 ml mand blight buffer (83 mM Tris–HCl, pH 7.5, 0.8 mM MgCl2, 66 mM KCl, 100 mM NaCl, and 10 mM dithiothreitol), and then this crude extract was centrifuged twice for 15 min at 15,000 × g (39). The supernatant was frozen in aliquots at −70°C. Viral extracts were prepared from strongly symptomatic leaves after the accumulation of viral RNAs was confirmed by RNA gel blot assays (data not shown).

Preparation of labeled dsRNA probes. Synthetic sRNAs were labeled with T4 polynucleotide kinase, and then the polynucleotide kinase was heat inactivated. To generate ds-sRNAs, unlabeled 5’-phosphorylated complementary-strand sRNAs in twofold molar excess were added to labeled single-stranded sRNAs, and then the mixture of labeled and unlabeled single-stranded sRNAs was heated for 94°C (1 min) and allowed to cool down slowly to room temperature. [α-32P]UTP-labeled in vitro RNA transcripts were used as long RNA probes. Labeled transcripts were produced from an M13-Forward/M13Reverse pBluescript KS PCR fragment of the T7 and T3 RNA polymerase, respectively. To generate long ds-sRNAs, a 1:1 mixture of labeled T7 and T3 in vitro transcripts was heated (1 min, 95°C) and annealed (2 h, 37°C). To remove single-stranded overhangs, annealed long ds-sRNAs were treated with RNAse A, extracted with phenol-chloroform, and precipitated. Labeled, annealed long ds-sRNAs were redissolved and diluted.

Gel mobility shift assay. In a binding reaction, labeled dsRNA (0.5 nM) was incubated with extract containing ~2 μg total protein. Binding reactions, mobility shift assays, and direct competition assays were carried out as described (39).
54) except that 0.02% Tween 20 was added to the binding buffer; 0.2 μg total protein was used for P15, TEV HC-Pro, and TCV CP competition experiments. Supershift assays were performed by adding 1 μg antibody (anti-c-Myc-peroxidase, Roche 1814150; anti-His, Amersham 274710-01; anti-HA, Roche 1583816) to the suppressor extracts 30 min before incubation with ds-sRNA probes.}

RESULTS

TCV CP binds long dsRNA in vitro and prevents sRNA accumulation in hairpin transcript-induced silencing assays. Theoretically, plant viruses can interfere with the silencing response by sequestering two different dsRNA components of the silencing system, long dsRNAs and ds-sRNAs. Long-dsRNA-binding proteins might compete with DCLs, thus reducing or delaying the accumulation of ds-sRNAs. Indeed, we have previously shown that PoLV P14 binds long dsRNA in vitro and prevents the accumulation of sRNA in hairpin-induced silencing assays. To test whether other viruses also express long-dsRNA-binding proteins, we prepared crude extracts (viral extracts) from plants which were infected with TAV, TEV, or wheat leaves (BSMV). Extracts prepared from mock-inoculated plants (−) and extracts isolated from plants that were infected with PΔ14, a mutant PoLV that was unable to express P14 suppressor protein, were used as negative controls. (B) Plant-expressed TCV CP binds long ds-sRNA. Extracts were prepared from noninoculated (−) N. benthamiana leaves and from leaves which were infiltrated with TCV CP (CP), BYV P21 (P21), PCV P15 (P15), TEV HC-Pro (HC-TEV), BSMV yB (yB), or reovirus Sigma3 (Reo). P14 and Reo were used as positive controls. Reo strongly binds long dsRNA but does not bind ds-sRNA. (C) High- and low-molecular-weight RNA gel blot analyses were carried out to study the effect of TCV CP and BYV P21 on accumulation of hairpin transcripts, GFP mRNAs, and sRNAs. N. benthamiana leaves were infiltrated (GFP-IR+35SGFP) with 35SGFP and with TCV CP (CP) or with BYV P21 (P21). GFP-ir indicates hairpin transcripts derived from GFP-IR, while GFP refers to GFP mRNA transcribed from 35SGFP (upper panel). Note that the probe we used for sRNA hybridization (bottom panel) detected both hairpin transcript- and GFP mRNA-derived sRNAs (GFP sRNA). (D) Effect of TCV CP and BYV P21 on accumulation of hairpin transcripts and hairpin-derived sRNAs. N. benthamiana leaves were infiltrated with GFP-IR (−) or cotransfected with GFP-IR and with TCV CP (CP) or with P21 (P21). Samples were taken at 3 d.p.i.
binding activity of suppressor extracts of other viruses (BYV P21, PCV P15, TEV HC-Pro, and BSMV γB) was also tested. PoLV P14 and reovirus Sigma3 were used as positive controls. As Fig. 1B shows, in addition to the positive controls, only the TCV CP extract bound long dsRNAs (Fig. 1B, lane 3). Moreover, the binding characteristics of TCV viral and CP suppressor extracts were similar. Both extracts caused accumulation of distinct shifted bands, suggesting that more than one CP molecule could bind to a single dsRNA.

To confirm that TCV CP also binds long dsRNA in vivo, we analyzed the effect of TCV CP on sRNA accumulation in hairpin-induced silencing assays. As a control, the BYV P21 suppressor was used. Coinfiltration of an Agrobacterium sp. that expresses GFP (GFP-IR) with another Agrobacterium sp. that expresses GFP (GFP-IR+35S) prevents transient GFP activity (Fig. 1C, upper panel), because GFP-IR-derived hairpin transcripts (GFP-ir) are rapidly processed to sRNAs, and then these sRNAs direct early degradation of GFP mRNAs (hairpin-induced silencing) (39). Coinfiltration of GFP-IR+35SGFP with the TCV CP or P21 suppressor resulted in strong green fluorescence (data not shown) and led to the accumulation of GFP mRNAs (Fig. 1C), indicating that both suppressors inhibited hairpin-induced silencing.

However, CP and P21 inhibit hairpin-induced silencing differently. In line with previous results (11, 43, 45), we found that in TCV CP-cointfiltrated samples GFP-ir accumulated to easily detectable levels, while sRNAs could not be detected (Fig. 1C). By contrast, in P21-cointfiltrated leaves GFP-ir transcripts could not be detected, whereas sRNAs were abundant (Fig. 1C). These data suggest that TCV CP binds hairpin transcripts, the long dsRNA precursors of sRNAs. These data are also consistent with previous suggestions that P21 suppresses silencing by binding hairpin-derived ds-sRNAs (11).

To further support the idea that CP directly inhibits hairpin-derived sRNA accumulation and that P21 is unable to prevent sRNA generation from hairpin transcripts, we cointfiltrated leaves with GFP-IR and with CP or with P21. As expected in GFP-IR- and CP-cointfiltrated leaves hairpin transcripts were detected, while sRNAs could not be found. By contrast, in GFP-IR- and P21-cointfiltrated samples sRNA accumulated to high levels, while hairpin transcripts could not be detected (Fig. 1D).

We also tested the effect of some other suppressors that were unable to bind long dsRNAs (PCV P15, TEV HC-Pro, and BSMV γB) in hairpin-induced silencing assays. We found that these suppressors operated like P21; they inhibited hairpin-induced silencing but failed to prevent the accumulation of hairpin-derived sRNAs (data not shown). These data suggest that the PCV P15, TEV HC-Pro, and BSMV γB suppressors, like P21, target hairpin-induced silencing downstream from the sRNA generation step.

Many plant viral silencing suppressors bind 21-nt ds-sRNA. Plant viruses can also inhibit silencing by expressing ds-sRNA-binding proteins. To test if ds-sRNA binding is a general silencing suppression strategy, the 21-nt ds-sRNA-binding activity of different viral extracts was analyzed.

As Fig. 2A shows, ds-sRNA-binding activity was identified in many but not in all viral extracts. Confirming our previous results (39), we could detect 21-nt ds-sRNA-binding activity in CymRSV and PoLV extracts (Fig. 2A, lanes 2 and 4) but not in extract that was isolated from mock inoculated plants (Fig. 2A, lane 1). Importantly, 21-nt ds-sRNA-binding activity could be also identified in BYV, PCV, TCV, TMV, TEV, and BSMV extracts (Fig. 2A, lanes 5, 6, 7, 13, 15 and 16). By contrast, extracts prepared from leaves infected with PVY, PVX, CMV, and TAV failed to bind ds-sRNA (Fig. 2A, lanes 8, 9, 12, and 14). Different viral extracts caused different mobility shifts, and therefore we concluded that the ds-sRNA-binding proteins were expressed by the viruses rather than by the infected hosts.

To test if the suppressors provided the ds-sRNA-binding activity for the viral extracts, the ds-sRNA-binding activity of CymRSV P19, PoLV P14, PCV P15, TCV CP, BYV P21, BSMV γB, and TEV HC-Pro suppressor extracts was analyzed. As controls, the ds-sRNA-binding activities of suppressor extracts of viruses (PVY HC-Pro, TAV 2b, and PVX P25) whose viral extracts did not contain ds-sRNA-binding activity were also tested. We found that PVY HC-Pro, TAV 2b, and PVX P25 suppressor extracts failed to form complexes with ds-sRNA (Fig. 2B, lanes 8, 10, and 11). Importantly, P19, P14, P15, TCV CP, P21, γB, and TEV HC-Pro extracts could bind ds-sRNAs (Fig. 2B). Moreover, similar shifts were obtained with the corresponding viral and suppressor extracts. These data suggest that (in addition to tombusvirus P19, aureusvirus P14, and closterovirus P21) TCV CP, PCV P15, BSMV γB, and TEV HC-Pro are also ds-sRNA-binding silencing suppressors and that the respective suppressors provided the 21-nt ds-sRNA-binding activity for both viral and suppressor extracts.

An unlikely alternative explanation would be that these suppressors activate host-encoded ds-sRNA-binding proteins in the infected cells and in the infiltrated patches. To distinguish between these two possibilities, antibody supershift assays were carried out with extracts isolated from leaves, in which epitope-tagged suppressors were expressed. Extracts were prepared from leaves that were infiltrated with agrobacteria expressing the HA-tagged P21 (P21-HA) or His-tagged TEV HC-Pro (His-TEV) suppressor and then the 21-nt ds-sRNA-binding activity of these extracts was tested in the absence of antibody, in the presence of HA antibodies, and in the presence of His antibodies.

In the absence of antibody, both the P21-HA and His-TEV extracts efficiently bound 21-nt ds-sRNA. As Fig. 2C shows, HA antibodies but not His antibodies caused a supershift on P21-HA extracts, while His antibodies but not HA antibodies resulted in a supershift on His-TEV extracts. Supershift assays were also conducted with extracts prepared from leaves in which Myc-tagged TCV CP (Myc-CP), P15 (Myc-P15), or BSMV γB (Myc-γB) suppressors were expressed. In the absence of antibody, the Myc-CP and Myc-γB extracts efficiently bound 21-nt ds-sRNA, whereas the Myc-P15 extract weakly bound 21-nt ds-sRNA. As Fig. 2D shows, the presence of Myc but not His antibodies resulted in strong supershifts for all three extracts (compare Fig. 2D, lane 2 to 3, lane 5 to 6, and lane 8 to 9). These data indicate that the suppressor proteins themselves bound the 21-nt ds-sRNA in the suppressor extracts.

P19 binds ds-sRNA as a dimer (62, 68). As other suppressor–ds-sRNA complexes (P14, P15, P21, etc.) also migrated more slowly than expected from the monomer, it is possible that...
these proteins also bind ds-sRNAs as dimers or as oligomers.

As the TCV CP and P14 suppressors bind both long dsRNAs and 21-nt ds-sRNAs, they will be referred to as size-independent dsRNA-binding suppressors. The P19, P21, P15, BSMV γB, and TEV HC-Pro suppressors bind 21-nt ds-sRNA but fail to bind long dsRNAs, and therefore these proteins will be referred to as size-selective ds-sRNA-binding suppressors. We could also detect a size-selective ds-sRNA-binding activity in TMV extract. As the replicate proteins are supposed to act as silencing suppressors in tobamoviruses (16, 30), we postulate that the TMV replicate is also a size-selective ds-sRNA-binding silencing suppressor.

We failed to detect dsRNA-binding activity in the extracts of PVX, CMV, TAV, and PVY. It is possible that (some of) these viruses also express dsRNA-binding protein, but these proteins are not present in an active form in either the viral or suppressor extract. For instance, it is likely that different HC-Pro suppressors inhibit silencing by targeting an identical step. Indeed, like TEV HC-Pro, *Turnip mosaic virus* HC-Pro is also a ds-sRNA-binding protein, although it binds ds-sRNA relatively weakly (E. Chapman and J. Carrington, personal communication). Therefore we assume that PVY HC-Pro could be a ds-sRNA-binding suppressor.

**P21 and P15 suppressors bind 21-nt ds-sRNAs preferentially.** In direct competition assays P14 binds both 26-nt ds-sRNA (having 24-nt duplex and 2-nt 3′ overhangs) and 21-nt ds-sRNA (having 19-nt duplex region and 2-nt 3′ overhangs), while P19 preferentially binds 21-nt ds-sRNA (39, 62, 68). As suppressors with different ds-sRNA-binding preferences can interfere with short- and long-sRNA-mediated silencing pathways differently (4, 62), we wanted to define whether other viral suppressors could discriminate between 21- and 26-nt ds-sRNAs.

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**FIG. 2.** Many plant viral silencing suppressors can bind ds-sRNA. (A) ds-sRNA-binding activity of plant viral proteins was studied in gel mobility shift assays. Labeled synthetic 21-nt RNAs were annealed to form 21-nt ds-sRNAs (having a 19-nt duplex region and 2-nt 3′ overhangs) and used as probes in binding studies. The 21-nt ds-sRNAs were incubated with crude viral extract. Extracts prepared from mock-inoculated (−) plants and extracts isolated from plants that were infected with PΔ14, a mutant PoLV that was unable to express P14, were used as negative controls. The ds-sRNA-binding activity of extracts prepared from PVX P14 and PVX P19 recombinant PVXs, which express the P14 and P19 suppressor, respectively, shows that ds-sRNA-binding proteins are also active if they are expressed by heterologous viruses. * indicates a host-encoded nonspecific ds-sRNA-binding activity that can be frequently detected in extracts from wild-type, mock-inoculated, agroinfiltrated, or virus-inoculated plants. (B) Plant-expressed viral silencing suppressors bind ds-sRNA. At 3 d.p.i. extracts were prepared from nontreated (−) *N. benthamiana* leaves and from leaves which were infiltrated with CymRSV P19 (P19), PoLV P14 (P14), PCV P15 (P15), TCV CP (CP), BYV P21 (P21), BSMV γB (γB), PVY HC-Pro (HC-PVY), TEV HC-Pro (HC-TEV), TAV 2b, or PVX P25 (P25). Suppressor extracts were probed with labeled 21-nt ds-sRNAs. (C and D) The suppressor proteins are the 21-nt ds-sRNA-binding factors in the suppressor extracts. Epitope-tagged suppressors were expressed in *N. benthamiana* leaves. To carry out antibody supershift assays, suppressor extracts were incubated with labeled 21-nt ds-sRNAs in the absence of antibody or in the presence of antibody specific against the respective epitope or in the presence of heterologous antibody. Note that Myc antibody-supershifted protein–ds-sRNA complexes stayed in the wells.
To this aim, direct competition assays were carried out with BYV P21, PCV P15, and TCV CP suppressor extracts. Labeled 26-nt ds-sRNAs were incubated with suppressor extracts and with increasing molar concentrations of unlabeled 21-nt and 26-nt ds-sRNA competitors (Fig. 3A, B, and C, upper panels). Competition experiments were repeated with labeled 21-nt ds-sRNAs (Fig. 3A, B, and C, bottom panels). As Fig. 3 shows, TCV CP bound ds-sRNA without obvious size specificity, because approximately the same molar excess of unlabeled 21-nt ds-sRNAs and 26-nt ds-sRNAs was required to outcompete either labeled 21- or 26-nt ds-sRNA (Fig. 3C). By contrast, P21 and P15 extracts bound 21-nt ds-sRNAs preferentially, and in both cases the 21-nt ds-sRNAs were much more efficient unlabeled competitors than the 26-nt ds-sRNAs (Fig. 3A and B).

It has been reported that *Escherichia coli*-expressed P21 is a general nucleic acid binding protein whose binding affinity increases with the length of the RNA (69). By contrast, our binding experiments strongly suggest that in planta-expressed P21 is a size-selective dsRNA-binding protein. As at a low P21-to-RNA ratio bacterially expressed P21 also acts as size-specific ds-sRNA-binding protein (Chapman and Carrington, personal communication), it is likely that these apparently conflicting results might be due to different assay conditions. Moreover, hairpin-inducing silencing experiments (Fig. 1C and D) suggest that in plant cells P21 fails to bind long dsRNAs but binds ds-sRNAs.

**Suppressors show different structural requirements for ds-sRNA binding.** P19 binds ds-sRNAs as a homodimer. The central and C-terminal β-strands of P19 contact the stem of the RNA (69). By contrast, our binding experiments strongly suggest that in planta-expressed P21 is a size-selective dsRNA-binding protein. As at a low P21-to-RNA ratio bacterially expressed P21 also acts as size-specific ds-sRNA-binding protein (Chapman and Carrington, personal communication), it is likely that these apparently conflicting results might be due to different assay conditions. Moreover, hairpin-inducing silencing experiments (Fig. 1C and D) suggest that in plant cells P21 fails to bind long dsRNAs but binds ds-sRNAs.

The P14 and TCV CP suppressors bound 19-nt dsRNAs and 21-nt ds-sRNAs equally well (compare Fig. 2B, lanes 3 and 5, with Fig. 4A, lanes 3 and 5), suggesting that these suppressors did not require 3′ overhangs for ds-sRNA binding. By contrast, the PCV P15 and BSMV γB extracts failed to bind 19-nt dsRNA (Fig. 4A, lanes 4 and 7), suggesting that the P15 and γB suppressors required the presence of 3′ overhangs for efficient ds-sRNA binding. To confirm these conclusions direct competition assays were carried out.

First, P19 and TEV HC-Pro extracts were studied to validate the system. We found that P19 extract bound 19-nt ds-sRNA preferentially (Fig. 4B), while TEV HC-Pro extract formed complexes more efficiently with 21-nt ds-sRNA (Fig. 4C). These findings were in line with the results obtained with purified P19 (62, 68) and TEV HC-Pro proteins (L. Lakatos et al., submitted for publication), indicating that suppressor extract can be used to define whether a suppressor requires 3′ overhangs for efficient ds-sRNA bindings or not. As Fig. 4 shows, competition assays confirmed that P14 and TCV CP bound 19-nt dsRNAs and 21-nt ds-sRNAs with similar efficiency (Fig. 4D and E) and that PCV P15 and BSMV γB extracts bound 21-nt ds-sRNAs much more efficiently than 19-nt ds-sRNAs (Fig. 4F and G). These data suggest that TCV
CP and P14 interact with the stem but not with the ends of ds-sRNAs. By contrast, the P15 and γB suppressors might interact directly with the 3' 2-nt overhangs of ds-sRNAs as these proteins require the presence of 3' overhangs for efficient ds-sRNA binding.

DISCUSSION

Here we show that many unrelated plant viral RNA silencing suppressors bind dsRNAs. These data strongly support the hypothesis that dsRNA binding is a widely used silencing suppression strategy which has evolved independently many times.

dsRNA-binding RNA silencing suppressors. In vitro binding studies revealed that extracts of CymRSV, PoLV, BYV, TEV, TMV, PCV, and BSMV plant RNA viruses bind dsRNAs. As in each case studied the suppressor extract binds dsRNAs similarly to the viral extract, we concluded that the silencing suppressors themselves form complexes with dsRNAs. The findings that the purified P19 (31, 54, 62, 68), P14 (39), P21 (11, 69), and TEV HC-Pro (L. Lakatos et al., submitted for publication) proteins can bind ds-sRNAs in vitro and that antibodies specifically affect 21-nt ds-sRNA binding of tagged suppressor extracts (Fig. 2C and D) support this conclusion.

dsRNA-binding silencing suppressors fall into two groups. P14 and TCV CP are size-independent dsRNA-binding proteins, while P19, P21, P15, BSMV γB, and TEV HC-Pro (and perhaps TMV replicase) are size-selective ds-sRNA-binding suppressors. Size selection is very effective; all size-selective suppressors tested, P19 (62), P21 and P15 (this study), and BSMV γB (Mérai et al., unpublished data), bind 21-nt ds-sRNAs more efficiently than 26-nt ds-sRNAs. Therefore we postulate that many if not all size-selective ds-sRNA-binding suppressors can discriminate between short and long sRNAs.

Competitive dsRNA-binding-based silencing suppression. Our findings that many unrelated suppressors bind dsRNAs suggest that dsRNA binding is a key element of silencing...
suppression. We propose that dsRNA-binding suppressors inhibit silencing by sequestering a dsRNA component of the silencing response and that size-selective and size-independent dsRNA-binding suppressors target different dsRNAs.

In plants, silencing generates only 21-nt ds-sRNAs from RNA viruses (40, 53), and hence expression of a protein that preferentially binds these molecules appears to be an ideal viral counterdefensive strategy. We suggest that size-selective dsRNA-binding suppressors interfere with silencing by sequestering ds-sRNAs, preventing the formation of active silencing effector complexes. Many different observations support the idea that all these suppressors act by binding ds-sRNAs. For instance, in the GFP-IR cofiltration assay the P19, P21, P15, BSMV γB, and TEV HC-Pro suppressors act similarly, and these proteins inhibit hairpin-induced silencing but fail to prevent ds-sRNA generation. Moreover, transgenic expression of P19, P21, HC-Pro, and P15 results in similar phenotypes (11, 19), likely because the endogenous sRNA (microRNA)-mediated silencing pathways were inhibited in each transgenic plant.

The binding data suggest that the P19, P21, HC-Pro, and P15 suppressors inhibit the formation of active RISCs in transgenic plants by binding the ds-sRNA intermediate molecules of microRNA biogenesis. Finally, the findings that in heterologous (Drosophila melanogaster or mammalian) systems, the P19 (31), P15 (19), P21 (L. Lakatos et al., submitted for publication), and TEV HC-Pro (46) suppressors inhibit both dsRNA- and ds-sRNA-induced silencing are also consistent with suggestions that these proteins act by sequestering ds-sRNAs. It has been experimentally proven that P19 can sequester viral ds-sRNAs in CymRSV-infected cells (31). Therefore, it is likely that other size-selective ds-sRNA-binding suppressors also inhibit virus-induced silencing by preventing the incorporation of viral sRNAs into effector complexes. Indeed, it has been shown that HC-Pro can also bind viral sRNAs in TEV-infected plants (L. Lakatos et al., submitted for publication).

We showed that TCV CP, like PoLV P14, is a size-independent dsRNA-binding protein. This explains apparently conflicting previous observations that CP prevents ds-sRNA accumulation in hairpin-induced silencing assays (11, 43, 45), while it suppresses ds-sRNA-induced silencing in mammalian cells (19). We suggest that size-independent dsRNA-binding suppressors as TCV CP and P14 prevent the generation of sRNAs from hairpin transcripts because they compete with DCLs for long dsRNAs, and they inhibit ds-sRNA-mediated silencing because they can also sequester ds-sRNAs. It is still not known if CP or P14 suppresses silencing by sequestering viral precursors of ds-sRNAs in virus-infected cells or inhibit silencing by binding ds-sRNAs.

As silencing also plays an antiviral role in animals, it is likely that many animal viruses suppress silencing by expressing dsRNA-binding proteins (32). Indeed, it has been demonstrated that size-independent dsRNA-binding proteins could act as silencing suppressors in insect and mammalian cells (5, 9, 14, 28, 33, 55). The flock house virus B2 suppressor is a dsRNA-binding protein which interacts with the backbone of dsRNA as a dimer (10, 35). B2 binds longer dsRNA more efficiently than ds-sRNA and inhibits antiviral silencing in C. elegans upstream of AGO, likely by binding the precursor of ds-sRNA (38). Thus, the molecular basis of silencing suppression might be similar for TCV CP, PoLV P14, and B2. Interestingly, a size-specific ds-sRNA-binding animal viral silencing suppressor has not been identified yet.

Evolution of dsRNA-binding silencing suppressors. Many dsRNA-binding suppressors are evolutionarily unrelated, suggesting that dsRNA-binding silencing suppressors have evolved independently many times. Convergent evolution results in remarkably similar dsRNA-binding characteristics of unrelated suppressors. For instance, both BSMV γB and TEV HC-Pro are size-selective ds-sRNA-binding silencing suppressors that require the presence of 3′ overhangs for efficient ds-sRNA-binding even though no significant sequence similarity can be found between these two proteins. Moreover, BSMV γB is a small protein whose only function is silencing inhibition, while TEV HC-Pro is a multifunctional protein.

Interestingly, although BSMV γB and HC-Pro are very dissimilar, both suppressors contain a cysteine-rich region that might form a zinc finger motif (1, 7, 17, 29, 52). It is tempting to speculate that zinc finger domains play important roles in the ds-sRNA binding of BSMV γB and HC-Pro. Moreover, predicted zinc fingers were also identified in suppressors which are distantly related to BSMV γB, such as PCV P15 (20), furovirus P19 (58), and tobravirus P16 (29, 46, 70), and in other suppressor proteins which are not related to either BSMV γB or HC-Pro, such as P23 of Citrus tristeza virus, a closterovirus (36, 37), or P10 of Grapevine virus A, a viivirus (13, 23). These zinc-finger-containing suppressors could also act by binding dsRNAs size independently or size selectively. Indeed, it was shown that citrus tristeza virus P23 binds dsRNA in vitro, although its size selectivity was not studied. Of course, not all dsRNA-binding suppressors contain zinc fingers: for example, zinc fingers could not be identified in P19, P21, or TCV CP.

Suppressors have evolved to target silencing with as little damage as possible to the host. Size-selective ds-sRNA binding is likely the result of such a dual selection, and these suppressors sequester sRNAs that play a role in antiviral response, while they do not interfere with endogenous long ds-sRNAs or long structured RNAs (4, 62). Due to this remarkable specificity of these suppressors, they can be expressed in all stages of infections. Interestingly, the expression of P14 and TCV CP size-independent silencing suppressors in infected plants might be downregulated by the virus (49, 71). The level of Sg2 RNA, from which P14 is translated, declines after 2 to 3 d.p.i. (39, 49, 50). In TCV-infected cells, the formation of capsid reduces available suppressor levels. It has been proposed that these are viral strategies to reduce symptoms, because infection with a mutant PoLV which expresses P14 constitutively or with a mutant TCV which expresses CP that fails to form capsid results in strong symptom accentuation (49, 71).

One can speculate that ancient dsRNA-binding suppressors were size-independent dsRNA-binding proteins that evolved in two ways to reduce unnecessary host damage. They could evolve into size-selective ds-sRNA-binding proteins or control the expression of size-independent dsRNA-binding suppressors.

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REFERENCES

1. Atreya, C. D., P. L. Atreya, D. W. Thornbury, and T. P. Pirone. 1992. Site-directed mutations in the potyvirus HC-Pro gene affect helper component activity, virus accumulation, and symptom expression in infected tobacco plants. Virology 191:106–111.

2. Baulcombe, D. 2005. RNA silencing. Trends Biochem. Sci. 30:290–293.

3. Baulcombe, D. 2004. RNA silencing in plants. Nature 431:356–363.

4. Baulcombe, D. C., and A. Molnar. 2004. Crystal structure of p19—a universal suppressor of RNA silencing. Trends Biochem. Sci. 29:279–281.

5. Bennesser, Y., S. Y. Le, M. Benkirane, and K. T. Jeang. 2005. Evidence that HIV-1 encodes an siRNA and a suppressor of RNA silencing. Immunity 22:607–619.

6. Blanco, S., V. W. Dolja, C. Llave, and T. P. Pirone. 1999. Histidine-tagging and purification of tobacco etch potyvirus helper component protein. J. Virol. Methods 77:11–15.

7. Bragg, J. N., D. M. Lawrence, and A. O. Jackson. 2004. The N-terminal 85 amino acids of the barley stripe mosaic virus Yb-pathogenesis protein contain functional RNA silencing suppressor activity. J. Virol. 78:3779–3785.

8. Brigneti, G., O. Voinnet, W. X. Li, L. H. Ji, S. W. Ding, and D. C. Baulcombe. 2003. Identification, subcellular localization and some properties of a cysteine-rich suppressor of gene silencing encoded by peanut clump viroid. J. Gen. Virol. 84:771–780.

9. Bennasser, Y., S. Y. Le, M. Benkirane, and K. T. Jeang. 2005. Evidence that HIV-1 encodes an siRNA and a suppressor of RNA silencing. Immunity 22:607–619.

10. Blouin, A., C. A. Mallory, D. P. Bartel, and H. Vauchere. 2005. Partially redundant functions of Arabidopsis DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs.Curr. Biol. 15:1494–1500.

11. Hamilton, A., O. Voinnet, L. Chappell, and D. Baulcombe. 2002. Two classes of virus-encoded RNA silencing suppressors. EMBO J. 21:4672–4680.

12. Harsányi, A., B. Rodki, K. Roka, A. Almasi, and R. Gaborjany. 2012. Abnormal etioplast development in barley seedlings infected with BSMV by seed transmission. Physiol. Plant. 141:143–155.

13. Himber, C., P. Duttenhofer, G. Moussallem, C. Renzenthaler, and O. Voinnet. 2003. Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. EMBO J. 22:4523–4533.

14. Johnson, K. L., B. D. Price, L. D. Eckerle, and L. A. Ball. 2004. Nodamura virus structural protein B2 encodes an RNA silencing suppressor that is essential for virus accumulation in both mammalian and insect cells. J. Virol. 78:6698–6704.

15. Koonin, E. V., V. P. Boyko, and V. V. Dolja. 1991. Small cysteine-rich proteins of different groups of plant RNA viruses are related to different families of nucleic acid-binding proteins. EMBO J. 10:2387–2393.

16. Kubota, K., S. Tsudo, A. Tamai, and T. Meshi. 2003. Tomato mosaic virus replication protein suppresses virus-targeted posttranscriptional gene silencing. J. Virol. 77:11016–11026.

17. Lakatos, L., G. Szittya, D. Silhavy, and J. Burgyán. 2004. Molecular mechanism of RNA silencing suppression mediated by p19 protein of tobravirus. EMBO J. 23:8674–8684.

18. Li, H. W., and S. W. Ding. 2005. Antiviral silencing in animals. FEBS Lett. 577:635–679.

19. Lu, X. W., H. Li, R. Lu, F. Li, M. Dus, P. Atkinson, E. W. Brydon, K. L. Johnson, A. Garcia-Sastre, L. A. Ball, P. Palese, and S. W. Ding. 2004. Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing. Proc. Natl. Acad. Sci. USA 101:1355–1359.

20. Lichten, Z., D. Silhavy, and J. Burgyán. 2003. Double stranded RNA-binding proteins could suppress RNA interference-mediated antiviral defenses. J. Gen. Virol. 84:975–980.

21. Lingel, A., B. Simon, E. Izaurralde, and M. Sattler. 2005. The structure of the flexuous virus B2 protein, a viral suppressor of RNA interference, shows a novel mode of double-stranded RNA recognition. EMBO Rep. 6:1149–1155.

22. Lopez, C., J. Navas-Castillo, S. Gowda, P. Moreno, and R. Flores. 2000. The 23-kDa protein coded by the TBSV terminal gene of citrus tristeza virus is a RNA-binding protein. Virology 269:462–470.

23. Meade, K., and C. J. Carrington. 2004. RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. Genes Dev. 18:179–186.

24. Moazed, D., and L. P. Burgin. 1991. Small cysteine-rich proteins of plant viruses and their role in viral infections. FEBS Lett. 290–293.

25. Merai, Z., Z. Kerenyi, A. Molnar, E. Barta, A. Valoczi, G. Bisztray, Z. Havelda, J. Burgyán, and D. Silhavy. 2005. Acreusivirus P14 is an efficient RNA silencing suppressor that binds double-stranded RNAs without size specificity. J. Virol. 79:7217–7226.

26. Meza, T., C. Sorbo, L. Lakatos, E. Varlalay, C. Lacomme, and J. Burgyán. 2005. Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. J. Virol. 79:7812–7818.

27. Prokhnknev, A. L., V. V. Peremenyslov, A. J. Napulí, and V. V. Dolja. 2002. Interaction between long-distance transport factor and H70-related movement protein of Beet Yellows Virus. J. Virol. 76:11003–11011.

28. Qi, Y., and G. J. Hannon. 2005. Uncovering RNAi mechanisms in plants: biochemistry enters the fray. FEBS Lett. 579:589–593.

29. Qi, Y., X. Zhong, A. Haya, and B. Ding. 2005. Dissecting RNA silencing in protoplasts uncovers novel effects of viral effectors on plant silencing at the pathway level. J. Cellular. Nucleic Acids Res. 32:e179.

30. Qui, F., and T. J. Morris. 2005. Suppressors of RNA silencing encoded by plant viruses and their role in viral infections. FEBS Lett. 579:595–596.

31. Qu, F., T. Ren, and T. J. Morris. 2003. The coat protein of turnip crinklw virus suppresses posttranscriptional gene silencing at an early initiation step. J. Virol. 77:511–522.

32. Reed, J. C., D. K. Kasschau, A. I. Prokhnevsky, K. Gopinath, G. P. Pogue, J. C. Carrington, and V. V. Dolja. 2003. Suppressor of RNA silencing encoded by beet Yellows virus B2 protein, a viral suppressor of RNA interference, shows a novel mode of double-stranded RNA recognition. EMBO Rep. 6:1149–1155.

33. Reed, J. C., J. A. Carrington, and V. V. Dolja. 2004. Evidence that BYV encodes a nucleic acid-binding protein and affects pathogenesis. Virus Genes 27:257–262.
52. Shi, X. M., H. Miller, J. Verchot, J. C. Carrington, and V. B. Vance. 1997. Mutations in the region encoding the central domain of helper component-proteinase (HC-Pro) eliminate potato virus X/potyviral synergism. Virology 231:35–42.

53. Silhavy, D., and J. Burgyan. 2004. Effects and side-effects of viral RNA silencing suppressors on short RNAs. Trends Plant Sci. 9:76–83.

54. Silhavy, D., A. Molnar, A. Lucioli, G. Szittya, C. Hornyik, M. Tavazza, and J. Burgyan. 2002. A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. EMBO J. 21:3070–3080.

55. Sullivan, C. S., and D. Ganem. 2005. A virus-encoded inhibitor that blocks RNA interference in mammalian cells. J. Virol. 79:7371–7379.

56. Szilassy, D., K. Salanki, and E. Balazs. 1999. Molecular evidence for the existence of two distinct subgroups in cucumber mosaic cucumovirus. Virus Genes 18:221–227.

57. Szittya, G., A. Molnar, D. Silhavy, C. Hornyik, and J. Burgyan. 2002. Short defective interfering RNAs of tombusviruses are not targeted but trigger post-transcriptional gene silencing against their helper virus. Plant Cell 14:359–372.

58. Te, J., U. Melcher, A. Howard, and J. Verchot-Lubicz. 2005. Soilborne wheat mosaic virus (SBWMV) 19K protein belongs to a class of cysteine rich proteins that suppress RNA silencing. Virol. J. 2:18.

59. Thole, V., T. Dalmay, J. Burgyan, and E. Balazs. 1993. Cloning and sequencing of potato virus Y (Hungarian isolate) genomic RNA. Gene 123:149–156.

60. Thomas, C. L., V. Leh, C. Lederer, and A. J. Maule. 2003. Turnip crinkle virus coat protein mediates suppression of RNA silencing in Nicotiana benthamiana. Virology 306:33–41.

61. Tomari, Y., and P. D. Zamore. 2005. Perspective: machines for RNAi. Genes Dev. 19:517–529.

62. Vargason, J., G. Szittya, J. Burgyan, and T. M. Hall. 2003. Size-selective recognition of siRNA by an RNA silencing suppressor. Cell 115:799–811.

63. Voinnet, O. 2005. Induction and suppression of RNA silencing: insights from viral infections. Nat. Rev. Genet. 6:206–220.

64. Voinnet, O., Y. M. Pinto, and D. C. Baulcombe. 1999. Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. Proc. Natl. Acad. Sci. USA 96:14147–14152.

65. Wang, M. B., and M. Metzlaff. 2005. RNA silencing and antiviral defense in plants. Curr. Opin. Plant Biol. 8:216–222.

66. Wasson, D. G. 2005. The role of the RNAi machinery in heterochromatin formation. Cell 122:13–16.

67. Xie, Z., L. K. Johansen, A. M. Gustafson, K. D. Kasschau, A. D. Lellis, D. Zielberman, S. E. Jacobsen, and J. C. Carrington. 2004. Genetic and functional diversification of small RNA pathways in plants. PLoS Biol. 2:E104.

68. Ye, K., L. Malinina, and D. J. Patel. 2003. Recognition of small interfering RNA by a viral suppressor of RNA silencing. Nature 426:874–878.

69. Ye, K., and D. J. Patel. 2005. RNA silencing suppressor p21 of beet yellows virus forms an RNA binding octameric ring structure. Structure 13:1375–1384.

70. Yelina, N. E., T. N. Erokhina, N. I. Lakhovitskaya, E. A. Minina, M. V. Schepetilnikov, D. E. Lesemann, J. Schiemann, A. G. Solovyev, and S. Y. Morozov. 2005. Localization of Poa semilatent virus cysteine-rich protein in peroxisomes is dispensable for its ability to suppress RNA silencing. J. Gen. Virol. 86:479–489.

71. Zhang, F., and A. E. Simon. 2003. Enhanced viral pathogenesis associated with a virulent mutant virus or a virulent satellite RNA correlates with reduced virion accumulation and abundance of free coat protein. Virology 312:8–13.

72. Zilberman, D., X. Cao, and S. E. Jacobsen. 2003. ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. Science 299:716–719.