

**Short Communication**    **Double-stranded RNA-binding proteins could suppress RNA interference-mediated antiviral defences**

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**RNA interference (RNAi) is a double-stranded (ds)RNA-inducible, sequence-specific RNA-degradation mechanism that operates as a natural antiviral system in plants and animals. Successful virus infection requires evasion or suppression of RNAi. Indeed, RNAi suppressor proteins have been identified in plant and animal viruses, although the molecular mechanism of silencing inhibition is still poorly understood. Because many RNA viruses encode dsRNA-binding proteins (dsRBPs) and as RNAi is triggered by the accumulation of dsRNAs, dsRBPs were examined to see if they inhibit RNAi. Here, it is shown that heterologous dsRBPs suppressed RNAi in plants, indicating that in natural host–virus interactions, pathogen-encoded dsRBPs could inactivate RNAi-mediated host defences.**

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Eukaryotes have evolved many different systems to resist virus infection. Identification of specific virus-encoded molecules or recognition of nucleic acid structures that are present only in infected cells could induce antiviral responses (Plasterk, 2002). As long double-stranded (ds)RNAs do not occur in the cytoplasm of eukaryotic cells, the accumulation of ds replicative intermediates of RNA viruses activates antiviral responses as RNA interference (RNAi) or translation inhibition and apoptosis. RNAi is an ancient defence mechanism that degrades dsRNAs and cognate mRNAs in a sequence-specific manner (Hannon, 2002; Voinnet, 2001; Zamore, 2001). Viral dsRNAs are first processed by an RNase III-like nuclease (DICER) into 21–26 nt dsRNAs (siRNAs) that guide another nuclease complex (RISC) to cleave homologous single-stranded (ss) viral RNAs. siRNAs also serve as guides for an RNA-dependent RNA polymerase to transform the target ssRNA into dsRNA (Lipardi *et al.*, 2001; Sijen *et al.*, 2001). RNAi was shown to act as an efficient antiviral system in plant (Matzke *et al.*, 2001; Vance & Vaucheret, 2001) and insect cells (Li *et al.*, 2002) and might also play an antiviral role in mammalian cells (Cullen, 2002). In higher plants, RNAi has evolved into a whole plant defence system. Cell-autonomous RNAi generates an unidentified mobile signal, thereby directing sequence-specific RNA degradation in distant tissues (Palauqui *et al.*, 1997; Voinnet & Baulcombe, 1997). To inhibit the antiviral effect of RNAi, plant (Li & Ding, 2001) and insect (Li *et al.*, 2002) viruses express different RNAi suppressor proteins. Although, the

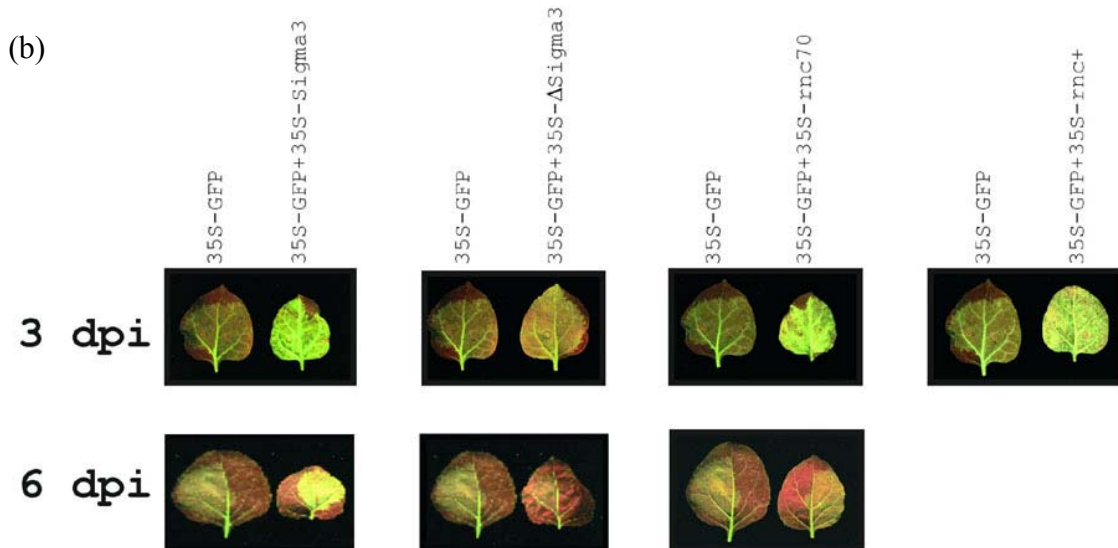
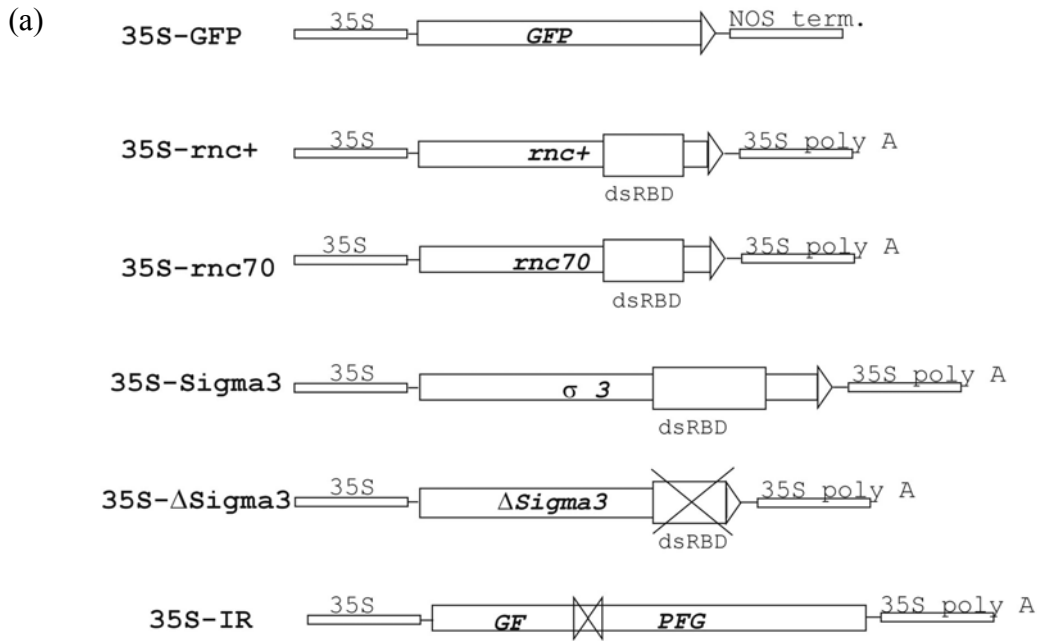
suppression of RNAi could be essential for efficient virus infection, the molecular mechanism of RNAi inhibition is still unknown.

In vertebrate cells, dsRNAs also activate RNA-dependent protein kinase (PKR)-mediated, non-specific antiviral responses, including inhibition of translation and induction of cell death. As a counterdefence strategy, many vertebrate viruses express dsRNA-binding proteins (dsRBPs) that prevent PKR activation by sequestering dsRNAs (Kaufman, 1999). As dsRNAs play a role in RNAi and since many non-vertebrate RNA viruses also express dsRBPs, it is possible that virus-encoded dsRBPs could operate as inhibitors of RNAi. To address this issue, we tested to see if dsRBPs could suppress RNAi in plants.

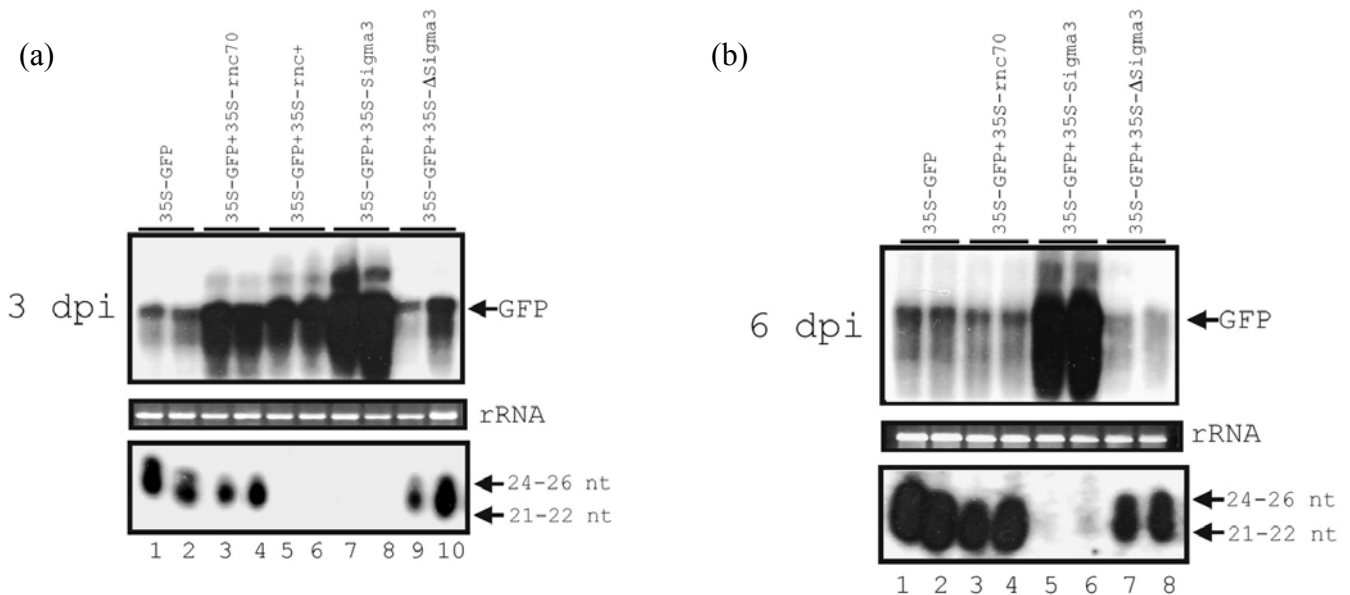
Transgene expression can also trigger RNAi. Since virus- and transgene-induced RNAi operate in overlapping pathways, virus-encoded RNAi suppressors inhibit transgene-triggered RNAi. As the mechanism of plant and animal RNAi is conserved, the *Agrobacterium tumefaciens* infiltration assay has been used to identify silencing suppressors encoded by both plant and animal viruses (Li *et al.*, 2002; Voinnet *et al.*, 1999). The infiltration of green fluorescent protein (GFP) transgenic *Nicotiana benthamiana* plants with *A. tumefaciens* carrying a vector in which the transcription of GFP is controlled by the 35S promoter (35S-GFP) not only results in transient GFP expression but also leads to the induction of GFP silencing. Cell-autonomous GFP silencing manifests as a weakening of green fluorescence, a decline in the level of GFP mRNA and an accumulation of GFP-specific siRNAs in the infiltrated patches (Brigneti *et al.*, 1998). siRNAs accumulate in two functionally different size classes. The 21–23 nt siRNA fraction guides RISC, while the 24–26 nt siRNA fraction is associated with systemic silencing (Hamilton *et al.*, 2002). If 35S-GFP is co-infiltrated with another *A. tumefaciens* expressing an RNAi suppressor, the levels of green fluorescence remain high, GFP mRNA levels do not decrease and siRNA accumulation is reduced in the infiltrated leaves (Voinnet *et al.*, 2000). *Escherichia coli* RNase III and the mammalian reovirus outer shell polypeptide  $\sigma 3$  are among the best-characterized dsRBPs; therefore, we tested the RNAi suppressor capacity of these proteins and their mutants. Both proteins carry conservative dsRNA-binding motifs and bind dsRNAs *in vitro* and *in vivo* (Dasgupta *et al.*, 1998; Denzler & Jacobs, 1994; Fierro-Monti & Mathews, 2000; Huismans & Joklik, 1976; Kharrat *et al.*, 1995; Nicholson, 1999; Yue & Shatkin, 1997). The postulated silencing suppressor capacity of *E. coli* RNase III, a mutant RNase III that binds dsRNA but lacks RNA cleavage activity (Rnc70) (Dasgupta *et al.*, 1998) and reovirus  $\sigma 3$  proteins were tested in the *Agrobacterium* co-infiltration assay. The *rnc+* (encodes RNase III) and *rnc70* (encodes Rnc70) genes were amplified by PCR from plasmids pACS21 and pSDF70 (Dasgupta *et al.*, 1998) with primers RNC START (5'-ATGAACCCCATCGTAAT-3') and RNC STOP (5'-TCATTCCAGCTCCAGTT-3'). The PCR products were then cloned into the *Sma*I-digested *Agrobacterium* binary vector BIN61S (Silhavy *et al.*, 2002) to create the constructs 35S-*rnc+* and 35S-*rnc70* (Fig. 1a). The S4 segment (encodes  $\sigma 3$ ) was amplified by PCR with primers S4START (5'-

ATGGAGTGTTGCTTGCC-3') and S4STOP (5'-TTAGCCAAGAATCATCGG-3') from plasmid pBC12BI (Giantini & Shatkin, 1989) and cloned into the *Sma*I site of BIN61S to create the construct 35S- $\sigma$ 3. As a negative control, a 35S- $\Delta\sigma$ 3 clone was constructed by PCR, amplifying the 5' first 846 nt segment of the S4 gene with primers S4START (5'-ATGGAGTGTTGCTTGCC-3') and  $\Delta$ S4STOP (5'-TTACATTTTACAGTTCCCAG-3'). Then, the PCR fragment was cloned into the *Sma*I-digested BIN61S plasmid. 35S- $\Delta\sigma$ 3 encodes a truncated protein that fails to bind dsRNAs (Miller & Samuel, 1992).

To examine whether dsRBPs suppress RNAi, GFP silencing was monitored in 35S-GFP infiltrated cells and in 35S-GFP+35S-*rnc*+, 35S-GFP+35S-*rnc*70, 35S-GFP+35S- $\sigma$ 3 and 35S-GFP+35S- $\Delta\sigma$ 3 co-infiltrated leaves of GFP transgenic *N. benthamiana* plants. *Agrobacterium* infiltration assays, GFP expression tests and GFP-specific RNA gel blot analyses were carried out as described previously (Silhavy *et al.*, 2002). In line with previous reports (Voinnet *et al.*, 2000), we found that, although green fluorescence was strong (Fig. 1b) and GFP mRNA expression was still high (Fig. 2a, top panel), the accumulation of GFP-specific siRNAs (Fig. 2a, bottom panel) in 35S-GFP infiltrated leaves at 3 days post-inoculation (p.i.) confirmed the early induction of GFP silencing. As expected, co-infiltration of 35S- $\Delta\sigma$ 3 with 35S-GFP did not affect GFP silencing (Fig. 1b and Fig. 2a). In contrast, co-infiltration of 35S-*rnc*+, 35S-*rnc*70 and 35S- $\sigma$ 3 with 35S-GFP suppressed the early effects of RNAi. GFP expression was stronger (Fig. 1b) and levels of GFP mRNA were higher (Fig. 2a, top panel), while the accumulation of GFP-derived siRNAs was reduced (Fig. 2a, bottom panel) in all three dsRBP co-infiltrated samples compared with 35S-GFP-injected and 35S-GFP+35S- $\Delta\sigma$ 3 co-infiltrated controls. These findings indicate that dsRBPs could act as RNAi suppressors. Different dsRBPs, however, suppressed transgene-induced RNAi to a different degree. GFP-derived siRNAs were not detected in 35S-GFP+35S-*rnc*+ or 35S-GFP+35S- $\sigma$ 3 co-infiltrated samples, while the presence of *Rnc*70 only reduced the levels of the siRNA accumulation (Fig. 2a, bottom panel). These data indicate that RNase III and  $\sigma$ 3 are strong RNAi suppressors, whereas *Rnc*70 acts as a weak inhibitor of silencing. By 6 days p.i., the degree of GFP silencing was similar in 35S-GFP+35S-*rnc*70 co-infiltrated samples to the 35S-GFP- and 35S-GFP+35S- $\Delta\sigma$ 3-injected controls (Fig. 1b and Fig. 2b), indicating that the weak RNAi suppressor could only delay the silencing-mediated degradation of GFP. In contrast, strong GFP expression (Fig. 1b) together with very low levels of GFP-specific siRNA indicated that the strong RNAi suppressor  $\sigma$ 3 inhibited GFP silencing in the 35S-GFP+35S- $\sigma$ 3 co-infiltrated leaves, at least to 6 days p.i. (Fig. 2b). As infiltration with 35S-*rnc*+ leads to local necrosis by 4–5 days p.i., 35S-*rnc*+ co-infiltrated leaves could not be analysed at 6 days p.i.



**Fig. 1.** (a) Schematic representation of constructs used in this work. dsRBD, dsRNA-binding domains; 35S and 35S poly(A), promoter and terminator regions of the 35S transcript encoded by *Cauliflower mosaic virus* (CaMV), respectively; NOS term, terminator region of the NOS transcript of *A. tumefaciens*. (b) Effect of dsRBPs on transient GFP expression. GFP transgenic *N. benthamiana* plants were infiltrated with 35S-GFP or co-infiltrated with 35S-GFP and dsRBPs as *E. coli* RNase III (35S-GFP+35S-rnc+), Rnc70 (35S-GFP+35S-rnc70) and reovirus  $\sigma$ 3 (35S-GFP+35S- $\sigma$ 3). Co-infiltration of 35S-GFP with the truncated version of  $\sigma$ 3 lacking dsRNA-binding activity (35S-GFP+35S- $\Delta$  $\sigma$ 3) was used as a control. Photographs of infiltrated leaves were taken under UV illumination.



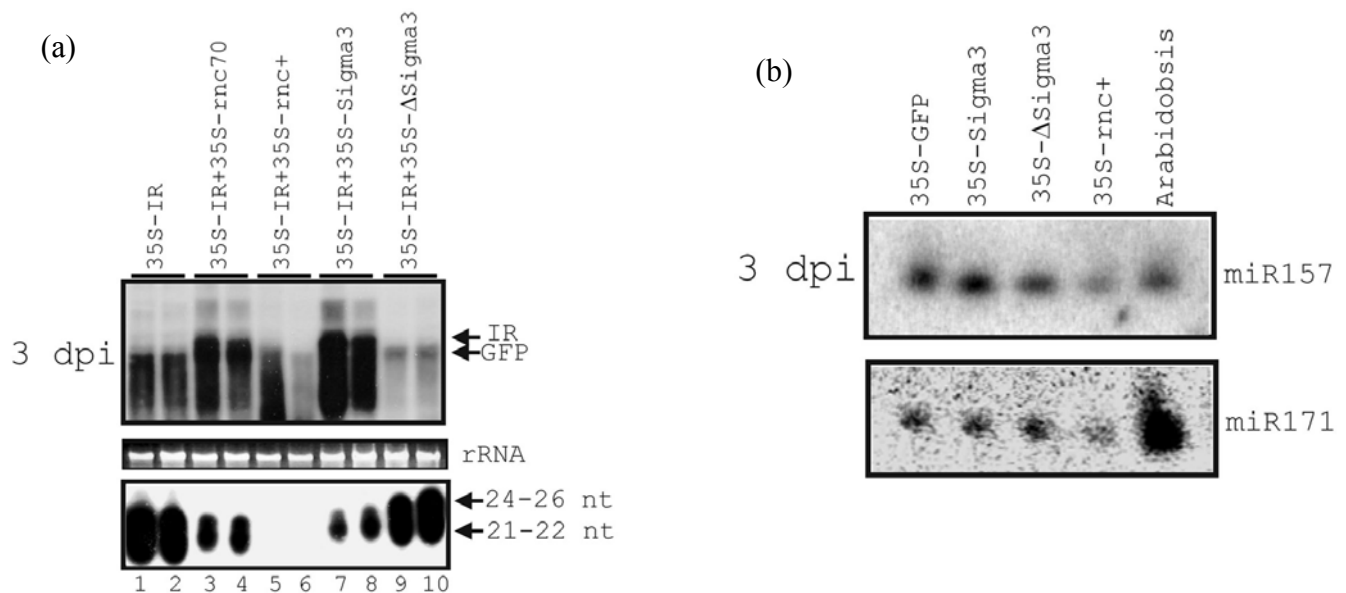
**Fig. 2.** Early (a) and late (b) effects of dsRBPs on transgene-induced RNAi. (a) Levels of GFP mRNA (top panel) and GFP-specific siRNA (bottom panel) in samples taken from infiltrated patches of 35S-GFP-injected or 35S-GFP+35S-rnc+, 35S-GFP+35S-rnc70, 35S-GFP+35S- $\sigma$ 3 or 35S-GFP+35S- $\Delta\sigma$ 3 co-injected leaves of GFP transgenic *N. benthamiana* plants. The same concentration of total RNA in the same volume ( $\sim 5 \mu\text{g}$ ) was used for mRNA and siRNA gel blot analyses. Ethidium bromide-stained rRNA is shown as a loading control. A radioactively labelled GFP PCR fragment generated by random priming was used as the probe for mRNA gel blot assays, while a labelled *in vitro* transcript corresponding to the antisense strand of GFP was used as the probe in Northern blot analyses of siRNAs.

Cell-autonomous GFP silencing generates signals that lead to systemic GFP silencing in non-infiltrated tissues of GFP transgenic *N. benthamiana* plants. Systemic GFP silencing can be monitored easily because chlorophyll autofluorescences red when no GFP is expressed. The formation of red fluorescence around the infiltrated area by 5–6 days p.i. in the 35S-GFP infiltrated leaves of GFP transgenic *N. benthamiana* plants showed the induction of systemic GFP silencing (data not shown) (Voinnet & Baulcombe, 1997). Because the accumulation of the long 24–26 nt GFP-specific siRNA fraction correlates with systemic silencing (Hamilton *et al.*, 2002) and because dsRBPs reduce the levels of both short and long siRNAs (Fig. 2, bottom panels), we expected that co-infiltration of dsRBPs with 35S-GFP would interfere with systemic silencing. Indeed, the development of red fluorescence was delayed by 1–2 days in 35S-GFP+35S-rnc70 co-infiltrated leaves and by 2–3 days in 35S-GFP+35S- $\sigma$ 3 co-infiltrated leaves (data not shown). As expected, co-infiltration of 35S- $\Delta\sigma$ 3 with 35S-GFP did not have an effect on systemic GFP silencing (data not shown).

dsRBPs inactivate PKR by depleting dsRNAs. If RNAi suppression of dsRBPs is also based on dsRNA sequestering, RNase III, Rnc70 and  $\sigma$ 3 should effectively bind dsRNAs in plant cells, thereby preventing the silencing-mediated degradation of dsRNAs. To test this hypothesis, silencing-mediated degradation of

dsRNA was analysed in the presence and absence of dsRBPs. GFP transgenic *N. benthamiana* leaves were infiltrated with *Agrobacteria* carrying a GFP inverted repeat (Fig. 1a); thus, the expressed mRNAs formed hairpin structures with a long stem (35S-IR) and could be digested by DICER. In line with previous reports (Johansen & Carrington, 2001) at 3 days p.i., siRNAs were very abundant in 35S-IR-infiltrated cells of GFP transgenic *N. benthamiana* (Fig. 3a, bottom panel), indicating that 35S-IR induced strong RNAi. As shown in Fig. 3(a), co-infiltration of 35S- $\Delta\sigma 3$  with 35S-IR did not influence RNAi-mediated dsRNA degradation, while dsRBPs inhibited 35S-IR-induced RNA silencing. siRNAs were not detected (35S-IR+35S-rnc+) or they accumulated to low levels (35S-IR+35S-rnc70 and 35S-IR+35S- $\sigma 3$ ) in 35S-IR and dsRBP co-infiltrated tissues (Fig. 3a). The accumulation of a higher molecular mass mRNA fraction that corresponds to IR mRNA in samples taken from 35S-IR+35S-rnc70 and 35S-IR+35S- $\sigma 3$  co-infiltrated leaves (Fig. 3a, top panel) suggests that dsRBPs prevented the degradation of IR dsRNA. The lack of this RNA fraction in control samples (Fig. 3a, top panel) could reflect the activity of DICER and other dsRNases. IR mRNAs were also absent in 35S-IR+35S-rnc+ co-infiltrated samples, even though siRNAs were not detected (Fig. 3a). These data suggest that *E. coli* RNase III degraded the co-expressed IR mRNAs.

In addition to siRNA, DICER also generates 21–25 nt long ss micro (mi)RNAs, which play a role in developmental regulation (Hutvagner *et al.*, 2001; Ketting *et al.*, 2001; Llave *et al.*, 2002; Reinhart *et al.*, 2002). miRNAs are produced from hairpin precursor RNAs transcribed from endogenous genes (Lee *et al.*, 2002). We examined the effect of heterologous dsRBPs on miRNA accumulation in the infiltrated leaves of *N. benthamiana* plants. Antisense oligonucleotides corresponding to miR157 (miR157 ANTISENSE, 5'-GTGCTCTCTATCTTCTGTCAA-3') and miR171 (miR171 ANTISENSE, 5'-GATATTGGCGCGGCTCAATCA-3') (Reinhart *et al.*, 2002) were radioactively labelled by T4 polynucleotide kinase and used as probes. RNA gel blot analysis revealed that miR157 and miR171 accumulated to equal levels in non-infiltrated controls (data not shown) and in 35S-GFP, 35S- $\Delta\sigma 3$ - and 35S- $\sigma 3$ -infiltrated leaves (Fig. 3b), while miRNA accumulation was reduced in 35S-rnc+ infiltrated samples (Fig. 3b). These data suggest that  $\sigma 3$  dsRBP failed to sequester miRNA precursors, although  $\sigma 3$  could sequester long dsRNA precursors of siRNAs. Indeed,  $\sigma 3$  binds dsRNAs efficiently only if they are longer than 32–45 bp (Yue & Shatkin, 1997). Because RNase III cleaves structured ssRNAs (Nicholson, 1999), it might also bind miRNA precursors, thereby reducing the accumulation of miRNAs. It is possible that certain virus-encoded dsRBPs, like RNase III, interfere with miRNA accumulation, thus contributing to the symptoms of virus infection.



**Fig. 3.** (a) Effects of dsRBPs on dsRNA-induced RNAi. RNA samples were isolated from GFP transgenic *N. benthamiana* plants infiltrated with *Agrobacteria* expressing hairpin dsGFP transcripts (35S-IR) or co-infiltrated 35S-IR with RNase III (35S-IR+35S-rnc+), Rnc70 (35S-IR+35S-rnc70), reovirus  $\sigma$ 3 (35S-IR+35S- $\sigma$ 3) and the truncated version of  $\sigma$ 3 lacking dsRNA-binding activity (35S-IR+35S- $\Delta\sigma$ 3). Levels of IR mRNAs (IR), endogenous GFP mRNAs (GFP) (top panel) and siRNAs (bottom panel) were analysed by RNA gel blots. The same concentration of total RNA in the same volume ( $\sim 5 \mu\text{g}$ ) was used for mRNA and siRNA gel blot analyses. Ethidium bromide-stained rRNA is shown as the loading control. A radioactively labelled GFP PCR fragment generated by random priming was used as the probe for mRNA gel blot assays, while a labelled *in vitro* transcript corresponding to the antisense strand of GFP was used as the probe in Northern blot analyses of siRNAs. (b) Effects of dsRBPs on accumulation of miRNAs. Labelled antisense oligonucleotides were used as probes for detecting levels of miR157 and miR171 in RNA samples isolated from infiltrated patches of wild-type *N. benthamiana* plants injected with 35S-GFP, 35S- $\sigma$ 3, 35S- $\Delta\sigma$ 3 and 35S-rnc+.

It is likely that certain virus suppressors target conserved elements of the RNAi machinery. Tombusvirus p19 RNAi suppressor binds ds siRNAs, thus inhibiting virus-induced systemic silencing in plants (Silhavy *et al.*, 2002). Other RNAi suppressors might target another conserved elements of RNAi, long dsRNAs. Indeed, we showed that heterologous dsRBPs could effectively suppress RNAi, presumably by sequestering dsRNAs. We propose that many virus-encoded dsRBPs play important roles in pathogenicity by interfering with RNAi-mediated cell-autonomous and systemic host defences. As effective silencing suppression likely requires early, abundant cytoplasmic expression of virus-encoded dsRBPs, we think that only a subset of virus-encoded dsRBPs could operate as natural RNAi suppressors. For instance, in reovirus- or vaccinia virus-infected mammalian cells, the expression of  $\sigma$ 3 or E3L might lead to inactivation of RNAi-mediated defences in addition to inhibition of PKR-mediated responses (Kaufman, 1999).

plants (Sano *et al.*, 1997; Watanabe *et al.*, 1995). RNase III- and Rnc70-expressing transgenic plants have shown virus resistance against viruses with segmented genomes (Langenberg *et al.*, 1997; Zhang *et al.*, 2001). However, finding that both RNase III and Rnc70 suppress RNA silencing suggests that the RNAi defence system of these transgenic plants could be compromised; therefore, these transgenic plants might be more susceptible to certain viruses.

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