EMBO reports

review

Viroids: an Ariadne's thread into the RNA labyrinth

José-Antonio Daròs, Santiago F. Elena & Ricardo Flores[+]

Instituto de Biología Molecular y Celular de Plantas (CSIC-UPV), Valencia, Spain

Viroids are structurally, functionally and evolutionarily different from viruses. Despite their small, non-protein-encoding, single-stranded circular RNA genome, viroids can infect higher plants and cause certain diseases. Members of the two viroid families, *Pospiviroidae* and *Avsunviroidae*, have evolved to usurp the transcriptional machinery of their host nuclei and chloroplasts, respectively, in which replication proceeds through a rolling-circle mechanism involving RNA polymerization, cleavage and ligation. Remarkably, viroids subvert certain DNA-dependent RNA polymerases to transcribe RNA templates, and, in the family *Avsunviroidae*, post-transcriptional cleavage is catalysed by hammerhead ribozymes. Viroids are models for studying RNA evolution and for analysing RNA transport in plants, because they can move intracellularly, intercellularly through plasmodesmata and to distal parts of the plant through the vascular system. Viroids elicit RNA-silencing phenomena, which might mediate some of their biological properties, including pathogenesis. As some viroids behave as catalytic RNAs, they are regarded as remnants of the RNA world.

Keywords: gene silencing; ribozymes; RNA transport; RNA world; viroids

EMBO reports (2006) **7,** 593–598. doi:10.1038/sj.embor.7400706

Introduction

During the past few years, small non-coding RNAs have become a topic of increasing interest in molecular biology after the discovery, in eukaryotes, of a series of widespread small RNAs that regulate gene expression, maintain genome stability and defend against invading viruses. However, a different class of small non-encoding RNAs, the viroids, has also been the object of intense research for about 40 years and is still an excellent model system that, similar to the mythic Ariadne's thread, can guide us towards the answers to fundamental questions in the labyrinth of RNA biology. Viroids are unique infectious agents that are restricted to the plant kingdom, and are composed solely of a non-protein-encoding, small (246–401 nucleotide (nt)), single-stranded circular RNA that is able to replicate autonomously in susceptible hosts (Diener, 2003; Flores *et al*, 2005a; Tabler & Tsagris, 2004). Viroids have similarities with two other classes of RNA replicon—namely, certain satellite RNAs of plant viruses (Mayo *et al*, 2005) and the RNA of hepatitis delta virus (HDV; Mason *et al*, 2005), because their genomes all have a circular structure and they replicate through a rolling-circle mechanism. However, the satellite RNAs are replicated and encapsidated by proteins of their helper viruses, and, although HDV antigenomic RNA encodes a protein (the δ-antigen) that regulates replication mediated by a host-encoded RNA polymerase, the coat protein of hepatitis B virus is needed for encapsidation. Therefore, the unique property of viroids is their ability to complete their infectious cycle without encoding any protein and without resorting to a helper virus.

Instituto de Biología Molecular y Celular de Plantas (CSIC-UPV), Universidad Politécnica de Valencia, Avenida de los Naranjos s/n, 46022 Valencia, Spain
[+]Corresponding author. Tel: +34 963 877 861; Fax: +34 963 877 859;
E-mail: rflores@ibmcp.upv.es

Submitted 15 February 2006; accepted 5 April 2006

The diversity of the viroidosphere

Potato spindle tuber viroid (PSTVd) was the first viroid to be identified (Diener & Raymer, 1967). Definitive proof of its physical nature was provided by the correlation of infectivity with a low-molecular-weight RNA (Diener, 1972). This work also revealed the strong secondary structure of PSTVd, a point that was further confirmed by electron microscopy (Sogo *et al*, 1973) and sequencing (Gross *et al*, 1978), which together predicted a lack of protein-encoding capacity and a rod-like conformation owing to extensive intramolecular base pairing in the RNA (Fig 1A). PSTVd was the first eukaryotic pathogen to be sequenced, which, in a way, initiated the genomic era.

Soon after, other viroids resembling PSTVd were characterized and, through comparative analysis, a model was proposed that divided the rod-like structure into five structural/functional domains (Keese & Symons, 1985). Prominent among these was the domain containing a central conserved region (CCR; Fig 1A). However, the characterization of *Avocado sunblotch viroid* (ASBVd; Hutchins *et al*, 1986) revealed the first exception: this viroid did not have a CCR, but presented the remarkable property that, in protein-free conditions, its strands of both polarities were able to self-cleave specifically at a single bond, generating 5′-OH and 2′,3′-phosphodiester termini. The discovery that the satellite RNA of a plant virus was endowed with a similar autolytic-processing ability (Prody *et al*, 1986) led to the first structural model of the common self-cleaving motif: the hammerhead structure. After the characterization of a second viroid RNA with hammerhead structures (Hernández & Flores, 1992), viroids were classified into two families: most of the approximately 30 known viroids belong to the family *Pospiviroidae* (type species PSTVd), the members of which have a CCR but cannot form hammerhead structures; by contrast, the four viroids belonging to the family *Avsunviroidae* (type species ASBVd), lack a CCR but undergo hammerhead-mediated self-cleavage (Flores *et al*, 2005b; Fig 1A,B). This classification scheme is further supported by another

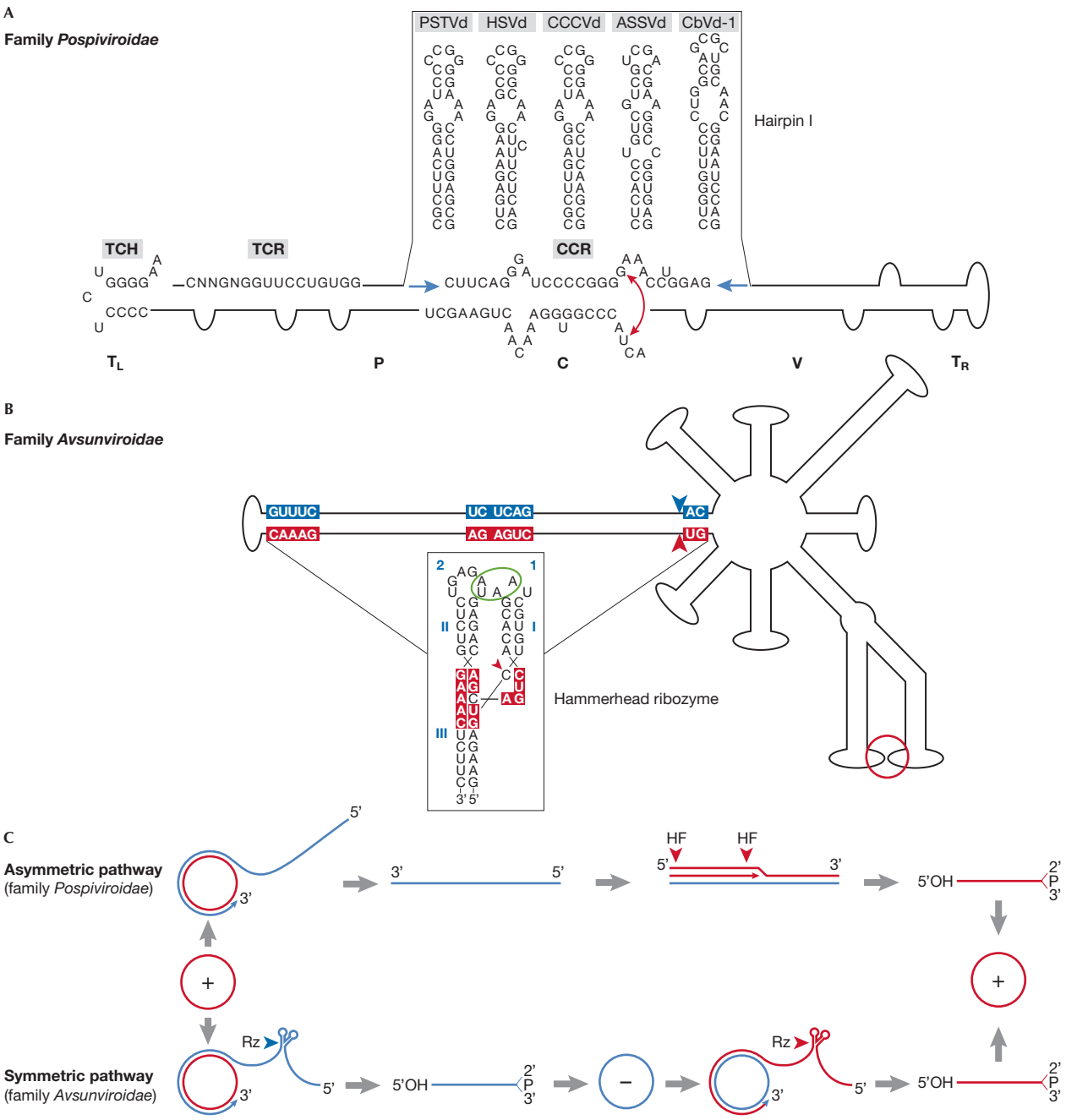

Fig 1 | Genome structure and replication mechanism of viroids. (**A**) Scheme of the rod-like genomic RNA that is characteristic of the family *Pospiviroidae* with the central (C), pathogenic (P), variable (V), and terminal left and right (TL and TR, respectively) domains. The central conserved region (CCR; genus *Pospiviroid*), the terminal conserved region (TCR; genera *Pospiviroid*, *Apscaviroid* and part of *Coleviroid*) and the terminal conserved hairpin (TCH; genera *Hostuviroid* and *Cocadviroid*) are shown. Blue arrows indicate the flanking sequences that, together with the upper strand of the CCR, form hairpin I, as depicted in the inset box for the type species of the five genera of the family *Pospiviroidae*: *Potato spindle tuber viroid* (PSTVd), *Hop stunt viroid* (HSVd), *Coconut cadang-cadang viroid* (CCCVd), *Apple scar skin viroid* (ASSVd) and *Coleus blumei viroid 1* (CbVd-1). The red double-headed arrow connects two residues of PSTVd linked after ultraviolet irradiation as a consequence of forming part of loop E. (**B**) Scheme of the branched genomic RNA of *Peach latent mosaic viroid* (PLMVd; family *Avsunviroidae*), in which the sequences conserved in most natural hammerhead ribozymes are shown on a red and blue background for (+) and (–) polarities, respectively, and the self-cleavage sites are indicated by arrowheads. The red circle denotes a kissing-loop interaction. The structure of the (+) hammerhead ribozyme is shown in the inset box, with Roman and Arabic numerals depicting helices I, II and III, and loops 1 and 2, respectively, and the arrowhead indicating the self-cleavage site. The green oval indicates a tertiary interaction between loops 1 and 2 that enhances catalytic activity. (**C**) Asymmetric and symmetric pathways of the rolling-circle replication mechanism that is used by members of the families *Pospiviroidae* and *Avsunviroidae*, respectively. Red and blue lines refer to (+) and (–) strands, respectively. Arrowheads point to cleavage sites of a host factor (HF) or ribozymes (Rz), and the resulting 5′ and 3′ groups are indicated.

demarcating criterion that has significant implications for viroid molecular biology: PSTVd and ASBVd replicate (and accumulate) in the nucleus and the chloroplast, respectively, and the same is probably true for the other members of both families. Indeed, the *Avsunviroidae* are the only pathogens able to enter and replicate in the chloroplast—an organelle with great potential in biotechnology (Daniell *et al*, 2002).

Because of their small size, viroids have always been viewed as models for studying RNA structure. The rod-like or quasi-rod-like secondary structure predicted for PSTVd and related viroids was confirmed by nuclease and bisulphite probing *in vitro* (Gross *et al*, 1978). The observation that certain sequence duplications or deletions maintain this type of structure also provided indirect support for its existence *in vivo*. However, during replication, these viroids can also adopt metastable secondary structures that contain hairpins, some of which, including hairpin I (Fig 1A), must be functionally relevant, because co-variations preserve their morphology in the *Pospiviroidae*. In addition, ultraviolet irradiation identified a motif in PSTVd termed loop E, which is formed by a complex array of non-Watson–Crick interactions and is also present in the eukaryotic 5S rRNA (Branch *et al*, 1985; Fig 1A). This motif has a role in the synthesis and delivery of 5S rRNA, and might also operate in PSTVd as a binding site for proteins involved in its replication, particularly in ligation, and in intranuclear transport from the nucleoplasm to the nucleolus (see below). The situation seems different in the *Avsunviroidae* because at least some of these viroids adopt a clearly branched conformation, both *in vitro* and *in vivo*, which is stabilized by an interaction between two kissing loops (Hernández & Flores, 1992; Bussière *et al*, 2000; Gago *et al*, 2005), and during replication they can alternatively form the catalytically active hammerhead structures (Fig 1B).

Masters of subverting their hosts' transcription machinery

Viroids replicate through a rolling-circle mechanism that involves only RNA intermediates (Branch & Robertson, 1984; Grill & Semancik, 1978). This mechanism is supported by the circular structure of the initial template and the presence in infected tissues of multimeric viroid RNAs of one or both polarities, which are the expected products of its reiterative transcription. The most abundant monomeric circular RNA, to which the (+) polarity is arbitrarily assigned, is transcribed by an RNA polymerase into oligomeric (–) and then into (+) strands. After cleavage by an RNase and ligation by an RNA ligase, these (+) strands generate the monomeric (+) circular RNA. This so-called asymmetric pathway of the rolling-circle mechanism is presumed to act during the replication of PSTVd and other members of its family, because the oligomeric (–) strands resulting from the first RNA–RNA transcription have been identified in infected tissues. However, because the monomeric (–) circular RNA has been found in ASBVd-infected avocado, an alternative symmetric pathway is assumed to operate in this and other members of the *Avsunviroidae*. It is thought that the oligomeric (–) strands are first processed to their monomeric circular counterparts, which then act as the template for the second round of RNA–RNA transcription (Fig 1C). As viroids are non-protein-encoding RNAs, the catalytic activities required for their replication were initially presumed to be provided by host enzymes. However, it is still unclear how viroids usurp the host nuclear and chloroplastic DNA-dependent RNA polymerases (DdRps) to transcribe their RNA genomes. The δ-antigen encoded in the antigenomic polarity of HDV RNA could facilitate the polymerase template switch for this virus, but the non-protein-encoding nature of viroids excludes a similar possibility for these pathogens.

The idea that DdRps, redirected to accept RNA templates, are the enzymes that catalyse elongation of viroid strands (and not the RNA-dependent RNA polymerases engaged in RNA silencing) derives mainly from studies with specific inhibitors. From the effects of α-amanitin *in vivo* and *in vitro*, the DdRp involved in the replication of PSTVd and related viroids seems to be the nuclear RNA polymerase II. Results obtained with a monoclonal antibody against a conserved domain of the larger subunit of RNA polymerase II also support this idea (Mühlbach & Sänger, 1979; Warrilow & Symons, 1999). Data for ASBVd suggest the involvement of the nuclear-encoded chloroplastic RNA polymerase (NEP), or another polymerase resistant to the inhibitor tagetitoxin. NEP, which is composed of a single polypeptide chain, is structurally similar to the T3 and T7 phage RNA polymerases, and has, similar to these polymerases, short (15–19 nt) promoters that seem ideally suited to fit in the small size of viroid genomes (Navarro & Flores, 2000). Moreover, T7 RNA polymerase can catalyse *in vitro* replication of two small (A+U)-rich single-stranded RNAs with compact secondary structures that resemble that of ASBVd (Konarska & Sharp, 1989). The lack of genomic tags for initiating transcription might have represented an advantage in the primitive RNA world, from which viroids are considered to be 'molecular fossils' (Diener, 1989; see below). However, evidence obtained for ASBVd and *Peach latent mosaic viroid* (PLMVd) by *in vitro* capping of the free 5′-triphosphate groups characteristic of chloroplastic primary transcripts, together with RNase-protection assays or RNA-ligase-mediated rapid amplification of cDNA ends (Delgado *et al*, 2005; Navarro & Flores, 2000), indicates that in their present cellular habitat, polymerization of viroid strands starts at specific sites. Data of this type for the *Pospiviroidae* are restricted to the (–) strand of PSTVd, with two initiation sites having been proposed (Tabler & Tsagris, 2004; Kolonko *et al*, 2006). Because RNA folding occurs during transcription, the initiation sites of nascent viroid strands might determine the adoption of transient metastable structures that are functionally relevant during replication (see below).

Remarkably, in the *Avsunviroidae*, cleavage of (+) and (–) multimers is autocatalytic, and is mediated by hammerhead structures (Prody *et al*, 1986; Hutchins *et al*, 1986; reviewed in Flores *et al*, 2001). These ribozymes—owing to their small size and easy manipulation to act *in trans* against specific RNAs—have been the subject of extensive structural studies. The results have revealed an intricate assortment of Watson–Crick and non-canonical interactions between the nucleotides that form their catalytic core (Fig 1B), which might explain why these residues are conserved in natural hammerhead ribozymes (Pley *et al*, 1994; Scott *et al*, 1995). More recent studies have uncovered additional interactions between peripheral regions of natural hammerhead ribozymes that increase their self-cleavage activity, particularly at the low magnesium concentrations that exist *in vivo* (de la Peña *et al*, 2003; Khvorova *et al*, 2003). The precise nature of these interactions is being dissected and they are being incorporated into a new generation of more efficient *trans*-acting artificial hammerhead ribozymes. In viroid replication, hammerhead ribozymes are only formed transiently during strand elongation, with the location of the transcription initiation sites being crucial for proper ribozyme folding and self-cleavage (Delgado *et al*, 2005). Moreover, some

hammerhead structures are catalytically active only in a double-hammerhead format, which can be adopted solely by the multimeric replicative intermediates, thereby restricting their activity to certain RNA contexts (Forster *et al*, 1988). Self-cleavage is probably assisted *in vivo* by host proteins acting as RNA chaperones (Daròs & Flores, 2002), with the resulting monomeric linear RNA adopting an alternative conformation that favours ligation. Therefore, regulation of RNA catalytic activity by a switch between alternative conformations resembles the conformational regulation of catalytic activity in proteins. Ligation in the *Avsunviroidae* has also been proposed to occur non-enzymatically (that is, self-ligation), giving rise to a 2′,5′-phosphodiester bond (Côté *et al*, 2001). If such an atypical bond exists in natural viroids, the RNA polymerase should be able to proceed through it. Moreover, replication in this family should be a largely RNA-based mechanism that only requires a host RNA polymerase. The involvement of an RNA ligase, however, cannot be dismissed, even though an enzyme of this class has never been documented in chloroplasts.

In the *Pospiviroidae*, *in vitro* assays with potato nuclear extracts and (+) PSTVd RNAs that are longer than unit length (Baumstark *et al*, 1997), and *in vivo* assays with *Arabidopsis thaliana* transformed with cDNAs that express dimeric (+) transcripts of representative members of this family (Daròs & Flores, 2004), have shown accurate processing to the monomeric (+) circular forms. These results suggest that the specificity for the cleavage reaction depends on a particular RNA conformation that makes a phosphodiester bond vulnerable to one or more host RNases; the findings also underline that the specific cleavage of the oligomeric intermediates of both viroid families, whether enzyme or ribozyme mediated, seems an inherent feature of the RNA. *In vitro* experiments are consistent with a cleavage reaction producing the monomeric (+) PSTVd linear RNA, which then switches to an extended conformation containing the loop E (Fig 1A) that promotes ligation by a host enzyme similar to the wheat germ RNA ligase (Baumstark *et al*, 1997). It is interesting to note that a dimeric (–) transcript of *Hop stunt viroid* (HSVd), a member of the *Pospiviroidae*, fails to be processed when expressed transgenically in *A. thaliana* (Daròs & Flores, 2004). Therefore, processing of viroid dimeric transcripts seems to be a polarity-intrinsic property, which dictates the susceptibility to, and the specificity of, the reactions mediated by the host enzymes. The finding that, in cultured cells and plants that have been infected, PSTVd (–) strands accumulate in the nucleoplasm, whereas the (+) strands are localized in the nucleolus as well as in the nucleoplasm (Qi & Ding, 2003), suggests that viroid (+) and (–) strands are transcribed by RNA polymerase II in the nucleoplasm. The (+) strands are then transferred and processed in the nucleolus, where processing of the ribosomal RNA and transfer RNA precursors also takes place. It is possible that specific motifs such as loop E, which are only present in PSTVd (+) strands, might be involved in this differential transport.

Models for analysing RNA transport in plants

Intranuclear transport is just one aspect of viroid movement. To infect a cell, the viroid must enter the nucleus or the chloroplast for replication (intracellular movement), exit to the cytoplasm, pass through the plasmodesmata to neighbouring cells (cell-to-cell movement) and finally reach the vasculature to invade systemically the most distal parts of the plant (long-distance movement). Studies in permeabilized cells and *in planta* have shown that PSTVd has a sequence or structural motif that has not yet been determined, which is required for nuclear import by a specific and saturable receptor through a cytoskeleton-independent route. This import is not coupled to the Ran GTPase cycle that mediates nuclear transport of many proteins and nucleic acids (Woo *et al*, 1999; Zhao *et al*, 2001). A PSTVd-binding protein with a nuclear localization signal has been proposed as a candidate for mediating viroid nucleocytoplasmic transport (Martínez de Alba *et al*, 2003). How members of the *Avsunviroidae* are internalized into the chloroplast remains an intriguing and challenging question, because no other foreign or cellular RNAs are known to be imported into this organelle. With regard to intercellular transport, microinjection experiments with PSTVd are consistent with this RNA having specific determinants for moving rapidly from cell to cell through plasmodesmata (Ding *et al*, 1997). Long-distance movement of the *Pospiviroidae* occurs through the phloem, probably by forming a complex with the RNA-binding phloem protein 2 (PP2), which seems to facilitate systemic movement and even translocation through intergeneric grafts (Ding *et al*, 2005; Gómez & Pallás, 2004). This phloem transport is linked to PSTVd replication and plant development, with the viroid restricted from entering the shoot apical meristem (SAM; Zhu *et al*, 2001). Conversely, a member of the *Avsunviroidae*, PLMVd, can invade cell layers close to the SAM (M.E. Rodio, S. Delgado, A. de Stradis, R.F. & F. Di Serio, unpublished data), suggesting differential interactions in both viroid families with the surveillance system that regulates the selective entry of RNA into the SAM (Foster *et al*, 2002). Viroids, therefore, can act as probes for studying the intracellular, intercellular and long-distance movement of RNA, with implications for plant development and defence.

Pathogenesis and RNA silencing

Renewed interest in RNA biology has been stimulated by the identification of members of a group of small RNAs as the effectors of a wide range of transcriptional and post-transcriptional silencing events, which are involved in development, genome maintenance and defence. Viroids have been instrumental in the discovery of RNA-directed *de novo* methylation of genomic sequences in plants (Wassenegger *et al*, 1994), which is a mechanism that mediates transcriptional silencing. Moreover, increasing evidence indicates that viroids are also inducers and targets of RNA silencing (Itaya *et al*, 2001; Martínez de Alba *et al*, 2002; Papaefthimiou *et al*, 2001; Vogt *et al*, 2004), and that processes of this kind could underlie distinct aspects of viroid biology, including pathogenesis and cross-protection. In tissues infected by members of the two families, small (21–25 nt) viroid-specific RNAs of both polarities have been identified, and these resemble small-interfering RNAs (siRNAs), which are the most reliable markers of RNA silencing (Itaya *et al*, 2001; Martínez de Alba *et al*, 2002; Papaefthimiou *et al*, 2001). The siRNAs result from the action of an RNase-III-like enzyme (Dicer or Dicer-like, DCL, in plants) on double-stranded RNA (dsRNA), which is the characteristic RNA-silencing inducer. There are four DCL isoenzymes with different subcellular locations, at least one of which (DCL-1) can also act on certain endogenous RNAs that have a hairpin secondary structure similar to that of viroids. Cleavage of these endogenous RNAs gives rise to microRNAs (miRNAs), another class of small non-protein-encoding RNAs of a size similar to siRNAs (Baulcombe, 2004). In the *Pospiviroidae*, viroid-specific siRNAs could therefore derive either from the dsRNA-replicative intermediates and the genomic RNA that accumulate in the nucleus, or from

the cytoplasmic genomic RNA that is moving from cell to cell. The latter alternative seems to be the only possibility for the *Avsunviroidae*, because RNA-silencing effects have not been reported in chloroplasts. Biochemical evidence also favours this pathway for PSTVd (Denti *et al*, 2004). As most of the RNA-silencing pathways in plants require an RNA-dependent RNA polymerase (Schiebel *et al*, 1998), it is likely that an enzyme of this class uses the siRNAs as primers and the viroid genomic RNA as a template to generate dsRNA. Secondary siRNAs could then be generated through the subsequent action of DCL, resulting in a signal-amplification cascade. The detection of viroid-specific siRNAs of different sizes in infected tissues (Itaya *et al*, 2001; Martínez de Alba *et al*, 2002; Papaefthimiou *et al*, 2001) is consistent with the involvement of more than one pathway in their genesis, the molecular details of which should be revealed by cloning and sequencing these RNAs.

The siRNAs and miRNAs are then incorporated into the RNA-induced silencing complex (RISC), and guide it for the cleavage or translational arrest of specific foreign or internal RNAs. It has been proposed that viroid-specific siRNAs, acting in the same way as endogenous miRNAs, could base pair with some host mRNAs, block their normal expression and induce disease (Papaefthimiou *et al*, 2001; Wang *et al*, 2004). Although this possibility is consistent with data showing that minor changes in the primary structure of members of both viroid families can convert a strain from severe to latent (Schnölzer *et al*, 1985; De la Peña *et al*, 1999; De la Peña & Flores, 2002), the candidate host mRNAs have not been identified. Therefore, the alternative hypothesis that the genomic viroid RNA acts as the primary pathogenic effector, by interacting with a host factor and distracting it from its normal role, cannot be ruled out. Cross-protection—the temporary attenuation of the viroid titre and the reduction in symptoms produced by a severe strain in plants previously inoculated with a mild strain of the same or a closely related viroid (De la Peña *et al*, 2002; Niblett *et al*, 1978)—could also be due to an RNA-silencing mechanism, assuming that the siRNAs derived from the mild strain bind to the RNA of the severe strain and target it for degradation. The lack of protein-encoding capacity of viroids poses the question of how they evade the RNA-silencing effects induced in their host, which is a problem that viruses have solved by encoding a range of proteins that suppress different steps of the silencing pathway. Viroids could have evolved their typical secondary structure to resist degradation (Wang *et al*, 2004), and, more specifically, as a trade-off between resistance to DCL and RISC, which act preferentially against RNAs with compact and relaxed secondary structures, respectively. Compartmentalization in organelles or association with proteins might also help viroids to elude RNA silencing.

Fossils of the RNA world and models of RNA evolution

The small size, circular structure, high G+C content, structural periodicities and, in particular, the catalytic activity exhibited by some viroids make them excellent candidates for remnants of the postulated ancestral RNA world (Diener, 1989). After the appearance of DNA-based cellular organisms, viroids would have evolved to parasitize certain cyanobacteria (the precursors of chloroplasts) followed by eukaryotic cells, with mutation and recombination events contributing to their subsequent divergence (Elena *et al*, 2001).

The processivity and fidelity of DdRps are probably affected when they are forced to transcribe viroid RNA templates. Supporting this idea, 'jumping' polymerases have been involved in generating sequence repetitions, deletions or insertions in single viroids, and in the emergence of viroids composed of a mosaic of sequences from others presumed to co-infect a common host (Hammond *et al*, 1989). Moreover, when inoculated as cDNA clones, viroids, particularly those of the *Avsunviroidae*, accumulate mutations rapidly, leading to complex quasi-species. This extreme plasticity also supports the *in vivo* significance of their predicted minimal free-energy RNA secondary structures, because mutations either map as single variations in the loops or as co-variations in the stems (de la Peña *et al*, 1999; de la Peña & Flores, 2002).

Questions about the evolution of mutational robustness and its evolvability, the evolutionary fate of genome duplications, the evolution of genome complexity and the role of neutrality in RNA replicons have been mostly addressed by analysing *in silico* models of RNA folding (Wagner, 2005). However, viroids offer a unique opportunity for addressing these issues experimentally. The small size of viroids ensures that the analysis covers the complete genome rather than a fragment, as is generally the case with viruses, and their only known phenotype—the RNA secondary structure—can be easily modelled using state-of-the art bioinformatic tools.

ACKNOWLEDGEMENTS

The authors are supported by grants from the Ministerio de Educación y Ciencia and the Generalitat Valenciana (Spain). S.F.E. is an EMBO Young Investigator.

REFERENCES

Baulcombe D (2004) RNA silencing in plants. *Nature* **431:** 356–363

Baumstark T, Schröder ARW, Riesner D (1997) Viroid processing: switch from cleavage to ligation is driven by a change from a tetraloop to a loop E conformation. *EMBO J* **16:** 599–610

Branch AD, Robertson HD (1984) A replication cycle for viroids and other small infectious RNAs. *Science* **223:** 450–454

Branch AD, Benenfeld BJ, Robertson HD (1985) Ultraviolet light-induced crosslinking reveals a unique region of local tertiary structure in potato spindle tuber viroid and HeLa 5S RNA. *Proc Natl Acad Sci USA* **82:** 6590–6594

Bussière F, Ouellet J, Côté F, Lévesque D, Perreault JP (2000) Mapping in solution shows the peach latent mosaic viroid to possess a new pseudoknot in a complex, branched secondary structure. *J Virol* **74:** 2647–2654

Côté F, Lévesque D, Perreault JP (2001) Natural 2′,5′-phosphodiester bonds found at the ligation sites of peach latent mosaic viroid. *J Virol* **75:** 19–25

Daniell H, Khan MS, Allison L (2002) Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. *Trends Plant Sci* **7:** 84–91

Daròs JA, Flores R (2002) A chloroplast protein binds a viroid RNA *in vivo* and facilitates its hammerhead-mediated self-cleavage. *EMBO J* **21:** 749–759

Daròs JA, Flores R (2004) *Arabidopsis thaliana* has the enzymatic machinery for replicating representative viroid species of the family Pospiviroidae. *Proc Natl Acad Sci USA* **101:** 6792–6797

De la Peña M, Flores, R (2002) Chrysanthemum chlorotic mottle viroid RNA: dissection of the pathogenicity determinant and comparative fitness of symptomatic and non-symptomatic variants. *J Mol Biol* **321:** 411–421

De la Peña M, Navarro B, Flores R (1999) Mapping the molecular determinant of pathogenicity in a hammerhead viroid: a tetraloop within the *in vivo* branched RNA conformation. *Proc Natl Acad Sci USA* **96:** 9960–9965

De la Peña M, Gago S, Flores R (2003) Peripheral regions of natural hammerhead ribozymes greatly increase their self-cleavage activity. *EMBO J* **22:** 5561–5570

Delgado S, Martínez de Alba E, Hernández C, Flores R (2005) A short double-stranded RNA motif of peach latent mosaic viroid contains the initiation and the self-cleavage sites of both polarity strands. *J Virol* **79:** 12934–12943

Denti MA, Boutla A, Tsagris M, Tabler M (2004) Short interfering RNAs specific for potato spindle tuber viroid are found in the cytoplasm but not in the nucleus. *Plant J* **37:** 762–769

Diener TO (1972) Potato spindle tuber viroid. VIII. Correlation of infectivity with a UV-absorbing component and thermal denaturation properties of the RNA. *Virology* **50:** 606–609

Diener TO (1989) Circular RNAs: relics of precellular evolution? *Proc Natl Acad Sci USA* **86:** 9370–9374

Diener TO (2003) Discovering viroids—a personal perspective. *Nat Rev Microbiol* **1:** 75–80
Diener TO, Raymer WB (1967) Potato spindle tuber virus: a plant virus with properties of a free nucleic acid. *Science* **158:** 378–381
Ding B, Kwon MO, Hammond R, Owens RA (1997) Cell-to-cell movement of potato spindle tuber viroid. *Plant J* **12:** 931–936
Ding B, Itaya A, Zhong X (2005) Viroid trafficking: a small RNA makes a big move. *Curr Opin Plant Biol* **8:** 606–612
Elena SF, Dopazo J, De la Peña M, Flores R, Diener TO, Moya A (2001) Phylogenetic analysis of viroid and viroid-like satellite RNAs from plants: a reassessment. *J Mol Evol* **53:** 155–159
Flores R, Hernández C, De la Peña M, Vera A, Daròs JA (2001) Hammerhead ribozyme structure and function in plant RNA replication. *Meth Enzymol* **341:** 540–552
Flores R, Hernández C, Martínez de Alba E, Daròs JA, Di Serio F (2005a) Viroids and viroid–host interactions. *Annu Rev Phytopathol* **43:** 117–139
Flores R, Randles JW, Owens RA, Bar-Joseph M, Diener TO (2005b) Viroids. In Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball AL (eds) *Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses*, pp 1145–1159. London: Elsevier/Academic
Forster AC, Davies C, Sheldon CC, Jeffries AC, Symons RH (1988) Self-cleaving viroid and newt RNAs may only be active as dimers. *Nature* **334:** 265–267
Foster TM, Lough TJ, Emerson SJ, Lee RH, Bowman JL, Forster RL, Lucas WJ (2002) A surveillance system regulates selective entry of RNA into the shoot apex. *Plant Cell* **14:** 1497–1508
Gago S, de la Peña M, Flores R (2005) A kissing-loop interaction in a hammerhead viroid RNA critical for its *in vitro* folding and *in vivo* viability. *RNA* **11:** 1073–1083
Gómez G, Pallás V (2004) A long-distance translocatable phloem protein from cucumber forms a ribonucleoprotein complex *in vivo* with hop stunt viroid RNA. *J Virol* **78:** 10104–10110
Grill LK, Semancik JS (1978) RNA sequences complementary to citrus exocortis viroid in nucleic acid preparations from infected *Gynura aurantiaca*. *Proc Natl Acad Sci USA* **75:** 896–900
Gross HJ, Domdey H, Lossow C, Jank P, Raba M, Alberty H, Sänger HL (1978) Nucleotide sequence and secondary structure of potato spindle tuber viroid. *Nature* **273:** 203–208
Hammond R, Smith DR, Diener TO (1989) Nucleotide sequence and proposed secondary structure of Columnea latent viroid: a natural mosaic of viroid sequences. *Nucleic Acids Res* **17:** 10083–10094
Hernández C, Flores R (1992) Plus and minus RNAs of peach latent mosaic viroid self-cleave *in vitro* via hammerhead structures. *Proc Natl Acad Sci USA* **89:** 3711–3715
Hutchins C, Rathjen PD, Forster AC, Symons RH (1986) Self-cleavage of plus and minus RNA transcripts of avocado sunblotch viroid. *Nucleic Acids Res* **14:** 3627–3640
Itaya A, Folimonov A, Matsuda Y, Nelson RS, Ding B (2001) Potato spindle tuber viroid as inducer of RNA silencing in infected tomato. *Mol Plant Microbe Interact* **14:** 1332–1334
Keese P, Symons RH (1985) Domains in viroids: evidence of intermolecular RNA rearrangements and their contribution to viroid evolution. *Proc Natl Acad Sci USA* **82:** 4582–4586
Khvorova A, Lescoute A, Westhof E, Jayasena SD (2003) Sequence elements outside the hammerhead ribozyme catalytic core enable intracellular activity. *Nat Struct Biol* **10:** 708–712
Kolonko N, Bannach O, Aschermann K, Hu KH, Moors M, Schmitz M, Steger G, Riesner D (2006) Transcription of potato spindle tuber viroid by RNA polymerase II starts in the left terminal loop. *Virology* **347:** 392–404
Konarska MM, Shap PA (1989) Replication of RNA by the DNA-dependent RNA polymerase of phage T7. *Cell* **57:** 423–431
Martínez de Alba AE, Flores R, Hernández C (2002) Two chloroplastic viroids induce the accumulation of the small RNAs associated with post-transcriptional gene silencing. *J Virol* **76:** 13094–13096
Martínez de Alba AE, Sägesser R, Tabler M, Tsagris M (2003) A bromodomain-containing protein from tomato specifically binds potato spindle tuber viroid RNA *in vitro* and *in vivo*. *J Virol* **77:** 9685–9694
Mason WS, Burrell CJ, Casey J, Gerlich WH, Howard CR, Newbold J, Taylor JM, Will H (2005) Deltavirus. In Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball AL (eds) *Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses*, pp 735–738. London: Elsevier/Academic
Mayo MA, Leibowitz MJ, Palukaitis P, Scholthof K-BG, Simon AE, Stanley J, Taliansky M (2005) Satellites. In Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball AL (eds) *Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses*, pp 1163–1169. London: Elsevier/Academic
Mühlbach HP, Sänger HL (1979) Viroid replication is inhibited by α-amanitin. *Nature* **278:** 185–188
Navarro JA, Flores R (2000) Characterization of the initiation sites of both polarity strands of a viroid RNA reveals a motif conserved in sequence and structure. *EMBO J* **19:** 2662–2670
Niblett CL, Dickson E, Fernow KH, Horst RK, Zaitlin M (1978) Cross-protection among four viroids. *Virology* **91:** 198–203
Papaefthimiou I, Hamilton AJ, Denti MA, Baulcombe DC, Tsagris M, Tabler M (2001) Replicating potato spindle tuber viroid RNA is accompanied by short RNA fragments that are characteristic of post-transcriptional gene silencing. *Nucleic Acids Res* **29:** 2395–2400
Pley HW, Flaherty KM, McKay DB (1994) Three-dimensional structure of a hammerhead ribozyme. *Nature* **372:** 68–74
Prody GA, Bakos JT, Buzayan JM, Schneider IR, Bruening G (1986) Autolytic processing of dimeric plant virus satellite RNA. *Science* **231:** 1577–1580
Qi Y, Ding B (2003) Differential subnuclear localization of RNA strands of opposite polarity derived from an autonomously replicating viroid. *Plant Cell* **15:** 2566–2577
Schiebel W, Pelissier T, Riedel L, Thalmeir S, Schiebel R, Kempe D, Lottspeich F, Sanger HL, Wassenegger M (1998) Isolation of an RNA-directed RNA polymerase-specific cDNA clone from tomato. *Plant Cell* **10:** 2087–2101
Schnölzer M, Haas B, Ramm K, Hofmann H, Sänger HL (1985) Correlation between structure and pathogenicity of potato spindle tuber viroid (PSTV). *EMBO J* **4:** 2181–2190
Scott WG, Finch JT, Klug A (1995) The crystal structure of an all-RNA hammerhead ribozyme: a proposed mechanism for RNA catalytic cleavage. *Cell* **81:** 991–1002
Sogo JM, Koller T, Diener TO (1973) Potato spindle tuber viroid. X. Visualization and size determination by electron microscopy. *Virology* **55:** 70–80
Tabler M, Tsagris M (2004) Viroids: petite RNA pathogens with distinguished talents. *Trends Plant Sci* **9:** 339–348
Vogt U, Pelissier T, Putz A, Razvi F, Fischer R, Wassenegger M (2004) Viroid-induced RNA silencing of GFP–viroid fusion transgenes does not induce extensive spreading of methylation or transitive silencing. *Plant J* **38:** 107–118
Wagner A (2005) Robustness, evolvability, and neutrality. *FEBS Lett* **579:** 1772–1778
Wang MB *et al* (2004) On the role of RNA silencing in the pathogenicity and evolution of viroids and viral satellites. *Proc Natl Acad Sci USA* **101:** 3275–3280
Warrilow D, Symons RH (1999) Citrus exocortis viroid RNA is associated with the largest subunit of RNA polymerase II in tomato *in vivo*. *Arch Virol* **144:** 2367–2375
Wassenegger M, Heimes S, Riedel L, Sänger HL (1994) RNA-directed *de novo* methylation of genomic sequences in plants. *Cell* **76:** 567–576
Woo Y-M, Itaya A, Owens RA, Tang L (1999) Characterization of nuclear import of potato spindle tuber viroid RNA in permeabilized protoplasts. *Plant J* **17:** 627–635
Zhao Y, Owens RA, Hammond RW (2001) Use of a vector based on potato virus X in a whole plant assay to demonstrate nuclear targeting of potato spindle tuber viroid. *J Gen Virol* **82:** 1491–1497
Zhu Y, Green L, Woo YM, Owens R, Ding B (2001) Cellular basis of potato spindle tuber viroid systemic movement. *Virology* **279:** 69–77

José-Antonio Daròs, Ricardo Flores & Santiago F. Elena