Chapter 5
Viroids

Robert A. Owens

Abstract Viroids are the smallest known agents of infectious disease – small, highly structured, single-stranded, circular RNA molecules that lack detectable messenger RNA activity. Whereas viruses supply some or most of the genetic information required for their replication, viroids are regarded as "obligate parasites of the cell's transcriptional machinery" and infect only plants. Four of the nearly 30 species of viroids described to date contain hammerhead ribozymes, and phylogenetic analysis suggests that viroids may share a common origin with hepatitis delta virus and several other viroid-like satellite RNAs. Replication proceeds via a rolling-circle mechanism, and strand exchange can result in a variety of insertion/deletion events. The terminal domains of potato spindle tuber and related viroids, in particular, appear to have undergone repeated sequence exchange and/or rearrangement. Viroid populations often contain a complex mixture of sequence variants, and environmental stress (including transfer to different hosts) has been shown to result in a significant increase in sequence heterogeneity. The new field of synthetic biology offers exciting opportunities to determine the minimal size of a fully functional viroid genome. Much of the preliminary structural and functional information necessary is already available, but formidable obstacles still remain.

Robert A. Owens
Molecular Plant Pathology Laboratory, United States, Department of Agriculture/Agricultural Research Service, Beltsville, MD 20705
robert.a.owens@ars.usda.gov

M.J. Roossinck (ed.), *Plant Virus Evolution*.

5.1 Introduction

The first viroid disease to be studied by plant pathologists was potato spindle tuber. Nearly 50 years were to elapse between the discovery of its infectious nature and ability to spread in the field that led Schultz and Folsom (1923) to group potato spindle tuber disease with several other "degeneration diseases" of potatoes and the demonstration by Diener (1971) that the molecular properties of its causal agent, *Potato spindle tuber viroid* (PSTVd), differed fundamentally from those of conventional plant viruses. In the discussion section, this seminal paper focuses on the possible origin and evolution of viroids – as relatives of conventional viruses or the "missing link" between viruses and host genes. Initially, it was the small size of the viroid RNA genome (10^5 Da or less) and the absence of a protein capsid that appeared to set viroids apart from conventional viruses. Later, the circular structure and noncoding nature of viroids was seen as evidence for only a distant relationship between viroids and viruses. Shortly after, *Avocado sunblotch viroid* (ASBVd) was discovered to possess catalytic (i.e., ribozyme) activity. Diener (1989) proposed that viroids might be *living fossils* of a prebiotic RNA world where RNA molecules functioned as both genotype and phenotype.

Since the first complete viroid nucleotide sequence (that of PSTVd; Gross et al. 1978) was published nearly 30 years ago, much has been learned about the molecular biology of viroid replication and other aspects of viroid–host interaction. More than 1,100 complete sequences of PSTVd and other viroids are now available online from the Subviral RNA Database (http://subviral.med.uottawa.ca), and a number of studies have examined different aspects of viroid evolution. By examining the conceptual framework underlying existing studies of viroid evolution, this chapter attempts to identify fruitful areas for future studies. For information concerning other aspects of viroid molecular biology, interested readers should consult the monograph by Hadidi et al. (2003) and a series of recent reviews (Tabler and Tsagris 2004; Flores et al. 2005a; Ding et al. 2005; Daros et al. 2006; Ding and Itaya 2007).

5.2 Genome Structure and Replication Strategy

The Eighth Report of the International Committee on the Taxonomy of Viruses officially recognizes 29 viroid species and divides them into two families (the *Pospiviroidae* and the *Avsunviroidae*) containing a total of seven genera (Flores et al. 2005b; Table 5.1). All species in the family *Pospiviroidae* have a rod-like secondary structure that contains five structural/functional domains (Keese and Symons 1985) and replicate in the nucleus. Three of the four members of the *Avsunviroidae* have a branched secondary structure, and all replicate/accumulate in the chloroplast. All members of the *Avsunviroidae* contain hammerhead ribozymes in both the infectious (+) strand and complementary (−) strand RNAs. Figure 5.1 compares the secondary structures of PSTVd (rod-like, *Pospiviroidae*) and *Peach*

Table 5.1 Officially recognized viroid species (Eighth Report of the of the International Committee on the Taxonomy of Viruses)

Genus[a]	Species	Sigla	Reported variants[b]	Length (nt)
Family *Pospiviroidae*				
Pospiviroid	Potato spindle tuber	PSTVd	109	341–364
	Chrysanthemum stunt	CSVd	19	348–356
	Citrus exocortis	CEVd	86	366–475
	Columnea latent	CLVd	17	359–456
	Iresine	IrVd	3	370
	Mexican papita	MPVd	6	359–360
	Tomato apical stunt	TASVd	5	360–363
	Tomato chlorotic dwarf	TCDVd	2	360
	Tomato planta macho	TPMVd	2	360
Hostuviroid	Hop stunt	HSVd	144	294–303
Cocadviroid	Coconut cadang-cadang	CCCVd	8	246–301
	Coconut tinangaja	CCCVd	2	254
	Citrus bark cracking	CBCVd	6	284–286
	Hop latent	HLVd	10	255–256
Apscaviroid	Apple scar skin	ASSVd	8	329–333
	Apple dimple fruit	ADFVd	2	306
	Apple fruit crinkle	AFCVd[c]	29	368–372
	Australian grapevine	AGVd	1	369
	Citrus bent leaf	CBLVd	24	315–329
	Citrus dwarfing	CDVd	53	291–297
	Grapevine yellow speckle 1	GYSVd-1	49	365–368
	Grapevine yellow speckle 2	GYSVd-2	1	363
	Pear blister canker	PBCVd	18	314–316
Coleviroid	*Coleus blumei*-1	CbVd-1	9	248–251
	Coleus blumei-2	CbVd-2	2	295–301
	Coleus blumei-3	CbVd-3	3	361–364
Family *Avsunviroidae*				
Avsunviroid	Avocado sun blotch	ASBVd	83	239–251
Pelamoviroid	Chrysanthemum chlorotic mottle	CChMVd	21	397–401
	Peach latent mosaic	PLMVd	168	335–351
Elaviroid	Eggplant latent	ELVd	9	332–335

[a]Names of viroid genera are derived from those of the respective type species (listed first)
[b]Sequences available online from the Subviral RNA Database (http://subviral.med. uottawa.ca)
[c]Provisional species (not officially recognized)

latent mosaic viroid (PLMVd; branched, *Avsunviroidae*). With the possible exception of PLMVd, viroids do not appear to contain any modified nucleotides or unusual phosphodiester bonds.

Nucleic acid extracts from infected leaf tissue contain a variety of viroid-related RNAs of both polarities. Some of these molecules – especially those having a complementary or (−) strand polarity – are considerably longer than the infectious circular viroid (+) strand. Northern analysis using strand-specific probes and/or primer extension has shown that these molecules represent the intermediates expected for a “rolling-circle” mechanism of replication.

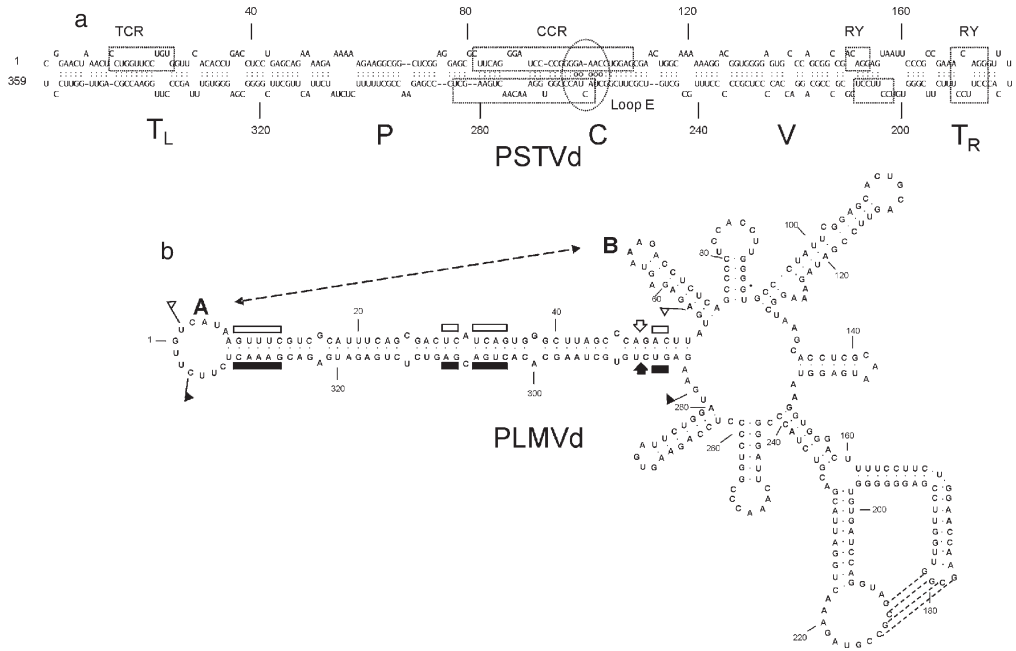

Fig. 5.1 **a** The rod-like secondary structure of *Potato spindle tuber viroid* (*PSTVd*; intermediate strain) showing the five domains characteristic of members of the family *Pospiviroidae:* the terminal left (T_L), pathogenicity (*P*), central (*C*), variable (*V*), and terminal right (T_R). The central conserved region (*CCR*) is located within the C domain and contains a UV-sensitive loop E motif with noncanonical base pairs (denoted by *circles*). The T_L domains of pospiviroids and apscaviroids contain a terminal conserved region (*TCR*), while those of hostuviroids and cocadviroids contain a terminal conserved hairpin (not shown). The T_R may also contain one or two copies of a protein-binding RY motif. **b** The branched secondary structure of *Peach latent mosaic viroid* (*PLMVd*: reference variant). (+) and (−) strand self-cleavage domains are indicated by *flags*, nucleotides conserved in most natural hammerhead structures by *bars*, and the self-cleavage sites by *arrows*. *Black symbols* and *white symbols* refer to plus and minus polarities, respectively. Nucleotides involved in a pseudoknot are indicated by *broken lines.* (Reproduced with permission from Malfitano et al. 2003)

Analysis of ASBVd-infected leaf tissue revealed the presence of monomeric circular RNAs of both polarities (Daros et al. 1994); thus, ASBVd (and presumably other avsunviroids) replicates via a symmetric rolling-circle mechanism (Fig. 5.2). Replication of PSTVd, in contrast, proceeds via an asymmetric rolling-circle mechanism in which progeny (+) strands are synthesized on a multimeric linear (−) strand template (Branch and Robertson 1984). The presence of hammerhead ribozymes in both strands of ASBVd allows its multimeric RNAs to cleave spontaneously, thereby releasing the corresponding linear monomers. Processing of longer-than-unit-length PSTVd (+) strand RNA requires that the central conserved region refold into a multihelix junction containing at least one GNRA tetraloop-hairpin followed by cleavage by an as-yet-unidentified host nuclease (Baumstark et al. 1997). A conserved loop E motif forms following the second cleavage reaction. Although evidence has been presented suggesting that monomeric linear PLMVd molecules can spontaneously circularize with the formation of a 2′,5′ phosphodiester linkage (Coté et al. 2001), circularization of most viroids appears to requires the action of a host RNA ligase.

A central question about viroid replication concerns the identity of the polymerase(s) involved. Inhibition of (+) and (−) strand PSTVd RNA synthesis by α-amanitin exhibits exactly the same dose–response effect in nuclear runoff experiments as host messenger RNA synthesis (Schindler and Muhlbach 1991), implicating host-DNA-dependent RNA polymerase II as the enzyme responsible for pospiviroid replication. Warrilow and Symons (1999) subsequently provided direct evidence for an association between RNA polymerase II and *Citrus exocortis viroid* (CEVd), showing that addition of a monoclonal antibody directed against the C-terminal domain of the largest subunit of RNA polymerase II results in immunoprecipitation of a nucleoprotein complex containing both (+) and (−) strand CEVd RNAs. Resistance of ASBVd RNA synthesis in permeabilized chloroplasts to

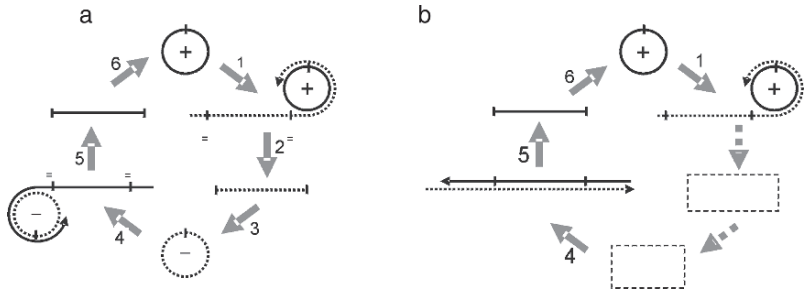

Fig. 5.2 Rolling-circle mechanism of viroid replication. **a** *Avocado sunblotch viroid* and other avsunviroids replicate via a *symmetric* mechanism in which cleavage of both multimeric (−) and (+) strand RNAs (steps 2 and 5) is mediated by ribozymes. Following cleavage, monomeric (-) strand RNA is circularized (step 3) before (+) strand synthesis begins. **b** PSTVd and other posiviroids replicate via an *asymmetric* mechanism in which multimeric (−) strand RNA synthesized by DNA-dependent RNA polymerase II (step 1) is not cleaved or ligated to prior to initiation of (+) strand synthesis (step 4)

tagetitoxin inhibition (Navarro et al. 2000) suggests that a nuclear-encoded RNA chloroplastic polymerase, and *not* the eubacteria-like RNA polymerase encoded by the plastid genome, is responsible for ASBVd strand elongation.

Although the existence of a defined cleavage site theoretically eliminates the need for a specific site for initiation of RNA synthesis, viroid replication appears to be promoter-driven. For ASBVd, both (+) and (−) strand synthesis initiate within AU-rich regions located in the terminal hairpin loops of the rod-like native structure. The nucleotide sequences around the ASBVd start sites bear a striking resemblance to the promoter sequences of certain chloroplast genes transcribed by the same nuclear-encoded RNA polymerase believed responsible for ASBVd replication (Navarro et al. 2000). Transcription of PSTVd (+) strands by RNA polymerase II starts at either position 359 or position 1 in the left terminal loop (Kolonko et al. 2006). Exactly how either of these viroids redirects the respective host-DNA-dependent RNA polymerase to accept its quasi-double-stranded RNA genome as a template is not known.

5.3 Evolutionary Relationships Among Subviral RNAs

Only two characters – an arbitrary level of less than 90% sequence similarity and differences in at least one biological property – are sufficient to distinguish the 29 officially recognized viroid species listed in Table 5.1. The broad outlines of this classification scheme first began to emerge in 1985, when Keese and Symons compared the sequences of eight viroids; i.e., one avsunviroid (ASBVd) and seven different pospiviroids. In the resulting model, PSTVd and other pospiviroids were proposed to contain five structural and functional domains. As shown in Fig. 5.1, these domains include (1) a central domain containing a conserved pair of inverted repeats and involved in viroid replication, (2) flanking pathogenicity and variable domains, and (3) two terminal domains that are interchangeable between viroids. The presence of partial sequence duplications in the right terminal domain of *Coconut cadang-cadang viroid* (CCCVd) was cited as evidence for the importance of RNA rearrangement/recombination in viroid evolution, suggesting that recombination with host RNAs may play an important role in the origin of viroids.

As nucleotide sequence information continued to accumulate, Elena and colleagues used phylogenetic analysis to examine two related topics, evolutionary relationships among viroids and the possible monophyletic origin of viroids and viroid-like satellite RNAs. Their initial study (Elena et al. 1991) included the viroid-like domain of hepatitis δ virus RNA and supported the previously proposed monophyletic origin for all subviral RNAs (Diener 1989). The resulting taxonomic classification contained both of the presently accepted viroid families as well as four of the five currently accepted *Pospiviroidae* genera (i.e., all except the coleviroids). When Jenkins et al. (2000) raised questions about the sequence alignment underlying this analysis, arguing that the sequence similarities needed to infer a reliable phylogeny were not present, Elena et al. (2001) reexamined these questions using improved techniques and a larger set of sequence data. Figure 5.3 shows the neighbor-joining tree produced by this reanalysis.

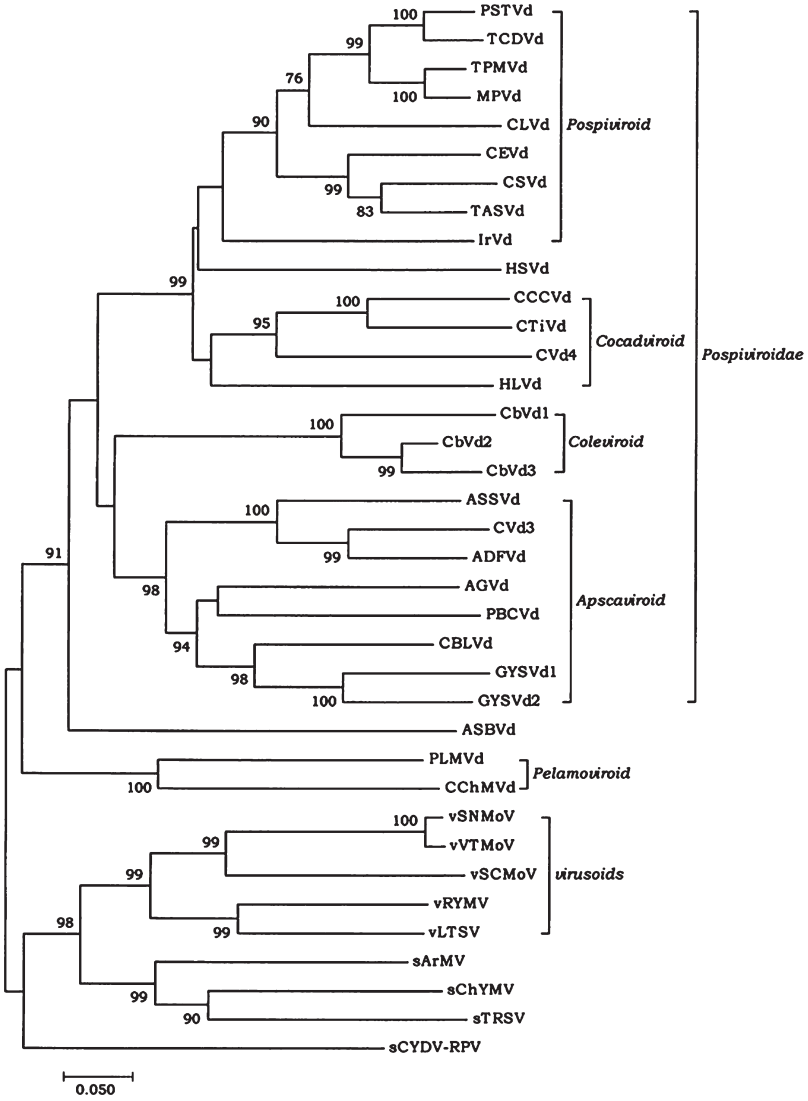

Fig. 5.3 Neighbor-joining phylogenetic tree obtained from an alignment of viroid and viroid-like RNA satellites. The distance matrix was obtained according to Hasegawa et al. (1985) but with gamma-distributed rates among sites (16 categories). Bootstrap values based on 1,000 random replicates, and only values greater than 70% are shown; less well supported branches can be collapsed. See Table 5.1 for viroid abbreviations. Viroid-like satellite RNAs: *vLTSV* (lucerne transient streak virus); *vRYMV* (rice yellow mottle virus); *vSCMoV* (subterranean clover mottle virus); *vSNMoV* (*Solanum nodiflorum* mottle virus); *vVTMoV* (velvet tobacco mottle virus); *sTRSV* (tobacco ringspot virus); *sArMV* (*Arabis* mosaic virus); *sChYMV* (chicory yellow mottle virus); *sCYDV-RPV* (cereal yellow dwarf virus-RPV). (Reproduced with permission from Elena et al. 2001)

The tree shown in Fig. 5.3 reproduces all groupings observed in the earlier analysis (Elena et al. 1991). Members of the family *Pospiviroidae* form three groups, one that includes the *Pospiviroid*, *Hostuviroid*, and *Cocadviroid* genera plus two other groups containing members of either the *Apscaviroid* or *Coleviroid* genera. For the autocatalytic subviral RNAs, viroids in the family *Avsunviroidae* can be seen to occupy a position intermediate between members of the *Pospiviroidae* and the viroid-like satellite RNAs. Additional support for these broad groupings was obtained by likelihood-mapping analysis, but key to the entire analysis was its two-stage alignment strategy. Sequences of viroids and viroid-like satellite RNAs were aligned separately using CLUSTAL-X and then manually edited to preserve local similarities; these partial alignments were then manually aligned before CLUSTAL-X was used to realign dissimilar regions and maximize overall similarity. An important anchor in this final alignment is a GAAA motif found in both the catalytic core of hammerhead ribozymes and the upper central conserved region of pospiviroids (Diener 1989).

5.4 Possible Roles of Conserved Sequence Motifs in Viroid Evolution

As shown in Fig. 5.1 and first described by Branch et al. (1985), the central domain of PSTVd (as well as other pospiviroids, apscaviroids, and possibly cocadviroids) contains a loop E motif similar to those found in a wide variety of other RNAs (Leontis and Westhof 1998). During rolling-circle replication, this loop E motif forms after cleavage of the nascent multimeric RNA and prior to monomer ligation. Loop E also contains a GAAA motif that is conserved among all hammerhead ribozymes. The terminal left domain of pospiviroids, apscaviroids, and coleviroids contains a terminal conserved region (Koltunow and Rezaian 1988), while those of hostuviroids and cocadviroids contain a terminal conserved hairpin (not shown). The terminal right domain of pospiviroids contains one or two copies of a protein-binding RY motif containing the sequence AGG/CUUUC (Gozmanova et al. 2003).

As first noted by Keese and Symons (1985), the terminal domains of pospiviroids appear to have been the target of repeated sequence exchange/rearrangement. For example, *Tomato apical stunt viroid* (TASVd) shares 73% overall sequence identity with CEVd, but the terminal right domains of these two viroids are only 46% similar. Although TASVd is less similar to PSTVd (only 64% overall similarity), their terminal right domains are highly (i.e., 90%) similar. Similar exchanges involving the terminal left domain of *Tomato planta macho viroid* (TPMVd) was also proposed. *Columnea latent viroid* (CLVd) appears to be a natural mosaic of viroid sequences that exhibits extensive sequence similarities to PSTVd and related viroids but has a central conserved region identical to that of *Hop stunt viroid*(HSVd; Hammond et al. 1989). These sorts of sequence rearrangements could be generated either by strand scission and ligation or, more likely, by discontinuous transcription.

Upon prolonged infection, both CCCVd (Haseloff et al. 1982) and CEVd (Szychowski et al. 2005) give rise to mixtures of larger RNA molecules containing duplications of part or all of the terminal right domain. In the case of CEVd, short AGCU tetrads provide possible sites for strand exchange by a "jumping polymerase," and the host plant plays a critical role in the origin and stability of specific duplications. Figure 5.4 shows the structures of CEVd-tomato and two of the longer CEVd-related RNAs, one containing an asymmetric duplication. Symmetrical sequence duplications like those found in CEVd D-87 create an additional binding site for Virp1, the viroid-binding host protein from tomato (Gozmanova et al. 2003). As shown in Fig. 5.1, wild-type (i.e., nonduplicated) PSTVd also contains two RY binding sites, and mutational analysis suggests that the ability of one or both of these sites to interact with Virp1 may be essential for some aspect of viroid transport (reviewed in Tabler and Tsagris 2004).

Spontaneous deletions have also been observed during viroid evolution in vivo. For example, constitutive expression of a noninfectious 350-nt PSTVd RNA containing a small deletion just upstream from the central conserved region in transgenic tobacco gave rise to a 341-nt PSTVd variant capable of independent replication in tomato (Wassenegger et al. 1994). The authors speculate that this rare event – observed in only a single plant expressing a (+) strand RNA transcript – was the result of either (1) host-DNA-dependent RNA polymerase being forced to jump over unpaired nucleotides in the quasi-double-stranded template or (2) a template repair process involving excision of unpaired nucleotides opposite the original deletion, followed by religation. Whether this was an all-or-none event is not known, but the net effect was to remove a large bulge loop in the opposing strand created by the original deletion in the PSTVd complementary DNA (cDNA).

As discussed by Schuster (2001), RNA molecules have many theoretical advantages over proteins for studies of the relationship between genotype and phenotype. These relationships are many-to-one and, thus, give ample room for neutrality. This is especially true for viroids where the rod-like (pospiviroids) or branched (avsunviroids) secondary structure predicted by computer calculation appears to be strongly conserved. Recently, Sanjuán et al. (2006) compared the "mutational robustness" of all 29 viroid species by calculating the structural effects of all possible single nucleotide point mutations. An evolutionary trend toward increased structural robustness was detected during viroid radiation, giving support to the presumed adaptive value of robustness. Genomic redundancy (see above) appears to contribute to structural stability, and the differences in robustness observed between the two viroid families can be explained by the relative fragility of the branched structure of members of the *Avsunviroidae*.

5.5 Structure of Viroid Quasispecies

Like many RNA viruses that infect plants or animals, individual viroids exist as complex populations of often closely related sequence variants in vivo. A number of studies have examined natural variability within viroid populations, and the

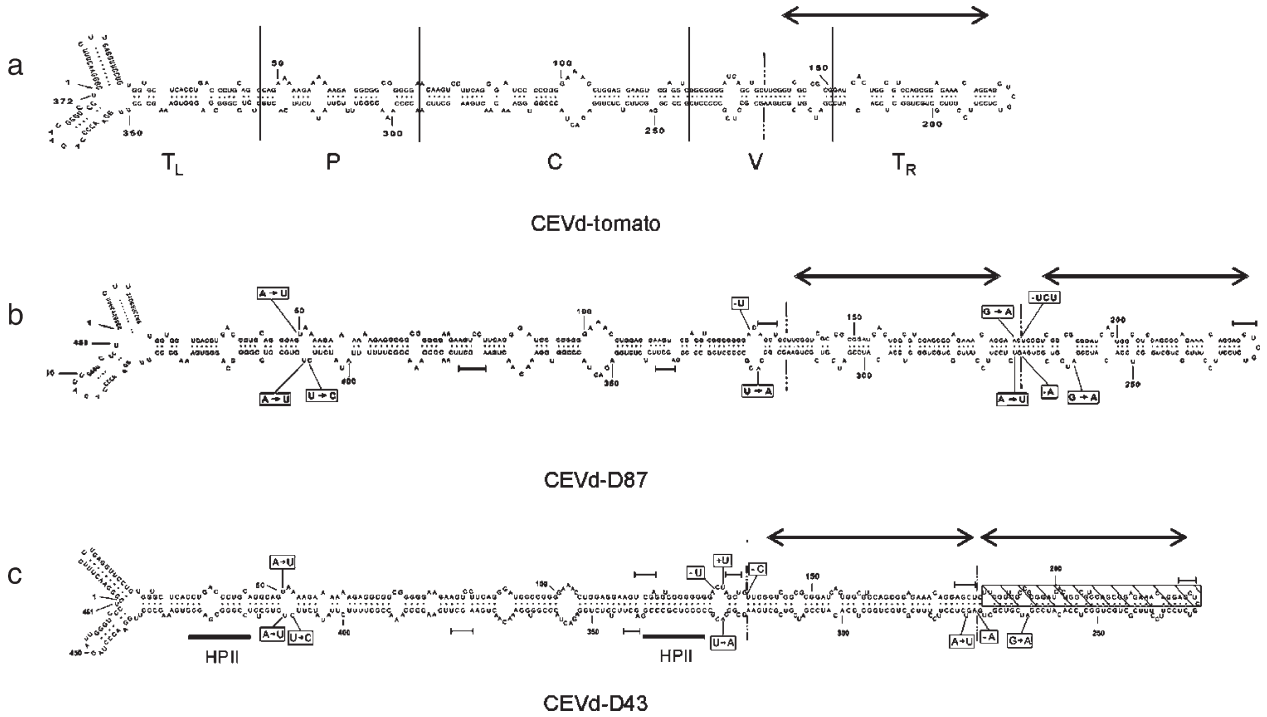

Fig. 5.4 Structures of selected CEVd sequence variants. **a** CEVd-tomato (the parental sequence); **b** CEVd D-87 containing a symmetric 87-nt duplication; and **c** CEVd D-43 containing an asymmetric 43-nt duplication. Nucleotides necessary to restore the symmetry of this asymmetric duplication are enclosed in a *cross-hatched box*. Positions of the five structural domains, terminal repeated regions (*horizontal arrows*), the AGCU tetrads (*horizontal bars*), and the stems of secondary hairpin II (*HPII*) are indicated. (Modified with permission from Fig. 2 in Szychowski et al. 2005)

Subviral RNA Database now contains the complete sequences of more than 1,100 viroid variants. In many cases, multiple sequence variants have been isolated from a single infected plant. Here, I focus on those viroids (e.g., HSVd, CEVd, PSTVd, and PLMVd) where (1) sequence information is abundant and (2) this information has been used to ask important questions about viroid–host interaction.

5.5.1 Origin of Viroid Diseases

As discussed by Diener (1996), several viroid diseases appear to be the result of chance transfer from an unknown (and possibly symptomless) wild host to large-scale monocultures of susceptible, genetically identical crop plants. The identity of these viroid reservoirs remains mysterious. Two groups of studies illustrate how the properties of different viroid variants have been used to explain the origin of specific viroid diseases.

The first example concerns hop stunt disease. Since its 1977 discovery as the causal agent of hop stunt disease, HSVd has been isolated from a wide variety of herbaceous and woody hosts (reviewed in Sano 2003). Two different groups have published phylogenetic analyses of known HSVd sequence variants (Kofalvi et al. 1997; Sano et al. 2001), and these can be divided into five clusters on the basis of the original host species:

1. A plum–peach–almond–apricot cluster
2. A German grapevine cluster
3. A general grapevine–hop cluster
4. A US citrus cachexia cluster
5. A general citrus–cucumber cluster

Figure 5.5 shows the neighbor-joining tree constructed by Sano et al. (2001), who used these relationships to propose a possible explanation for the origin of hop stunt disease in Japan.

Hops are native to the Mediterranean and Caucasus regions and were initially introduced to Japan from Europe and the USA at the end of the nineteenth century. Modern Japanese cultivars have been developed from these parental materials by crossing and selection. Hop stunt disease was first recognized in Nagano and Fukushima prefectures in the 1940s–1950s. So far, it has not been observed in either Europe or the USA. Thus, it would appear that the causal agent of hop stunt disease was introduced into hop from another host approximately 40–50 years after the introduction of hops into Japan. The close relationship between hop and grapevine isolates of HSVd shown in Fig. 5.5 strongly suggests that hop stunt disease originated when a sequence originally infecting grapevine invaded a new host (i.e., hop). Because phylogenetic analysis indicates that most HSVd-hop sequences form a single clade, the movement of HSVd-g from grapevine to hop must have been a unique (or at least an extremely rare) event. Hop gardens are often found adjacent to vineyards in Nagano and Fukushima prefectures, and farmers there seem to like

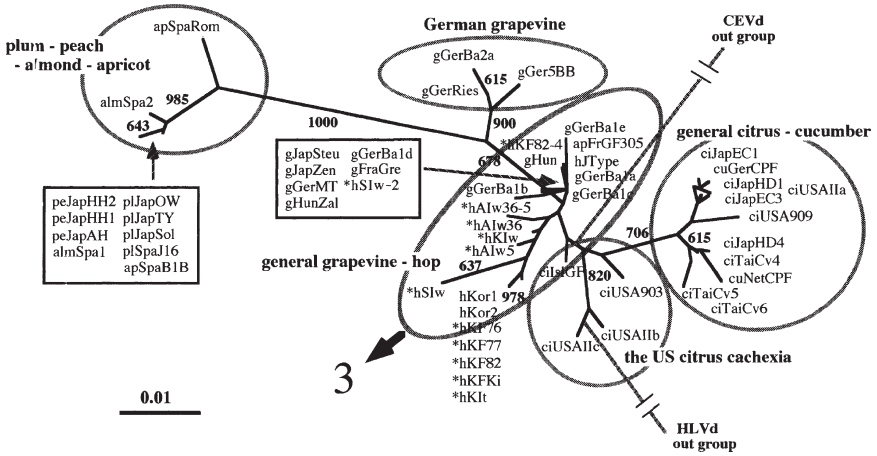

Fig. 5.5 Neighbor-joining analysis of HSVd variants recovered from a variety of hosts. CEVd and HLVd have been used as outgroups, and names of individual isolates are abbreviated. Lowercase letters indicate the original host plant: *h* hop, *g* grapevine, *ci* citrus, *pl* plum, *pe* peach, *al* almond, *ap* apricot, and *cu* cucumber; isolate names or numbers follow. Values on the nodes are confidence level estimates from bootstrap sampling (1,000 replicates). (Reprinted with permission from Sano et al. 2001 who sequenced the isolates denoted by *asterisks*)

planting a grapevine in a corner of their plowed field or hop garden. Once established, the viroid would be easily transmitted mechanically from hop to hop through contaminated tools because the titer in hops is more than 10 times higher than that in grapevine.

Another viroid that is 93–98% similar to AFCVd has been isolated from hops growing in Akita Prefecture, where apple cultivation is widespread. As described by Sano et al. (2004), phylogenetic analysis suggests that accumulation of host-specific sequence variation following isolation in different host species may be leading to the divergence of two viroid species from a common ancestor. A similar scenario has been proposed to explain several outbreaks of PSTVd in commercially grown tomatoes.

In 1990, Puchta et al. reported the isolation of a new strain of PSTVd from tomatoes being grown in two experimental greenhouses in the Netherlands. PSTVd-N contained only 356 nt and differed in sequence from the intermediate strain of PSTVd at 21 of 360 positions in a pairwise alignment (i.e., 5.8%). The same viroid was detected in healthy-looking pepino plants (*Solanum muricatum*) being grown in the same greenhouses; the seeds of these pepinos had been imported from New Zealand and Crete. Shortly thereafter, Behjatnia et al. (1996) reported the isolation of a very similar PSTVd variant from a wild *Solanum* sp. coinfected with two geminiviruses related to the Australian strain of *Tomato leaf curl virus* and growing in the Northern Territory of Australia. In recent years, infections of both field-grown (New Zealand) and greenhouse-grown (UK) tomatoes involving additional PSTVd variants related to PSTVd-N have been reported. Unlike the several

host-specific groupings observed with HSVd (Fig. 5.5), unpublished phylogenetic analyses indicate that the PSTVd variant population is relatively unstructured. The one exception is PSTVd-N and the small group of related variants that appear to have originated in Oceania. It appears likely that one or more *Solanum* spp. act as a symptomless reservoir from which these variants periodically emerge to infect tomato (Verhoeven et al. 2004).

A final example involves the possible origin of potato spindle tuber disease. As described by Martínez-Soriano et al. (1996), the cultivated potato (*Solanum tuberosum* L.) originated in the Andes of South America, but attempts to isolate PSTVd or a similar viroid from other solanaceous species growing in this area, including some known to have been used in potato breeding, have been unsuccessful. Solanaceous plants native to Mexico have also been used in potato breeding, and one such species (i.e., *Solanum cardiophyllum* Lindl.) was shown to harbor *Mexican papita viroid* (MPVd), a previously unknown pospiviroid most closely related to TPMVd. These authors suggest that potatoes grown in the USA may have become infected by chance transfer of MPVd or a related viroid from endemically infected wild solaneous germplasm that was imported from Mexico in the late nineteenth century in efforts to identify sources of resistance to late blight. For this scenario to be correct, sequence evolution must have proceeded very rapidly after transfer to potato, however. While the two most distantly related variants in the PSTVd species differ by approximately 6%, PSTVd and MPVd exhibit only 80% sequence identity. The original host of PSTVd has yet to be identified.

5.5.2 Molecular Conformation and Disease Induction

Mild and severe strains of PSTVd (as well as several other viroids) differ only slightly in nucleotide sequence (Dickson et al. 1979), and much effort has been expended to determine how as few as one or two substitutions can have such dramatic biological effects. Less widely appreciated is the variation in sequence complexity exhibited by different viroid isolates. Pioneering studies by Visvader and Symons (1985) showed that both mild and severe isolates of CEVd contain a complex mixture of sequence variants, and characterization of three phenotypically different isolates of PSTVd yielded similar results (Góra et al. 1994). Additional studies have described the structure of ASBVd (Rakowski and Symons 1989; Schnell et al. 2001), CDVd (Owens et al. 2000), GYSVd (Rigden and Rezaian 1993; Polivka et al. 1996), *Hop latent viroid* (HLVd; Matousek et al. 2001) and HSVd (Palacio-Bielsa et al. 2004) populations. Arguably, the quasispecies concept has had the greatest influence on studies of PLMVd pathogenicity.

As discussed in Sec. 5.4, theoretical studies indicate that the branched secondary structure of PLMVd is more susceptible to disruption by point mutations than the rod-like conformation of members of the *Pospiviroidae* (Sanjuán et al. 2006). Naturally occurring isolates of PLMVd are divided into severe or latent strains depending on the appearance of leaf symptoms on seedlings of the peach indicator host GF-305 under

greenhouse conditions. Extensive sequencing studies with PLMVd have been carried out by two different groups, and phylogenetic analysis of over 100 variants indicates that they can be divided into two groups sharing 85% sequence identity (Fig. 5.6). Group II variants include 99 variants showing 93% or more identity and can be further subdivided into three subgroups, each showing specific structural features. Variants in subgroup IIb appear to be the product of RNA recombination between members of subgroups IIA and IIC during coinfection (Hassen et al. 2007).

Taking advantage of this extensive sequence variation, both the Spanish and Canadian groups have used covariation analysis to refine their proposed secondary structure models for PLMVd. As shown in Fig. 5.1b, the right side of PLMVd contains a pseudoknot detected by nuclease probing in vitro (Bussière et al. 2000). Covariation analysis provides strong support for the existence of (1) several hairpin stems within this complex, highly branched region, and (2) a second pseudoknot that crosses the extended left side of the molecule containing the (+) strand (−) strand ribozymes (Ambros et al. 1998; Pelchat et al. 2000; Hassen et al. 2007). Induction of an extensive leaf chlorosis known as "peach calico" requires the presence of a 12–13-nt insertion in the hairpin loop containing positions 337 and 1. Although direct evidence is lacking, the presence of this insertion does not appear to interfere with pseudoknot formation; furthermore, the insertion spontaneously reappears upon prolonged passage of PLMVd molecules from which it had been deleted (Malfitano et al. 2003).

5.5.3 Stability of Natural Viroid Populations

Viroid isolates from woody perennials such as grapevine (Polivka et al. 1996) or citrus (Owens et al. 2000) may contain complex mixtures of sequence variants, likely reflecting the extended opportunity for sequence drift, RNA recombination, and/or reinfection. These naturally occurring viroid isolates (i.e., those not derived from cloned cDNAs) are usually quite stable when passaged in a single host under controlled conditions. On at least one occasion, however, a severe (and more rapidly replicating) sequence variant of PSTVd was observed to sweep through the quasispecies surrounding the intermediate green strain under house conditions (Gruner et al. 1995). Although the spontaneous conversion of PSTVd-Int to RG1 requires only three substitutions, mutational analysis suggests that PSTVd-Int occupies a comparatively steep peak within the fitness landscape (Owens et al. 2003).

In the case of CEVd, sequential passage of an isolate from a single sweet orange source through a series of alternate hosts (Etrog citron, *Gynura aurantiaca*, a *Lycopersicon esculentum* × *L. peruvianum* hybrid tomato, and disorganized hybrid tomato callus) resulted in a series of isolates differing in symptom expression, titer, and electrophoretic mobility (Semancik et al. 1993). Sequence analysis suggested the existence of a "tomato signature," a pattern of sequence changes shared by all isolates derived from hybrid tomato tissues. Two of these changes are located in the loop E motif of CEVd.

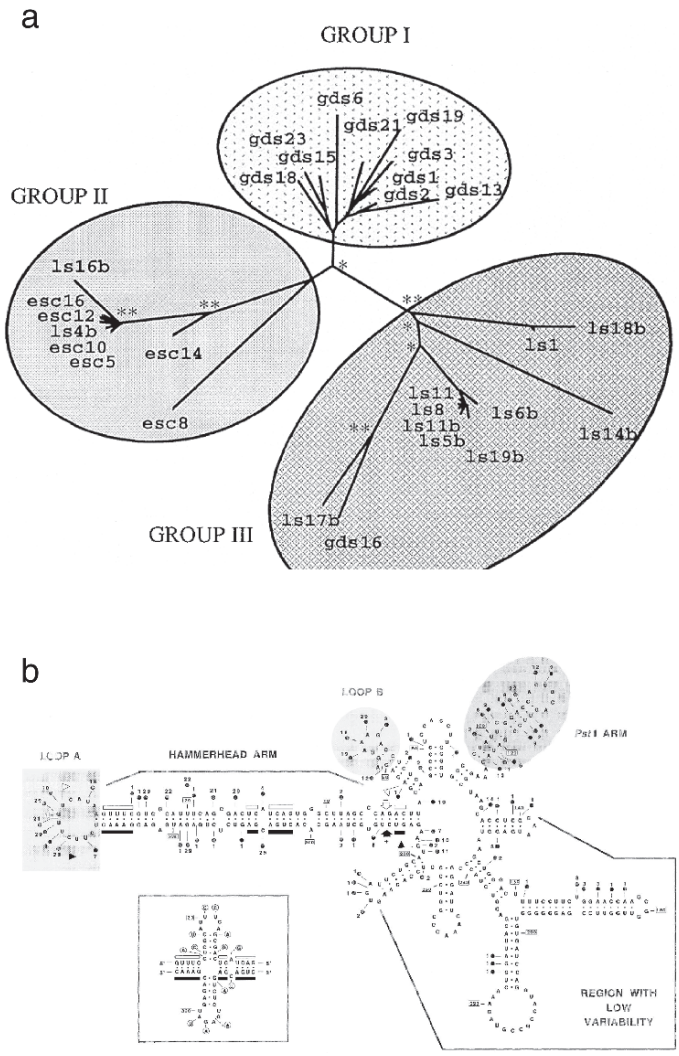

Fig. 5.6 Relationships among PLMVd sequence variants. **a** Dendrogram based on the genetic distances calculated between the 29 PLMVd sequence variants. Three groups are delineated, and *asterisks near nodes* indicate their statistical significance as determined by bootstrap analysis (100 replicates). *Double asterisks* node detected in 90–100% of replicates; *single asterisks* node detected in more than 50% of replicates. **b** Distribution of polymorphic positions along the secondary structure of PLMVd. Sequence changes (substitutions, insertions, or deletions) are marked by *circles*, and the number of variants in which each specific position is affected is indicated. Regions involved in forming (+) and (−) strand hammerhead structures are flanked by *flags*, the conserved nucleotides present in most natural hammerhead structures are indicated by *bars*, and the self-cleavage sites are indicated by *arrows*. *Black symbols* and *white symbols* refer to (+) and (−) polarities, respectively. The reference sequence is marked every 20 nt with *boxed numbers*. *Inset*: Alternative cruciform conformation for the hammerhead arm in the most stable secondary structure. (Reproduced with permission from Ambros et al. (1998))

Transfer of PSTVd from tomato (its most common experimental host) to tobacco (*Nicotiana tabacum* L.) results in the appearance of new sequence variants. Some of these variants contain a C/U substitution at position 259 (Wassenegger et al. 1996); another contained a U/A substitution at position 257 (Zhu et al. 2002). Both of these changes are located within the conserved core of loop E motif in PSTVd. A subsequent study by Zhong et al. (2006) considers the effects of these and other mutations in loop E on PSTVd replication and transport in light of the need to maintain its overall conformation. Because loop E motifs in cellular RNAs are well known to serve as important protein binding sites, it is easy to imagine how sequence changes in this portion of PSTVd or CEVd could result in dramatic shifts in quasispecies composition.

Thermotherapy is widely used to produce virus-free plant material (Mink et al. 1998), but the underlying mechanisms are not understood. Possibilities include (1) inactivation of intact virus particles and/or (2) an inhibition of virus replication/transport that allows the actively growing shoot apex to "outgrow" the much-reduced rate of virus spread. Viroid populations are also sensitive to heat stress. Using a combination of temperature gradient gel electrophoresis and DNA heteroduplex analysis to examine the effect of thermal stress on viroid populations in HLVd-infected hops (Matoušek et al. 2001) and PSTVd-infected *N. benethamiana* (Matoušek et al. 2004), Matoušek et al. showed that heat treatment is followed by a significant increase in sequence polymorphism (Fig. 5.7). Many variants contained multiple mutations, suggesting an accumulation of mutations during successive replication cycles; furthermore, approximately two thirds of all sequence changes in HLVd or PSTVd were located in the left side of secondary structure.

All HLVd variants tested were infectious, and all gave rise to complex progeny populations in hop. Most interesting were the populations of HLVd or PSTVd "thermomutants" observed after transfer to alternative hosts. Although no evidence of systemic infection could be detected when *Arabidopsis thaliana* was biolistically inoculated with PSTVd-Int strain, biolistic inoculation with a population of PSTVd thermomutants passaged through the intermediate host *Raphanus sativus* was followed by progeny accumulation to levels approximately 0.3% of those observed in tomato (i.e., detectable only by reverse-transcription PCR). Many of these molecules contained sequence changes in the upper portion of the central conserved region (a region known to play a key role in replication/cleavage), but these changes were not stably maintained when the individual variants were returned to tomato. Similar experiments involving transfer of HLVd thermomutants to tomato or *N. benthamiana* resulted in progeny accumulation to levels detectable by molecular hybridization (Matoušek 2003). Clearly, much remains to be learned about how the genetic diversity generated by thermal (and other possibly other environmental) stress may contribute to changes in viroid host range and speciation.

At high mutation rates, the fittest organisms are not necessarily those that replicate most quickly but rather those that show the greatest robustness against the generally deleterious effects of mutations. This phenomenon is sometimes termed "survival of the flattest." A recently published study by Codoñer et al. (2006) compared the ability of two viroids to compete against one another in coinfected chrysanthemum plants. One of the viroids tested (i.e., *Chrysanthemum stunt viroid*,

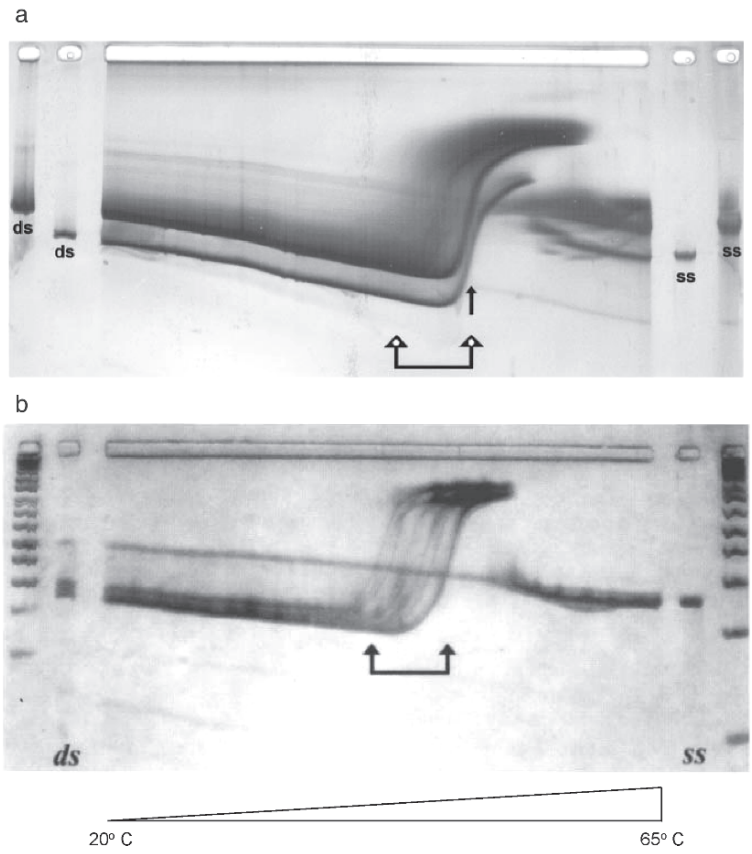

Fig. 5.7 Heat-induced accumulation of HLVd sequence variants. **a** Temperature gradient gel electrophoresis (TGGE) analysis of double-stranded HLVd complementary DNAs (cDNAs) prepared from viroid recovered from control and heat-treated hops. The control sample was loaded first; after 15 min of electrophoresis at 10°C, the sample from heat-treated plants was applied. The melting temperature of the major HLVd variant is indicated by the *single arrow*; additional cDNA species with melting temperatures in the range 42–48°C are indicated by the *double arrows*. These variants are present only in HLVd cDNA from heat-treated hop. **b** TGGE analysis of an HLVd population following heat treatment and three passages in *Nicotiana benthamiana*. HLVd cDNA was synthesized by reverse-transcription PCR, denatured, and allowed to form heteroduplexes before analysis. The range of melting temperatures of HLVd heteroduplexes is indicated by the *arrows*. A temperature gradient of 20–65°C was used for both analyses, and nucleic acids were visualized by silver staining. Double- and single-stranded portions of each gel pattern are indicated by *ds* and *ss*, respectively. (Modified with permission from Matousek et al. 2001 and Matousek 2003)

CSVd) replicates rapidly in the nucleus, and the progeny are genetically homogeneous. *Chrysanthemum chlorotic mottle viroid* (CChMVd), in contrast, replicates at a slower rate in the chloroplast, and progeny populations are highly variable. Under optimal growth conditions, CSVd outcompetes CChMVd as predicted by the Darwinian "survival of the fittest" paradigm. When the mutation rate is increased by exposure to UV radiation, however, the slowly growing (but more robust)

CChMVd predominates. These experimental results are consistent with computer simulations of competing viroid populations.

5.5.4 Generation of Populations From Individual Viroid Variants

Several different approaches have been used to monitor the genetic stability of individual viroid sequence variants in vivo. These include (1) inoculation with recombinant plasmid DNAs (Góra-Sochacka et al. 1997; Ambrós et al. 1999), (2) *Agrobacterium*-mediated introduction of nondisarmed recombinant Ti plasmids (Hammond 1994), and (3) *Agrobacterium*-mediated plant transformation (Wassenegger et al. 1994). When working with highly debilitated variants that are only weakly infectious, constitutive expression from an integrated transgene provides an effective means to detect the rare events that can restore viroid infectivity.

Góra et al. (1994) used a reverse-transcription PCR strategy to generate, in a single step, infectious full-length cDNAs from three phenotypically dissimilar isolates of PSTVd. When this method was applied to a "mild" isolate, only a single sequence variant was recovered. "Intermediate" and "severe" isolates yielded three and four variants, respectively. Not all of the variants recovered from the severe isolate produced severe symptoms when inoculated onto Rutgers tomato; thus, the presence of milder variants in a mixed inoculum may be masked by variants that are more severe. Follow-up studies (Góra-Sochacka et al. 1997) revealed that many of these naturally occurring PSTVd sequence variants were unstable when inoculated alone – sometimes disappearing within a single 5–6-week passage in tomato. This finding supports one of the basic tenets of the quasispecies theory, that mixtures of variants can complement each other, and hence the whole population is in essence a single entity analogous to an individual with thousands of alleles rather than just two. In most cases, the new variants detected induced symptoms that were less severe than those of the parent. The number of sequence changes detected in both studies was relatively limited, confined almost exclusively to the pathogenicity and variable domains (Fig. 5.1) with only a few changes located in the terminal right domain.

A similar study carried out by Ambrós et al. (1999) with PLMVd yielded very different results. As shown in Fig. 5.6, sequences comprising the PLMVd species can be divided into three groups. When GF-305 peach seedlings were slash-inoculated with a series of four cDNAs derived from PLMVd variants of differing pathogenicities, the resulting progeny contained a total of 33 sequence variants. The structure of the viroid populations derived from single PLMVd sequences differed according to the observed phenotype, and mutation frequencies were considerably higher than those reported for PSTVd (see above). While Malfitano et al. (2003) have shown that spontaneous mutational changes in PLMVd can include the appearance of a 12–13-nt insertion in the loop closing the hammerhead arm (Fig. 5.1b), the majority of changes observed were single nucleotide substitutions.

As discussed by the authors, this rapid evolution of PLMVd may be due to the involvement of a different (more error-prone?) RNA polymerase in its replication that contributes to the fluctuating symptoms observed with naturally occurring severe isolates of PLMVd. Polymorphisms appear to be limited by the need to maintain ribozyme activity and certain other structural and functional elements of PLMVd.

One important advantage of screening assays that involve mechanical inoculation of full-length viroid cDNAs or RNA transcripts is that the results are usually available within a few weeks. Many point mutations in PSTVd and other viroids, however, appear to abolish infectivity via mechanical inoculation. In some cases, these mutations have been shown to inhibit replication; in other cases, cell-to-cell or long-distance transport is disrupted (Qi et al. 2004). Placement of appropriately designed PSTVd cDNAs under the control of a constitutive or tissue-specific promoter followed by introduction of the resulting transgene into the nuclear genome of the host has made it possible to study the process by which severely debilitated variants regain systemic infectivity. No comparable experiments involving *Avsunviroidae* mutants and plastid transformation have been reported.

PSTVd mutants with changes in the terminal loops are not infectious when tomato cotyledons are mechanically inoculated with either cDNA or RNA transcripts (Hammond and Owens 1987). Nevertheless, when *Agrobacterium*-mediated inoculation was used to introduce a PSTVd mutant containing several changes in the right terminal loop into stem tissue, both replicative intermediates and viroid progeny could be recovered from gall and root tissues (Hammond 1994). Progeny were only occasionally detected in newly developing leaves, and sequence analyses revealed that the initial mutations in PSTVd-R were not stably maintained. Mutations in the right terminal loop do not abolish the ability of the PSTVd to replicate; rather, they appear to alter interaction with specific host components (e.g., VirP1; Gozmanova et al. 2003) and restrict the normal pattern of intercellular transport. Their phenotype resembles that of mutations elsewhere in the molecule that regulate transport across the bundle sheath–mesophyll boundary (Qi et al. 2004).

In a further refinement of this transgene-based approach, Wassenegger et al. (1994) used transformed tobacco to study the evolution of a second noninfectious PSTVd mutant *in planta*. The mutant contained a 9-nt deletion located immediately adjacent to the upper portion of the central conserved region in PSTVd-Int, and 20 independent primary transformants were screened by northern hybridization. Only one plant proved to be PSTVd-infected, and sequence analyses of the progeny revealed a surprising result. Instead of the 350-nt PSTVd variant encoded by the transgene, the infectious replicon contained only 341 nt. In addition to a complementary 9-nt deletion that restored the integrity of the rod-like native structure, the progeny also contained five additional point mutations. One of these point mutations was the same C259/U change in the loop E motif that is found in tobacco mechanically inoculated with PSTVd isolate KF440-2 (Wassenegger et al. 1996). Because the defective PSTVd RNA is continually produced in every cell nucleus and is available for RNA–RNA replication catalyzed by RNA polymerase II, it can be continuously mutated with selection acting at each step in the replicative pathway.

Unfortunately, the stochastic nature of this "repair" process makes it difficult to capture in its entirety.

5.6 Origin and Evolution of Viroids

Several possible origins for viroids have been proposed. Viroids could be primitive ancestors or highly degenerate derivatives of conventional viruses, but as discussed by Diener (1989), their unusual molecular structure and biological properties together with a lack of sequence similarity. Evolution argue against this possibility, of viroids from transposable elements, plasmids, or introns has also been proposed. At the present time, the balance of evidence suggests that viroids could represent "relics of precellular RNA evolution", and several reviews exploring this area have been published (Diener 1996, 2001, 2003).

In essence, the argument for viroid origin in the RNA world is straightforward: RNA is the only known biological macromolecule that can function as both genotype and phenotype, allowing evolution to occur in the absence of DNA or protein. As described by Diener (1989), a simple hammerhead ribozyme similar to those found in ASBVd and other members of the *Avsunviroidae* is theoretically capable of performing all the polymerization, cleavage, and ligation steps required for viroid replication. The circular structure of the viroid genome and the rolling-circle mechanism of replication eliminate the need for replication to initiate at a specific site; likewise, the apparently polyploid nature of viroid genomes (Juhasz et al. 1988) would have favored their survival under the error-prone conditions of the prebiotic world.

Figure 5.8 compares the structure of the first intermediate in the PSTVd cleavage-ligation pathway (Baumstark et al. 1997) with those of the hammerhead and hairpin ribozymes.The upper portion of the pospiviroid central conserved region contains a short sequence motif (GAAA) that is also present in hammerhead ribozymes (Diener 1989). Moving from the level of RNA primary/secondary structure to tertiary structure, however, one can see that pospiviroids share an even greater degree of similarity with ribozymes. The hairpin ribozyme found in (−) strand satellite RNA of *Tobacco ringspot virus* contains two domains that interact in the transition state. Like the central conserved region of pospiviroids, the loop B domain of the hairpin ribozyme also contains a loop E motif. Loop E motifs are found in many different contexts, often acting as "organizers" for multihelix loops in ribosomal RNAs (Leontis and Westhof 1998; Hendrix et al. 2005); in the case of the hairpin ribozyme, a conformational change in the loop E motif accompanies domain docking and is essential for catalysis (Hampel and Burke 2001). In addition to sequence-specific cleavage, the hairpin ribozyme also catalyzes RNA ligation. Recent experimental work with the hammerhead and hairpin ribozymes suggests that they have are more similar than previously thought (Burke 2002), and the possibility that viroids are "relics of precellular evolution" continues to be very much alive.

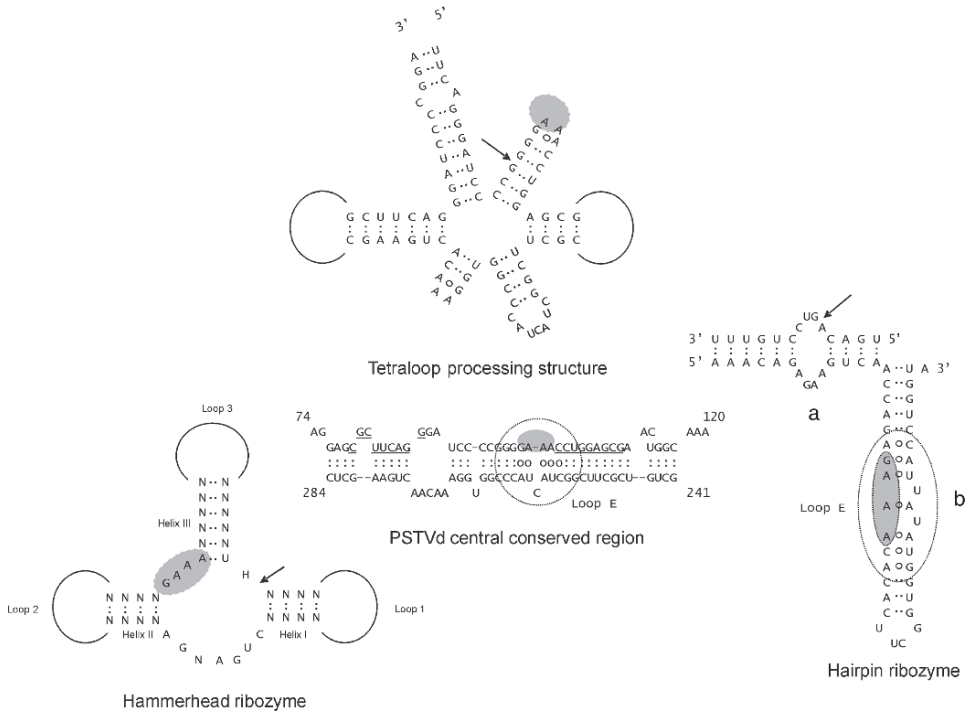

Fig. 5.8 Possible evolutionary relationships between viroids and ribozymes. The central conserved region of PSTVd and other *Pospiviroidae* is defined by a pair of inverted repeats (*underlined*) and contains a loop E motif (*dashed circle*). Loop E is not an internal loop but rather a highly structured array of non-Watson–Crick base pairs (denoted by *open circles*). Pospiviroids and hammerhead ribozymes (*lower left*) both contain a short conserved GAAA (shaded) sequence, suggesting to Diener (1989) the possible existence of a common ancestor in the prebiotic RNA world. The presence of a loop E motif in the hairpin ribozyme (*lower right*) provides additional support for such a relationship. The processing structure involved in the initial PSTVd cleavage (*above*) does not contain a loop E motif, however. Cleavage sites in the respective RNAs are denoted by *arrows*

5.7 Concluding Remarks

The now-routine determination of the nucleotide sequences of entire microbial genomes has led to the development of *synthetic biology*, a new field in which it is possible to ask, "How few parts (i.e., proteins and RNAs) does it take to construct a cell?" Recent experimental studies involving *Mycoplasma genitalium* (an obligate parasite requiring relatively little adaptive capability) suggest that the answer is 43 RNA-encoding and 400 or fewer protein-coding genes (Glass et al. 2006; Koonin 2000). The genomes of known viroids are approximately 1,000–2,000 times smaller than those of mycoplasmas, ranging in size from 246 to 401 nt. Has the time now come to ask, "What is the minimal size of the viroid genome?" Perhaps so, for much of the necessary preliminary information is either already in hand or is currently the subject of active investigation.

The existence of five structural domains in members of the *Pospiviroidae* was first proposed more than 20 years ago on the basis of the comparison of only eight different viroids (Keese and Symons 1985), and much effort has since been devoted to probing the biological role(s) of individual domains by a variety of techniques. The Subviral RNA Database now contains the complete sequences of more than 1,100 viroid variants, information that can be used to compare the evolutionary constraints operating on viroids. A series of RNA structural studies carried out by Detlev Riesner and colleagues and largely focused on PSTVd have identified several alternative interactions such as secondary hairpin II that are essential for viability (reviewed in Steger and Riesner 2003). Multimeric PSTVd RNAs undergo a complicated series of structural rearrangements during conversion into monomeric progeny but, a recent study by Schrader et al. (2003) clearly showed that a "miniRNA" containing only sequences from the central domain of PSTVd can be efficiently processed in a nuclear extract. Interactions with host protein(s) are almost certainly required for other steps in the replication pathway as well. For example, interaction of PSTVd with Virp1, an RNA-binding protein from tomato containing a nuclear localization signal, involves a discrete sequence/structural motif in the right terminal loop (Martínez de Alba et al. 2003). Following transport into the nucleus, synthesis of PSTVd (–) strands by RNA polymerase II initiates in the left terminal loop at either position 359 or position 1 (Kolonko et al. 2006).

Several studies have described the expression of potentially infectious viroid RNAs from transgenes inserted into the nuclear genomes of both host and nonhost plant species. This is a particularly powerful experimental strategy, because it allows time for an initial variant that is only very weakly infectious to mutate and evolve in vivo. As described earlier, Wassenegger and colleagues have used this strategy to demonstrate (1) a role for the loop E motif in determining PSTVd host range as well as (2) the evolution of a PSTVd variant lacking a large portion of the pathogencity domain. Such an error-correction mechanism will be very important in future efforts to design a minimal viroid genome capable of autonomous replication, because much about viroid–host interaction remains only poorly understood. Viroid replication appears to be relatively resistant to RNA silencing (Wang et al. 2004) but unanticipated interactions between individual

structural domains provide a particular challenge. Sequence changes in all but the terminal right domain have been shown to influence symptom development (Sano et al. 1992; Qi and Ding 2003), and a series of five sequence changes in pathogenicity and variable domains of PSTVd disrupts transport from the bundle sheath to the leaf mesophyll (Qi et al. 2004). Even small deletions are likely to have unpredictable effects.

References

Ambrós S, Hernandez C, Desvignes JC, Flores R (1998) Genomic structure of three phenotypically different isolates of peach latent mosaic viroid: implications of the existence of constraints limiting the heterogeneity of viroid quasispecies. J Virol 72:7397–7406

Ambrós S, Hernandez C, Flores R (1999) Rapid generation of genetic heterogeity in progenies from individual cDNA clones of peach latent mosaic viroid in its natural host. J Gen Virol 80:2239–2252

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

Behjatnia SAA, Dry IB, Krake LR, Condé BD, Connelly MI, Randles JW, Rezaian MA (1996) New potato spindle tuber viroid and tomato leaf curl geminivirus strains from a wild *Solanum* sp. Phytopathology 86:880–886

Branch AD, Robertson HD (1984) A replication cycle for viroids and other small infectious RNA's. Science 223:450–455

Branch AD, Benenfeld BJ, Baroudy B.M, Wells FV, Gerin JL, Robertson HD (1985) Ultraviolet light-induced cross-linking 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

Burke JM (2002) Hairpin and hammerhead ribozymes: how different are they? Biochem Soc Trans 30:1115–1118

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

Codoñer FM, Daròs J-A, Solé RV, Elena SF (2006) The fittest versus the flattest: Experimental confirmation of the quasispecies effect with subviral pathogens. PLoS Pathog 2:1187–1193

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

Daros JA, Marcos JF, Hernandez C, Flores R (1994) Replication of avocado sunblotch viroid: evidence for a symmetric pathway with two rolling circles and hammerhead ribozyme processing. Proc Natl Acad Sci USA 91:12813–12817

Daros J-A, Elena SF, Flores R (2006) Viroids: an Ariadne's thread into the RNA labyrinth. EMBO Rep 7:593–588

Dickson E, Robertson HD, Niblett CL, Horst RK, Zaitlin M (1979) Minor differences between nucleotide sequences of mild and severe strains of potato spindle tuber viroid. Nature 277:60–62

Diener TO (1971) Potato spindle tuber "virus" IV. A replicating, low molecular weight RNA. Virology 45:411–428

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

Diener TO (1996) Origin and evolution of viroids and viroid-like satellite RNAs. Virus Genes 11:119–131

Diener TO (2001) The viroid: biological oddity or evolutionary fossil? Adv Virus Res 57:137–184

Diener TO (2003) Discovering viroids – a personal perspective. Nat Rev Microbiol 1:75–80

Ding B, Itaya A (2007) Viroid: a useful model for studying the basic principles of infection and RNA biology. Mol Plant Microbe Interact 20:7–20

Ding B, Itaya A, Zhong XH (2005) Viroid trafficking: a small RNA makes a big move. Curr Opin Plant Biol 8:606–612

Elena SF, Dopazo J, Flores R, Diener T, Moya A (1991) Phylogeny of viroids, viroidlike satellite RNAs, and the viroidlike domain of hepatitis δ virus RNA. Proc Natl Acad Sci USA 88:5361–5364

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, Hernandez C, Martinez de Alba AE, Daros J 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. VIIII report of the International Committee on Taxonomy of Viruses. Elsevier/Academic, London, pp 1145–1159

Glass JI, Assad-Garcia N, Alperovich N, Yooseph S, Lewis MR, Maruf M, Hutchison CA 3rd, Smith HO, Venter JC (2006) Essential genes of a minimal bacterium. Proc Natl Acad Sci USA 103:425–430

Góra A, Candresse T, Zagórski W (1994) Analysis of the population structure of three phenotypically different PSTVd isolates. Arch Virol 138:223–245

Góra-Sochacka A, Kierzek A, Candresse T, Zágorski W (1997) The genetic stability of potato spindle tuber viroid (PSTVd) molecular variants. RNA 3:68–74

Gozmanova M, Denti MA, Minkov IN, Tsagris M, Tabler, M (2003) Characterization of the RNA motif responsible for the specific interaction of potato spindle tuber viroid RNA (PSTVd) and the tomato protein Virp1. Nucleic Acids Res 31:5534–5543

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

Gruner R, Fels A, Qu F, Zimmat R, Steger G, Riesner D (1995) Interdependence of pathogenicity and replicability with potato spindle tuber viroid. Virology 209:60–69

Hadidi A, Flores R, Randles JW, Semancik JS (eds) (2003) Viroids. CSIRO, Collingwood

Hammond RW (1994) *Agrobacterium*-mediated inoculation of PSTVd cDNAs onto tomato reveals the biological effect of apparently lethal mutations. Virology 201:36–45

Hammond RW, Owens RA (1987) Mutational analysis of potato spindle tuber viroid reveals complex relationships between structure and infectivity. Proc Natl Acad Sci USA 84:3967–3971

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

Hampell KJ, Burke JM (2001) A conformational change in the "loop E-like" motif of the hairpin ribozyme is coincidental with domain docking and is essential for catalysis. Biochemistry 40:3723–3729

Haseloff J, Mohamed NA, Symon RH (1982) Viroid RNAs of cadang-cadang disease of coconuts. Nature 299:316–321

Hassen IF, Massart S, Motard J, Roussel S, Kummert J, Fakhfakh H, Marrakchi M, Perreault JP, Jijakli MH (2007) Molecular features of new peach latent mosaic viroid variants suggest that recombination may have contributed to the evolution of this infectious RNA. Virology 360:50–57

Hendrix DK, Brenner SE, Holbrook SR (2005) RNA structural motifs: building blocks of a modular biomolecule. Q Rev Biophys 38:221–243

Jenkins GM, Woelk CH, Rambaut A, Homes EC (2000) Testing the extent of sequence similarity among viroids, satellite RNAs, and the hepatitis delta virus. J Mol Evol 50:98–102

Juhasz A, Hegyi H, Solymosy F (1988) A novel aspect of the information content of viroids. FEBS Biochim Biophys Acta 950:455–458

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

Kofalvi SA, Marcos JF, Canizares MC, Pallas V, Candresse T (1997) Hop stunt viroid (HSVd) sequence variants from *Prunus* species: evidence for recombination between HSVd isolates. J Gen Virol 78:3177–3186

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

Koltunow AM, Rezaian MA (1988) Grapevine yellow speckle viroid: structural features of a new viroid group. Nucleic Acids Res 16:849–864

Koonin EV (2000) How many genes can make a cell: the minimal-gene-set concept. Annu Rev Genomics Hum Genet 1:99–116

Leontis NB, Westhof E (1998) A common motif organizes the structure of multi-helix loops in 16S and 23 S ribosomal RNAs. J Mol Biol 283:571–583

Malfitano M, Di Serio F, Covelli L, Ragozzino A, Hernández C, Flores R (2003) Peach latent mosaic viroid variants inducing peach calico (extreme chlorosis) contain a characteristic insertion that is responsible for this symptomatology. Virology 313:492–501

Martínez de Alba AE, Sägasser 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

Martínez-Soriano JP, Galindo-Alonso J, Maroon CJ, Yucel I, Smith DR, Diener TO (1996) Mexican papita viroid: putative ancestor of crop viroids. Proc Natl Acad Sci USA 93:9397–9401

Matoušek J (2003) Hop latent viroid (HLVd) microevolution: an experimental transmission of HLVd "thermomutants" to solanaceous species. Biol Plant 46:607–610

Matoušek J, Patzak J, Orctova L, Schubert J, Vrba L, Steger G, Riesner D (2001) The variability of hop latent viroid as induced upon heat treatment. Virology 287:349–358

Matoušek J, Orctova L, Steger G, Skopek J, Moors M, Dedic P, Riesner D (2004) Analysis of thermal stress-mediated PSTVd variation and biolistic inoculation of progeny of viroid "thermomutants" to tomato and *Brassica* species. Virology 323:9–23

Mink GI, Wample R, Howell WE (1998) Heat treatment of perennial plants to eliminate phytoplasmas, viruses and viroids while maintaining plant survival. Breeding for resistance to plant viruses. In: Hadidi A, Kheterpal RK, Koganezawa H (eds) Plant virus disease control. APS, St Paul, pp 332–345

Navarro JA, Vera A, Flores R (2000) A chloroplastic RNA polymerase resistant to tagetitoxin is involved in replication of avocado sunblotch viroid. Virology 268:218–225

Owens RA, Yang G, Gundersen-Rindal D, Hammond RW, Candresse T, Bar-Joseph M (2000) Both point mutation and RNA recombination contribute to the sequence diversity of citrus viroid III. Virus Genes 20:243–252

Owens RA, Thompson SM, Kramer M (2003) Identification of neutral mutants surrounding two naturally-occurring variants of *Potato spindle tuber viroid*. J Gen Virol 84:751–756

Palacio-Bielsa A, Romero-Durban J, Duran-Vila N (2004) Characterization of citrus HSVd isolates. Arch Virol 149:537–552

Pelchat M, Levesque D, Ouellet J, Laurendeau S, Levesque S, Lehoux J, Thompson DA, Eastwell KC, Skreczkowski LJ, Perrault JP (2000) Sequencing of peach latent mosaic viroid variants from nine North American peach cultivars shows that this RNA folds into a complex secondary structure. Virology 271:37–45

Polivka H, Staub U, Gross HJ (1996) Variation of viroid profiles in individual grapevine plants: novel grapevine yellow speckle viroid 1 mutants show alterations of hairpin I. J Gen Virol 77:155–161

Puchta H, Herold T, Verhoeven K, Roenhorst A, Ramm K, Schmidt-Puchta W, Sänger HL (1990) A new strain of potato spindle tuber viroid (PSTVd-N) exhibits major sequences differences as compared to all other PSTVd strains sequenced so far. Plant Mol Biol 15:509–511

Qi Y, Ding B (2003) Inhibition of cell growth and shoot development by a specific nucleotide sequence in a noncoding viroid RNA. Plant Cell 15:1360–1374

Qi Y, Pélissier T, Itaya A, Hunt E, Wassenegger M, Ding B (2004) Direct role of a viroid RNA motif in mediating directional RNA trafficking across a specific cellular boundary. Plant Cell 16:1741–1752

Rakowski AG, Symons RH (1989) Comparative sequence studies of variants of avocado sunblotch viroid. Virology 173:352–356

Rigden JE, Rezaian MA (1993) Analysis of sequence variation in grapevine yellow speckle viroid 1 reveals two distinct alternative structures for the pathogenic domain. Virology 193:474–477

Sanjuán R, Forment J, Elena SF (2006) *In silico* predicted robustness of viroids (sic) RNA secondary structures. I. The effect of single mutations. Mol Biol Evol 23:1427–1436

Sano T (2003) Hop stunt viroid. In: Hadidi A, Flores R, Randles JW, Semancik JS (eds) Viroids. CSIRO, Collingwood, pp 207–212

Sano T, Candresse T, Hammond RW, Diener TO, Owens RA (1992) Identification of multiple structural domains regulating viroid pathogenicity. Proc Natl Acad Sci USA 89:10104–10108

Sano T, Mimura R, Ohshima K (2001) Phylogenetic analysis of hop and grapevine isolates of hop stunt viroid supports a grapevine origin for hop stunt disease. Virus Genes 22:53–59

Sano T, Yoshida H, Goshono M, Monma T, Kawasaki H, Ishizaki K (2004) Characterization of a new viroid strain from hops: evidence for viroid speciation by isolation in different host species. J Gen Plant Pathol 70:181–187

Schindler I-M, Mühlbach H-P (1992) Involvement of nuclear DNA-dependent RNA polymerases in potato spindle tuber viroid replication: a re-evaluation. Plant Sci 84:221–229

Schnell RJ, Olano CT, Kuhn DN (2001) Detection of avocado sunblotch viroid variants using fluorescent single-strand conformation polymorphism analysis. Electrophoresis 22:427–432

Schrader O, Baumstark T, Riesner D (2003) A mini-RNA containing the tetraloop, wobble-pair and loop E motifs of the central conserved region of potato spindle tuber viroid is processed into a minicircle. Nucleic Acids Res 31:988–998

Schultz ES, Folsom D (1923) Transmission, variation, and control of certain degeneration diseases of Irish potatoes. J Agric Res 25:43–118

Schuster P (2001) Evolution *in silico* and *in vitro*: the RNA model. Biol Chem 382:1301–1314

Semancik JS, Szychowski JA, Rakowski AG, Symons RH (1993) Isolates of citrus exocortis viroid recovered by host and tissue selection. J Gen Virol 74:2427–2436

Szychowski JA, Vidalakis G, Semancik JS (2005) Host-directed processing of *Citrus exocortis viroid*. J Gen Virol 86:473–477

Steger G, Riesner D (2003) Molecular characteristics. In: Hadidi A, Flores R, Randles JW, Semancik JS (eds) Viroids. CSIRO, Collingwood, pp 15–29

Tabler M, Tsagris M (2004) Viroids: petite RNA pathogens with distinguished talents. Trends Plant Sci 9:339–348

Verhoeven JTJ, Jansen C, Willemen TM, Kox LFF, Owens RA, Roenhorst JW (2004) Natural infections of tomato by *Citrus exocortis viroid*, *Columnea latent viroid, Potato spindle tuber viroid* and *Tomato chlorotic dwarf viroid*. Eur J Plant Pathol 110:823–831

Visvader JE, Symons RH (1985) Eleven new sequence variants of citrus exocortis viroid and the correlation of sequence with pathogencity. Nucleic Acids Res 13:2907–2920

Wang MB, Bian XY, Wu LM, Liu LX, Smith NA, Isenegger D, Wu RM, Masuta C, Vance VB, Watson JM, Rezaian A, Dennis ES, Waterhouse PM (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 R (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, Sänger HL (1994) An infectious RNA replicon evolved from an *in vitro*-generated non-infectious viroid deletion mutant via a complementary deletion *in vivo*. EMBO J 13:6172–6177

Wassenegger M, Spieker RL, Thalmeir S, Gast FU, Riedel L, Sänger HL (1996) A single nucleotide substitution converts potato spindle tuber viroid (PSTVd) from a noninfectious to an infectious RNA for *Nicotiana tabacum*. Virology 226:191–197

Zhong X, Leontis N, Qian S, Itaya A, Qi Y, Boris-Lawrie K, Ding B (2006) Tertiary structural and functional analyses of a viroid RNA motif by isostericity matrix and mutagenesis reveal its essential role in replication. J Virol 80:8566–8581

Zhu Y, Qi Y., Xun R, Owens R, Ding B (2002) Movement of potato spindle tuber viriod reveals regulatory points of phloem-mediated RNA traffic. Plant Physiol 130:138–146