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VIROIDS AND VIROID-HOST INTERACTIONS

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■ **Abstract** Although they induce symptoms in plants similar to those accompanying virus infections, viroids have unique structural, functional, and evolutionary characteristics. They are composed of a small, nonprotein-coding, single-stranded, circular RNA, with autonomous replication. Viroid species are clustered into the families *Pospiviroidae* and *Avsunviroidae*, whose members replicate (and accumulate) in the nucleus and chloroplast, respectively. Viroids replicate in three steps through an RNA-based rolling-circle mechanism: synthesis of longer-than-unit strands catalyzed by host RNA polymerases; processing to unit-length, which in the family *Avsunviroidae* is mediated by hammerhead ribozymes; and circularization. Within the initially infected cells, viroid RNA must move to its replication organelle, with the resulting progeny then invading adjacent cells through plasmodesmata and reaching distal parts via the vasculature. To carry out these movements, viroids must interact with host factors. The mature viroid RNA could be the primary pathogenic effector or, alternatively, viroids could exert their pathogenic effects via RNA silencing.

INTRODUCTION

The closest reference term to viroid is virus, reflecting the intimate historical links between viruses, discovered at the end of the nineteenth century in plants, and viroids, also discovered in plants some 70 years later. The first viroid, *Potato spindle tuber viroid* (PSTVd) (25, 27), was identified during an attempt to characterize the virus presumed to cause a potato disease. These and subsequent results obtained for the same disease (97) as well as for another disease that was also presumed to have a virus etiology but which turned out to be induced by *Citrus exocortis viroid* (CEVd) (86, 96), established the viroid concept on solid experimental ground. Viroids and viruses cannot, in principle, be discriminated according to the

phenotypic effects that they incite in their hosts, which range from severe symptoms to latent infections. However, both entities differ radically in structure, function, and evolutionary origin, and if they are collected together in taxonomic schemes like the one endorsed by the International Committee for Taxonomy of Viruses (ICTV) (36), it is for historical and practical reasons. (Prions are also included in this scheme, even though there is an even more drastic difference between them and viruses). In this review we focus on the interactions of viroids with their host plants but first we briefly describe the properties of these unique pathogens and their classification (for a detailed coverage of different aspects of viroids, see 42).

TAXONOMY OF THE *VIROIDAE*: FAMILIES, GENERA, SPECIES

Figure 1 summarizes the structural characteristics of viroids. Because their mechanisms of replication and pathogenesis are discussed in detail below, here we only highlight other properties that distinguish them from viruses. Viroids are tiny single-stranded RNAs (246–401 nt), approximately tenfold smaller than the genome of the smallest RNA viruses; they have a circular structure and high degree of self-complementarity that promotes compact folding (87). As a functional reflection of these singular features, viroids, in contrast to viruses, do not appear to code for specific proteins (reviewed in 26), hence they must rely almost entirely on host factors to complete their infectious cycle. This means that the parasitism developed by viroids and viruses also differs: Viruses and viroids can essentially be regarded as parasites of the translation and transcriptional apparatus of their hosts, respectively. However, some viroids are catalytic RNAs and "code" for hammerhead ribozymes that mediate the self-cleavage of the multimeric RNAs generated in their replication through a rolling-circle mechanism. Apart from certain plant satellite RNAs and the RNA of human hepatitis delta virus (reviewed in 35, 101), which resemble viroids in their circular structure and rolling-circle replication mode mediated by ribozymes, no other virus-related RNAs have been characterized as catalytic RNAs. This property constitutes the strongest argument in support of the idea that viroids may have an ancient evolutionary origin independent of viruses, going back to the RNA world postulated to have preceded the present world on Earth based on DNA and proteins (reviewed in 26).

Most of the nearly 30 viroid species known (36) (Table 1) belong to the family *Pospiviroidae*, type species PSTVd (25, 41), and adopt in vitro a rod-like or quasi-rod-like secondary structure of minimal free energy (29, 87) with five structural-functional domains (52, 89) (Figure 1*a*). The central conserved region (CCR), within the C domain, is formed by two stretches of conserved nucleotides, in which those of the upper strand are flanked by an inverted repeat. Depending of the nature of the CCR, and on the presence or absence of a terminal conserved region (TCR) and a terminal conserved hairpin (TCH), members of this family are allocated to five genera (36) (Table 1). The other four viroids, *Avocado sunblotch*

TABLE 1 Viroid species with their abbreviations, accession numbers of typical sequence variants, sizes, and genus and family to which they belong

Viroid species	Abbreviation	Accession	Size (nt)	Genus	Family
Potato spindle tuber	PSTVd	V01465	359	Pospiviroid	Pospiviroidae
Tomato chlorotic dwarf	TCDVd	AF162131	360	Pospiviroid	Pospiviroidae
Mexican papita	MPVd	L78454	360	Pospiviroid	Pospiviroidae
Tomato planta macho	TPMVd	K00817	360	Pospiviroid	Pospiviroidae
Citrus exocortis	CEVd	M34917	371	Pospiviroid	Pospiviroidae
Chrysanthemum stunt	CSVd	V01107	356	Pospiviroid	Pospiviroidae
Tomato apical stunt	TASVd	K00818	360	Pospiviroid	Pospiviroidae
Iresine 1	IrVd-1	X95734	370	Pospiviroid	Pospiviroidae
Columnea latent	CLVd	X15663	370	Pospiviroid	Pospiviroidae
Hop stunt	HSVd	X00009	297	Hostuviroid	Pospiviroidae
Coconut cadang-cadang	CCCVd	J02049	246	Cocadviroid	Pospiviroidae
Coconut tinangaja	CTiVd	M20731	254	Cocadviroid	Pospiviroidae
Hop latent	HLVd	X07397	256	Cocadviroid	Pospiviroidae
Citrus IV	CVd-IV	X14638	284	Cocadviroid	Pospiviroidae
Apple scar skin	ASSVd	M36646	329	Apscaviroid	Pospiviroidae
Citrus III	CVd-III	AF184147	294	Apscaviroid	Pospiviroidae
Apple dimple fruit	ADFVd	X99487	306	Apscaviroid	Pospiviroidae
Grapevine yellow speckle 1	GVYSd-1	X06904	367	Apscaviroid	Pospiviroidae
Grapevine yellow speckle 2	GVYSd-2	J04348	363	Apscaviroid	Pospiviroidae
Citrus bent leaf	CBLVd	M74065	318	Apscaviroid	Pospiviroidae
Pear blister canker	PBCVd	D12823	315	Apscaviroid	Pospiviroidae
Australian grapevine	AGVd	X17101	369	Apscaviroid	Pospiviroidae
Coleus blumei 1	CbVd-1	X52960	248	Coleviroid	Pospiviroidae
Coleus blumei 2	CbVd-2	X95365	301	Coleviroid	Pospiviroidae
Coleus blumei 3	CbVd-3	X95364	361	Coleviroid	Pospiviroidae
Avocado sunblotch	ASBVd	J02020	247	Avsunviroid	Avsunviroidae
Peach latent mosaic	PLMVd	M83545	337	Pelamoviroid	Avsunviroidae
Chrysanthemum chlorotic mottle	CChMVd	Y14700	399	Pelamoviroid	Avsunviroidae
Eggplant latent*	ELVd	AJ536613	333	Elaviroid	Avsunviroidae

*Pending ICTV approval; whether Apple fruit crinkle and Citrus viroid original source should be considered as new viroid species of genus *Apscaviroid* is also pending.

viroid (ASBVd) (47), *Peach latent mosaic viroid* (PLMVd) (45), *Chrysanthemum chlorotic mottle viroid* (CChMVd) (65), and *Eggplant latent viroid* (ELVd) (30), do not have the conserved CCR, TCR, and TCH motifs but, remarkably, both their polarity strands self-cleave through hammerhead ribozymes; they form the second family, *Avsunviroidae* (reviewed in 33), whose type species is ASBVd (formal inclusion of ELVd in this family is pending ICTV approval) (Figure 1*b*). Apart from the core nucleotides conserved in their hammerhead structures, no extensive sequence similarities exist between them, but PLMVd and CChMVd are grouped in one genus because of their branched secondary structure (21, 45, 65), which is stabilized by a pseudoknot (10; S. Gago, M. De la Peña & R. Flores, unpublished results) (Figure 1*b*), and their insolubility in 2 M LiCl (65). ASBVd, the only viroid with a high A + U content (62%) (47), forms a monospecific genus, and ELVd, whose properties fall between those of the members of the other two genera, has been proposed to constitute its own genus (30). This classification scheme is further supported by phylogenetic reconstructions with entire viroid sequences (36) and by the different subcellular replication (and accumulation) sites of the type members of both families, with available data indicating that in this respect other viroids behave like their corresponding type species. Within each genus, the criteria to demarcate viroid species are an arbitrary level of below 90% sequence similarity and distinct biological properties. Viroids, like viruses, propagate in their hosts as populations of closely related sequence variants (quasi-species), although one or more may predominate in the population. Heat stress may significantly alter the structure of viroid quasi-species (62). Some viroid variants with minor changes affecting certain regions are directly related to specific diseases (57, 71, 83) or to dramatic alterations in symptom severity (21, 93, 103).

BIOLOGY

Host Range and Host Specificity

Viroids are the etiologic agents of a number of diseases affecting economically important herbaceous and ligneous plants including potato, tomato, cucumber, hop, coconut, grapevine, several subtropical and temperate fruit trees (avocado, peach, apple, pear, citrus, and plum), and some ornamentals (chrysanthemum and coleus). *Coconut cadang-cadang viroid* (CCCVd) and *Coconut tinangaja viroid* (CTiVd) infect monocotyledons, whereas the others infect dicotyledons. Some viroids, among which the most instructive example is *Hop stunt viroid* (HSVd), have wide host ranges but others, exemplified by those forming the family *Avsunviroidae*, are mainly restricted to their natural hosts. A single nucleotide substitution converts PSTVd from noninfectious to infectious for *Nicotiana tabacum* (108).

Although most viroids are transmitted mechanically and some through seed or pollen, with only *Tomato planta macho viroid* (TPMVd) known to be aphid-transmissible under specific ecological conditions, the most efficient transmission

route for viroids is vegetative propagation of infected material. This explains why certain grapevine and, specifically, citrus cultivars propagated on infected cultivars or rootstocks contain complex mixtures of different viroids.

Symptoms and Ultrastructural Effects

Some viroids have destructive consequences, as illustrated by CCCVd that has killed millions of coconut trees in the Philippines, whereas others affect leaves (chlorosis in spots or covering the whole blade, epinasty, rugosity, and necrosis), stems (pitting, internode shortening, and dwarfing), bark (scaling, cracking, cankers), flowers (size reduction, broken lines on petals), fruits (discolorations and skin deformations, suture cracking), seeds (enlarged stones), and reserve organs (malformations), as well as less conspicuous effects including delays in foliation, flowering and ripening, and growing pattern (open habit) of mature trees (Figure 2). Certain viroids only induce symptoms in a particular organ (bark or fruit), whereas others have more general effects. Infections caused by a few viroids result in very mild or no symptoms. Absence of symptoms is common in naturally infected wild plants, which can act as reservoirs. Symptom expression is generally favored by high light intensity and, particularly, high temperature. Viruses, by contrast, have a broader range of environmental conditions for optimal symptom expression. These differences may explain why viroids mainly affect crops grown in tropical or subtropical areas (and in greenhouses), and also why curing some viroid infections is recalcitrant to thermotherapy.

Ultrastructural studies on the cytopathic effects induced by members of the family *Pospiviroidae* have shown paramural bodies known as plasmalemmasomes and aberrations of the thylakoid membranes of chloroplasts (94). Parallel studies with members of the family *Avsunviroidae* have revealed grossly disorganized chloroplasts and membranous bodies in the yellow regions of ASBVd-infected avocado leaves, while in completely chlorotic ("bleached") leaves some chloroplasts looked similar to proplastids (23). This latter observation has been reproduced in PLMVd-infected leaves displaying extensive chlorosis (peach calico), which in the most dramatic instances covers most of the leaf area (Figure 2*h*) (M.E. Rodio, A. Destradis, R. Flores & F. Di Serio, unpublished results).

Cross-Protection

The phenomenon of cross-protection refers to observations that the ability of members of both families to infect a host may be influenced by previous infections by other strains of the same or closely related viroid (53, 68). More specifically, when a plant pre-infected with a mild viroid strain is challenge-inoculated with a severe strain of the same viroid, the typical symptoms of the second strain and the accumulation level of its RNA are blocked or attenuated for a certain time. On this basis, even before PSTVd, PLMVd, and CChMVd were identified as viroids, the existence of mild or nonsymptomatic strains thereof was postulated and used to develop cross-protection bioassays. It was also suggested that the nature of

CChMVd was uncharacteristic (68). The mechanisms underlying cross-protection have not been determined but may be related to RNA silencing (see below).

REPLICATION

Circular and Oligomeric RNA Templates: Support for a Rolling-Circle Mechanism

On finding that in PSTVd-infected tomato the most abundant viroid circular RNA, arbitrarily considered as having (+) polarity, is accompanied by oligomeric (−) RNAs, it was proposed that the latter were replicative intermediates resulting from reiterative transcription of the former (6). The existence of CEVd (−) RNA sequences in infected *Gynura aurantiaca* (40) had already indicated that viroid replication was an RNA-based process and undermined the idea of participation of DNA intermediates. Moreover, differential centrifugation studies showing that PSTVd (24) and its complementary strands (99) accumulate in the nucleus strongly suggested the involvement of a nuclear RNA polymerase in replication. Re-examination of this question with PSTVd and other members of its family using finer approaches (in situ hybridization and confocal laser scanning and transmission electron microscopy) confirmed (5, 44) and extended these observations (81).

In contrast, parallel experiments first with ASBVd (4, 56, 63) and then with PLMVd (9) showed the preferential accumulation of their (+) and (−) strands in the chloroplast, indicating involvement of the enzymatic machinery of this organelle in the replication of members of the family *Avsunviroidae*.

Rolling-Circle Mechanism: Asymmetric and Symmetric Pathways

Figure 3 displays the two alternative pathways: the asymmetric, first proposed to account for replication of PSTVd (6, 7, 31) and HSVd (48), and the symmetric, initially advanced on theoretical grounds (6) and then experimentally supported for ASBVd (18, 46) and PLMVd (9). Other members of both families appear to replicate following the same pathway as their type species. The difference discriminating both pathways is the (−) template: The monomeric (−) circular RNA has been detected in ASBVd-infected avocado (18, 46) and PLMVd-infected peach (9), but not in PSTVd-infected tissues (6, 7, 31), or in electroporated protoplasts (79) in which oligomeric (−) strands accumulate. Thus, cleavage and ligation occur in (+) and (−) strands in the symmetric pathway with two rolling circles, but only in (+) strands in the asymmetric pathway with a single rolling circle.

The three catalytic activities required—RNA polymerase, RNase, and RNA ligase (Figure 3)—were initially presumed to reside in host proteins. However, in the family *Avsunviroidae*, the second activity is now known to be mediated by hammerhead ribozymes embedded in both polarity strands, a finding with far-reaching implications. In the context of the first activity, the question of how

viroids redirect the template specificity of certain host DNA-dependent RNA polymerases to transcribe RNA is one of the most interesting challenges unresolved.

RNA Polymerization, Cleavage, and Ligation: Role of Enzymes and Ribozymes

In light of recent reviews (34, 100), here we summarize only the main features, novel data, and some unresolved issues regarding RNA polymerization, cleavage, and ligation. In vivo and in vitro transcription analyses in the presence of α-amanitin indicate that RNA strand elongation in representative members of the family *Pospiviroidae* is abolished by nanomolar concentrations of this fungal toxin, which typically inhibit the nucleoplasmic RNA polymerase II (32, 64, 91). In line with this view, a chromatin-enriched fraction from CEVd-infected tomato, purified by affinity with a monoclonal antibody to the carboxy-terminal domain of the largest subunit of RNA polymerase II, has been shown to contain the viroid (+) and (−) strands (106). Similar analyses in the presence of tagetitoxin support the role of a nuclear-encoded chloroplastic RNA polymerase (NEP), or another polymerase resistant to this bacterial inhibitor, in ASBVd strand elongation (67). In vitro studies with PLMVd and RNA polymerase of *Escherichia coli* suggest the participation of the eubacterial-like plastid-encoded polymerase (PEP) (75), although this is a nonphysiological system. Moreover, synthesis (and accumulation) of PLMVd is particularly active in areas exhibiting peach calico, in which development of proplastids into chloroplasts and processing of chloroplastic rRNA precursors (and, consequently, translation of plastid-encoded proteins) appear to be impaired (M.E. Rodio, R. Flores & F. Di Serio, unpublished results). These latter observations are more consistent with the involvement of a NEP-like enzyme in PLMVd replication and suggest that other chloroplastic factors mediating this process (see below) are also nuclear-encoded.

To determine whether initiation of viroid RNAs is site-specific (promoter-driven) or occurs at random (also allowing complete transcription of the circular template), data obtained by in vitro capping with [α^{32}P]GTP and guanylyltransferase, which specifically labels the free 5′-triphosphate group characteristic of chloroplastic primary transcripts, and RNase protection assays have mapped the initiation of ASBVd (+) and (−) RNAs isolated from infected avocado at similar A + U-rich terminal loops in their predicted quasi-rod-like secondary structures (66). Primer-extension analysis of the 5′ termini of PLMVd subgenomic RNAs from infected peach, presumed to be replication by-products, and in vitro transcription studies with truncated PLMVd RNAs and RNA polymerase of *E. coli* suggest that the initiation sites of this viroid also map at terminal loops (75). However, in vitro capping studies to map the 5′-triphosphate termini of PLMVd (+) and (−) linear RNAs isolated from infected tissue indicate alternative initiation sites (S. Delgado, A.E. Martínez de Alba, C. Hernández & R. Flores, unpublished results). Still controversial are the initiation sites in the family of nuclear viroids

(*Pospiviroidae*), an issue that has been addressed by in vitro transcription of the PSTVd monomeric (+) circular RNA with a nuclear extract from potato or with purified RNA polymerase II from wheat germ and tomato (100).

Correct processing to the monomeric (+) circular forms has been demonstrated through assays in vitro with potato nuclear extracts and longer-than-unit (+) PSTVd RNAs (2, 102) and in vivo with *Arabidopsis thaliana* transformed with cDNAs expressing dimeric (+) transcripts of five representative members of the family *Pospiviroidae* (17). These results indicate that the RNase activity catalyzing cleavage of longer-than-unit (+) strands is a host enzyme(s) whose site-specificity is determined by a particular RNA folding. Specifically, cleavage of PSTVd (+) strands is proposed to be driven by a branched structure with a GNRA tetraloop, which subsequently switches to an extended conformation with an E loop promoting ligation (2). However, this mechanism may not apply to other members of the family *Pospiviroidae* that are unable to form the GNRA tetraloop and the loop E. On the other hand, it has been found that a dimeric (−) transcript of HSVd expressed transgenically in *A. thaliana* fails to be processed (17) and that in infected cultured cells and plants PSTVd (−) strands accumulate in the nucleoplasm, whereas the (+) strands are localized in the nucleolus as well as in nucleoplasm (Figure 3) (see below). Such findings suggest that viroid (+) strands are processed in the nucleolus where processing of the rRNA and tRNA precursors also occurs. Which factors determine this differential traffic of viroid (+) and (−) strands remain an intriguing issue.

In members of the family *Avsunviroidae*, however, the RNase activity is due not to an enzyme but to a hammerhead ribozyme, a small RNA motif embedded in both polarity strands that, through a transesterification in the presence of Mg^{2+}, self-cleaves at a specific phosphodiester bond producing 5′-OH and 2′,3′-cyclic phosphodiester termini (47, 78; reviewed in 35). Hammerhead ribozymes are formed by a central core of conserved sequences flanked by three double-stranded regions with loose sequence requirements that are capped by loops. X-ray crystallography has revealed a complex array of non-canonical interactions between the nucleotides of the central core, explaining why they are strictly conserved in natural ribozymes and illustrating that the actual shape does not resemble a hammerhead but rather an inverted Y in which the stems III and II are almost co-linear (Figure 1*b*, inset). There is firm evidence supporting the proposal that hammerheads mediate the in vivo self-cleavage of the multimeric viroid RNAs wherein they are inserted, and that the reaction is under strict control through a conformational switch between the active ribozyme, which is transiently formed during replication, and an alternative folding that promotes ligation (34). In contrast to the accepted view, recent data show that modifications of loops 1 and 2 of natural hammerheads induce a severe reduction in their catalytic activity, indicating that these peripheral regions play a critical role in catalysis through tertiary interactions between some of their nucleotides that may favor the active site at the low magnesium concentration existing in vivo (20, 54) (Figure 1*b*, inset). These interactions could be stabilized by chloroplastic proteins behaving as RNA chaperones, which would explain why they facilitate the hammerhead-mediated self-cleavage of a viroid RNA (16).

Data on the third replication step are limited, but in PSTVd and nuclear viroids the reaction is presumably catalyzed by a host enzyme similar to the wheat germ RNA ligase. Support for this hypothesis comes from data in vitro showing that this enzyme mediates circularization of the monomeric linear PSTVd RNA isolated from infected tissue (8) and that a potato nuclear extract can process longer-than-unit PSTVd RNAs to the monomeric circular forms (2, 102), and in vivo indicating that *A. thaliana* seems to have a similar enzyme able to circularize the monomeric linear forms of representative members of the family *Pospiviroidae* (17). Still unclear is whether a chloroplastic RNA ligase exists for members of the family *Avsunviroidae* or, alternatively, the reaction is autocatalytic. Support for this latter view is based on the in vitro self-ligation of the monomeric linear PLMVd RNAs resulting from hammerhead-mediated self-cleavage, which mostly leads to 2′′,5′′- instead of the conventional 3′,5′-phosphodiester bonds (14) and the proposal that these atypical bonds are present in circular PLMVd RNAs isolated from infected tissue and impede their in vitro self-cleavage (15). In line with these results, early in vitro studies with RNA of human hepatitis delta virus (HDV) which, like viroids, also replicates through a rolling-circle mechanism mediated by ribozymes (albeit of a non-hammerhead class), showed ligation of ribozyme-cleaved sequences in protein-free conditions (reviewed in 101). However, these conditions were far from physiological, and more recent studies have shown that a host-specific function is needed for ligation of the RNAs resulting from cleavage by a wide variety of ribozymes, which include hammerhead and HDV ribozymes (84). Furthermore, the hammerhead-generated 5′-OH and 2′,3′-cyclic phosphodiester termini are those typically required by a wheat germ-like RNA ligase, a feasible alternative to self-ligation and, in contrast to a previous report (15), the circular PLMVd RNAs isolated from infected tissue appear to self-cleave in vitro (S. Delgado, C. Hernández & R. Flores, unpublished results).

MOVEMENT

Viroid movement received scant attention in early studies on these peculiar pathogens but an increasing number of recent reports are now elucidating the mechanisms and putative host factors involved in this step of the viroid infectious cycle. This area has been further promoted by the possible relevance of these results to the general processes of RNA transport in plants, including mRNAs and mobile silencing signals (see below). In contrast to viruses, which encode their own movement proteins, viroids must interact directly with host factors for mobility. Viroids therefore offer a unique system to dissect RNA traffic in plants.

Intracellular Movement

After entering a cell, the viroid RNA must move to its replication site, either the nucleus or the chloroplast, to generate the progeny for release to the cytoplasm to invade neighboring cells. Import of fluorescein-labeled PSTVd transcripts into

the nucleus has been studied in permeabilized tobacco cells and shown to be a cytoskeleton-independent process mediated by a specific and saturable receptor (109). This process was demonstrated to be sequence or structure specific because no import was observed for mRNA fragments of the same size or for two viroids that replicate and accumulate in the chloroplast. These results were validated in planta with an RNA virus vector expressing a green fluorescent protein (GFP) reporter gene bearing an intron that was either unmodified or contained an embedded full-length PSTVd copy. Inoculation with these cytoplasmic replicating viral constructs caused fluorescence only with the PSTVd-containing vector, indicating that the viroid sequence targeted the recombinant RNA to the nucleus where the intron was removed, with the spliced mRNA returning to the cytoplasm where it was translated (110).

Overall, these data suggest that nuclear import of PSTVd results from interaction of a viroid sequence or structural motif with cellular factors that may lead to the formation of a ribonucleoprotein complex, which would shuttle the viroid to the nucleus. Pertinent to this point is the identification of a bromodomain-containing protein from tomato that binds specifically PSTVd in vitro and in vivo (61). This Viroid RNA-binding Protein 1 (VirP1), which was isolated from an RNA-ligand screening through its ability to interact with PSTVd (+) RNA, is a member of a family of transcriptional regulators associated with chromatin remodeling. As expected, VirP1 contains a nuclear localization signal and is a candidate to mediate PSTVd transfer (and that of other related viroids) into or out of the nucleus. Further work has mapped the RNA motif responsible for the specific interaction between VirP1 and PSTVd to an asymmetrical internal loop (termed RY motif owing to its base composition), within the right terminal domain, which is present twice in this viroid and in most members of the genus *Pospiviroid*, once in HSVd, and is partially conserved in the genus *Cocadviroid* (39, 58). Mutations in any of the two RY motifs of PSTVd abolished infectivity, a finding that supports its biological relevance.

The general picture of the intracellular viroid movement becomes more complex when analyzed at the organelle level. Using improved sample preparation and in situ hybridization, it has recently been shown that the PSTVd (−) strand localizes in the nucleoplasm of infected tomato and *N. benthamiana* plants or cultured cells, whereas the (+) strand localizes in the nucleolus as well as in the nucleoplasm (Figure 3), with distinct spatial patterns that may represent successive stages of the viroid RNA migration or processing (81). These results suggest that after synthesis of PSTVd (+) and (−) RNAs in the nucleoplasm, the latter stays anchored to this compartment while the (+) strand is transported to the nucleolus. This finding points to the existence of highly specialized machinery in eukaryotic cells able to discriminate the opposite strands of an RNA and may have implications in gene regulation and pathogen infection.

As regards intracellular movement in members of the family *Avsunviridae*, the transfer mechanism to the chloroplast has yet to be investigated. Novel transport pathways may well be discovered in plant cells, given that no other alien or cellular RNAs have been reported to traffic inside this organelle.

Cell-to-Cell Movement

Once it has infected the first cells, a viroid has to colonize adjacent cells prior to invading the more distal plant parts. Proteins and nucleic acids, either endogenous or of viral origin, have been reported to move cell-to-cell via the plasmodesmata (PD), the plant organella providing cytoplasmic connections between cells. Studies with PSTVd suggest that viroids follow the same pathway for intercellular movement (28). PSTVd was retained when microinjected into symplasmically isolated guard cells of mature tomato and tobacco leaves; however, when injected into symplasmically connected mesophyll cells, the viroid moved quickly from cell to cell. Remarkably, the fusion to PSTVd aided the transport through PD of an otherwise nonmobile RNA, suggesting that the viroid contains a motif for PD transport.

Long-Distance Movement

Systemic spread of viroids occurs through the vasculature and allows their access to tissues far away from those initially infected. As in viruses, this transport involves loading the viroid into the phloem and follows the flow of photoassimilates from the photosynthetic source to sink tissues/organs of the plant (72, 111). Evidence of this movement at the cellular level has been provided for PSTVd by in situ hybridization, which also showed that trafficking within the phloem is probably sustained by viroid replication and tightly regulated by plant developmental and cellular factors (111). The viroid was not detected in shoot apical meristems (SAM) of infected *N. benthamiana* or tomato plants, whereas it was present in the vascular tissues (most probably the procambium and/or protophloem) below the SAM, suggesting that PD at some cellular boundaries between the SAM and the rest of plant body restrict PSTVd trafficking into the SAM. Moreover, PSTVd was absent in developing flowers, whereas mature flowers contained the viroid in parenchyma cells of sepals but, intriguingly, not in petals, stamens, styles, or ovaries, although the phloem connections were already established. The absence of PSTVd in some floral organs of *N. benthamiana* may be attributable to restricted traffic in these organs and not to replication suppression (112). This restriction could be nonoperative under certain conditions because PSTVd is seed-transmissible in tomato and therefore able to infect ovules and/or pollen eventually. In contrast to the strong vascular tropism that PSTVd shows below the SAM in tomato, PLMVd appears able to invade cell layers very close to the SAM in peach (M.E. Rodio, S. Delgado, M.D. Gómez, R. Flores & F. Di Serio, unpublished results), suggesting that different mechanisms regulate movement in the two viroid families.

Viroid translocation via the phloem is most probably facilitated by host proteins. Evidence has been obtained in vitro and in vivo of the formation of a ribonucleoprotein complex between HSVd and one of the most abundant phloem polypeptides of cucumber, the dimeric lectin known as Phloem Protein 2 (PP2) (37, 38, 69). This protein fulfils the requirements of an RNA chaperone protein involved in systemic movement of HSVd: RNA-binding activity probably mediated

by a double-stranded RNA binding motif, ability to interact with PD and increase their exclusion limit, and competence for long-distance translocation. Specifically, this protein is able to move and aid HSVd movement through intergeneric grafts involving a host rootstock and a nonhost scion, although the viroid remains largely confined within the vascular tissue of the scion, suggesting that additional host factors are required for HSVd to leave the phloem efficiently (38). Indeed, phloem entry and exit are probably mediated by different motifs, as revealed by the identification of two PSTVd mutants able to enter and replicate in the phloem of *N. tabacum* but unable to leave the vascular tissue (112). The same viroid variants infected all cells of the upper uninoculated leaves in *N. benthamiana*, indicating that the regulation mechanisms of phloem-mediated RNA traffic may differ in distinct plant species. Moreover, in PSTVd, a motif has recently been identified that mediates trafficking from the bundle sheath into the mesophyll but not in the opposite direction (82).

It has been suggested that VirP1 from tomato, like PP2 from cucumber, is involved in the systemic spread of PSTVd in this host, given that a PSTVd mutant defective for VirP1 binding is unable to spread systemically (58). However, in contrast to PP2, the ability of VirP1 to interact with PD and move long distance has yet to be proved. In conclusion, viroids must have evolved to exploit existing transport routes and, given their peculiar characteristics, one may expect these pathogens to be instrumental in elucidating structural traits, mechanisms, and cellular factors that mediate RNA traffic in plants.

PATHOGENESIS

Lacking protein-coding capacity, viroids must incite disease by direct interaction of their genomic RNA or derivatives thereof with host factors (proteins or nucleic acids). This primary interaction triggers a cascade of events, still poorly understood, that eventually lead to macroscopic symptoms.

Is the Mature Viroid RNA the Direct Pathogenic Effector?

In PSTVd, mutations of 3–4 nucleotides, with marked effects on symptoms, have been mapped at a virulence-modulating (VM) region that overlaps a premelting (PM) region within a domain of the rod-like structure (93). Because a similar situation was observed in CEVd (103), this domain was termed pathogenic (P) (52) (Figure 1*a*). Although an inverse correlation was found between the thermodynamic stability of the PM region and virulence in tomato for some PSTVd strains (93), other data do not support this correlation (70). Alternatively, from comparisons of the most stable secondary structures of PSTVd variants of different pathogenicity and considerations about bending of RNA helices, it has been advanced that major differences in their VM region geometry and concomitant alterations in RNA-protein interactions are the primary cause of viroid pathogenicity (70, 92). In this context, a correlation between the virulence of PSTVd strains and

the activation of certain protein kinases has been reported (reviewed in 26). It is also possible that differential interactions with host proteins involved in viroid replication, movement, or accumulation could be the initial event in viroid pathogenesis (Figure 4). Moreover, other data indicate the contribution of additional domains to symptom expression (89), including the C domain, in which a specific nucleotide (A257) in PSTVd induces severe growth stunting and flat top of the tomato shoot (80). Pathogenicity determinants outside the P domain have also been identified in other members of the family *Pospiviroidae* such as CCCVd (85) and HSVd (71, 83).

Within the family *Avsunviroidae*, motifs involved in pathogenesis have also been recognized. ASBVd variants slightly diverging in the poly-A right terminal loop have been associated with different leaf symptoms (95), although difficulties inherent to bioassays in avocado have precluded a direct analysis. In CChMVd, a tetraloop in the in vivo branched RNA conformation has been mapped as the major pathogenicity determinant because site-directed mutagenesis and bioassays have shown that the change from UUUC to GAAA converts a variant from severe into latent without altering the final viroid titer (19, 21). A similar methodology was used to demonstrate that PLMVd variants containing a 12–13-nt insertion incite peach calico. This insertion is always found in the same position, has limited sequence variability, and folds itself into a hairpin (57). Moreover, the pathogenicity determinant might be restricted to the U-rich tetraloop of this hairpin. Although the precise role of this loop in symptom development is not known, electron microscopy observations have revealed that chloroplast differentiation is blocked in symptomatic tissues (M.E. Rodio, S. Delgado, R. Flores & F. Di Serio, unpublished results). The identification of pathogenicity determinants with a similar structure (a U-rich tetraloop) in CChMVd and PLMVd is intriguing.

Instead of proteins as the primary host target, base-pair interactions between PSTVd and host RNAs, resulting in interference with rRNA maturation, mRNA splicing, or 7S RNA assembly into the signal recognition particle, were proposed as possible molecular events initiating pathogenesis (reviewed in 26). Although these hypotheses fail to explain the differential symptoms induced by the same viroid variant on closely related species (26), the observation that the PSTVd (+) strand is able to migrate to the nucleolus and cause a redistribution of the small nucleolar RNAs has led to the suggestion that PSTVd could incite deleterious effects on rRNA processing and related events by competing for certain nucleolar factors (81).

Early studies on the signal-transduction cascade leading to symptom expression showed that viroids activate a general plant defense system based on pathogenesis-related (PR) proteins. This system is also induced by other pathogens including viruses, bacteria and fungi, which modify the concentrations of certain hormones and metabolites such as polyamines (13). More recently, a macroarray-based approach has shown that PSTVd infection alters the gene expression pattern in tomato and that the regulation of specific genes, which are not involved in the host reaction

to other pathogens, may be affected in a strain-dependent manner (50). The role of these genes in pathogenesis is unknown, except in tomato plants infected with a PSTVd strain with the U257A substitution; here the stunted growth is caused by restricted cell expansion, but not cell division or differentiation, and has been correlated with the down-regulated expression of an expansin gene, LeExp2 (80).

Although it is generally accepted that viroid diseases are induced by specific interference with regulation of host gene expression, many questions about the underlying mechanism remain unanswered. The discovery that a group of small antisense RNAs, namely the small interfering RNAs (siRNAs) and the microRNAs (miRNAs) (reviewed in 1, 11), mediate a regulatory layer of host gene expression in eukaryotes has led to new attractive hypotheses for viroid pathogenesis.

Do Viroids Exert their Pathogenic Effects via Specific siRNAs?

miRNAs (21–24 nt long) negatively regulate the expression of genes involved mainly in development (11). They are generated by an enzyme of the RNase III class (Dicer) (3), Dicer-like (DCL) in plants, acting on miRNA precursors, which are endogenous nonprotein-coding transcripts adopting a hairpin conformation with partially double-stranded regions. Depending on the degree of sequence complementarity with their cognate mRNAs, miRNAs guide the RNA-induced silencing complex (RISC) (43) to degrade the target RNA or, less frequently in plants, to repress its translation. Plant siRNAs (21–26-nt long) are associated with both transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS) (reviewed in 1). TGS controls gene expression negatively by siRNA-directed DNA methylation of certain promoters, a process that is linked to histone modification in plants. PTGS, a sequence-specific RNA-degradation mechanism, was first identified in plants as a defense response against invading RNAs from transgenes, transposons, and viruses (reviewed in 1, 55). siRNAs play a key role in this system because, like miRNAs, they are loaded into RISC and guide it to degrade specific RNAs. siRNAs also resemble miRNAs in that they are generated by Dicer, although acting on double-stranded RNA (dsRNA) in this case. Most transgenic and transposon dsRNAs derive from transcription of inverted repeats in the nucleus, with other dsRNAs that accumulate in the cytoplasm resulting from replication of single-stranded RNA (ssRNA) viruses. To counteract this antiviral defense, plant viruses code for specific proteins, called silencing suppressors, that inhibit PTGS (reviewed in 55).

The possibility that viroids may influence host gene expression at both post-transcriptional and transcriptional levels and, indirectly, induce symptoms (see below) is supported by the recent identification of viroid-specific siRNAs in plants infected by members of the families *Pospiviroidae* (49, 59, 73) and *Avsunviroidae* (59, 60), together with the previous finding that replicating PSTVd induces de novo methylation of PSTVd sequences transgenically inserted in the plant genome (107). Where and how these viroid-specific siRNAs are generated is unresolved. Since DCL isoenzymes with nuclear localization have been described (reviewed

in 1) (Figure 4), a nuclear origin for the siRNAs in the family *Pospiviroidae* seems plausible. In contrast, because no chloroplastic DCL has been reported, siRNA generation in the cytoplasm during viroid cell-to-cell movement seems more likely in the family *Avsunviroidae.* This possibility could also apply to nuclear viroids, given that PSTVd-derived siRNAs accumulate in the cytoplasm (22) (Figure 4). Regarding the templates, the primary candidates for siRNA generation in the family *Pospiviroidae* are the nuclear dsRNA intermediates of viroid replication. However, the mature genomic forms (structurally similar to miRNAs precursors) could also be degraded by DCL-1 in the nucleus or, alternatively, serve as templates for the synthesis of secondary dsRNAs catalyzed by a cytoplasmic RNA-dependent RNA polymerase (RdRp) (90) (Figure 4). Indeed, although PSTVd and ASBVd monomeric forms are resistant in vitro to human Dicer (12), they appear to be sensitive to wheat germ DCL (A.E. Martínez de Alba and R. Flores, unpublished results). In this framework, direct characterization of the small RNAs derived from replicating PSTVd indicates that they are predominantly of the (+) polarity resembling, as a population, miRNAs rather than siRNAs (B. Ding, personal communication).

Are viroid-specific siRNAs relevant in pathogenesis or are they just by-products of the RNA silencing machinery? The in vivo concentration of these siRNAs cannot explain differences in symptom development because their levels are essentially the same in infections induced by severe, mild, or latent strains of PSTVd and CChMVd (60, 73). However, an inverse correlation exists in chloroplastic viroids between the accumulation of genomic viroid forms and their corresponding siRNAs: the in vivo PLMVd and CChMVd concentrations are low but their siRNAs are easily detectable, whereas in tissues accumulating a very high ASBVd concentration the corresponding siRNAs are undetectable (60) or accumulate poorly (59). This finding is consistent with the siRNA participation in a PTGS defense response of certain hosts aimed at attenuating the in vivo viroid titer (60); this response should take place in the cytoplasm where RISC is assumed to act. Other data support this view indirectly. First, PSTVd infection of transgenic plants, expressing a reporter gene 3′ fused to partial-length PSTVd sequences, activates degradation of the recombinant transcript (and methylation of the nuclear transgenic viroid DNA) (104), probably via the siRNAs that result from PSTVd replication. Second, preliminary observations indicate that the infectivity of some viroids is reduced when they are coinoculated with an excess of their cognate dsRNAs (A. Carbonell, S. Gago & R. Flores, unpublished results), suggesting that the latter are processed by DCL in vivo and the resulting siRNAs target the genomic forms for RISC-mediated degradation (Figure 4). And third, accumulation of PSTVd-specific siRNAs precedes tomato plant recovery from severe symptoms of PSTVd infection (88). These results argue against the proposal that viroids are resistant to RNA silencing–mediated degradation and might have evolved their compact secondary structure to escape this host defense pathway (105). Such folding might also have appeared to provide resistance against RNases or certain inactivating proteins that target RNA single-stranded regions (74).

However, some viroid-specific siRNAs might also direct host DNA methylation (76) or act like endogenous miRNAs targeting host mRNAs for degradation (59, 73, 105) (Figure 4). Supporting this idea, transgenic tomato plants expressing inverted repeats of an almost complete (noninfectious) PSTVd sequence showed symptoms similar to, though milder than, those induced by infectious PSTVd RNA. These transgenic plants accumulated PSTVd-derived siRNAs, whereas neither siRNAs nor typical symptoms were detected in plants expressing direct repeats of the same viroid sequence (105). However, the effects of expressing sequences from a mild PSTVd strain were not determined, very few small RNAs derived from replicating PSTVd correspond to domains responsible for symptom expression (B. Ding, personal communication) and, most important, the predicted targeted host mRNAs remain untested. Furthermore, on a genome-size basis, the probability of generating siRNAs complementary to host endogenous mRNAs is significantly higher for RNA viruses than for viroids. Although virus-derived siRNAs and virus-induced gene silencing have been observed in plant virus infections (reviewed in 1, 55), no endogenous mRNA targets of virus derived-siRNAs have been identified; the miRNAs encoded by the genomic DNA of an animal virus (77) are bona fide miRNAs and not siRNAs acting as miRNAs. Symptom induction by plant viruses can be partially explained by the interference of viral protein suppressors with miRNA-regulated pathways (51). Inasmuch as most plant viruses have evolved PTGS suppressors as a counter defense strategy (reviewed in 55), how do viroids cope with RNA silencing? Protection could be afforded by their compact conformation (105), but only partially (see above), or by compartmentation in organella or association with proteins. Alternatively, they could interfere with the host inborn immune system because viroid infection has been associated with induced tolerance to a subsequent infection by a fungus (98).

In summary, viroid titer could be regulated by the concerted action of DCL and RISC, with DCL processing the genomic viroid RNA (or its replicative intermediates) and the resultant siRNAs priming RISC and targeting additional viroid RNAs for degradation (Figure 4). A similar mechanism could account for the cross-protection phenomena observed in both families (19, 53, 68), with the siRNAs from the first inoculated strain targeting the RNA of the challenging strain. Whether viroid-specific siRNAs also target degradation of endogenous mRNAs (PTGS) or methylation of their promoters (TGS), causing disease through this route, remains an intriguing but unproved possibility. In the meantime, the alternative view that the genomic viroid RNA is the primary pathogenic effector must be also entertained.

CONCLUDING REMARKS AND PERSPECTIVES

Research into the structure and replication of viroids has uncovered a subviral world and established what, in a certain way can be regarded as the lowest step of complexity, and probably one of the oldest on the biological scale ("the frontier

of life"). Additionally, it has led to discoveries transcending virology and the plant world: the hammerhead ribozyme, with deep functional, biotechnological, and evolutionary repercussions (47, 78; reviewed in 35), and RNA-dependent DNA methylation (107) that mediates transcriptional silencing. This illustrates, once again, how unveiling apparently marginal details of a specific system can illuminate far-reaching questions. But this may not be the end. Recently, viroids have become a tool for dissecting aspects of transcriptional and posttranscriptional gene silencing, as well as for understanding how minimal RNAs can elicit disease (reviewed in 26, 34, 100). Moreover, viroids are being used as probes to study the factors governing the intracellular, cell-to-cell, and long-distance movement of RNAs (81, 109, 111, 112). Within this context, studies on chloroplast viroids could elucidate how a foreign RNA manages to cross the membrane surrounding this organelle. Last but not least, whether viroids exist out of the plant kingdom is still a challenging and exciting question. After all, since viruses were initially found in plants and subsequently in most types of other organisms, why should viroids be restricted to the green world?

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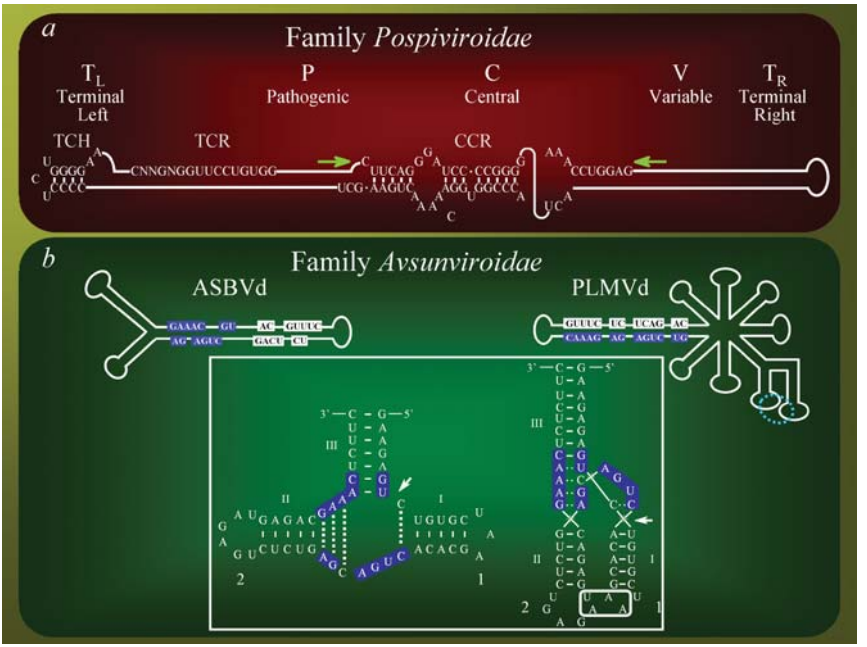

Figure 1 Structural features of viroids. (*a*) Rod-like secondary structure of members of the family *Pospiviroidae*. Domains C (*central*), P (*pathogenic*), V (*variable*), and T_L and T_R (*terminal left and right*, respectively) are indicated, as well as the CCR (*central conserved region*, here displayed for the genus *Pospiviroid*), TCR (*terminal conserved region*, present in the genera *Pospi-* and *Apscaviroid*, and in the two largest members of the genus *Coleviroid*) and TCH (*terminal conserved hairpin*, present in the genera *Hostu-* and *Cocadviroid*). Arrows indicate flanking sequences that together with the upper CCR strand form a hairpin, and the S-shaped line connects the residues linked after UV irradiation as a consequence of forming part of the loop E. (*b*) Quasi-rod-like and branched secondary structures of ASBVd and PLMVd, respectively (family *Avsunviroidae*). Sequences conserved in most natural hammerhead structures are shown within boxes with blue and white backgrounds for (+) and (−) polarities, respectively. Broken oval in PLMVd denotes a kissing-loop interaction. (*Inset*) PLMVd (+) hammerhead structure represented according to the original scheme (*left*) and to X-ray crystallography data obtained with artificial hammerhead structures (*right*), in which a proposed tertiary interaction between loops 1 and 2 enhancing the catalytic activity is indicated. Nucleotides conserved in most natural hammerhead structures are depicted as above. Arrows mark the self-cleavage site, and continuous and broken lines denote Watson-Crick and noncanonical pairs, respectively.

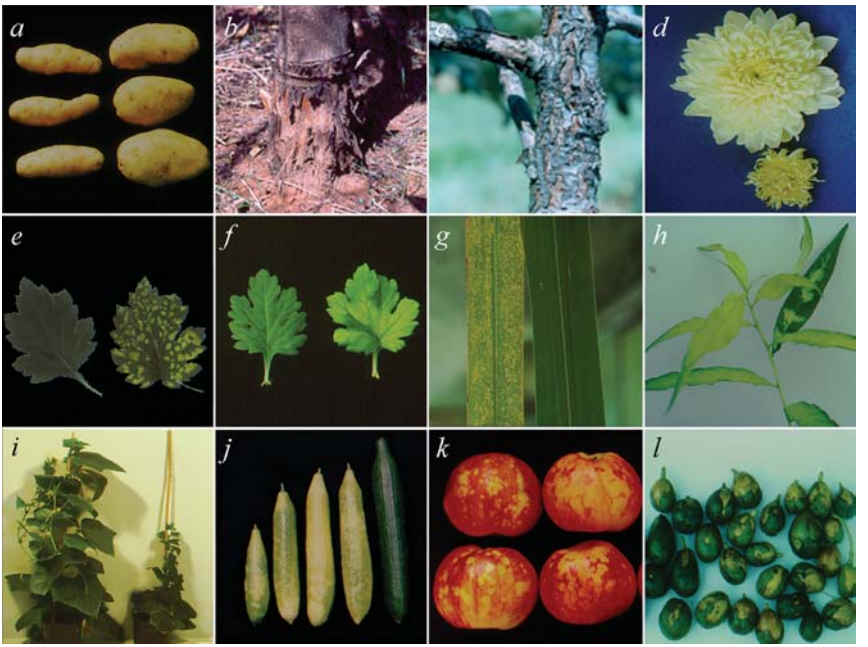

Figure 2 Alterations in different organs accompanying infections by some representative viroids. (*a*) Symptoms of PSTVd on potato tubers (*left*) and healthy controls (*right*) (courtesy of T.O. Diener). (*b*) Symptoms of CEVd on a trifoliate orange rootstock (courtesy of N. Duran-Vila and P. Moreno). (*c*) Symptoms of PBCVd on pear A20 (courtesy of J.C. Desvignes). (*d*) Flower symptoms of CSVd on chrysanthemum (*bottom*) and a healthy control (*top*) (courtesy of J.M. Bové). (*e*) Leaf symptoms of CSVd on chrysanthemum (*right*) and a healthy control (*left*). (*f*) Leaf symptoms of CChMVd on chrysanthemum (*right*) and a healthy control (*left*). (*g*) Leaf symptoms of CCCVd on coconut (*left*) and a healthy control (*right*). (*h*) Extreme leaf chlorosis (peach calico) induced by PLMVd. (*i*) Internode shortening induced by HSVd on cucumber (*right*) and a healthy control (*left*). (*j*) Symptoms of HSVd on cucumber fruits (*left*) and a healthy control (*right*) (courtesy of H.L. Sänger). (*k*) Fruit symptoms (dapple apple) induced by ASSVd (courtesy of J.C. Desvignes). (*l*) Fruit symptoms of ASBVd on avocado (courtesy of P.R. Desjardins).

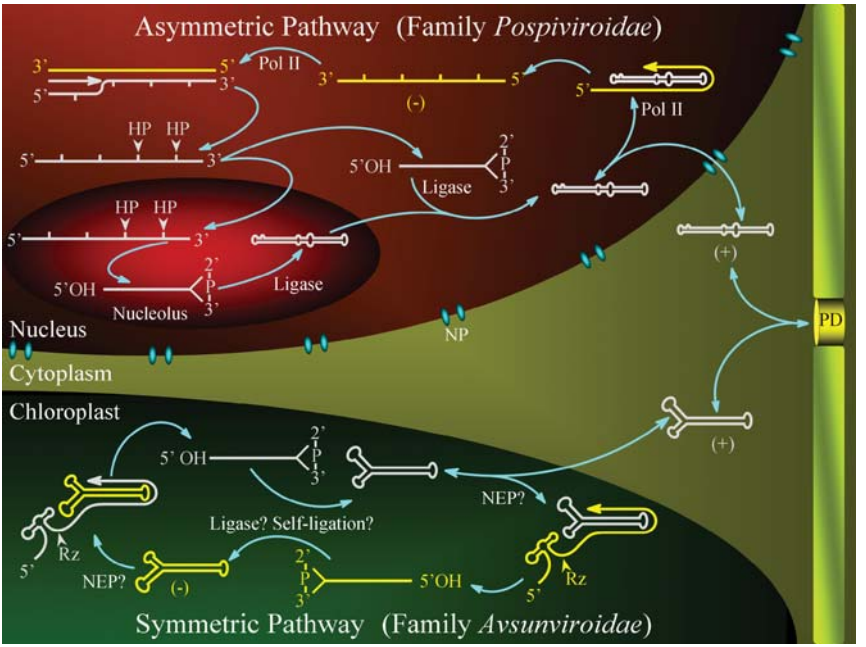

Figure 3 Rolling-circle mechanism proposed for viroid replication. The asymmetric pathway, with one rolling circle, is followed by members of the family *Pospiviroidae* and takes place in the nucleus. The symmetric pathway, with two rolling circles, is followed by members of the family *Avsunviroidae* and takes place in the chloroplast. White and yellow lines indicate plus (+) and minus (−) strands, respectively, and cleavage sites are marked by arrowheads. Self-cleavage mediated by hammerhead ribozymes (Rz) leads to linear monomeric RNAs with 5′-hydroxyl and 2′-3′-cyclic phosphodiester termini; cleavage catalyzed by a host protein (HP) probably generates the same termini. Pol II refers to RNA polymerase II and NEP to nuclear-encoded RNA polymerase. PD and NP are abbreviations for plasmodesmata and nuclear pores, respectively.

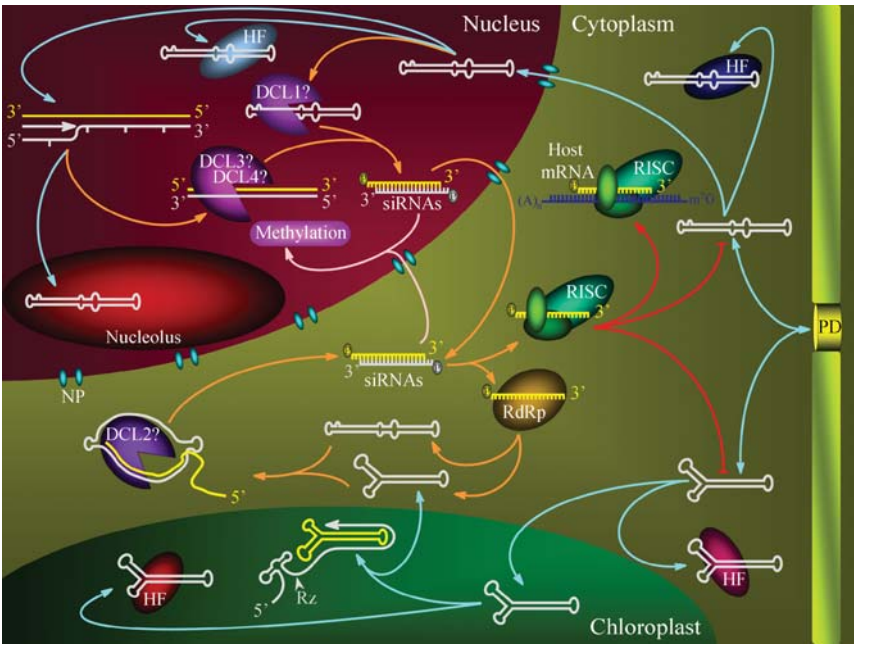

Figure 4 Hypothetical mechanisms of viroid pathogenesis. Symptoms could result from direct interaction between the genomic viroid RNAs and a host factor (HF, protein or RNA), either in the organelle where the viroid replicates and accumulates or in the cytoplasm during viroid movement. Alternatively, RNA silencing could mediate symptom development. In the family *Pospiviroidae* viroid-specific siRNAs could accumulate either in the nucleus (from the action of DCL1 on the genomic viroid RNAs, or of DCL3 and/or DCL4 on the dsRNAs formed during replication), or in the cytoplasm (from the action of DCL2 on the aberrant RNAs generated by a cytoplasmic RdRp, or on the genomic viroid RNAs, not shown). In the family *Avsunviroidae* viroid-specific siRNAs should be generated only in the cytoplasm because no chloroplastic DCL has been characterized. siRNAs would then load RISC for degradation or translation repression of host mRNAs with complementary sequences, or for direct methylation of host DNA. siRNAs could also target genomic viroid RNAs and regulate their titer through a feed-back mechanism. The sizes of the different molecules are not proportional. Both hypothetical mechanisms should not be necessarily exclusive. PD, plasmodesmata; NP, nuclear pores.

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