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Review

Viroids: unusual small pathogenic RNAs

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Viroids are small (about 300 nucleotides), single-stranded, circular, non-encapsidated pathogenic RNA molecules. They do not code for proteins and thus depend on plant host enzymes for their replication and other functions. They induce plant diseases by direct interaction with host factors but the mechanism of pathogenicity is still unknown. They can alter the expression of selected plant genes important for growth and development. Viroids belong to two families, the *Avsunviroidae* and the *Pospiviroidae*. Viroids of the *Avsunviroidae* family adopt a branched or quasi rod-like secondary structure in their native state. Members of the *Pospiviroidae* family adopt a rod-like secondary structure. In such native structures five structural/functional domains have been identified: central (C), pathogenicity, variable and two terminal domains. The central conserved region (CCR) within the C domain characterizes viroids of the *Pospiviroidae*. Specific secondary structures of this region play an important role in viroid replication and processing. Viroids of the *Avsunviroidae* family lack a CCR but possess self-cleaving properties by forming hammerhead ribozyme structures; they accumulate and replicate in chloroplasts, whereas members of the *Pospiviroidae* family have a nuclear localization. Viroid replication occurs *via* a rolling circle mechanism using either a symmetric or asymmetric pathway in three steps, RNA transcription, processing and ligation.

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Abbreviations: C domain, central domain; CCR, central conserved region; ExM, extended middle structure; HP, hairpin; NEP, nuclear-encoded chloroplastic RNA polymerase; P, pathogenicity; SRP, signal recognition particle; TL, terminal left; TR, terminal right; U snRNAs, uridine-rich small nuclear RNAs; V, variable; VM, virulence modulating; abbreviations of viroid species names are listed in Table 1.

VIROID DISCOVERY

The spindle tuber disease of potato was first described by Martin in 1922 (mentioned in Diener, 1987). The affected plants were smaller and their leaves much narrower and pointed than typical leaves. Tubers from affected plants were long, narrow, smooth skinned and showed more eyes. The disease was readily transmitted by contact of healthy plants with diseased plants, by contaminated cultivating equipment, and through the seed and pollen (Diener, 1987). For over 40 years the agent causing this potato disease had been thought to be a plant virus. Later it was shown that the infectious agent has a low sedimentation coefficient and is sensitive to treatment with ribonuclease but insensitive to treatment with deoxyribonuclease, phenol, chloroform, n-butanol, and ethanol. Because of those properties it was concluded that the infectious agent designated PSTVd (for abbreviations see Table 1) is a short, free RNA molecule. When the physico-chemical parameters of this pathogen had been clarified, the term "viroid" was proposed (Diener, 1971) in order to differentiate these small, protein-free infectious RNAs from conventional viruses with an encapsidated genome (reviewed in Diener, 2001). The next plant pathogens recognized as viroids were CEVd and CSVd. To date, over 30 different viroid species have been detected, the majority causing diseases of economically important crop plants including potato, tomato, hop, coconut, grapevine, citrus, avocado, peach, apple, pear, chrysanthemum and coleus.

This review presents the most important characteristics of viroid biology. Intensive work on the physico-chemical properties of PSTVd has led to the discovery of its primary and secondary structures; later many sequence variants of PSTVd and of other new viroids were discovered. What is intriguing is that these pathogens replicate autonomously in host plants, spread systemically, and cause disease symptoms as do viruses. However, as opposed to viruses, viroids have a particularly simple structure and no protein coding capacity. The details of some of their biological functions such as trafficking or pathogenicity are still not clear. Undoubtedly, they use the cellular host enzymatic machinery and depend on host cellular mechanisms. Thus, because of their properties, they constitute excellent tools to study such mechanisms.

For further general information on viroids the reader may wish to consult other recent reviews (Diener, 2001; Flores, 2001).

VIROID CLASSIFICATION

The scheme of viroid classification (reviewed in Diener, 2001) is presented in Table 1. According to this classification viroids are divided in two families, the *Avsunviroidae* and the *Pospiviroidae*. Members of the *Avsunviroidae* family are able to catalyze self-cleavage of multimers produced during replication and do not possess a central conserved region (CCR). Members of the *Pospiviroidae* family possess a CCR and have no self-cleaving properties.

The species are primarily defined on the basis of sequence data. An arbitrary level of 90% sequence identity is accepted as separating species from variants. The presence and type of CCR serve to define the genus.

STRUCTURE OF VIROIDS

The first viroid sequence determined was the sequence of the PSTVd intermediate strain (PSTVd-DI; Gross *et al.*, 1978). This single-stranded covalently-closed RNA molecule consists of 359 nucleotides. By convention this RNA is referred to as (+) RNA. The authors proposed a model of a unique rod-like structure with a serial arrangement of double-helical sections and small internal loops (Figs. 1 and 2). This secondary structure was later proposed for most other *Pospiviroidae*. However,

Table 1. Viroid classification (Flores *et al.*, 1998).

Family *Avsunviroidae*		
Species name	Abbreviation	Genus
Avocado sunblotch viroid	ASBVd	*Avsunviroid*
Chrysanthemun chlorotic mottle viroid	CChMVd	
Peach latent mosaic viroid	PLMVd	*Pelamoviroid*
Eggplant latent viroid	ELVd	*Elaviroid*
Family *Pospiviroidae*		
Species name	Abbreviation	Genus
Apple dimple fruit viroid	ADFVd	
Apple scar skin viroid	ASSVd	
Apple/citrus junos fruit viroid	ACJVd	
Australian grapevine viroid	AGVd	
Citrus bent leaf viroid	CBLVd	
Citrus viroid II	CVd-II	
Citrus viroid I LSSI	CVd-LSS	*Apscaviroid*
Citrus viroid III	CVd-III	
Citrus viroid OS	CVd-OS	
Grapevine yellow speckle viroid-1	GYSVd-1	
Grapevine yellow speckle viroid-2	GYSVd-2	
Japanease citrus viroid I	JCVd	
Pear blister canker viroid	PBCVd	
Coleus blumei viroid	CbVd	
Coleus blumei viroid-1	CbVd-1	*Coleviroid*
Coleus blumei viroid-2	CbVd-2	
Coleus blumei viroid-3	CbVd-3	
Citrus viroid IV	CVd IV	
Coconut cadang-cadang viroid	CCCVd	*Cocadviroid*
Coconut tinangaja viroid	CTiVd	
Hop latent viroid	HLVd	
Hop stunt viroid	HSVd	*Hostuviroid*
Chrysanthemum stunt viroid	CSVd	
Citrus exocortis viroid	CEVd	
Columnea latent viroid	CLVd	
Iresine viroid	IRVd	
Mexican papita viroid	MPVd	*Pospiviroid*
Potato spindle tuber viroid	PSTVd	
Tomato apical stunt viroid	TASVd	
Tomato chlorotic dwarf viroid	TCDVd	
Tomato planta macho viroid	TPMVd	

A compilation of viroid sequences including their accession numbers is available on the World Wide Web: http://subviral.med. uottawa.ca/cgi-bin/home.cgi (Pelchat *et al.*, 2003)

viroids of the *Avsunviroidae* family differ in this regard (Fig. 1). ASBVd assumes a quasi-rod-like structure, containing two terminal hairpins in the left part of the molecule (Flores *et al.,* 2000). A new member of this family, ELVd, can also adopt a quasi rod-like secondary structure (Fadda *et al.,* 2003). Two members of this family, PLMVd (Bussière *et al.,* 2000) and CChMVd (Navarro & Flores, 1997), adopt *in vitro* and most likely *in vivo,* complex branched conformations. Unlike other viroids, PLMVd and CChMVd are insolu-

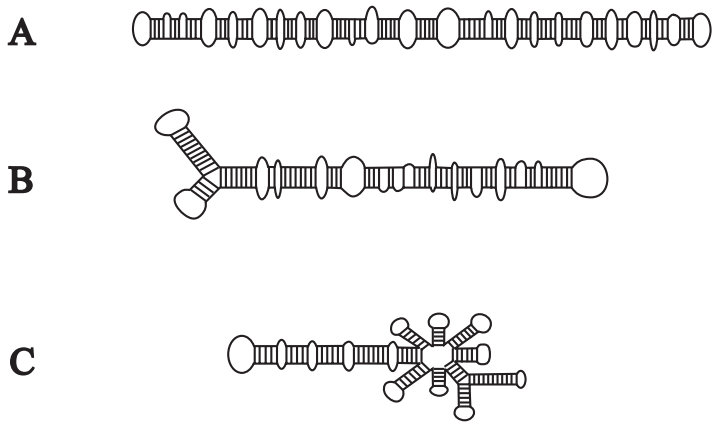

Figure 1. Schematic models of viroid structures.

(A) Rod-like secondary structure proposed for PSTVd, the type member of family *Pospiviroidae*. (B) Quasi rod-like secondary structure proposed for ASBVd, the type member of family *Avsunviroidae*. (C) Complex branched conformation proposed for PLMVd.

ble in 2 M LiCl (Navarro & Flores, 1997). This fact confirms that their secondary structures are different from those of all other viroids.

A comparative pairwise sequence analysis of members of the PSTVd group (Table 1) indicated the presence of five structural domains (Fig. 2) whose boundaries were defined by very sharp changes in sequence homology (Keese & Symons, 1985). The domain model was developed for viroids of the PSTVd group (the only ones available at that time), but was soon generalized to all viroids of the *Pospiviroidae* family.

The CCR within the C domain (Fig. 2) consists of conserved nucleotides (core sequences) located in the upper and lower strands. Specific secondary structures of the CCR are crucial for the models proposed for viroid replication and processing (Keese & Symons, 1985; Diener, 1986; Baumstark & Riesner, 1995; Baumstark *et al.,* 1997).

Domain P is associated with symptom expression and is characterized by an oligo (A_{5-6}) sequence present in all viroids of the PSTVd family.

Domain V shows the highest sequence variability between closely related viroids. The only significant sequence relationship between viroids in the V domain appears to be the presence of an oligopurine:oligopyrimidine helix, usually with a minimum of three G:C pairs.

Domains TR and TL are interchangeable between viroids; thus their role in RNA rear-

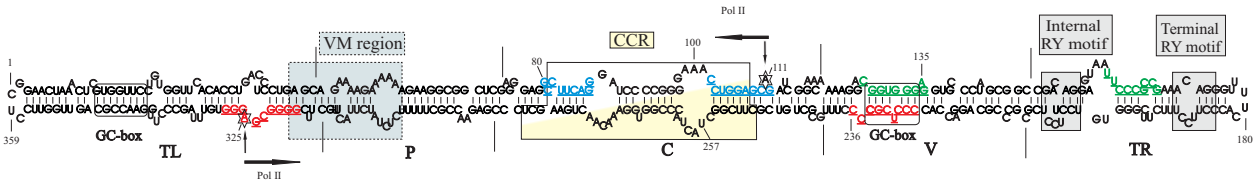

Figure 2. Primary and secondary structure of the type member of *Pospiviroidae* family, PSTVd intermediate strain.

TL, P, C, V, and TR are the terminal left, pathogenicity, central, variable and terminal right structural domains. Underlined are nucleotides forming HP I (blue), HP II (red) and HP III (green). Positions of the replication start sites A111 and A325 are highlighted by stars. The CCR, VM region, and RY motifs (described in "Viroid trafficking") are shaded.

rangements during viroid evolution has been suggested. These domains may play a role in viroid movement in plants (Hammond, 1994; Maniataki *et al.,* 2003).

Viroids of the *Avsunviroidae* family lack CCRs but contain sequences which can assemble into self-cleaving hammerhead structures.

As described above, under native conditions PSTVd forms a rod-like structure with a high degree of intramolecular base pairing. However, being single-stranded RNA molecules viroids have a high potential to form a large variety of structures and to undergo structural transitions. Indeed, during thermal denaturation, several structural transitions from a rod-like conformation to a single-stranded circle was observed (reviewed in Diener, 1987). During the major thermal transition phase all base pairs of the native structure are disrupted and a metastable structure with three hairpins (HP I, HP II and HP III) is observed. HP I is formed in the CCR of the PSTVd-type molecule. HP II is formed by base pairing of sequences of the pathogenicity (P) and variable (V) domains (Figs. 2 and 3); it is present in the majority of the *Pospiviroidae*. HP III is only found in PSTVd. The position, length and GC content of the regions forming HP I and HP II are conserved. Therefore, it was argued that the ability to form these hairpins is not coincidental and results from biological selection. There is now ample evidence that HP II is part of a metastable structure within the (–) strand oligomer and is formed during viroid replication (Schröder & Riesner, 2002). Mutations affecting the core of HP II either abolish infectivity or revert to wild-type sequences (Loss *et al.*, 1991). Moreover, in our experiments it was shown that in non-infectious recombinant viroid restoration of correct base pairing in the HP II structure leads to the recovery of infectivity (Candresse *et al.*, 2001). During replication, (–) strands are generated as multimeric intermediates with a metastable structure containing HP II (Fig. 3). These intermediates serve only as templates for (+) strand synthesis. HP II exhibits a strong similarity in sequence and three-dimensional structure to certain DNA GC-clusters found in the 5'-upstream regions of some eukaryotic genes (Loss *et al.*, 1991); based on reports of the regulatory activity of the GC-rich segments and in particular of their function as transcription factor binding sites, it was suggested that an unidentified host transcription factor binds to HP II in the oligomeric (–) strand RNA.

VIROID REPLICATION

Subcellular localization of mature viroids and replication intermediates

Infectivity tests had led to the early suggestion that viroids are associated with nuclei and/or membranes. Applying improved fractionation techniques and quantitative analyses of viroid concentration, it was shown that approximately 95% of the viroids present in PSTVd-infected tomato leaves are associated with the nucleolar fraction obtained from purified nuclei (Schumacher *et al.*, 1983). Later, the localization was investigated by *in situ* hybridization with immunological labelling techniques and fluorescence microscopy (Harders *et al.*, 1989). In plants infected with PSTVd, viroid (+) strand was mostly found in the nucleoli, confirming the results of previous fractionation studies (Schumacher *et al.*, 1983). A similar distribution was found for (–) strand replication intermediates of PSTVd. Circular viroid RNA and replication intermediates were also found in the nucleoplasm although at much lower concentrations than in the nucleolus. Recently, a differential subnuclear localization of the (–) and (+) strands of PSTVd has been reported (Qi & Ding, 2003a). In infected cultured cells of *Nicotiana benthamiana* and in infected tomato and *N. benthamiana* plants, the (–) strand RNAs are localized in the nucleoplasm, whereas the (+) strands are observed in the nucleolus as

well as in the nucleoplasm with distinct spatial patterns. Furthermore, the presence of (+) PSTVd RNA in the nucleolus causes redistribution of small nucleolar RNAs such as U3 and U14.

The nucleolar localization of PSTVd has to be considered in the context of its replication. It is assumed that the synthesis of (–) and (+) strands oligomers is carried out by the host DNA-dependent RNA polymerase II. In the cell, this enzyme is responsible for mRNA synthesis and is partly associated with chromatin. This means that RNA polymerase II or the viroid, including its replication intermediates, may shuttle between the nucleoplasm and the nucleolus (Harders *et al.*, 1989). A recent report on the different localizations of mature viroids and (–) strand replication intermediates indicates intranuclear trafficking of the (+) strand RNA of PSTVd but not of the (–) strand RNA (Qi & Ding, 2003a). According to the model proposed (Qi & Ding, 2003a), synthesis of the (–) and (+) strands occurs in the nucleoplasm. The (–) strands are anchored in the nucleoplasm but the (+) strands are transported selectively into the nucleolus. Two alternative pathways for the processing and trafficking of the (+) strands are possible. Multimeric (+) PSTVd migrates into the nucleolus where its cleavage to monomers and circularisation occurs. The second possibility is that multimeric (+) PSTVd is cleaved and circularised in the nucleoplasm, after which some circular molecules migrate into the nucleolus. In either pathway, circular monomers migrate into the cytoplasm and then to neighbouring cells through the plasmodesmata.

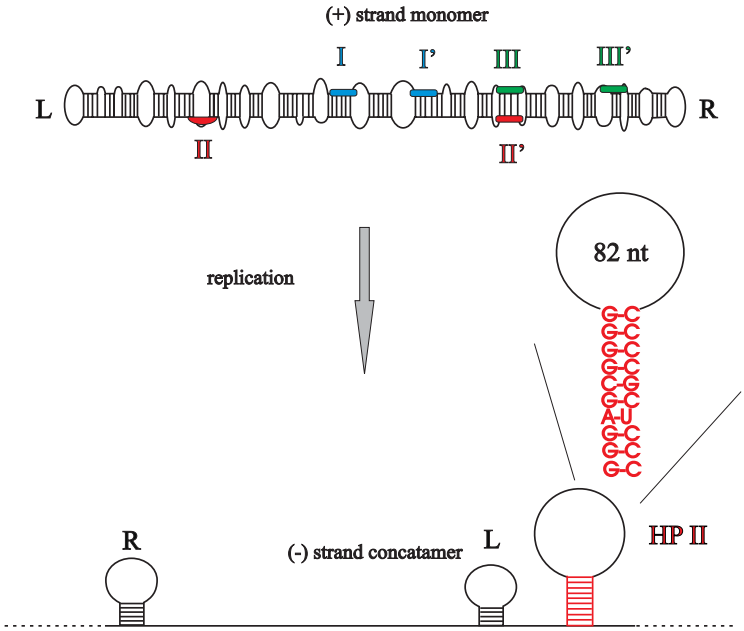

Figure 3. Formation of metastable RNA structure containing HP II during PSTVd replication.

Complementary sequences I and I', II and II', III and III' which can form the stems of HP I, HP II, and HP III, respectively, are indicated on the rod-like structure in different colours. R and L, right and left terminal hairpin. Sequential folding during synthesis of concatameric (–) strands leads to metastable conformations including HP II. Such a structure serves as template for the synthesis of concatameric (+) RNAs which are rearranged into metastable structures containing two tetraloops (see Fig. 5). Adapted from Schröder and Riesner (2002).

CEVd, CCCVd and presumably other members of the *Pospiviroidae* family also have a nuclear localization. In contrast, the members of the *Avsunviroidae* family, ASBVd, PLMVd and presumably CChMVd and ELVd replicate and accumulate in chloroplasts (reviewed in Diener, 2001).

Host RNA polymerases: candidates for viroid replicases

Since viroids do not code for proteins their replication must depend entirely on host enzymatic systems. Inhibitors of RNA transcription such as α-amanitin and actinomycin D can help to distinguish between the different host RNA polymerases involved in viroid replication. Such experiments indicate that for PSTVd and CEVd, and presumably other viroids of the *Pospiviroidae* family, DNA-dependent RNA polymerase II is involved in viroid replication (Diener, 1987). A role for RNA polymerase II in viroid replication *in vivo* was supported by the isolation of an active replication complex of CEVd as a chromatin-enriched fraction in which viroid RNA was associated with the largest subunit of RNA polymerase II (Warrilow & Symons, 1999).

A study of ASBVd replication, the type species of the *Avsunviroidae* family, clearly yielded different results. Marcos and Flores (1992) demonstrated that synthesis of ASBVd RNAs is not affected by the concentrations of α-amanitin which typically inhibit RNA polymerases II and III, suggesting that either polymerase I or an unidentified RNA polymerase activity resistant to α-amanitin might be involved in ASBVd replication. These results are not surprising if one considers that ASBVd replicates and accumulates in chloroplasts. Later experiments with tagetitoxin suggested that the nuclear-encoded chloroplastic RNA polymerase (NEP) is involved in ASBVd replication, although another tagetitoxin-resistant RNA polymerase can not be excluded (Navarro *et al.,* 2000).

Replication initiation

Studies of *in vitro* replication using (+) strand circular PSTVd as template in a nuclear extract from healthy potato cells (Fels *et al.*, 2001) indicate that synthesis of (–) strand PSTVd predominantly starts at two sites: nucleotides A111 (C domain) and A325 (TL domain) (Fig. 2). The results were obtained using RT-PCR and identifying the 5' ends by primer extension. The sequences of the first seven nucleotides transcribed are very similar, 105GGAGCGA111 and 319GGGGCGA325. GC-boxes are located at a distance of 15 and 16 nucleotides upstream, respectively, in the native viroid structure. The GC-boxes may have a function similar to the GC-rich hairpin II in (–) strand intermediates (Loss *et al.*, 1991; Schröder & Riesner, 2002).

Different initiation properties have been shown for ASBVd RNA. By *in vitro* capping of linear monomeric ASBVd RNA isolated from infected tissue and by RNase protection assays, U121 in the (+) strand and U119 in the (–) strand were recognized as unique initiation sites (Navarro & Flores, 2000). The sites are embedded in A+U rich regions of terminal hairpin loops of the predicted quasi rod-like structures of monomeric circular ASBVd RNAs (Fig. 4). The sequences around the initiation sites are similar to the promoters of NEP, supporting the involvement of an NEP-like activity in ASBVd replication.

Models of viroid replication

Based on the detection of different replication intermediates, replication of viroids has been proposed to occur by a rolling circle mechanism (reviewed in Diener, 2001). Two variants of this mechanism have been proposed, involving in the first (asymmetric) variant, and in the second (symmetric) variant, two types of rolling circles. In the first variant the infectious circular (+) RNA is copied continuously by RNA polymerase into a concatameric (–) strand. The concatameric (–) RNA then serves as a template for the production of concatameric (+) RNA strands that are cleaved to monomers, and finally produce circular progeny. In this pathway (asymmetric) a single type of rolling circle is detected. PSTVd and presumably other members of the *Pospiviroidae* family replicate according to this pathway.

In the second variant, concatameric (–) RNA strands created as in the first mechanism are cleaved producing monomers that are circularized and then copied to yield concatameric viroid (+) RNA molecules. Specific cleavage of these strands produces (+) monomers that are circularized to yield the progeny RNA. This symmetric pathway with two types of rolling circles is proposed for members of the *Avsunviroidae* family, ASBVd and PLMVd (reviewed in Diener, 2001).

Processing of replication intermediates

Replication by a rolling circle mechanism implicates high specificity cleavage of oligomers into genome-length units. For ASBVd, CChMVd and PLMVd self-cleavage as an RNA-mediated reaction was demonstrated *in vitro*, and most likely operates *in vivo* (reviewed by Diener, 2001). The oligomeric RNAs of both polarities assume hammerhead structures catalyzing the self-cleavage reactions (Fig. 4). Recently it was found that a chloroplast protein which binds ASBVd *in vivo* may facilitate hammerhead-mediated self-cleavage of RNA (Darbòs & Flores, 2002). Here UV cross-linking was used to characterize viroid-protein interactions in ribonucleoprotein complexes. UV-irradiated avocado leaves infected with ASBVd were screened for host proteins directly interacting with the viroid *in vivo*. In this manner several ASBVd-host protein adducts were identified.

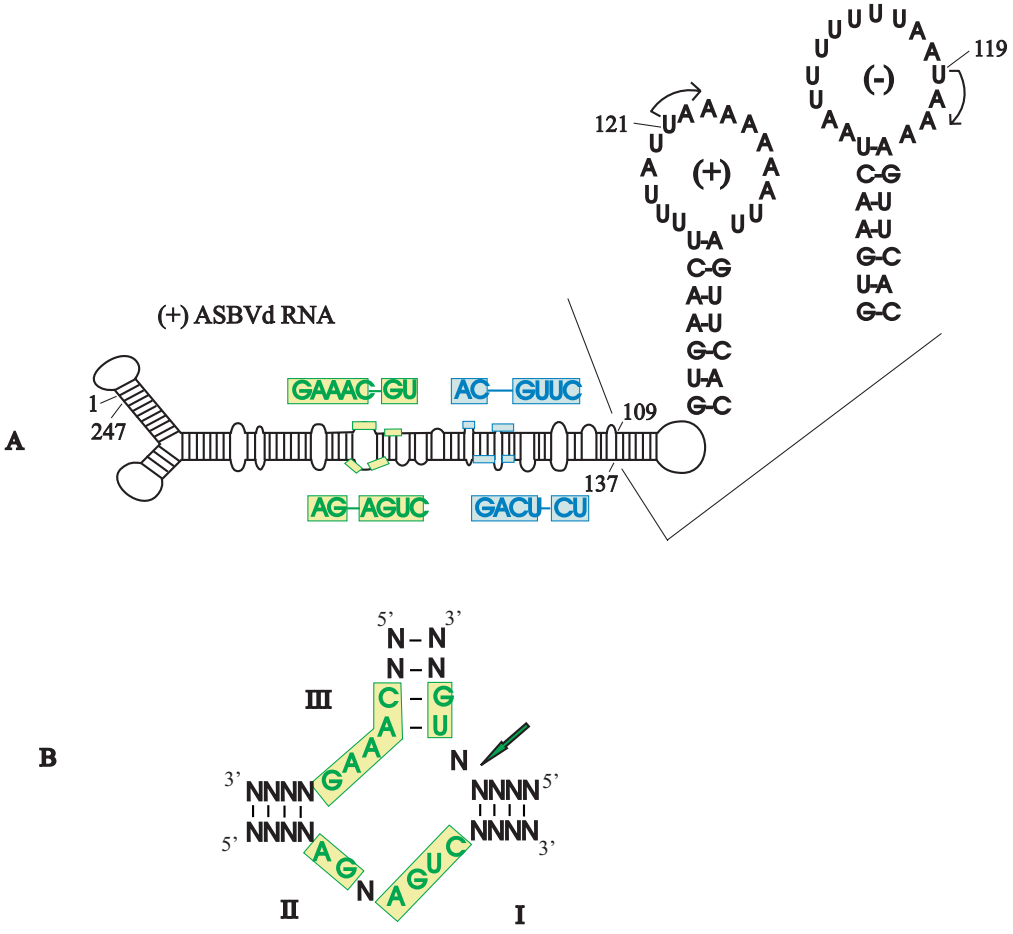

Figure 4. Hammerhead structure.

(A) Quasi rod-like structure proposed for ASBVd; conserved nucleotides characteristic for hammerhead structures are shown within boxes for (+) polarity (green colour) and (–) polarity (blue). The A+U-rich terminal loops of the ASBVd (+) and (–) strand are shown enlarged with the initiation sites marked with arrows. (B) Consensus hammerhead structure with conserved nucleotides shown in boxes. N indicates non-conserved nucleotide. The central core is flanked by three helices, I, II and III. The self-cleavage site is indicated by an arrow.

Tandem mass spectrometry analysis of the most abundant cross-linked species identified two chloroplast proteins, PARBP33 and PARBP35. These RNA-binding proteins belong to a family of nuclear-encoded chloroplast proteins whose members are involved in stabilization, maturation and editing of chloroplast transcripts. PARBP33 and PARBP35 contain a putative N-terminal transit peptide potentially directing the protein into the chloroplast, an acidic domain that forms the N-terminal region in the mature protein and two consecutive RNA-binding domains of the RRM (RNA recognition motif) class. It was shown that PARBP33 may behave as a chaperone stimulating *in vitro,* and possibly *in vivo,* the hammerhead-mediated self-cleavage of concatameric ASBVd transcripts. Hence, despite its RNA-based mechanism, the cleavage of ASBVd is facilitated by proteins. Other additional functions of PARBP33 and PARBP35, such as escorting the viroid into the chloroplast are also possible (Daròs & Flores, 2002).

After cleavage, the monomeric strands are circularized into progeny viroids. It is not clear whether the circularization is a process dependent on host enzymes or whether it is a self-ligation reaction. Self-ligation (in low yield) *via* the formation of 2',5'-phosphodiester bonds of a PLMVd transcript has been observed *in vitro* (Lafontaine *et al.*, 1995). This, however, does not exclude the possibility that a host RNA ligase may catalyze the ligation steps *in vivo*. More recently the presence of 2',5'-phosphodiester bonds at the ligation site of circular PLMVd strands isolated from infected peach leaves was reported (Côte *et al.,* 2001). These bonds serve to close linear replication intermediates, yielding circular molecules. Furthermore, these bonds are shown to stabilize the replicational circular templates, resulting in a significant advantage in terms of viroid viability. Self-ligation of PLMVd *in vivo* has been proposed (Côte *et al.,* 2001).

As described above for representatives of the *Avsunviroidae* family, self-cleavage reactions have been demonstrated. However, for viroids of the *Pospiviroidae* family no such data exist. Attempts to detect nonenzymatic processing of oligomeric PSTVd have revealed only very low (Robertson *et al.*, 1985) or negligible (Tsagris *et al.,* 1987) activity as measured by final product accumulation. Later it was demonstrated that longer than unit-length PSTVd RNA transcripts can be cleaved and ligated *in vitro* to circular molecules by RNase T1 from *Aspergillus oryzae* (Tsagris *et al.,* 1991), therefore the involvement of a plant endoribonuclease in viroid processing was postulated. These studies were performed with longer-than-unit-length PSTVd RNA transcripts flanked by several nucleotides from the upper part of the CCR duplicated at the 5' and 3' ends. The site of cleavage and religation of the (+) strand RNA transcript by RNase T1 was located at G80.

Infectivity studies either with RNA transcripts or with cloned viroid cDNA revealed that longer-than-unit-length transcripts are infectious when they contain a short duplication of the upper part of the CCR ($_{92}$CCCGGGGA$_{99}$) (Candresse *et al.,* 1990; Tabler & Sänger, 1984). From these findings it was concluded that processing of viroids proceeds in this region. Additional evidence for the existence of this cleavage site comes from site-directed mutagenesis within the CCR of PSTVd and CEVd cDNAs. Since the upper part of the CCR (UCCR) and the neighbouring sequences on either side are extensively self-complementary, two UCCRs (present in the oligomer) are able to form a very stable tri-helical region of 28 base pairs interrupted by two small internal loops (reviewed in Diener, 2001).

In several reports it was suggested that formation of the so-called tri-helical structure is a prerequisite for viroid RNA processing (Diener, 1986; Hecker *et al.*, 1988). However, later Steger *et al.* (1992) demonstrated that

the structure essential for *in vitro* processing by RNase T1 is not the tri-helical structure, but a thermodynamically less stable ExM structure. The influence of the secondary structure of the RNA on the substrate activity of a longer-than-unit-length transcript for processing to circular viroids was also studied in a nuclear extract from potato cell suspensions that simulates *in vivo* conditions more closely (Baumstark & Riesner, 1995). The transcript analysed contained a repeat of 17 additional nucleotides in the CCR. Only one of the four possible secondary structures of the CCR, the so called ExM structure, was processed to proper viroid circles (Fig. 5). Improved investigations on PSTVd transcript processing in nuclear extracts led to the localization of the cleavage and ligation sites (Baumstark *et al.*, 1997). These sites were only one base pair apart from an internal loop in the CCR (positions 97–102 and 256–262) similar to loop E of eukaryotic 5S rRNA whose three-dimensional structure was solved by NMR. Actually, a highly conserved loop E motif is apparent in quite different RNAs such as 16S, 23S rRNA, group I and II introns, RNase P and the hairpin ribozyme (Gast *et al.*, 1996). The loop is involved in RNA–RNA as well as RNA–protein interactions. A loop E-like motif in viroids was deduced from a characteristic UV cross link between two bases G98 and U260. Loop E contains an unusual G·A base-pair, a reverse-Hoogsteen A·U pair, and an extrahelical C which may form a nucleotide triplet with this A·U.

It has been shown that the region containing loop E (Fig. 5) can be rearranged into an alternative conformation. The PSTVd transcript analysed was correctly processed in a potato nuclear extract only if this region was folded into a structure containing at least one GNRA (N = any base; R = A or G) tetraloop-hairpin (Fig. 5). After cleavage, the RNAs are either ligated autocatalytically with low efficiency, or enzymatically by host plant RNA ligases with high efficiency, yielding mature circular products (Baumstark *et al.,* 1997).

VIROID TRAFFICKING

Systemic viroid infection consists of two major steps, replication in plant cells and movement throughout the whole plant. Cell-to-cell movement of PSTVd occurs through plasmodesmata and appears to be an active process mediated by specific sequence or by structural motifs (Ding *et al.,* 1997; Zhu *et al.*, 2001). Long-distance trafficking of PSTVd takes place in the phloem. Such trafficking is likely sustained by viroid replication in the phloem and is governed by plant developmental and cell factors (Owens *et al.,* 2001; Zhu *et al.*, 2001). Analysis of PSTVd trafficking in *N. benthamiana* showed that PSTVd movement within sieve tubes does not simply follow mass flow from source to sink. Viroids are rather transported into selective sink organs (Zhu *et al.,* 2002). It was demonstrated that in tobacco two mutants can enter the phloem to spread systemically but can not exit the phloem. This suggests that the PSTVd genome contains multiple structural motifs for traffic; phloem entry and exit appear to be mediated by different motifs. Most likely, these viroid motifs mimic endogenous plant RNA motifs such that they may be recognized by cell factors for trafficking (Zhu *et al.,* 2002).

The involvement of phloem proteins in systemic transport of viroids in cucumber plants was investigated. Two research groups independently demonstrated an interaction *in vitro* between HSVd and phloem protein 2, an abundant lectin present in phloem exudates (Gómez & Pallás, 2001; Owens *et al.,* 2001). As phloem protein 2 possesses RNA-binding properties and an ability to move from cell to cell *via* plasmodesmata as well as at long distances, its involvement in viroid movement has been suggested.

Recently, a viroid binding protein VirP1 from tomato was identified applying an RNA ligand screening procedure (Gozmanova *et al.,* 2003). VirP1 is expressed in different tissues of healthy plants including those that are

normally not infected by PSTVd. This protein contains a nuclear localization signal and a bromodomain. Analysis of its sequence revealed that VirP1 belongs to a family of transcription regulators associated with chromatin remodelling. It was demonstrated that VirP1 interacts specifically with monomeric and oligomeric PSTVd (+) strand transcripts. Moreover, a complex of VirP1 with PSTVd was identified *in vitro* (Martinez de Alba *et al.*, 2003). The role of VirP1 in viroid infection is not known. However, given its properties, VirP1 may play a role in several steps in the life cycle of the viroid. First, because VirP1 contains a nuclear localization signal it may help in transferring the viroid to the nucleus. Second, it may participate in exporting viroid from the nucleus, e.g. through phosphorylation-dephosphorylation of the serine-rich domain at its carboxy terminus. Third,

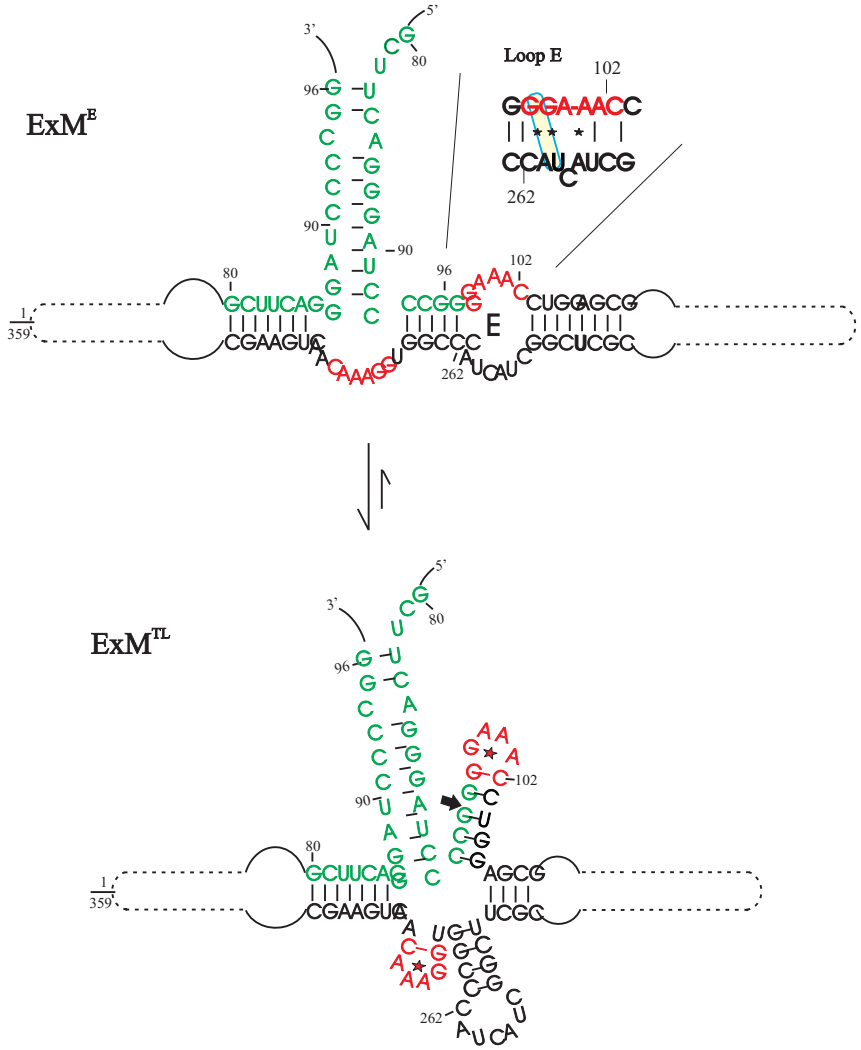

Figure 5. PSTVd transcript in possible alternative conformations of the ExM structure.

One conformation ExM^{E} contains loop E, the alternative ExM^{TL} comprises two tetraloops and a regular hairpin loop. The 5′-cleavage in the upper tetraloop is indicated by an arrow; stars indicate non-canonical base-pairs; bases characteristic of tetraloops of the GNRA family are red. The duplicated sequence between nucleotides 80 and 96 is given in green. Circled bases G98 and U260 in the enlargement of the loop E, denote the loop E-specific UV crosslink. Adapted from Baumstark *et al.* (1997).

through interaction of its bromodomain it may be involved in tethering the viroid to the polymerase II transcription complex. In addition, a possible role in systemic spread of the viroid can not be excluded (Maniataki *et al.,* 2003; Martinez de Alba *et al.,* 2003).

Applying the yeast three-hybrid system, the TR domain of PSTVd was identified as the region binding VirP1 (Maniataki *et al.,* 2003). Within the TR domain two asymmetric internal loops, each composed of the sequence elements 5'-ACAGG and UCCUUCC-5', were found to be responsible for specific RNA-protein interaction (Gozmanova *et al.,* 2003). This structural element was called an 'RY' motif (Fig. 2) because of its nucleotide composition, the lower strand being composed of pyrimidines (Y) and the top strand of purines (R) with the exception of a C residue. The terminal RY motif located close to the terminal right loop of the PSTVd secondary structure has an about 5 fold higher binding affinity than the more centrally located internal RY. Each motif can bind VirP1 independently of the other; however, a cooperative effect is not excluded. A single nucleotide exchange introduced simultaneously into both RY motifs almost completely abolished the specific binding to VirP1. On the other hand, a two-nucleotide mutation in the terminal right hairpin loop, leaving the terminal RY motif intact, had only a moderate effect on the binding affinity and no effect on infectivity. Compensatory mutations in the terminal RY motif that maintained the overall PSTVd secondary structure did not restore the binding activity. In view of this observation it seems that VirP1 recognizes a domain with a specific RNA sequence, in addition to a secondary structure. The RY motif is phylogenetically conserved in several genera of *Pospiviroidae* indicating its functional role. It seems that strong interaction with VirP1 is beneficial for propagation of the viroid. It has been suggested that the RY motif could determine the host range of the viroid (Gozmanova *et al.,* 2003).

VIROID PATHOGENICITY

Similarity with cellular RNAs and affinity for host proteins

Despite their small size and lack of mRNA activity, viroids can induce disease symptoms similar to those induced by plant viruses, and their interference with cell functions must stem from interaction between the viroid and certain host constituents. Targets for such interactions may be assigned to either host proteins or nucleic acids. Based on the sequence similarity between (+) and (–) viroid strands and various cellular RNAs such as U1 snRNA, U3B snRNA and U5 snRNA, it was proposed that viroids may interfere with mRNA splicing or pre-rRNA processing (Diener, 1987). Similarly, the signal recognition particle (SRP) RNA from tomato shows a notable sequence similarity with a fragment of the P domain of PSTVd (nucleotides 277–309; reviewed in Diener, 2001). Together with six polypeptides, SRP RNA (also termed 7S RNA) forms the cytoplasmic SRP directing membrane-bound and soluble proteins into or across the membranes of the endoplasmic reticulum. Potential hybrid formation between PSTVd RNA and SRP RNA suggests that viroids may interfere with the SRP-dependent intracellular protein transport in an 'antisense-like' manner.

It was shown that PSTVd binds to a 43 kDa protein present in nuclear extracts of healthy and infected plants. A similar complex is formed *in vivo* in nuclei of infected cells but its biological significance is still unclear (Diener, 2001).

Hiddinga *et al.* (1988) identified a host-encoded 68 kDa protein phosphorylated to different extents in extracts from PSTVd-infected and mock-inoculated tissues. Preliminary evidence suggests that the protein is immunologically related to the mammalian P68 protein, a serine/threonine kinase induced by interferon and activated by dsRNA. Activation of P68 triggers a chain of reactions that

culminates by impeding the initiation of protein synthesis. In an *in vitro* system, mammalian P68 was activated by autophosphorylation in the presence of purified PSTVd and the activation potential was higher for viroid strains inducing severe or intermediate symptoms than for a strain inducing mild symptoms (Diener *et al.*, 1993). More recently during a study of the molecular basis of the response of tomato cv. Rutgers to PSTVd infection, a specific protein kinase gene *pkv* was identified (Hammond & Zhao, 2000). This gene is transcriptionally activated in plants infected with either intermediate or severe strains of PSTVd. In plants inoculated with a mild strain the activation is lower and in the case of mock-inoculated plants activation of the *pkv* gene is not detectable. A full-length copy of this gene encoding a 55 kDa viroid-induced protein kinase reveales significant homologies to cyclic nucleotide-dependent protein kinases.

Recently, a comprehensive analysis of the gene expression pattern of tomato infected with two PSTVd strains and *Tobacco mosaic virus* (TMV) was investigated by Itaya *et al.*, (2002). These PSTVd strains inducing mild and more severe symptoms differ only by three nucleotides. It was demonstrated that the PSTVd strains examined, induced and suppressed a tomato gene mostly starting at 17 days post inoculation and reaching a maximum at day 25. In contrast, TMV induced tomato gene expression most strongly at 5 to 10 days post inoculation. The severe PSTVd strain altered the expression of a total of 52 genes. Ten of them were also altered by the mild strain, six by TMV, and nine by both PSTVd mild and TMV infections. The remaining 27 genes were specifically induced only by the severe strain. In contrast, infection with the mild PSTVd strain altered the expression of one unique gene which is homologous to the gene encoding the mitochondrial 34 kDa porin. TMV infection altered expression of 27 genes; among them 10 were unique. Therefore, PSTVd strains of different severity, and TMV can induce or suppress expression of common as well as unique genes.

It was found that PSTVd infection altered expression of genes encoding products involved in the defense/stress response, cell wall structure, chloroplast function, protein metabolism and others. The expression level of genes such as those encoding catalase 1, chitinase, cyclophilin, PR-1b and heat shock proteins was higher in the case of infection by a severe PSTVd strain than by a mild strain. Therefore the expression level of these genes seems to be correlated with the appearance of disease symptoms (Itaya *et al.*, 2002).

The mechanism underlying altered tomato gene expression during PSTVd infection is not clear. It was suggested that viroids may alter the patterns of host gene expression at the transcriptional or post-transcriptional level resulting in disease formation. However translational or post-translational regulations of host gene expression are also possible (Itaya *et al.*, 2002).

Sequences involved in viroid pathogenicity

Early PSTVd sequence comparisons led to a model in which symptom severity was specifically correlated with the part of the P domain called the “virulence modulating” (VM) region (Fig. 2), the segment formed by nucleotides 42–60 and its counterpart in the secondary structure, nucleotides 300–319 (Schnölzer *et al.*, 1985). The authors proposed that decreasing the thermal stability of this region might be correlated with increasing virulence of PSTVd strains. In the CEVd sequence, variations were also observed and analysed (Visvader & Symons, 1985). However, in contrast to PSTVd, the calculated stability of the corresponding region was considerably higher in sequence variants with severe symptoms than in those with mild symptoms (Visvader & Symons, 1985). Further studies with additional strains indicated that the thermodynamic stability of the VM region and the entire molecule is not correlated with

the pathogenicity of the viroid strain tested (Owens *et al.*, 1996). Instead, on the basis of thermodynamic considerations a new structural model explaining viroid pathogenicity was proposed. An increase in bending of the VM regions from intermediate to severe strains was suggested to be involved. In mild strains the bending appeared to be in the opposite direction. This model was tested by experimentally probing the three-dimensional structure of the VM region of six PSTVd strains (Schmitz & Riesner, 1998). It was proposed that the differences observed in the bending of the VM region might affect the affinity of viroids for host proteins. The strength of this interaction would determine the pathogenic response of the infected plants.

The various PSTVd isolates inducing different symptoms in infected plants differ mostly in the sequence of their P domain. However, the effect of mutations in the V (Hu *et al.*, 1996) and the TL (Hu *et al.*, 1997) domains was also demonstrated. To investigate the role of individual structural domains in viroid pathogenicity, a series of different chimeras was constructed and analyzed. Sano *et al.* (1992) constructed interspecific chimeras by exchanging the TL and/or P domain between CEVd inducing mild symptoms and TASVd inducing severe symptoms. The analysis demonstrated that the P domain plays an important role in pathogenicity; however, the TL domain and the V+TR domains may also influence the severity of symptoms (Sano *et al.*, 1992). In another study six intramolecular chimeras were constructed by exchanging the P and V domains between mild and severe PSTVd isolates (Góra *et al.*, 1996). Infectivity studies showed that the P domain is directly responsible for the severity of symptoms. The results also indicated that symptom severity was not a simple function of varying viroid accumulation.

In a study with CCCVd mutants it was demonstrated that both the P domain and the CCR influenced the severity of the symptoms (Rodriguez & Randles, 1993). Later it was shown that also in the case of PSTVd the CCR may play a role in pathogenicity. In 1996 Wassenegger and coworkers reported that a mutation in loop E of the CCR, C259U, converted the lethal tomato PSTVd–KF-440-2 strain into a strain that is infectious in tobacco. Similarly, the C259U or U257A substitution in a PSTVd intermediate strain conferred infectivity in tobacco (Zhu *et al.*, 2002). A replication assay in cultured cells of tobacco demonstrated that the U257A, U257C and C259U substitutions each enhanced PSTVd replication (Qi & Ding, 2002). The results indicated that specific mutations in this region may determine replication levels for host adaptation. More recent studies on these mutations indicated that the CCR is not only involved in replication and host range determination but could also alter symptom severity of PSTVd in tomato (Qi & Ding, 2003b). In this study the infectivity experiments were performed on tomato plants inoculated with *in vitro* transcripts of a PSTVd intermediate strain carrying specific mutations in the loop E region. PSTVd carrying mutations C259U and U257C caused symptoms similar to those caused by the non mutated PSTVd intermediate strain. In contrast, plants inoculated with PSTVd carrying mutation U257A showed severe growth stunting, relatively small leaves with necrosis, and characteristic flat top of the shoot (called "flat-top" symptom). In addition, this mutation also caused premature plant death.

Another alteration at this nucleotide position, U257G, caused growth stunting intermediate between that caused by C259U and U257A. Furthermore, the double mutant C257A/C259U caused symptoms of intermediate severity indicating that the pathogenic effect of the U257A substitution was abolished by the C259U substitution.

To test the general importance of A257 in pathogenicity the substitution U257A was introduced in three other PSTVd variants of different virulence. These variants, PSTVd-mild,

PSTVd-KF440-2 (lethal), and PSTVd-RG1 (lethal) differ in nucleotide sequences in the VM region of the P domain. In each case infected plants developed characteristic flat-top symptoms and growth stunting. Thus the results indicate that A257 plays a specific role in conferring the flat-top symptom. However, sequence variations in the P domain allowed infected plants to develop different severity levels of the final symptoms. Therefore, it was concluded that A257 is a new pathogenicity determinant which may function independently and synergistically in symptom expression with the P domain. Furthermore, it was shown that A257 did not alter viroid secondary structure, replication levels, or tissue tropism. Infection with PSTVd carrying the U257A mutation specifically caused restricted cell expansion but not cell division and differentation. This led to plant stunting. The observations are in agreement with the finding that expression levels of *LeExp2* were severely repressed in leaves and young stems of tomato infected by the PSTVd intermediate strain with the U257A mutation. *LeExp2* is expressed in rapidly growing parts of hypocotyls, leaves and stems. This gene encodes an expansin that possibly functions to loosen cell walls to permit cell expansion. It was suggested that *LeExp2* is one of the many genes whose expression is altered during infection with the PSTVd mutant carrying the U257A substitution. Altered expression of *LeExp2* may occur downstream of the cascade of altered gene expression that leads to the flat-top symptom (Qi & Ding, 2003).

As described above the loop E motif in PSTVd plays a role not only in viroid replication and processing but also in pathogenicity. Its function in pathogenicity seems to be similar to the role played by a tetraloop in pathogenicity of CChMVd, a member of the *Avsunviroidae* family. The four-nucleotide UUUC→GAAA substitution at nucleotide positions 82–85 converted the symptomatic strain CChMVd-S to a nonsymptomatic strain, CChMVd-NS without altering the final accumulation level of the viroid (De la Peña *et al.,* 1999). These four nucleotides form a tetraloop capping the stem of the hairpin. It should be stressed that GAAA tetraloops belong to the GNRA tetraloop family (see chapter "Processing of replication intermediates"). Such a GAAA tetraloop is implicated in PSTVd processing, but a similar role for the CChMVd tetraloop can be dismissed, because processing in this viroid occurs in another region and is mediated by a hammerhead ribozyme. More recently, a detailed study of this tetraloop in CChMVd by site directed mutagenesis was reported (De la Peña & Flores, 2002). In the first experiment two different tetraloops of the UNCG family, UCCG and UUCG, were introduced. Fifteen to 20 days post inoculation no symptoms appeared, in contrast to control plants infected with the symptomatic CChMVd strain where severe symptoms were observed. Therefore, both tetraloop changes entailed a reversion of the phenotype from symptomatic to non-symptomatic. However, one month after inoculation, the plants showed mild symptoms. Dot-blot analysis revealed that viroid accumulation was similar in all cases including in control plants. Therefore the phenotype was not a consequence of different viroid accumulation levels in the infected plants. Analysis of the progeny sequence showed a high instability of the tetraloop sequence. The parental tetraloops UCCG and UUCG were not detected. Finally, three months after inoculation the plants showed symptoms of chlorotic mottle. Sequence analyses revealed the reversion of the mutated tetraloop to UUUC typical for the symptomatic strain. Therefore the GAAA tetraloop characteristic of the CChMVd-NS strain is not functionally interchangeable for other stable tetraloops of the UNCG family. This suggests that selection pressure acts on the sequence and not on the structure. The same results were obtained with constructs carrying the UUAA tetraloop mutation where reversion to the UUUC tetraloop was observed. An analogous situation

was observed when the tetraloop was changed either to a UAA triloop or UUUU tetraloop.

Another kind of pathogenicity determinant was found in PLMVd. This viroid is able to replicate either symptomlessly or to induce a broad variety of symptoms; prominent among them is extensive chlorosis of peach known as calico (PC). Sequence analyses of PLMVd of the PC isolate revealed two groups of variants (Malfitano *et al.,* 2003). The size of the first group (336–338 nt) was similar to that of typical PLMVd variants of nonsymptomatic and mosaic-inducing isolates. The second group was longer (348–351 nt) due to an insertion of 12–13 nt. This insertion was found in the hairpin loop capping the hammerhead arm, it had a limited sequence variability and folded into a hairpin. PLMVd variants containing this insertion caused PC symptoms. To show a direct relationship between the insertion and severe symptoms, a PC-inducing variant in which the insertion was removed was constructed. Such a deleted variant was able to replicate without inducing symptom formation (Malfitano *et al.,* 2003).

VIROIDS AS QUASI-SPECIES

Since the determination in 1978 of the first nucleotide sequence of PSTVd, the sequence of over 80 different PSTVd sequence variants have been established. For other viroids a variety of sequences also have been reported, for example 150 sequences for HSVd, 88 for ASBVd, 115 for PLMVd, and 11 for CLVd (Pelchet *et al.,* 2003). Sequence heterogeneity has been widely observed in natural viroid isolates. In addition, numerous reports indicate that *in vitro*-constructed PSTVd mutants may rapidly revert to wild-type or accumulate new compensatory mutations (reviewed in Diener, 2001). This indicates a high plasticity of the viroid genome. It is now clear that viroids as also other RNA pathogens, propagate in the host as a population of similar but nonidentical sequences composing a quasi-species (Ambrós *et al.,* 1999; Eigen, 1993; Góra *et al.,* 1996). New sequence variants arise as a result of high error rates during RNA replication and competition between the arising new variants.

POSSIBLE ORIGIN OF VIROIDS

Since the discovery of viroids, many of their properties such as their molecular structure, replication mechanism, possible evolutionary relationships among themselves and with satellite RNAs have been elucidated. However, the origin of viroids remains a mystery. This problem is closely linked with the recognition of natural viroid reservoirs as a source of crop plant infection.

Numerous viroid diseases have been detected in the last century (reviewed by Riesner & Gross, 1985). For example, the diseases caused by PSTVd and CEVd were first observed in the early 1920s, whereas those caused by HSVd were first described as late as 1963–1970. It appears that human activities such as the introduction of vast monocultures, may have contributed to the generation and spread of viroids and viroid diseases. Since viroids often do not cause obvious symptoms in wild plants, it was proposed that viroids of crop plants originate from unknown wild host plants for which they are not pathogenic. Indeed, several variants of a novel viroid, designated Mexican papita viroid (MPVd) have been isolated from *Solanum cariophyllum* Lindl. plants growing wild in the Mexican state of Aguascalientes (Martinez-Soriano *et al.,* 1996). The nucleotide sequence of this viroid is closely related to those of TPMVd and PSTVd. The discovery of wild, viroid-infected, but symptomless *S. cariophyllum* plants growing in rural Mexico and the close phylogenetic relationship of MPVd with TPMVd and PSTVd suggest that MPVd may represent an ancestor of the PSTVd-group of viroids causing crop diseases. The results suggest that MPVd or a

similar viroid from endemically infected wild solanaceous plants in Mexico was transferred to potato-growing areas with infected germplasm (Martinez-Soriano *et al.*, 1996).

Several hypotheses have been advanced to explain the origin of viroids (reviewed in Diener, 2001). For example, it has been suggested that viroids may have originated from retroviruses or transposable elements by deletion of interior sequences, or that they may represent escaped introns. It was also speculated that viroids may have evolved from prokaryotic RNAs during infection of higher plants by prokaryotes. This hypothesis is based on the finding that not only eukaryotic RNA polymerases, but also RNA and DNA-polymerases from *Escherichia coli* are able to transcribe viroids *in vitro* into complementary RNA and DNA copies, respectively. However, viroid-related sequences have not been found in prokaryotes.

With the demonstration that some RNAs have catalytic properties, the idea that RNA preceded DNA as a carrier of genetic information has gained support. Models for self-replicating precellular RNAs suggest the existence of primitive RNA enzymes with properties similar to those of self-splicing introns. Because members of the *Avsunviroidae* family, the viroid-like satellite RNAs, and the viroid-like domain of Hepatitis delta RNA (Pelchat *et al.,* 2003) are self-cleaving, it is equally plausible to consider these RNAs as relics of the RNA world. This leads to an alternative hypothesis on the origin of viroids and viroid-like satellite RNAs: one can suppose that these RNAs have evolved from "free-living" molecules and have assumed an intracellular existence during early evolution of cellular organisms, as have introns.

This proposal raises the possibility that all viroids and viroid-like RNAs may derive from a common ancestor. Indeed, phylogenetic studies of these RNAs revealed their monophyletic origin. The phylogenetic tree separates autonomously replicating, but not self-cleaving viroids from self-cleaving, but not autonomously replicating satellite RNAs. Members of the *Avsunviroidae* family that display functional similarities to both groups (self-cleaving and autonomous replication) may represent an evolutionary link between viroids and satellite RNAs (Elena *et al.*, 1991).

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