Chapter 13
Viroids: Small Noncoding Infectious RNAs with the Remarkable Ability of Autonomous Replication

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Abstract Viroids are infectious agents of plants, constituted exclusively by a noncoding small (246–401 nucleotides) circular RNA molecule. When this RNA manages to enter a cell of an appropriate host plant, it moves to the subcellular replication site and replicates through an RNA-to-RNA rolling circle mechanism. Viroid progeny is then able to move cell-to-cell through plamodesmata and long distances through the phloem to invade distal parts of host plants. Two types of viroids exist, classified into the families *Pospiviroidae* and *Avsunviroidae*. They replicate in the nucleus (*Pospiviroidae*) and chloroplast (*Avsunviroidae*), hijacking host enzymes. Members of the family *Pospiviroidae* recruit host DNA-dependent RNA polymerase II, RNase III and DNA ligase 1, while members of the *Avsunviroidae* (which contain embedded hammerhead ribozymes for self-cleavage) use host nuclear-encoded chloroplastic RNA polymerase and the chloroplastic isoform of tRNA ligase. Viroids are mainly transmitted mechanically from plant to plant, and frequently exert a pathogenic effect on infected plants. Some symptoms in viroid infections are induced by the viroid-derived small RNAs produced by the host defensive RNA silencing machinery. Interestingly, viroids are targets of the host Dicer-like and RNA-dependent RNA polymerase enzymes, but are particularly resistant to the action of the RNA-induced silencing complex.

13.1 Introduction

Viroids are a particular type of pathogens that affect plants, since they exclusively consist of a small circular single-stranded RNA molecule. In the species known to date, this molecule ranges from 246 to 401 nucleotides (nt); indeed very few

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A. Wang, X. Zhou (eds.), *Current Research Topics in Plant Virology*,
DOI 10.1007/978-3-319-32919-2_13

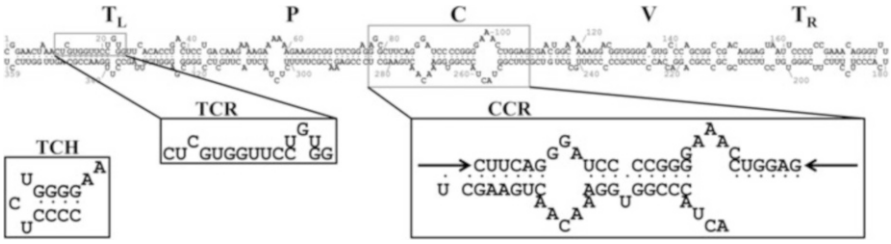

Fig. 13.1 Sequence of PSTVd (sequence variant U23058) and secondary structure as obtained by the Mfold algorithm. The division in domains T_L, P, C, V and T_R domains is indicated. Conserved motifs (CCR and TCR) are boxed. TCH motif, not present in PSTVd but conserved in viroids belonging to the genera *Hostuviroid* and *Cocadviroid* within the family *Avsunviroidae*, is also indicated

sequence variants are longer in length, but contain block-duplications of parts of their genomes. Viroid RNAs are extensively base-paired and adopt compact secondary structures of minimum free energy (Fig. 13.1). Most viroid molecules can adopt rod-like or quasi-rod-like conformations (Giguère et al. 2014b), as confirmed in viroid preparations under electron microscopy (Sogo et al. 1973). However, very few viroids adopt branched conformations of minimum free energy, which also include tertiary structure elements (Giguère et al. 2014a). Remarkably, there is no evidence that viroid RNAs code for proteins, which means that with only a small RNA molecule, viroids are able to replicate when they enter the appropriate host cell, move cell-to-cell, move long distances through the plant, and somehow, circumvent the host defensive response. How they manage to do this is still a mystery that scientists have been trying to decipher since their discovery in the 1960/1970s.

During this time, many articles and some books have exhaustively reviewed different aspects of viroid biology. The following are some recent examples Diener 2003; Tabler and Tsagris 2004; Ding et al. 2005; Flores et al. 2005; Daròs et al. 2006; Ding and Itaya 2007; Tsagris et al. 2008; Ding 2009; Sano et al. 2010; Navarro et al. 2012b; Palukaitis 2014; Flores et al. 2015. This chapter is a personal overview of the field and attempts to highlight the classic findings that have shaped current knowledge about viroids and recent tendencies in research into these fascinating pathogens.

13.2 Viroid Discovery

While working at the U.S. Department of Agriculture in Beltsville, Maryland (USA), Theodor O. Diener found that the properties of the causal agent of potato spindle tuber disease, at that time believed to be a virus, were not conventional at all. In late 1960s early 1970s, he published a series of thorough articles that pinpointed the atypical properties of this pathogen (see the following as examples Diener and Raymer 1967; Diener 1972). The causal agent of potato spindle tuber

disease behaved as a single naked RNA molecule, which was far too small to contain sufficient genetic information for an autonomously replicating virus. The infectivity of the agent was not affected by DNase, protease or phenol treatment, but was inactivated by RNase treatment under low ionic strength conditions. In 1971 he proposed the term "viroid" to designate the new class of pathogens (Diener 1971a). Soon the viroid concept was reinforced by the characterization of the causal agent of exocortis in citrus (Semancik and Weathers 1972). A few years later, Heinz L. Sänger (Justus Liebig-Universitität, Giessen, Germany) and collaborators first discovered that viroids were covalently closed RNA molecules, which existed as highly base-paired rod-like structures (Sänger et al. 1976). They were also able to elucidate the full sequence and secondary structure of *Potato spindle tuber viroid* (PSTVd) (Gross et al. 1978), the first pathogen of a eukaryotic organism from which the complete molecular structure was established. An analysis of the PSTVd sequence reinforced previous results, which suggested that viroids might not encode proteins (Davies et al. 1974), and this notion has not been refuted to date. In 1981 at the University of Adelaide (Australia), Robert H. Symons determined the sequence of *Avocado sunblotch viroid* (ASBVd) and observed its very low homology with the other viroid sequences known at the time (Symons 1981) (Fig. 13.2). A few years later, he and his collaborators showed that ASBVd strands of both polarities contained ribozymes, which were able to self-cleave dimeric transcripts of this viroid (Hutchins et al. 1986) (Fig. 13.2). Interestingly, these small noncoding

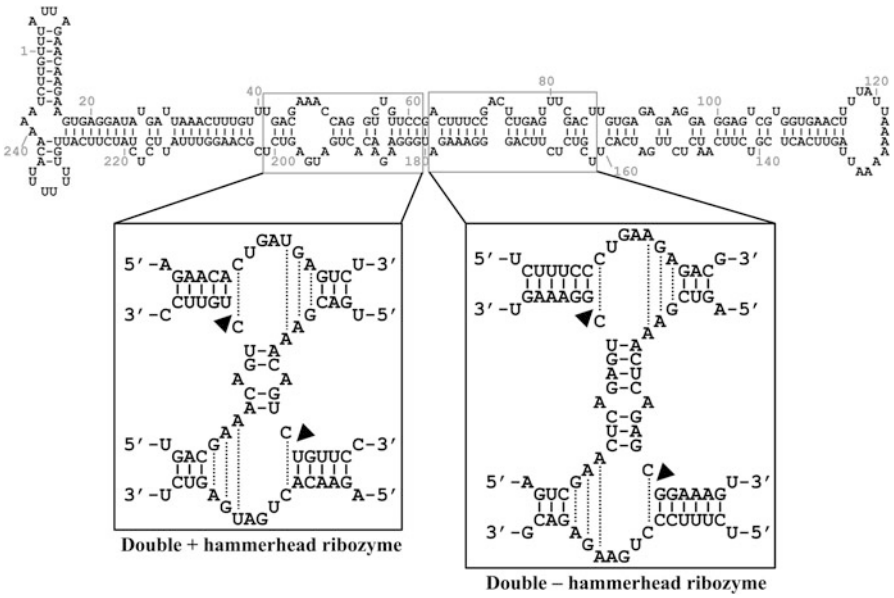

Fig. 13.2 Sequence of ASBVd (sequence variant X52041) and secondary structure as obtained by the Mfold algorithm. The domains forming the double hammerhead ribozyme structures in oligomeric strands of + and – polarities are boxed. In the ribozyme schemes, *arrowheads indicate* the self-cleavage sites and *dotted lines* non-canonical interactions

RNAs, able to replicate and move autonomously in plants, remained as an oddity in biology until recent years, when a myriad of small and long noncoding RNAs have been discovered, which play crucial roles in the regulation of almost all biological processes (Morris and Mattick 2014).

13.3 Viroid Species

The 9th report the International Committee on Taxonomy of Viruses (ICTV) currently recognizes 32 different viroid species (Owens et al. 2012a) (Table 13.1), and many sequence variants have been described to belong to most of these species. Viroid sequence variants have been collected together in the Subviral RNA Database (http://subviral.med.uottawa.ca) (Rocheleau and Pelchat 2006). Initially, a criterion of less than 90 % sequence identity along the whole molecule was arbitrarily established to demarcate species in viroid taxonomy. However, to better adapt viroid taxonomy to currently accepted criteria in virus taxonomy, different and non-overlapping biological properties are also required to establish new species. This change led to two previously established species, *Tomato chlorotic dwarf viroid* (TCDVd) and *Mexican papita viroid* (MPVd) (Table 13.1), to be currently under consideration (Di Serio et al. 2014).

An early analysis of viroid phylogeny showed two clearly different viroid lineages (Elena et al. 1991). These two lineages are currently recognized as two different viroid families, *Pospiviroidae* and *Avsunviroidae* (Table 13.1), named after the type species PSTVd (Fig. 13.1) and ASBVd (Fig. 13.2), respectively. Most viroids known to date belong to the family *Pospiviroidae*. A common property shared by these viroids is the presence of a conserved region, approximately at the center of their structures of minimum free energy, known as the central conserved region (CCR) (Fig. 13.1). This is a quasi-double-stranded structure with an upper and a lower strand flanked by two imperfect inverted repeats that allow the formation of a cruciform structure, an alternative to that of minimum free energy. Specific sequences in the CCR, and the presence or absence of two other conserved elements in the molecule, the terminal conserved region (TCR) and the terminal conserved hairpin (TCH) (Fig. 13.1), are used to allocate different species to five genera: *Pospiviroid* (type member PSTVd), *Hostuviroid* (type member *Hop stunt viroid*, HSVd), *Cocadviroid* (type member *Coconut cadang-cadang viroid*, CCCVd), *Apscaviroid* (type member *Apple scar skin viroid*, ASSVd) and *Coleviroid* (type member *Coleus blumei viroid 1*, CbVd-1) (Table 13.1). Keese and Symons (1985) proposed a model for the presence of five structural domains in viroids. This model has survived to date for family *Pospiviroidae* members: terminal-left (T_L), pathogenic (P), central (C), variable (V) and terminal-right (T_R) (Fig. 13.1). The CCR is in the C domain and the alternative TCR or TCH are in the T_L domain.

Unlike many species in the family *Pospiviroidae*, only four species are presently recognized in the family *Avsunviroidae* (Flores et al. 2000; Fadda et al. 2003). The

Table 13.1 Viroid species recognized by ICTV in its 9th report

Family	Genus	Species	Acronym
Pospiviroidae	***Pospiviroid***	*Chrysanthemum stunt viroid*	CSVd
		Citrus exocortis viroid	CEVd
		Columnea latent viroid	CLVd
		Iresine viroid 1	IrVd-1
		Mexican papita viroid	MPVd
		Pepper chat fruit viroid	PCFVd
		Potato spindle tuber viroid	PSTVd
		Tomato apical stunt viroid	TASVd
		Tomato chlorotic dwarf viroid	TCDVd
		Tomato planta macho viroid	TPMVd
	Hostuviroid	*Hop stunt viroid*	HpSVd[a]
		Dahlia latent viroid[b]	DLVd
	Cocadviroid	*Citrus bark cracking viroid*	CBCVd
		Coconut cadang-cadang viroid	CCCVd
		Coconut tinangaja viroid	CTiVd
		Hop latent viroid	HpLVd
	Apscaviroid	*Apple dimple fruit viroid*	ADFVd
		Apple scar skin viroid	ASSVd
		Australian grapevine viroid	AGVd
		Citrus bent leaf viroid	CBLVd
		Citrus dwarfing viroid	CDVd
		Citrus viroid V	CVd V
		Citrus viroid VI	CVd VI
		Grapevine yellow speckle viroid 1	GYSVd-1
		Grapevine yellow speckle viroid 2	GYSVd-2
		Pear blister canker viroid	PBCVd
		Apple fruit crinckle viroid[b]	AFCVd
		Grapevine yellow speckle viroid 3[b]	GYSVd-3
		Persimmon latent viroid[b]	PLVd
	Coleviroid	*Coleus blumei viroid 1*	CbVd-1
		Coleus blumei viroid 2	CbVd-2
		Coleus blumei viroid 3	CbVd-3
		Coleus blumei viroid 4[b]	CbVd-4
		Coleus blumei viroid 5[b]	CbVd-5
		Coleus blumei viroid 6[b]	CbVd-6
Avsunviroidae	***Avsunviroid***	*Avocado sunblotch viroid*	ASBVd
	Pelamoviroid	*Chrysanthemum chlorotic mottle viroid*	CChMVd
		Peach latent mosaic viroid	PLMVd
	Elaviroid	*Eggplant latent viroid*	ELVd

Type species of the different genera are highlighted on *gray background*
[a]Although official acronym is HpSVd, this species is commonly abbreviated HSVd
[b]Species not yet approved by ICTV

molecules of these viroids do not contain a CCR, rather embedded hammerhead ribozyme structures in their strands of both polarities (Fig. 13.2), which are able to self-cleave the viroid oligomeric RNAs. The four viroids in the family *Avsunviroidae* are allocated to three genera, *Avsunviroid* (type species ASBVd), *Pelamoviroid* (type species *Peach latent mosaic viroid*, PLMVd) and *Elaviroid* (type species *Eggplant latent viroid*, ELVd), based on specific sequences in hammerhead domains, guanosine plus cytosine (G + C) content, and solubility in 2 M LiCl, which most probably reflects how compact tertiary structure is.

New viroid species are continuously being discovered. Some recent examples include: *Citrus viroid V* (CVd-V), found in the citrus relative *Atalantia citroides* (Serra et al. 2008); two viroids that infect Japanese and American persimmon (Nakaune and Nakano 2008; Ito et al. 2013), *Pepper chat fruit viroid* (PCFVd), *Dahlia latent viroid* (DLVd), and a symptomless viroid related to *Iresine viroid 1* (IrVd-1) that has been isolated from portulaca (Verhoeven et al. 2009, 2013, 2015); a new grapevine viroid isolated from China (Jiang et al. 2009); two tentative new species of coleviroids (CbVd-5 and CbVd-6) found in *Coleus blumei* (Hou et al. 2009a, b). Deep sequencing approaches are greatly influencing the identification of new viroid and viroid-like RNAs. The full genomic sequence of a viroid that resembles *Apple dimple fruit viroid* (ADFVd) has been assembled from a library of small RNAs obtained from fig (Chiumenti et al. 2014). Hence algorithms (PFOR, and its improved version PFOR2) have been recently developed to assemble the circular genomes of viroid and viroid-like RNAs from deep sequencing data. These algorithms are homology-independent and can reveal viroid and viroid-like genomes that do not necessarily resemble any currently known species, like *Grapevine latent viroid* (GLVd) and two viroid-like RNAs with hammerhead ribozymes from grapevine and apple (Wu et al. 2012; Zhang et al. 2014).

13.4 Viroid Relatives

Viroid properties are quite unique. In fact some such properties suggest that they might be survivors from the RNA world (Diener 1989; Flores et al. 2014). However, some other RNAs share properties with viroids. The main one is human hepatitis delta virus (HDV), a satellite RNA virus. HDV consists of a single-stranded circular RNA with ribozymes (not of the hammerhead-type) in the strands of both polarities, and very much resemble viroids (Flores et al. 2012; Taylor 2014). The main differences are that HDV depends on a helper virus, hepatitis B virus (HBV), in whose virions it is encapsidated, and it encodes a protein in the antigenomic strand, the delta antigen. Some satellite RNAs of plant viruses probably relate more to viroids, and are also noncoding RNAs; interestingly however, the coding properties of the satellite RNA of *Rice yellow mottle virus* have been recently reported (AbouHaidar et al. 2014). Some of these satellite RNAs are circular, or undergo a circular phase during replication, and they contain ribozymes (hammerhead-type, but not only hammerhead-type) (Hu et al. 2009; Rao and

Kalantidis 2015). Circular satellites are known as virusoids, although the ICTV no longer supports this category.

Another RNA that is related to viroids, with a somewhat puzzling biological nature, is cherry small circular RNA (cscRNA). This is a viroid-like RNA with hammerhead ribozymes in the strands of both polarities (Di Serio et al. 1997). Recent research has suggested that it is a satellite RNA of the mycoviruses that induce the leaf scorch disease of cherries (Minoia et al. 2014b). What is even more puzzling is the biological nature of a retroviroid-like element found in carnations. This circular viroid-like RNA, with hammerhead ribozymes in the strands of both polarities, cannot be transmitted from plant to plant, but a DNA counterpart has been found to be directly fused to DNA sequences of *Carnation etched ring virus*, a pararetrovirus, most likely in the form of an extrachromosomal element that is transmitted to descendants (Daròs and Flores 1995).

13.5 Viroid Replication

Viroids replicate through an RNA-to-RNA rolling circle mechanism. Since viroids do not code for proteins, the polarity of viroid RNAs is arbitrarily assigned. In most species, the viroid molecule with the highest accumulation in infected tissues is circular. This form is considered the viroid genome and is attributed positive, or plus (+), polarity. Consequently, complementary forms are considered to take negative, or minus (−), polarity. Viroid RNAs of + and – polarities accumulate asymmetrically in infected tissues. Strands of + polarity accumulate in larger amounts than those of – polarity. Differential accumulation depends on viroid species and is generally greater in the members of *Pospiviroidae* than in *Avsunviroidae*. However, two species of *Avsunviroidae*, PLMVd and, particularly *Chrysanthemum chlorotic mottle viroid* (CChMVd; both of which belong to the genus *Pelamoviroid*), are peculiar because monomeric linear forms are predominant, probably due to very active hammerhead ribozymes.

13.5.1 Replication Mechanism

One major finding to help decipher viroid replication was the detection of *Citrus exocortis viroid* (CEVd) RNAs of – polarity in *Gynura aurantiaca* infected tissues (Grill and Semancik 1978) and lack of evidence of viroid DNA intermediates. Oligomeric RNAs of + polarity were also detected in both PSTVd-infected potato cells (Spiesmacher et al. 1983) and tissues infected by ASBVd, CEVd and CCCVd (Hutchins et al. 1985). Another main observation was the unambiguous detection of monomeric circular viroid RNAs of – polarity in avocado tissues infected by ASBVd, unlike what occurred in tissues infected by PSTVd and other members of its family (Hutchins et al. 1985; Daròs et al. 1994). In order to understand the

mechanism of viroid replication, presence of hammerhead ribozymes in the RNAs of both polarities in all the viroids of family *Avsunviroidae* is highly relevant (Hutchins et al. 1986; Flores et al. 2001). These ribozymes are also considered to likely act *in vivo* during replication because linear RNAs opened at the ribozyme self-cleavage site have been identified in ASBVd-infected avocado tissue (Daròs et al. 1994). For all these reasons, two different versions of a rolling-circle mechanism have been proposed to explain viroid replication in the members of *Pospiviroidae* and *Avsunviroidae* (Branch and Robertson 1984; Branch et al. 1988).

Members of the family *Pospiviroidae* are considered to replicate via an asymmetric rolling circle mechanism (Fig. 13.3). In this mechanism, viroid circular RNA of + polarity acts as a template for the synthesis of oligomeric RNAs of – polarity by reiterative transcription. Oligomeric – RNAs act as templates to produce complementary oligomeric RNAs of + polarity. According to the asymmetric model, only + oligomeric RNAs are cleaved to monomers which, in the last instance, are ligated to render viroid + circular progeny (Fig. 13.3). The symmetric model explains the replication of the viroids belonging to the family *Avsunviroidae*. In this model (Fig. 13.3), viroid oligomeric – RNAs are also produced from a circular template of + polarity. However, these oligomeric RNAs undergo self-cleavage through hammerhead ribozymes and the resulting monomeric RNAs are circularized to produce monomeric circular viroid RNAs of – polarity. This species is the template to produce oligomeric + RNAs in a second rolling circle, which is symmetrical to the first (Fig. 13.3). Oligomeric + RNAs, which also contain hammerhead ribozymes, self-cleave to produce monomers that are finally circularized.

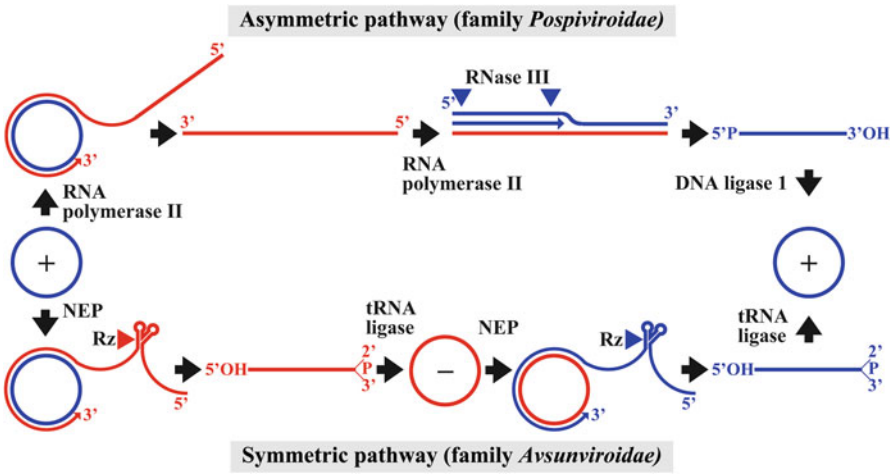

Fig. 13.3 Schematic representation of the viroid RNA-to-RNA rolling circle replication mechanism. Asymmetric and symmetric pathways are followed by the members of the family *Pospiviroidae* in the nucleus and by the members of the *Avsunviroidae* in chloroplasts, respectively. Viroid RNAs of + and – polarities are represented with *blue* and *red lines*, respectively. *Arrowheads* represent cleavage sites. P and OH indicate phosphoester and hydroxyl terminal groups

13.5.2 Replication Site

One important question is where exactly all these replication steps take place. Early works localized PSTVd and its replication intermediates in the nuclei of infected cells (Diener 1971b; Spiesmacher et al. 1983), and all evidence obtained to date indicates that the replication and accumulation of the members of *Pospiviroidae* occur in this subcellular location. More specifically, an *in situ* hybridization analysis of *Nicotiana benthamiana* tissues infected with PSTVd has revealed that viroid – strands localize in the nucleoplasm, while + strands localize in both the nucleoplasm and nucleolus (Qi and Ding 2003). In contrast, an *in situ* hybridization analysis with electron microscopy has indicated that ASBVd localizes in the chloroplasts of infected cells, mostly on thylakoid membranes (Bonfiglioli et al. 1994; Lima et al. 1994). The same occurred with PLMVd in infected peach leaves (Bussière et al. 1999). Multistranded ASBVd complexes, considered viroid replication intermediates, have also been localized in chloroplasts (Navarro et al. 1999). Thus, chloroplasts are accepted as the replication and accumulation site of *Avsunviroidae*. However, a recent intriguing research conducted with ELVd has shown that this viroid RNA is able to traffic from the cytoplasm to the nucleus, and from the nucleus to the chloroplast (Gómez and Pallás 2012), so reality could be more intricate.

13.5.3 RNA Transcription

Another important question is what enzymes are involved in all these replication steps. This is a particularly interesting question, because viroids, unlike viruses, do not encode any replication protein. Pioneering viroid transcription analyses, done with replication complexes that were partially purified from infected tissues, have demonstrated that the synthesis of HSVd and CEVd strands is not affected by DNase or actinomycin D, but is sensitive to fungal toxin α-amanitin at the low concentrations that typically inhibit eukaryotic DNA-dependent RNA polymerase II (Mühlbach and Sänger 1979; Flores and Semancik 1982). This conclusion is quite outstanding because RNA polymerase II typically uses a DNA template in host cells. However, viroids somehow manage to subvert its activity to recognize an RNA template. Involvement of DNA-dependent RNA polymerase II in the transcription of the members of the family *Pospiviroidae* has been further supported through an immunoprecipitation analysis done with an antibody against the carboxy-terminal domain of this enzyme (Warrilow and Symons 1999), and by showing direct binding between the enzyme and PSTVd in a tomato nuclear extract (Bojic et al. 2012). By also analyzing the effect of a fungal toxin, this time tagetitoxin, upon transcription using partially purified replication complexes from avocado chloroplasts infected by ASBVd, it has been concluded that the polymerase which mediates the synthesis of these viroid RNAs is chloroplastic nuclear-

encoded polymerase (NEP) (Navarro et al. 2000). This is a single-subunit enzyme that resembles bacteriophage RNA polymerases. However, *in vitro* analyses, which used PLMVd RNAs and purified *Escherichia coli* RNA polymerase, have suggested that bacterial-like RNA polymerase from peach chloroplasts may catalyze PLMVd replication (Pelchat et al. 2001). This is a plastid-encoded polymerase (PEP) that consists in several subunits and resembles prokaryotic RNA polymerases. In any case, both host enzymes are DNA-dependent RNA polymerases, which means that the members of the *Pospiviroidade* and *Avsunviroidae* are capable of changing the substrate specificity of some of their replication enzymes.

Another intriguing question in viroid replication is whether viroid transcription starts at random in the circular template or if, on the contrary, transcription promoters exist in viroid molecules. A pioneering research work by Navarro and collaborators, which labeled the 5′-triphosphate groups of linear ASBVd RNAs of both polarities isolated from infected avocado tissues, has demonstrated that this viroid transcripts start with a UAAAA sequence, which maps to similar A + U-rich terminal loops in their predicted quasi-rod-like secondary structures. Moreover, the sequences around initiation sites have been highlighted as being similar to the promoters used by chloroplastic NEP (Navarro and Flores 2000), which further supports the involvement of this polymerase in ASBVd replication Other studies done into PLMVd have also suggested that the transcription of + and − strands starts at definite positions in the corresponding templates. More specifically, they map at similar double-stranded motifs, which contain the conserved GUC triplet that precede the self-cleavage site in both polarity strands (Delgado et al. 2005; Motard et al. 2008). For the members of the family *Pospiviroidae*, an analysis that employed PSTVd molecules and a potato nuclear extract, and which allowed the *de novo* synthesis of viroid transcripts, has revealed that – strands also start at a single site located in the hairpin loop of the viroid left terminal region (Kolonko et al. 2006). Finally, although viroid RNA turnover has not received much attention to date, a recent research work, which used PSTVd-infected eggplant tissues, has provided a mechanistic insight into how viroid decay may proceed *in vivo* during replication (Minoia et al. 2015).

13.5.4 Viroid RNA Cleavage

The oligomeric transcripts of both polarities in the viroids of the family *Avsunviroidae* self-cleave through the hammerhead ribozymes embedded in these molecules (Hutchins et al. 1986; Prody et al. 1986; Flores et al. 2001). Self-cleavage occurs in the absence of proteins. However, host RNA chaperons, like proteins PARBP33 and PARBP35 from avocado chloroplasts, may facilitate self-cleavage *in vivo* (Daròs and Flores 2002). Interestingly, tertiary interactions between peripheral regions in hammerhead structures have proven crucial for activity at the low magnesium concentrations which exist *in vivo* (De la Peña et al. 2003; Khvorova et al. 2003). During replication, viroid oligomeric transcripts

self-cleave very efficiently. In fact, a mutational analysis that used ELVd + hammerhead ribozyme has suggested that natural viroid ribozymes have been evolutionary selected to cleave RNAs co-transcriptionally (Carbonell et al. 2006). Then, after cleavage and circularization, viroids must have some kind of regulatory mechanism to avoid the cleavage of circular viroid progeny. An early work conducted after the discovery of the hammerhead ribozyme has demonstrated that ASBVd achieves this regulation thanks to thermodynamically unstable hammerhead ribozymes that contain short helices III capped with short loops. The cleavage of ASBVd hammerhead ribozymes is efficient by the formation of double hammerhead structures only in oligomeric transcripts, while single hammerhead ribozymes are poorly active (Forster et al. 1988; Davies et al. 1991). PLMVd and CChMVd may regulate the activity of their hammerhead ribozymes in circular progeny by engaging their corresponding sequences in thermodynamically very stable quasi-double-stranded arms. Nonetheless, these two viroids, particularly CChMVd, have the lower ratio of circular to linear forms in infected tissues. Finally, ELVd hammerhead ribozyme of + polarity shows efficient co-transcriptional cleavage, as mentioned above, and a poor self-cleavage rate constant after transcription (Carbonell et al. 2006).

Despite a thorough search along this lines (Tsagris et al. 1987), there is no evidence to prove that the oligomeric RNAs of the members of the family *Pospiviroidae* undergo autocatalytic cleavage. This means that a specific host enzyme must recognize the replication intermediate of + polarity and cleaves precisely to produce the monomeric linear RNAs. A pioneering work that used CEVd has identified the upper CCR strand as the processing site and advanced hairpin I, or an alternative double-stranded palindrome structure formed by two contiguous hairpins I, as the RNA motifs that direct cleavage (Visvader et al. 1985). It was not long before a viroid processing model, which involved this thermodynamically double-stranded structure that can be adopted by the oligomers of all the members of the *Pospiviroidae*, was soon proposed (Diener 1986). A research work, done with PSTVd RNAs and nuclear extracts from potato cell suspensions, has mapped an equivalent cleavage site in the upper strand of the CCR, but also proposed a multi-branched structure that would undergo conformational transition to mediate viroid RNA cleavage and ligation (Baumstark et al. 1997). It is noteworthy that the sequence motifs which support this last model do not exist beyond the genus *Pospiviroid*. Gas and collaborators used an *Arabidopsis thaliana* experimental system in which viroid replication intermediates were expressed (Daròs and Flores 2004), to map the processing site of three viroids (CEVd, ASSVd and HSVd) that belong to three different genera in the family *Pospiviroidae*. They found equivalent positions in loop capping hairpin I, more specifically between the third and fourth nucleotides of this tetraloop (Gas et al. 2007). From the effect on the cleavage and ligation of a series of mutations around the CEVd processing site, it has been concluded that the substrate for cleavage is the double-stranded structure adopted by the hybridization of two hairpin I domains, which belong to two contiguous units in viroid oligomeric intermediate, whereas ligation is determined by loop E and the flanking nucleotides of the two CCR strands (Gas et al. 2007).

Another analysis of monomeric linear CEVd RNAs isolated from *A. thaliana* plants expressing oligomeric + RNAs has identified 5′-phosphomonoester and 3-′-hydroxyl terminal groups in this replication intermediate. The nature of these termini and the double-stranded structure, previously proposed to be the substrate for cleavage *in vivo*, suggests that a type III RNase catalyzes cleavage, and an RNA ligase that recognizes these termini, promotes circularization (Gas et al. 2008). All these results led to a model to explain RNA processing during the replication of the members of the *Pospiviroidae* (Fig. 13.4). In this model the four nucleotides in the loops of two contiguous hairpins I in the oligomeric replication intermediate –the sequence of this tetraloop in all known members of *Pospiviroidae* is palindromic– interact to trigger a conformational transition that forms the double-stranded intermediate. This intermediate that contains the two cleavage sites in opposite strands, separated by two nucleotides in a 3′ protruding manner, is the substrate for host RNase III that produces the monomers with 5′-phosphomonoester and 3′-hydroxyl terminal groups. Finally, these monomers undergo conformational transition to form a ligation intermediate that is sealed by an RNA ligase activity (DNA ligase 1, see below) recognizing these 5′-phosphomonoester and 3′-hydroxyl terminal groups (Fig. 13.4).

13.5.5 Viroid RNA Circularization

With the genuine monomeric linear replication intermediate at hand (which in the case of PSTVd is opened at position G95-G96 and contains 5′-phosphomonoester and 3′-hydroxyl terminal groups), Nohales and collaborators purified a tomato protein fraction capable of efficient circularizing this RNA (Nohales et al. 2012a). A mass spectrometry analysis of this fraction highlighted the presence of tomato DNA ligase 1. A recombinant version of this protein produced in *E. coli* has demonstrated the efficient circularization of representative viroids in the family *Pospiviroidae*, opened at their physiological processing sites. Finally a virus-induced gene silencing (VIGS) approach has demonstrated the involvement of this host enzyme in viroid circularization in PSTVd-infected *N. benthamiana* plants (Nohales et al. 2012a). This remarkable finding indicates that, similarly to what occurs in transcription, viroids (*Pospiviroidae*) also subvert a DNA enzyme to mediate an RNA reaction in the last replication step (Fig. 13.3).

Unlike the monomeric linear replication intermediates of the members of the *Pospiviroidae*, those from *Avsunviroidae* are produced by the activity of hammerhead ribozymes, and contain 5′-hydroxyl and 2′,3′-cyclic phosphodiester termini. These are the typical terminal groups recognized by tRNA ligase, an enzyme conserved in all the eukaryotes involved in tRNA maturation (Abelson et al. 1998). After considering a work showing that this enzyme in plants, in addition to the nucleus, also localizes in the cytoplasm and chloroplast (Englert et al. 2007), Nohales and collaborators cloned the cDNA corresponding to this enzyme from eggplant and produced a recombinant version of the protein in *E. coli*. This recombinant protein

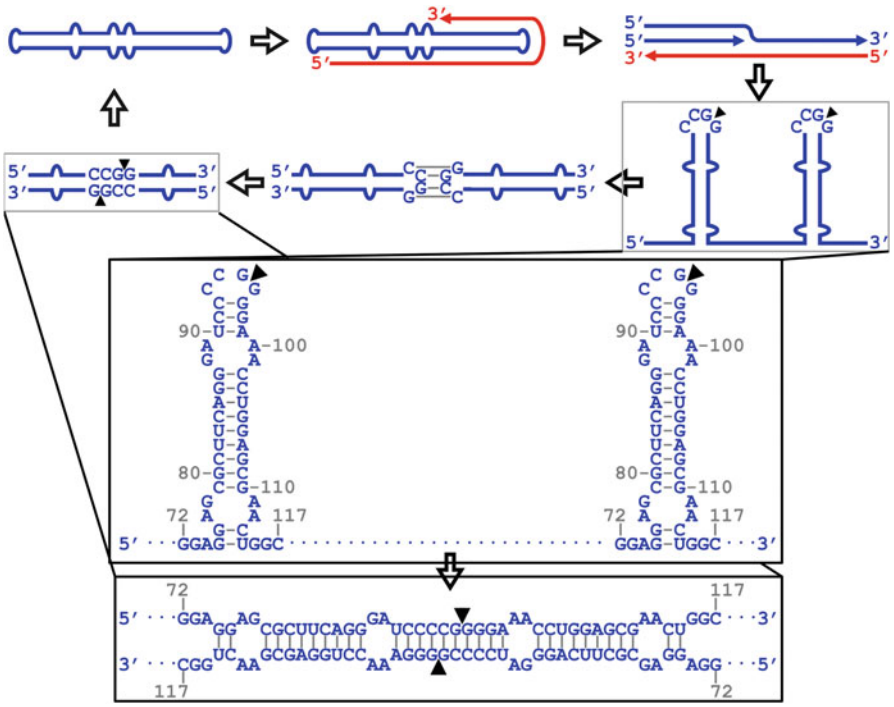

Fig. 13.4 Model to explain RNA processing during the replication of the viroids belonging to the family *Pospiviroidae*. The nucleotides in the loop of two contiguous hairpins I in the oligomeric replication intermediate interact to trigger formation of a palindromic quasi-double-stranded structure, which is the substrate for a host RNase III. After cleavage, linear monomers refold to a conformation recognized by DNA ligase 1. *Blue* and *red lines* and sequences correspond to + and – polarities, respectively. *Gray lines* indicate the kissing loop interaction and *arrowheads* indicate the processing sites. Sequences and numbering correspond to PSTVd sequence variant U23058

efficiently circularizes all the monomeric linear forms of both polarities of the four species in the family *Avsunviroidae*. A VIGS assay has been done to silence *N. benthamiana* endogenous tRNA ligase, and it also supports the involvement of this enzyme in ELVd circularization in plants (Nohales et al. 2012b).

13.6 Viroid Traffic

When viroid molecules manage to enter the host cell, they must move towards the appropriate subcellular location for replication which entails, according to current knowledge, the nucleus for the members of the family *Pospiviroidae* and chloroplasts for the members of the *Avsunviroidae*. Then the viroid progeny, like that of plant viruses, should move to neighboring cells to continue replication, and then to distal plant parts once they reach vascular tissue (Ding et al. 2005). There is a remarkable difference between viroids and viruses in terms of the systemic invasion

of host plants. Viroid infections can be considered slow (several weeks) when compared to the pace of most plant viruses, which can reach distal plant parts in just a few days. The molecular mechanisms that underlie this difference are currently unknown.

Analyzing viroid movement is extremely difficult when compared to plant viruses. There are no viroid-encoded proteins to which reporter moieties, like green fluorescence protein (GFP), can be fused for tracking purposes. What makes things worse is that viroid genomes are highly compact and do not admit the insertion of reporter genes while maintaining viability. Nonetheless, an ingenious experimental approach, which used *Potato virus X* (PVX) as a vector to launch PSTVd fragments embedded in an intron-containing GFP mRNA has demonstrated that hairpin I, the palindromic structure present in the upper strand of the C domain in all the species of the family *Pospiviroidae*, suffices to mediate entry of RNA into the nucleus of *N. benthamiana* cells (Zhao et al. 2001; Abraitiene et al. 2008). This import is independent of the cytoskeleton, uncoupled to the Ran GTPase cycle and facilitated by a receptor (Woo et al. 1999). A bromodomain-containing protein, which also interacts with PSTVd, has recently proposed to mediate nuclear importation of satellite RNA of *Cucumber mosaic virus* (CMV) (Chaturvedi et al. 2014). As mentioned above, satellite RNAs differ from viroids in which the former need a helper virus to assist some steps in the infection process. On the other side, fusion of the ELVd sequences in the 5′ untranslated region (5′UTR) of a GFP reporter construct expressed in *N. benthamiana* tissues using *Agrobacterium tumefaciens* leads to GFP translation and accumulation in chloroplasts, thus evidencing the remarkable capability of ELVd RNA to translocate into chloroplasts (Gómez and Pallás 2010). Further research with ELVd, which incorporated the intron-containing PVX expression tool this time, has indicated that the scenario of *Avsunviroidae* intracellular movement might be more complex than initially expected because upon entry into the cell, this viroid may move first from the cytoplasm to the nucleus, and then from the nucleus to the chloroplast for replication, to finally move back to the nucleus and the cytoplasm to continue its systemic spread through the plant (Gómez and Pallás 2012).

Viroids are considered to move cell-to-cell through plasmodesmata. When they reach vascular tissue, they translocate into the phloem to move long distance through the plant. Thanks to a genome-wide mutational analysis, Zhong and collaborators have revealed a series of motifs along the PSTVd molecule involved in systemic movement (Zhong et al. 2008). With more details, a bipartite motif that included U201 and U309, together with U47/A313 of the PSTVd molecule, has been found to be involved in viroid movement from bundle sheath to mesophyll cells (Qi et al. 2004). Bulge 7 was shown to be involved in the translocation from bundle sheath cells into the phloem (Zhong et al. 2007), and bulge 6 in the movement from palisade to spongy mesophyll cells (Takeda et al. 2011). Once inside the vascular tissue, phloem protein 2 has been proposed to be the host factor to mediate viroid movement, as this protein has been reported to interact with HSVd RNA *in vitro* and *in vivo* (Gómez and Pallás 2001; Owens et al. 2001; Gómez and Pallás 2004). Graft experiments with citrus viroids have also highlighted the presence of a translocatable factor from Etrog citron that is capable of mediating

viroid invasion of nonvascular tissues (Bani-Hashemian et al. 2015). *N. tabacum* protein Nt-4/1 has also been suggested to be involved in PSTVd mobility (Solovyev et al. 2013).

13.7 Viroid Pathogenesis

Viroids infect angiosperm plants, monocotyledonous and dicotyledonous. To date no viroid that infects gymnosperms, pteridophytes, bryophytes or algae has been found. Among angiosperms, all kinds of plants are infected, including herbaceous and ligneous. Most of the viroids known to date are associated with cultivated plants, although this may reflect only the effect of agricultural practices on amplifying and spreading viroids worldwide. Viroids are particularly prevalent in plants cultivated in tropical and subtropical regions. This can once again be an effect of agricultural practices, or could indicate an advantage of warmer climates in viroid replication and spread. It is worth mentioning that citrus plants have the infamous honor of being hosts of many viroid species (Murcia et al. 2009), and that despite efforts made to set up such an experimental system, no complete viroid infection has been described in plant model *A. thaliana* (Daròs and Flores 2004). The effect of viroid diseases range from devastating, like cadang-cadang which killed millions of coconut palms in South East Asia (Randles et al. 1988) to asymptomatic. Some examples of the so called latent viroids exist, like ELVd, that have no apparent effect on host plants (Fadda et al. 2003). Yet in many instances, symptoms depend on the host –PSTVd sequence variants, which are strongly symptomatic in tomato, are almost symptomless in *N. benthamiana*– or interestingly on viroid sequence variants, and some examples of well characterized pathogenicity determinants exist (De la Peña and Flores 2002; Malfitano et al. 2003; Murcia et al. 2011; Wu et al. 2013).

Typical symptoms in viroid diseases are leaf chlorosis, internode shortening, bark cracking, flower discoloration, fruit skin deformation or tuber malformation. Plant stunting and leaf epinasty (downward bending from growth at the top) are common expressions of many viroid diseases. The molecular mechanisms that underlie viroid symptoms have been a mystery for a long time, and most symptoms in viroid diseases are possibly the result of a complex combination of molecular effects. However, a recent research by Navarro and collaborators has demonstrated that two viroid-derived small sRNAs (vd-sRNAs, see below), which arise from the – strand of a PLMVd variant that induces intense albinism (peach calico), target the mRNA coding for chloroplast heat-shock protein 90 (cHSP90). This protein is a molecular chaperone involved in chloroplast development and its down-regulation may abort chloroplast maturation to produce the albino phenotype (Navarro et al. 2012a). It is also interesting to note that PLMVd has been shown to accumulate to higher titers in albino sections of infected peach leaves. Consequently, targeting host cHSP90 with vd-sRNA through an RNA silencing down-regulating mechanism might be a viroid strategy to increase its progeny. One good example of

how symptoms in infected plants can arise from RNA silencing mechanisms also stems from the satellite RNA of CMV. Infection with the yellow satellite RNA (Y-sat) of CMV induces a small interfering RNA (siRNA) that down-regulates a chlorophyll biosynthetic gene (CHLI), which, in turn, promotes leaf yellowing (Shimura et al. 2011). Interestingly, the hypothesis that vd-sRNAs trigger symptoms in viroid infections was anticipated years ahead of these discoveries (Wang et al. 2004) and led to heated debate as to whether viroids, whose genomes are at least 10 times shorter than those of plant viruses, may target host genes (Navarro et al. 2012b). Another observation to support this hypothesis came from the expression of PSTVd sequences as artificial microRNAs (amiRNAs) in *N. tabacum* and *N. benthamiana*. One amiRNA, which corresponds to the virulence modulating region of this viroid and targets host soluble inorganic pyrophosphatase, induces abnormal phenotypes that closely resemble PSTVd symptoms in these plants (Eamens et al. 2014).

Despite all these examples, most symptoms in viroid diseases may still be the result of a complex combination of molecular effects. Viroids hijack host proteins to mediate replication and movement, and some symptoms may result from detracting these proteins from their physiological roles in host plants. HSVd infection has been shown to cause a significant imbalance in the expression of phenylpropanoid metabolite-affecting genes via a complex mechanism (Füssy et al. 2013). HSVd infection has also been show to induce changes in the dynamic DNA methylation of ribosomal RNA (rRNA) genes. In infected plants, some rRNA genes are demethylated and transcriptionally reactivated (Martinez et al. 2014). Moreover, viroid infections are known to induce a strongly altered gene expression in the host plants (Itaya et al. 2002; Owens et al. 2012b; Rizza et al. 2012; Lisón et al. 2013).

13.8 Viroids and RNA Silencing

Plants use RNA silencing pathways to defend themselves from invading viruses, but viruses display RNA silencing suppressors to counteract this defensive mechanism (Ding 2010). The relation between viroids and RNA silencing has been, ever since this mechanism was discovered, intriguing for several reasons. First, viroid molecules strongly resemble the structured RNAs that are substrates of Dicer-like (DCL) enzymes and trigger RNA silencing. Viroid replication also produces, at least transitorily, double-stranded RNAs. Second, as viroids are noncoding RNAs, they cannot display the repertoire of proteins with RNA silencing suppressor activity found in plant viruses (Li and Ding 2006). Third, and in quite the opposite direction, highly structured viroid molecules may be particularly resistant to the down-regulating activity of the RNA-induced silencing complex (RISC). Fourth and finally, the subcellular localization of viroid molecules (nucleus and chloroplast) very much dissembles that of RNA viruses, and could also be important to interpret how viroids circumvent RNA silencing.

Pioneering analyses have shown that plant tissues infected with viroids, regardless of them belonging to the families *Pospiviroidae* or *Avsunviroidae*, accumulate vd-sRNAs, similarly to what occurs in virus infections (Itaya et al. 2001; Papaefthimiou et al. 2001; Martínez de Alba et al. 2002). Moreover, these vd-sRNAs have been seen to be phosphorylated and methylated at the 5′ and 3′ ends, respectively, like genuine virus-derived small RNAs, which supports an origin from RNA silencing pathways (Martín et al. 2007). More recently, these vd-sRNAs have also been shown to be loaded by Argonaute (AGO) proteins (Minoia et al. 2014a). Furthermore, vd-sRNAs have also been reported to be functional *in vivo* down-regulating reporter genes fused to viroid sequences (Vogt et al. 2004; Gómez and Pallás 2007). However, mature viroid molecules have been reported to exhibit a remarkable resistance (Gómez and Pallás 2007; Itaya et al. 2007), but not complete immunity (Carbonell et al. 2008; Kasai et al. 2013), to RNA silencing. All these observations suggest that viroids may be able to maintain a delicate equilibrium between triggering and being targets of the plant antiviral RNA silencing pathways.

Analyses performed by deep sequencing have demonstrated that vd-sRNAs are homogeneously distributed along both strands of viroid RNAs, which suggests the involvement of RNA-dependent RNA polymerases (RDR) in the production of secondary vd-sRNAs that amplify the silencing signal (Di Serio et al. 2009; Navarro et al. 2009; Bolduc et al. 2010; Diermann et al. 2010; Martínez et al. 2010). RDR are cytoplasmic enzymes, which indicates that plants may take advantage of viroid traffic through the cytoplasm to trigger RNA silencing. In this context, RDR6 has been shown to preclude meristem invasion by PSTVd in *N. benthamiana* (Di Serio et al. 2010). One interesting and important aspect of RNA silencing, RNA-directed DNA methylation, was first discovered in viroid-infected plants (Wassenegger et al. 1994; Dalakouras et al. 2013). However, a recent work has revealed that despite PSTVd replication induces RNA-directed DNA methylation, it fails to trigger posttranscriptional gene silencing in the nucleus, the organelle where this viroid replicates (Dalakouras et al. 2015).

13.9 Viroid Transmission

The main form of viroid plant-to-plant transmission seems to be mechanical inoculation, which may be accidentally caused in some agricultural practices, such as grafting or pruning, or may be naturally occurring through injuries that result from physical contact between plants. It is worth noting that root grafting may occur naturally in high-density plantations. Seed and pollen transmission of viroids is not common, although substantial rates have been reported for some viroids like ASBVd or ELVd (Flores et al. 2000; Fadda et al. 2003). Bearing this in mind, it is clear that managing free-of-viroid germplasms is crucial to avoid spreading viroid disease, and that the exchange of contaminated material has probably been the main cause of the worldwide spread of some viroid diseases. Tool disinfection in

agricultural practices to prevent mechanical transmission is therefore important (Li et al. 2015). Attention should also be paid to plants in which viroids can occur latently, as shown in some ornamental plants (Verhoeven et al. 2008; Singh et al. 2009). All these recommendations stress the importance of viroid diagnoses in managing viroid diseases.

Fortunately, viroid transmission by vectors (beyond human beings) is not considered significant, although some cases of insect transmission have been reported for *Tomato apical stunt viroid* (TASVd) (Antignus et al. 2007) and TCDVd (Matsuura et al. 2010), and a recent worrying work has localized two viroids, PSTVd and TASVd, in stylets and stomachs of aphids feeding on infected plants (Van Bogaert et al. 2015).

13.10 Viroid Diagnosis

Reliably diagnosing viroid diseases is crucial for managing free-of-viroid vegetative material in the plant industry, and to eradicate infected plants and trees in orchards and plantations to avoid transmission. Viroid detection in plant tissues entails another difficulty when compared to viruses because viroid-encoded proteins are lacking, which precludes the use of immunological techniques like the enzyme-linked immunosorbent assay (ELISA), otherwise widely used in plant health programs.

It was only possible to accomplish early efforts made in viroid diagnoses by means of biological assays with indicator hosts, which showed characteristic infection symptoms. Biological assays are slow and costly, but very sensitive and informative, and are still used nowadays in some instances, particularly in research (Murcia et al. 2011). The analysis of RNA preparations by polyacrylamide gel electrophoresis (PAGE) has revolutionized viroid diagnoses when combining separation under two different conditions, first native and then denaturing (Schumacher et al. 1983). This technique takes advantage of the fact that viroids are circular molecules, which migrate likewise to their linear host counterparts of a similar molecular weight under native conditions, and quickly slow down migration under denaturing conditions. In this way, they are easily separated and detected in the second denaturing gel. Double or sequential PAGE, with some modifications made to the original design, is still greatly appreciated in research, particularly for the identification of new viroids, because its results are sequence-independent (Verhoeven et al. 2013). Molecular hybridization techniques have been widely used for viroid diagnoses in both dot-blot and northern versions. Using polyprobes allows the simultaneous detection of several viroids (Lin et al. 2011; Torchetti et al. 2012). Recent developments in molecular hybridization techniques have resorted to microarray chips as they are able to simultaneously detect hundreds of species of viruses, satellite RNAs and viroids (Nam et al. 2014; Adams et al. 2015). Finally, a wide variety of very sensitive reverse transcription-polymerase chain reaction (RT-PCR) techniques, including one-step, multiplex and quantitative

approaches, as well as RT loop-mediated isothermal amplification (RT-LAMP), has been also proposed for viroid diagnoses (Hajizadeh et al. 2012; Botermans et al. 2013; Thanarajoo et al. 2014; Malandraki et al. 2015).

13.11 Biotechnological Applications of Viroids

A classic biotechnological application of viroids has been cross-protection. Even before this phenomenon even began to be understood (Ziebell and Carr 2010), researchers realized that asymptomatic or mild strains of some viroids, like viruses, were able to protect plants from more severe symptoms caused by aggressive strains (Niblett et al. 1978; Khoury et al. 1988). Viroids have also been used to induce desirable agronomic traits in plants, particularly dwarfing. Citrus trees infected by certain viroid strains show some dwarfing properties that facilitate cultivation, and do not apparently affect fruit production and quality (Tessitori et al. 2013). An interesting example is an elite Brazilian cultivar of Tahiti acid lime, whose properties, which include not only tree size, but also fruit quality, are thought to be induced by a particular combination of viroids (Eiras et al. 2010). Viroids are certainly considered very interesting tools for basic research. Their unique properties have made them ideal experimental systems in many research works into the structure-function relationship, RNA replication and processing, RNA movement through the plant, evolution of RNA pathogens and, of course, RNA silencing. However, some viroid properties, mainly a very compact molecule packed with functions, have so far precluded their use as biotechnological tools. Our recent research, however, shows how the combined expression of ELVd (used as a molecular scaffold) and eggplant tRNA ligase allows production of large amounts of recombinant RNA in *E. coli* cultures (Daròs et al. 2014).

Acknowledgement This work was supported by grants AGL2013-49919-EXP and BIO2014-54269-R from Ministerio de Economía y Competitividad (MINECO, Spain).

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