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Viral Suppressors of Gene Silencing

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Glossary

Dicer, or Dicer-like (DCL) enzymes RNAse III or RNAse-III-like enzymes responsible for digesting the noncoding regions of mRNAs to produce 21–24 nt single-strand RNAs known as miRNAs and siRNAs.
Green fluorescent protein (GFP) This is derived from jellyfish and fluorescence green. Excitation wavelength is 488 nm and emission is above 520 nm. Fusions involving GFP are often used to study protein subcellular targeting or distribution in tissues.
MicroRNAs (miRNAs) Single-strand RNAs that are 21–24 nt in length are found in eukaryotes and arise from noncoding regions of transcripts. These are produced by nucleolytic processing by DICER, and RNAse-III-like enzyme. These are crucial components of the RNAi pathway.
RNA interference (RNAi) Similar to post-transcriptional gene silencing. More specifically, cellular or synthetic small RNA molecules can target homologous mRNA for degradation thereby preventing gene expression.
RNA silencing or post-transcriptional gene silencing (PTGS) Mechanism regulating gene expression by regulating RNA accumulation after transcription. Mechanism involves RNA degradation machinery to shut off gene expression.
Short interfering RNAs (siRNAs) Double-strand RNAs that are 21–24 nt in length which are generated by DICER or Dicer-like enzymes. SiRNAs can spread systemically in *C. elegans* and may cause silencing in distal organs. Some single-strand RNAs are made double-strand by RNA-dependent RNA polymerases. These double-stranded products are then cleaved by DICER.
Transcriptional gene silencing (TGS) Silencing of genes in the nucleus. A small RNA molecule triggers *de novo* DNA methylation thereby blocking transcription. Small RNA typically is homologous to the target gene.
Virus-induced gene silencing (VIGS) Viral RNAs can trigger for PTGS similar to small RNAs. Several plant viruses have been engineered as vectors for use in experiments shutting off gene expression by PTGS. Fragments of genes, antisense RNAs, small RNAs can be introduced into the viral vector and silencing is induced upon inoculation with the recombinant virus.

Introduction

RNA silencing, also known as post-transcriptional gene silencing (PTGS) or RNA interference (RNAi) is a mechanism regulating gene expression in a wide range of eukaryotes. RNA silencing is a mechanism in which small RNAs block gene expression by targeting homologous mRNAs without impacting nuclear DNA. Andrew Hamilton and David Baulcombe first showed that short antisense RNAs of 20–25 nt which share homology with target mRNAs are produced in silenced tissues. Since 1999 additional short RNAs have been identified, including microRNAs and siRNAs, which target homologous RNA sequences either for sequence specific degradation or, in some instances, function to repress mRNA translation.

PTGS is induced by double-stranded RNAs in most eukaryotic systems. Since most RNA viruses form double-stranded RNA replication intermediates, replicating viruses often trigger PTGS, which subsequently degrades all homologous RNAs within the cell. This has led researchers to suggest that PTGS may have originally evolved as an antiviral defense mechanism. The ability of PTGS to target viral RNAs for degradation was demonstrated in the early 1990s when transgenic plants expressing untranslatable transcripts of the viral coat protein or replicase gene were found to be resistant to infection by the homologous virus while remaining susceptible to unrelated viruses. Virus resistance was also reported in experiments using transgenic plants which failed to accumulate detectable levels of the transgenically expressed coat protein RNA.

The laboratory of William Dougherty provided the first reports of transgenic plants recovering from virus infection. A set of transgenic tobacco plants expressing a nontranslatable tobacco etch virus (TEV) coat protein RNA were initially susceptible to virus infection but then recovered and became highly resistant to secondary inoculation. Viral RNA was undetectable and transgene RNAs showed lower steady-state level accumulation in recovered leaves indicating that an RNA degradation mechanism was triggered. Contemporary studies by Rob Goldbach's laboratory showed that nontranslatable transcripts for the tomato spotted wilt virus N protein-protected transgenic plants from infection by the virus. The idea that viruses trigger a cellular antiviral defense pathway which degrades homologous RNAs was further supported by experiments in the laboratory of

David Baulcombe showing that recovery can occur during infection of nontransgenic tobacco plants. *Nicotiana clevelandii* inoculated with the nepovirus tomato black ring virus (TBRV) strain W22 initially showed clear virus symptoms but later recovered. Plants were resistant to secondary inoculation with the same W22 strain but were susceptible to inoculation with the heterologous nepovirus, tobacco ringspot virus (TRSV), and showed partial protection to secondary infection with the TBRV strain BUK. Insertion of a fragment of the TBRV genome into the potato virus X (PVX) genome was sufficient to block infection of PVX in recovered plants indicating that plants contain an inducible sequence-specific degradation mechanism that may be a component of the plant immune response to RNA virus infection.

This same PTGS mechanism also provides cross-protection against secondary virus infection seen in the TBRV and PVX experiments described above and in experiments showing that plants recovered from infection with tobacco rattle virus containing GFP (TRV-GFP) were resistant to secondary infection with PVX-GFP. Technology based on PTGS has been developed to engineer virus resistance in transgenic plants.

Viral-induced gene silencing (VIGS) in transgenic plants can result in methylation of the cognate transgene or nuclear gene. It has been suggested that *de novo* DNA methylation triggered by transcriptional gene silencing (TGS) or PTGS is driven by siRNAs imported into the nucleus, which recruit DNA methyltransferases to similar target sequences. Thus, RNA viruses, such as PVX or tobacco rattle virus (TRV), have been engineered as vectors used for knocking out host gene expression. These are termed VIGS vectors. Entire genes or fragments of genes can be inserted into the viral vector and following inoculation, induce silencing of the cognate endogenous gene. One of the earliest examples of VIGS targeting an endogenous gene was insertion of the phytoene desaturase gene (PDS) into PVX. PDS is involved in carotenoid biosynthesis and affects plants' susceptibility to photobleaching. Virus-induced PDS silencing causes leaves to lose all green color and bleach white under normal lighting conditions. The most popular research tools to study TGS and PTGS are transgenic plants expressing the green fluorescent protein (GFP) and PVX or TRV vectors containing fragments of the GFP coding sequence. GFP expression can be monitored using a UV lamp and over time GFP expression disappears throughout the entire plant. In 1997, Olivier Voinnet and David Baulcombe first reported GFP-transgene silencing by an RNA virus and also demonstrated the silencing signal can spread systemically to suppress gene expression in distal regions. Thus, the use of VIGS to suppress expression of endogenous genes has become an important tool for analysis of gene function.

DNA viruses can also be used as gene silencing vector. The chalcone synthase gene, which is involved in flower pigmentation in *Petunia hybrida* flowers, was inserted into tobacco yellow dwarf virus genome and flower pigmentation was completely altered in virus infected petunia. Cabbage leaf curl virus (CabLCV), a member of the genus *Begomovorus* of the family *Geminiviridae*, has been engineered to express any endogenous targeted gene of *Arabidopsis* and proved to be an efficient tool for scientists studying gene expression in this model plant. Similarly, the African cassava mosaic virus (ACMV), another begomovirus, modified to be a silencing vector, was demonstrated to be able to silence a variety of endogenous genes in cassava, thereby providing a useful tool to breeders for that crop.

Viral-Derived Short Interfering RNAs

Small RNAs of approximately 21–24 nt are found in all eukaryotes and belong to two general classes: microRNAs (miRNAs) and short interfering RNAs (siRNAs). miRNAs are 21–24 nt ssRNAs and arise from nonprotein coding regions of transcripts which are nucleolytically processed by an RNAse-III-like enzyme called DICER in animals and *Caenorhabditis elegans*, or DICER-like (DCL) in *Arabidopsis*. siRNAs similar to miRNAs range in size from 21 to 24 nt, but are dsRNAs derived from longer double-stranded RNA (dsRNA), including RNAs containing inverted repeats or replicative forms of RNA viruses.

Animals and *C. elegans* encode only a single DICER while *Arabidopsis* encodes four DCL proteins. While studies in *Drosophila* and plants show that DICER plays a role in antiviral defense, currently there is no direct evidence that RNA silencing acts as a natural antiviral defense mechanism in vertebrates. Dicer 2 mutants in *Drosophila* are hypersusceptible to virus infection. All four DCL proteins in *Arabidopsis* are involved in generating siRNAs from DNA and RNA viruses. DCL1 and DCL4 proteins produce 21 nt siRNAs, DCL2 produces 22 nt siRNAs, and DCL3 produces 24 nt siRNAs. While reports show all four DCL proteins contribute to the production of siRNAs from geminiviruses (CaLCuV) and pararetroviruses (cauliflower mosaic virus; CaMV) in plants, DCL2 and DCL4 generate siRNAs for defense against RNA viruses.

Cellular RNA-dependent RNA polymerases (RDR) also contribute to the formation of siRNAs. Single-stranded RNAs are made double stranded by cellular RDRs and then are cleaved by DICER to produce siRNAs. *HEN1* encodes a methyltransferase which acts alongside the DCL proteins to methylate the 3′-terminal nt protecting siRNAs from degradation. siRNAs then guide sequence-specific RNA-induced silencing complexes (RISCs) to target sequences for degradation. The RISC is comprised of several proteins including *ARGONAUTE* (*AGO*), which bind siRNAs or target sequences. RDR proteins use the siRNAs as primers for

synthesis of dsRNAs from target viral RNAs. *Arabidopsis* encodes three *RDR* genes named *RDR1*, *RDR2*, and *RDR6*. *RDR1* is salicylic acid inducible. *Arabidopsis* plants showing defects in RDR1 show increase susceptibility to TMV and TRV. *RDR6* is also known as *SDE1* or *SGS2* and is required for transgene-generated short RNAs. *SDE5* is a factor recently identified which acts with *RDR6* to generate dsRNAs. *SDE5* may function in the nuclear transport of dsRNAs produced by *RDR6*. Mutant plants deficient in *RDR6* show increased susceptibility to cucumber mosaic virus (CMV) but not to other viruses.

Research in plants and in *C. elegans* first showed that a systemic silencing signal which spreads to distal organs is likely to be an siRNA or dsRNA. Research has shown that 21 nt siRNAs can spread 10–15 cells in tobacco and *Arabidopsis* plants. For more extensive movement, additional rounds of signal amplification are needed to produce a second generation of siRNAs. A model of transitive RNA silencing was proposed by Voinnet and colleagues in 2003 in which dsRNAs are synthesized *de novo* by RDR6, which are then cleaved by DICER to generate the second generation of 21 nt siRNAs. Cycles of siRNA propagation and movement into neighboring tissues lead to general silencing throughout surrounding tissues. Systemic silencing spread was proposed to rely on vascular transport of longer 25 nt siRNAs.

RNAi-Based Antiviral Therapies

Viral siRNAs accumulate to significant levels in plants and insects but have not been characterized in human cells infected with any RNA viruses. However, synthesized siRNAs have been shown to block replication and accumulation of a wide range of animal RNA and DNA viruses in cell cultures and vertebrate systems. There are examples of siRNAs targeted to specific sequences in the genomes of viruses including poliovirus (PV), foot and mouth disease virus (FMDV), hepatitis virus A (HVA), influenza virus, SARS-COV, HIV, and hepatitis virus B (HVB) which reduce virus titer and inhibit replication in cell cultures and in mice. Researchers using synthesized RNAs targeting different viral RNA sequences reported this strategy to be a successful form of antiviral therapy. For monopartite RNA viruses such as PV, FMDV, HVA, short RNAs targeting conserved sequences corresponding to genes encoding structural proteins or the viral replicase have been successful.

One of the important issues in developing RNAi therapeutics has been the pressure on target sequences to mutate which causes the virus to escape the suppressive activity of the siRNA molecule. To address this concern researchers have relied on bioinformatics tools and GenBank database to search entries of virus sequences to identify highly conserved nucleic acid elements ranging from 21 to 25 nt in length. Comparisons among virus isolates have been crucial for determining the most conserved regions. Among viruses, which have high mutation rates, therapies combining synthesized short RNA molecules targeting several conserved sequences have been most effective at reducing the occurrence of escape viruses.

The polyomavirus Simian virus 40 (SV40) as well as members of the family *Herpesviridae* including Epstein–Barr virus (EBV), herpes simplex virus 1 (HSV-1), Kaposi's sarcoma-associated virus (KSHV), and were found to encode miRNAs. At least 23 miRNAs have been identified in EBV-infected lymphocytes, many of which map to the BART gene. All BART gene miRNAs accumulate mainly during latency suggesting that they likely play a role in this stage of infection. KSHV is also associated with lymphomas and 12 miRNAs have been identified in latent infected cells. For KSHV a different set of miRNAs are seen during lytic infection suggesting that specific miRNAs are expressed during different stages of the viral life cycle. Viral-encoded miRNAs function to regulate viral gene expression and to downregulate host transcription. Since many of the herpesviral miRNAs associate with latency, they likely play a role in enabling the virus to evade the host immune system for many years. Further research is needed to find out if viral miRNAs affect tumorogenesis and if RNAi technology can be used to alter the onset of cancer.

Viral Suppressors of RNA Silencing Counter Cellular Defenses to Promote Infection

Plant and insect viruses encode silencing suppressor proteins which inhibit one or more steps in the miRNA or siRNA degradation pathway, thus countering the antiviral defense machinery. Many silencing suppressors bind dsRNA, siRNAs, and miRNAs. The potyvirus HC-Pro blocks the RISC from acting on target RNAs (**Figure 1**). HC-Pro was the first identified suppressor of RNA silencing in plants and was discovered by researchers studying viral synergistic diseases. Synergism is a phenomenon in which one virus shows increased titer and symptom induction due to the presence of a second but unrelated virus. Many, but not all, examples of synergy involve co-infection in which one of the viral partners is a potyvirus. Building on the early studies of PVX/PVY synergy, Vicki Vance, James Carrington, and their colleagues carried out further investigations of PVX/PVY synergy at the molecular level. They examined virus-specific RNA production in doubly infected plants and used PVX-derived vectors and transgenic plants to express segments of PVY and other potyviral genomes. Transgenic plants expressing the potyviral P1/HC-Pro sequence developed the same synergistic response when inoculated with unrelated

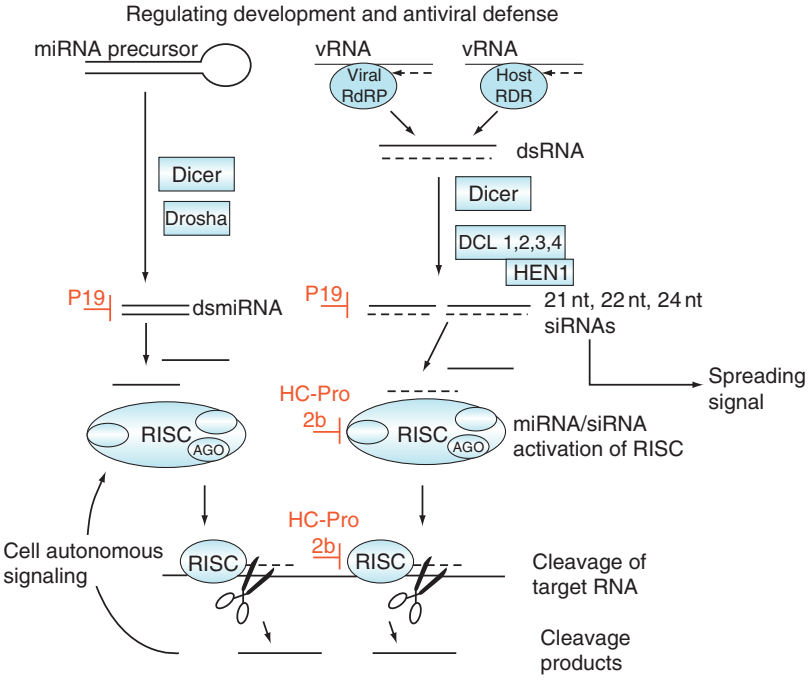

Figure 1 Model for RNA silencing updated from Himber C, Dunoyer P, Moissiard G, Ritzenthaler C, and Voinnet O (2003) Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. *EMBO Journal* 22(17): 4523–4533; Matzke MA and Matzke AJ (2004) Planting the seeds of a new paradigm. *PLoS Biology* 2: E133; Blevins T, Rajeswaran R, Shivaprasad PV, *et al.* (2006) Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. *Nucleic Acids Research* 34(21): 6233–6246. Double-stranded RNAs (dsRNAs) are produced either by hairpin interactions or by viral and host RNA-dependent RNA polymerases which synthesize complementary RNAs using viral RNA (vRNA) or mRNA templates. Dicer or Dicer-like proteins cleave the dsRNAs to smaller miRNAs or siRNAs which then guide the RISC complex to the target RNAs. Target RNAs are cleaved into short RNAs which can interact with the RISC complex to continue the cycle of cell autonomous RNA degradation. Some siRNAs are diverted prior to the RISC complex and move cell-to-cell and long distance to perpetuate silencing in surrounding and distal tissues. Examples of viral silencing suppressors are identified in red which block silencing by acting on the siRNAs or RISC complex.

viruses such as PVX, TMV, and CMV. P1/HC-Pro silencing suppression was demonstrated by crossing the transgenic plants with transgenic plants that were silencing for the GUS gene. The progeny lines showed restored GUS expression indicating that P1/HC-Pro suppressed transgene silencing. In a second set of experiments the GFP transgene was silencing by PVX vector containing GFP. When P1/HC-Pro was added, GFP expression was restored. Contemporaneously with this work, it was shown that CMV is also able to counteract RNA silencing and that this is mediated by the CMV 2b protein.

A reversal of silencing assay was developed for identification of viral silencing suppressor proteins. This assay uses the GFP expressing *Nicotiana benthamiana* line 16c which is susceptible to GFP silencing by infiltrating leaves with a suspension of *Agrobacterium* carrying the GFP gene. This induces silencing of GFP throughout the entire plant. GFP expression is restored to silenced plants following infection with PVX containing a gene for a silencing suppressor. Variations on this approach include grafting of transgenic plants that are silenced for reporter gene expression to scions that are transgenic for the same reporter gene and for the candidate silencing suppressor protein. This approach was used to demonstrate that the CMV 2b protein inhibits systemic silencing in plants. Using this approach a wide number of viral silencing suppressor proteins have been identified (**Table 1**). Remarkably, some viruses encode more than one silencing suppressor. For example, citrus tristeza virus encodes at least three proteins with silencing suppressor activity, which can confound attempts by the host at generating resistance to the virus.

In general, silencing suppressor proteins inhibit production of siRNAs. Many silencing suppressor proteins bind siRNAs preventing their incorporation into the RISC. The tombusvirus p19 is one example whose crystal structure was recently described. *In vitro* assays show that p19 binds 21 nt siRNAs. The potyvirus P1/HC-Pro acts to block the RISC reducing siRNA accumulation while enhancing miRNA accumulation. The cucumovirus 2b inhibits the spread of the silencing signal but also accumulates in the nucleus where it interferes with silencing-induced DNA methylation.

Several viral silencing suppressor proteins have cross-kingdom activity, that is, they are able to suppress RNA silencing in both insect and plant cells. In a seminal

Table 1 Viral silencing suppressors (April 2007)

Host	*Genome*	*Virus genus*	*Virus name(s)*	*Suppressor(s)*	*Other name/function(s)*
Plant	DNA	*Begomovirus*	Tomato leaf curl virus	C2	Transcriptional activator
			Tomato yellow leaf curl virus	C2	Adenosine kinase inhibitor
			African cassava mosaic virus	AC2, AC4	
			Mungbean yellow mosaic virus	AC2	
			Tomato golden mosaic virus	AL2	
		Curtovirus	Beet curly top virus	L2	
	(+) ssRNA	*Aureusvirus*	Pothos latent virus	P14	
		Benyvirus	Beet necrotic yellow vein virus	P14	Regulate RNA2, CP accumulation
				P31	Enhance P14 activity in roots
		Carmovirus	Turnip crinkle virus	P38	CP
			Hibiscus chlorotic ringspot virus	CP	
		Closterovirus	Beet yellows virus	P21	
			Beet yellow stunt virus	P22	
			Citrus tristeza virus	P20	
				P23	
				CP	
			Grapevine leafroll-associated virus-2	P24	
		Crinivirus	Sweet potato chlorotic stunt virus	P22	
				RNAse 3	
		Comovirus	Cowpea mosaic virus	Small CP	
		Cucumovirus	Cucumber mosaic virus	2b	Systemic movement virulence
			Tomato aspermy virus	2b	
		Furovirus	Soil-borne wheat mosaic virus	19K	
		Hordeivirus	Barley stripe mosaic virus	γb	
			Poa semilatent virus		
		Ipomovirus	Cucumber vein yellowing virus	P1	Protease
		Pecluvirus	Peanut clump virus	P15	
		Polerovirus	Beet western yellows virus		
			Cucurbit aphid-borne yellows virus		
			Potato leafroll virus		
		Potexvirus	Potato virus X	TGBp1	Intercellular movement
		Potyvirus	Potato virus Y	HC-Pro	Systemic movement, protease, transmission
			Tobacco etch virus		
			Turnip mosaic virus		
			Zucchini yellow mosaic virus		
		Sobemovirus	Rice yellow mottle virus	P1	Systemic movement
			Cocksfoot mottle virus		
		Tobamovirus	Tobacco mosaic virus	126K	Replicase component
			Tomato mosaic virus		
		Tobravirus	Tobacco rattle virus	16K	Seed transmission
		Tombusvirus	Artichoke mottled crinkle virus	P19	Virulence, movement
			Carnation Italian ringspot virus		
			Cymbidium ringspot virus		
			Tomato bushy stunt virus		
		Tymovirus	Turnip yellow mosaic virus	P69	Virulence, movement
		Vitivirus	Grapevine virus A	P10	
	(−) ssRNA	*Tenuivirus*	Rice hoja blanca virus	NS3	
		Tospovirus	Tomato spotted wilt virus	NS_S	
	dsRNA	*Phytoreovirus*	Rice dwarf virus	Pns10	
	Viroid	*Pospiviroid*	Potato spindle tuber viroid	RNA secondary structure	
		Avsunviroid	Avocado sunblotch viroid		
Fungus	dsRNA	*Hypovirus*	Cryphonectria hypovirus 1-EP713	p29	Protease

Continued

Table 1 Continued

Host	*Genome*	*Virus genus*	*Virus name(s)*	*Suppressor(s)*	*Other name/function(s)*
Animal	DNA	*Adenovirus*	Adenovirus	VA1 RNA	
		Poxvirus	Vaccinia	E3L	Interferon antagonist
	(+) ssRNA	*Flavivirus*	Hepatitis C virus	Core protein	
		Nodavirus	Flock house virus Nodamura virus Striped jack nervous necrosis virus Greasy grouper nervous necrosis virus	B2	
		Picorna-like	Cricket paralysis virus	N-terminal domain of non-structural protein	
	(−) ssRNA	*Orthomyxovirus*	Influenza A, B, C viruses	NS1	Interferon antagonist
		Orthobunyavirus	La Crosse virus	NS_S	
	Retrovirus	*Lentivirus*	Human immunodeficiency virus-1	Tat	
		Spumavirus	Primate foamy virus type 1	Tas	
	dsRNA	*Orthoreovirus*		σ3	Outer shell protein
	Viroid-like	*Deltavirus*	Hepatitis delta virus	RNA secondary structure	

Updated from tables collated by Bucher E, Lohuis D, van Poppel PM, Geerts-Dimitriadou C, Goldbach R, and Prins M (2006) Multiple virus resistance at a high frequency using a single transgene construct. *Journal of General Virology* 87: 3697–3701; Li F and Ding SW (2006) Virus counterdefense: Diverse strategies for evading the RNA-silencing immunity. *Annual Review of Microbiology* 60: 503–531; Palukaitis and MacFarlane (2006); and Silhavy D and Burgyan J (2004) Effects and side-effects of viral RNA silencing suppressors on short RNAs. *Trends in Plant Science* 9: 76–83.

study, the group of S.W. Ding used both transgenic plants harboring silenced reporter genes and cultured insect cells to demonstrate that the B2 protein of flock house virus (FHV), an insect-infecting virus, was a silencing suppressor. Since then, a variety of insect and vertebrate-infecting, as well as fungus-infecting, viruses have been shown to encode proteins with RNA silencing activity under various assay conditions (Table 1). However, the results obtained by assaying viral proteins for silencing suppression in nonhost systems need to be interpreted with caution. For example, the influenza virus NS1 protein, which inhibits the interferon system in human cells, also inhibits RNA silencing in plants and in drosophila cells but not in HeLa cell cultures.

Recently, Deleris and colleagues created lines of *Arabidopsis thaliana* plants carrying single or multiple mutant alleles of the *DCL* genes. These plants offer an additional and less ambiguous method for identifying silencing suppressors, at least for plant viruses. This is illustrated by experiments using a turnip crinkle virus (TCV) derivative in which the coat protein gene, which also functions as a silencing suppressor, was replaced by a *GFP* gene. Compared to wild-type TCV, this modified virus is compromised in movement and symptom induction. However, symptom induction and systemic movement were restored in double *dcl2–dcl4* mutant plants; to the same extent seen in plants constitutively expressing a transgene encoding TCV coat protein.

Viroids are infectious small circular RNAs which do not encode a protein and rely on host DNA-dependent RNA polymerases for replication. Biao Ding's laboratory detected small 21 nt RNAs that are seen in PSTVd-infected plants and are active in RISC-mediated cleavage of target RNAs containing the GFP coding sequence fused to a homologous RNA segment, but the small RNAs do not impact PSTVd accumulation. While further experiments found no indication that PSTVd RNA suppresses silencing, experiments showed that PSTVd secondary structure blocks RISC-mediated cleavage. Thus, instead of suppressing RNA silencing, secondary structure of the PSTVd genome provides protection against degradation by the silencing machinery.

Silencing Suppressors Affect Plant Development

Many plant viral suppressors of RNA silencing have the ability to cause disease by altering the normal course of plant development. This was revealed by analysis of mutant viruses. For example, naturally occurring mutations in the gene for the TMV 126 kDa replicase protein result in a masked (symptomless) phenotype which relates to decreased viral silencing suppression activity and rate of systemic movement. Site-specific mutations introduced into the tobacco vein mottling virus (a potyvirus) HC-Pro gene also altered symptom expression. More drastic forms of mutagenesis, such as complete deletion of the 2b protein gene, created CMV strains that did not induce symptoms.

Transgenic plants expressing known or candidate viral silencing suppressor genes have been used to characterize the effects of the protein on plant gene expression, metabolism, and development, in the absence of virus infection. In most cases, stable expression of RNA silencing suppressors such as potyviral P1/HC-Pro proteins, tombusviral P19 proteins, beet yellows virus P21 protein, TCV CP and ACMV or SLCMV AC4 proteins, strongly disrupted plant development. This was most often seen as stunting of the plants and deformation of the stem, petioles, leaves, and flowers.

In the case of the CMV 2b protein, the strength of the altered phenotype in 2b-transgenic *Arabidopsis* plants corresponded with the severity of the symptoms induced by the strain from which the gene had been obtained. Transgenic expression of a 2b protein from a severe strain strongly inhibited growth of primary roots while both mild and severe strain 2b proteins enhanced the elongation of lateral roots. Overall, the effects of severe strain 2b proteins expressed as transgenes mimicked the phenotypes resulting from mutations in genes regulating the auxin response pathway and in *ago1* mutant plants.

Viral Suppression of the miRNA Pathway

It has been assumed that silencing suppressors alter plant development because they target elements that are common to, or highly similar between, the antiviral siRNA pathway and miRNA-directed regulation of host mRNA accumulation and translation (**Figure 1**). Since miRNAs negatively regulate the mRNA levels of several factors with roles in development (e.g., scarecrow-like factors, auxin response factors etc.), silencing suppressors which have the ability to interfere with components of small RNA-directed pathways can trigger or inhibit aspects of plant development.

Northern analysis has been used to study changes in the levels of miRNAs and target mRNAs accumulation in transgenic *Arabidopsis* plants expressing viral silencing suppressors. This method of analysis has provided evidence that some viral silencing suppressors disrupt of miRNA-regulated gene expression. The technique has also been used to monitor of levels of longer dsRNA species, miRNA precursor transcripts, miRNA duplexes, as well as the mRNA targets and their breakdown products (**Figure 1**). In combination with *in vivo* or *in vitro* studies of RNA or protein binding by silencing suppressor proteins, comparisons of steady-state levels of these various RNA species in nontransgenic versus transgenic plants expressing the silencing suppressors have helped reveal the target(s) of silencing suppressors within the miRNA pathway.

Is interference with miRNA-regulated gene expression entirely due to a case of mistaken identity, in which silencing suppressors accidentally or incidentally inhibit common targets or similar steps within the miRNA and siRNA-directed pathways? In some cases, for example where the mode of action of a silencing suppressor is to bind dsRNAs in a relatively nonsize selective manner, such as the TCV CP or the aureusvirus P14 protein, there would be a greater potential for cross-inhibition between the two pathways. In cases where the silencing suppressor acts by size-selective RNA binding, or can bind selectively to protein components of the silencing pathways (e.g., binding only to specific members of the AGO or DCL protein families), there is the possibility that silencing suppressors discriminate between components of the two RNA silencing pathways (**Figure 1**). Speculatively, this ability to discriminate suggests the possibility that natural selection operates on the genes for silencing suppressor to produce factors that minimize damage to host plants, or produce developmental changes that in some way favor the replication, spread, or transmission of the virus.

A recent breakthrough in animal virus research revealed that HIV-1 infection suppresses the microRNA pathway in a manner that promotes HIV-1 infection, but it is not evident whether this phenomenon depends on a viral silencing suppressor protein. Knockdown of *Dicer* and *Drosha* in HIV-1 infected cells showed that these two RNAse III enzymes contribute to suppression of HIV-1 infection. Microarray experiments identified miRNAs that were upregulated or induced only in HIV-1 infected cells. miR-17/92 is a polycistronic miRNA cluster which is downregulated during HIV-1 replication. miR-17/92 cluster includes miR17–5p and miR20a which target the histone acetylase PCAF, a cofactor for Tat in HIV-1. Thus, the miRNAs do not target HIV-1 but cellular factors necessary for HIV-1 gene expression. Thus, HIV-1 suppression of miR-17/92 cluster ensures a necessary supply of PCAF for virus replication.

See also: Plant Resistance to Viruses: Natural Resistance Associated with Recessive Genes; Plant Resistance to Viruses: Engineered Resistance; Plant Resistance to Viruses: Natural Resistance Associated with Dominant Genes; Virus Induced Gene Silencing (VIGS).

Further Reading

Blevins T, Rajeswaran R, Shivaprasad PV, *et al.* (2006) Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. *Nucleic Acids Research* 34(21): 6233–6246.

Bucher E, Lohuis D, van Poppel PM, Geerts-Dimitriadou C, Goldbach R, and Prins M (2006) Multiple virus resistance at a high frequency using a single transgene construct. *Journal of General Virology* 87: 3697–3701.

Cai X, Schafer A, Lu S, *et al.* (2006) Epstein–Barr virus microRNAs are evolutionarily conserved and differentially expressed. *PLoS Pathogens* 2(3): e23.

Deleris A, Gallego-Bartolome J, Bao JS, Kasschau KD, Carrington JC, and Voinnet O (2006) Hierarchical action and inhibition of plant Dicer-like proteins in anti-viral defense. *Science* 313(5783): 68–71.
Galiana-Arnoux D, Dostert C, Schneemann A, Hoffmann JA, and Imler JL (2006) Essential function *in vivo* for Dicer-2 in host defense against RNA viruses in drosophila. *Nature Immunology* 7(6): 590–597.
Gitlin L, Stone JK, and Andino R (2005) Poliovirus escape from RNA interference: Short interfering RNA-target recognition and implications for therapeutic approaches. *Journal of Virology* 79(2): 1027–1035.
Hamilton A, Voinnet O, Chappell L, and Baulcombe D (2002) Two classes of short interfering RNA in RNA silencing. *EMBO Journal* 21 (17): 4671–4679.
Hamilton AJ and Baulcombe DC (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 286(5441): 950–952.
Himber C, Dunoyer P, Moissiard G, Ritzenthaler C, and Voinnet O (2003) Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. *EMBO Journal* 22(17): 4523–4533.
Li F and Ding SW (2006) Virus counterdefense: Diverse strategies for evading the RNA-silencing immunity. *Annual Review of Microbiology* 60: 503–531.
Li HW, Li WX, and Ding SW (2002) Induction and suppression of RNA silencing by an animal virus. *Science* 296(5571): 1319–1321.
Marathe R, Anandalakshmi R, Smith TH, Pruss GJ, and Vance VB (2000) RNA viruses as inducers, suppressors and targets of post-transcriptional gene silencing. *Plant Molecular Biology* 43(2–3): 295–306.
Matzke MA and Matzke AJ (2004) Planting the seeds of a new paradigm. *PLOS Biology* 2: E133.
Pfeffer S, Sewer A, Lagos-Quintana M, *et al.* (2005) Identification of microRNAs of the herpesvirus family. *Nature Methods* 2(4): 269–276.
Pfeffer S and Voinnet O (2006) Viruses, microRNAs and cancer. *Oncogene* 25(46): 6211–6219.
Segers GC, van Wezel R, Zhang XM, Hong YG, and Nuss DL (2006) Hypovirus papain-like protease p29 suppresses RNA silencing in the natural fungal host and in a heterologous plant system. *Eukaryotic Cell* 5(6): 896–904.
Silhavy D and Burgyan J (2004) Effects and side-effects of viral RNA silencing suppressors on short RNAs. *Trends in Plant Science* 9: 76–83.

Viroids

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Glossary

Catalytic RNA RNA molecules that are able to catalyze, in a protein-free medium, specific reactions involving the formation or breakage of covalent bonds. In nature, these reactions are usually transesterifications (self-cleavage and ligation) affecting the catalytic RNA itself.
Hammerhead structure The conserved secondary/tertiary structure shared by the smallest class of natural ribozymes. Most have been found in one or both strands of certain viroid and viroid-like satellite RNAs where they mediate self-cleavage of multimeric intermediates arising from replication through a rolling-circle mechanism.
Ribozyme RNA motif responsible for the catalytic activity of certain RNA molecules. In nature, they are found embedded within catalytic RNAs.

Introduction

Viroids are the smallest known agents of infectious disease – small (246–401 nt), highly structured, circular, single-stranded RNAs that lack detectable messenger RNA activity. While viruses have been described as 'obligate parasites of the cell's translational system' and supply some or most of the genetic information required for their replication, viroids can be regarded as 'obligate parasites of the cell's transcriptional machinery'. Thus far, viroids are known to infect only plants.

The first viroid disease to be studied by plant pathologists was potato spindle tuber. In 1923, its infectious nature and ability to spread in the field led Schultz and Folsom to group potato spindle tuber disease with several other 'degeneration diseases' of potatoes. Nearly 50 years were to elapse before Diener's demonstration in 1971 that the molecular properties of its causal agent, potato spindle tuber viroid (PSTVd), were fundamentally different than those of conventional plant viruses.

Genome Structure

Efforts to understand how viroids replicate and cause disease without the assistance of any viroid-encoded polypeptides have prompted detailed analysis of their structure. Viroids possess rather unusual properties for single-stranded RNAs (e.g., a pronounced resistance to digestion by ribonuclease and a highly cooperative thermal denaturation profile), leading to an early realization that they might have an unusual higher-order structure.

To date, the complete sequences of 29 distinct viroid species plus a large number of sequence variants have