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Vicki Vance, *et al.*

Science **292**, 2277 (2001);
DOI: 10.1126/science.1061334

Science (print ISSN 0036-8075; online ISSN 1095-9203) is published weekly, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005.

REVIEW

RNA Silencing in Plants—Defense and Counterdefense

Vicki Vance[1]* and Hervé Vaucheret[2]

RNA silencing is a remarkable type of gene regulation based on sequence-specific targeting and degradation of RNA. The term encompasses related pathways found in a broad range of eukaryotic organisms, including fungi, plants, and animals. In plants, it serves as an antiviral defense, and many plant viruses encode suppressors of silencing. The emerging view is that RNA silencing is part of a sophisticated network of interconnected pathways for cellular defense, RNA surveillance, and development and that it may become a powerful tool to manipulate gene expression experimentally.

RNA silencing was first discovered in transgenic plants, where it was termed cosuppression or posttranscriptional gene silencing (PTGS). Sequence-specific RNA degradation processes related to PTGS have also been found in ciliates, fungi (quelling), and a variety of animals from *Caenorhabditis elegans* to mice (RNA interference RNAs (siRNAs) [for recent reviews on RNA silencing, see (*1*–*3*)]. A key feature uniting the RNA silencing pathways in different organisms is the importance of double-stranded RNA (dsRNA) as a trigger or an intermediate. The dsRNA is cleaved into small interfering RNAs (siRNAs) (21 to 25 nucleotides) of both polarities, and these are thought to act as guides to direct the RNA degradation machinery to the target RNAs (*4*, *5*). An intriguing aspect of RNA silencing in plants is that it can be triggered locally and then spread via a mobile silencing signal (*6*, *7*). The signaling molecule is currently unknown but is expected to contain a nucleic acid component to account for the sequence-specificity. Systemic spread of silencing also occurs in other organisms, though the mechanism may not be the same as in plants. Finally, in plants, RNA silencing is correlated with methylation of homologous transgene DNA in the nucleus (*8*, *9*). Other types of epigenetic modifications may be associated with silencing in other organisms. Three major avenues of research have contributed to a recent burst of information about these different aspects of RNA silencing. (i) Mutant analyses have identified a number of genes that are required for RNA silencing in multiple organisms. (ii) Plant viral suppressors of silencing have provided a tool to identify steps in the pathway and an alternate approach to find cellular proteins that are involved in the process. (iii) The development of an in vitro RNA silencing system from *Drosophila* has allowed a biochemical analysis of some steps in the pathway.

RNA Silencing as a Defense Against Viruses

Several lines of research indicate that RNA silencing is a general antiviral defense mechanism in plants. The first indication came from studies of pathogen-derived resistance (PDR) in plants. In PDR, resistance to a particular virus is engineered by stably transforming plants with a transgene derived from the virus. Eventually, it became clear that one class of PDR was the result of RNA silencing of the viral transgene. Once RNA silencing of the transgene had been established, all RNAs with homology to the transgene were degraded, including those derived from an infecting virus (*10*). Thus, plant viruses could be the target of RNA silencing induced by a transgene. The same work demonstrated that plant viruses could also induce RNA silencing. Virus-induced gene silencing (VIGS) can be targeted to either transgenes or endogenous genes (*11*) and the technique has been used to screen for gene function using libraries of endogenous sequences cloned into a viral vector.

The idea that RNA silencing is an antiviral defense pathway is strengthened by observations of natural plant-virus interactions. First, plants recover from certain plant viral infections, and the recovered plants are resistant to reinfection by the initial virus (and to closely related viruses) because of an RNA silencing mechanism (*12*, *13*). Second, many plant viruses encode proteins that suppress RNA silencing (*14*), suggesting a coevolution of defense and counterdefense between the host and the invading viruses. These viral suppressors target different steps in the silencing pathway and have provided a new approach to understand the mechanism of RNA silencing in plants. It remains to be seen if viruses of fungi or animals use a similar counterdefensive strategy against RNA silencing, or indeed, if RNA silencing serves as a defense against viruses in organisms other than plants.

In plants, RNA silencing can be induced locally and then spread throughout the organism (*6*, *7*), and this aspect of the process likely reflects its role in viral defense. Plant viruses generally enter a cell at a small wound, replicate within that cell, and then move cell-to-cell until they reach the vascular tissue, which serves as a conduit to all parts of the plant body. The movement of the mobile silencing signal in the plant parallels that of the virus, traveling in the vascular tissue and spreading out from the veins (Fig. 1). Thus, an invading virus enters a race with the host. If the virus moves faster, it can establish a systemic infection. If the silencing signal goes faster, then the virus will enter systemic tissues only to find RNA silencing already established, and the infection will be aborted. Given the defensive nature of the mobile silencing signal, it is not surprising that some viruses encode proteins that interfere with the production or movement of the signal (*15*). The mechanism for systemic spread of silencing is one of the big unanswered questions in the field and also one of the most difficult to address experimentally. It may well be that plant viral suppressors will provide a powerful set of tools to examine this aspect of silencing.

Recent studies of two plant viral suppressors, the helper component–proteinase (HC-Pro) of potyviruses and the p25 protein encoded by potato virus X (PVX), represent two different viral strategies to suppress silencing. HC-Pro is a highly effective suppressor of silencing that can enhance the accumulation of a broad range of unrelated plant viruses, likely accounting for the large number of potyvirus-associated synergistic diseases in plants (*16*). It prevents both VIGS and transgene-induced RNA silencing (*17*), and it reverses an already established RNA silencing of a transgene (*18*). HC-Pro suppression of transgene-induced RNA silencing is reversed at a step that eliminates the accumulation of the siRNAs (*19*, *20*), but fails to eliminate the mobile silencing signal as assayed by grafting experiments (*20*). In contrast, PVX p25 is much less effective in blocking silencing than HC-Pro, and it appears to target and interfere with systemic silencing (*15*).

[1]Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA. [2]Laboratoire de Biologie Cellulaire, INRA, 78026 Versailles Cedex, France.

*E-mail: vance@biol.sc.edu or vauchere@versailles.inra.fr

Viral suppressors provide an approach to identifying cellular factors involved in RNA silencing. A tobacco gene called *rgs-CaM*, the product of which interacts with the potyvirus HC-Pro protein in a yeast two-hybrid system, is the first identified cellular suppressor of RNA silencing (*21*). VIGS of a green fluorescent protein (GFP) transgene is impaired when the *rgs-CaM* gene is overexpressed from a transgene. Furthermore, *rgs-CaM* expression is induced in plants after infection by a potyvirus and in transgenic plants expressing the potyviral HC-Pro protein, suggesting that the rgs-CaM protein acts as a relay for potyvirus-mediated suppression of PTGS (*21*). Because calmodulin and related proteins normally act by the binding of calcium and subsequent activation of target proteins, HC-Pro suppression of silencing possibly occurs via activation of *rgs-CaM* and its unknown target protein.

Cellular Proteins Involved in RNA Silencing in Plants

Several genes controlling RNA silencing in plants have been identified through genetic screens of *Arabidopsis* mutants impaired in transgene-induced RNA silencing. They encode a putative RNA-dependent RNA polymerase (RdRP) [*SGS2/SDE1* (*22*, *23*)], a coiled-coil protein of unknown function [*SGS3* (*23*)], a protein containing PAZ and Piwi domains [*AGO1* (*24*, *25*)], and an RNA helicase [*SDE3* (*26*)]. Genes encoding related proteins are involved in RNA silencing in *C. elegans* and *Neurospora* or *Chlamydomonas*. Indeed, the putative RdRp SGS2/SDE1 is related to QDE-1 [*Neurospora* (*27*)] and EGO-1 [*C. elegans* (*28*)]; the PAZ/Piwi protein AGO is related to QDE-2 [*Neurospora* (*29*)] and RDE-1 [*C. elegans* (*30*)]; and the RNA helicase SDE3 is related to SMG-2 [*C. elegans* (*31*)] and MUT-6 [*Chlamydomonas* (*32*)]. Conversely, there are no proteins related to SGS3 encoded by the genomes of *C. elegans* and *Drosophila* (which both undergo RNA silencing), raising the possibility that the function of *SGS3* could be plant-specific (*23*).

Studies in *C. elegans* and in a *Drosophila* in vitro silencing system have identified two ribonucleases involved in RNA silencing in those organisms and suggest additional components of the RNA silencing pathway in plants. The *MUT-7* gene of *C. elegans* (*33*) encodes a protein similar to RNaseD (which displays 3′→5′ exonuclease activity), whereas the *Drosophila DICER* gene (*34*) encodes a protein similar to RNase III (which displays dsRNA endonuclease activity). In the *Drosophila* in vitro RNA silencing system, the input dsRNA is cleaved by the RNase III–like enzyme (DICER) into 21 to 25 nucleotide RNAs of both polarities (siRNAs). The siRNAs incorporate into a multicomponent silencing complex (termed RISC in the *Drosophila* system), where they act as guides to target complementary RNAs. An *Arabidopsis* ortholog of the *DICER* gene (called either *CAF*, *SIN1*, or *SUS1*) has been identified (*35*, *36*). Unfortunately, a knockout in this gene is lethal to the embryo, indicating that it is absolutely required for plants. Whether hypomorphic mutants like *caf* or *sin1* are defective for RNA silencing is not yet known.

Studies in *Arabidopsis* and *Neurospora* indicate that changes at the DNA level are required for transgene-induced RNA silencing in plants and fungi. Using reverse genetics, *Arabidopsis* mutants called *ddm1* and *met1* were shown to be impaired in the triggering (*ddm1*) or maintenance (*met1*) of silencing of an exogenous 35S-GUS transgene (*37*). The corresponding *DDM1* and *MET1* genes encode a SNF2/SWI2 chromatin remodeling factor and a maintenance DNA-methyltransferase, respectively (*38*, *39*). Although DNA methylation seems to be dispensable for quelling, because it occurs efficiently in the *Neurospora dim-2* methylation mutant (*40*), a putative DNA helicase (QDE-3) is required for quelling (*41*) and could play a role similar to that played by DDM1 in *Arabidopsis*.

A Branched Model of RNA Silencing—Different Ways to Make dsRNA?

RNA silencing is induced in plants at varying efficacies by transgenes designed to produce either sense or antisense transcripts. Furthermore, transgenes engineered to produce self-complementary transcripts (dsRNA) are potent and consistent inducers of RNA silencing (*42*, *43*). Finally, replication of plant viruses, many of which produce dsRNA replication intermediates, very effectively causes a type of RNA silencing called VIGS. Whether VIGS, and the different types of transgene-induced RNA silencing in plants result from similar or distinct mechanisms is still a matter of debate. However, recent genetic evidence raises the possibility that the RNA silencing pathway is branched and that the branches converge in the production of dsRNA. Here, we propose a model for sense transgene-mediated RNA silencing in plants and the possible convergence of other branches of the pathway at dsRNA (Fig. 2).

In this model of sense transgene–induced RNA silencing, transcription of the transgene locus produces aberrant RNA because of changes in chromatin structure that require DDM1 and QDE-3–like proteins. These aberrant transcripts form a local duplex structure (*44*) that is used as substrate by a plant RdRP (SGS2/SDE1) in combination with a coiled-coil protein (SGS3), an RNA helicase (SDE3), and a PAZ/Piwi protein (AGO1) to synthesize longer dsRNA. This complex containing the longer dsRNAs then recruits a DICER-like dsRNase (CAF/SIN1/SUS1), thus generating the siRNAs that are channeled into the RISC-like silencing complex where they serve as guides to target complementary RNAs for destruction. The siRNAs (or precursors of the siRNAs) could also direct methylation of transgene DNA (maintained by MET1), in this way further promoting production of aberrant RNA.

In our model, silencing mediated by dsRNA and by viral RNA are separate branches of the pathway that merge with the sense transgene–silencing branch at the step of dsRNA accumulation. Evidence for these separate branches of the pathway comes from studies of the effect of dsRNA continuously produced by a transgene or delivered exogenously by a virus into silencing-defective mutants of *Arabidopsis*. VIGS occurs in *Arabidopsis* RdRP (sgs2/sde1) and RNA helicase (sde3) mutants impaired in silencing of a sense transgene (*22*, *26*). Similarly, silencing mediated by transgenes producing dsRNA occurs in RdRP (sgs2/sde1), coiled-coil (sgs3), and PAZ/Piwi (ago1) mutants (*45*). Studies of RNA silenc-

Fig. 1. RNA silencing on the move. Movement of the mobile silencing signal in a GFP-expressing tobacco plant is visualized as a red trail where GFP has been silenced, revealing red fluorescence of chlorophyll in the background of green fluorescence from GFP.

ing–defective mutants of *C. elegans* indicated that the putative RdRp EGO1 is needed only for RNAi against some genes in the germ line (*28*) and that the PAZ/Piwi protein RDE-1 is required only for initiation of RNAi and is dispensable for RNA silencing by transgenes. These observations suggest that the genetic requirements for RNA silencing depend on how the process is triggered. Alternatively, the different genetic requirements for RNA silencing triggered by sense RNA, dsRNA, or viruses could reflect that paralogs are involved in these different branches of the pathway. Indeed, many of the genes involved in RNA silencing belong to multigene families, and different family members might fulfill similar roles in the different branches. For example, it has long been known that virus infection in plants induces cellular RdRp activity, and recently it has been reported that an RdRp is induced by salicylic acid (SA) in tobacco and results in resistance against viruses (*46*). The *Arabidopsis* gene with highest homology to this tobacco SA-inducible RdRp is also SA-inducible and is not the *SGS2/SDE1* gene. These results raise the possibility that the SA-inducible RdRp substitutes for the *SGS2/SDE1*-encoded RdRp in VIGS.

Silencing of gene expression by antisense transgenes might be more complex and occur by different mechanisms in individual transgenic lines. Antisense transgene loci arranged as inverted repeats efficiently silence homologous endogenous mRNA (*47*, *48*). Thus, as shown in our model, some cases of antisense inhibition may result from entry into the RNA silencing pathway at the dsRNA or aberrant RNA (abRNA in Fig. 2) step. However, whether antisense transgene RNA can actually trigger degradation of homologous endogenous RNA directly (as shown for sense RNA silencing) is still not known. Indeed, it has frequently been reported that single-copy antisense transgenes are weak silencers (*48*). In addition, sense RNA and antisense RNA expressed from unlinked loci can accumulate together without being degraded (*49*, *50*), suggesting that dsRNA may not be formed efficiently in such cases. These results raise the possibility that some cases of antisense inhibition work through a mechanism that is distinct from RNA silencing and produces only weak suppression.

RNA Silencing as Part of a Network of Defenses and/or RNA Surveillance

Plants have evolved a complex set of defense mechanisms, as well as a likely equally complex set of mechanisms to ensure proper functioning of RNA-dependent processes. Several lines of evidence suggest that RNA silencing partially overlaps some of these other defense and RNA surveillance pathways. First, dsRNA, a key inducer of RNA silencing, also induces transcriptional gene silencing (TGS) (*51*), a process that acts as a defense against certain transposable elements. Furthermore, dsRNA-induced TGS results in the accumulation of small si-like RNAs. Previously such RNAs were thought to be unique to (and indicative of) RNA silencing (*52*). In addition, *ddm1* and *met1* mutants of *Arabidopsis,* which are impaired in TGS of endogenous sequences derived from degenerated retrotransposons (*53*), are also defective in RNA silencing (*37*). Similarly, transposons are mobilized in certain silencing impaired mutants in *C. elegans* and *Chlamydomonas* (*30*, *32*, *33*). Together, these results suggest that TGS and RNA silencing are partially overlapping mechanisms, both serving as a defense against mobile genetic elements.

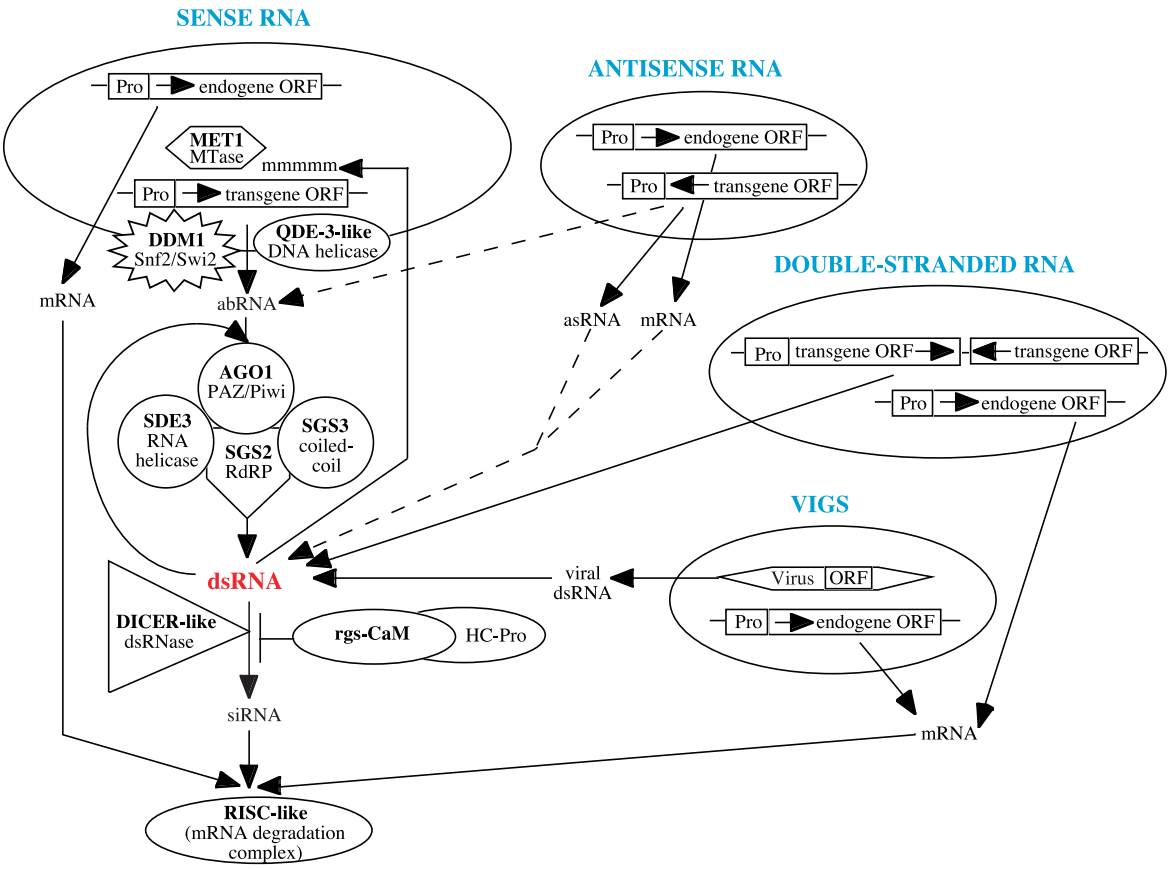

Fig. 2. A branched model for RNA silencing in plants. RNA silencing of an endogenous gene by a viral vector or by different types of transgenes is depicted. dsRNA is proposed to be the common intermediate linking the various ways of initiating RNA silencing. Viruses, as well as transgenes, arranged as inverted repeats, can directly produce dsRNA, whereas transgenes with a single copy sense orientation produce aberrant (ab) transcripts that serve as a substrate for producing dsRNA. Transgenes expressing antisense RNA could potentially enter the pathway either at the dsRNA or abRNA steps or not at all (see text). Small interfering RNAs derived from dsRNA (siRNA) incorporate into the RISC-like silencing complex to mediate sequence-specific RNA degradation. Proposed action of gene products known so far to be involved in RNA silencing is indicated.

Second, the cucumber mosaic virus 2b protein, a suppressor of RNA silencing (*18*), also suppresses an SA-mediated defense in tobacco (*54*). Further evidence for a connection between these two defense pathways is the discovery that an RdRp is induced by SA in tobacco and results in resistance against viruses (*46*).

Third, RNA silencing has some genetic requirements in common with nonsense-mediated decay (NMD), a highly conserved pathway in eukaryotes that targets and destroys RNAs containing a premature stop codon. Seven genes are required for NMD in *C. elegans*, three of which were also required for persistence of the silenced state [*smg* 2, 5, and 6 (*31*)]. *Smg-2* is homologous to yeast *Upf1*, encoding an adenosine triphosphatase with RNA-binding and helicase properties, whereas the required SMG-5 and SMG-6 proteins dephosphorylate SMG-2 (*31*).

Although RNA silencing appears to be an ancient response to dsRNA and part of an interconnected network of pathways involved in protection against aberrant or pathogenic nucleic acids, the role of this mechanism in adult mammals is not clear. Sequence-specific RNA degradation can be triggered in mouse embryos by injection of dsRNA. However, the technique is much less successful in adult animal cells (*55*, *56*), where dsRNA is a potent trigger of two other defense pathways: PKR-mediated apoptosis and the 2′-5′ oligoadenylate/RNase L pathway (*57*, *58*). It may be that RNA silencing is masked in mammals by the dominance of these other defense pathways, again pointing to the interconnected nature of defense responses. One possibility is that RNA silencing is not generally used in mammals except perhaps early in development or in a tissue-specific manner. Recent work raises the possibility that RNA silencing could be experimentally induced in mammals by direct introduction of small RNAs that mimic those produced by DICER (*59*). These may be able to incorporate into the RNA degradation complex and to induce RNA silencing, but they would be too short to trigger the competing defense responses that lead to apoptosis (*59*).

RNA Silencing—a Role in Development?

Many mutants impaired in RNA silencing in plants, fungi, and animals have no obvious phenotype, suggesting that the corresponding genes, as well as silencing itself, are dispensable for normal development (*22*, *23*, *26*, *27*, *30*). In contrast, other silencing mutants exhibit developmental abnormalities (*ago1*, *caf/sin1/sus1* in *Arabidopsis*, *ego-1*, *mut-7* in *C. elegans*), [(*24*, *35*, *28*, *33*), respectively], suggesting that these genes play independent roles in development and silencing. The recent identification of *ago1* alleles that show almost normal development but that are as deficient in RNA silencing as a null mutant supports the idea that AGO1 participates independently in silencing and development (*50*). Furthermore, plants that express high levels of the viral silencing suppressor HC-Pro or that overexpress the endogenous suppressor rgs-CaM also show abnormal development (*21*). This raises the possibility that the suppression of RNA silencing mediated by these proteins works via interaction with factors that have a dual role in silencing and development. Thus, development appears to be another of the interconnected network of pathways of which RNA silencing is a part.

RNA Silencing—Practical Implications

Until recently RNA silencing was viewed primarily as a thorn in the side of plant molecular geneticists, limiting expression of transgenes and interfering with a number of applications that require consistent, high-level transgene expression. With our present understanding of the process, however, it is clear that RNA silencing has enormous potential for engineering control of gene expression, as well as for use as a tool in functional genomics. It can be experimentally induced with high efficiency (*42*, *43*) and targeted to a single specific gene or to a family of related genes. Likewise, dsRNA-induced TGS may have similar potential to control gene expression. Unwanted RNA silencing, on the other hand, can be alleviated using viral suppressor technology or mutants impaired in silencing. The ability to manipulate RNA silencing thus sets the stage for realizing a wide variety of practical applications of biotechnology ranging from molecular farming to possibly even gene therapy in animals.

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60. We thank C. Beclin, L. Bowman, M. Fagard, and G. Pruss for comments on the manuscript and D. C. Baulcombe, Z. Chen, and S. W. Ding for sending manuscripts in press.