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Potato spindle tuber viroid (PSTVd)

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ABSTRACT

Potato spindle tuber viroid (PSTVd) is the type strain of the largest viroid family *Pospiviroidae*. PSTVd is a circular, single-stranded RNA molecule with a sequence length of about 359 nt and a rod-like native structure that causes infectious diseases in solanaceous plants. PSTVd does not code for any protein but replicates autonomously in the nucleus of infected plants and is systemically transported by host proteins using functional motifs encoded in its genome. Here we summarize the present knowledge about these motifs and their functional relationship to replication, processing, transport, and cause of symptoms.

Keywords: DNA-dependent RNA polymerase II, Loop E, metastable structures, silencing RNAs, structural elements, transcription
Abbreviations: CCR, central conserved region; **nt**, nucleotide(s); **NMR**, nuclear magnetic resonance spectroscopy; **PM**, premelting region; **PSTVd**, potato spindle tuber viroid; **TL**, terminal left region; **TR**, terminal right region; **V**, variable region; **VM**, virulence modulating region

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INTRODUCTION

The name viroid is derived from virus-like; that is, on the one hand viroids do behave like plant viruses in certain respects, but on the other they differ from viruses in many biological features. Mature viroids consist of a covalently closed circular RNA that ranges in size from 246 to 399 bases. Because viroids do not code for any peptide or protein, they have to utilize proteins of the host for most biological functions like replication, processing or transport. Therefore, viroids can be regarded as minimal parasites of the host machinery. To do so, viroids have to present to the host machinery the appropriate signals that have to be based either on the sequence or the structure of viroid RNA.

Viroids replicate by a rolling circle mechanism using either an asymmetric or a symmetric pathway. Different enzymes are involved in the different types of the replication cycle. In either case replication includes a processing step of oligomeric replication intermediates to molecules of unit length. This step proceeds either by a viroid-internal ribozyme or by proteinaceous RNAse(s) of the host. According to this feature and others, viroids are classified (Flores *et al.* 1998) into two families:

Pospiviroidae do possess a thermodynamically stable rod-like secondary structure with a "central conserved region" (CCR) and do not self-cleave. They replicate via an asymmetric rolling circle mechanism and are located in the nucleus. According to sequence and structural homology, *Pospiviroidae* may be divided into three subfamilies and several genera, which are named according to the respective type member: pospiviroids, potato spindle tuber (PSTVd); hostuviroids, hop stunt; cocadviroids, coconut cadang cadang; apscaviroids, apple scar skin; and coleviroids, *Coleus blumei*.

Avsunviroidae are named after avocado sun blotch viroid, they do not possess a CCR and self-cleave by a hammerhead ribozyme. They replicate in a symmetric rolling circle mechanism and are located in the chloroplast. This family consists of four members, avocado sunblotch, peach latent mosaic, chrysanthemum chlorotic mottle, and eggplant latent viroid.

In the late 1960s Ted Diener (Diener and Raymer 1967, 1969) discovered the infectious agent as a novel pathogen; the name viroid was termed in the early 1970s (Stollar and

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Invited Review

Diener 1971; Diener 1972). The definition and basic features still make viroids exceptional entities, even more so at the end of the 1960s when viroids were described first as naked pathogens (for a personal view see Diener 2003). PSTVd was the first known circular RNA. It is smaller by a factor of 10 than the smallest virus. PSTVd was the largest RNA sequenced in 1978 by Gross *et al*.

For an overview on viroid-related topics see the recent reviews and books (Hadidi *et al.* 2003; Tabler and Tsagris 2004; Flores *et al.* 2005; Ding *et al.* 2005; Daros *et al.* 2006; Ding and Itaya 2007). In this review we will concentrate on PSTVd.

HOST RANGE AND SYMPTOMS OF PSTVd

Potato spindle tuber disease was described first in North America in the early 1920s (Martin 1922); effects are stunted potato plants with elongated tubers. Symptom expression depends, among other things, on the PSTVd strain and on temperature: different strains, named for example mild, intermediate, and severe, reduce yields of tubers from about 15 up to 100%; high temperatures increase symptoms. PSTVd can be transmitted by seed and pollen; efficient transmissions are possible mechanically by sap contamination. There is evidence for heterologous encapsidation of PSTVd in particles of potato leafroll virus and transmission by aphid vectors (Querci *et al.* 1997; Syller 2000). Natural infections of PSTVd have been reported on pepino (*Solanum muricatum*), avocados (*Persea americana*) and a range of wild *Solanum* spp.. Most other solanaceous species (f.e. *Solanum*, *Nicotiana*, *Petunia* spp.) have been infected under experimental conditions. The standard propagation host for PSTVd is tomato (*Lycopersicon esculentum*) cv. 'Rutgers', which develops symptoms like stunting and epinasty, depending on the severity of the infecting PSTVd strain (**Fig. 1**). Other tomato genotypes (f.e. cvs. 'Harzfeuer' and 'Moneymaker') are more tolerant to PSTVd infection compared to cv. 'Rutgers'. *Nicotiana benthamiana* is referred to as a symptomless host but biolistic transfer of lethal strain AS1 RNA led to induction of strong stunting (Matouek *et al.* 2007). A single nucleotide substitution in the loop E region (see below) of PSTVd converted the lethal strain KF440-2 from a noninfectious to an infectious RNA for *Nicotiana tabacum* (Wassenegger *et al.* 1996). For further details on these topics see Diener (1987), Hadidi *et al.* (2003) and Flores *et al.* (2005). Unfortunately (for scientific reasons) *Arabidopsis* is not a host for PSTVd or any other viroid

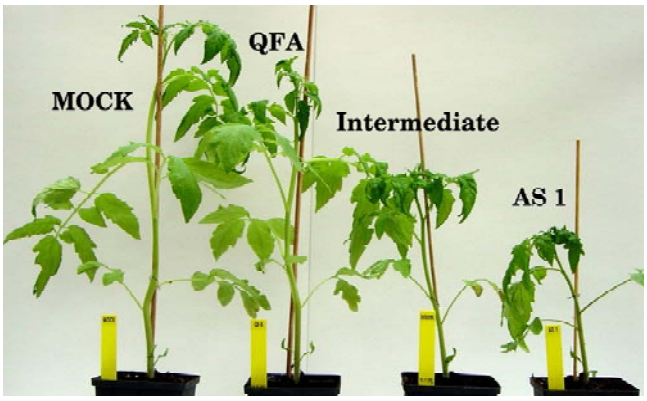


Fig. 1 Symptoms of different PSTVd strains. Tomato plants (*L. esculentum* cv. 'Rutgers') of identical age and duration of infection are shown: the first is non-inoculated (mock), the others are inoculated with a mild (QFA; Gruner *et al.* 1995), the type strain "intermediate (Diener)" (Gross *et al.* 1978), and a strong (AS1; Matoušek *et al.* 2007) symptoms producing PSTVd strain. Besides the standard stunted habitus of PSTVd-infected tomato plants, PSTVd-AS1 shows symptoms up to necrosis of stem and leaves.

(Daros and Flores 2004; Matoušek *et al.* 2004b). According to the analysis by Daros and Flores (2004) *Arabidopsis* has the enzymatic machinery for replicating viroid species of *Pospiviroidae* but viroids are unable to move to distant plant parts in *Arabidopsis*. Biolistic infection of *Arabidopsis* with a large pool of PSTVd mutants, generated by thermal stress in *Nicotiana benthamiana* and passaged through *Raphanus sativa*, gave rise to a new mutant progeny, but its level was ~300 times lower than compared to tomato (Matoušek *et al.* 2004b).

PSTVd IS REPLICATED IN AN ASYMMETRIC ROLLING-CIRCLE MECHANISM

The circular PSTVd is defined to have (+)polarity. It is the template for transcription into oligomeric linear (-)strands (**Fig. 2**). These (-)intermediates are template for the synthesis of oligomeric linear (+)intermediates. They are enzymatically processed by cleavage into molecules of unit length and ligated to the mature circles. This transcription process excludes circles of (-)polarity (Branch *et al.* 1988), which are present in the symmetric rolling-circle mechanism of

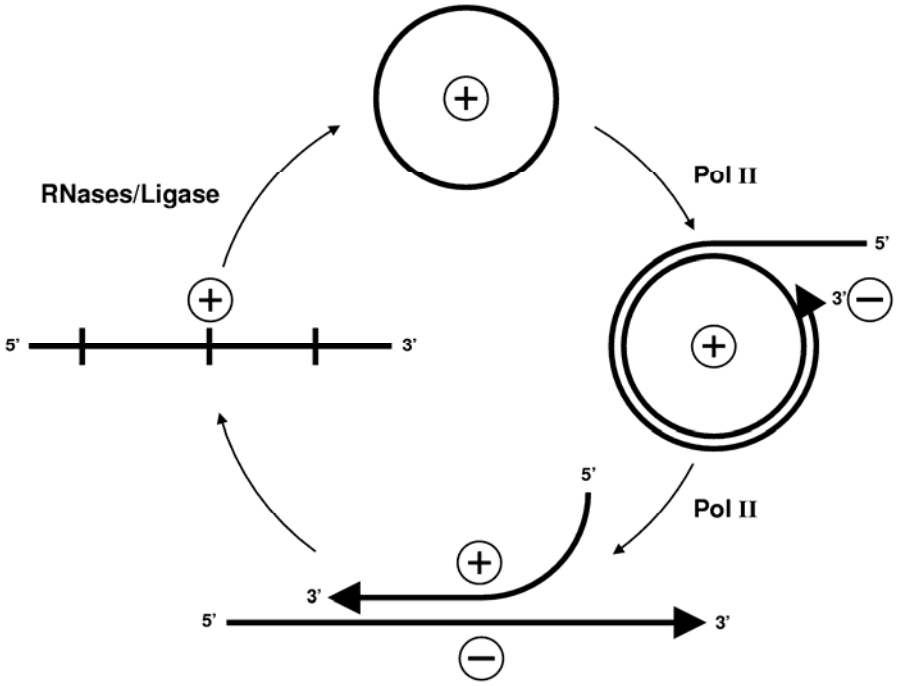


Fig. 2 Replication cycle of PSTVd. According to Branch and Robertson (1984) and Branch *et al.* (1988) the mature circular (+)strand (top) is transcribed into oligomeric (-)strands (right, bottom). These are template for synthesis of oligomeric (+)strands (bottom, left). The latter are enzymatically processed to monomers and ligated to circles (Baumstark and Riesner 1995; Baumstark *et al.* 1997). Both transcription steps are catalyzed by DNA-dependent RNA polymerase II (polII) (Mühlbach and Sänger 1979; Schindler and Mühlbach 1992).

Avsunviroidae. Furthermore, an autocatalytic cleavage and ligation step – for example by hammerhead ribozymes as in *Avsunviroidae* – could not be shown for PSTVd or any member of the *Pospiviroidae* family (Tsagris *et al.* 1987); for further details on the enzymatic processing see below.

By inhibition studies with α-amanitin and actinomycin D it was demonstrated that synthesis of (-)- as well as (+)-strand intermediates strongly depends on the activity of DNA-dependent RNA polymerase II and that RNA polymerases I and III are unlikely to be responsible for PSTVd replication (Mühlbach and Sänger 1979; Schindler and Mühlbach 1992).

The reason why PSTVd is a template for transcription by polII might be based on a structural similarity of GC-rich regions in PSTVd with GC-rich DNA-promoter regions of house-keeping genes: GC-rich regions in DNA can easily form non-B-form double helices (Heinemann *et al.* 1987; Wong *et al.* 2007), which may act as transcription factor binding sites. Details on such GC-rich motifs in (+) and (-)PSTVd are given below.

PSTVd IS LOCALIZED IN NUCLEI OF INFECTED CELLS

The subnuclear distribution of PSTVd (+)- and (-)-strand sequences was analyzed by fractionation of subcellular compartments from leaf tissue of tomato plants (Stollar and Diener 1971; Schumacher *et al.* 1983; Hecker *et al.* 1988) and later by *in situ* hybridization using fluorescence-labeled PSTVd transcripts as probes (Harders *et al.* 1989; Qi and Ding 2003). The majority of PSTVd molecules of both polarities is located in nuclei of infected cells. In chloroplasts – where viroids from the *Avsunviroidae* family are located – practically no PSTVd molecules were detected. The average number of PSTVd copies per nucleus is about 10^4; the copy number in individual cells is as high as $5 \cdot 10^5$, because only up to ≈20% of cells seem to be infected. Inside of the nuclei, about equal numbers of (+)-stranded PSTVd molecules are located in the nucleo-plasm and in nucleoli; that is, concentration of (+)PSTVd is highest in nucleoli. According to Harders *et al.* (1989) these distributions do hold for PSTVd molecules of both polarities; according to Qi and Ding (2003) only (+)strands are located in nucleoli whereas (-)strands are exclusively located in the nucleoplasm. Both would fit to the replication cycle of PSTVd and the involvement of polII, which is located in the nucleoplasm: circular PSTVd as well as (-)intermediates are transcribed in the nucleoplasm and at least the mature PSTVd is transported to nucleoli.

INTRA-PLANT TRANSPORT OF PSTVd

After inoculation with an infectious PSTVd strain of a host plant by either using Carborundum as abrasive or biolistically using a gene gun (Matoušek *et al.* 2004a), PSTVd has to infect at least a first cell and then to move through the plant for systemic infection. Woo *et al.* (1999) showed that infectious PSTVd transcripts are actively imported from cytoplasm into nuclei. In contrast, mRNAs of similar size, 70 kDa dextran, and two viroids from the *Avsunviroidae* family, which replicate in chloroplasts, remained in the cytoplasm. That is, (+)PSTVd has to contain a sequence or structure motif that enable its transport into the nucleus (Zhao *et al.* 2001).

For movement from cell to cell, plant viruses use virus-encoded 'movement proteins', which are not available to viroids. By micro-injection of PSTVd transcripts Ding *et al.* (1997) showed that the PSTVd moved in a few minutes from the injected cells via plasmodesmata to neighboring cells and accumulated in the corresponding nuclei. PSTVd cDNA was also able to mediate intracellular transport.

Long-distance movement of PSTVd parallels in part photoassimilation transport (Palukaitis 1987; Hammond 1994; Stark-Lorenzen *et al.* 1997); that is, after inoculation of a third leaf of a tomato plant, PSTVd replicates and moves into the shoot tip and leaves adjacent to the tip and then in other leaves between the inoculated leaf and the shoot tip as well as in the root, but not into the leaves below the inoculated leaf. PSTVd replication takes place in companion and phloem parenchyma cells during movement through the phloem sieve cells (Zhu *et al.* 2001). PSTVd is either not present or its concentration is below the detection limit of *in situ* hybridization in petals, stamens and ovary of tomato flowers (Zhu *et al.* 2001). This is in conflict with reports on the seed transmissability of PSTVd (see Singh *et al.* 2003).

THE NATIVE STRUCTURE OF PSTVd IS ROD-LIKE

Already prior to knowledge of the first PSTVd sequence (Gross *et al.* 1978) it was obvious from biophysical experiments that the native structure of PSTVd had to consist of a linear, rod-like arrangement of short helices and loops (**Fig. 3**; Langowski *et al.* 1978). This was corroborated by further biophysical and biochemical analyses like:

- melting curves (either optically registered or by temperature-gradient gel electrophoresis; Henco *et al.* 1977; Rosenbaum and Riesner 1987; Schmitz and Steger 1992)
- chemical and enzymatic mapping (Gross *et al.* 1978)
- binding of tRNAs to loops (Wild *et al.* 1980)
- UV cross-linking of loop E *in vivo* (Wang *et al.* 2007b)
- dye-binding
- electron microscopy
- determination of dimensions and stiffness by analytical ultracentrifugation (Riesner *et al.* 1982)
- secondary structure prediction (Steger *et al.* 1984)
- measurements (Henco *et al.* 1979) and prediction (Schmitz and Steger 1996) of kinetic folding

including knowledge of the sequence (Riesner *et al.* 1979). "Native" structure means that no structural variation could be detected under various salt conditions (from 0 to 10 mM Mg), which excluded any tertiary structure (in the sense of the L-conformation of tRNA or pseudoknots). In other words, the rod-like structure of PSTVd is the structure under thermodynamic equilibrium.

The relatively high stability of circular PSTVd against nucleolytic degradation might be based on the missing 5′/3′ ends, which prohibit access for exonucleases, and on the mixture of short helices and short loops, which make PSTVd a bad substrate for both double and single strand-specific endonucleases.

METASTABLE BRANCHED STRUCTURES ARE CRITICAL FOR PSTVd

From equilibrium and kinetic denaturation measurements (Henco *et al.* 1979; Riesner *et al.* 1979; Steger *et al.* 1984), a detailed mechanism of the reversible denaturation-renaturation was derived for circular PSTVd. Three characteristic regions – the premelting regions 1, 2, and 3 (PM1–3; cf. **Fig. 3**) – show lowest stability and denature 5-10°C below the main transition (49.5°C in 11 mM or 73°C in 1 M ionic strength). During the main transition all base pairs are disrupted and at least two particularly stable hairpins I (nt 79–87/102–110; $f_{G:C} = 0.67$) and II (nt 227–236/319–328; $f_{G:C} = 0.9$), which are not part of the native structure, are newly formed. At higher temperatures the stable hairpins denature according to their individual stabilities. Of course such temperatures of structural rearrangements are irrelevant to any biological process, but according to kinetic analysis the stable hairpins do form as metastable structural elements by sequential folding (Boyle *et al.* 1980; Nussinov and Tinoco 1981) during synthesis of (+) as well as (-) strands (Schmitz and Steger 1996; Repsilber *et al.* 1999). By structure-specific oligonucleotide-mapping hairpin II-containing (-)-strand intermediates could be detected *in vivo* (Schröder and Riesner 2002).

From carefully designed mutants of PSTVd, analysis of the mutants' infectivity, genetic stability and/or time cour-

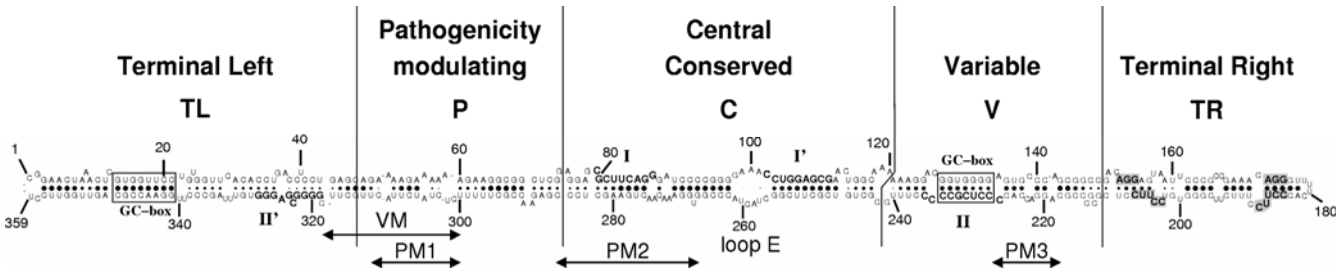


Fig. 3 Native secondary structure of PSTVd. The size of dots connecting base pairs is based on thermodynamic prediction of a consensus structure for 45 PSTVd variants; the sequence is given for the PSTVd variant intermediate (Gross *et al.* 1978). Nucleotides that are part of the extrastable hairpins I and II are in bold face. GC-rich regions are boxed. The five domains of pospiviroids are marked as proposed by Keese and Symons (1985). The two shaded 5′-AGG/CUUCC-5′ motifs in the TR domain are involved in Virp1 binding (Gozmanova *et al.* 2003).

ses of reversion to the wild-type sequence, Loss *et al.* (1991) and Qu *et al.* (1993) concluded (i) that hairpin II is a functional element in the (-)-strand replication intermediate, generated due to sequential folding during synthesis, and that it is essential for template activity of (+)-strand synthesis; (ii) that G:U pairs are tolerated transiently in (-)-strand hairpin II; the lower stability of such a hairpin II is compensated by additional mutations outside hairpin II which suppress the competition of a rod-like structure; and (iii) that the reversions are generated spontaneously during (-)-strand synthesis.

STRUCTURAL DOMAINS OF PSTVd

On the basis of sequence homology between seven pospiviroids, Keese and Symons (1985) proposed a model of five structural and functional domains in the rod-like structure (**Fig. 3**). These are the terminal left (TL) and right (TR), the pathogenicity-modulating (PM), central conserved (CC), and variable (V) domain. Features of these domains will be discussed below. Take note that these domains consist of structural elements from the rod-like PSTVd structure, whereas already hairpin II, described in the previous section, depends on sequence regions that are not close neighbors in the native structure.

To obtain results described in the previous and following sections, synthetic mutants were used to analyze interdependence of specific sequence positions or structural elements with biological features or functions of PSTVd. In this context remember that most positions in PSTVd serve several functions: base pairing in (+)- and (-)-strand equilibrium and metastable structures is critical to present sequence and/or structural elements to the host machinery. As a consequence most multiple mutations are lethal to PSTVd whereas single-site mutants do revert often quite easily during the first plant passage. For examples see Loss *et al.* (1991), Owens *et al.* (1991), Qu *et al.* (1993), and Hu *et al.* (1997). Two simple strategies do help to get progeny from synthetic mutants: the infectivity of circular RNA is superior to that of linear molecules (Feldstein *et al.* 1998) and biolistic inoculation with a gene gun has a higher efficiency of inoculation than conventional mechanical inoculation using Carborundum (Matoušek *et al.* 2004a). A further alternative are transgenic plants. For example, Wassenegger *et al.* (1994) agrotransformed *Nicotiana tabacum* plants with a non-infectious PSTVd cDNA with a deletion of 9 nt (nt 70–78); *in planta* a mutant evolved that had a 9 nt deletion (nt 282–290) opposite in secondary structure to the original deletion plus additional five single nucleotide changes. The evolved 341 nt long mutant was infectious and genetically stable in tomato cv 'Rentita'.

The TL region contains the start site of (-)strand replication intermediates

The TL region of PSTVd contains a repeat of eight nucleotides (nt 15–21 and 352–359; **Fig. 4**) like most other members of *Pospiviroidae* except the Iresine viroid. This duplication allows for two alternative structures, an elongated and a bifurcated one (Riesner *et al.* 1979; Gast *et al.* 1996). According to structure prediction, consensus structure prediction for viroids of *Pospiviroidae*, and biophysical analysis by temperature-gradient gel electrophoresis, optical melting curves and nuclear magnetic resonance spectroscopy (NMR), the elongated structure is highly favored over the bifurcated structure (Dingley *et al.* 2003).

Kolonko *et al.* (2006) describe an *in vitro* system for (-)-strand PSTVd synthesis in a nuclear extract from a non-infected cell culture of the host plant *Solanum tuberosum*. (-)strands, which were synthesized after addition of circular PSTVd as template, were purified by affinity chromatography. Primer-extension analysis of the *de novo*-synthesized (-)strands revealed a single start site located in the TL hairpin loop at either U359 or C1. This fits to the earlier observation of Goodman *et al.* (1984) that wheat germ pol II binds to the terminal loops of PSTVd. A differentiation between the two positions was not possible due to low resolution of the sequencing gels. A mutant C1G was less infectious than the wild-type sequence but this mutant as well as mutant C1U (Owens *et al.* 1991) were genetically stable. A mutant U359G reverted to wild type within the first plant passage. This finding suggests that U359 is the start site of transcription and that the nucleotide identity of this first position is essential for transcription initiation; such a dependence is known from other polymerases. Located nearby to the start site is a GC-rich region (marked as GC-box in **Fig. 3**), which may act to facilitate initiation (Fels *et al.* 2001) similar to the GC-rich hairpin II in the (-)-strand intermediate.

The start site of (+)strands from (-) to (+)transcription is not known.

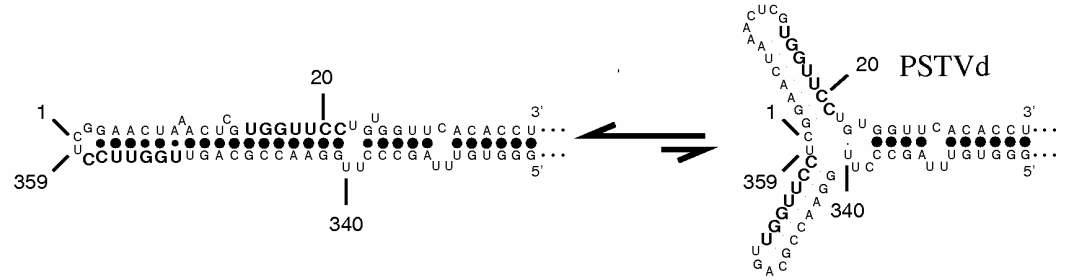


Fig. 4 PSTVd's TL region in rod-like and bifurcated conformation. Nucleotides in bold-face highlight the two repeat units that give rise to the structural alternatives. Size of dots connecting base pairs is proportional to their predicted pairing probability.

Pathogenicity-modulating region

In 2007 at least 100 natural and synthetic sequence variants of PSTVd are known (Rocheleau and Pelchat 2006). Most variants differ by only a few mutations. In many cases small sequence variations in the PM region lead to variations in symptoms of infected tomato cv. 'Rutgers'. Schnölzer *et al.* (1985) determined the sequences of three such pathogenicity variants called "mild" (KF6), "severe", and "lethal" (KF440). The name "lethal" was chosen to depict the increased symptoms in comparison to the variant "severe", but the lethal variants lead not to death of the host. Including the standard strain "intermediate (DI)" the important sequence differences were located in the "virulence-modulating" (VM) region (**Fig. 3**). This region includes the "pre-melting loop 1" (Steger *et al.* 1984) and all four variants fitted to a model in which thermodynamic stability of the VM region decreased with increasing symptoms. This hypothesis pointed to a PSTVd/nucleic acid interaction as the primary trigger for symptom production.

The thermodynamic model of a pathogenicity trigger was obsoleted by an increasing number of PSTVd variants with a stability of the VM, PM or P region that did not fit to their symptoms. At first an interdependence between pathogenicity and replicability was shown (Gruner *et al.* 1995); that is, more severe PSTVd variants do overgrow milder variants. It has been shown, however, that severity of symptoms is not a direct consequence of PSTVd titers (Góra *et al.* 1996; Owens *et al.* 1996). From three-dimensional structure prediction for 12 PSTVd variants producing different symptoms (Owens *et al.* 1996) and gel-electrophoretic determination of degree of bending and flexibility in the VM region of six out of these 12 PSTVd variants (Schmitz and Riesner 1998) a model was put forward that mild variants possess a straight and rigid VM region whereas the angle of bending increases monotonically with the pathogenicity of the variant. This model favors protein-binding to the VM region as the primary pathogenic event.

In the mean time a further PSTVd variant was found (AS1; Matoušek *et al.* 2007) that does produce more severe symptoms than the other "lethal" strains but its sequence does not fit to either of the above models.

RNA interference may provide a different explanation for PSTVd pathogenicity. The low molecular weight RNA fraction of the cytoplasm from tomato plants inoculated with PSTVd contains a population of 22 and 23 nt long PSTVd-specific RNAs of both polarities (Itaya *et al.* 2001; Papaefthimiou *et al.* 2001; Denti *et al.* 2004). Such RNAs are characteristic of post-transcriptional gene silencing; that is, PSTVd might trigger RNA silencing. The levels of small PSTVd-specific RNAs were enhanced in symptomatic tomato plants after biolistic infection with PSTVd variant AS1 in comparison to the mild QFA variant (Matoušek *et al.* 2007). According to Itaya *et al.* (2007) the small RNAs are mostly derived from single-stranded PSTVd (**Fig. 5C**), and PSTVd replication was resistant to RNA silencing presumably to its stable secondary structure. That is, PSTVd behaves more like a pri- or pre-miRNA (Wang *et al.* 2007a) than giving rise to siRNAs via a double-stranded replication intermediate, which could never be identified for PSTVd.

The small PSTVd-specific RNAs, isolated, cloned and sequenced by Itaya *et al.* (2007), were derived mainly from (+)PSTVd and there particularly from the upper part of TL and V domains and from the lower part of TR domain (**Fig. 5C**).

A computational approach to search for potential targets of PSTVd-derived small RNAs proceeded as follows (Teune, Junge, Steger, unpublished): The 359 nt long PSTVd sequences of variants KF5 (mild) and RG1 ("lethal") was cut into 359 overlapping fragments of length 23 nt; these were searched for in the genome of *Arabidopsis thaliana*. As matches were counted complementary fragments with one mismatch, one insertion and/or one deletion. In total 108 matches were found for KF5 and 206 for RG1, respectively (**Fig. 5A, 5B**). In the *Arabidopsis* genome about 50% of matches are located in regions annotated as protein-coding. In the PSTVd sequences 90% of matches are located in the upper TL to upper P regions and in the lower P region; these are marked by black arrows in the secondary structure of PSTVd (**Fig. 5C**). A statistical significance of this result is not easy to evaluate due to the nucleotide bias of the P region, which contains an oligo-purine stretch in the upper and an oligo-pyrimidine stretch in the lower part.

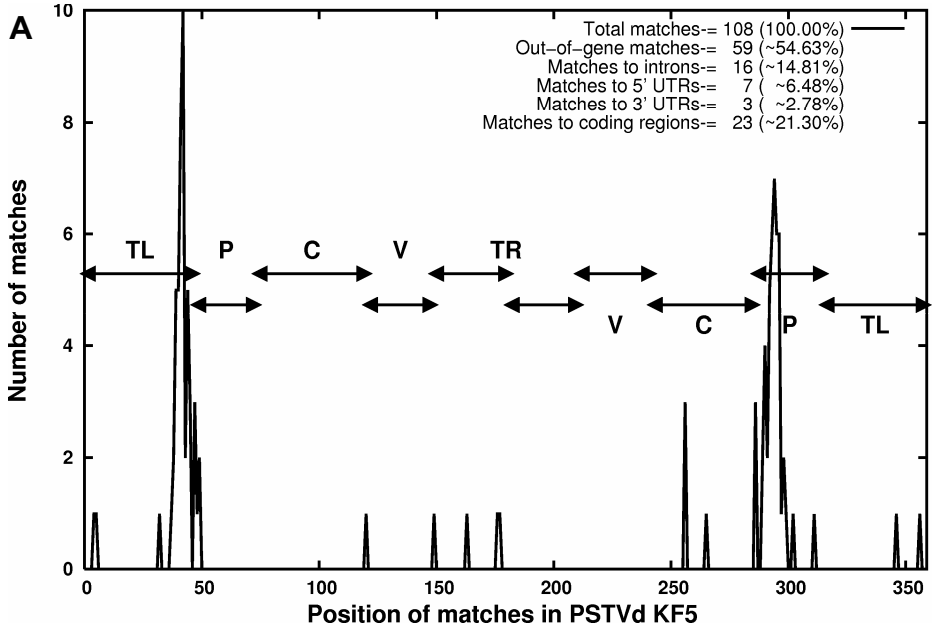
Host proteins that might be affected by the PSTVd-derived small RNAs are presently unknown. During disease development of a PSTVd-infected host many protein concentration alterations are found. An example is a "pathogenesis-related" protein of 14 kDa that is drastically increased in concentration; its induction, however, is not viroid-specific because it is also accumulating after viral and fungal infections (Camacho Henriquez and Sänger 1984). Itaya *et al.* (2002) analyzed the altered gene expression in tomato cv. 'Rutgers' after infection with either a mild or a severe PSTVd strain. Genes with altered expression encode products involved in defense/stress response, cell wall structure, chloroplast function, protein metabolism, and other diverse functions; the expression of some of these genes was also altered by TMV infection.

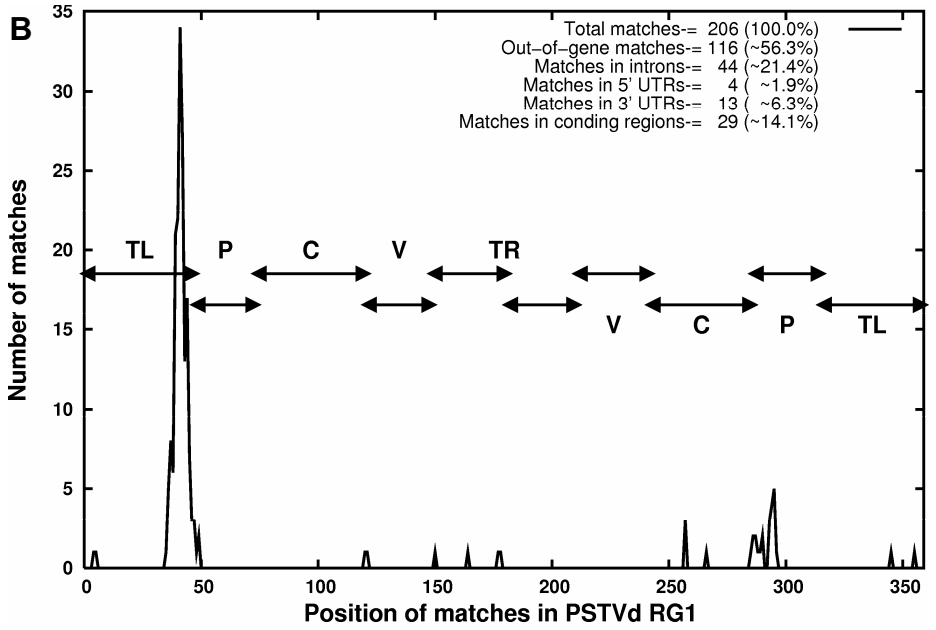
The central conserved region is involved in processing of (+)intermediates

From sequence comparison among the early available viroid sequences, the presence of a highly conserved region has been described in both the upper and the lower central domain of PSTVd and related viroids (Gross *et al.* 1982; Diener 1983; Keese and Symons 1985). In PSTVd, the upper conserved region contains the sequence that forms hairpin I during the main melting transition (Henco *et al.* 1979; Steger *et al.* 1984). The same hairpin is also prominently observed in simulations of the kinetically controlled folding as well as sequential folding of PSTVd (Schmitz and Steger 1996).

The C region contains a structure motif that has been described in a variety of RNAs, especially eukaryotic 5 S rRNA, the large subunit rRNAs from all kingdoms of life, and the hairpin ribozyme. This so-called loop E motif (**Fig. 6**) consists of five and six nucleotides in the two strands forming the loop, which is enclosed by Watson-Crick base pairs. All eleven loop nucleotides are involved in non-Watson-Crick base pairs and stacking interactions. The loop E motif in PSTVd was proposed from sequence similarity to the loop E motif from eukaryotic 5S rRNA and the sarcin-ricin loop motif from the large subunit rRNAs. High structural similarity to these motifs was verified by UV-induced cross-linking between nucleotides G98 and U260 residing on opposite strands of the internal loop motif (Branch *et al.* 1985). This cross link arises from the particular cross-strand purine stack conformation of nucleotides G98-A99 and U260-A261, which appears preserved in PSTVd although the stabilizing base triple formed by the bulged nucleotide 259 and the A99:U260 pair is absent in PSTVd. The structural similarity between known loop E motifs and the PSTVd loop E has been confirmed by chemical modification studies (Owens and Baumstark 2007). Replacement of the bulged nucleotide C259 by G results in a marked stabilization of the loop motif (Schrader *et al.* 2003), which is in excellent agreement with data obtained on the sarcin-ricin loop motif (Seggerson and Moore 1998).

The central conserved region of PSTVd, including the loop E motif plays an important role in the processing reaction that converts multimeric, linear (+)polarity viroid transcripts into monomeric, infectious circular progeny (Steger *et al.* 1986; Candresse *et al.* 1990; Steger *et al.* 1992). The following model (**Fig. 7**) was proposed by Baumstark (1995, 1997), and mutational analysis (Schrader *et al.* 2003) has been instrumental in highlighting the importance of the involved motifs for correct processing. An





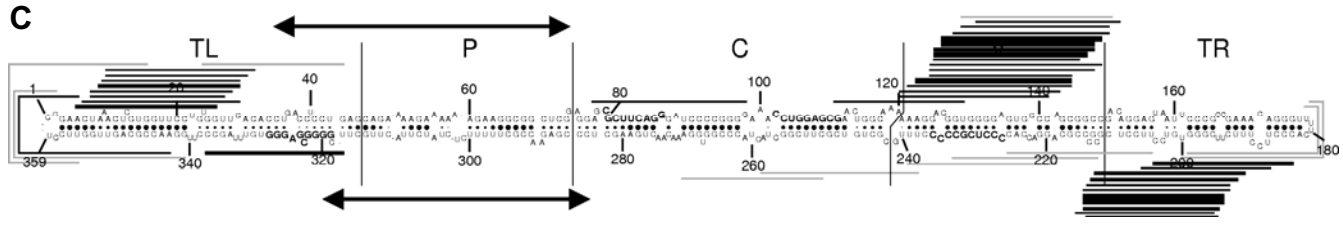


Fig. 5 Complementarity of PSTVd fragments and the *Arabidopsis thaliana* genome. The locations of fragments complementary to the *Arabidopsis* genome are shown in (A) for PSTVd variant KF5 (mild) and in (B) for PSTVd variant RG1 ("lethal"); take note of the scalings of the y axis in both graphs. The two regions with most hits are marked by black arrows in the secondary structure of PSTVd (**C**). By black and grey lines are marked viroid-specific small RNAs with (+) and (-)sequence, respectively, identified in PSTVd-intermediate-infected tomato cv. Rutgers (Itaya *et al.* 2007). For labeling of PSTVd structural regions compare **Fig. 3**.

alternative conformation of the upper strand of loop E in a longer-than-unit length PSTVd (+)intermediate, forming a hairpin capped by a GAAA tetraloop is recognized by a host enzyme (Klümper 2002) in the first step of cleaving the 5′ end of the primary transcript (**Fig. 7A**). The hairpin stem is destabilized by this cleavage, and the structure of the C region rearranges to form the loop E motif (**Fig. 7B**). This rearrangement is driven by the stability inherent in the compact loop E structure. The second step of processing results in cleaving off the 3′ portion of the primary transcript, resulting in a monomeric linear RNA (**Fig. 7C**). This second cleavage step occurs at a distance from the loop E motif that is similar to the distance between the α-sarcin cleavage position and the loop E motif in the sarcin-ricin loop. The subsequent ligation reaction is catalyzed by an enzyme that has been characterized as similar to a tRNA synthetase (Klümper 2002). This step is inhibited by the presence of a stable base triple between G259 and A99: U260 in a mutant (Schrader *et al.* 2003).

A number of mutations have been reported that influence infectivity and levels of replication of PSTVd in both host and non-host plants (Wassenegger *et al.* 1996; Qi and Ding 2002). All naturally occurring mutants preserve the key structural features of the loop E motif, most notably the cross-strand purine stack and the absence of a base triple involving position 259 (as concluded from different thermodynamic stabilities between C259G and all other mutants). Based on assumptions on isosteric replacement of non-canonical base pairs (Leontis *et al.* 2002), a model for the non-canonical pairings in PSTVd loop E has been proposed (Zhong *et al.* 2006). This model is not entirely free from contradiction to experimental data. It does not take

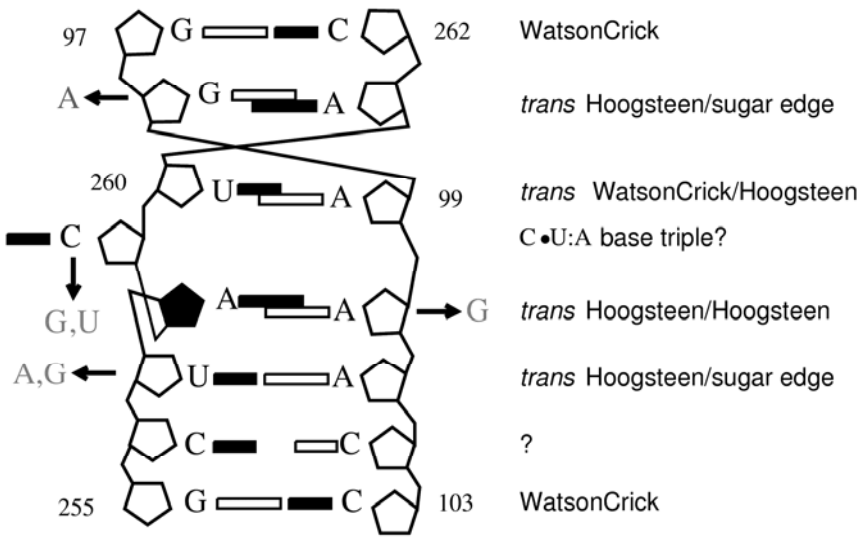


Fig. 6 Loop E motif in the CCR of PSTVd. On the left is shown schematically the structure; on the right are named the proposed pairing interactions. Arrows point to mutations described in the text. The central base triple, which is G·A:U in the ricin-sarcin loop motif, does not form due the C259. For an overview on non-Watson-Crick base pairs see f.e. Leontis *et al.* (2002).

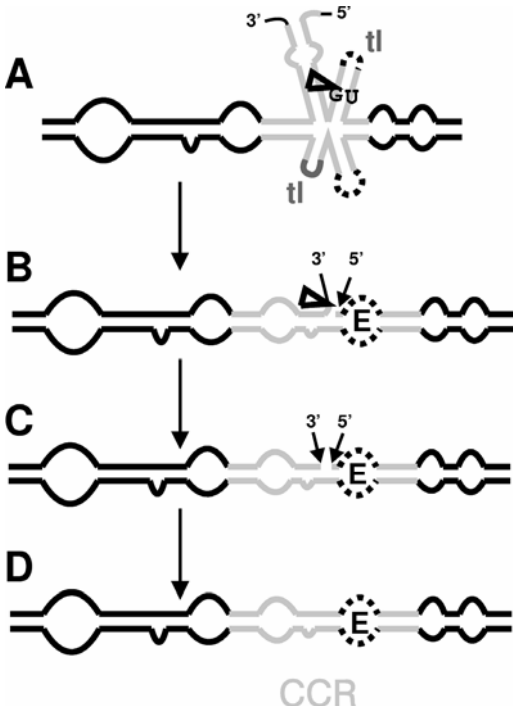


Fig. 7 PSTVd processing: a switch from cleavage to ligation is driven by a change from a tetraloop to a loop E conformation. The central section of the rod-like PSTVd structure is shown schematically. Sequence regions belonging to the CCR are shown in grey; those forming loop E in the native structure by stippled lines; the hollow triangle points to the cleavage sites. For further details see text.

into account the fact that mutant A100G does not preserve the UV cross link, hence does not support the loop E structure (Schrader *et al.* 2003). A G100:A258 that is 'near-isosteric' to A100:A258 evidently is not sufficiently similar to support loop E structure formation. Nonetheless, analysis of the mutation data from a base pair isostericity point of view results in a picture of this crucial structure element of PSTVd that is consistent with most experimental data, and opens the question of functional differences caused by mutations that have a strictly conservative effect on structure. On this background, the role of key positions like C259 and U257 may be in protein interaction rather than loop structure.

The variable domain

The name of this domain stems from its low degree of sequence conservation between otherwise closely related pospiviroids (Keese and Symons 1985). It includes premelting loop 3 (PM3), the 5′ part of highly conserved hairpin II, and a GC-box. Hu *et al.* (1996) designed a series of single- and double-site mutations in PM3 and analyzed infectivity at 26 and 32°C, and thermodynamic stability of the original mutants and additional, *in planta* successively appearing mutants. In general PSTVd accumulates to higher concentrations at higher temperatures, and the higher temperature allows for appearance, replication, and selection of otherwise inhibitory mutants (Gruner *et al.* 1995; Matoušek *et al.* 2004b). During these plant passages, some of the original mutations were retained but additional, spontaneous mutations appeared that obviously had to compensate for some defects of the original mutants. Remember that an increased stability of PM3, and thus an increased stability of the equilibrium structures of complete (+) as well as (-)strands, leads to decreasing possibility for formation of hairpin II. Hu *et al.* (1996) concluded that selective pressures arising from the interaction of assay temperature and structural stability *in vivo* appear capable of moving PSTVd populations between peaks of relatively high fitness, and that selection may occur at the level of either the (+) or the (-)strand, depending on the exact nature and location of the mutation.

The terminal right domain

By mutations in the terminal hairpin loop, Hammond (1994) identified this element as critical for trafficking: after *Agrobacterium*-mediated inoculations the PSTVd mutant was restricted to the gall and root tissues of the plant.

Using a phage-based screening system (Sägesser *et al.* 1997), Martínez de Alba *et al.* (2003) identified a "viroid binding protein" (Virp1) from tomato that interacts *in vivo* and *in vitro* with PSTVd. Virp1 contains a nuclear localization signal and a bromodomain, which is found in a variety of proteins thought to play a role in dynamic chromatin; a host function of Virp1 is not known (Kociaska *et al.* 2005). Gozmanova *et al.* (2003) identified a 5′-AGG-3′/3′-CUU CC-5′ motif and the neighboring asymmetric internal loop close to the right hairpin loop as critical for Virp1-binding to PSTVd (**Fig. 3**); a similar motif closer to the variable region also contributes to binding. Mutations in any of both motifs abolished infectivity of PSTVd mutants.

SUMMARY

PSTVd consists of overlapping sequence and structural domains which are necessary for PSTVd to parasite on the host system. The knowledge about the interacting partners of the host is low; this might be due to the problem that these envisaged interactions have not to be of the high specificity as for the co-evolved interactions between RNAs and proteins of the host. Furthermore, the high concentra-

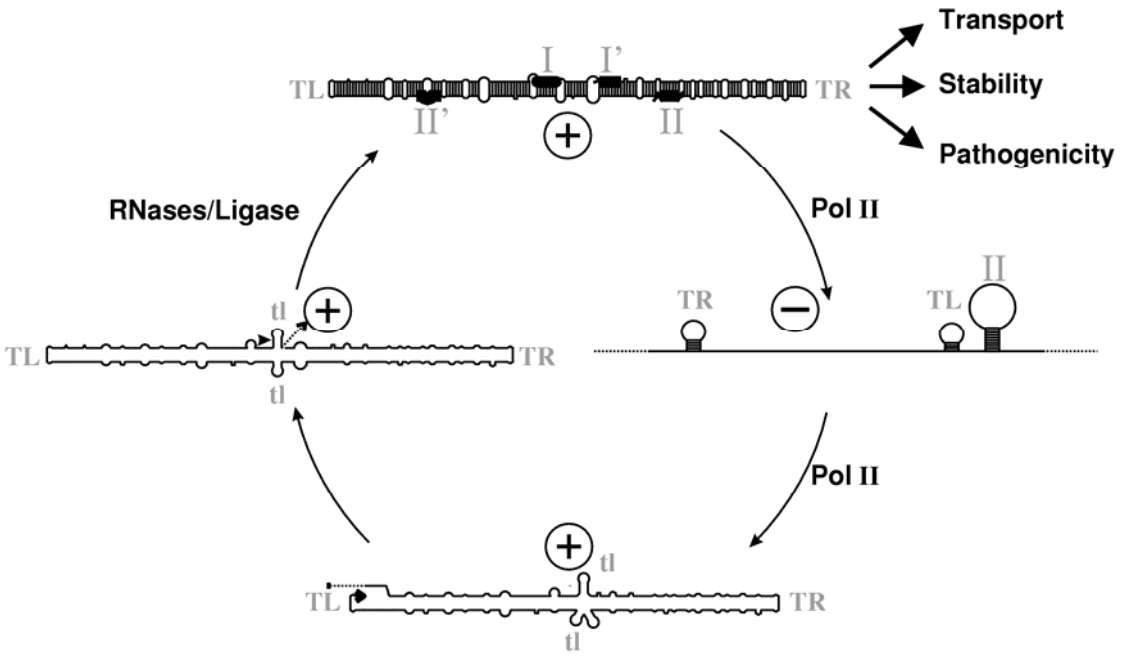


Fig. 8 The replication cycle of PSTVd is a cycle of structures.

tion of PSTVd in nucleoli (up to a few hundred μM) does not force very high binding constants. Despite this problem a few proteins are known to interact with PSTVd (**Fig. 8**):

- polII is responsible for transcription;
- a (unidentified) RNAse is responsible for 5′ cleavage and
- a further (unidentified) RNAse for 3′ cleavage of (+)oligomeric replication intermediates to unit-length molecules (Klümper 2002),
- a ligase for closure of the unit-length molecules to the mature circles,
- and Virp1 might be involved in trafficking.

These interactions and the capability of PSTVd to arrange into thermodynamically stable as well as metastable structures led to the hypothesis that the replication cycle of PSTVd (and other viroids) is not only a cycle of sequences of both polarities – as shown in **Fig. 2** – but a cycle of different structures (**Fig. 8**) which increases the number of structural elements available for interactions with the host machinery.

Several host RNAs were proposed as partners interacting with PSTVd (for examples see Gross *et al.* 1982; Dinter-Gottlieb 1986; Haas *et al.* 1988; Meduski and Velten 1990); the interactions were shown, however, by computer predictions and *in vitro* experiments with partial sequences, but do not hold for full sequences. For example, denaturation plus renaturation of PSTVd in the presence of tomato 7S RNA did not give rise to any PSTVd/7S RNA complexes (Gruner 1992). Neither processing of ribosomal RNA nor assembly of ribosomes is influenced by PSTVd infection of tomato cv. 'Rutgers', and PSTVd does not cosediment with ribosomes from infected tomato plants (Thiel 1999). One remaining host RNA is 5 S rRNA which might be involved in intercellular transport of PSTVd (Aschermann 2001).

PSTVd is not only of interest as a special pathogen but played also a significant role during development of RNA technology during the last 25 years: the need for large and pure amounts of PSTVd for biophysical analyses forced development of anion exchange columns (Colpan *et al.* 1983) that led to founding of the company Qiagen, the circular PSTVd with its structural alternatives forced development of bioinformatic tools for structure prediction for circular RNAs, for suboptimal structures (Steger *et al.* 1984) and for kinetic structure formation (Schmitz and Steger 1996), structural analysis of co-existing structures of PSTVd and in crude extracts asked for development of temperature-gradient gel electrophoresis (Rosenbaum and Riesner 1987), and NMR analysis of PSTVd's large TL region led to detection of magnetic coupling through hydrogen bonds (Dingley and Grzesiek 1998). The complexity of PSTVd, however, begs for further techniques and advancement of knowledge in related plant processes.

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