Current Biology, Vol. 14, R198–R200, March 9, 2004, ©2004 Elsevier Science Ltd. All rights reserved. DOI 10.1016/j.cub.2004.02.021

Plant RNAi: How a Viral Silencing Suppressor Inactivates siRNA

Dispatch

Phillip D. Zamore

The three-dimensional structure of an siRNA bound to the tombusvirus p19 protein — a suppressor of gene silencing — provides a first glimpse into how plant viruses can defeat their host's anti-viral RNAi defenses.

The consequences of T cells fighting a virus are known to every cold sufferer. T cells mediate the cellular adaptive immune response, identifying virally infected cells using a surface-bound immunoglobulin-like protein called the T-cell receptor. Plants also catch colds, but they lack protein-based adaptive immunity. Instead, the burdens of anti-viral defense and systemic immune surveillance fall on an ancient adaptive response, the nucleic acid-based RNA silencing pathway.

RNA silencing encompasses post-transcriptional gene silencing in plants, 'quelling' in fungi, and RNA interference (RNAi) in animals [1–3]. Common to all RNA silencing pathways is the production of ~21 nucleotide RNAs which pair to create a 19 base-pair double helix with two-nucleotide, 3′ single-stranded tails. These small RNAs are said to 'silence', because they block expression of genes or RNA transcripts to which they are complementary in sequence. When small RNAs are generated from sources of long double-stranded RNA, such as viruses, transgenes, or transposons, they are called small interfering RNAs (siRNAs). siRNAs are produced by Dicer enzymes, members of a family of ~200 kDa endoribonucleases which contain an amino-terminal RNA helicase domain, a central 'PAZ' motif, and carboxy-terminal tandem ribonuclease III domains.

After their production by Dicer, siRNAs are loaded into a multi-protein effector complex, the RNA-induced silencing complex (RISC). Within the RISC, siRNAs direct the cleavage of a single phosphodiester bond in target RNAs to which they are sufficiently complementary [4]. The siRNA-programmed RISC is similar in concept to the mammalian T cell: it finds the virus using a specificity adaptor — an siRNA, rather than a T-cell receptor — and then destroys it, by cleaving viral mRNA, rather than eliminating the infected cell. A unique feature of RNAi-based anti-viral defense is that its specificity determinant derives directly from viral RNA; the viral genome is turned against itself.

RNA silencing also provides a systemic anti-viral defense system for plants. Plant viruses first infect a small region of the plant, such as a spot on a leaf, but the local infection soon spreads as viral 'movement' proteins distribute the virus through the plant vasculature to more distant regions of the plant [5]. As a defense, the plant uses virus-specific nucleic acid molecules, perhaps siRNAs themselves, to spread the news of the infection from cell to cell and through the vasculature. Systemic silencing is possible because plants — like nematodes, but unlike flies or mammals — contain enzymes that copy RNA into RNA, allowing the systemic silencing signal to be amplified by cycles of intracellular copying and extracellular transport [6]. This relay system may let distant regions prepare RNA silencing defenses ahead of the viral onslaught.

The analogy to mammalian viral defenses extends further still. Plant viruses, like their mammalian counterparts, encode counter-measures intended to thwart the host defenses. In plants, these protein-based viral counter-defenses are called silencing suppressors [7,8]. Two papers [9,10] now report the first three-dimensional structures of a viral silencing suppressor, the tombusvirus p19 protein. These structures provide the first molecular details of how a virus can block an RNA silencing response.

Tombusviruses are positive-strand RNA viruses. Their picturesque names, Cymbidium ringspot virus, Carnation Italian ringspot virus (CIRV), Tomato bushy stunt virus, belie their toxic effect on infected plants. The tombusvirus p19 protein blocks the spread of RNA silencing defenses beyond the site of local infection [11], allowing the virus to infect the entire plant. Burgyán and colleagues have shown that p19 binds double-stranded siRNAs *in vitro* [12] and *in vivo* [11], suggesting that p19 thwarts systemic RNA silencing by sequestering siRNA. In fact, p19 binding is remarkably strong, with a dissociation constant of about 170 pM, and highly specific for active siRNAs [10] — the two p19 structures show why.

The core of the p19 dimer contains basic and polar residues that contact phosphate groups along the binding face of the siRNA double helix, as well as a b-sheet that forms the dimer interface. The core provides p19 its capacity for strong, sequence-independent binding to double-stranded RNA, but not to DNA. Intriguingly, the p19 core uses the same three-dimensional strategy to bind double-stranded siRNA as the L1 protein of the large ribosomal subunit uses to bind a double-stranded RNA stem of the 23S rRNA [10].

The p19 protein binds siRNA, and not simply double-stranded RNA, because it measures double-helical length. In addition to the core of the dimer, each monomer contains an a-helix, the 'reading head' that measures siRNA length. Within this helix, a tryptophan residue — Trp42 in CIRV p19 — stacks on top of the first base of the siRNA strand. That is, Trp42 'caps' the 5′ end of each strand of the siRNA duplex. The distance between the Trp42 residues from each monomer corresponds to the length of a 19 base pair double helix, the size of the double-stranded portion of a canonical 21 nucleotide siRNA. Helices longer than 19 base pairs can bind the p19 dimer, but binding to the 21 nucleotide

Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA.
E-mail: phillip.zamore@umassmed.edu

double helix of a 23 nucleotide siRNA is about 20-fold less strong, and longer helices bind more weakly still. These longer RNA duplexes are likely accommodated because the reading head is linked by a flexible tether to the core. But binding to helices longer than 19 base pairs may require breaking a salt bridge between residues Glu41 and Arg75. This salt bridge appears to fix the position of the reading head; breaking it is predicted to reduce the siRNA binding energy. Plants produce two distinct classes of siRNAs: 'short', 21–23 nucleotide siRNAs, and 'long', 24–25 nucleotide siRNAs [13]. The finding that p19 binds more strongly to short siRNAs may explain why the levels of the long siRNA class are not significantly altered in some studies with p19 transgenic plants [14].

Active siRNAs bear 5′ phosphate groups [15,16]. In addition to its role in end capping, Trp42 contacts the 5′ phosphate group via its imidazole nitrogen; siRNAs lacking 5′ phosphates bind about 20-fold less tightly to p19. In animals, and likely in plants, the 5′ phosphate licenses siRNA for entry into the RISC assembly pathway, suggesting that p19 leaves the siRNA no route for escape: if the siRNA has a phosphate, and so can function in silencing, it is a good substrate for p19 binding; a dephosphorylated siRNA may escape p19 binding, but is unlikely to trigger anti-viral RNA silencing.

A second tryptophan residue, Trp39, also defines the optimal 19 base pair helical RNA length for binding. Trp42 and Trp39 are essential for p19 function: mutation of either abrogates p19 function in whole plants [10]. Unlike Trp42, Trp39 stacks not on the end of the siRNA, but rather on the 3′ face of the last base of the helical part of the siRNA, forcing bases 20 and 21 — the siRNA 3′ single-stranded 'tails' — out of the way. In fact, purified p19 binds a bit more tightly to a blunt 19-mer than to a canonical 21 nucleotide siRNA, suggesting that accommodating the single-stranded tails distorts the protein just a little.

The structure of the p19 dimer bound to siRNA helps explain why some p19-producing plants accumulate blunt-ended 19 base-pair siRNAs: the tails of a p19-bound siRNA may be readily accessible to intracellular nucleases. An alternative explanation, that siRNAs denuded of their tails by a cellular nuclease accumulate because they subsequently bind p19, seems unlikely; such siRNAs are never seen in the absence of p19, yet truncated siRNAs represent as much as half the siRNAs in some p19-producing plants. Blunt, 19 base-pair RNA helices do not trigger RNAi in *Drosophila* embryo lysates [17]. It is not known if blunt, 19 base-pair RNA duplexes are active in plants, but it is tempting to speculate that p19 first sequesters siRNA, then promotes its conversion to a tailless, inactive form.

The p19 protein may also sequester other small RNA duplexes that act in the RNA silencing pathway. MicroRNAs (miRNAs) are single-stranded 21–25 nucleotide RNAs that regulate gene expression. miRNAs are cut from longer stem–loop precursors by Dicer and other members of the ribonuclease III family [18–20]. Unlike siRNAs, the precursors to miRNAs are usually transcribed from non-coding genes separate from the genes they regulate. In plants, most miRNAs

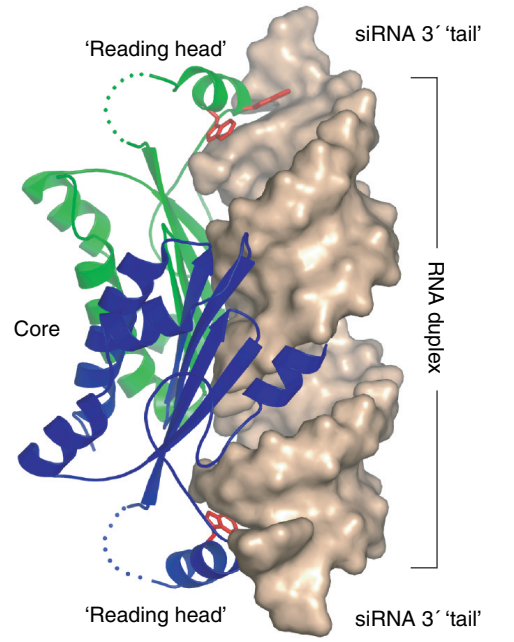

Figure 1. The structure of the p19 silencing suppressor bound to siRNA.

The p19 dimer binds one face of an siRNA duplex (brown). Contacts between the 'core' and the RNA phosphate groups contribute to the protein's high affinity for double-stranded RNA, while a pair of tryptophan residues (red), Trp42 and Trp39, in the 'reading head' measure siRNA length. Because each p19 monomer (blue and green) contributes a reading head, the protein has been described as a 'molecular caliper' that sizes up double-stranded RNA so as to bind best to canonical siRNAs. (Figure courtesy J. Vargason and T.M. Tanaka Hall.)

function like siRNAs to direct endonucleolytic cleavage of their target mRNAs. Although miRNAs are single-stranded, they derive from siRNA-like duplexes in which one of the two strands is destined to become the mature miRNA and the other degraded. p19 likely binds these miRNA duplexes, because cytoplasmic p19 causes at least one miRNA, miR-159, to be shortened by two nucleotides [14]. p19-producing plants are small, flower early, and have serrated leaves and poor fertility. Perhaps p19 disrupts normal plant development because it traps miRNAs in a double-stranded state, preventing them from maturing into the single-stranded form required for their function as developmental regulators [14].

Virally encoded suppressors of silencing have also become tools for dissecting RNA silencing pathways. The advances in understanding the molecular basis of p19 action provide reassurance that transgenic p19 can be used with exquisite specificity to chelate the small RNA duplexes that trigger RNA silencing. In plants, p19 expressed in the nucleus versus the cytoplasm was recently used to infer the intracellular site of siRNA and miRNA biogenesis [14]. In animals, p19 blocks assembly of siRNA into RISC in lysates of *Drosophila* embryos, but has no effect on the activity of Dicer or of RISC assembled before the addition of p19 [11].

Together with quantitative binding assays, analyses *in planta*, and the new structures of p19 bound to siRNA, these *in vitro* experiments suggest that p19 acts simply by binding siRNA duplexes, blocking their assembly into RISC. A key prediction of this hypothesis is that throughout the course of viral infection, the intracellular concentration of p19 should be greater than both its dissociation constant for siRNA and the intracellular concentration of siRNA. Measuring the concentration of both p19 and anti-viral siRNAs in tombusvirus infected plant cells remains a key challenge for the field. Nonetheless, p19 is now poised to become a central tool to dissect the biological roles of small RNAs at particular developmental stages and in individual cell lineages in both plants and animals.

References

1. van der Krol, A.R., Mur, L.A., Beld, M., Mol, J.N.M., and Stuitji, A.R. (1990). Flavonoid genes in petunia: Addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell *2*, 291-299.
2. Napoli, C., Lemieux, C., and Jorgensen, R.A. (1990). Introduction of a chimeric chalcone synthase gene into *Petunia* results in reversible co-suppression of homologous genes *in trans*. Plant Cell *2*, 279-289.
3. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature *391*, 806-811.
4. Elbashir, S.M., Lendeckel, W., and Tuschl, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. *15*, 188-200.
5. Voinnet, O., and Baulcombe, D.C. (1997). Systemic signalling in gene silencing. Nature *389*, 553.
6. Himber, C., Dunoyer, P., Moissiard, G., Ritzenthaler, C., and Voinnet, O. (2003). Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. EMBO J. *22*, 4523-4533.
7. Voinnet, O., Pinto, Y.M., and Baulcombe, D.C. (1999). Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. Proc. Natl. Acad. Sci. USA *96*, 14147-14152.
8. Li, W.X., and Ding, S.W. (2001). Viral suppressors of RNA silencing. Curr. Opin. Biotechnol. *12*, 150-154.
9. Ye, K., Malinina, L., and Patel, D.J. (2003). Recognition of small interfering RNA by a viral suppressor of RNA silencing. Nature *426*, 874-878.
10. Vargason, J.M., Szittya, G., Burgyan, J., and Tanaka Hall, T.M. (2003). Size selective recognition of siRNA by an RNA silencing suppressor. Cell *115*, 799-811.
11. Lakatos, L., Szittya, G., Silhavy, D., and Burgyan, J. (2004). Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses. EMBO J. in press.
12. Silhavy, D., Molnar, A., Lucioli, A., Szittya, G., Hornyik, C., Tavazza, M., and 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.
13. Hamilton, A., Voinnet, O., Chappell, L., and Baulcombe, D. (2002). Two classes of short interfering RNA in RNA silencing. EMBO J. *21*, 4671-4679.
14. Papp, I., Mette, M.F., Aufsatz, W., Daxinger, L., Schauer, S.E., Ray, A., van der Winden, J., Matzke, M., and Matzke, A.J. (2003). Evidence for nuclear processing of plant micro RNA and short interfering RNA precursors. Plant. Physiol. *132*, 1382-1390.
15. Chiu, Y.-L., and Rana, T.M. (2002). RNAi in human cells: basic structural and functional features of small interfering RNA. Mol. Cell *10*, 549-561.
16. Nykänen, A., Haley, B., and Zamore, P.D. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell *107*, 309-321.
17. Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W., and Tuschl, T. (2001). Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. EMBO J. *20*, 6877-6888.
18. Hutvágner, G., McLachlan, J., Pasquinelli, A.E., Balint, É., Tuschl, T., and Zamore, P.D. (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science *293*, 834-838.
19. Grishok, A., Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkun, G., and Mello, C.C. (2001). Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. Cell *106*, 23-34.
20. Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, T., Hannon, G.J., and Plasterk, R.H. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev. *15*, 2654-2659.