

Viroids and RNA silencing

Mechanism, role in viroid pathogenicity and development of viroid-resistant plants

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Viroids are autonomously replicating, small single-stranded circular RNA pathogens that do not code for proteins and may cause diseases in infected, susceptible plants. They have the ability to induce both RNA-mediated transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS), or RNA silencing, in infected plants. Their induced RNA silencing has also been demonstrated in a wheat germ extract system. A possible role of RNA silencing in viroid pathogenicity and evolution has been discussed. It is suggested that RNA silencing can be employed to engineer plants for viroid resistance and attempts to produce these plants have been also discussed.

Introduction

A decade ago, the knowledge of how dsRNA suppresses target gene expression by sequence-specific manner was lacking. We now have good understanding of this process, known as RNA silencing. RNA silencing or RNAi is an important mechanism of gene regulation, which takes place naturally in several organisms, including protozoa, fungi, plants and animals. The process can be divided into RNA-mediated transcriptional gene silencing (TGS) and post transcriptional gene silencing (PTGS); both of which are triggered by endogenous or exogenous, perfect or nearly perfect double-stranded RNA (dsRNA).¹

Genetic and biochemical experiments have established a general mechanistic model for a set of related pathways in RNA silencing and identified factors that are required for the process in a variety of organisms. The process is triggered by conversion of double-stranded or hairpin RNAs to various classes of small RNAs whose sizes ranging between 18–26 nucleotide (nt) duplexes, designated short-interfering RNAs (siRNAs), by

a ribonuclease III (RNase III)-like enzyme termed Dicer in animals and Dicer-like (DCL) in plants.²⁻⁶

The production of siRNAs by Dicer is an ATP-dependent step^{3,4} and likely involves interactions with other proteins, including an Argonaute-like protein, a dsRNA binding protein, and an RNA helicase.⁷ At least four Dicer homologues have been found in both rice and Arabidopsis genomes.⁸ The siRNAs produced from a fully dsRNA substrate by Dicer have distinctive characteristics: they represent both polarities and have two nucleotide 3' overhangs with 5' phosphate and 3' hydroxyl groups.^{5,9} In another ATP-dependent step,¹⁰ the siRNAs are denatured and one of the strands is incorporated into a multi-subunit, endonuclease protein silencing complex, called the RNA-induced silencing complex (RISC).¹¹ Within the activated RISC, siRNAs act as guides to bring the complex into contact with complementary mRNAs, resulting in their degradation.^{3-5,11,12} In some organisms, including plants and nematodes, the siRNA can serve as a primer for an RNA-dependent RNA polymerase 6 (RDR6) to generate dsRNAs, which are in turn processed by another DCLs into siRNAs, thereby creating abundant siRNAs.¹³⁻¹⁵ The action of an RDR6 provides an amplification phenomenon in the sense that only a few dsRNA molecules are required to degrade a much larger population of RNAs. According to this mechanism, RNA silencing is believed to play a role in the host defense against pathogen infection, as well as in inactivating the expression of undesired host genes.¹⁴

Most known plant viruses and viroids contain RNA genomes and replicate via dsRNA intermediates, thereby serving as potent inducers and targets of RNA silencing; i.e., RNA silencing provides a multi-layer defense system which protects plants from invasion by exogenous RNA replicons such as viruses and viroids.¹⁶⁻¹⁹

Viroids are the smallest known infectious agents and induce disease in a wide range of plant hosts that includes many crop species.^{20,21} They are single-stranded, circular, non-coding RNAs with the size ranging 246–401 nt. Viroid replication is entirely dependent on transcriptional and processing machinery supplied

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by the host, and transport of the resulting progeny utilizes pre-existing cellular pathways. The more than 30 species of viroids are classified into two families: members of the family Pospiviroidae contain a central conserved region and replicate in the nucleus; members of the family Avsunviroidae lack a central conserved region but exhibit hammerhead ribozyme activity and replicate in the chloroplast.^{18,22-24} Although replication occurs in different subcellular compartments, members of both families of viroids induce RNA silencing and the accumulation of viroid-specific small RNAs following infection.²⁵⁻²⁸ Plants infected by viroids contain high levels of viroid-specific small RNAs, but the circular genomic RNAs themselves appear relatively resistant to RNA silencing—raising the possibility that viroid replication may also be resistant.²⁹⁻³¹

Induction of Transcriptional Gene Silencing (TGS) in Viroid-Infected Plants

That viroids could exert their pathogenicity through RNA-directed gene silencing was proposed by Sanger et al.³² The experimental trigger of their working hypothesis was the finding that Potato spindle tuber viroid (PSTVd)-specific cDNA integrated into the genome of *Nicotiana tabacum* SRI becomes specifically methylated as soon as an autonomous viroid RNA-directed RNA replication has taken place in these plants.³³ From these observations it was inferred that RNA, in general, is capable of inducing and directing sequence-specific de novo methylation of genomic DNA. Since DNA methylation has been associated with gene silencing, it was conceivable that a viroid-induced RNA-directed DNA methylation resulted in a subsequent plant gene silencing. In this case PSTVd induces RNA-mediated transcriptional gene silencing.

Induction of Post Transcriptional Gene Silencing (PTGS) in Viroid-Infected Plants and in Wheat Germ Extract

During the last few years several reports have demonstrated that viroids induce PTGS.^{18,25-27,29-31,34-39}

Itaya et al.²⁵ reported the detection of small RNAs of approximately 25 nt with sequence specific to PSTVd as an indication of the presence of PTGS, or RNA silencing. Papaefthimiou et al.²⁶ also reported the detection of PSTVd-specific small RNAs with the sizes 22–23 nt of both polarities covering different regions of the PSTVd molecule in viroid-infected tomato plants. Detection of viroid-specific small RNAs of approximately 21–23 nt were also reported from Peach latent mosaic viroid (PLMVd)-infected peach trees and Chrysanthemum chlorotic mottle viroid (CChMVd)-infected chrysanthemum.²⁷ Nuclear viroids as well as chloroplastic viroids can be inducers for viroid-specific RNA silencing. At the same time of the discovery, initial analyses have been done by focusing on the roles of viroid-specific RNA silencing in relation to viroid pathogenicity; i.e., quantitative analyses of viroid-specific small RNAs induced by viroid strains with differential pathogenicity. However, they did not appear to be responsible for the difference

in symptoms in case of tomato plants infected with PSTVd isolates with different pathogenicity. No significant difference in the RNA silencing response to strains of CChMVd of different virulence was also observed as the accumulation levels of the small RNAs were essentially the same, at least in quantity. Thus, as with the case of PSTVd in the family Pospiviroidae, RNA silencing does not seem to be involved in symptom development in viroids in the family Avsunviroidae.

However, these primary observations were depended on quantitative analysis of viroid-specific small RNAs accumulation induced by strains with different virulence. Considering the fact that RNA silencing is nucleotide sequence-specific gene regulation mechanism, different viroid strains with different nucleotide sequence can produce qualitatively (but not quantitatively) different viroid-specific small RNAs in the infected plant, which, in turn, can target the host sequences with various efficiencies and may lead to differential severity of symptom expressions. In fact, as presented below and also discussed in the next section, the influence of viroid-induced RNA silencing to the severity of symptom development can be different depending on viroid-host combinations. For example, tissues with different symptom expressions, characterized by the presence of different predominant Avocado sunblotch viroid (ASBVd) variants, were found to induce PTGS at differential levels, and detection of the PTGS-associated small interfering RNAs as well as their relative concentration was also related to viroid titer.²⁸ In contrast, PTGS induced in *Gynura aurantiaca* infected with two closely-related variants of Citrus exocortis viroid (CEVd) was not directly related to viroid titer with initiation of symptoms.²⁸ Furthermore, ASBVd accumulates at very high levels in infected avocado tissue but does not accumulate detectable levels of ASBVd-specific small RNAs, whereas PLMVd and CChMVd, which reach only lower titers in their infected peach and chrysanthemum tissues, respectively, accumulate detectable levels of viroid-specific small RNAs.⁴⁰ This inverse correlation between the viroid accumulation levels and the presence and/or the absence of the small RNAs may suggest the involvement of small RNAs in a defense response via RNA silencing of the host; i.e., RNA silencing would attenuate the detrimental effect of viroids in the family Avsunviroidae by lowering their in vivo titer as suggested by Martinez de Alba et al.²⁷ The possibility of whether the RNA silencing is involved in viroid pathogenicity is still debatable and will be discussed later in this review.

Sequencing analysis of viroid-induced small RNAs in viroid-infected plants have been done or are now extensively underway using several viroid-host combinations such as PSTVd-tomato,^{29,31} CEVd-tomato³⁰ and PLMVd-peach.⁴¹ The analyses revealed that the majority of viroid-specific small RNAs are derived from some restricted regions in the viroid molecule. The latest large-scale nucleotide sequencing analysis on two each of viroids grouped in the family Pospiviroidae; Hop stunt viroid (HSVd) and Grapevine yellow speckle viroid 1, and in the family Avsunviroidae; CChMVd and PLMVd also confirmed that the large majority of viroid-specific small RNAs of 21 to 24nt are derived from a few specific regions (hotspots) of the plus and minus strand of viroid RNAs,^{42,43} (Figs. 1 and 2, the center).

Figure 1. Viroid replication and viroid-induced RNA silencing. After invading host plant cells, viroid replicates in the nuclei (Pospiviroidae; presented in this figure) or in the chloroplast (Avsunviroidae; not included in this figure) via rolling circle mechanism, in which plus-strand (blue in the figure) viroid molecule (the upper most) is transcribed into minus-strand (red) by host transcriptional machinery (shown in the clockwise rotation), and plus-strand is again transcribed from the minus-strand, processed into a unit-length (blue molecule with red star at both ends), and finally circularized to produce mature viroid molecule. During the RNA-directed RNA replication process, double-stranded RNA structures are formed between the plus- and minus-strand molecules of viroid replication intermediates, as well as highly base-paired viroid molecule itself, induce viroid-specific RNA silencing, are attacked as the targets, and are diced into small pieces with the length ca. 21–24 nucleotides called viroid-specific small RNAs.

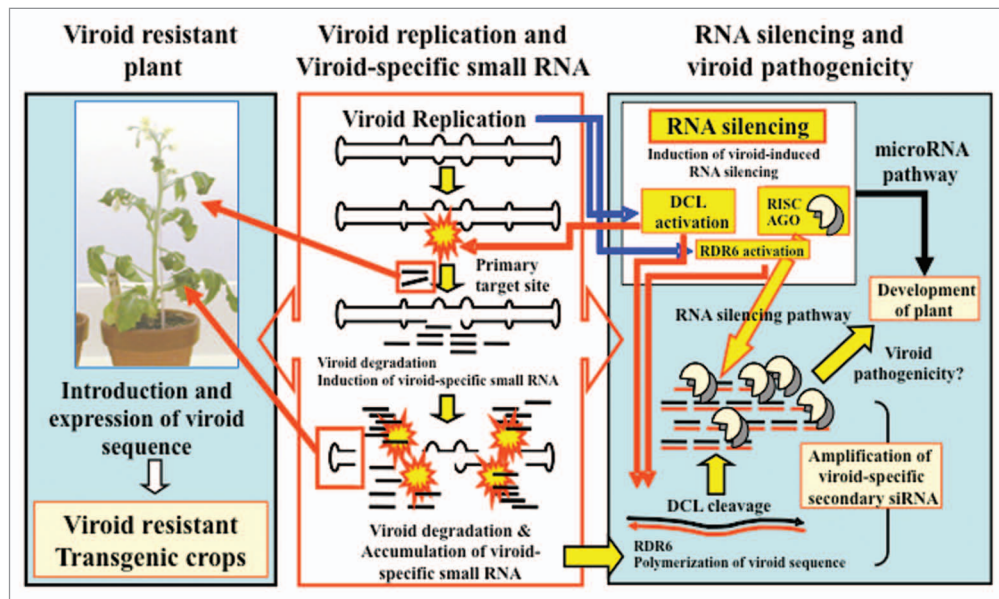
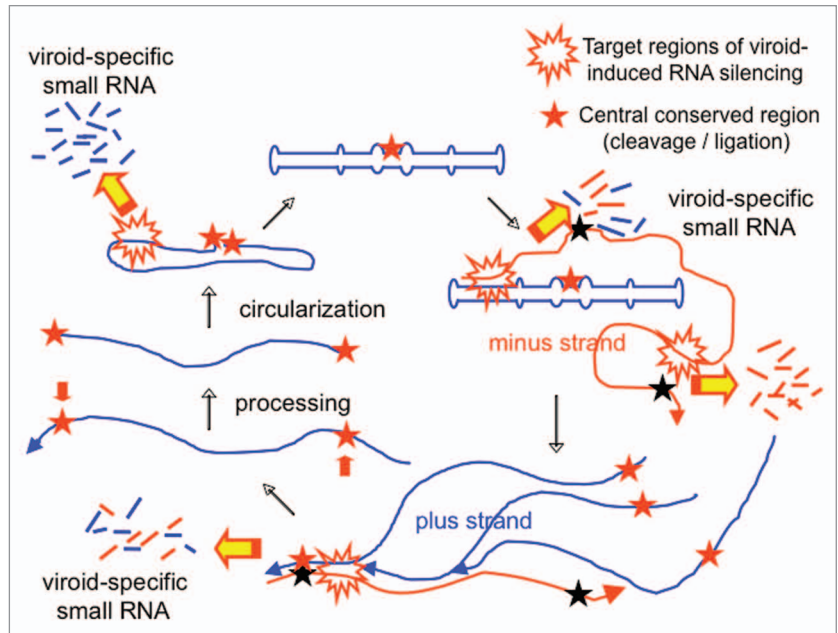


Figure 2. Schematic representation of viroid replication, viroid-specific small RNAs, RNA silencing and viroid-resistant transgenic plant. The center panel; Replication of viroid in infected host cells induces viroid-specific RNA silencing. Targeted by RNA silencing, viroid molecule is diced into various lengths of viroid-specific small RNAs consisting of ca. 21–24 nucleotides from several hotspot regions in the molecule, and as a result, a large amount of viroid-specific small RNAs are accumulated in infected plant cells. The right; After induction of viroid-specific RNA silencing, host RNA silencing machineries consist of Dicer-like (DCL) enzyme, RNA-induced silencing complex (RISC), Argonaute (AGO) proteins, RNA-dependent RNA polymerase 6 (RDR6), etc., work co-operatively to protect plants from viroid infection by degrading invaded viroid and amplifying viroid-specific small RNAs (secondary siRNA) to enforce RNA silencing targeting viroid replication. However, accumulation of the viroid-derived small RNAs in the infected host plant, as the side effect, could give rise to inconceivable adverse effects on certain normal host regulation genes in a sequence-specific manner, which may result in viroid pathogenicity. The left; Viroid-specific RNA silencing could be used for the creation of viroid-resistance transgenic plants. By introducing viroid-derived sequence designed to form hairpin or dsRNAs after expression in the transgenic plant, the pre-expressed viroid-derived sequence induces artificial viroid-specific RNA silencing and is expected to act as defense against invaded viroid replication.

PSTVd is known to replicate and accumulate in nuclei; however, PSTVd-specific small RNAs were detected in the cytoplasm but not in the nuclei.³⁵ The cytoplasmic localization of viroid-specific small RNAs could be the result of the dsRNA replication intermediates and/or highly structured viroid RNA itself are either (1) exported first in the cytoplasm and then cleaved by DCL enzyme(s) such as DCL2 and DCL3 that have been associated with cytoplasmic small RNA biogenesis or (2) cleaved first into small RNAs in the nuclei by DCL enzyme(s) such as DCL1 and then exported into cytoplasm, which is similar to the nuclear processing of plant miRNAs.⁴⁴⁻⁴⁶ In plants, furthermore, the small RNAs produced can subsequently be used as primers by RNA dependent RNA polymerase such as RDR6 for converting the target RNA template into dsRNA to produce more substrate for DCL enzyme(s).⁴⁵⁻⁴⁸ (Fig. 2, right).

Landry and Perreault,³⁸ using the chloroplastic viroid PLMVd and wheat germ extract experiments, showed dsRNA complexes formed by intermolecular base pairing of PLMVd strands of both polarities as well as highly structured plus and minus strand itself can serve as substrate for DCL enzyme(s) and identified the P11 hairpin of PLMVd, which is known to be implicated in its replication, as the domain recognized by DCL enzyme(s), thereby initiating RNA silencing. Previous *in vivo* studies indicated that accumulated small RNAs in infected plants form a population of sequences representing the entire PLMVd genome.^{27,37} Recent analysis of PLMVd-specific small RNA sequence also revealed that the majority of the small RNAs are derived from several regions in the molecule.^{41,43} In the wheat germ extract experiments,³⁸ the cleavage of plus and minus strand produced different size profiles of small RNAs, suggesting that small structural differences between the RNA species generated different products or recruited different DCLs. This seems to be also the case in tomato plants infected with PSTVd; i.e., time course analysis of PSTVd-specific small RNAs accumulation revealed the presence of at least two size classes of small RNAs in infected host plant and the size distribution became more heterogeneous with time, suggesting the involvement of two or more DCLs.³¹ Consequently, either the viroid replicating in nuclei or in chloroplast, the process by which viroid-specific small RNAs are generated is diverse, probably involving multiple DCL activities, viroid RNA substrates and subcellular compartments.³¹ Qualitative analysis of viroid-induced RNA silencing using large-scale nucleotide sequencing to map viroid-specific small RNAs in various viroids (or strains)—host combinations may solve this intriguing question.

Possible Role of Gene Silencing in Viroids Pathogenicity and Evolution

Viroid pathogenicity has long been analyzed in relation to the unique highly base-paired structure of the genomic RNA. PSTVd and related viroids contain five structural domains.⁴⁹ For years, it was believed that viroids induce disease by interacting with an unknown host factor (i.e., protein), thereby disrupting normal cell function. This hypothesis was proposed in the mid-1980s when a region of the viroid genome was identified as the

“virulence-modulating” (VM) region; i.e., changes of nucleotide within the region of PSTVd have dramatic effects on the virulence.⁵⁰ It is important to note, however, that changes in any of the other four structural domains can also have significant effects on symptom development.⁵¹⁻⁵⁶ The mechanistic details of this viroid-host interaction remained a mystery. During the last few years it was suggested that, perhaps viroids act as small regulatory RNAs to influence gene expression. Various models involving the primary sequence or secondary structural features of viroid genomes have been proposed to account for their pathogenicity.⁵⁷

Recently, an alternative pathogenicity model for viroids via RNA silencing mechanism has been proposed. In the earlier analyses, a positive correlation between symptom severity and the accumulation of viroid-specific small RNAs was found in avocado infected with ASBVd.²⁸ Symptom expression may also decrease in PSTVd-infected tomato plants late in the infection in relation to viroid-induced RNA silencing.³⁴ It has been suggested that viroid-specific small RNAs are probably involved in cross protection against viroids.⁴ In HSVd-infected *Nicotiana benthamiana*, symptom expression is dependent upon the activity of RNA-directed RDR6, an enzyme known to be involved in small RNA biogenesis and RNA silencing.⁵⁸ RDR6 plays a key role in the synthesis of trans-acting small interfering RNAs (ta-siRNAs), catalyzing the conversion of single-stranded RNA fragments generated by miRNA-mediated cleavage of specific Pol II transcripts into double-stranded RNAs that are themselves substrates for DCL(s). Finally, the first indirect indication that RNA silencing may play an important role in symptom expression was provided by Wang and colleagues,³⁶ in which, transgenic tomato lines engineered to express a non-infectious form of PSTVd hairpin RNA displayed symptoms similar to those observed in PSTVd-infected plants. The significance of these observations was recently called into question, however, when the T3 progeny from certain of the lines failed to develop similar symptoms.⁵⁹ Thus, further careful analysis are needed to be carried out.

siRNA-directed degradation requires a minimum sequence identity of about 19nt between the siRNA and the cognate target RNA.^{60,61} The pathogenicity of viroids is generally determined by the nucleotide sequence within particular small (about 20nt) regions of the viroid genome,^{62,63} such as the defined virulence modulating (VM) region of PSTVd (nucleotides no. 45–68).^{50,63} A BLAST search with the full-length sequence of PSTVd-RG1 revealed numerous sequences from several plant species that have 19–20-nt identities with the PSTVd genome.³⁶ Almost all of these 19–20-nt sequences correspond to the A + G-rich VM region of PSTVd, suggesting that small RNAs derived from this region of PSTVd may target the silencing of host regulatory genes. Recently, however, the nucleotide sequence of PSTVd- or CEVd-specific small RNAs revealed that they are not derived evenly from PSTVd or CEVd whole molecule but from several hot spots and that the viroid VM region is not included in the hot spots,^{29-31,39} although more comprehensive analysis such as the one based on large-scale nucleotide sequencing is definitely required.

Viroids are unencapsidated, circular RNA molecules and do not encode any functional proteins and yet are capable of accumulating at high levels in plants. Wang et al.³⁶ suggest that viroids

have developed a nuclease resistance strategy to protect themselves against degradation by RNA silencing.³⁶ In fact, viroid replication appeared somewhat resistant to RNA silencing,^{29-31,59} suggesting that RNA silencing is an important selection pressure shaping the evolution of their secondary structures. A likely explanation for the resistance of viroids to RNA silencing is that their extensive intramolecular base-pairing renders them less accessible to the RISC complex for degradation. Additionally, the presence of mismatches in the duplex regions restricts perfectly paired regions of the predicted secondary structures of viroid RNAs⁶⁴ to no longer than 14 nt.³⁶ This would be sufficient to protect them against cleavage by DCL, which requires a minimum of about 19 bp of dsRNA.⁶⁵ Chang et al.⁶⁶ reported that PSTVd or viroid-like RNAs are highly resistant to DCL cleavage in an in vitro system. The resistance of viroids to RNA silencing-mediated degradation implies that RNA silencing may have directed the evolution of viroids. Thus RNA silencing in plants may play a central role in the pathogenicity of viroids and the evolution of their secondary structure. Like viruses, the evolutionary pathway that viroids appear to have adopted allows them not only to use the host functions for their replication but also to evade host defenses and to elicit pathogenic reactions. Whereas viruses achieve these functions by means of an array of encoded proteins called RNA silencing suppressor, viroids appear to ensure their evolutionary survival using an exclusively sequence and structure-based strategy.

RNA Silencing and Viroid Resistant Transgenic Plants

Viroids may infect some susceptible hosts asymptotically. These hosts may serve as reservoirs for viroid infection that causes diseases of economically important crops, such as the case of hop stunt disease caused by HSVd which is transmitted from the symptomless grapevines.⁶⁷ A variety of crops, including fruit trees, are vegetatively propagated as scions or cuttings for widespread distribution. With the globalization of agriculture, it is easier for viroids to become widely distributed and introduced into new environments and induce disease epidemics. Recently, symptomless infection of several Pospiviroids; PSTVd, Tomato chlorotic dwarf viroid, CEVd and Chrysanthemum stunt viroid in ornamentals of the family Solanaceae have been reported and have become a major threat in plant quarantine world-wide.⁶⁸⁻⁷⁰ PSTVd for example, is an extremely damaging pathogen for potato production,^{20,21,24} therefore, it is essential to develop reliable control measures to protect crops from viroid infection.

In the last two decades, attempts have been made to create transgenic plants resistant to viroid infection mainly based on antisense RNA, ribozyme and double-strand RNA-specific ribonuclease.⁷¹⁻⁷⁶ Those technologies have been shown as useful tools for blocking the expression of target RNA in plants or animals. In addition to blocking endogenous genes, many reports described the use of antisense RNA technology for generating antiviral activity. Matousek et al.⁷¹ first reported the use of antisense RNA to produce viroid resistant transgenic plants. They created two

constructs of transgenic plants expressing short antisense RNA (18 nt) complementary to the upper central conserved region (CCR) of PSTVd plus-strand, and a long antisense RNA (173 nt) complementary to the left hand half of PSTVd minus-strand. Though viroid infection was not protected efficiently, however, the replication in the transgenic plants was delayed significantly. Namely, the resistance mediated by antisense RNA was limited. The attempts to use antisense and ribozyme RNAs for producing viroid resistant transgenic plants was also reported,⁷² in which transgenic plants expressing two each of ribozyme and antisense RNAs targeting plus- and minus-strand of CEVd were generated. Transgenic seedlings expressing antisense construct targeting the minus-strand CEVd slightly suppressed CEVd accumulation, however, those expressing the ribozyme targeting the CEVd plus-strand showed higher levels of CEVd accumulation than in the wild-type; i.e., ribozyme targeting viroid sequence has a certain possibility to suppress viroid infection, but the apparent enhancement of CEVd replication in the transgenic plants expressing the ribozyme targeting the CEVd plus-strand remains mystery.

Using a combination of antisense RNA and hammerhead ribozyme by the improved design, Yang et al.⁷³ constructed a hammerhead ribozyme with short antisense sequences which targets the minus-strand of PSTVd. The tandem-repeated ribozyme with short (9–11 nt) antisense sequences of 49-nt long was designed to cleave the 3'-terminal of a GUC trinucleotide within the T1 domain of PSTVd minus-strand, a putative binding site for DNA-dependent RNA polymerase II. The transgenic potato lines expressing the ribozyme-antisense construct inhibited PSTVd infection or suppressed the viroid accumulation at a low level.

Viroids are RNA replicons and are reproduced via RNA-RNA rolling circle replication. Viroids not only form a temporarily double stranded RNA in replication, as well as the majority of plant viruses, but also show high intermolecular base pairing and form double stranded RNA-like rod-structure. Since PSTVd was digested with double-stranded RNA specific ribonuclease (*pac 1*) in vitro, Sano et al.⁷⁴ produced transgenic potato lines (cv. Russet Burbank) transformed with *pac1*. The transgenic potato lines expressing *pac1* gene product inhibited viroid infection and suppressed its accumulation.

More recently, accumulated knowledge on the mechanisms of RNA silencing revealed that RNA silencing directs antiviral immunity in plants.⁷⁷ Based on the concept, several attempts have just begun to use RNA silencing to engineer plants for viroid resistance. Since its highly ordered hairpin RNA structure, mature viroid RNA molecule can be a strong inducer for RNA silencing targeting viroid replication. Several investigators have noted the striking similarity between the rod-like structure of viroids like PSTVd and those of primary host microRNA (pri-miRNA) transcripts; indeed, incubation of RNA transcripts derived from either PLMVd³⁸ or PSTVd²⁹ with a partially-purified DCL preparations results in the release of small 21–24 nt RNAs. Carbonell et al.⁷⁸ reported that viroid-specific small RNAs, homologous to members of the two families of nuclear- and chloroplast-replicating viroid, co-delivered mechanically interfered with one of the viroids. The interference was

sequence-specific, temperature-dependent and, in some cases, also dependent on the dose of the co-inoculated dsRNA or viroid-specific small RNAs, suggesting the susceptibility of viroid RNAs to RNAi. Schwind et al.⁵⁹ reported that transgenic tomato plants expressing a hairpin RNA (hpRNA) construct derived from PSTVd sequences exhibit resistance to PSTVd infection. Since the resistance seems to be correlated with high-level accumulation of hpRNA-derived short interfering RNAs (siRNAs) in the plant, hpRNA-derived siRNAs (hp-siRNAs) appear to effectively target the mature viroid RNA, suggesting that RNAi can be employed to engineer plants for viroid resistance, as has been well established for viruses.

A recent study, however, revealed that plants infected with viroids contain high levels of viroid-specific small RNAs, but the circular highly structured viroid RNAs themselves appear relatively resistant to RNA silencing, raising the possibility that viroid replication may also be resistant to RNA silencing.^{29-31,79} In addition, as we have discussed in the section on possible role of gene silencing on viroid pathogenicity, viroid-induced RNA silencing may interfere with a certain host gene expression through viroid-derived small RNAs. In case it's true, we may need to solve the potential phenotype (i.e., disease symptoms) of the transgenic plants expressing the hairpins homologous to viroid sequences. Taking into consideration the fact that viroid-specific small

RNAs are generated from several hotspots in the viroid molecule and some of them are derived from the pathogenicity domain like VM region which may interfere by sequence-specific manner with a certain host gene expression, we may need to identify hotspot sequence(s) without showing any considerable sequence homology to the host genome. Based on the sequence(s), we will be able to design and produce transgenic plant expressing hairpin constructs which trigger "pin-point RNA silencing" targeting only the specific hotspot region(s) of viroid molecule. The mechanism underlying this viroid nature of resistance/tolerance to RNA silencing has not yet been understood,⁷⁸ but the report by Schwind et al.⁵⁹ that certain transgenic tomato lines expressing high levels of hp-siRNA-derived small viroid RNAs exhibited certain levels of resistant to viroid infection will be promising for further investigation on this exciting research field.

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References

- Hannon GJ. RNA interference. *Nature* 2002; 418:16-9.
- Hamilton AJ, Baulcombe DC. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 1999; 286:950-2.
- Zamore PD, Tuschl T, Sharp PA, Bartel DP. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 2000; 101:25-33.
- Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 2001; 409:363-6.
- Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes and Development* 2001; 15:188-200.
- Hamilton A, Voinnet O, Chappell L, Baulcombe D. Two classes of short interfering RNA in RNA silencing. *EMBO J* 2002; 21:4671-9.
- Tabara H, Yigit E, Siomi H, Mello CC. The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1 and a DExH-box helicase to direct RNAi in *C. elegans*. *Cell* 2002; 109:861-71.
- Finnegan EJ, Margis R, Waterhouse PM. Posttranscriptional gene silencing is not compromised in the Arabidopsis Varpel factory (DicerLike 1) mutant, a homolog of Dicer-1 from *Drosophila* [sic]. *Current Biology* 2003; 13:236-40.
- Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T. Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J* 2001; 20:6877-88.
- Nykanen A, Haley B, Zamore PD. ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* 2001; 107:309-21.
- Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* 2000; 404:293-6.
- Hammond SM, Caudy AA, Hannon GJ. Post-transcriptional gene silencing by double-stranded RNA. *Nat Rev Genet* 2001; 2:110-9.
- Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double stranded RNA in *Caenorhabditis elegans*. *Nature* 1998; 391:806-11.
- Baulcombe D. Amplified silencing. *Science* 2007; 315:199-200.
- Sijen T, Steiner FA, Thijssen KL, Plasterk RHA. Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. *Science* 2007; 315:244.
- Baulcombe D. RNA silencing in plants. *Nature* 2004; 431:356-63.
- Ding SW, Li H, Lu R, Li F, Li WX. RNA silencing: a conserved antiviral immunity of plants and animals. *Virus Res* 2004; 102:109-15.
- Daros JA, Elena SF, Flores R. Viroids: Ariadne's thread into the RNA labyrinth. *EMBO Rep* 2006; 7:593-8.
- Ruiz-Ferrer V, Voinnet O. Roles of plant small RNAs in biotic stress responses. *Annu Rev Plant Biol* 2009; 60:485-510.
- Diener TO. Biological properties, 9-35. *In: Diener TO, (ed.), The viroids*. Plenum Press, New York USA 1987.
- Hadidi A, Flores R, Randles JW, Semancik J. Viroids. CSIRO Publishing, Collingwood, Victoria, Australia 2003; 370.
- Flores R, Hernández C, Martínez de Alba AE, Daros JA, Di Serio F. Viroids and viroid-host interactions. *Annu Rev Phytopathol* 2005; 43:117-39.
- Ding B, Itaya A. Viroid: a useful model for studying the basic principles of infection and RNA biology. *Mol Plant Microbe Interact* 2007; 20:7-20.
- Ding B. The biology of viroid-host interactions. *Annu Rev Phytopathol* 2009; 47:105-31.
- Itaya A, Folimonov A, Matsuda Y, Nelson RS, Ding B. Potato spindle tuber viroid as inducer of RNA silencing in infected tomato. *Mol Plant Microbe Interact* 2001; 14:1332-4.
- Papaefthimiou I, Hamilton AJ, Denti MA, Baulcombe DC, Tsagris M, Tabler M. Replicating potato spindle tuber viroid RNA is accompanied by short RNA fragments that are characteristic of post-transcriptional gene silencing. *Nucleic Acids Res* 2001; 29:2395-400.
- Martinez de Alba AE, Flores R, Hernandez C. Two chloroplastic viroids induce the accumulation of small RNAs associated with posttranscriptional gene silencing. *J Virol* 2002; 76:13094-6.
- Markarian N, Li HE, Ding SW, Semancik JS. RNA silencing as related to viroid-induced symptom expression. *Arch Virol* 2004; 149:397-406.
- Itaya A, Zhong X, Bundschuh R, Qi Y, Wang Y, Takeda R, et al. A structured viroid RNA serves as a substrate for dicer-like cleavage to produce biologically active small RNAs but is resistant to RNA-induced silencing complex-mediated degradation. *J Virol* 2007; 81:2980-94.
- Martín R, Arenas C, Daròs JA, Covarrubias A, Reyes JL, Chua NH. Characterization of small RNAs derived from citrus exocortis viroid (CEVd) in infected tomato plants. *Virology* 2007; 367:135-46.
- Machida S, Shibuya M, Sano T. Enrichment of viroid small RNAs by hybridization selection using biotinylated RNA transcripts to analyze viroid induced RNA silencing. *J Gen Pl Pathol* 2008; 74:203-7.
- Sänger HL, Schiebel L, Riedel T, Péliissier T, Wassenegger M. The possible links between RNA-directed DNA methylation (RdDM), sense and antisense RNA, gene silencing, symptom-induction upon microbial infections and RNA-directed RNA polymerase (RDRP). *Proc 8th Intern Symp Molecular Plant-Microbe Interactions*. Tennessee 1996.
- Wassenegger M, Heimes S, Riedel L, Sänger L. RNA-directed de novo methylation of genomic sequences in plants. *Cell* 1994; 76:567-76.
- Sano T, Matsuura Y. Accumulation of short interfering RNAs characteristic of RNA silencing precedes recovery of tomato plants from severe symptoms of Potato spindle tuber viroid infection. *J Gen Pl Pathol* 2004; 70:50-3.
- Denti M, Boula A, Tsagris M, Tabler M. Short interfering RNAs specific for potato spindle tuber viroid are found in the cytoplasm but not in the nucleus. *Plant J* 2004; 37:762-9.
- Wang MB, Bian XY, Wu LM, Liu LX, Smith NA, Isenegger D, et al. On the role of RNA silencing in the pathogenicity and evolution of viroids and viral satellites. *Proc Natl Acad Sci USA* 2004; 101:3275-80.

37. Landry P, Thompson D, Perreault J-P. The role of viroids in gene silencing: the model case of Peach latent mosaic viroid. *Can J Plant Pathol* 2004; 26:31-8.
38. Landry P, Perreault J-P. Identification of a Peach latent mosaic viroid hairpin able to act as a Dicer-like substrate. *J Virol* 2005; 79:6540-3.
39. Machida S, Yamahata N, Watanuki H, Owens RA, Sano T. Successive accumulation of two size classes of viroid-specific small RNA in potato spindle tuber viroid-infected tomato plants. *J Gen Virol* 2007; 88:3452-7.
40. Flores R, Daros JA, Hernandez C. The Avsunviroidae family: viroids containing hammerhead ribozymes. *Adv Virus Res* 2000; 55:271-323.
41. Patrick St-Pierre I, Hassen F, Thompson D, Perreault JP. Characterization of the siRNAs associated with peach latent mosaic viroid infection. *Virology* 2009; 383:178-82.
42. Navarro B, Pantaleo V, Gisel A, Moxon S, Dalmay T, et al. Deep sequencing of viroid-derived small RNAs from grapevine provides new insights on the role of RNA silencing in plant-viroid interaction. *PLoS ONE* 2009; 4:7686.
43. Di Serio F, Gisel A, Navarro B, Delgado S, Martínez de Alba Á-E, et al. Deep sequencing of the small RNAs derived from two symptomatic variants of a chloroplastic viroid: Implications for their genesis and for pathogenesis. *PLoS ONE* 2009; 4:7539.
44. Papp I, Mette MF, Aufsatz W, Daxinger L, Schauer SE, Ray A, et al. Evidence for nuclear processing of plant micro RNA and short interfering RNA precursors. *Plant Physiol* 2003; 132:1382-90.
45. Voinnet O. Use, tolerance and avoidance of amplified RNA silencing by plants. *Trends Plant Sci* 2008; 13:317-28.
46. Xie Z, Qi X. Diverse small RNA-directed silencing pathways in plants. *Biochemica et Biophysica Acta* 2008; 1779:720-4.
47. Dillin A. The specifics of small interfering RNA specificity. *Proc Natl Acad Sci USA* 2003; 100:6289-91.
48. Voinnet O. RNA silencing bridging the gaps in wheat extracts. *Trends Plant Sci* 2003; 8:307-9.
49. Keese P, Symons RH. Domains of viroids: Evidence of intermolecular RNA rearrangements and their contribution to viroid evolution. *Proc Natl Acad Sci USA* 1985; 82:4582-6.
50. Schnölzer M, Haas B, Ramm K, Hofmann H, Sängler HL. Correlation between structure and pathogenicity of potato spindle tuber viroid (PSTVd). *EMBO J* 1985; 4:2181-90.
51. Visvader JE, Symons RH. Eleven new sequence variants of citrus exocortis viroid and the correlation of sequence with pathogenicity. *Nucleic Acids Res* 1985; 13:2907-20.
52. Sano T, Candresse T, Hammond RW, Diener TO, Owens RA. Identification of multiple structural domains regulating viroid pathogenicity. *Proc Natl Acad Sci USA* 1992; 89:10104-8.
53. Rodriguez MJB, Randles JW. Coconut cadang-cadang viroid (CCCVd) mutants associated with severe diseases vary in both the pathogenicity domain and central conserved region. *Nucleic Acids Res* 1993; 21:2771.
54. Reanwarakorn K, Semancik JS. Correlation of hop stunt viroid variants to cachexia and xyloporosis diseases of citrus. *Phytopathology* 1999; 89:568-74.
55. Sano T, Ishiguro A. Viability and pathogenicity of intersubgroup viroid chimeras suggest possible involvement of the terminal right region in replication. *Virology* 1998; 240:238-44.
56. Qi Y, Ding B. Inhibition of cell growth and shoot development by a specific nucleotide sequence in a noncoding viroid RNA. *Plant Cell* 2003; 15:1360-74.
57. Semancik JS. Pathogenesis. In *Viroids*, Hadidi A, Flores R, Randles JW, Semancik JS, eds. CSIRO Publishing, Collingwood, Australia 2003; 61-6.
58. Gómez G., Martínez G., Pallas V. Viroid-induced symptoms in Nicotiana benthamiana plants are dependent on RDR6 activity. *Plant Physiol* 2008; 148:414-23.
59. Schwind N, Zwiebel M, Itaya A, Ding B, Wang MB, Krczal G, Wassenegeger M. RNAi-mediated resistance to Potato spindle tuber viroid in transgenic tomato expressing a viroid hairpin RNA construct. *Mol Plant Pathol* 2009; 10:459-69.
60. Zamore PD. RNA interference: listening to the sound of silence. *Nat Struct Mol Biol* 2001; 8:746-50.
61. Vanitharani R, Chellappan P, Fauquet CM. Short interfering RNA-mediated interference of gene expression and viral DNA accumulation in cultured plant cells. *Proc Natl Acad Sci USA* 2003; 100:9632-6.
62. Owens RA, Steger G, Hu Y, Fels A, Hammond RW, Riesner D. RNA structural features responsible for potato spindle tuber viroid pathogenicity. *Virology* 1996; 222:144-58.
63. Steger G, Riesner D. In *Viroids*, Hadidi A, Flores R, Randles JW, Semancik JS, eds. CSIRO Publishing, Collingwood, Australia 2003; 15-29.
64. Palcher M, Rocheleau L, Perreault J, Perreault J-P. Subviral RNA: a database of the smallest known auto-replicable RNA species. *Nucleic Acids Res* 2003; 31:444-5.
65. Yu JY, DeRuite SL, Turner DL. RNA interference by expression of short-interfering RNA and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci USA* 2002; 99:6047-52.
66. Chang J, Provost P, Taylor JM. Resistance of human hepatitis delta virus RNAs to dicer activity. *J Virol* 2003; 77:11910-7.
67. Kawaguchi-Ito Y, Li S-F, Tagawa M, Araki H, Goshono M, Yamamoto S, et al. Cultivated grapevines represent a symptomless reservoir for the transmission of Hop stunt viroid to hop crops: 15 Years of Evolutionary Analysis. *PLoS ONE* 2009; 4:8386.
68. Serio FD. Identification and characterization of potato spindle tuber viroid infecting *Solanum jasminoides* and *S. nantonnetii* in Italy. *J Plant Pathol* 2007; 89:297-300.
69. James T, Mulholland V, Jeffries C, Chard J. First report of Tomato chlorotic dwarf viroid infecting commercial petunia stocks in the United Kingdom. *Plant Pathol* 2008; 57:400.
70. Verhoeven JThJ, Jansen CCC, Roenhorst JW. First report of popsviroids infecting ornamentals in the Netherlands: Citrus exocortis viroid in *Verbena* sp., Potato spindle tuber viroid in *Brugmansia suaveolens* and *Solanum jasminoides*, and Tomato apical stunt viroid in *Cestrum* sp. *Plant Pathol* 2008; 57:399.
71. Matousek J, Schroder ARW, Trnena L, Reimers M, Baumstark T, Dedic P, et al. Inhibition of viroid infection by antisense RNA expression in transgenic plants. *Biol Chem Hoppe-Seyler* 1994; 375:765.
72. Atkins D, Young M, Uzzell S, Kelly L, Fillatti J, Gerlach WL. The expression of antisense and ribozyme genes targeting citrus exocortis viroid in transgenic plants. *J Gen Virol* 1995; 76:1781-90.
73. Yang X, Yie Y, Zhu F, Liu Y, Kang L, Wang X, Tien P. Ribozyme-mediated high resistance against potato spindle tuber viroid in transgenic potatoes. *Proc Natl Acad Sci USA* 1997; 94:4861-5.
74. Sano T, Nagayama A, Ogawa T, Ishida I, Okada Y. Transgenic potato expressing a double-stranded RNA-specific ribonuclease is resistant to potato spindle tuber viroid. *Nat Biotechnol* 1997; 15:1290-4.
75. Ogawa T, Toguri T, Kudoh H, Okamura M, Momma T, Yoshioka M, et al. Double-stranded RNA-specific ribonuclease confers tolerance against Chrysanthemum stunt viroid and Tomato spotted wilt virus in transgenic chrysanthemum plants. *Breed Sci* 2005; 55:49-55.
76. Ishida I, Tukahara M, Yoshioka M, Ogawa T, Kakitani M, Toguri T. Production of anti-virus, viroid plants by genetic manipulations. *Pest Manag Sci* 2002; 58:1132-6.
77. Ding S-W, Voinnet O. Antiviral immunity directed by small RNAs. *Cell* 2007; 130:413-26.
78. Carbonell A, Martínez de Alba AE, Flores R, Gago S. Double-stranded RNA interferes in a sequence-specific manner with the infection of representative members of the two viroid families. *Virology* 2008; 371:44-53.
79. Gomez G, Pallas V. Mature monomeric forms of Hop stunt viroid resist RNA silencing in transgenic plants. *Plant J* 2007; 51:1041-9.
80. Barba M, Hadidi A. RNA silencing and viroids. *J Plant Pathol* 2009; 91:243-7.