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dsRNA with 5′ overhangs contributes to endogenous and antiviral RNA silencing pathways in plants

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In plants, SGS3 and RNA-dependent RNA polymerase 6 (RDR6) are required to convert single- to double-stranded RNA (dsRNA) in the innate RNAi-based antiviral response and to produce both exogenous and endogenous short-interfering RNAs. Although a role for RDR6-catalysed RNA-dependent RNA polymerisation in these processes seems clear, the function of SGS3 is unknown. Here, we show that SGS3 is a dsRNA-binding protein with unexpected substrate selectivity favouring 5′-overhang-containing dsRNA. The conserved XS and coiled-coil domains are responsible for RNA-binding activity. Furthermore, we find that the V2 protein from *tomato yellow leaf curl virus*, which suppresses the RNAi-based host immune response, is a dsRNA-binding protein with similar specificity to SGS3. In competition-binding experiments, V2 outcompetes SGS3 for substrate dsRNA recognition, whereas a V2 point mutant lacking the suppressor function *in vivo* cannot efficiently overcome SGS3 binding. These findings suggest that SGS3 recognition of dsRNA containing a 5′ overhang is required for subsequent steps in RNA-mediated gene silencing in plants, and that V2 functions as a viral suppressor by preventing SGS3 from accessing substrate RNAs.

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Introduction

In plants, short-interfering RNAs (siRNAs) are part of an innate immune response that targets invading viruses through post-transcriptional gene silencing. The conserved plant-specific proteins SGS3 and RNA-dependent RNA polymerase 6 (RDR6), required for viral immunity, are thought to convert single-stranded RNA (ssRNA) transcripts of sense transgenes and viral genomes into double-stranded RNA (dsRNA). DICER-like proteins cleave the resulting dsRNAs into siRNAs, which then assemble into RNA-induced silencing complexes (RISCs) and guide cleavage of the complementary viral transcripts. SGS3 and RDR6 may also be involved in secondary siRNA production from cleaved viral transcripts (Mourrain *et al*, 2000; Beclin *et al*, 2002; Vaistij *et al*, 2002; Chapman *et al*, 2004; Muangsan *et al*, 2004; Zamore, 2004; Vaucheret, 2006). In addition to their roles in viral defense, SGS3 and RDR6 are crucial for biogenesis of natural antisense transcript-derived siRNAs (nat-siRNAs, ~24 nt) and trans-acting siRNAs (ta-siRNAs, ~21 nt) (Peragine *et al*, 2004; Vazquez *et al*, 2004; Allen *et al*, 2005; Borsani *et al*, 2005; Gasciolli *et al*, 2005; Xie *et al*, 2005; Yoshikawa *et al*, 2005; Montgomery *et al*, 2008).

Although RDR6 has been demonstrated to possess the enzymatic activity required to convert ssRNA substrates into dsRNA (Curaba and Chen, 2008), SGS3 remains functionally uncharacterised. SGS3 consists of zinc finger, XS and coiled-coil domains (Figure 1A), a domain structure found within a class of uncharacterised plant proteins (Bateman, 2002). The zinc finger and coiled-coil domains indicate possible roles in nucleic acid recognition and protein–protein interactions by analogy to other proteins, whereas the function of the conserved XS domain is unknown. On the basis of genetic evidence, SGS3 has been proposed to enhance RDR6 reactivity by protecting or stabilising ssRNA transcripts, by physically interacting with RDR6 and/or by recruiting RDR6 to substrates (Mourrain *et al*, 2000; Beclin *et al*, 2002; Muangsan *et al*, 2004; Yoshikawa *et al*, 2005; Vaucheret, 2006).

Underscoring its importance in plant antiviral defense, SGS3 is the target of a small protein called V2 from *tomato yellow leaf curl virus* that has been shown recently to function as a suppressor of host RNA silencing (Zrachya *et al*, 2007; Glick *et al*, 2008). On the basis of yeast two-hybrid data and fluorescence microscopy, V2 was proposed to interact with tomato (*Solanum lycopersicum*) SGS3 (SlSGS3) in infected plant cells (Glick *et al*, 2008). Such a mechanism contrasts with that of other known plant virus-encoded suppressor proteins that inhibit host RNA silencing by binding directly to virus-derived siRNAs or to components of the RISC such as AGO1 (Silhavy *et al*, 2002; Vargason *et al*, 2003; Ye *et al*, 2003; Chapman *et al*, 2004; Zamore, 2004; Zhang *et al*, 2006; Ding and Voinnet, 2007). Mutation of two cysteine residues in the zinc finger motif of V2 eliminated its suppressor activity and disrupted the interaction with SlSGS3, suggesting that the V2–SlSSG3 interaction is important for immune suppression by V2 (Zrachya *et al*, 2007; Glick *et al*, 2008).

To determine the biochemical function of SGS3 and the mechanism of V2-based suppression of its activity, we analysed recombinant *Arabidopsis thaliana* SGS3 (AtSGS3),

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S. lycopersicum SGS3 (SlSGS3) and the viral suppressor V2 *in vitro*. We show that SGS3 is a structure-selective RNA-binding protein that specifically recognises dsRNA substrates containing a single-stranded 5′ overhang. These results imply a new kind of substrate as a critical intermediate in the RNAi pathway in plants. Binding is sequence independent but requires the junction of a 5′ single-stranded segment and a dsRNA region. The binding behaviour of truncated forms of SGS3 suggests that the XS domain is responsible for RNA recognition, and thus may represent a previously uncharacterised RNA-binding module. The V2 suppressor protein shares the same RNA-binding specificity as SGS3. Its ability to out-compete SGS3 for RNA-substrate binding, and the reduction of this capability in a nonfunctional V2 mutant, explain its capacity to block SGS3-mediated RNA interference during viral infection.

Results

SGS3 selectively binds to dsRNA substrates containing 5′ single-stranded overhangs

To test the hypothesis that SGS3 is a nucleic acid-binding protein, we analysed recombinant full-length and N-terminally truncated versions of *Arabidopsis* and tomato SGS3 for RNA and DNA binding using an electrophoretic mobility shift assay (EMSA). The *Arabidopsis* (AtSGS3) and tomato (SlSGS3) full-length proteins (aa 1–625, 72 kD; aa 1–633, 73 kD) consisted of the complete three-domain structure, whereas the N-terminal truncations (AtSGS3ΔN: aa 290–625, 40 kD; SlSGS3ΔN: aa 299–632, 40 kD) included the XS and coiled-coil domains (Figure 1). Inductively coupled plasma analysis revealed that AtSGS3 but not AtSGS3ΔN contains zinc, suggesting that the zinc-finger domain in SGS3 binds a zinc ion (data not shown).

Twenty one different forms of RNA, DNA and DNA–RNA hybrids with varying sequences were prepared, including ssRNA, ssDNA, dsRNA, dsDNA and DNA–RNA hybrids with or without 5′ or 3′ overhangs (Figure 2). First, we tested RNA and DNA substrates of forms 1–11, all of which are

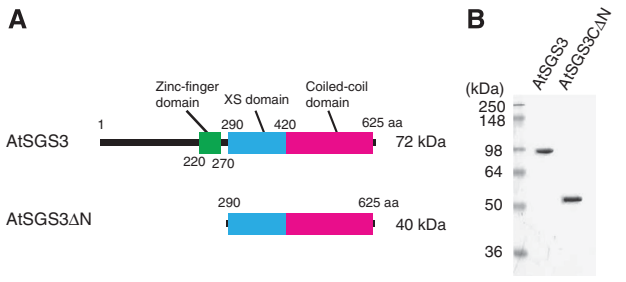

Figure 1 AtSGS3 constructs. (**A**) Schematic diagram of AtSGS3 constructs. (**B**) Coomassie brilliant blue-stained SDS–PAGE gel of purified AtSGS3 and AtSGS3ΔN.

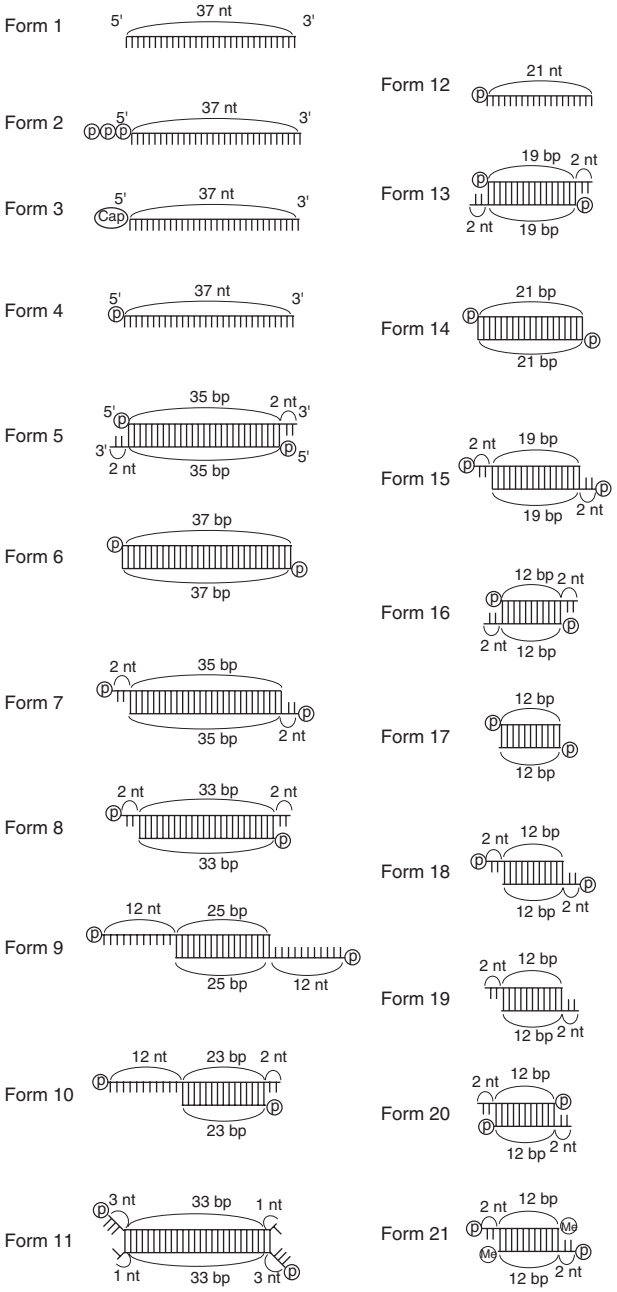

Figure 2 RNA/DNA structures used in this study. Cartoon representations for the RNA/DNA structures used in this study. Forms 1–4 is a 37 nt single strand. Form 5 is a 35 bp long double-strand with 2 nt 3′ overhang on each strand. Form 6 is a 37 bp double-strand without overhang. Form 7 is a 35 bp long double-strand with 2 nt 5′ overhang on each strand. Form 8 is a 33 bp long double-strand with both 2 nt 5′ overhang and 2 nt 3′ overhang on one strand. Form 9 is a 25 bp long double-strand with 12 nt 5′ overhang on each strand. Form 10 is a 23 bp long double-strand with 12 nt 5′ overhang and 2 nt 3′ overhang on each strand. Form 11 is a 33 bp long double-strand with both 3 nt 5′ overhang and 1 nt 3′ overhang on each strand. Form 11 is designed by breaking terminal base pairs in form 4. Form 9 is a 21 nt single strand. RNA form 12 represents a typical siRNA/miRNA single strand. Form 13 is a 19 bp long double-strand with 2 nt 3′ overhang on each strand. RNA form 14 represents a typical siRNA duplex. Form 14 is a 21 bp double-strand without overhang. Form 15 is a 19 bp long double-strand with 2 nt 5′ overhang on each strand. Form 16 is a 12 bp long double-strand with 2 nt 3′ overhang on each strand. Form 17 is a 12 bp double-strand without overhang. Form 18 is a 12 bp long double-strand with 2 nt 5′ overhang on each strand. Form 1 has no modification at 5′ end. Form 2 has 5′ triphosphate. Form 3 has 5′ cap modification. Forms 4–18 has 5′ terminal monophosphate on each strand. Form 19 is same as form 18, except that it has no terminal phosphate on either strand. Form 20 is same as form 19 except that it has 3′ terminal phosphate on each strand. Form 20 is same as form 18, except that it has 3′ end methylation modification at ribose 2′ position. See Materials and methods for the sequences.

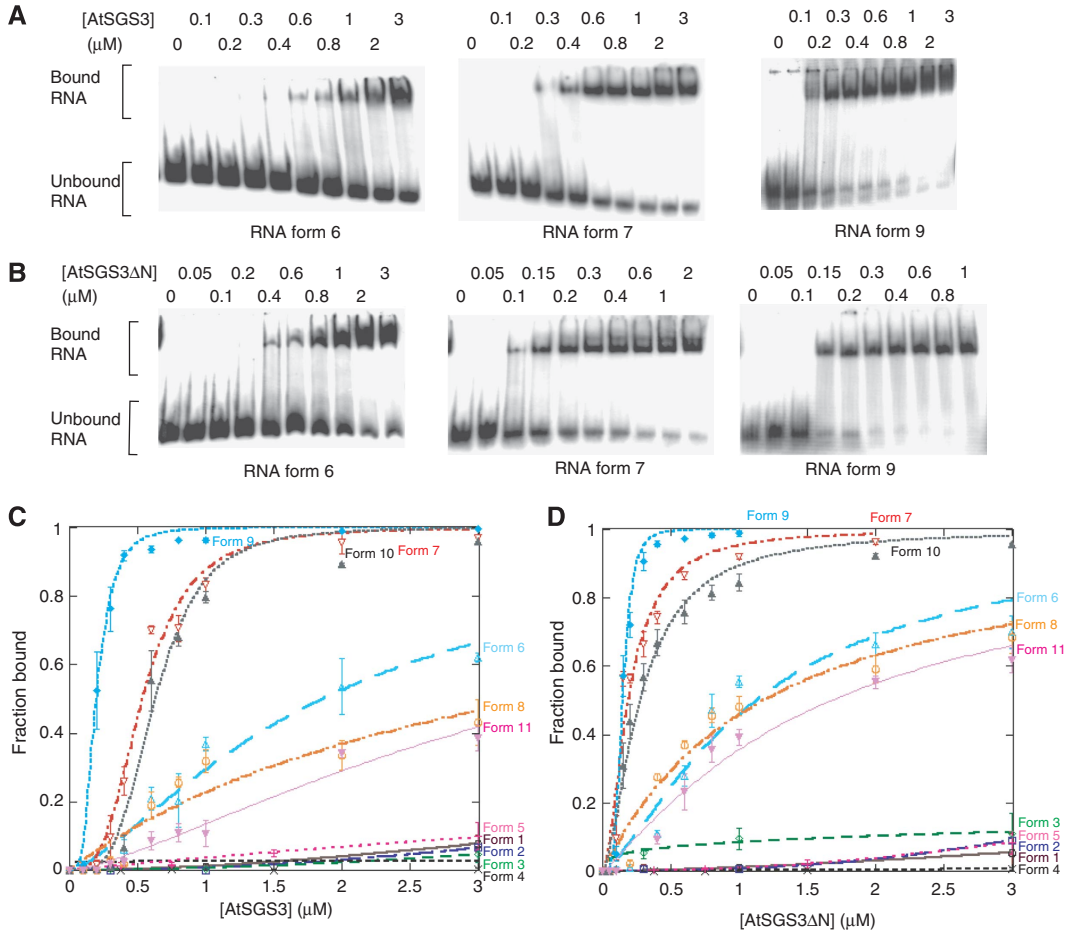

Figure 3 RNA-binding study of AtSGS3 and AtSGS3ΔN by electrophoretic gel mobility shift analyses (EMSA). (**A**, **B**) EMSAs of binding of AtSGS3 (A) and AtSGS3ΔN (B) to RNAs form 6, 7 and 9. (**C**, **D**) RNA-binding curves for AtSGS3 (C) and AtSGS3ΔN (D) to RNAs form 1–11, determined by EMSAs. Note that K_d was determined according to protein monomer concentrations, which are shown. See Figure 2 for the RNA structures.

~37 nts in length. AtSGS3 exhibited highest binding affinity for RNA of form 7 (dsRNA with 2 nt 5′ overhang on each strand) and form 9 (dsRNA with 12 nt 5′ overhang on each strand), with K_ds of 0.54 and 0.21 μM, respectively (Figure 3A and C; Table I). Hill constants were 3.2 and 4.0, respectively, indicating that binding is cooperative. AtSGS3 showed affinity for RNA form 10 (dsRNA with 12 nt 5′ overhang and 2 nt 3′ overhang on one strand), with K_ds and Hill constants of 0.63 μM and 3.9, respectively. In contrast, AtSGS3 bound weakly (K_d = 2–3 μM) to dsRNA substrates having short or frayed overhangs (forms 6, 8 and 11). No binding was detected to ssRNA with no 5′-modification (form 1), ssRNA with 5′-triphosphorylation (form 2), 5′-cap (form 3) or 5′-monophosphorylation (form 4), to DNA (forms 4–9) or to siRNA-like dsRNA containing 2 nt 3′ overhangs on each strand (form 5) (Figure 3C; Table I). Although AtSGS3 showed some affinity for a DNA–RNA hybrid (form 9; K_d = 1.2 μM), it did not bind to DNA–RNA hybrids of forms 5–7. These data show that AtSGS3 preferentially binds dsRNA containing a 5′ overhang. Furthermore, lower affinity of RNA form 11 as compared with RNA form 7 indicates that stable terminal base pairing at the junction between the double- and single-stranded regions of the substrate is important for high-affinity AtSGS3 binding. Thus, SGS3 likely recognises the junction of the dsRNA and the 5′ overhanging region.

The relative binding behaviour of the N-terminally truncated form of AtSGS3 and AtSGS3ΔN was similar to that of AtSGS3 (Figure 3B and D; Table I). Notably, however, binding affinities were consistently 2–5-fold stronger for the truncated protein. Hill constants ranged from 2 to 4, and no detectable binding was observed to RNAs of forms 1–5, DNAs of forms 4–9 and DNA–RNA hybrids of forms 5–7. These results, surprisingly, imply that the XS domain and/or the coiled-coil domain, but not the zinc-finger domain, are responsible for the RNA-binding activity and selectivity. We found no evidence that AtSGS3 and AtSGS3ΔN possess nuclease or phosphatase activity with any form of RNA, DNA and DNA–RNA hybrid tested, as no cleavage fragments or reduction of the total amount of 5′ end-labelled nucleic acid was observed in any of these assays.

Next, we examined the binding activity of AtSGS3 and AtSGS3ΔN with shorter RNAs of ~21 nt (RNAs form 12–15) or ~14 nt (RNAs form 16–18) overall length (Figure 4A and B; Table I). AtSGS3 bound with highest affinity to RNA form 15 (dsRNA with 2 nt 5′ overhang on each strand) and RNA form 18 (dsRNA with 2 nt 5′ overhang on each strand), with K_ds of 0.56 and 0.33 μM, respectively, and Hill constants of

Table I Summary of binding affinities of AtSGS3, AtSGS3ΔN, SlSGS3, SlSGS3ΔN, V2 and V2SS to RNA, DNA–RNA hybrid and DNA

	K_d (μM)		
	AtSGS3	AtSGS3ΔN	V2
RNA form 1	>3	>3	>3
RNA form 2	>3	>3	>3
RNA form 3	>3	>3	>3
RNA form 4	>3	>3	>3
RNA form 5	>3	>3	>3
RNA form 6	1.88 ± 0.19	1.13 ± 0.03	>3
RNA form 7	0.54 ± 0.02	0.19 ± 0.01	1.72 ± 0.07
RNA form 8	>3	1.21 ± 0.13	>3
RNA form 9	0.21 ± 0.02	0.15 ± 0.02	0.77 ± 0.11
RNA form 10	0.63 ± 0.04	0.28 ± 0.01	1.50 ± 0.16
RNA form 11	>3	1.70 ± 0.04	>3
DNA–RNA hybrid form 5	>3	>3	>3
DNA–RNA hybrid form 6	>3	>3	>3
DNA–RNA hybrid form 7	>3	>3	>3
DNA–RNA hybrid form 9	1.22 ± 0.01	2.06 ± 0.06	1.54 ± 0.14
DNA form 4	>3	>3	>3
DNA form 5	>3	>3	>3
DNA form 6	>3	>3	>3
DNA form 7	>3	>3	>3
DNA form 9	>3	>3	>3
RNA form 12	>3	>3	>3
RNA form 13	>3	>3	>3
RNA form 14	>3	1.32 ± 0.22	>3
RNA form 15	0.56 ± 0.08	0.33 ± 0.05	1.99 ± 0.07
RNA form 16	>3	>3	>3
RNA form 17	2.78 ± 1.18	1.52 ± 0.19	>3
RNA form 18	0.33 ± 0.05	0.11 ± 0.01	0.32 ± 0.05
RNA form 21	0.26 ± 0.02	0.24 ± 0.05	0.47 ± 0.04
		K_d (μM)	
	SlSGS3	SlSGS3ΔN	V2SS
RNA form 18	0.19 ± 0.07	0.20 ± 0.01	0.64 ± 0.05

Each K_d was determined based on monomer protein concentration. Standard errors are shown. K_d is shown as >3, when the averaged ($n = 3$ or 4) value for fraction bound is less than 0.5 at the 3-μM protein condition. See Figure 2 and Materials and methods for the structures and sequences of RNA, DNA–RNA hybrid and DNA.

2.1 and 2.8, respectively. Binding to RNA form 14 (dsRNA without overhang) and RNA form 17 (dsRNA without overhang) was weaker (K_ds ⩾ 3 μM). Likewise, AtSGS3ΔN showed highest affinity for RNA forms 15 and 18 (K_ds = 0.33 and 0.11 μM, respectively, and Hill constants of 1.5 and 2.8, respectively). Neither AtSGS3 nor AtSGS3ΔN bound detectably to RNA form 12 (ssRNA) or RNA forms 13 or 16 (dsRNA with 2 nt 3′ overhang on each strand).

We also examined the binding affinity of SlSGS3 and SlSGS3ΔN for RNA form 18 and obtained K_ds of 0.19 and 0.20 μM, with Hill constants of 2.3 and 2.9, respectively (Table I). Note that RNA form 12 and RNA form 13, which have structures typical of single- or double-stranded siRNA, had no appreciable affinity for any of the SGS3 constructs tested. These results show that in contrast to the PAZ domain characteristic of Dicer and Argonaute proteins or the P19 viral siRNA suppressor protein, which bind to siRNAs with 3′ single-stranded overhangs, SGS3 selectively recognises dsRNA molecules of variable length containing a 5′ single-stranded overhang.

The presence of a 3′ terminal phosphate inhibits SGS3 binding

To further define the recognition properties of SGS3, we examined whether the presence of a 5′ or 3′ terminal phosphate on a dsRNA molecule affects SGS3 binding. RNAs of form 18–20 share the same nucleotide sequence and structure but differ in their phosphorylation state (Figure 2). As detected by electrophoretic gel mobility shift analysis, AtSGS3 binds RNA form 18 (5′ phosphates) and RNA form 19 (no phosphates) but not RNA form 20 (3′ phosphates) (Figure 4C). Similar results were obtained for AtSGS3ΔN (Figure 4D). Thus, the presence of 3′ terminal phosphates on a dsRNA molecule that otherwise binds to SGS3 is sufficient to prevent detectable binding.

The presence of a 2′-O-methyl modification on the 3′ terminal ribose does not significantly affect SGS3 binding

In plants, miRNA, siRNA and ta-siRNA molecules are methylated at the 3′-terminal ribose 2′-position (Yu *et al*, 2005). We tested whether 3′ end methylation affects binding affinity to SGS3. AtSGS3 and AtSGS3ΔN bound to RNA form 21 (dsRNA with 2 nt 5′ overhang and 2′-O-methylation at the 3′ terminal ribose on each strand), with K_ds of 0.26 and 0.24 μM, respectively (Figure 2; Table I). These K_ds are comparable with those measured for the RNA without 3′ end methylation (form 18, 0.33 and 0.11 μM, respectively), showing that 3′ end methylation does not significantly affect SGS3 binding.

V2 shares RNA-binding selectivity with SGS3

Data from previous yeast two-hybrid and cell-based assays suggested that SGS3 is a target of the plant viral protein V2, which suppresses siRNA-based viral immunity in infected plants. To elucidate the molecular function of V2, we analysed recombinant versions of both the wild-type V2 and the nonfunctional C84/C86S double-mutant (V2SS) proteins (Figure 5A). The V2SS mutant lacks suppressor activity for host cell RNA silencing *in vivo* (Zrachya *et al*, 2007). No evidence for a direct interaction between SGS3 and V2 was obtained using an N-terminally GST-tagged variant of wild-type V2 in attempts to co-purify AtSGS3, AtSGS3ΔN, SlSGS3 or SlSGS3ΔN using glutathione-coupled beads (data not shown). Similar negative results were obtained using an N-terminally GST-tagged version of SlSGS3 in efforts to co-purify V2 (data not shown). Similarly, we did not detect indirect interaction mediated by RNA (forms 9 or 18) (data not shown).

Next, we tested V2 and V2SS for nucleic acid binding using the same set of RNA, DNA and DNA–RNA hybrid substrates as for SGS3 (Figure 2). V2 bound to dsRNA of form 7, RNA form 9 and RNA form 10, with K_ds of 0.8–1.5 μM and Hill constants of 2.2–2.7 (Figure 5B; Table I). It also bound to shorter dsRNAs of form 15 and RNA form 18, with K_ds of 2 and 0.3 μM and Hill constants of 3.5 and 4.3 (Figure 5C and D; Table I). V2 did not bind detectably to RNAs of form 1–6, 8, 11–14, 16 and 17, DNAs of form 4–9 and DNA–RNA hybrids of form 5–7 (Figure 5B and C; Table I). Thus, similar to SGS3, V2 preferentially binds cooperatively to dsRNA containing a 5′ overhang. As for SGS3, the terminal base pair at the end of the dsRNA region is important for V2 recognition.

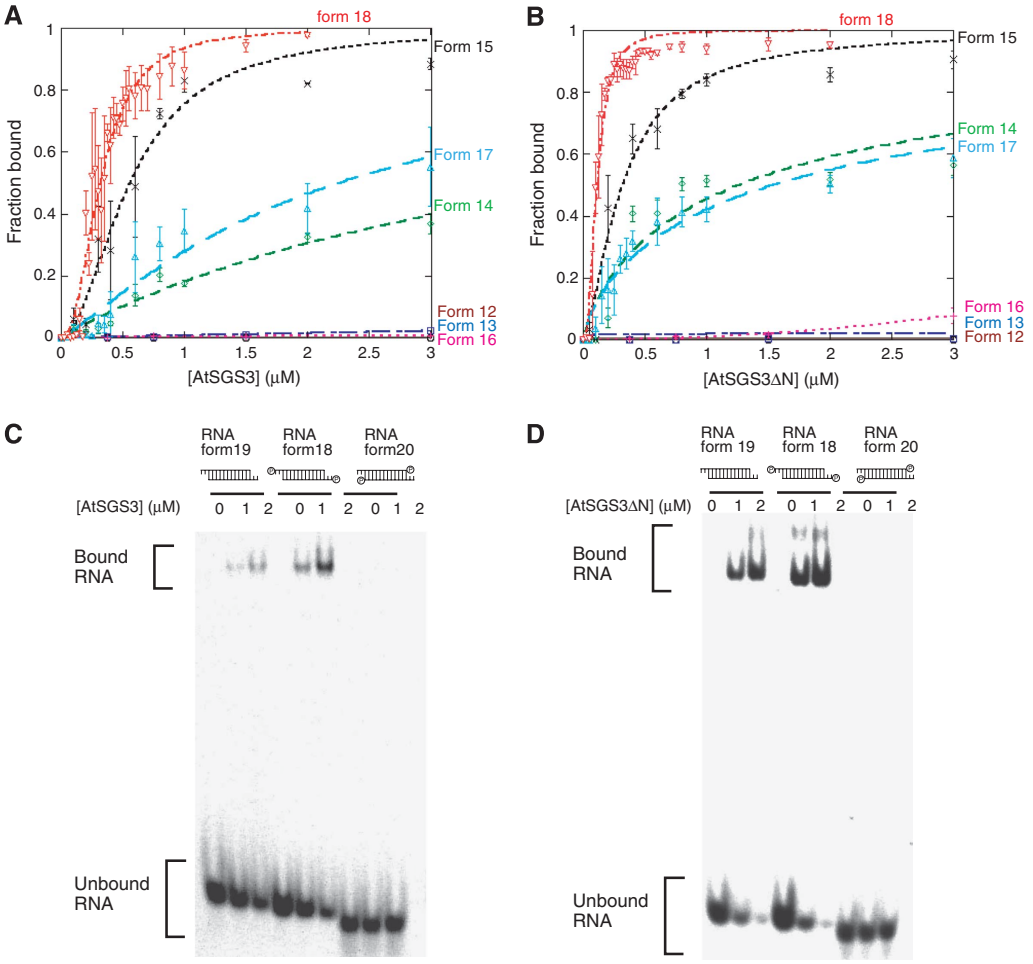

Figure 4 RNA-binding study of AtSGS3 and AtSGS3ΔN by gel mobility shift analyses with shorter RNA and analyses of the effect of terminal phosphate. (**A**, **B**) RNA-binding curves for AtSGS3 (A) and for AtSGS3ΔN (B) to RNAs form 12–18, determined by EMSAs. (**C**, **D**) EMSAs of binding of AtSGS3 (C) and AtSGS3ΔN (D) to RNAs form 18–20. The non-radioactive RNAs (1 μM) were used, and the gels were stained with SYBR Gold. Protein monomer concentrations are shown.

V2SS bound to RNA form 18, with a K_d of 0.64 μM and a Hill constant of 5.3 (Figure 5C and D; Table I). Thus, V2SS has a two-fold weaker equilibrium-binding affinity for this substrate RNA relative to V2. Because the double mutations in V2SS disrupt Zn binding as detected by inductively coupled plasma analysis (data not shown), the intact zinc-binding motif and a bound zinc ion may be important for the higher affinity binding of wild-type V2 to a dsRNA substrate.

Binding assays using dsRNAs containing 5′ or 3′ terminal phosphates showed that V2 can bind to RNAs of form 18 and 19 but not form 20 (Figure 5E). These results show that the presence of a 3′ terminal phosphate on dsRNA inhibits V2 binding, as was the case for SGS3. Together with the previous data, these findings indicate that similar to SGS3, V2 recognises and binds to the junction of a dsRNA region and a 5′ overhanging strand.

V2 showed affinity for RNA form 21 (dsRNA with 2 nt 5′ overhang and 3′ end methylation modification on the terminal ribose 2′-position on each strand), with K_d of 0.47 μM, which is comparable with that for the RNA without 3′ end methylation (form 18, 0.32 μM) (Figure 2; Table I). Therefore, similar to SGS3, 3′ end methylation modification does not significantly affect V2 binding.

RDR6 activity is not affected by the presence of SGS3 and V2

To test the hypothesis that SGS3 and V2 might directly or indirectly interact with RDR6, we overexpressed and purified the wild-type *A. thaliana* RDR6 protein (AtRDR6) from baculovirus-infected insect cells. Attempts to immunoprecipitate AtRDR6 after incubation with N-terminally HA-tagged AtSGS3 and anti-HA antibody-conjugated beads were unsuccessful, suggesting that AtRDR6 and AtSGS3 do not form a stable direct interaction *in vitro* (data not shown). Similar immunoprecipitation experiments in the presence of dsRNA substrates (RNA forms 9 or 18) were likewise unsuccessful, providing no evidence for an RNA-mediated interaction between AtRDR6 and N-terminally HA-tagged AtSGS3 (data not shown).

Next, we examined the effects of SGS3 and V2 on the RNA-dependent RNA polymerase activity of RDR6. RDR6 catalyses primer-independent, but not primer-dependent, RNA polymerisation reactions *in vitro* (Curaba and Chen, 2008). We performed RDR6-catalysed RNA polymerisation reactions using ssRNA (RNA form 4) as a template, in the presence or absence of AtSGS3, AtSGS3ΔN or V2 (Figure 6). The amount of template-directed RNA synthesised by AtRDR6

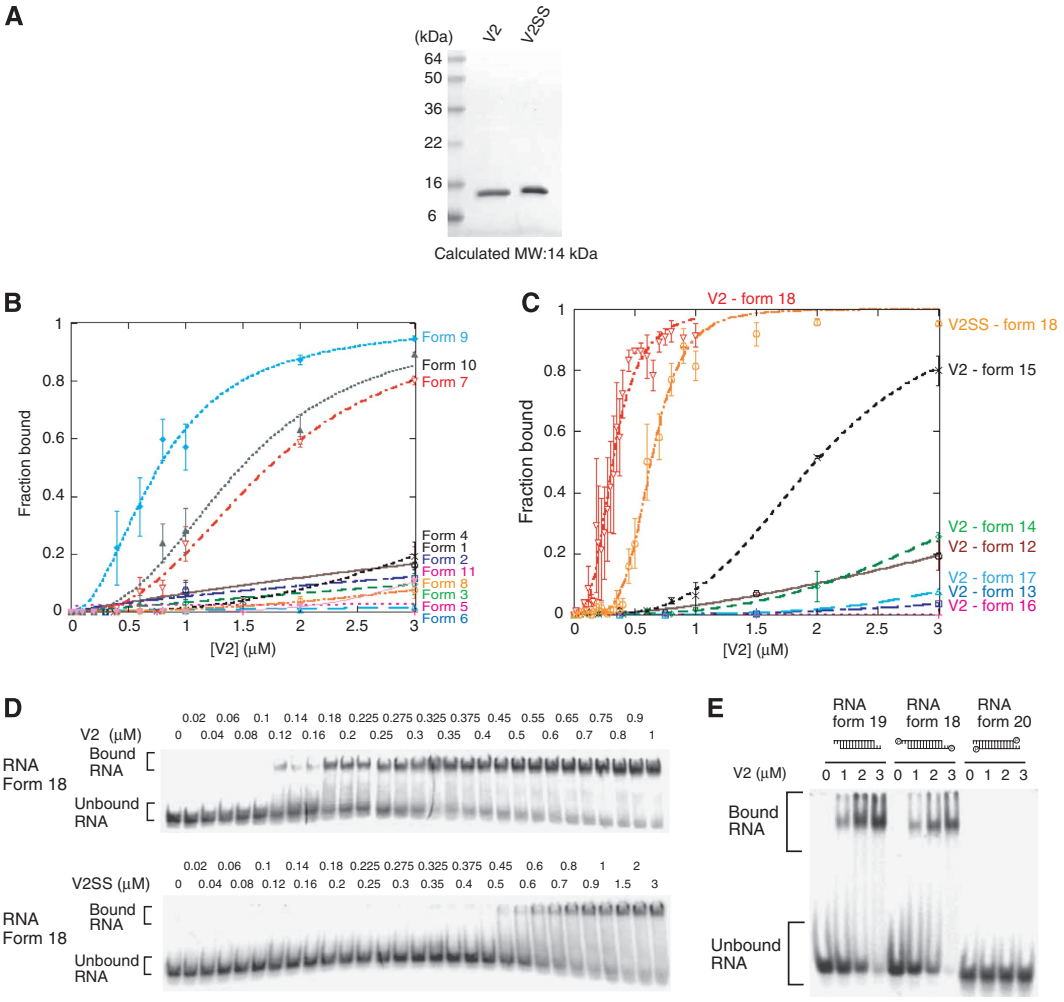

Figure 5 RNA-binding analyses of V2. (**A**) Coomassie brilliant blue-stained SDS–PAGE gel of purified wild-type V2 and the C84S/C86S mutant V2 (V2SS). (**B**) RNA-binding curves for V2 to RNAs form 1–11, determined by EMSAs. (**C**) RNA-binding curves for V2 to RNAs form 12–18 and for V2SS mutant to RNA form 18, determined by EMSAs. (**D**) EMSAs of binding of V2 (top panel) and V2SS mutant (bottom panel) to RNA form 18. (**E**) EMSAs of binding of V2 to RNAs form 18–20. The non-radioactive RNAs (1 μM) were used, and the gels were stained with SYBR Gold. Protein monomer concentrations are shown.

increased linearly over time and was unaffected by the presence of AtSGS3, AtSGS3ΔN or V2. In control reactions, SGS3 and V2 were not observed to have any RNA-dependent RNA polymerase activity.

We also tested whether AtRDR6 catalyses RNA polymerisation using dsRNA as a template, in the presence and absence of AtSGS3, AtSGS3ΔN or V2. We used RNA form 9 as a template, which has 5′ overhangs on each strand and was shown to be bound by SGS3 and V2 in our prior experiments (Figure 2; Table I). No product RNA, such as an ~37 nt RNA produced by unprimed polymerisation or an ~49 nt RNA derived from primed elongation of the partially overlapped substrate dsRNA, was detected under any condition tested (data not shown). These results imply that RDR6 does not use dsRNA as a polymerisation template, in the presence and absence of either SGS3 or V2. We conclude that neither SGS3 nor V2 interacts directly or indirectly with RDR6 to alter its ability to bind or copy RNA templates.

V2 out-competes SGS3 for binding to RNA substrates

The discovery that SGS3 and V2 share RNA recognition specificity, and that neither protein affects RDR6 function, suggested that V2 might suppress RNAi-based plant viral immunity by competing for substrates normally bound by SGS3. To test this idea, we performed binding competition assays to analyse the ability of SGS3 or V2 to bind dsRNA form 18 in the presence of the other protein. K_ds of AtSGS3, SlSGS3 and V2 for RNA form 18 are all similar (0.2–0.3 μM; Table I). We incubated 5′ radiolabelled RNA form 18 with 1 μM V2 alone, 1 μM SGS3 alone or 1 μM each V2 and SGS3. Note that the concentration of each protein was 3–5-fold higher than the K_d values. In binding reactions including both proteins, five different orders of protein addition and incubation times were tested (Figure 7). Because gel mobility of the V2–RNA and the SGS3–RNA complexes are different, relative amounts of V2- and SGS3-bound RNA were readily distinguishable (Figure 7A and B, left panel). In these experiments, the unbound RNA was not retained on the gels.

Regardless of the order of protein addition or incubation time, V2 was able to out-compete AtSGS3 and SlSGS3 for binding to substrate RNA. The equilibrium-binding constants of V2 are similar or higher (i.e., weaker) than each of the SGS3 proteins tested (Table I). Thus, one possible explanation for these results is that binding kinetics differ for these

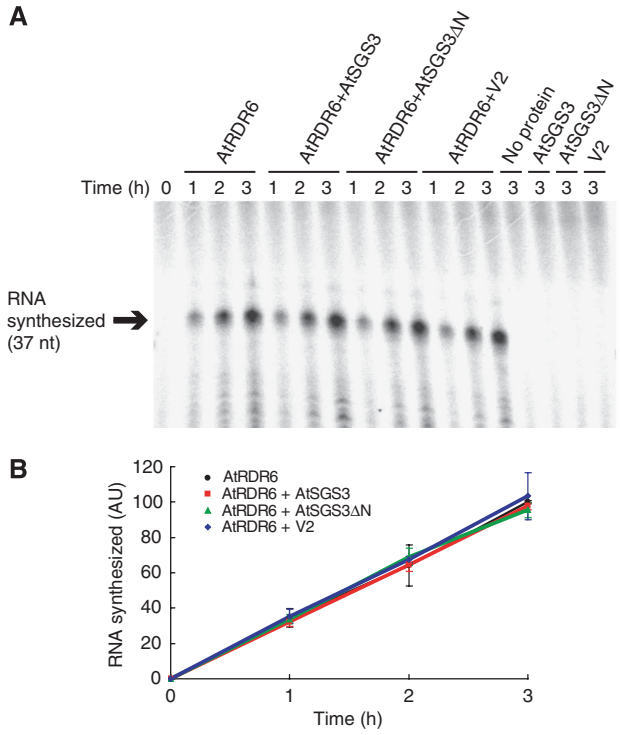

Figure 6 RDR6 activity assay in the presence and absence of AtSGS3, AtSGS3ΔN or V2. (**A**) Complementary RNA strand synthesised by RDR6 from ssRNA template was resolved by denaturing polyacrylamide gel electrophoresis. The synthesised RNA is shown by arrow. (**B**) Band intensities of the synthesised RNAs were quantified by phosphoimager and the averaged amounts ($n=3$, error bar represents standard error) were plotted. The arbitrary unit is used for synthesised RNA amount, where the averaged value for AtRDR6-only condition at 3 h is defined as 100.

proteins. V2 may have a slower off-rate relative to SGS3, enabling V2 to remain bound to dsRNA once it captures the substrate. In this way, V2 appears to deprive SGS3 of its dsRNA substrate through a kinetic rather than a thermodynamic property. No super-shifted band corresponding to dsRNA bound simultaneously by both SGS3 and V2 was observed, arguing against a direct interaction between SGS3 and V2 in the presence of the bound dsRNA.

Next, we performed the same binding-competition assays between V2SS and AtSGS3 or SlSGS3. Regardless of the order of protein addition or incubation time, AtSGS3 and SlSGS3, but not V2SS, predominantly bound to the substrate RNA under all conditions tested (Figure 7A and B, right panel). Thus, V2SS lacks the ability to efficiently out-compete AtSGS3 and SlSGS3 for binding to substrate dsRNA. This finding implies that V2 deprives SGS3 of its natural substrate RNA, providing a plausible explanation for the V2-mediated anti-RNAi response and implicating dsRNA with 5′ single-stranded overhangs in the siRNA-based antiviral defense pathway in plants.

Discussion

SGS3 and V2 are dsRNA-binding proteins with similar unexpected substrate specificities

In this paper, we demonstrate that SGS3 and V2 are dsRNA-binding proteins that preferentially recognise dsRNA substrates containing 5′ single-stranded overhangs. This specificity is entirely distinct from that of any other known RNAi-related RNA-binding proteins, and thus suggests a new kind of dsRNA substrate as part of the RNAi-mediated gene silencing pathway in plants. No evidence was obtained in this study to support enzymatic activities of SGS3 and V2, including nuclease, phosphatase, RNA-dependent RNA polymerase, nucleotidyltransferase or ligase activity. SGS3 and V2 were also not observed to interact directly or indirectly through dsRNA with each other, or with RDR6, and neither protein affected the RNA-dependent RNA polymerase activity of RDR6.

The sequences, lengths of 5′ overhanging regions and overall duplex length varied among the four different dsRNA substrates with 5′ overhangs on each end tested in this study, yet the equilibrium-binding constants measured for each of these RNAs with SGS3 or SGS3ΔN differed by only ~3-fold. This indicates that SGS3 recognises the structure of the substrate—the ssRNA–dsRNA junction with 5′ overhang—rather than its sequence or length. Such a recognition specificity is consistent with the observation that SGS3 is involved in several RNA silencing pathways, where the sequence and the length of substrate RNAs, including those used by RDR6, are variable (Mourrain *et al*, 2000; Peragine *et al*, 2004; Vazquez *et al*, 2004; Allen *et al*, 2005; Borsani *et al*, 2005; Gasciolli *et al*, 2005; Xie *et al*, 2005; Yoshikawa *et al*, 2005; Montgomery *et al*, 2008).

Consistently, dsRNA substrates containing 5′ overhangs on both strands bound with higher affinity than those with 5′ and 3′ overhangs on the same strand to SGS3, SGS3ΔN and V2 (compare RNA form 7 with RNA form 8 and RNA form 9 with RNA form 10; Figure 3C, D and 5B; Table I). This implies that a 3′ overhang at one end inhibits SGS3 or V2 binding to dsRNA even when the substrate includes an otherwise favourable 5′ overhang at the other end of the duplex. If binding is cooperative, as suggested by the Hill constants of >1 determined in this study, SGS3 or V2 recognition of each end of the duplex may favour binding of another SGS3 or V2 molecule or complex to the opposite end.

The XS domain is likely responsible for unique dsRNA recognition

The C-terminal fragment of SGS3 containing the XS and the coiled-coil domains, but not the N-terminal zinc-finger domain, were necessary for dsRNA-binding affinity and specificity. The molecular function of the zinc-finger domain, therefore, remains to be elucidated. Neither the XS domain nor the coiled-coil domain was stable when expressed separately (data not shown), hindering the further functional dissection of each domain. However, considering that coiled-coil domains often function in oligomerisation (Burkhard *et al*, 2001; Lupas and Gruber, 2005) and that both AtSGS3 and AtSGS3ΔN appear to oligomerise in solution based on analytical gel filtration behaviour (data not shown), the dsRNA-binding activity is likely attributable to the XS domain. The sequence of the XS domain shares no homology with that of known RNA- or DNA-binding proteins. Thus, we propose that the XS domain is a new kind of dsRNA-binding domain with novel substrate specificity.

Several other functionally uncharacterised plant proteins contain an XS domain (Bateman, 2002). Our results suggest the possibility that these proteins are also RNA-binding proteins. In these proteins, the XS domain is always followed

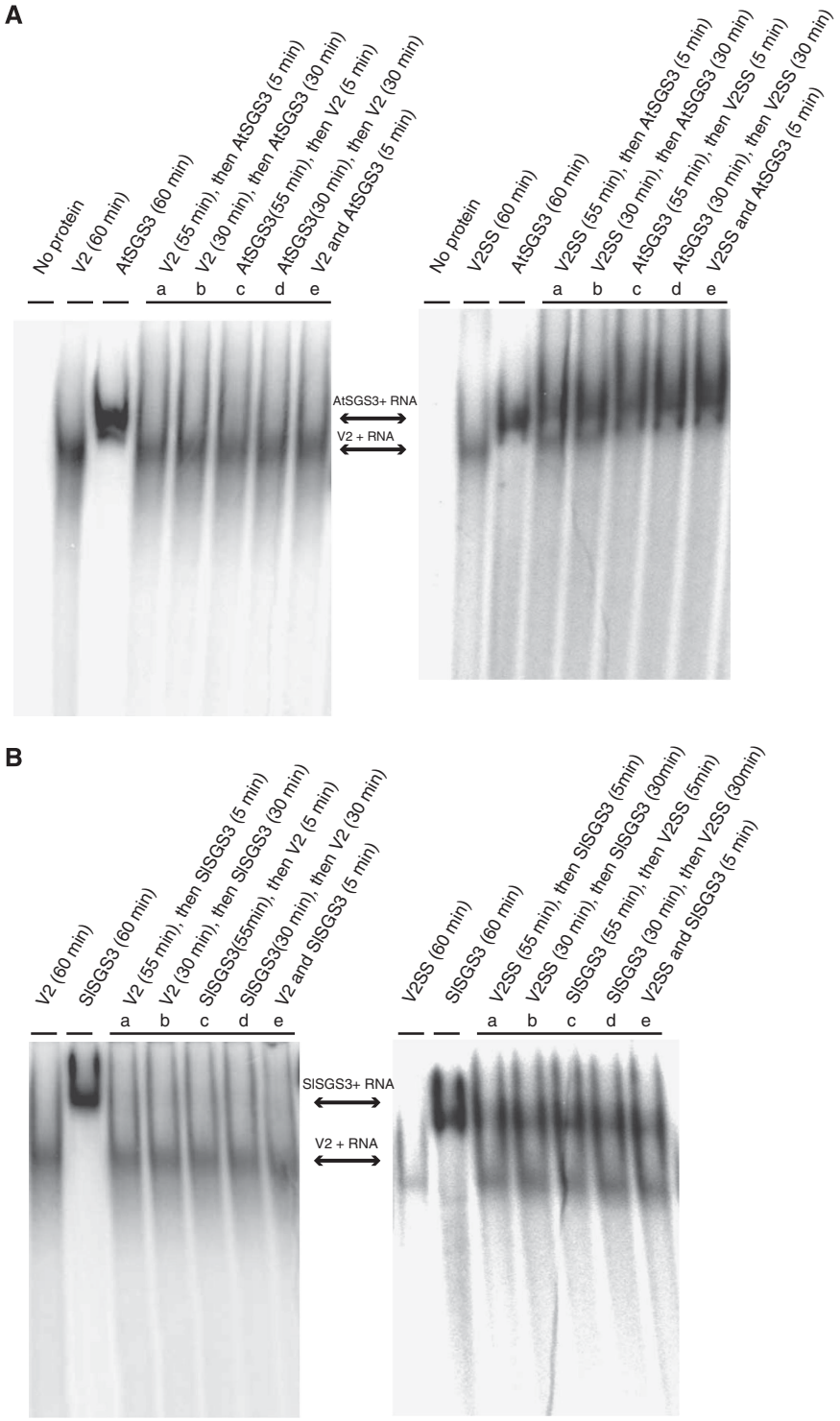

Figure 7 RNA-binding competition analyses between V2 and SGS3 proteins. EMSAs of binding of V2/SGS3 proteins to RNA form 18. (**A**) Binding competition between AtSGS3 and V2 (right panel) or V2SS (left panel). (**B**) Binding competition between SlSGS3 and V2 (right panel) or V2SS (left panel). A measure of 1 μM each of indicated protein was incubated with RNA form 18. For reaction condition with single protein (V2-alone, AtSGS3-alone, SlSGS3-alone or V2SS-alone), the reaction mixtures were incubated for 1 h at 4 °C after protein addition. For reaction conditions with two proteins (V2/V2SS + AtSGS3/SlSGS3), five different orders of protein additions and incubation time were tested. (a) Add V2 and incubate for 55 min, then add SGS3 protein and incubate for 5 min more; (b) add V2 and incubate for 30 min, then add SGS3 protein and incubate for 30 min more; (c) add SGS3 protein and incubate for 55 min, then add V2 and incubate for 5 min more; (d) add SGS3 and incubate for 30 min, then add V2 and incubate for 30 min more; (e) add both V2 and SGS3 at the same time and incubate for 5 min. The samples were run on the gel for long enough so that the shifted bands can be resolved; unbound RNA was not retained on the gel.

by a coiled-coil domain, as in SGS3. Furthermore, the coiled-coil domain is followed by an XH domain in every case except for SGS3. The XH domain is also specific to plants, and its function is unknown. It is possible that one or more proteins containing XS and XH domains mediate an indirect interaction between SGS3 and RDR6 and/or V2.

What are the natural SGS3 substrate RNAs and the role of binding by SGS3?

Although this study establishes the RNA-binding specificity of SGS3, its natural substrates and the role of binding have yet to be determined. In the case of nat-siRNA biosynthesis, dsRNAs generated by convergent transcription contain a 5′ overhang on each strand, to which SGS3 is proposed to bind. SGS3 may prevent degradation of these dsRNAs, alter their location, and/or mark them for further processing. Convergent viral transcripts might likewise give rise to dsRNAs with 5′ overhangs on each strand.

In the case of ta-siRNA biogenesis, SGS3 was proposed to stabilise the *TAS* precursor transcript ssRNA (Yoshikawa *et al*, 2005). This idea was based on the finding that an *A. thaliana rdr6* knockout mutant, but not an *sgs3* knockout mutant or an *sgs3/rdr6* double knockout mutant, accumulated both 5′ and 3′ fragments of the *TAS1* and *TAS2* precursor transcripts that are produced by AGO1 cleavage directed by miR173. In the present study using purified SGS3, we did not detect significant binding activity using ssRNA substrates with no, monophosphorylation, triphospholylation or cap modification (RNA forms 1–4, 12). We also found that SGS3 did not bind detectably to ssRNA oligonucleotides with sequences corresponding to miR173 or miR390, or to several regions within the *TAS1* and *TAS3* precursor that were selected to mimic the states before and after cleavage (data not shown). Therefore, we conclude that at least on its own, SGS3 does not bind to the ssRNA region of the *TAS* precursor transcript. It is possible that *TAS* precursor RNA forms a partially dsRNA segment with a 5′ overhang through self-folding or interaction with miRNA(s) or antisense transcripts and that this structure binds to SGS3. It is also possible that initial ta-siRNA transcripts may hybridise to *TAS* precursors that they derive from, giving rise to 5′ overhang-containing dsRNAs. Such structures could then bind to SGS3, triggering additional production of ta-siRNAs. Whether SGS3 contributes to strand selection in such substrates should be addressed in future work.

V2 inhibits RNAi by competitive binding of SGS3 substrates

V2 functions as an RNA-silencing suppressor that inhibits the innate immune response of the host plant (Zrachya *et al*, 2007). V2 was proposed to interact with SGS3 and thus inhibit its function, based on the results of yeast two-hybrid and fluorescence microscopy experiments in plant cells (Glick *et al*, 2008). The double-mutant protein V2SS has no RNA-silencing suppressor activity and FRET signals interpreted as indicating direct interaction with SGS3 were significantly reduced for V2SS (Zrachya *et al*, 2007; Glick *et al*, 2008). Although both V2 and V2SS localised to the cytoplasm along with SGS3, a physical interaction between V2 and SGS3 had not been established.

In this study, we were unable to detect binding of V2 to SGS3 in the absence or presence of RNA (RNA forms 9 or 18). Instead, our data suggest that these proteins compete for binding to the same dsRNA substrates. We do not exclude the possibility that they interact directly or indirectly through RNA only under certain conditions. For example, V2 and SGS3 might bind to each junction of dsRNA and 5′ overhang within a single dsRNA with 5′ overhangs on each strand, if the RNA is long enough. Such an indirect interaction might explain the results of the yeast two-hybrid and fluorescence microscopy experiments in plant cells (Glick *et al*, 2008).

As V2 and SGS3 share similar dsRNA-binding specificity, the mechanism of RNA silencing suppression by V2 may be that V2 deprives SGS3 of its substrate RNA. In this model, the V2-bound RNA cannot be used for antiviral siRNA production. Whereas V2 out-competes SGS3 for binding to substrate dsRNA form 18, the nonfunctional V2SS mutant did not stably compete with SGS3 despite binding to RNA, with an equilibrium-binding constant only two-fold higher (weaker) than the wild-type V2. These results suggest that there may be a functionally important difference in binding kinetics, rather than thermodynamics, between V2 and SGS3, such that V2 traps dsRNA substrates as they are released from SGS3. There may also be differences in binding cooperativity between the two proteins that help account for the observed competition behaviour.

The substrate specificity of V2 that favours 5′ overhang-containing dsRNAs is distinct from any known RNA silencing suppressors, for example, the viral RNAi-suppressor proteins p19 of tombusviruses and p21 of *Beet yellow virus* bind and sequester siRNAs (Silhavy *et al*, 2002; Vargason *et al*, 2003; Ye *et al*, 2003; Chapman *et al*, 2004; Lakatos *et al*, 2004). These proteins recognise the length and terminal structure of target siRNA duplexes by binding to the central helix and 2 nt 3′ overhangs at the ends. In another example, the B2 protein of Flock House virus binds to long dsRNA substrates and inhibits siRNA formation (Chao *et al*, 2005; Lu *et al*, 2005). It binds to the one face of an A-form RNA helix and does not recognise a specific end structure or length.

Taken together, our findings strongly indicate that V2 blocks host cell RNA silencing by targeting a unique step of the RNA interference pathway. Furthermore, these results imply a new kind of substrate as a critical intermediate in the RNAi pathway in plants. The discovery that dsRNA substrates containing 5′ overhangs have an important function in antiviral defense in plants suggest the potential for new approaches to combating viral infection of crops.

Materials and methods

Construction, expression and purification of proteins

The gene encoding AtSGS3 was amplified from an *A. thaliana* cDNA library (ATCC) and was cloned into a pFastBacHTc plasmid (Invitrogen), using SalI and NotI restriction sites. The gene for AtSGS3ΔN was subcloned into the modified pcoldI vector (Takara) containing the HRV3C protease recognition site to cleave off the N-terminal His-tag, using SalI and NotI restriction sites. cDNA clone plasmids of V2 and SlSGS3 were a gift from Yedidya Gafni (Volcani Center, Israel). The gene for V2 was subcloned into the modified pcoldI vector. C84S/C86S mutation was introduced by PCR. The gene for SlSGS3 was subcloned into the modified pET28c vector (Novagen) containing N-terminal His-tag, MBP and the HRV3C protease recognition site to cleave off the His-tag and MBP, using SalI and NotI restriction sites. The gene for SlSGS3ΔN was subcloned into the modified pcoldI vector. The cDNA clone plasmid of AtRDR6 was a kind gift from Xuemei Chen (University of California, Riverside). The gene for AtRDR6 was subcloned into the pFascBacHTc using SalI and NotI restriction sites.

N-terminally His-tagged AtSGS3 and AtRDR6 were expressed in Sf9 cells infected with baculovirus generated with pFastBacHTc according to product instructions. N-terminally His-tagged AtSGS3ΔN, SlSGS3ΔN, V2, V2SS and N-terminally His-tagged MBP fusion SlSGS3 were expressed in Rosseta2(DE3) *Escherichia coli* cells. The proteins were purified using HisTrap Ni^{2+}-affinity column (GE Healthcare), followed by TEV/HRV3C protease

cleavage to remove the His-tag and His-MBP and purification over a HiTrapQ anion exchange column (GE Healthcare) and/or a HiLoad 16/60 Superdex200 gel filtration column (GE Healthcare). The proteins expressed from pFastBacHTc (AtSGS3 and AtRDR6), the modified pcoldI (AtSGS3ΔN, SlSSG3ΔN, V2 and V2SS) and the modified pET28c (SlSGS3) have vector-derived non-native amino acids (GAMGIRNSKAYVD, GPVD and GPMDVD, respectively) at their N terminus after protease-mediated tag removal.

RNA and DNA substrates

The following sequences are the RNA/DNA oligos (synthesised by Integrated DNA Technologies) used in this study (p represents a terminal phosphate):

RNAoligo 4, 5′-pAAUCGUGAACUUUCAAACUAUACAACCUACUACCUCA-3′;
RNAoligo 5, 5′-pAGGUAGUAGGUUGUAUAGUUUGAAAGUUCACGAUUCA-3′;
RNAoligo 6, 5′-pUGAGGUAGUAGGUUGUAUAGUUUGAAAGUUCACGAUU-3′;
RNAoligo 7, 5′-pAAUGAGGUAGUAGGUUGUAUAGUUUGAAAGUUCACGA-3′;
RNAoligo 8, 5′-pAGGUAGUAGGUUGUAUAGUUUGAAAGUUCACGA-3′;
RNAoligo 9, 5′-pGAGCAUUCCCAAUGAGGUAGUAGGUUGUAUAGUUUGA-3′;
RNAoligo 10, 5′-pAGGTAGTAGGTTGTATAGTTTGA-3′;
RNAoligo 11, 5′-pAAAGAGGUAGUAGGUUGUAUAGUUUGAAAGUUCACGU-3′;
RNAoligo 12, 5′-pGAUUUCUUCCCUUCUGAUGUU-3′;
RNAoligo 13, 5′-pCAUCAGAAGGGAAGAAAUCAA-3′;
RNAoligo 14, 5′-pAACAUCAGAAGGGAAGAAAUC-3′;
RNAoligo 15, 5′-pCUAACAUCAGAAGGGAAGAAA-3′;
RNAoligo 16, 5′-pCGAGCAUGCUCGCC-3′;
RNAoligo 17, 5′-pCGAGCAUGCUCG-3′;
RNAoligo 18, 5′-pCCCGAGCAUGCUCG-3′;
RNAoligo 19, 5′-CCCGAGCAUGCUCG-3′;
RNAoligo 20, 5′-CCCGAGCAUGCUCGp-3′;
RNAoligo 21, 5′-pCCCGAGCAUGCUCGme-3′;
DNAoligo 4, 5′-pAATCGTGAACTTTCAAACTATACAACCTACTACCTCA-3′;
DNAoligo 5, 5′-pAGGTAGTAGGTTGTATAGTTTGAAAGTTCACGATTCA-3′;
DNAoligo 6, 5′-pTGAGGTAGTAGGTTGTATAGTTTGAAAGTTCACGATT-3′;
DNAoligo 7, 5′-pAAATGAGGTAGTAGGTTGTATAGTTTGAAAGTTCACGA-3′;
DNAoligo 9, 5′-pAGGTAGTAGGTTGTATAGTTTGAAAGTTCACGA-3′;

RNAoligo 4, RNAoligo 12, DNAoligo 4 were used as RNA form 4, RNA form 12 and DNA form 4, respectively. To make RNAs form 5–11, RNAoligos 5–11 were annealed to RNAoligo 4, respectively. To make RNAs form 13–15, RNAoligos 13–15 were annealed to RNAoligo 12, respectively. RNAoligos 16–20 are self-complementary and are used as RNAs form 16–20, respectively. To make DNAs form 5–7, 9, DNAoligos 5–7, 9 were annealed to DNAoligo 4, respectively. To make DNA–RNA hybrids form 5–7,9, RNAoligos 5–7, 9 were annealed to DNAoligo 4, respectively. For 5′ end labelling, the phosphate was removed by alkaline phosphatase, followed by ^{32}P labelled phosphoryration by T4 polynucleotide kinase. For forms 5–11, 13–15, one strand was 5′ end labelled.

RNA form 1, 5′-GGGCGUGAACUUUCAAACUAUACAACCUACUACCUCA-3′;
RNA form 2, 5′-pppGGGCGUGAACUUUCAAACUAUACAACCUACUACCUCA-3′; RNA form 3, 5′-Cap-GGGCGUGAACUUUCAAACUAUACAACCUACUACCUCA-3′;

RNA form 2 was transcribed using T7 polymerase with α-labelled nucleotide. RNA form 1 was prepared from RNA form 2 by alkaline phosphatase. RNA form 3 was transcribed using T7 polymerase with α-labelled nucleotide and a cap analogue (NEB).

Electrophoretic mobility shift assay

About 0.05–0.6 nM of 5′ end-labelled RNA/DNA/DNA–RNA hybrid was incubated at 4 °C for 1 h in a 10-μl reaction volume containing 30 mM Hepes-NaOH buffer (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 3% (v/v) glycerol and varied concentration of proteins. The products of binding reactions were run on 6% polyacrylamide gels in 1 × TBE at 4 °C. The gels were dried and then analysed by phosphorimaging. The data points were fit using the Hill formalism by KaleidaGraph (Synergy Software, Reading, PA), where fraction bound = $1/(1+(K_d^n/[P]^n))$ and n represents the Hill constant. Three or four independent experiments were performed and averaged K_d values and standard errors were shown. In the graphs in the figures, the data points represent the averaged values and the curves were fit using the averaged values.

For analysis of the effect of the terminal phosphate, 1 μM of non-radioactive RNA was used, and the gels were stained with SYBR Gold (Invitrogen). The binding and gel running conditions were the same as those used for the 5′ end-labelled experiments.

RDR6 activity assay

Assays were conducted at 25 °C in 20 μl reaction mixtures containing 50 mM Tris–HCl, 200 mM NaCl, 20 mM NH_4OAc, 2% (w/v) PEG4000, 8 mM M_gCl_2, 0.1 mM EDTA, 0.1 unit/μl of RNasein, 1 mM ATP, 1 mM CTP, 1 mM UTP, 0.1 mM GTP and 0.5 mCi/μl [α-^{32}P]GTP, with or without 1 μM AtSGS3, AtSGS3ΔN or V2. 5 μM RNAoligo 4 was used as RDR reaction template. Reactions were initiated by adding final 300 nM of AtRDR6. A measure of 4-μl aliquots were taken at each time points, and the reactions were stopped by adding 4 μl of 2 × loading dye containing 7 M urea and heating for 3 min at 95 °C. The samples were resolved on 12% polyacrylamide, 7 M urea gels. The gels were dried and analysed by phosphorimaging. Three independent experiments were performed.

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References

Allen E, Xie Z, Gustafson AM, Carrington JC (2005) microRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell* **121:** 207–221

Bateman A (2002) The SGS3 protein involved in PTGS finds a family. *BMC Bioinformatics* **3:** 21

Beclin C, Boutet S, Waterhouse P, Vaucheret H (2002) A branched pathway for transgene-induced RNA silencing in plants. *Curr Biol* **12:** 684–688

Borsani O, Zhu J, Verslues PE, Sunkar R, Zhu JK (2005) Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis. *Cell* **123:** 1279–1291

Burkhard P, Stetefeld J, Strelkov SV (2001) Coiled coils: a highly versatile protein folding motif. *Trends Cell Biol* **11:** 82–88

Chao JA, Lee JH, Chapados BR, Debler EW, Schneemann A, Williamson JR (2005) Dual modes of RNA-silencing suppression by Flock House virus protein B2. *Nat Struct Mol Biol* **12:** 952–957

Chapman EJ, Prokhnevsky AI, Gopinath K, Dolja VV, Carrington JC (2004) Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. *Genes Dev* **18:** 1179–1186

Curaba J, Chen X (2008) Biochemical activities of Arabidopsis RNA-dependent RNA polymerase 6. *J Biol Chem* **283:** 3059–3066

Ding SW, Voinnet O (2007) Antiviral immunity directed by small RNAs. *Cell* **130:** 413–426

Gasciolli V, Mallory AC, Bartel DP, Vaucheret H (2005) Partially redundant functions of Arabidopsis DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. *Curr Biol* **15:** 1494–1500

Glick E, Zrachya A, Levy Y, Mett A, Gidoni D, Belausov E, Citovsky V, Gafni Y (2008) Interaction with host SGS3 is required for

suppression of RNA silencing by tomato yellow leaf curl virus V2 protein. *Proc Natl Acad Sci USA* **105:** 157–161

Lakatos L, Szittya G, Silhavy D, Burgyan J (2004) Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses. *EMBO J* **23:** 876–884

Lu R, Maduro M, Li F, Li HW, Broitman-Maduro G, Li WX, Ding SW (2005) Animal virus replication and RNAi-mediated antiviral silencing in Caenorhabditis elegans. *Nature* **436:** 1040–1043

Lupas AN, Gruber M (2005) The structure of alpha-helical coiled coils. *Adv Protein Chem* **70:** 37–78

Montgomery TA, Howell MD, Cuperus JT, Li D, Hansen JE, Alexander AL, Chapman EJ, Fahlgren N, Allen E, Carrington JC (2008) Specificity of ARGONAUTE7-miR390 interaction and dual functionality in TAS3 trans-acting siRNA formation. *Cell* **133:** 128–141

Mourrain P, Beclin C, Elmayan T, Feuerbach F, Godon C, Morel JB, Jouette D, Lacombe AM, Nikic S, Picault N, Remoue K, Sanial M, Vo TA, Vaucheret H (2000) Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* **101:** 533–542

Muangsan N, Beclin C, Vaucheret H, Robertson D (2004) Geminivirus VIGS of endogenous genes requires SGS2/SDE1 and SGS3 and defines a new branch in the genetic pathway for silencing in plants. *Plant J* **38:** 1004–1014

Peragine A, Yoshikawa M, Wu G, Albrecht HL, Poethig RS (2004) SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in Arabidopsis. *Genes Dev* **18:** 2368–2379

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

Vaistij FE, Jones L, Baulcombe DC (2002) Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. *Plant Cell* **14:** 857–867

Vargason JM, Szittya G, Burgyan J, Hall TM (2003) Size selective recognition of siRNA by an RNA silencing suppressor. *Cell* **115:** 799–811

Vaucheret H (2006) Post-transcriptional small RNA pathways in plants: mechanisms and regulations. *Genes Dev* **20:** 759–771

Vazquez F, Vaucheret H, Rajagopalan R, Lepers C, Gasciolli V, Mallory AC, Hilbert JL, Bartel DP, Crete P (2004) Endogenous trans-acting siRNAs regulate the accumulation of Arabidopsis mRNAs. *Mol Cell* **16:** 69–79

Xie Z, Allen E, Wilken A, Carrington JC (2005) DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in Arabidopsis thaliana. *Proc Natl Acad Sci USA* **102:** 12984–12989

Ye K, Malinina L, Patel DJ (2003) Recognition of small interfering RNA by a viral suppressor of RNA silencing. *Nature* **426:** 874–878

Yoshikawa M, Peragine A, Park MY, Poethig RS (2005) A pathway for the biogenesis of trans-acting siRNAs in Arabidopsis. *Genes Dev* **19:** 2164–2175

Yu B, Yang Z, Li J, Minakhina S, Yang M, Padgett RW, Steward R, Chen X (2005) Methylation as a crucial step in plant microRNA biogenesis. *Science* **307:** 932–935

Zamore PD (2004) Plant RNAi: how a viral silencing suppressor inactivates siRNA. *Curr Biol* **14:** R198–R200

Zhang X, Yuan YR, Pei Y, Lin SS, Tuschl T, Patel DJ, Chua NH (2006) Cucumber mosaic virus-encoded 2b suppressor inhibits Arabidopsis Argonaute1 cleavage activity to counter plant defense. *Genes Dev* **20:** 3255–3268

Zrachya A, Glick E, Levy Y, Arazi T, Citovsky V, Gafni Y (2007) Suppressor of RNA silencing encoded by Tomato yellow leaf curl virus-Israel. *Virology* **358:** 159–165