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Identification and RNA Binding Characterization of Plant Virus RNA Silencing Suppressor Proteins

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A B S T R A C T

Suppression is a common mechanism employed by viruses to evade the antiviral effects of the host's RNA silencing pathway. The activity of suppression has commonly been localized to gene products in the virus, but the variety of mechanisms used in suppression by these viral proteins spans nearly the complete biochemical pathway of RNA silencing in the host. This review describes the agrofiltration assay and a slightly modified version of the agro-infiltration assay called co-infiltration, which are common methods used to observe RNA silencing and identify viral silencing suppressor proteins in plants, respectively. In addition, this review will provide an overview of two methods, electrophoretic mobility shift assay and fluorescence polarization, used to assess the binding of a suppressor protein to siRNA which has been shown to be a general mechanism to suppress RNA silencing by plant viruses.

1. Introduction

RNA silencing is a conserved biochemical pathway that results in the sequence-specific degradation of RNA. Regulation of gene expression associated with the degradation of RNA in the pathway has been shown to play an important role in organism development [1] and genome integrity [2]. In addition, RNA silencing serves as a defense mechanism in higher plants and insects when it is triggered by dsRNA from invading viruses [3–5]. dsRNA created during viral replication and dsRNA-like molecules formed by single-stranded viral RNAs or aberrant RNAs that fold into dsRNA segments potentially with mismatched and/or unpaired bases are processed in plants by the host's Dicer-like proteins (DCLs) into viral siRNAs (21–24 nucleotides) that accumulate in the infected cells [6–9]. These viral siRNAs are subsequently incorporated into the host's RNA-induced silencing complex (RISC) and one of the proteins of the complex, a member of the Argonaute (AGO) family of proteins, guides these viral siRNAs to a complementary viral RNA sequence and catalyzes the hydrolysis of the phosphate backbone [10–13]. In addition to the accumulation of viral siRNAs during the primary infection, many viral infections result in an amplification of the silencing signal likely using viral aberrant ssRNA and the host's RNA-dependent RNA polymerases (RDRs) resulting in viral dsRNAs which are processed by DCLs [14–17].

Many viruses have evolved counter defense mechanisms that suppress the antiviral effects of RNA silencing and these mechanisms have typically been linked to one or more proteins produced by each virus. Viral RNA silencing suppressor proteins have been identified in many viruses that attack hosts from each of the kingdoms, they display little to no sequence homology, and their mechanisms of suppression span the complete pathway of RNA silencing [18,19]. Consequently, protein suppressors of RNA silencing show a lack of conserved sequence homology across viral genera. As a result, they have been grouped into the following categories: (1) inhibition of the binding and processing of viral dsRNA into viral siRNA, (2) inhibition of the assembly of RISC, (3) inhibition of the targeting of viral dsRNAs, and (4) inhibition of the amplification of viral siRNAs (reviewed in [18,19]). Several of the mechanisms used by the suppressor proteins use dsRNA binding as a general strategy of suppression [20]. Some silencing suppressors have been shown to bind long hairpin derived or inverted repeat dsRNA and prevent them from being processed into siRNAs [21,22], others bind and sequester siRNAs and prevent their incorporation into RISC [23,24], and still others have been shown to compete with RDRs for binding dsRNA with 5′ ssRNA overhangs to prevent the amplification of viral siRNAs [25].

This review will first focus on one of the techniques used to visualize RNA silencing in plants, the agroinfiltration assay. Specifically, we will address the theory behind the procedure and provide an overview of the protocol. Second, we will provide the theory and procedure for the agro-coinfiltration assay that has been used to visualize and identify RNA silencing suppression in

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plants. Finally, we will provide an overview of two techniques, the electrophoretic mobility shift assay and fluorescence polarization, used to characterize the affinity and specificity of siRNA binding by RNA silencing suppressor proteins.

2. Identification of RNA silencing suppressor proteins

2.1. Agro-infiltration assay to visualize RNA silencing

RNA silencing in plants is commonly induced and visualized using the agroinfiltration assay in transgenic plants. This assay is usually performed in a commonly studied plant (*Arabidopsis thaliana* or *Nicotiana benthamiana*) with a stably integrated and transiently expressed reporter gene, usually encoding *Escherichia coli* β-glucuronidase (GUS) or *Aequorea victoria* green fluorescent protein (GFP) [26,27]. In GUS transgenic plants, the presence of the reporter is visualized histochemically by introducing a chromogenic glucuronide-based substrate (e.g. 4-methyl umbelliferyl glucuronide or 5-bromo-4-chloro-3-indolyl β-D-glucuronide). The substrate is introduced into the plant by abrading the underside of the leaf by rubbing with a carborundum (silicon carbide) powder and vacuum infiltration or by fixing a section of the plant in formaldehyde and vacuum filtration. In GFP transgenic plants the presence of the reporter is visualized directly under UV light as a green color in contrast to the normal red color of the plant due to the fluorescence of chlorophyll. While both reporter constructs have been used, the GFP reporter is commonly used because it is easily visualized, it is generally less destructive than histochemistry, and it allows the observer to follow gene expression over time. Therefore, this review of agroinfiltration will focus on the use of GFP as a reporter.

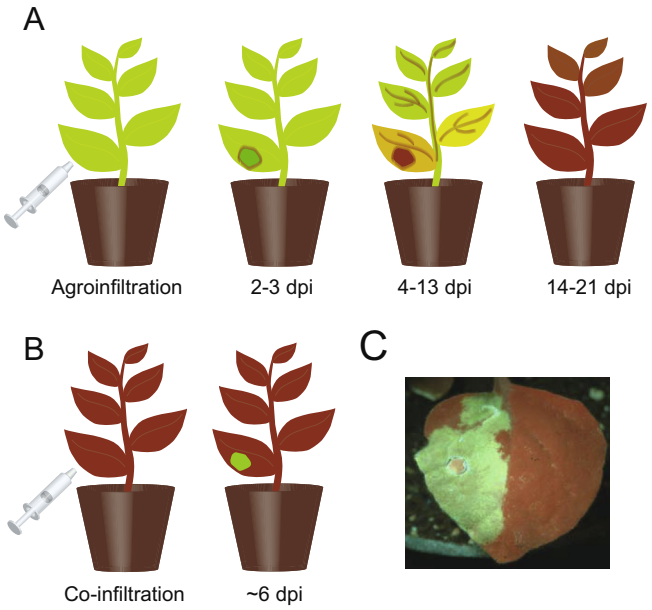

Fig. 1. Agroinfiltration and coinfiltration assays to visualize RNA silencing and suppression of RNA silencing. (A) GFP transgenic plants were injected with an agrobacterium containing a 35S-GFP inverted repeat construct. As the GFP mRNA is silenced and the GFP subsides, the plant changes color from green to yellow to red fluorescence under UV light. The RNA silencing travels systemically through the vascular system of the plant until the GFP is inhibited in the entire plant as 14–21 days post-inoculation (dpi). (B) Wild-type plants were co-injected with an agrobacterium containing a 35S-GFP inverted repeat construct and an agrobacterium containing a 35S-suppressor protein. The effects of RNA silencing are suppressed allowing for the production of GFP mRNA and GFP. Suppression results in green fluorescence at the site of inoculation in the plant as visualized under UV light. (C) Leaf of a wild-type *N. benthamiana* plant coinfiltrated with an agrobacterium containing a 35S-GFP construct and an agrobacterium containing a 35S-Carnation Italian Ringspot Virus p19 suppressor construct at 6 dpi (Reprinted from Ref. [55], with permission from Elsevier).

RNA silencing is initiated in the assay by introducing foreign DNA into the transgenic plant by infiltrating a leaf with a tumor causing plant bacterium called *Agrobacterium tumefaciens*. This agrobacterium naturally carries a >200 kilobase tumor-inducing (Ti) plasmid that is transferred to the plant and a segment of its DNA called transfer-DNA (T-DNA) is integrated into the plant genome [28]. T-DNA can be modified so that a gene of interest can be integrated into the plant. In addition to the T-DNA, the Ti plasmid carries virulence genes (*vir*) responsible for transduction of the plasmid into plant cells. However, the T-DNA and *vir* are not required to be on the same vector and in many instances a binary system is used. The *vir* region is carried on a larger plasmid with no T-DNA and is called the helper plasmid, and the T-DNA is carried on a smaller plasmid without *vir* and is called the binary plasmid. This smaller binary plasmid contains a more general origin of replication used by both *E. coli* and *A. tumefaciens* that allows for maintenance, cloning, and amplification of the T-DNA in *E. coli* [29,30]. In the agroinfiltation RNA silencing assay, the GFP gene is typically cloned into the T-DNA segment of this vector coupled with the 35S promoter from the common plant virus Cauliflower Mosaic Virus to promote heterologous expression of GFP [31].

A. tumefaciens containing a GFP construct is infiltrated into the GFP transgenic plant and causes RNA silencing of the GFP transgene. The agrobacterium is grown in buffered Luria broth along with acetosyringone which is naturally released during plant tissue damage and recognized by the receptor encoded by the *vir*A gene [32]. The addition of acetosyringone has been shown to increase the transformation rate of the Ti vector(s) [33]. Infiltration is facilitated by poking holes in the underside of the leaf with a razor blade followed by injection of the agrobacterium with a needleless syringe [34]. During the early post-infiltration period (2–3 days post infiltration [dpi]) the transgenic plants exhibit a stronger GFP expression against the weaker transient GFP expression as indicated by a green color in and around the site of infiltration under UV light with a reddish-yellow boundary indicating initial silencing of the GFP mRNA (Fig. 1A). Several days after infiltration the GFP transgene silencing is observed in upper leaves and axillary shoots as indicated by a transition from green, through yellow to a red color in these tissues under UV light [35]. In addition, Northern blot analysis is performed to detect the presence of GFP mRNA and confirm the suppression of RNA silencing [36]. Given enough time (14–21 dpi), RNA silencing spreads systemically throughout producing a plant that appears completely red with little to no detectable GFP mRNA.

2.2. Co-infiltration assay to visualize and identify suppressors of RNA silencing

Some viruses have the ability to reverse pre-existing GFP RNA silencing in transgenic plants, but reversal of silencing is enhanced with the expression of certain viral gene products that correlate with the pathogenicity of the virus [5,37]. Other viruses require the addition of certain virulence factor proteins to achieve suppression of pre-existing GFP RNA silencing [38–40]. Potato virus Y and cucumber mosaic virus both encode suppressors of RNA silencing (HC-Pro and 2b, respectively) that facilitate the reversal of a silenced GFP transgene. However, there is no suppression of transgenic GFP in potato virus X infected *N. benthamiana*. Incorporation of the HC-Pro gene from potato virus Y or the 2b gene of cucumber mosaic virus into the potato virus X genome and expression of the gene products during infection results in the suppression of GFP silencing. This was perhaps the first observation of distinct mechanisms of RNA silencing suppression used by different virulence factor proteins in different viruses and has

been implicated in the varying degrees of virulence in different viruses. The identification of these RNA silencing suppressors has used a slightly modified form of the agroinfiltration assay called co-infiltration.

In the co-infiltration assay, two distinct agrobacterium cultures carrying separate gene constructs are infiltrated into GFP transgenic plants or non-transgenic plants in equal issues at equal optical densities to initiate RNA silencing suppression [41–44]. The first agrobacterium is transformed with a 35S construct that contains GFP followed by an inverted repeat of GFP. The inverted repeat of GFP promotes dsRNA formation and serves as a substrate for the DCLs to initiate RNA silencing of GFP upon plant co-infiltration. The second agrobacterium is transformed with a 35S-putative suppressor protein construct that suppresses GFP RNA silencing. The two cultures are co-infiltrated either simultaneously at the same place on the leaf to suppress the local silencing of GFP or at different locations on the same leaf to suppress the systemic silencing of GFP by preventing the signal of silencing from moving through the plant's vascular system [45,46]. The concentration of infiltrated cells is adjusted to avoid toxicity caused by the RNA silencing suppressor protein interfering with the biochemical pathways of the plant. Visualization of RNA silencing suppression in these assays is also done by observing GFP under UV illumination and by GFP mRNA levels. In non-transgenic plants, the site of co-infiltration displays a green fluorescence under UV light against the red background of the plant (Fig. 1B and C). In contrast, transgenic plants show a brighter green fluorescence at the site of co-infiltration against a weaker green background from the transiently expressed GFP transgene.

3. Suppressor protein affinity and specificity for siRNA

A common approach used to measure the affinity and specificity of suppressor protein interactions with siRNA is through equilibrium binding assays. In these experiments, an *in vitro* equilibrium titration experiment is designed by adding samples of purified suppressor protein and a labeled siRNA together. For direct binding experiments, samples of the suppressor protein at varying concentration are added to labeled siRNA samples at a constant concentration. The concentration of the labeled siRNA is usually chosen to be 10-fold lower than the apparent dissociation constant (K_d). Under these conditions, the concentration of the free suppressor protein is nearly equivalent to the total suppressor protein under all conditions of the titration. These conditions are achieved with high affinity suppressors (i.e. low K_d values) using a radioactive label like ^{32}P, which provides high sensitivity in low concentrations. The titration samples are allowed to reach equilibrium and the amount of bound and free siRNA or the fraction of siRNA bound is measured. The fraction of bound, labeled siRNA (θ) is plotted versus free suppressor protein concentration, [P], and the data are fit to the hyperbolic equation.

$$\theta = \frac{[P]}{K_d + [P]} \tag{1}$$

to calculate an apparent K_d. Alternatively, if the labeled siRNA concentration is close to the K_d, then free suppressor protein is not equal to the total suppressor protein. In this case the positive, physically realizable root of the quadratic equation is used.

$$\theta = \frac{(P_t + R_t + K_d) - \sqrt{(P_t + R_t + K_d)^2 - 4P_tR_t}}{2R_t} \tag{2}$$

where P_t is total suppressor protein concentration and R_t is total labeled siRNA concentration.

For competition binding experiments, samples of suppressor protein at varying concentrations are added to a mixture of a labeled siRNA samples at a constant concentration and an unlabeled competitor siRNA at varying concentrations. The fraction of bound, labeled siRNA is plotted versus competitor siRNA concentration using the equation.

$$\theta = \frac{1}{2R_t}\left[K_d + \frac{K_d}{K_c}C_t + P_t + R_t - \sqrt{\left(K_d + \frac{K_d}{K_c}C_t + P_t + R_t\right)^2 - 4R_tP_t}\right] \tag{3}$$

where C_t is total competitor siRNA concentration and K_c is the apparent dissociation constant for the competitor siRNA [47,48]. In competition experiments, the value reported is usually the apparent relative dissociation constant, $K_{rel} = K_c/K_d$. The measurement of free and plant viral suppressor protein bound dsRNA/siRNA or the fraction of bound dsRNA/siRNA to determine apparent K_d has been performed with several methods including, but not limited to, electrophoretic mobility shift assay (EMSA), isothermal titration calorimetry (ITC), direct fluorescence or fluorescence polarization (Fluorescence), surface plasmon resonance (SPR), and total internal reflection fluorescence spectroscopy (TIRFS) shown in Table 1.

3.1. Electrophoretic mobility shift assay

The most frequently used equilibrium binding assay to establish suppressor protein affinity for siRNA is the electrophoretic mobility shift assay. This assay allows for the measurement of free and siRNA bound suppressor protein using gel electrophoresis to separate the two species. In addition to quantitatively measuring the apparent K_d shown in Table 1, this method has been used as a qualitative diagnostic tool to determine if suppressor proteins bind siRNA [44,62]. The requirements of this assay are high concentrations of purified protein and labeled siRNA.

Suppressor proteins are expressed and purified in bacterial expression systems as glutathione S-transferase (GST) or histidine fusion proteins separated by a protease site. The protein is purified with affinity chromatography using the fused tag. Hydrolysis of the protein fusion with the associated protease is advantageous to prevent any influence with siRNA binding or the potential dimerization of the proteins, a common feature of the structurally characterized suppressors [50,55,63]. Ion exchange and/or gel filtration chromatography are used for purification followed by centrifugal concentration to produce suppressor protein for binding assays.

Milligram quantities of the individual strands of siRNA are chemically synthesized and one of the two strands is labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase, annealed to the complementary strand, and purified by gel electrophoresis. For the kinase reaction, 20–50 pmols of ssRNA with a free 5′ hydroxyl group is added to 0.3 μM [γ-^{32}P] ATP and 20 U of T4 polynucleotide kinase in a Tris-buffered solution at pH 7.6. The reaction is incubated at 37 °C for 30 min, then it is incubated at 70 °C for 10 min to inactivate the kinase. A solution with equal molar concentration of the complementary strand containing a 5′ phosphate is added and the strands are annealed in a thermocycler for 1 min. at 90 °C followed by slow cooling (1 °C/3 min.) until the strands reach 25 °C. The slow cooling helps to minimize intra-strand base pairing and hairpin formation. The annealed duplex is run on a non-denaturing 20% tris-borate-ethylenediaminetetraacetic acid (TBE) polyacrylamide gel using 1× TBE running buffer containing 18 mM Tris Base, 18 mM boric acid, and 0.4 mM EDTA pH 8.3. The band containing the labeled siRNA is cut from the gel and placed in a solution of 0.5 M sodium acetate and 1 mM EDTA at pH 6.0 and left at 4 °C overnight to elute the RNA. The gel fragments are removed, the labeled siRNA is precipitated with ethanol at −20 °C, and the sample is lyophilized.

Table 1
dsRNA binding suppressor proteins encoded by plant viruses characterized by quantitative equilibrium binding assays.

Viral family	Virus	Suppressor	Method	Refs.
Aureusvirus	Pothos latent virus	p14	EMSA	[22]
Cucumovirus	Cucumber mosaic virus	2b	EMSA/SPR	[49]
	Tomato Aspermy virus	2b	SPR	[49]
			ITC	[50]
			TIRFS	[51]
Closterovirus	Beet yellows virus	P21	EMSA	[20,24]
Hordeivirus	Barley yellow mosaic virus	γB	EMSA	[20]
	Poa semilatent hordeivirus	γB	EMSA	[52]
Pecluvirus	Peanut clump virus	p15	EMSA	[20]
Potyvirus	Tobacco etch virus	HC-Pro	EMSA	[20,24]
	Potato virus Y	HC-Pro	SPR	[49]
	Turnip yellow virus	HC-Pro	SPR	[49]
	Cucumber vein yellowing virus	P1b	EMSA	[53,54]
Tombusvirus	Tomato bushy stunt virus	p19	EMSA/SPR	[49]
	Cymbidium ringspot virus	p19	EMSA	
	Carnation Italian ringspot virus	p19	EMSA	[24,55]
			Fluorescence	[56,57]
Tenuivirus	Rice hoja blanca virus	NS3	EMSA	[58]
	Rice stripe virus	NS3	EMSA	[59]
Tobamovirus	Tobacco mosaic virus	p122	EMSA	[60]
Tospovirus	Tomato spotted wilt virus, Ground nut ringspot virus, Impatiens necrotic spot virus, Tomato yellow ring virus	NSs	EMSA	[61]

The suppressor protein and siRNA titration samples are prepared, equilibrated, and run on a gel to separate bound and free siRNA. For direct binding experiments, samples are prepared with decreasing concentration of suppressor protein using serial dilution. Briefly, an initial tube containing buffer, the highest concentration of suppressor protein, labeled siRNA, and water is prepared, and half of this solution (for a twofold dilution) is diluted in a tube containing an equal issue and concentration of buffer, labeled siRNA, and water only. Half of the issue of this new solution (for a twofold dilution) is diluted in another tube with equal issue and concentration of buffer, labeled siRNA, and water. The procedure is repeated until the concentration of the suppressor protein is diluted to between one and two orders of magnitude below the apparent K_d. The 1× tris-buffered saline binding buffer used in these experiments contains Tris–HCl (20 mM), a reducing agent, 1 mM dithiothreitol (DTT), β-mercaptoethanol (BME), or tris(2-carboxyethyl) phosphine (TCEP) if it is used in the purification of the suppressor protein without chelators (e.g. EDTA) [64], and a low concentration of a surfactant, usually 0.01–0.02% Tween-20 to block non-specific binding of protein/siRNA to the pipette tips and microcentrifuge tubes. For competition binding experiments, the constant concentration of suppressor protein is chosen to give 80–90% bound siRNA based on the direct binding experiments. The labeled siRNA at constant concentration is added simultaneously with decreasing concentrations of the unlabeled siRNA. Briefly, an initial tube containing buffer, suppressor protein, the highest concentration of competitor siRNA, labeled siRNA, and water is prepared, and half of this solution (for a twofold dilution) is diluted in a tube containing an equal issue and concentration of buffer, suppressor protein, labeled siRNA, and water only. Half of the issue of this new solution (for a twofold dilution) is diluted in another tube with equal issue and concentration of buffer, suppressor protein, labeled siRNA and water. The samples from both direct and competition binding experiments are equilibrated at room temperature for one hour and the samples are adjusted to a final concentration of 7.5% (w/v) with Ficoll 400. Samples are loaded onto a running 6% TBE polyacrylamide gel in 0.5× TBE running buffer. A blank lane containing buffered Ficoll 400 and bromophenol blue is used to monitor the progress of the gel. The siRNA bound to suppressor protein moves through the gel slower and will be shifted relative to the unbound siRNA.

The gel is dried and exposed to a storage phosphor screen followed by imaging of the screen and quantification of the free and bound siRNA bands. After the gels are dried, they are exposed to the phosphor screen for 1–5 days depending on the amount of labeled siRNA used. For high affinity interactions with low apparent K_d, the concentration of the labeled siRNA is low and requires longer exposure to the phosphor. The phosphor screens are imaged with photostimulated luminescence, the bands associated with bound and free siRNA are quantified by integrating the band intensities, and the data are fit to the appropriate equation for equilibrium binding conditions [65].

The electrophoretic mobility shift assay is perhaps the most commonly used technique especially for its sensitivity, but there are several disadvantages. The assay requires a separation of bound and free siRNA during gel electrophoresis which disrupts the equilibrium binding conditions. This disruption is most apparent where the binding affinity between siRNA and suppressor protein is low. In addition, the equilibrated samples are either added to electrophoresis loading buffer usually containing TBE and Ficoll 400 and run in 0.5× TBE thereby disrupting equilibrium, or the samples are allowed to equilibrate in the gel-loading buffer and run in 0.5× TBE. In both conditions, the equilibrium buffer conditions are dictated to some extent by the method rather than experimenter.

3.2. Fluorescence polarization

Spectroscopic equilibrium binding assays like fluorescence polarization measure siRNA species while still in equilibrium rather than separating bound and free siRNA and allow for flexibility in the conditions of the binding solution. In fluorescence polarization experiments, the fraction of free and suppressor bound fluorescently labeled siRNA is measured indirectly based on their different tumbling properties in solution. The fluorophore is excited with vertically polarized light and the intensity of both vertically and horizontally polarized emitted light is measured to determine the amount of depolarization caused by tumbling or molecular rotation in solution during the fluorescence lifetime. Polarization is calculated as

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}} \tag{4}$$

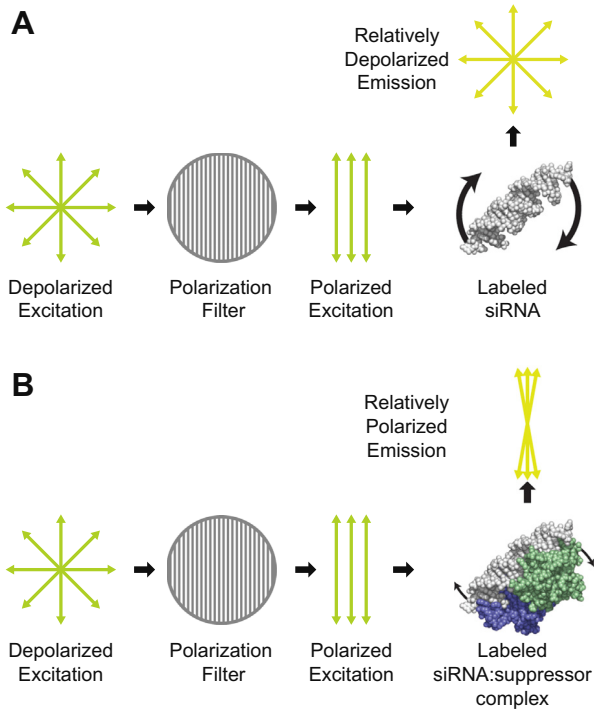

Fig. 2. Fluorescence polarization of siRNA and siRNA:suppressor protein complex. (A) Free siRNA (white) is excited with vertically polarized light and rotates during the fluorescence lifetime causing the emitted light to be largely depolarized and resulting in an I_{VH} and I_{VV} that are similar in magnitude. (B) Suppressor protein (blue and green dimer) bound siRNA (white) is excited with vertically polarized light, but rotation of the larger complex is slow relative to the fluorescence lifetime allowing the emitted light to stay largely polarized resulting in a greater I_{VV} relative to I_{VH} as compared to free siRNA.

where I_{VV} and I_{VH} are the vertically and horizontally polarized intensities of emitted light when excited with vertically polarized light, and G, the grating correction factor, is the ratio of the vertically to horizontally polarized emitted light when excited with horizontally polarized light ($G = I_{HV}/I_{HH}$). The grating correction factor is instrument dependent and corrects for differences in the detection of vertically and horizontally polarized light [66,67]. In these experiments, a fluorophore-labeled siRNA is excited by polarized light, and the emitted light will be depolarized due to molecular rotation of the siRNA during the fluorescence lifetime (Fig. 2A). In contrast, when the labeled siRNA is bound to the suppressor protein and is excited by polarized light, the emitted light will remain relatively polarized due to the slower molecular rotation of the larger complex (Fig. 2B).

The fluorophore label is chosen and attached to the ssRNA during chemical synthesis. Two common fluorophores that have been incorporated into phosphoramidites for RNA synthesis are Cy3 and Dy547. Both are based on cyanine dyes and have similar excitation (~550 nm) and emission (~570 nm) maxima [68]. One of the RNA strands composing the double-stranded siRNA is chemically synthesized with the fluorophore attached at the 5′ end. Since the label could interfere with binding, it is necessary to perform a competition experiment with a standard, unlabeled siRNA (e.g. 19-bp, 2 nucleotide 3′ overhangs, and 5′ phosphates) as a control. siRNA annealing and preparation of equilibrated samples are performed in the same manner as in the electrophoretic mobility shift assay for both direct and competition binding experiments. Fluorescence polarization is measured at room temperature in a fluorometer with manual or automated large aperture polarizers for emission and excitation. Polarization is plotted with respect to suppressor protein concentration in direct binding experiments or with respect to competitor siRNA concentration in competition binding experiments and fit to the appropriate equation for equilibrium binding conditions.

4. Perspectives

The identification of many RNA silencing suppressor proteins with the agroinfiltration assay has further elucidated the mechanism of the associated viral infections. Virulence of several plant viruses has been directly linked to the expression of a suppressor protein. As a result, it has been shown that artificial microRNAs (miRNAs) produced by transgenic plants targeting a viral suppressor can serve as a counter–counter defense strategy to provide plants with virus-specific resistance [69]. In addition, small molecule inhibitors of silencing suppressors have been identified which could be adapted to be used as anti-viral agents [57].

The characterization of the specificity of these suppressors for different dsRNA including siRNA and miRNA using equilibrium binding experiments has laid the groundwork for their use as biomolecular tools. The p19 viral silencing suppressor has been used to study organism development and RNA silencing in heterologous systems [21]. During development, miRNAs with various unpaired or mismatched bases are transcribed by an organism and have been shown to control gene expression. Transgenic expression of p19 results in binding and sequestration of miRNAs controlling that portion of organism development that is dependent on RNA silencing regulated gene expression. As an extension of p19's ability to bind to miRNAs, it has been used to both sequester and immunoprecipate miRNAs *in vivo* [70,71], and it has been used to bind and quantify miRNAs in blood serum using affinity chromatography [72].

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References

[1] W. Boerjan, G. Bauw, M. Van Montagu, D. Inze, Plant Cell 6 (1994) 1401–1414.
[2] F.F. Assaad, K.L. Tucker, E.R. Signer, Plant Mol. Biol. 22 (1993) 1067–1085.
[3] S.W. Ding, O. Voinnet, Cell 130 (2007) 413–426.
[4] S.N. Covey, N.S. Al-Kaff, A. Langara, D.S. Turner, Nature 385 (1997) 781–782.
[5] F. Ratcliff, B.D. Harrison, D.C. Baulcombe, Science 276 (1997) 1558–1560.
[6] A.J. Hamilton, D.C. Baulcombe, Science 286 (1999) 950–952.
[7] A. Molnar, T. Csorba, L. Lakatos, E. Varallyay, C. Lacomme, J. Burgyan, J. Virol. 79 (2005) 7812–7818.
[8] G. Szittya, S. Moxon, V. Pantaleo, G. Toth, R.L. Rusholme Pilcher, V. Moulton, J. Burgyan, T. Dalmay, PLoS Pathog. 6 (2010) e1000838.
[9] H. Shimura, V. Pantaleo, Biochim. Biophys. Acta 1809 (2011) 601–612.
[10] D. Baulcombe, Nature 431 (2004) 356–363.
[11] H. Vaucheret, F. Vazquez, P. Crete, D.P. Bartel, Genes Dev. 18 (2004) 1187–1197.
[12] J.J.W. Harvey, M.G. Lewsey, K. Patel, J. Westwood, S. Heimstadt, J.P. Carr, D.C. Baulcombe, PLoS ONE 6 (2011) e14639.
[13] H.B. Scholthof, V.Y. Alvarado, J.C. Vega-Arreguin, J. Ciomperlik, D. Odokonyero, C. Brosseau, M. Jaubert, A. Zamora, P. Moffett, Plant Physiol. 156 (2011) 1548–1555.
[14] F. Qu, Mol. Plant Microbe Interact. 23 (2010) 1248–1252.
[15] G. Szittya, A. Molnar, D. Silhavy, C. Hornyik, J. Burgyan, Plant Cell 14 (2002) 359–372.
[16] X.B. Wang, Q. Wu, T. Ito, F. Cillo, W.X. Li, X. Chen, J.L. Yu, S.W. Ding, Proc. Natl. Acad. Sci. USA 107 (2010) 484–489.
[17] D. Garcia, S. Garcia, D. Pontier, A. Marchais, J.P. Renou, T. Lagrange, O. Voinnet, Mol. Cell 48 (2012) 109–120.
[18] T. Csorba, V. Pantaleo, J. Burgyan, Adv. Virus Res. 75 (2009) 35–71.
[19] J. Burgyan, Z. Havelda, Trends Plant Sci. 16 (2011) 265–272.
[20] Z. Merai, Z. Kerenyi, S. Kertesz, M. Magna, L. Lakatos, D. Silhavy, J. Virol. 80 (2006) 5747–5756.
[21] P. Dunoyer, C.H. Lecellier, E.A. Parizotto, C. Himber, O. Voinnet, Plant Cell 16 (2004) 1235–1250.
[22] Z. Merai, Z. Kerenyi, A. Molnar, E. Barta, A. Valoczi, G. Bisztray, Z. Havelda, J. Burgyan, D. Silhavy, J. Virol. 79 (2005) 7217–7226.
[23] L. Lakatos, G. Szittya, D. Silhavy, J. Burgyan, EMBO J. 23 (2004) 876–884.

[24] L. Lakatos, T. Csorba, V. Pantaleo, E.J. Chapman, J.C. Carrington, Y.P. Liu, V.V. Dolja, L.F. Calvino, J.J. Lopez-Moya, J. Burgyan, EMBO J. 25 (2006) 2768–2780.
[25] R. Fukunaga, J.A. Doudna, EMBO J. 28 (2009) 545–555.
[26] J. Haseloff, K.R. Siemering, D.C. Prasher, S. Hodge, Proc. Natl. Acad. Sci. USA 94 (1997) 2122–2127.
[27] R.A. Jefferson, T.A. Kavanagh, M.W. Bevan, EMBO J. 6 (1987) 3901–3907.
[28] M.D. Chilton, M.H. Drummond, D.J. Merio, D. Sciaky, A.L. Montoya, M.P. Gordon, E.W. Nester, Cell 11 (1977) 263–271.
[29] A. Hoekema, P.R. Hirsch, P.J.J. Hooykaas, R.A. Schilperoort, Nature 303 (1983) 179–180.
[30] A.J. de Framond, K.A. Barton, M.D. Chilton, Nat. Biotechnol. 1 (1983) 262–269.
[31] J.T. Odell, F. Nagy, N.H. Chua, Nature 313 (1985) 810–812.
[32] Y.W. Lee, S. Jin, W.S. Sim, E.W. Nester, Gene 179 (1996) 83–88.
[33] S.N. Sheikholeslam, D.P. Weeks, Plant Mol. Biol. 8 (1987) 291–298.
[34] J.J. English, G.F. Davenport, T. Elmayan, H. Vaucheret, D.C. Baulcombe, Plant J. 12 (1997) 597–603.
[35] O. Voinnet, D.C. Baulcombe, Nature 389 (1997) 553.
[36] E. Mueller, J. Gilbert, G. Davenport, G. Brigneti, D.C. Baulcombe, Plant J. 7 (1995) 1001–1013.
[37] G. Pruss, X. Ge, X.M. Shi, J.C. Carrington, V. Bowman Vance, Plant Cell 9 (1997) 859–868.
[38] G. Brigneti, O. Voinnet, W.X. Li, L.H. Ji, S.W. Ding, D.C. Baulcombe, EMBO J. 17 (1998) 6739–6746.
[39] R. Anandalakshmi, G.J. Pruss, X. Ge, R. Marathe, A.C. Mallory, T.H. Smith, V.B. Vance, Proc. Natl. Acad. Sci. USA 95 (1998) 13079–13084.
[40] K.D. Kasschau, J.C. Carrington, Cell 95 (1998) 461–470.
[41] O. Voinnet, C. Lederer, D.C. Baulcombe, Cell 103 (2000) 157–167.
[42] L.K. Johansen, J.C. Carrington, Plant Physiol. 126 (2001) 930–938.
[43] P. Dunoyer, S. Pfeffer, C. Fritsch, O. Hemmer, O. Voinnet, K.E. Richards, Plant J. 29 (2002) 555–567.
[44] A. Hamilton, O. Voinnet, L. Chappell, D. Baulcombe, EMBO J. 21 (2002) 4671–4679.
[45] H.S. Guo, S.W. Ding, EMBO J. 21 (2002) 398–407.
[46] P. Dunoyer, G. Schott, C. Himber, D. Meyer, A. Takeda, J.C. Carrington, O. Voinnet, Science 328 (2010) 912–916.
[47] S.Y. Lin, A.D. Riggs, J. Mol. Biol. 72 (1972) 671–690.
[48] K.M. Weeks, D.M. Crothers, Biochemistry 31 (1992) 10281–10287.
[49] H. Shimura, T. Fukagawa, A. Meguro, H. Yamada, M. Oh-Hira, S. Sano, C. Masuta, FEBS Lett. 582 (2008) 4047–4052.
[50] H.Y. Chen, J. Yang, C. Lin, Y.A. Yuan, EMBO Rep. 9 (2008) 754–760.
[51] U.J. Rashid, J. Hoffmann, B. Brutschy, J. Piehler, J.C.H. Chen, Biochemistry 47 (2008) 12655–12657.
[52] D.V. Rakitina, N.E. Yelina, N.O. Kalinina, FEBS Lett. 580 (2006) 5077–5083.
[53] A. Valli, G. Dujovny, J.A. Garcia, J. Virol. 82 (2008) 974–986.
[54] A. Valli, J.C. Oliveros, A. Molnar, D. Baulcombe, J.A. Garcia, RNA 17 (2011) 1148–1158.
[55] J.M. Vargason, G. Szittya, J. Burgyan, T.M. Tanaka Hall, Cell 115 (2003) 799–811.
[56] J. Cheng, S.M. Sagan, Z.J. Jakubek, J.P. Pezacki, Biochemistry 47 (2008) 8130–8138.
[57] S.M. Sagan, R. Koukiekolo, E. Rodgers, N.K. Goto, J.P. Pezacki, Angew. Chem. Int. Ed. 46 (2007) 2005–2009.
[58] H. Hemmes, L. Lakatos, R. Goldbach, J. Burgyan, M. Prins, RNA 13 (2007) 1079–1089.
[59] M. Shen, Y. Xu, R. Jia, X. Zhou, K. Ye, J. Mol. Biol. 404 (2010) 665–679.
[60] T. Csorba, A. Bovi, T. Dalmay, J. Burgyan, J. Virol. 81 (2007) 11768–11780.
[61] E. Schnettler, H. Hemmes, R. Huismann, R. Goldbach, M. Prins, R. Kormelink, J. Virol. 84 (2010) 11542–11554.
[62] K. Ye, L. Malinina, D.J. Patel, Nature 426 (2003) 874–878.
[63] J.A. Chao, J.H. Lee, B.R. Chapados, E.W. Debler, A. Schneemann, J.R. Williamson, Nat. Struct. Mol. Biol. 12 (2005) 952–957.
[64] E.B. Getz, M. Xiao, T. Chakrabarty, R. Cooke, P.R. Selvin, Anal. Biochem. 273 (1999) 73–80.
[65] R.F. Johnston, S.C. Pickett, D.L. Barker, Electrophoresis 11 (1990) 355–360.
[66] T. Azumi, S.P. McGlynn, J. Chem. Phys. 37 (1962) 2413–2420.
[67] R.F. Chen, R.L. Bowman, Science 147 (1965) 729–732.
[68] L.A. Ernst, R.K. Gupta, R.B. Mujumdar, A.S. Waggoner, Cytometry 10 (1989) 3–10.
[69] T. Ai, L. Zhang, Z. Gao, C.X. Zhu, X. Guo, Plant Biol. 13 (2011) 304–316.
[70] J. Cheng, D.C. Danielson, N. Nasheri, R. Singaravelu, J.P. Pezacki, Biochemistry 50 (2011) 7745–7755.
[71] J.M. Calabrese, P.A. Sharp, RNA 12 (2006) 2092–2102.
[72] N. Khan, J. Cheng, J.P. Pezacki, M.V. Berezovski, Anal. Chem. 83 (2011) 6196–6201.