The role of viroids in gene silencing: the model case of *Peach latent mosaic viroid*

Patricia Landry, Dan Thompson, and Jean-Pierre Perreault

Abstract: In plants, RNA silencing plays important roles in antiviral defense, host genome integrity and development. The process can be divided into RNA-mediated transcriptional and posttranscriptional gene silencing. The ability of viroids, which are small circular RNAs that infect higher plants and cause significant economic losses in agriculture, to induce both of these mechanisms has been demonstrated. Consequently, these RNA genomes appear to be suitable for the study of RNA silencing. In presenting useful strategies for detecting viroid-associated posttranscriptional gene silencing, as well as original results suggesting how *Peach latent mosaic viroid* induces it, this work reviews the current understanding of RNA silencing, a process which constitutes the latest revolution in molecular biology. RNA silencing has gained a lot of attention as a tool for the selective inhibition of gene expression. Clearly, we are only beginning to appreciate the complexity of this mechanism, as well as its biological diversity and potential applications.

Key words: RNA interference, viroid, RNA genome, posttranscriptional gene silencing, *Peach latent mosaic viroid*.

Résumé : Chez les plantes, l'atténuation de l'ARN joue un rôle important dans la défense antivirale, l'intégrité du génome et le développement. Ce processus peut être divisé en atténuation génique transcriptionnelle et post-transcriptionnelle, médiée par l'ARN. L'habileté des viroïdes, de petits ARN circulaires pathogènes des plantes supérieures causant des pertes significatives en agriculture, à induire ces deux mécanismes a été démontrée. Conséquemment, ces génomes d'ARN sont d'excellents candidats pour l'étude de l'atténuation de l'ARN. En présentant des stratégies utiles pour la détection d'atténuation génique post-transcriptionnelle associée aux viroïdes, ainsi que des résultats originaux suggérant comment le viroïde de la mosaïque latente du pêcher (PLMVd) l'induit, ce travail résume la compréhension actuelle de l'atténuation de l'ARN, un processus qui constitue la dernière révolution en biologie moléculaire. L'atténuation de l'ARN a gagné beaucoup d'attention comme outil pour inhiber de façon sélective l'expression génique. Il est clair que nous ne commençons qu'à apprécier la complexité de ce mécanisme, sa diversité biologique ainsi que son potentiel d'application.

Mots clés : interférence à l'ARN, viroïde, génome à ARN, atténuation génique post-transcriptionnelle, viroïde de la mosaïque latente du pêcher.

Introduction

The engineering of transgenic petunias, performed several years ago with the goal of altering their pigmentation, led to the discovery of the latest revolution in molecular biology: RNA silencing. The introduction of exogenous transgenes did not deepen the flower colour as expected. Instead, flowers showed varied pigmentation, with some completely lacking pigment production (for a review, see Jorgensen 1990). The outcome of these experiments was that not only were the transgenes inactive, but also that the added DNA sequences somehow affected the expression of the endogenous loci. Explaining these results led to the discovery of a novel gene-regulation mechanism, which was named cosuppression or posttranscriptional gene silencing (PTGS).

RNA silencing, which takes place naturally in several organisms, including protozoa, fungi, plants, and mammals, has gained a lot of attention as a tool for selective gene silencing. In plants, RNA silencing plays an important role in antiviral defense, genome integrity, and development. It can be divided into RNA-mediated transcriptional gene silencing (TGS) and PTGS. RNA-mediated TGS occurs when double-stranded RNA (dsRNA) with sequence homology to a promoter is produced, leading to de novo DNA methylation of the promoter region of the structural gene (Wassenegger et al. 1994); while PTGS reduces the steady-state levels of targeted host or viral cytoplasmic RNA (for a review, see Hannon 2002). The ability of plant viroids to induce both mechanisms has been demonstrated, and conse-

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quently, these RNA genomes appear to be suitable for the study of RNA silencing.

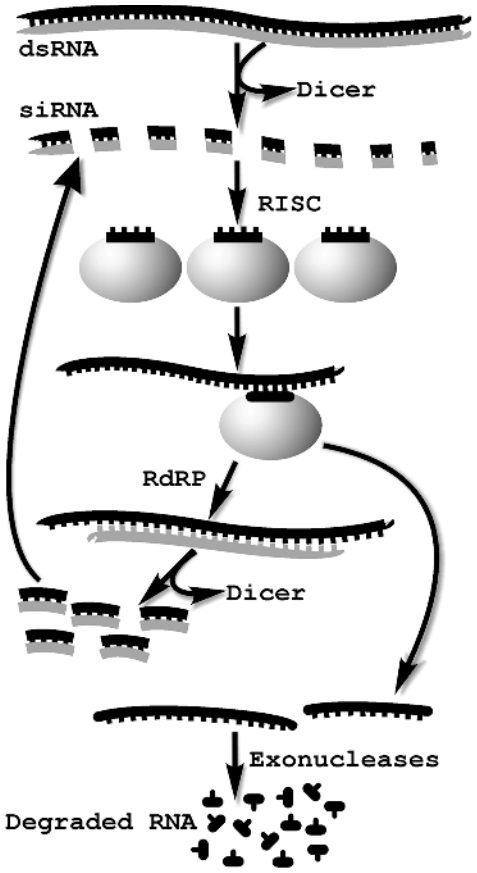
Overview of the posttranscriptional gene-silencing mechanism

The molecular details of RNA silencing are becoming better understood because of the results from a combination of biochemical and genetic approaches (for reviews, see Dillin (2003) and Tijsterman et al. (2002); see also Tabara et al. 2002). In plants, a biochemical framework for RNA silencing, using wheat germ extract, was reported recently (Tang et al. 2003; Voinnet 2003). A key feature uniting the RNA-silencing pathways in different organisms is the importance of dsRNA either as a trigger or as an intermediate (Fig. 1). The dsRNA is cleaved by a ribonuclease of the RNase III family (called Dicer) into small interfering RNAs (siRNAs), of both polarities. In vitro experiments with wheat germ extract showed that plant siRNAs arose as two distinct species, 21 and 25 nucleotides (nt) in length, confirming earlier in vivo observations in tobacco and *Arabidopsis* (Hamilton et al. 2002). These RNA species are double-stranded and possess two nucleotides long 3′ overhangs and 5′ phosphates that are the hallmarks of Dicer cleavage products. The two siRNA classes are most likely produced by two distinct Dicers (Tang et al. 2003). At least four Dicer homologues have been found in both the rice and *Arabidopsis* genomes (Finnegan et al. 2003). The siRNAs produced by Dicer are incorporated into a multisubunit protein complex, the RNA-induced silencing complex (RISC), which acts as a guide directing the RNA degradation machinery to the target RNAs. Consequently, a plant virus can be both the inducer and the target of RNA silencing. In some organisms, including plants and nematodes, the siRNA can serve as a primer for an RNA-dependent RNA polymerase (RdRP), thereby creating many more siRNAs (Fire et al. 1998). The action of an RdRP provides an amplification phenomemon 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.

Viroids and PTGS

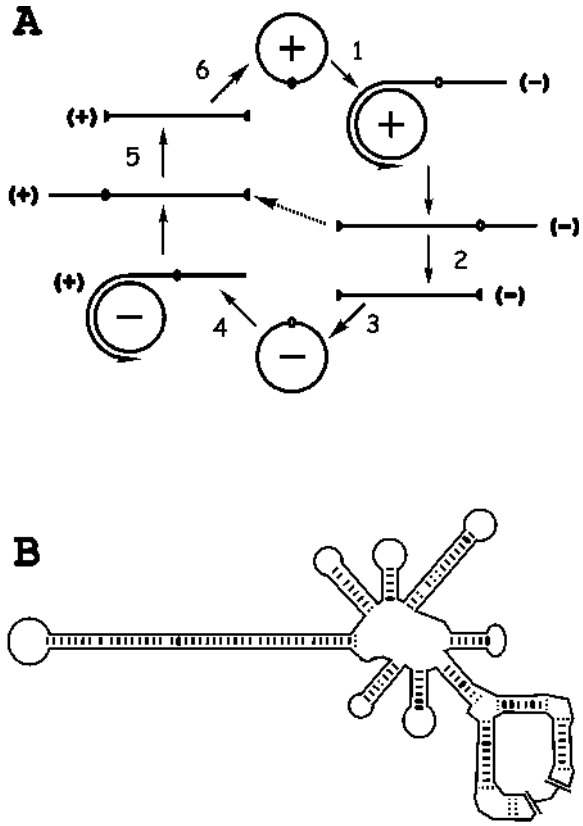
Viroids are the smallest nucleic acid based pathogens currently known (for a review, see Flores et al. 2000). They are small (~240–460 nt), single-stranded, circular RNAs that infect higher plants and cause significant economic losses in agriculture. In infected cells, viroids replicate in a DNA-independent manner through a rolling-circle mechanism that adopts either a symmetric or an asymmetric mode (Bussière et al. 1999). According to the symmetric mode (Fig. 2), the infecting circular monomer (which is assigned plus polarity by convention) is replicated into linear multimeric minus strands that are then cleaved and ligated, yielding minus circular monomers. Using the latter RNA as template, the same three steps are then repeated to produce the progeny. In contrast, in the asymmetric mode, the linear multimeric minus strands serve directly as the template for

Fig. 1. Overview of the posttranscriptional gene-silencing mechanism. Double-stranded RNAs (ds-RNAs) are cleaved by Dicer into 21- to 25-nt small interfering RNAs (siRNAs), which are then complexed with the RNA-induced silencing complex (RISC). Once the siRNA–RNA template hybrids form, the target RNA can be cleaved, and the products degraded by exonucleases. Alternatively, a copy of the target can be synthesized by the RNA-dependent RNA polymerase (RdRP) by extension of the siRNA, which, therefore, leads to the production of more siRNAs.



the synthesis of linear multimeric plus strands that are then cleaved and ligated to produce the progeny. Viroids have been classified into two families (*Pospiviroidae* and *Avsunviroidae*), primarily according to sequence conservation (Flores et al. 2000), as well as biological and molecular hallmarks (Bussière et al. 1999). Pospiviroidae members, which are frequently represented by the *Potato spindle tuber viroid* (PSTVd), share a conserved central region. These viroids replicate in the nucleus via the asymmetric mode, in a process that appears to involve host proteins catalyzing each step (Bussière et al. 1999). Conversely, avsunviroidae members replicate in chloroplasts via the symmetric mode. They are represented by *Peach latent mosaic viroid* (PLMVd), a viroid of 338 nt that is the causal agent of peach latent mosaic disease (Hernandez and Flores 1992). The multimeric intermediates of PLMVd replication are processed by self-catalytic hammerhead motifs, while the circularization of the monomeric linear strands has been suggested to result from a nonenzymatic self-ligation (Côté et al. 2001). These mechanisms have led to the proposal

Fig. 2. *Peach latent mosaic viroid* (PLMVd) rolling-circle replication and schematic secondary structure. (A) The polymerization, hammerhead mediated self-cleavage and ligation steps are numbered 1–4, 2–5, and 3–6, respectively. The polarities of the strands are indicated in parentheses. The dotted arrow shows the different steps of the asymmetric mode. In the latter case, the multimeric RNA strands of minus polarity are used as templates for the synthesis of the plus polarity strands without further processing. (B) The schematic secondary structures for the PLMVd strands, according to the model published previously (Bussière et al. 2000; Pelchat et al. 2000).



that the replication of avsunviroidae members relies primarily on a viroid RNA-based mechanism, rather than on the involvement of host proteins (Côté et al. 2001). The differential subcellular localization of the pospiviroidae and avsunviroidae viroids has been suggested to be the source of their distinct replication mechanisms (Bussière et al. 1999).

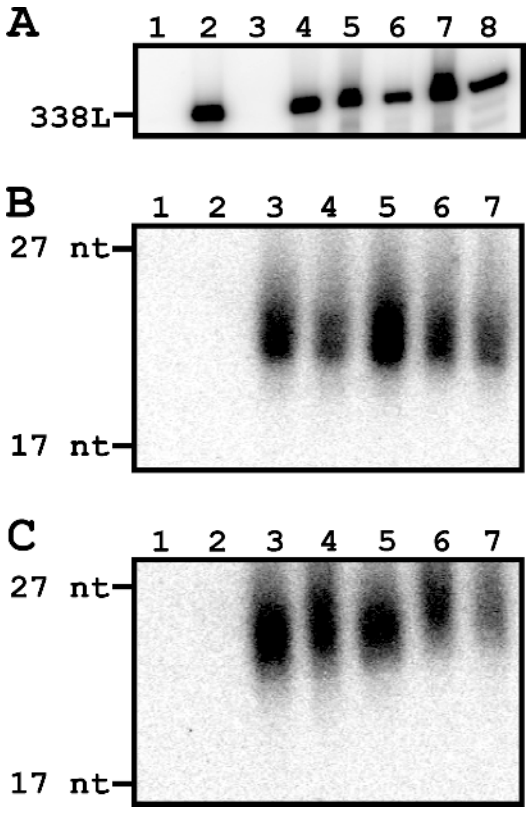
Based on the detection of the characteristic siRNAs of 21–25 nt, representing different domains of the viroid genome, species of both pospiviroidae and avsunviroidae groups were suggested to induce PTGS (Itaya et al. 2001; Martinez de Alba et al. 2002; Papaefthimiou et al. 2001). Based on the subcellular compartmentation of viroid replication, it has been suggested that PTGS might take place in both the nucleus and the chloroplasts (Martinez de Alba et al. 2002). The later proposition appears highly improbable, yet all evidence, including the localization of the Dicer enzyme (Provost et al. 2002), indicates that PTGS occurs via a cytoplasmic mechanism. Clearly, the detailed mechanism of how a viroid induces PTGS remains to be revealed. By presenting useful strategies to detect viroid-associated PTGS, and for how PLMVd induces it, this work reviews the role of these infectious RNAs in gene silencing.

Detection of viroid-associated siRNAs

The initial step of any study that aims to demonstrate the induction of PTGS by a viroid must be the detection of both the viroid and the siRNAs, which are the hallmarks of this biological mechanism, in infected leaves. This is best performed by Northern blot hybridization with isolated RNA samples fractionated by polyacrylamide gel electrophoresis. For example, RNA samples from one healthy and five PLMVd-infected peach cultivars grown on the west coast of Canada were isolated according to the procedure by Pelchat et al. (2000). They were fractionated on polyacrylamide gels of varying acrylamide concentrations to allow distinction of RNA species (21–25 and 338 nt). The nucleic acids were transferred to positively charged nylon filters and then probed as described previously (Bussière et al. 1999). In all cases, the riboprobes of both the plus and minus polarities used for the Northern blot hybridizations were full-length ^{32}P-labelled internally linear PLMVd transcripts. These were synthesized with the StripEZ transcription kit (Ambion, Austin, Tex.) to obtain probes that can be stripped under mild washing conditions, thereby permitting multiple probings of the same membrane. The transcription reactions were performed in the presence of 50 μCi (1 Ci = 37 Gbq) of [α-^{32}P]GTP (3000 Ci·mmol^{-1}), using the recombinant plasmid pPD1 that possesses two tandemly repeated PLMVd sequences inserted between the RNA T7 and T3 promoters (Beaudry et al. 1995). During transcription, RNAs of both polarities possessing hammerhead sequences were produced and self-cleaved efficiently, yielding 338-nt monomeric transcripts. The Northern membranes were analyzed by autoradiography.

Typical examples of the autoradiograms obtained are illustrated in Fig. 3. A concentration of 5% acrylamide was employed for the fractionation of the replicational intermediates, which are 338 nt in size. The linear PLMVd strands were found to be the predominant conformers accumulating in the leaves (Fig. 3A, lanes 4–8; see also Bussière et al. 1999). This autoradiogram corresponds to a hybridization performed with the PLMVd strands of plus polarity as probe, meaning that we are detecting the accumulated PLMVd monomeric strands of minus polarity within the leaves. Lane 1 shows that no cross-hybridization occurs, even if PLMVd is composed of several complementary domains. Similar results were obtained using a probe of minus polarity with which the PLMVd strands of plus polarity are detected (data not shown). The 21- to 25-nt siRNAs were detected on 12% polyacrylamide gels. The probe of plus polarity allowed the detection of siRNAs of minus polarity and vice et versa (Figs. 3B and 3C, respectively), indicating that both strands of the viroid participate in the mechanism. siRNA species were only detected in the samples isolated from the PLMVd-infected peach leaves. These complex mixtures of RNA species, which vary in terms of size (21–25 nt) and sequence composition, have varying electrophoretic mobilities, resulting in the observation of thin bands. Interestingly, lane 2 corresponds to a mixture of synthetic PLMVd strands of either minus or plus polarity (comple-

Fig. 3. Autoradiograms of Northern blot hybridizations with RNA samples isolated from either healthy or *Peach latent mosaic viroid* (PLMVd)-infected leaves. (A) RNA samples were fractionated by polyacrylamide gel electrophoresis (PAGE) on a 5% denaturing gel prior to being transferred to a nylon filter. The hybridization was performed with ^{32}P-labelled PLMVd strands of plus polarity. Only the portion showing the monomeric linear strands, according to the position of synthetic, linear PLMVd transcripts (338L) used as a size marker, is illustrated. Lanes 1 and 2 are synthetic, monomeric PLMVd strands of either plus or minus polarity, showing the absence of cross-hybridization. Lane 3 is an RNA sample from healthy leaves. Lanes 4–8 are RNA samples from PLMVd-infected leaves of peach 'Harrow Beauty', 'Agua', 'Redgold', 'Siberian C', and 'Hardired', respectively. (B–C) RNA samples were fractionated by PAGE on 12% denaturing gel prior to being transferred to nylon filters. The hybridizations were performed with ^{32}P-labelled PLMVd strands of both plus (B) and minus (C) polarity, respectively. Only the portions showing the small RNA species are shown. For both autoradiograms, the samples loaded on the gel were identical: lanes 1 and 2, RNA samples from healthy leaves either with or without synthetic, monomeric PLMVd transcripts of polarity complementary to the probe, respectively; lanes 3–7, RNA samples from PLMVd-infected leaves of peach 'Harrow Beauty', 'Agua', 'Redgold', 'Siberian C', and 'Hardired', respectively. Adjacent to the gel, the positions of deoxyoligonucleotides of 17 and 27 nt used as markers are indicated.



mentary to the probe) added to the RNA sample isolated from a healthy plant during the extraction procedure. In this case, no small RNA species were detected, indicating that the siRNAs were not simple degradation products of longer species. More importantly, this experiment demonstrated that replicating PLMVd is accompagnied by siRNAs of both polarities. Since the presence of the small RNAs maybe considered as an indicator of PTGS in plants, these results suggest that the PLMVd is responsible for the initiation of the silencing mechanism; however, a definitive confirmation remains to be ascertained.

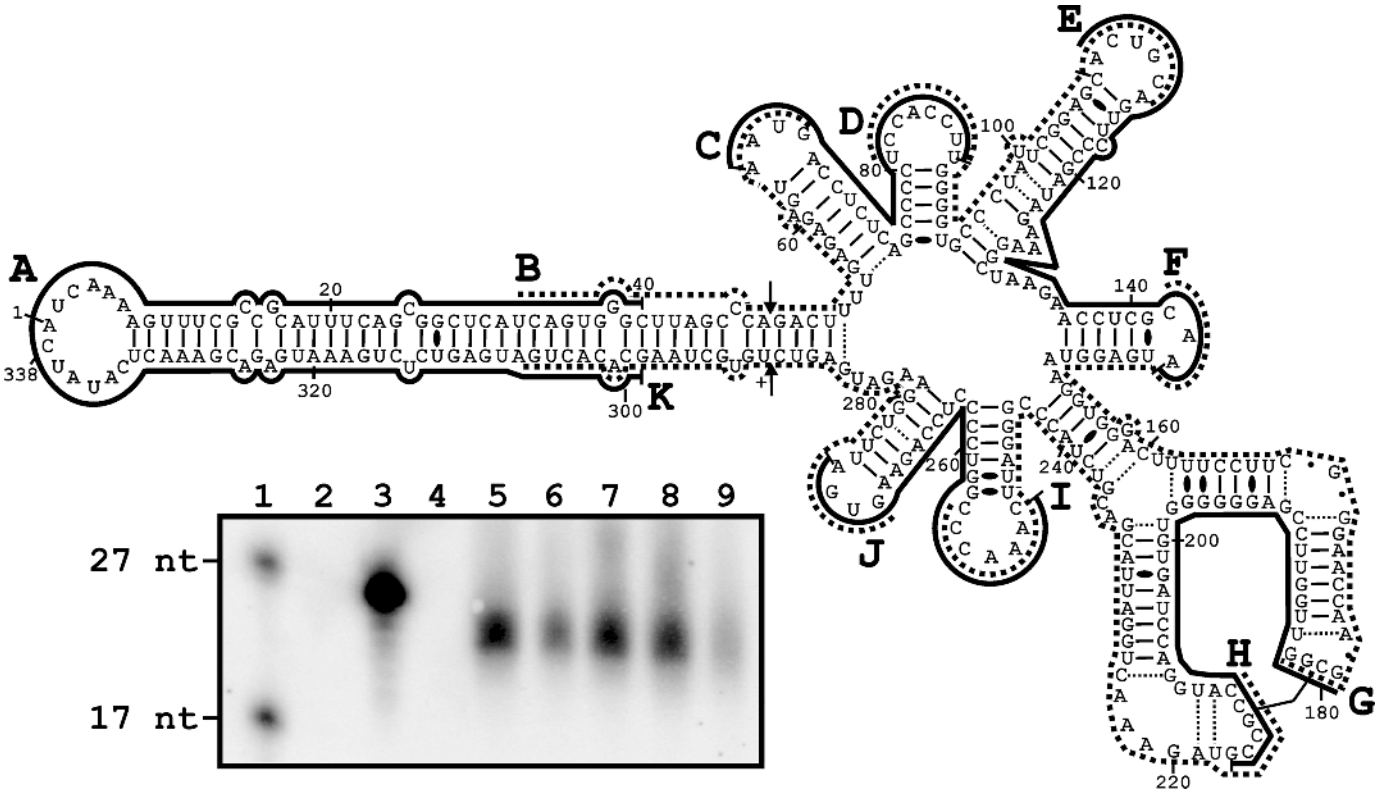
To investigate whether or not a specific region, or the entire viroid molecule, induces the siRNA mechanism, Northern blot hybridizations were performed using PLMVd-derived oligodeoxynucleotides of both polarities, of sizes varying from 25 to 40 nt. The ensemble of these oligodeoxynucleotides completely covers the PLMVd RNAs (Fig. 4). When ^{32}P-labelled at their 5′ end, all of these oligodeoxynucleotides detected siRNAs in RNA samples extracted from PLMVd-infected peach trees, but not from healthy ones (see Fig. 4, inset; compare lane 4 with lanes 5–9). Clearly, accumulated siRNAs form a population of sequences that are not restricted to a specific PLMVd region(s), but rather represent the entire viroid molecule. This is in agreement with the demonstration that two different PLMVd-derived probes detected siRNAs in one PLMVd-infected sample (Martinez de Alba et al. 2002). Furthermore, this conclusion receives physical evidence from the cloning of siRNAs species isolated from PLMVd-infected samples, which permitted the identification of several sequences corresponding to different domains of the viroid (data not shown). This experiment also revealed that the populations of small RNAs were composed of 21- to 25-nt species, supporting the implication of more than one Dicer-like enzyme.

Together, these experiments constitute the original demonstration that PTGS induced by a viroid is not restricted to only one PLMVd variant (the five tested differed in terms of sequence; see Pelchat et al. 2000), but rather appears to be generalized to all variants. We attempted, unsuccessfully, to establish various correlations between PLMVd replication, the levels of siRNAs detected, and the accumulation of the siRNAs (in terms of quantity with the polarity of the PLMVd probes). However, this does not exclude that these correlations may exist, for example between the silencing RNA mechanim and the pathogenesis.

Double-stranded PLMVd hybrids are substrates for Dicer

The RNA interference mechanism is induced by the presence of nonspecific dsRNA structures that are substrates for the ribonuclease Dicer. The dsRNA structures should be composed of at least 21 consecutive base pairs. The secondary structure of PLMVd strands of plus polarity has been determined by computer prediction and analysis of base-pair covariation, using an extensive collection of natural variants, and has been further confirmed in vitro by nuclease mapping experiments coupled with binding shift assays with oligonucleotides (see Fig. 4; Bussière et al. 2000; Pelchat et al. 2000). Visual inspection of this secondary structure did not indicate the presence of any strech of more than 12 consecutive base pairs. Similarly, the analysis

Fig. 4. Analysis of the small interfering RNA (siRNA) composition, using *Peach latent mosaic viroid* (PLMVd)-derived oligonucleotides as probe. Eleven oligonucleotides, ranging from 25 to 40 nt, covering the full length of the PLMVd RNA of plus polarity, as well as the corresponding oligonucleotides of minus polarity, were synthesized and individually used to probe the Northern blots of the siRNAs retrieved from PLMVd-infected RNA samples isolated from peach leaves. The sequences of the oligonucleotides are represented by the full and dotted lines on the secondary structure of PLMVd shown, while the letters identify the oligonucleotides. In the inset is a typical example of an autoradiogram obtained with the oligonucleotide I of plus polarity. Lane 1 is two radioactive deoxyoligonucleotides of 17 and 27 nt, respectively, which served as electrophoretic mobility controls. Lanes 2 and 3 correspond to the oligonucleotide I of either plus or minus polarity, respectively. Lane 4 is an aliquot of RNA isolated from healthy leaves, while lanes 5–9 are samples isolated from PLMVd-infected leaves of different cultivars.



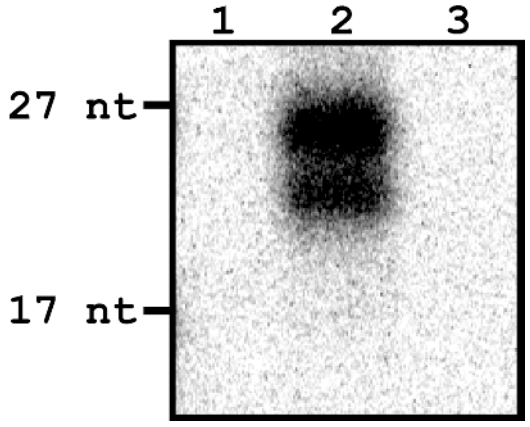
of computer-assisted secondary structure predictions of the minus polarity RNA strands did not suggest the presence of any double-stranded region long enough to activate Dicer (data not shown). Therefore, it appears likely that dsRNA complexes formed by intermolecular base-pairing of PLMVd strands of both plus and minus polarities must serve as the substrates for Dicer.

To verify this hypothesis, we adopted an assay based on the wheat germ extract containing Dicer-like enzymes that convert dsRNA into siRNAs (Tang et al. 2003). Initially, ^{32}P-labelled internally PLMVd transcripts of both plus and minus polarities synthesized in vitro were denatured by heat at 85 °C for 2 min and then slowly cooled to room temperature to favor the formation of dsRNA. An aliquot of the resulting mixture was incubated either with or without the wheat germ extract, according to the manufacturer's recommendations (Promega, Madison, Wisc.) for 3 h at 25 °C. The reaction was stopped by proteinase-K treatment (15 min at 65 °C) followed by phenol extraction, and the nucleic acids were then precipitated in ethanol and fractionated on denaturing (7 mol·L^{-1} urea) polyacrylamide (8%) gels. The double-stranded PLMVd structure induced Dicer activity, resulting in the formation of 21- to 25-nt siRNAs (Fig. 5, lane 2). If the wheat germ extract was inactivated by heat denaturation (5 min in boiling water) prior to the addition of the double-stranded PLMVd, no product was detected, even after overexposure of the gel (Fig. 5, lane 3). Together, these observations indicate that if PLMVd strands of both plus and minus polarities fold into a double-stranded duplex in a cellular context, then the resulting dsRNA might become a substrate for Dicer. However, the results of this experiment do not exclude definitively that a PLMVd strand alone (i.e., either of plus or minus polarity) may also be a substrate for the Dicer enzymes.

How do viroids overcome gene silencing?

The answer to this question remains a mystery; however, several hypotheses can be considered. First, the subcellular localization of a viroid might allow it to evade PTGS induction. Attempts to detect PSTVd- and PLMVd-related siRNAs in tomato nuclear and peach chloroplast RNA preparations, respectively, have failed so far (Papaefthimiou et al. 2001; and data not shown). Dicer enzyme had been localized in the cytoplasm (Provost et al. 2002), while pospiviroidae and avsunviroidae viroids have been shown to

Fig. 5. Autoradiogram of a polyacrylamide gel electrophoresis (PAGE) of Dicer activity assays performed on a wheat germ extract. ^{32}P-labelled internally *Peach latent mosaic viroid* transcripts of both plus and minus polarities synthesized in vitro were denatured by heat at 85 °C for 2 min and then slowly cooled to room temperature. An aliquot of the resulting mixture was then incubated either without, or with, the wheat germ extract, according to the manufacturer's recommandations (Promega, Madison, Wisc.) for 3 h at 25 °C (lanes 1 and 2, respectively). The reactions were halted by proteinase-K treatment (15 min at 65 °C) followed by phenol extraction, and the nucleic acids were precipitated in ethanol and fractionated by PAGE on a 8% denaturing gel (7 mol·L^{-1} urea). The conditions in lane 3 were identical to those of lane 2, except that the wheat germ extract was deactivated by heat treatment prior to the incubation. Adjacent to the gel, the positions of deoxyoligonucleotides of 17 and 27 nt used as markers are indicated.



replicate in the nucleus and chloroplasts, respectively, (Bussière et al. 1999). Therefore, it is reasonable to believe that PTGS induction has to wait for the cytoplasmic phase of the viroid life cycle to occur. The viroid RNA is present in the cytoplasm as it moves from cell to cell and spreads systemically in the entire plant. Second, viroids exist primarily as single-stranded circular RNAs that fold into highly stable secondary structures. As shown above for PLMVd, such a structure does not contain any dsRNA stretches of 21 nt or greater in length; therefore, the final form of the viroid would be an unlikely substrate for a Dicer-like enzyme. Formation of PLMVd dsRNA hybrids, which have been shown to be substrates for Dicer activity, is highly improbable (i.e., almost accidental) since the single-stranded structure of the viroid is almost as stable as the double-stranded conformer. It would be surprising for the formation of dsRNA hybrids to be related to the viroid titer since it has been demonstrated that infection by *Avocado sunblotch viroid*, in which the dsRNA hybrid accumulates in large concentrations, did not result in the detection of siRNAs (Martinez de Alba et al. 2002). The reason why this viroid infection did not lead to siRNA accumulation remains to be determined. Third, viroids may be bound to cellular proteins, which would protect them from Dicer hydrolysis. Finally, viroids may actively suppress RNA silencing, either by activating host protein suppressors of RNA silencing or by directly silencing host genes in the pathway. Such a mechanism has only been described for viral and cellular proteins so far. For example, coat protein of the *Turnip crinkle virus* has been demonstrated to mediate the suppression of RNA silencing in *Nicotiana benthamiana* Domin., while a protein of the replication mechanism of the *Tomato mosaic virus* has been shown to possess an equivalent activity (Thomas et al. 2003; Kubota et al. 2003). Therefore, viruses are not only a potential target for RNA silencing, they can also activate the process and encode protein suppressors. Viroids do not encode proteins; consequently, it is unclear how they might suppress PTGS. However, it is possible to imagine that a viroid might either interact with a host component (i.e., a protein or a nucleic acid) or modulate (i.e., up or down regulate) the expression of a host component, yielding a suppression of RNA silencing.

Transmission of the silencing signal

Another important issue of the PTGS mechanism is the transmission of the silencing signal, if indeed such a phenomenon exists. So far, we refer solely to a mechanism that involves cell-autonomous amplification of the silencing signal. In other words, the viroid has to replicate in the cell to eventually induce the Dicer enzyme and PTGS. Such a mechanism does not involve transmission of a silencing signal, but rather transmission of the viroid itself. Conversely, the possibility cannot be excluded that the siRNAs themselves, or double-stranded viroid hybrids, might be transmitted between cells. Furthermore, two types of transmission must be considered (for a review, see Hannon 2002). The first is short-range, cell-to-cell transmission. The plant cells are connected through cytoplasmic bridges called plasmodesmata. The movement of RNA and proteins via these cell–cell junctions is well known, and it is likely that either viroids (Zhu et al. 2002), long dsRNA, or siRNAs could pass through these connections. The silencing signal might also pass through the plant vasculature (Voinnet et al. 1998). Studies of the viral silencing inhibitor Hc-Pro have provided evidence against siRNAs being critical for the systemic silencing in plants. It has been shown that Hc-Pro expression in a silenced rootstock relieves silencing and inhibits siRNA production, but that a systemic signal can still be passed from this rootstock to an engrafted section lacking Hc-Pro expression (Kasschau et al. 2003). Clearly, the spread of viroids and the silencing signal in plants are intriguing questions that need to be investigated.

PTGS as source of many more discoveries

Many questions concerning the PTGS mechanism remain unsolved, and many aspects have not yet been investigated. Regardless, RNA interference is becoming a powerful tool. Because target identification depends upon Watson-Crick base-pairing interactions, the PTGS machinery can be both flexible and exquisitely specific. For example, it may be used for specific gene function targeting. In the nematode *Caenorhabditis elegans*, testing of the functions of individual genes by RNA interference has now been extended to analysis of nearly all of the worm's predicted approximately 19 000 genes (Hannon 2002). Similar strategies are being

pursued in other organisms, including plants. Furthermore, cells adopt a similar strategy to control mRNA expression. Plants contain many small transcripts, called miRNAs (micro RNAs), which have identical, or nearly so, complementary sequences in their mRNA collection (Rhoades et al. 2002). Interestingly, the majority of the target mRNAs are transcription factors that regulate developmental events, and the region complementary to the miRNA is almost invariably placed within the coding region. These observations lead to speculation that plant miRNAs function as siRNAs and mediate the specific destruction of target mRNAs. It remains to be experimentally shown whether or not this is indeed the case. Therefore, genetic engineering to manipulate gene expression, based on the use of siRNAs, has the potential of providing new characteristics such as virus resistance to plant cells through mRNA destruction. In conclusion, we are just beginning to appreciate the mechanistic complexity of RNA silencing and its biological diversity and potential in plants.

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