

Viroids: petite RNA pathogens with distinguished talents

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Viroids are small, circular, single-stranded RNA molecules that cause several infectious plant diseases. Viroids do not encode any pathogen-specific peptides but nonetheless, the subviral pathogens replicate autonomously and spread in the plant by recruiting host proteins via functional motifs encoded in their RNA genome. During the past couple of years, considerable progress has been made towards comprehending how viroids interact with their hosts. Here, we summarize recent findings on the structure–function relationships of viroids, their strategies and mechanisms of replication and trafficking, and the identification and characterization of interacting host proteins. We also describe the impact of the RNA silencing machinery of plants on viroid RNAs and how this has started to influence our models of viroid replication and pathogenicity.

Viroids have always been puzzling exceptions to the rules, ever since their name was coined by Ted Diener [1] to characterize an infectious, virus-like, low molecular weight RNA. At first sight, viroid diseases resemble regular viral infections in many aspects. However, at the molecular level, viroids are clearly distinct. Viroids consist of nothing but a discrete molecular species of a single-stranded, albeit covalently closed, circular RNA molecule whose major characteristics are summarized in **Box 1**. It is not only the circular RNA nature that is exceptional but also the absence of any encapsidation or any other form of a protective coat. Thus, the viroid is an infectious ‘naked’ RNA. Even more surprising is the absence of any functional open reading frame, which classifies the viroids as non-coding RNAs, unlike viral RNAs. Moreover, viroids do not replicate in the cytoplasm like conventional plant RNA viruses do but in either the nucleus or the chloroplast.

Box 1. Major similarities and differences among viroids

Common properties

- Viroids cause infectious diseases in distinct host plants.
- Viroids are RNA replicons with genetic information for replicability, host specificity and transportability.
- Viroids consist of a single species of a single-stranded but covalently closed circular RNA molecule, which ranges from 246 to 401 nucleotides, depending on the viroid species.
- Each viroid species assumes a characteristic secondary structure and can be divided into structural and functional domains.
- Viroid RNA is ‘naked’ (i.e. it is not encapsidated in a protein coat).
- There is no evidence for RNA translation, therefore viroids are classified as non-coding RNAs.
- Viroids replicate via linear, oligomeric RNA intermediates – of both polarities – using host RNA polymerases.
- Viroid infection causes the generation of specific short interfering RNAs (siRNAs).
- Viroids spread systemically in host plants.

Differences between viroids

Viroids can be classified into two major families, the *Pospiviroidae* [type species potato spindle tuber viroid RNA (PSTVd)] and the *Avsunviroidae* [type species avocado sunblotch viroid (ASBVd)], which are subdivided into several genera that are phylogenetically related [90] and for which online information is available [91]. Most viroids belong to *Pospiviroidae*; there are five genera and >24 species. The *Avsunviroidae* comprise two genera, with three species in total.

Pospiviroidae are replicated in the nucleus by host-dependent RNA polymerase II in an asymmetric replication cycle (Figure 2). The rod-like RNA secondary structure can be divided into several functional domains (Figure 1). There is no self-cleavage domain and the processing reaction probably involves host enzymes.

Avsunviroidae are replicated in the chloroplast by the nucleus-encoded RNA polymerase in a symmetric replication cycle that includes a circular RNA of (–) polarity (Figure 2). The secondary structure is branched (particularly in two of the three species). RNA strands of both polarities self-cleave autolytically via a hammerhead ribozyme domain.

To illustrate the major differences between the two major families of viroids, the basics of PSTVd and ASBVd replication are briefly summarized in Figure 2.

Pospiviroidae mentioned in this article

- Potato spindle tuber viroid RNA (PSTVd).
- Citrus exocortis viroid (CEVd).
- Hop latent viroid (HLVd).
- Hop stunt viroid (HSVd).
- Iresine viroid.

Avsunviroidae mentioned in this article

- Avocado sunblotch viroid (ASBVd).
- Chrysanthemum chlorotic mottle viroid (CChMVd).
- Peach latent mosaic viroid (PLMVd).

Non-coding does not mean non-functional. On the contrary, the viroid genome has the genetic information necessary for a replicon: first, it copies itself and proliferates autonomously (i.e. without the support of a helper virus); second, it provides information for host specificity and movement from cell to cell and long distance; and, third, it is able to induce a specific pathogenic effect in its host. To perform these functions, conventional RNA viruses require not only a genome that is at least ten times larger but also the expression of several specific proteins. The key to the biological activity of viroids is the self-complementary circular RNA and its resulting secondary structure, which must either be functional or provide several binding signals to host factors that directly or indirectly serve the life cycle of the infectious agent. Viroids can be considered as compact minimalists that do not need to even encode their own pathogen-specific proteins. Because of these features and because some viroids combine structure and function in the form of a ribozyme with self-cleaving properties, viroids have been considered to be 'RNA fossils' of a former RNA world. We think this is unlikely because viroids depend on a highly developed host. Rather than fossils, viroids should be considered as highly gifted, modern RNA parasites that have eliminated all the dispensable 'extravagance' of viral pathogens to make use of the host in the most sophisticated manner, regardless of whether they originate from satellite viruses or retroviroid-like DNA sequences [2], introns or other sources.

Over the past two decades, a lot of knowledge about viroid pathogens has accumulated and they are among the best-studied RNA molecules. Surprisingly, past controversies about who discovered what first are still on the agenda [3–5]. From a historic perspective, the principal milestones were the discovery of viroids, including the invention of the specific term, by Ted Diener [6], the proof of circularity by Heinz Sanger *et al.* [7], the direct RNA sequencing of the first viroid [potato spindle tuber viroid RNA (PSTVd)] by Hans Gross *et al.* [8] and the identification of the hammerhead self-cleavage structure by Bob Symons *et al.* [9]. Acknowledging the outstanding contributions of the first generation of viroid researchers, a new generation of molecular biologists – concerned not so much about competition as about attracting funding for this topic – has now started to shift the focus to a more integrated understanding of the viroid diseases. The centre of attention is moving slowly away from the RNA molecule (although there is still progress, as outlined below) towards the interaction with the host plant. In spite of their molecular differences to conventional infectious agents, viroids follow the classical scheme of infection, initial replication, spread and replication in young tissues, thereby causing a pathogenic effect, which is always the result of interference with host factors and their normal function. There are some key questions:

- What are the enzymes and cofactors used in replication?
- How is the switch from (–) to (+) RNA synthesis achieved?
- Which proteins protect the circular viroid and facilitate its movement through the host?

- How does the viroid escape the host's defence, particularly the defence against invading nucleic acids?
- What is the relationship of the viroid to epigenetic modifications of the chromatin?

These problems are far from being solved, although significant progress has been made lately, which we try to summarize here.

The purpose of this article is to review selected recent developments in the viroid field rather than to provide an updated overview. A personal perspective on the discovery of viroids was recently given by Ted Diener [6] and, for a more detailed current and general overview of viroids, including the original references, see Refs [10–12].

Structure–function relationships

As already mentioned, the viroid RNA molecule has many different biological functions that are condensed in a surprisingly short sequence. From previous studies of the viroid molecule over the past two decades, it seemed that almost every nucleotide is functional and under selection. In a continuation of projects on the analysis of viroid RNA sequences, several studies have further substantiated and extended this finding and have helped to define the specific functional domains of various viroids.

Analysis of PSTVd progeny of tomato plants inoculated with cloned cDNA showed that there could be sequence variability in some positions after a single plant passage [13]. This flexibility allows the viroid pathogen to adapt to environmental changes and environmental changes can, in turn, increase sequence diversity. For example, heat treatment of hop plants infected with hop latent viroid (HLVd) resulted in reduction of the viroid titre but was accompanied by a strong increase in sequence variability and complexity [14]. In spite of the flexibility of viroid sequences, there is also a sufficient degree of conservation. Analysis of 16 new sequence variants of hop stunt viroid (HSVd) belonging to different subgroups and originating from four Mediterranean countries, showed conserved regions, including a non-functional hammerhead-like domain, suggesting that HSVd is an evolutionary link between *Pospiviroidae* and *Avsunviroidae* [15]. To analyse the selective principles, Robert Owens *et al.* [16] randomized an infectious cDNA of an intermediate PSTVd isolate at five bases, including positions 46, 47 and 317, which represent the only difference to the highly severe strain RG1 [17]. Of the 768 possible variants, 23 were recovered, including the natural variant RG1 and two other known variants. Using numerical simulations, the authors deduced that the recovered variants represent more than 80% neutral mutants and that the underlying sequence PSTVd-Int occupies a comparatively steep peak within the fitness landscape.

A few nucleotide exchanges affect not only the pathogenicity of PSTVd but also its host range. Several years ago, it was reported that a single C to U change at position 259 allows efficient replication of the KF440-2 isolate of PSTVd in tobacco (*Nicotiana tabacum*) [18]. Yijun Qi and Biao Ding observed that the same mutation of the PSTVd-Int, as well as the change of U257 to either C or A (but not G) resulted in a five- to tenfold higher replication rate in tobacco protoplasts [19]. In an ensuing study, the

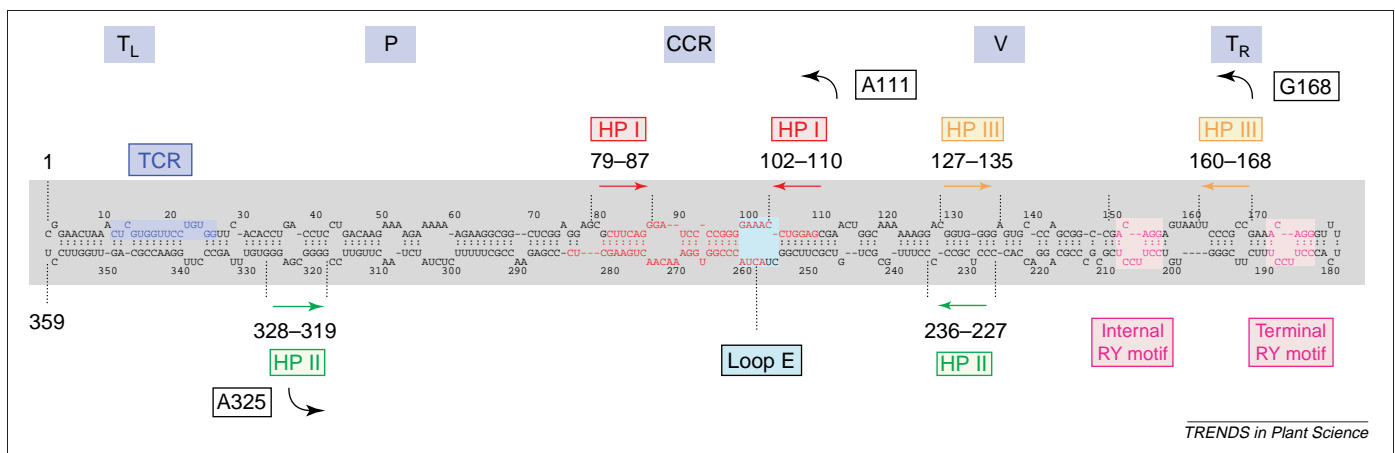


Figure 1. Conserved and functionally active structural elements of potato spindle tuber viroid RNA (PSTVd). This is the secondary structure of strain KF440-2 calculated by Mfold [92] at 10°C. The PSTVd molecule is divided into five structural domains – terminal left (T_L), which contains the terminal conserved region (TCR) found in the genus *Pospiviroid*, the pathogenicity domain (P) [which contains the central conserved region (CCR)], the variable (V) and the terminal right (T_R) domains. The nucleotides of the CCR domain are depicted in red. The upper strand of the CCR is in large parts palindromic. The border of the other domains are not strictly defined. A few sequence changes in the pathogenicity domain are sufficient to alter the severity of disease symptoms [93]. This modulation is not caused by apparent changes in viroid titres but by the sequence changes themselves. Reverse genetics is possible because cloned cDNA is infectious. However, many mutations – if infectious at all – result in revertants, illustrating that the PSTVd RNA is under strong selection. This scheme details the sequences (with nucleotide numbers) that form during the denaturation of the three hairpin loops of PSTVd RNA, HP I–HP III, in metastable structures [36]. The three reported start sites for transcription initiation are indicated by bent arrows; the first template nucleotide and the direction of transcription are indicated. Loop E is involved in the processing of PSTVd and the two RY motifs are the binding domain of tomato protein Virp1.

authors demonstrated that the U257 to A change (U257A) converted the intermediate strain PSTVd-Int into a lethal strain when assayed on tomato [20]. However, the lethal effect can be compensated by the mutation C259U. This is interesting because it is the first example of changes in PSTVd pathogenicity caused by a nucleotide alteration outside the pathogenicity domain. The mutation did not influence the PSTVd secondary structure, neither the replication level nor tissue tropism. The stunting in the infected tomato plants was not influenced by altered cell division or differentiation but resulted from restricted cell expansion, correlating with a downregulation of *LeExp2*, an expansin gene. All these mutations that either alter the host specificity or pathogenicity are located within the loop E motif (Figure 1). This motif is involved in RNA–RNA and RNA–protein interactions, and found in a wide range of RNAs. The loop E motif is also part of the central conserved region (CCR) of PSTVd, which is the functional domain for the processing reaction of longer-than-unit-length PSTVd (+) RNA transcripts [21]. However, the processing structure is not the loop E motif but a kinetically favoured multibranch structure with four helices, with a cleavage–ligation site within, between nucleotides G95 and G96. In the processing structure, the cleavage site is located within a stem that is closed by a tetraloop containing the phylogenetically conserved sequence GAAA that is also part of loop E [21]. Elegant and ultimate proof that the loop E region contains the processing domain was provided by Ole Schrader *et al.*, who recapitulated the correct processing reaction with a linear 148 nucleotide RNA covering the core CCR domain and including a 17 nucleotide duplication of the upper strand [22]. This precursor was converted *in vitro* into a mini-circle.

Another proof of functionality has been established for the hairpin II (HP II), which is a thermodynamically metastable structure that is essential for infectivity [23]

and whose restoration can establish infectivity of a non-viable recombinant viroid [24]. Using an RNA oligonucleotide designed for specific binding to the transient (–) strand intermediate of PSTVd, the formation of the predicted HP II has been shown during *in vitro* transcription by T7 RNA polymerase and during viroid replication [25].

Progress has also been made in identifying the pathogenicity determinants for *Ausunviroidae*. Two of its three family members are distinct from all other viroids by their overall branched secondary structure, which does not resemble the rod shape typical for *Pospiviroidae*. Using reverse genetics it has been shown that the nature of the tetraloop sequence that closes the stem formed between nucleotides 62 and 100 determines the symptom expression of chrysanthemum chlorotic mottle viroid (CChMVd) [26]. The tetraloop sequences GAAA (non-symptomatic) and UUUC (symptomatic) represent the two major fitness peaks. Peach calico, an extensive form of chlorosis, is caused by a subgroup of peach latent mosaic viroid (PLMVd) [27]. Analysis of 16 full-length cDNA clones showed that symptomatic strains have a 12–13 nucleotide insertion compared with the non-symptomatic strains. In PLMVd and CChMVd, the loop nucleotides that close helices I and II, which are located outside the hammerhead domain, contribute to the faster *cis* RNA cleavage rather than that reported for artificial *trans*-cleaving hammerhead ribozymes [28]. Apparently, these nucleotides are under selection to allow fast, efficient autolytic self-cleavage.

Replication of viroids

It was established more than a decade ago that both strands of PSTVd RNA are synthesized by the DNA-dependent RNA polymerase II (Pol II) [29], but the chloroplast RNA polymerase that replicates avocado sunblotch viroid (ASBVd) was not identified until 2000 [30]. Using preparations from purified protoplasts,

José-Antonio Navarro *et al.* performed transcription assays in combination with the inhibitor tagetitoxin. In this way, they showed that ASBVd is transcribed by the single-unit nuclear-encoded polymerase (NEP), rather than the plastid-encoded polymerase (PEP). Further support for the role of NEP comes from the observation that the (+) and (-) RNA strands of ASBVd both begin with the sequence UAAAA, which is a 5' sequence element typically found in NEP-encoded RNA transcripts and agrees with the mapping of the precise initiation sites for (-) and (+) RNA strand synthesis [31]. In spite of evidence for the involvement of NEP rather than PEP in ASBVd replication, the DNA-dependent RNA polymerase from *E. coli*, which is structurally related to PEP, uses a hairpin structure of PLMVd as an RNA promoter initiating *in vitro* transcription [32]. However, it remains to be seen whether PLMVd, which is more structured than ASBVd, is replicated by PEP. At present, it cannot be completely ruled out that the two genera of chloroplastic *Avsunviroidae* are replicated by different polymerases.

In addition to the polymerases, further proteins have been identified that might play a role in the replication of ASBVd. The two related chloroplast proteins PARBP33 and PARBP35 were found as adducts to the viroid RNA in ultraviolet-irradiated avocado leaves [33]. Proteins of this family are involved in interacting with chloroplast RNA transcripts, thereby facilitating maturation and editing. Therefore, these proteins might indirectly serve the replication and proliferation of ASBVd, particularly given that PARBP33 was found to have chaperone function supporting the autolytic hammerhead-mediated self-cleavage reaction of the multimeric ASBVd transcripts.

Also for PSTVd, the Pol-II-mediated transcription start site was mapped using a transcription extract prepared from an uninfected potato culture [34]. Using reverse-transcription PCR as the detection method, at least two start sites (A111 and A325) were identified for the initiation of (-) strand synthesis. However, these start sites are at variance with the start site G168, which had been identified much earlier using purified Pol II from tomato and wheat germ to assay the nascent PSTVd (-) strand directly via the incorporation of radioactively labelled cordycepin [35], which might be less sensitive to artefacts than PCR detection methods. Interestingly, all three reported initiation sites are located in one of the three hairpin loop regions that play an important role during denaturation and renaturation of PSTVd [36] (Figure 1).

Viroid movement and viroid-binding proteins

Like any infectious viral entity, viroids need to spread in their host. Several phases can be distinguished. In the originally infected cell, the viroid must move to either the nucleus or the chloroplast, and replication must be successful and sufficiently fast to avoid host defence mechanisms. The next step is the invasion of neighbouring cells or more distant tissues, followed ultimately by transmission to an uninfected plant. For plant viruses, it is often observed that viral titres in the inoculated leaf are rather low. However, if the virus reaches the vascular tissue, it can be transported to young, growing tissues,

where it replicates to large amounts. Short- and long-distance movements of viral particles and/or RNA are therefore crucial phases for the infection process and most viruses encode specific proteins to ensure a successful passage. The viroid RNA must move without the support of a pathogen-encoded movement protein, as used by many RNA viruses. Clearly, the circularity of the RNA and the resulting secondary structure provides some protection, but other intrinsic RNA signals must play a role as well.

Cell-to-cell movement of PSTVd probably proceeds via plasmodesmata because PSTVd injected into symplastically connected mesophyll cells moves rapidly to neighbouring cells, unlike when injected into symplastically isolated guard cells [37]. Moreover, fusion of PSTVd RNA causes the movement of another otherwise unmovable unrelated RNA [37]. Thus, it is likely that the viroid RNA recruits supporting protein factors.

Intracellular processes have been characterized using an infectious fluorescein-labelled PSTVd (F-PSTVd) [38]. The F-PSTVd was transported in permeabilized tobacco protoplasts to the nucleus, unlike other RNAs of similar size and unlike two chloroplastic viroids. Inclusion of unlabelled PSTVd impaired uptake but unrelated RNA did not, which suggests a specific, saturable nuclear import for PSTVd RNA. The import was not dependent on GTP and therefore the mechanism must be distinct to the RAN-GTPase pathway. Likewise, inhibitors of microtubule and actin filaments did not inhibit nuclear transport. Collectively, these experiments suggested that PSTVd contains a specific signal that directs it to the nucleus. This was confirmed by an elegant study by Yan Zhao *et al.* [39], who engineered an intron with an embedded full-length copy of PSTVd RNA into the coding region of green fluorescent protein (GFP). This construct was virally expressed in the cytoplasm and GFP expression was seen only for the recombinant RNA with a PSTVd-containing intron, which provided the signal for nuclear import, thus allowing splicing to a functional mRNA.

Using improved *in situ* hybridization techniques and strand-specific probes, Yijun Qi and Biao Ding recently showed that PSTVd (-) was localized in the nucleoplasm but not in the nucleolus of infected *Nicotiana benthamiana* plants and cultured cells, whereas PSTVd (+) RNA was detectable in the nucleoplasm as well as in the nucleolus [40]. Four distinct spatial localization patterns of PSTVd (+) RNA within the nucleolus can be distinguished, potentially reflecting different stages of viroid RNA maturation or storage. The authors stress that the nuclear environment can discriminate the two PSTVd strands of opposite polarity. Because *in situ* hybridization cannot discriminate between linear and circular forms of PSTVd, the circular structure might also be the determinant for the trafficking of the (+) strand to the nucleolus. The functional significance for the nucleolar phase of PSTVd (+) RNA remains to be determined.

To address the general long-distance trafficking pattern, Yali Zhu *et al.* [41] inoculated PSTVd to the stem of tomato or *N. benthamiana* followed by *in situ* hybridization of various tissues. First, they provided cytological evidence that PSTVd was transported to sink but not to

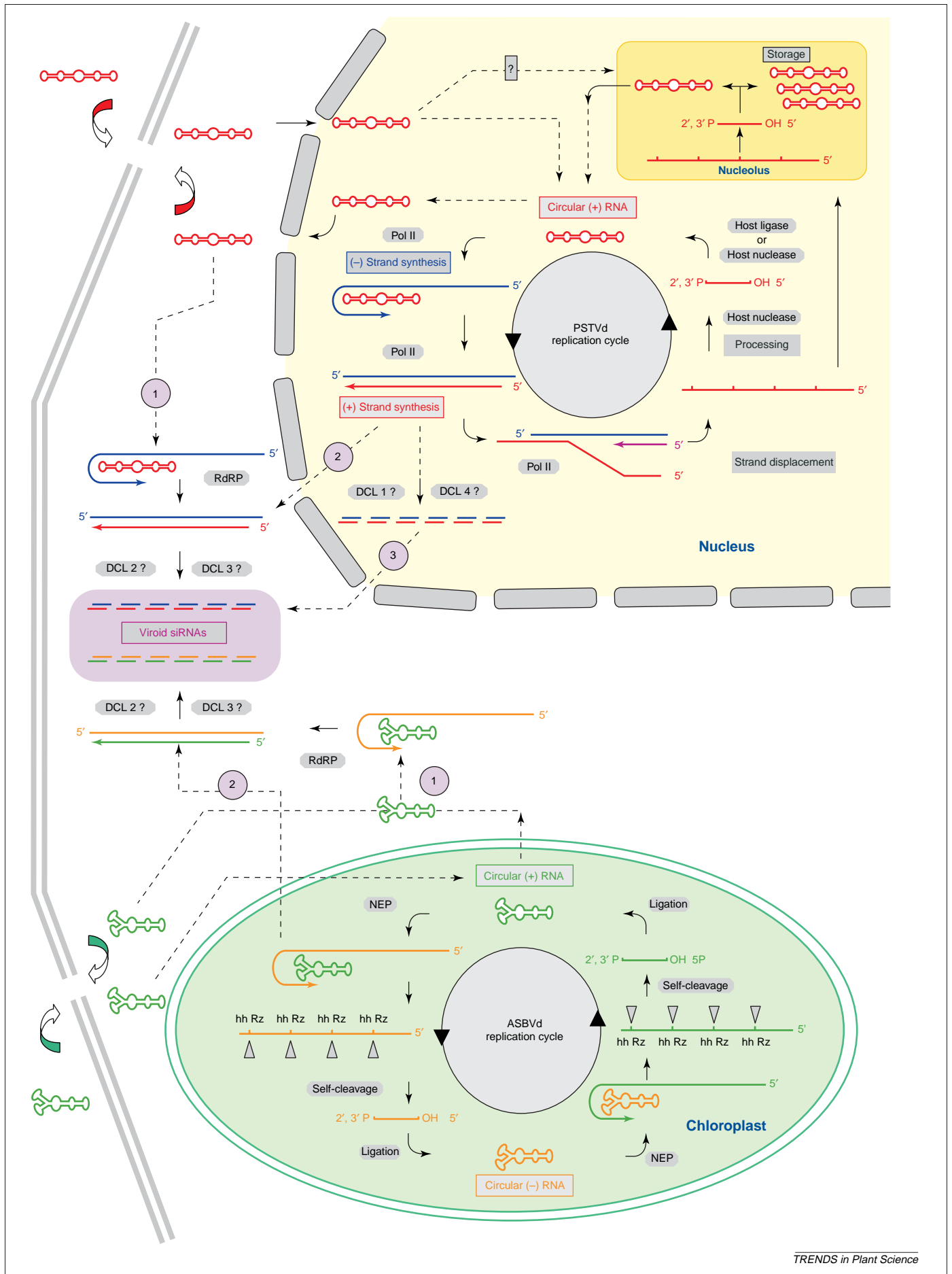
source leaves, confirming a long-standing finding [42] that systemic long-distance movement of viroids proceeds via the phloem. They also detected some replicative (–) forms of PSTVd. Furthermore, PSTVd did not enter shoot apical meristems and, in flowers, the viroid RNA was detectable only in sepals, being absent from petals, stamens and ovary, although small quantities should be there given that PSTVd can be transmitted by seed in tomato [43]. In accordance with long-distance movement in the phloem, two independent studies showed the formation of a ribonucleoprotein complex between HSVd and phloem protein 2 in cucumber phloem exudates; phloem protein 2 is a dimeric lectin and the most abundant protein in phloem [44,45]. These data suggest that phloem protein 2 alone or in conjunction with further proteins facilitates the long distance movement of viroids and possibly other RNAs, potentially including the mobile signal that is responsible for systemic spread of RNA silencing. Further evidence that transportation of viroids in the phloem does not simply follow the mass flow but is instead an active and regulated transportation process came from an ensuing study [46]. Using *N. benthamiana* as a host, the authors identified PSTVd mutants that also replicate in *N. tabacum*. In this host, mutated PSTVd can enter the phloem and spread in it, but the RNA cannot exit the phloem. This suggests that specific RNA motifs and host factors are required for systemic spreading of viroids via the phloem.

It is likely that proteins that interact specifically with viroid RNA are directly or indirectly involved in cellular or intercellular transportation processes. It was therefore of interest when Viroid RNA-binding protein 1 (Virp1) was identified via RNA-ligand screening [47] from a tomato cDNA expression library by its property to interact with PSTVd (+) RNA [48]. Virp1 interacts specifically with PSTVd and related viroids [citrus exocortis viroid (CEVd) and HSVd] but not with any other RNA tested. Virp1 contains a bromodomain and is a member of a family of transcriptional regulators associated with chromatin remodelling, but is the first of its kind for which RNA binding properties have been shown. Immunoprecipitation from infected tomato leaves has been used to document the interaction between Virp1 and PSTVd *in vivo* [48]. In accordance with the presumed function as a chromatin-interacting protein, Virp1 exhibits a nuclear localization signal. Therefore, Virp1 might transfer the viroid RNA to the nucleus and bring it into contact with transcription units associated with chromatin. Hence, it might also have a function during viroid RNA replication. The specific interaction of Virp1 and PSTVd was confirmed via the yeast three-hybrid system, which also revealed an interaction with HSVd [49]. The site of binding activity was mapped to the T_R domain (Figure 1) of PSTVd, for which certain mutant variants had been described earlier [50]. These mutant sequences were not able to infect plants unless they were delivered via *Agrobacterium*-mediated inoculation. However, the progeny consisted of new sequence variants (revertants), neither the wild type nor the originally inoculated mutant sequence. It was found that the mutant sequence had impaired interaction with Virp1, whereas the naturally selected variants derived

from that mutant regained some binding activity. This suggested that the interaction of PSTVd with Virp1 was required for an infection process and pointed towards a certain sequence element as determinant for Virp1 binding [49]. This motif was finally identified as an internal loop, now termed the RY motif in view of its base composition (Figure 1) [51]. In PSTVd and most representatives of the genus *Pospiviroid* (except CEVd and iresine viroid), the RY motif is present twice, but mutational analysis has shown that the terminal RY motif contributes more strongly to the specific interaction than the internal RY motif does. The RY motif can also be found in HSVd and some sequence elements of it are also present in the genus *Cocadviroid*, suggesting that it is, with Virp1, a determinant of host range. Disruption of either of the RY motifs abolishes infectivity [51], possibly by interfering with transport processes into or out of the nucleus (or even within) or cell-to-cell transport. How the switch between nuclear import–export and cell-to-cell transport is regulated, is not clear. This is not a trivial process, as illustrated by geminiviruses, which also replicate in the nucleus and require two viral proteins to shuttle between the nucleus of one cell to the nucleus of the other cell [52]. The acetylation of hepatitis delta virus small antigen (S-HDAg) is another interesting regulatory mechanism for nuclear localization of a viroid-like pathogen [53].

Viroids and RNA silencing

Mechanism of silencing and experimental observations
Post-transcriptional gene silencing (PTGS) [called RNA interference (RNAi) in animals] is the major molecular defence strategy of plants against RNA viruses. Many reviews have summarized the mechanisms of this process [54,55]. The key feature is the occurrence of double-stranded RNA that is cleaved by one form of the RNase-III-like enzyme Dicer into short double-stranded RNA fragments with single-stranded 3' protruding ends, which are called short interfering RNAs (siRNAs). The siRNAs are then incorporated into a multiprotein complex termed RNA-induced silencing complex (RISC). RISC gets activated and, by a helicase function, the two siRNA strands separate so that the remaining strand guides the sequence-specific cleavage of single-stranded complementary target RNA. In plants, RNA silencing is more complicated because different classes of siRNAs can be distinguished [56] and silencing is not cell autonomous but can spread from cell to cell and long distance, mediated by an unidentified mobile signal [57]. During an infection by a conventional RNA virus, the entire process of RNA silencing is probably cytoplasmic. The general trigger is believed to be the double-stranded replicative intermediate, which gets cleaved (or processed) into siRNA. Specific degradation of single-stranded (+) or (–) viral RNA occurs by RISC-mediated cleavage, also in the cytoplasm. Moreover, with the aid of the RNA-directed RNA polymerase (RdRP), further double-stranded RNA might be produced, resulting in the generation of secondary siRNAs. This might result also in the spread of silencing to neighbouring genes, a process that is called transitive silencing [58]. The first cellular RdRP was purified from tomato and analyzed



a decade ago by Winfried Schiebel and colleagues [59,60] for a potential role in viroid replication.

Like viruses, viroids also replicate via double-stranded RNA intermediates, which are located in either the nucleus (*Pospiviroidae*) or the chloroplast (*Avsunviroidae*) (Figure 2). Therefore, it is remarkable that both classes of viroids can generate specific siRNAs [61–63]. Originally, no siRNAs were detected for ASBVd [63] but, in a separate study, short RNAs were also seen for the type viroid of *Avsunviroidae* [64]. In PSTVd, strands of both polarities are equally represented and from all parts of the viroid molecule [61]. Viroids of different pathogenicity generated comparable titres of siRNAs [61]. Time-course analysis showed that siRNA could be detected at about the time that circular and linear RNAs are first detectable, and the titre follows more or less the replication rate (M. Denti *et al.*, unpublished).

Potential role of viroid-specific siRNAs

The detection of viroid-specific siRNAs is puzzling because their occurrence fits neither the conventional model of RNA silencing nor the biology of viroids: their double-stranded replicative intermediates are not generated in the cytoplasm, nor are there single-stranded cytoplasmic viroid-specific RNAs that could act as a target for PTGS. The major questions are in which cellular compartment the viroid-specific siRNAs are made and where they accumulate, and whether they are part of a PTGS response that will impair viroid replication. This relates to the question of whether there are PTGS-related processes in other cellular compartments than the cytoplasm. Certainly, some PTGS processes can proceed in the nucleus. For example, two of the four Dicer enzymes that have been identified in the *Arabidopsis* genome, Dicer-like 1 (DCL1) and DCL4, contain a nuclear localization signal [65]. Although *Arabidopsis* is not a host for viroids, it is reasonable to assume that also other plants have related Dicer enzymes. Thus, the processing reaction of double-stranded PSTVd RNA might proceed in the nucleus (Figure 2, pathway 3). If this pathway is correct, processing should be done by DCL4 because DCL1 seems to be responsible exclusively for the processing of pre-micro-RNAs (pre-miRNAs) into miRNAs [66], another class of small regulatory RNAs that are single-stranded and are generated from endogenous precursors [67]. The miRNAs are believed to control and modulate the expression of transcription factors and genes that play

an important role during plant development [68] and the first target genes have been identified [69,70]. The processing of plant miRNAs proceeds in the nucleus [71], which is at variance with the mammalian pathway, in which only polycistronic pri-miRNAs are processed in the nucleus to pre-miRNA [72], which are then exported and thereafter processed to the mature miRNAs [73]. However, even if the processing of double-stranded PSTVd RNA proceeds in the nucleus, can the resulting siRNAs be incorporated into a nuclear RISC complex to degrade specifically single-stranded viroid RNAs? This question cannot be answered conclusively; nuclear RISC has recently been reviewed [74]. By contrast, there is no evidence for any RNA silencing in the chloroplast, suggesting that at least *Avsunviridae*-derived siRNAs are generated in the cytoplasm. If there is such a pathway for the cytoplasmic conversion of chloroplastic viroid RNA or replicative intermediates to siRNA, this should also be possible for the family *Pospiviroidae*. One should also consider the possibility that siRNAs of both viroid families might be produced only in specific cell types, for example the vascular tissue, during exit or entry of the viroid in these tissues, a step that is common to both groups of viroids.

To address this question, it was recently shown in a biochemical analysis that PSTVd-derived siRNAs accumulate in the cytoplasm [75]. The siRNAs might be generated in the nucleus and then exported, but it is also possible that double-stranded PSTVd RNAs are constantly exported to the cytoplasm and then cleaved by any of the Dicer nucleases (Figure 2, pathway 2). The exported double-stranded form might remain undetectable because it is permanently degraded. This interpretation would explain the relatively high concentration of siRNAs in the cytoplasm [the amount of (–) detectable in siRNAs seems to exceed the amount of detectable replicative (–) intermediates] and would be reminiscent of the expression of double-stranded RNA from an engineered transgene, for example, for virus protection. Also, in some transgenic lines, only the siRNAs are detectable, not the double-stranded RNA precursors [76]. Exportation of double-stranded RNA intermediates to the cytoplasm might also be possible for *Avsunviroidae* (Figure 2, pathway 2). Similar to the export of duplex RNA, single-stranded PSTVd or ASBVd RNAs might be exported as well. However, given the existence of the cytoplasmic siRNAs, they would remain undetectable because of RISC-mediated

Figure 2. Replication scheme of the two major families of viroids and the possible pathways to viroid-specific short interfering RNAs (siRNAs). Potato spindle tuber viroid RNA (PSTVd) is the type species of the family *Pospiviroidae*, which replicates in the nucleus. The monomeric circular RNA assumes a rod-shaped secondary structure, although a branched left domain has also been discussed recently [94], although this does not seem to play a dominant role [95]. The circular genome (red) is transported into the nucleus and ‘transcribed’ by DNA-dependent RNA Pol II in a rolling-circle-like mechanism into an oligomeric complementary RNA that is by convention called (–) RNA (blue), even though the genomic (+) RNA does not serve an mRNA function. The oligomeric (–) RNA is again transcribed by RNA Pol II into oligomeric (+) RNA, which is then processed into the monomeric circular PSTVd unit. According to a recent model by Yijun Qi and Biao Ding [40], the oligomeric (+) RNA is also transported into the nucleolus for processing. Avocado sunblotch viroid (ASBVd) is the type species of the family *Avsunviroidae*. Members of this family have a secondary structure with a different degree of branching. ASBVd (green) replicates in the chloroplast and RNA is transcribed by the nucleus-encoded RNA polymerase. Unlike PSTVd, the oligomeric (–) strand is also converted into monomeric circular units. Therefore, the replication scheme is ‘symmetrical’. Moreover, at variance with PSTVd, both oligomeric forms of RNA [(+) as well as (–)] are cleaved autolytically via a hammerhead domain. Because of this, text books and ribozyme articles often state that ‘hammerhead ribozymes are derived from viroids’. However, such a general statement is not entirely correct because (i) the hammerhead domain is not found in the *Pospiviroidae*, which represents most viroids, and (ii) *Avsunviroidae* share the ribozyme feature with certain satellite RNAs [9], for example, the satellite of tobacco ring spot virus, which contains domains for the hammerhead and hairpin ribozymes. Satellite RNAs are also subviral RNAs but, unlike viroids, they require the RNA polymerase of a helper virus for their replication. Satellite RNAs can be linear single-stranded RNAs but some of them that assume a circular structure like viroids are called ‘virusoids’. The replication cycles of both families of viroids (not to be interpreted as a simultaneous infection in the same cell) result in the accumulation of viroid-specific siRNAs. Broken arrows indicate hypothetical pathways: the hypothetical pathways numbered 1–3 explain how siRNA might be generated from double-stranded precursors.

cleavage. In that sense, viroid-specific siRNAs might be part of a PTGS response. Owing to the lack of a protective coat, single- and double-stranded forms of *Pospiviroidae* and *Avsunviroidae* are highly vulnerable in the cytoplasm. As a consequence, they might have developed different strategies for escaping the PTGS-mediated RNA degradation by 'retreating' to either the nucleus or the chloroplast, two cellular sites, where the viroid RNA is safe. The monomeric circular RNA might be the only viroid form that can pass through the cytoplasm, a necessity for successful cell-to-cell and long-distance transport. The circular forms are not cleavable by human Dicer [77], and RISC-mediated degradation should also be impaired because the circularity in combination with the strong secondary structure should hamper strand displacement so that the siRNA within an activated RISC cannot guide hybridization to the RNA circle. However, the cytoplasmic circular RNA of both families of viroids might be converted by the RdRP into double-stranded RNAs (Figure 2, pathway 3), which are then converted into siRNAs. This would be in agreement with the postulated function of RdRP in RNA silencing – the conversion of cytoplasmic aberrant RNA into double-stranded RNA. However, viroid RNA might be an inefficient substrate for RdRP because there is evidence that viroid sequences prevent transitive silencing [78].

A low concentration of nuclear PSTVd-specific siRNA is also remarkable in view of their postulated role in heterochromatin formation [79,80]. In pioneering work, Michael Wassenegger *et al.* [81] showed that viroid RNA can direct DNA methylation of homologous sequences, and it was later found that the expression of double-stranded RNA might also result in the methylation and transcriptional inactivation of homologous promoter sequences [82] and that a DNA target of 30 bp is sufficient for RNA-directed DNA methylation [83]. However, RNA-directed DNA methylation can be initiated in 35S-GFP transgenic plants following infection with plant RNA viruses carrying GFP sequences [84]. Therefore, siRNAs that are generated in the cytoplasm might induce nuclear DNA methylation as well.

Viroid-specific siRNA might also modulate the expression of endogenous host genes via transitive silencing. In principle, a match of one of the 359 conceivable PSTVd-derived siRNAs with a host mRNA would be sufficient to downregulate the corresponding gene. This idea has been discussed in two studies [61,83] and it is now clear that the match would not need to be perfect because miRNAs with only a partial complementarity can also negatively modulate gene expression and it was shown that siRNA might enter the miRNA pathway and vice versa [85,86]. Is this the key to viroid pathogenicity? It might be part of the story because the occurrence of PSTVd-specific siRNAs just precedes the development of disease symptoms. During the review of this manuscript, Ming-Bo Wang *et al.* [87] provided experimental evidence supporting this hypothesis. They engineered transgenic tomato that expressed most (nucleotides 16–355) of the PSTVd strain RG1 sequence in the form of either a hairpin construct or a direct repeat. Because the viroid sequence is incomplete, no real infection can take place. However, the hairpin

plants generated siRNAs and induced symptoms such as abnormal, dark-green leaves, whereas the direct repeat construct was free of siRNA and of detectable abnormalities. This suggests that the PSTVd-derived siRNAs might be responsible for the occurrence of disease symptoms. Similar processes might also account for other pathogenic RNAs. This hypothesis would also predict that PTGS-deficient host plants might replicate viroids but would not react with disease symptoms. Moreover, one would postulate that the few sequence changes in a severe strain of PSTVd would generate siRNAs that would better match an unknown target gene. This hypothesis could be tested by expressing short RNA sequences of mild and severe PSTVd strains. The differential gene expression profiles of tomato plants infected with two PSTVd isolates of different pathogenicity have been analyzed at various stages of infection [88]. As a control, tomato plants infected with tobacco mosaic virus were included in the analysis. The authors identified 55 genes that were expressed differently depending on whether plants were infected with PSTVd; the genes were not expressed when infected with tobacco mosaic virus. More intriguing, this group of genes included seven that were specifically induced by the severe strain, whereas the mild strain induced one specific gene. There was no indication that gene suppression was caused by PTGS based on homology to PSTVd sequences. However, this cannot be ruled out because it might be sufficient to alter the expression of a regulatory gene whose change in titre might be undetected owing to a generally low concentration.

Future directions

The full complexity of viroids, which is squeezed into a remarkably short RNA sequence, requires further research into the interplay with their respective host factors, for example, by using knockouts of these genes. It is encouraging that a good basis for such future studies has recently been established. Further understanding of the molecular biology of viroids should parallel and mutually influence the advancement of knowledge in related plant processes, such as viral pathogenicity and mobile RNA signals. A further challenge is to find out whether it is significant that viroids and viroid-like pathogens are found in organisms (plants and mammals) in which DNA methylation plays an important regulatory role in development and environmental adaptation, and whether viroids can interfere with factors involved in chromatin modifications. Studying the strategies that viroids use to infect a plant also helps to disclose basic molecular and cellular processes. For example, the understanding of RNA-mediated chromatin remodelling is likely to lead to new prospects for antiviral therapy and gene therapy via viral vectors. It would not be the first time that research on viroids has unexpectedly generated surprises that significantly influence the general picture of cellular and molecular biology of plants and even other organisms. In the past, viroid research has resulted in the discovery of hammerhead ribozymes [9] and prompted the biochemical identification and characterization of the RdRP [59,60], which is important for RNA silencing. Viroid RNA enabled Michael Wassenegger *et al.* [89] to discover RNA-directed

DNA methylation, the first indication that RNA can direct chromatin changes. So, there is reason to assume that further surprises will follow if we analyze the remarkable abilities of viroids to survive in the complex environment of higher plants in spite of their simple RNA nature. In that sense, viroids are truly modern parasites, regardless of how ancient their origin might be.

Note added in proof

While this manuscript was in press, José-Antonio Daròs and Ricardo Flores [96] have shown that some basic steps of the replication cycle of Avsunviroidae and Pospiviroidae, such as RNA-RNA transcription and processing into the monomeric circular (+) RNA, can proceed in the non-host plant *Arabidopsis thaliana*, suggesting that deficiencies in movement limit the infectivity of viroids in the model plant. Using insertion mutants, Zhixin Xie *et al.* [97] characterized the role of different Dicer enzymes on the formation of different classes of siRNA. GFP-fusions showed that DCL2 and DCL3 are also found in the nucleus and, therefore, they are also candidate enzymes for processing nuclear double-stranded viroid RNA (Figure 2).

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