

Molecular Mechanisms of Viroids

Subjects: **Virology**

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Viroids are one of the most inscrutable single-stranded, structured, circular RNA pathogens of plants as well as the smallest infectious agents ever known. Despite being incapable of coding for any proteins, viroids affect susceptible plant hosts with visually discernible symptoms resembling those induced by several plant viruses. Diener, 1967, 1971 discovered and exemplified the Potato Spindle Tuber Viroid (PSTVd), the first viroid ever known. He coined the term “viroid” to represent this diminutive, naked, protein-free, circular RNA plant pathogen. This conceptualization of the viroid was further substantiated by Sanger, 1972 as well as Semancik and Weathers, 1972, who identified the citrus exocortis viroid (CEVd) that is responsible for causing the citrus exocortis disease. Another viroid, the chrysanthemum stunt viroid (CSVd) is also one of the viroids initially identified.

viroids

replication

rolling circle

pathogenicity

1. Biology of Viroids

Discovered in 1971, viroids are the smallest and simplest plant pathogens, ranging from 250 to 400 nucleotides of RNA, and are known as “living fossils of the hypothetical RNA World” ^[1]. These autonomous infectious agents are devoid of any protein coding capability and can survive without any protected membrane around their single-stranded, circular RNA genomes. Viroids can not only fight the host cellular mechanisms of RNA degradation but can also successfully replicate themselves using factors from the host plant, resulting in their spread throughout the plants and disease symptoms ^[2]. Angiosperms are reported as the only natural hosts of viroids ^[3].

The processes involved in viroid infection after entry into cells includes ^{[4][5][6][7]}.

(a)entry into a subcellular organelle (chloroplast or nucleus according to the type of viroid),

(b)rolling circle replication within chloroplast or nucleus,

(c)release out of the cell following replication,

(d)transport into nearby cells,

(e)entry into and within the phloem,

(f) invasion of nonvascular cells from the phloem and,

(g)repeat of the infection cycle.

The viroid genome does not encode for any protein, but instead spreads throughout the plant by recruiting host proteins using their own RNA folded structure. Functional motifs within the RNA structure are required for cellular trafficking in host plants [8]. The nucleotide sequence of the viroid is also important for interacting with the genome of the host. RNA-directed DNA methylation (RdDM) has been reported in viroid-infected plants [9][10] and examples of silencing of functional genes by individual viroid derived small RNA (vd-sRNA) have been reported [11][12][13].

Viroids replicate in host plants using host RNA polymerases that interact with the viroid RNA template, resulting in a higher than usual rate of errors in comparison to DNA replication. Hence, a given replicating viroid produces several mutants along with its original sequence, the mix being termed quasispecies [14][15]. These closely related quasispecies exhibit vast variation in their host invading capability, demonstrating that the property of infecting the host depends mainly on nucleotide sequence of the viroids. Furthermore, the cause of variability in symptoms of viroid infections also depends on viroid-host-environment interactions.

The cytopathology of family Pospiviroidae include cavity formation within the cell membrane and cell wall thickening [16]. Peach latent mosaic viroid (PLMVd) infections were demonstrated to lead to chloroplast malformations [17]. Tracking viroid cytopathic effects represents a promising approach to understand the links between disturbances at the cellular level and macroscopic symptoms [18][19].

Viroids are distinguished on the basis of their transmission within host plants following either vertical or horizontal patterns [20]. Pollen and ovules of a plant are responsible for transmission of viroid infections from parental plants through seeds and then seedlings to the next generation in the vertical mode of transmission; an example is the PSTVd. During horizontal transmission (an example is Tomato planta macho viroid), infection is transmitted to the next generation through the ovaries of a plant getting infected from physical contact of another plant without fertilization [21]. The degree of vertical and/or horizontal transmission of viroids depends on the molecular interaction of the viroid with the host plant, resulting in recognition and elimination of viroid RNA in the male gametophyte [22].

Mediator (MED) is a conserved protein complex in plants. According to recent reports, MED subunits exhibited differential expression patterns against different viroids, suggesting that the connection of the MED subunit transcriptional reprogramming with viroid infections resulted in differences in symptom development for different viroids [23].

2. Taxonomy and Classification of Viroids

According to the International Committee on Virus Taxonomy (ICTV), “rules concerned with the classification of viruses shall also apply to the classification of viroids” [24].

Viroid classification is based on secondary structure. Currently 32 species of viroids are recognized by ICTV. The observation of quasispecies poses an important taxonomic question for considering related viroid variants as of the same or different species. Less than 90% genome sequence identity was the first criterion taken into consideration and to be accompanied later by the possession of another distinguishing biological feature, in order to classify any two viroid species as distinct, as per the ICTV classification requirements [25].

Viroids are divided into two families: Avsunviroidae and Pospiviroidae [3][4][5][7][26][27][28]. Family Avsunviroidae contain three genera with members resembling ASBVd with branched secondary structures chloroplast-based symmetric rolling-circle replication mechanism and most importantly having ribozyme activities. On the other hand, family Pospiviroidae with five genera has PSTVd-like members, consisting of five major domains as secondary structures, asymmetric rolling-circle replication that occurs in the nucleus and are commonly devoid of any ribozyme activities. Avsunviroidae infect only dicotyledonous plants, both herbaceous and woody [29] while viroids of the family Pospiviroidae can infect both monocot and dicot plants [30].

The three genera of family Avsunviroidae are: Avsunviroid, Pelamoviroid, and Elaviroid. Species in the genus Avsunviroid, have a low content of G + C with rod-like secondary structure. Species of genus pelamoviroids have high G + C content with branched secondary structures. The single species of genus elaviroid have intermediate structural properties that are between those of the other two genera of the family [19].

Five genera of family Pospiviroidae are: Apscaviroid, Cocadviroid, Coleviroid, Hostuviroid and Pospiviroid. Each genus has a characteristic central conserved region (CCR) with least modifications, while the species of genera apscaviroids and pospiviroids have terminal conserved regions (TCR) and those of cocadviroids and hostuviroids have terminal conserved hairpins (TCH). The Coleviroid species are devoid of TCR and TCH [31].

3. Viroid Structure and Replication

PSTVd was the first viroid structure ever determined [32]; electron microscopy revealed that PSTV forms a secondary structure, and this was confirmed in 1978 [33][34].

Most of the known viroid species belong to the *Pospiviroidae* family, named after the type member, the PSTVd [4]. The Pospiviroidae adopt circular, internally base-paired, rod-like structures and their genomes consist of five distinct domains: The terminal left (TL), terminal right (TR), central (C), pathogenic (P) and variable (V) domains. The pathogenicity domain possesses relatively low thermodynamic stability (or pre-melting) due to the presence of an oligopyrimidine stretch in most of the *Pospiviroidae*, the V domain is the most variable region, and the C domain is most highly conserved.

To date, only four viroids have been identified as belonging to the *Avsunviroidae* family, named after ASBVd. These types of viroids lack a conserved central region but retain rod-like and branched regions like Pospiviroidae viroids. However, unlike Pospiviroidae viroids, *Avsunviriodae* members can form hammerhead ribozyme motifs in both

polarities which mediate cleavage of their replication intermediates, while RNA cleavage within *Pospiviroidae* family members takes place using host enzymes [4].

The replicative mechanism of viroids operates through two alternative pathways depending in whether it is mediated by one or two rolling circles [35]. The first pathway is called asymmetric, typical of *Pospiviroidae* and is mediated by a single rolling circle wherein the incoming circular positive sense viroid RNA genome is repeatedly transcribed to form multimeric, linear (–) strands. Thereupon, in the next step, these (–) sense concatemers serve as templates for the synthesis of multimeric, linear (+) strand concatemers which subsequently are cleaved into monomeric (+) circles. In the alternate second pathway called symmetric, typical of *Avsunviroidae* [36], replication operates through two rolling circles wherein the multimeric linear (–) strands generated from the (+) sense RNA genome of the viroid undergo cleavage and ligation to generate circular (–) strand RNA monomers. These (–) strand circles then act as templates for the subsequent generation of linear, multimeric (+) strands which then cleave into (+) sense monomer genomes.

For PSTVd, the type member of *Pospiviroidae*, the absence of circular monomeric (–) sense RNA in plants after natural infection supports the asymmetric model of replication [37][38]. On the other hand, for the ASBVd (the type member of *Avsunviroidae*) it has been shown by in vitro studies that both (+) and (–) dimeric RNAs are able to self-cleave, thus supporting the model of symmetric replication [39][40]. Additionally, it has been observed in ASBVd-infected avocado that both monomeric circular (+) and (–) strands occur in multistranded complexes substantiating that ASBVd replication occurs through two rolling circles [41][42].

Members of the *Pospiviroidae* replicate in the nucleus [43]. Replication is initiated from a precise site, thus implying the possibility of existing viroid promoters. The processes of cleavage and ligation in the *Pospiviroidae* family are thought to be catalyzed by a host enzyme similar to RNase III and an RNA ligase that supports the circularization process. The two enzymes can circularize the viroid by covalent fusion of both 5' and 3' termini. Researchers have not identified whether chloroplastic RNA ligase is necessary for the circulation process or whether autocatalysis takes place.

Avsunviroidae replication takes place within chloroplasts. The mode of entry and exit into the chloroplasts is still debatable as it is not well identified [43].

The accumulation of *Avsunviroidae* (+) and (–) strands in the chloroplast indicates the involvement of enzymatic machinery of the chloroplast in the replication cycle; in contrast, accumulation of the *Pospiviroidea* RNA strands in the nucleus suggests the involvement of nuclear RNA polymerase and other cellular enzymes in their replication cycle [35].

4. Movement and Systemic Trafficking of Viroid RNAs

Viroids are subviral pathogens that cause infection in several crop plants, leading to considerable yield losses [44]. Within the plants, viroid RNA moves to adjacent cells through plasmodesmata and, via the phloem, to distant sink

organs [45]. Viroids have recently emerged as ideal model systems to study RNA transport within and between cells [45]. Conventional viroid infection of a host plant comprises a series of coordinated steps that involve both intracellular movement and intercellular movement.

Mutational experiments of viroids have identified RNA motifs within the viroid genome that are important for cell-to-cell trafficking. For PSTVd, this consists of 27 RNA loop motifs separated by short helices [41][46][47]. An RNA motif of PSTVd was found to be essential for trafficking from bundle-sheath cells into mesophyll cells when the viroid was exiting in the phloem of young tobacco leaves [48]. Whereas in *Nicotiana benthamiana*, a different RNA motif was required for movement of PSTVd from the bundle sheath cells into the phloem [49]. Furthermore, 11 out of 27 loops of PSTVd RNA motifs are important for cell-to-cell movement and intercellular spread and these RNA motifs could also be involved in the trafficking of viral and cellular RNAs [50]. Loop 19 was identified for viroid movement from palisade to spongy mesophyll cell of *N. benthamiana* [51], while loop 6 had previously been shown to be essential for palisade-to-spongy mesophyll trafficking [52]. These studies enlighten the potential functions of plasmodesmata (PD), as different RNA motifs are required to transit PD at different cell-to-cell interfaces. It was also identified that different RNA motifs can be used to transit across the same cellular interface.

Various experiments have been performed to identify viroid RNA movement. Microinjection experiments using infectious RNA transcripts and labeled with the fluorescent dye TOTO-1 iodide have shown that PSTVd can move rapidly from cell to cell via the plasmodesmata in tobacco mesophyll cells [53]. It was also observed that PSTVd RNA accumulates in the nuclei of both the injected cell and neighboring cells. Using dot-blot hybridization to monitor PSTVd distribution in infected tomato seedlings, it was found that the movement pattern of PSTVd was indistinguishable from that of most plant viruses at the whole plant level [53].

5. Seed, Pollen and Insect Transmission of Viroids

Most of the viroids are disseminated through human activities during planting and through trade in materials of the plants, such as seeds and tissue culture stocks, but some viroids have evolved specific mechanisms exploiting the plants' processes of reproduction and are transmitted via seed and/or pollen [54][55][56]. Pollen is an important breeding biological tool of germplasm naturally found to be associated with number of viroids, known as being pollen-transmitted [57]. Transmission of viroids by pollen can be horizontal, contaminating a fertilized flower, or can be vertical which is more common when infected pollen fertilize and infect the resulting seed [58]. Pollen transmitted PSTVd and PCFVd have been detected in tomato (*Solanum lycopersicum*) crops [59].

The rate at which infected seeds produce infected plants is called the seed transmission rate. The lower rate of seed-transmission in PSTVd could be due to the restricted movement of viroids in floral organs. In *Nicotiana benthamiana* and *Solanum lycopersicum*, the accumulation of PSTVd was not observed in the petals, ovary and stamens, but detected only in the sepals [60]. In petunia, PSTVd is seed-transmitted either through viroid-carrying pollen grains or embryo sacs.

Tomato chlorotic dwarf viroid (TCDVd) of the genus Pospiviroid infects *Solanum lycopersicum* and is distributed in the ovary and ovules but not in the shoot apical meristem [61][62]. Matsushita et al. [62] reported that TCDVd was found on the surface of the seed coat.

In *Capsicum annuum* L. PCFVd, another Pospiviroid, was reported to be seed-transmitted [63]. The presence of the Pospiviroids GYSVd 1 and HSVd were found in *Vitis vinifera* seedlings [64].

ASBVd is an example of Avsunviroid transmitted from infected trees to the seeds of the next generation [65][66][67]. Another species of family Avsunviroidae genus Pelamoviroid, PLMVd, is not reported to be pollen transmitted and is not seed-transmitted [68][69][70]. The only known species of genus Elaviroid in the family Avsunviroidae Eggplant latent viroid (ELVd), which is reported to be seed-transmitted via eggplants [71][72].

Additionally, insect-transmitted mechanical inoculation of viroids was demonstrated to be a source of infection through plants via pollen [73]. Insect pests are known for their tendency to transmit PSTVd to *Solanum tuberosum* (potato) [74]. *Myzus persicae*, commonly known as green peach, aphid-transmitted PSTVd from other plants coinfecting with viruses [75][76][77]. Apple scar skin viroid (ASSVd) was found to be transmitted by *Trialeurodes vaporariorum* (whitefly) from viroid-infected plants to cucumber, bean, tomato and pea plants [78].

6. Pathogenicity of Viroids

The first complete sequence of the PSTVd was reported by Gross et al. [33]. However, it was assumed that viroid pathogenicity was due to direct interaction with one or more of the host cellular constituents and or by indirect interaction via RNA silencing. Viroid pathogenesis has been shown to be increased due to the interaction with nuclear and cytoplasmic RNAs which results in the activation of protein kinases. It has been reported by Hiddinga et al. [79] that a 68-kDa protein extracted from viroid infected tissues is differentially phosphorylated, and that dsRNA dependent protein kinases similar to their equivalents in mammalian cells are involved in the regulation of viroid synthesis. In this case, the binding of protein kinases enhanced viroid pathogenicity. The PSTVd strains produced different symptoms with only 3–4 nucleotides changed [80] and this change could alter the protein binding site resulting in abnormal function [81]. Infectious cDNA clones were first constructed for PSTVd [82] and then for other viroids to determine their pathogenic determinants [83][84][85]. It is also noticeable that in some cases, viroid accumulation to high titers is observed although the plants are asymptomatic or while on the contrary, other viroids at low titers cause severe symptoms, which represents the contribution of alternative mechanisms [86].

At the microscopic level, the cytopathogenic effects of viroids on host cellular structures have been reported. For example, for some viroid infections, there is an abnormal development of cytoplasmic membranes to form “plasmalemmasomes”, irregular thickening of cell walls [87], chloroplast abnormalities and electron-dense deposits in the cytoplasm and chloroplasts [18].

In recent studies, various molecular mechanisms are involved in the induction of viroid diseases and in this context, RNA silencing has a crucial role in viroid pathogenesis. RNA silencing was first identified in plants and then in other

eukaryotes, where it provides other novel regulatory roles in addition to translation. There is very strong evidence that the combined action of the structure-specific dicer-like proteins and the sequence-specific RNA induced silencing complex curb plant RNA and DNA virus infections as well as viroid infections [\[88\]\[89\]\[90\]\[91\]\[92\]\[93\]](#).

7. Viroid-Host Interactions

Being naked, noncoding RNA molecules, viroids induce disease through direct interaction of their genome with some of the host factors. Despite their simplicity in both genome and structure, viroids elicit complex responses in their host plants wherein even with minor changes in their nucleotide sequence, they can induce entirely different symptoms in their host plants based on the cultivar [\[94\]](#). In general, all viroids elicit “pathogenesis-related” [PR] proteins during their infection cycle [\[95\]](#).

Earlier, the PSTVd was shown to associate with several nuclear and histone proteins of tomato [\[96\]](#). Initial investigations of the leaf proteins of tomato plants infected with PSTVd revealed a significant increase in the levels of the PR protein, P14 [\[97\]](#). Introspection into the expression of genes involved in stress response, defense response, chloroplast function and cell wall structure revealed altered expression depending on severity of PSTVd infection [\[98\]\[99\]](#). In another study, the wheat germ RNA polymerase II was shown to interact with terminal loops of the PSTVd [\[100\]](#). Martínez de Alba et al. [\[101\]](#), demonstrated that the tomato Virp1 bromodomain-containing protein bound to the PSTVd 71-nucleotide bulged TR hairpin structure and that this association played a significant role in systemic spread of the PSTVd [\[102\]](#). The tomato protein, p68 involved with the ds-RNA-induced protein kinase activity was shown to be differentially phosphorylated by the PSTVd [\[103\]\[104\]](#). Moreover, the PSTVd was demonstrated to differentially activate the p68 protein based on the severity of the viroid strain, thus implicating the p68 protein in viroid pathogenesis. Interestingly, this differential p68 activation was detected with strains of PSTVd that varied only by a single two-nucleotide inversion within the lower pathogenicity domain of the PSTVd which caused minor variations in their secondary structures. Hammond and Zhao, [\[105\]](#) reported increased transcription of a novel protein kinase (PKV protein) in tomatoes infected by PSTVd depending on the severity of infection. Additionally, they also showed down-regulation of genes responsible for chloroplast biogenesis as well as impacts on the mRNA levels involved in gibberellin biosynthesis and those of some of the signaling hormones. The PSTVd replicates in the nucleus for which the replication start site is present within the hairpin loop of the TL region of its secondary structure and transcription is mediated by the host DNA-dependent RNA polymerase II [\[106\]](#). In *Arabidopsis thaliana*, the transcription factor IIIA [TFIIIA] and the ribosomal protein L5 [RPL5] have been shown to play a role in PSTVd replication by binding to the (+) strand of the viroid [\[107\]](#). Additionally, in *Nicotiana benthamiana*, the canonical 9-zinc finger [ZF] Transcription Factor IIIA [TFIIIA-9ZF] as well as its variant TFIIIA-7ZF were demonstrated to interact with the PSTVd (+) strand while only the latter recognized the PSTVd (–) strand. Plus, the expression levels of TFIIIA-7ZF directly correlated with viroid replication [\[108\]](#). PSTVd reportedly recruits the RPL5 splicing regulator to interact with the CCR that plays a critical role in its replication [\[109\]\[110\]](#).

The *Cucumis sativus* Phloem Protein 2 RNA binding protein [CsPP2] was shown to be associated with long distance movement of HSVd RNA by forming a ribonucleoprotein complex [\[111\]\[112\]](#). The CsPP2 was also demonstrated to enhance the efficiency of transfer of the ASSVd through the *Trialeurodes vaporariorum* [Tv]

Whitefly [78]. To-date, the Virp1 and CsPP2 proteins are the best elucidated factors shown to be involved in translocation of the Pospiviroidae. Among Avsunviroidae, the ASBVd infection in avocado revealed the involvement of PARBP33 and PARBP35 chloroplast RNA-binding proteins with the self-cleavage of ASBVd multimer transcripts mediated through hammerhead ribozyme [113].

Tomato plants infected with CEVd showed changes in levels of proteins involved in translation [114]. Additionally, CEVd reportedly induced as well as reduced in vitro phosphorylation of a wide range of proteins when infecting its host plants particularly, at the beginning of symptom appearance [115]. These changes in phosphorylation were enhanced in the presence of Mn^{2+} , demonstrating the significance of Mn^{2+} -dependent protein kinase action on the varied phosphorylation patterns. In this context, Hidding et al., 1988 [79] reported enhancement in the phosphorylation of a 68 kDa host protein homologous to the human ds-RNA-dependent protein kinase in tomato plants infected with PSTVd. Cottilli et al., 2019 [116] demonstrated changes in the translational machinery of tomato plants infected with the CEVd. They detected the presence of the CEVd within the ribosomal fractions and the CEVd impacted the polysome profiles, specifically causing the accumulation of the 40S ribosomal subunit. The CEVd was also shown to alter ribosome biogenesis and 18S rRNA maturation. Further, the levels of the ribosomal stress mediator NAC082 was increased in infected leaves. These changes correlated with the extent of disease symptoms caused by CEVd. Therefore, these findings showed that in tomato plants CEVd causes defective ribosome biogenesis and impacts the machinery of translation resulting in ribosomal stress.

Viroids, even without having any ability to code for proteins, impact the translational machinery. The CEVd has been demonstrated to cause changes in the accretion of ribosomal proteins such as S3, S5 and L10 in tomato plants [114]. It also impacts the levels of the eEF1A, eEF2 and eIF5A eukaryotic translation factors in these plants. Certain viroids have been reported to associate with the eIF1A or with the L5 ribosomal protein [107][114][117]. The HSVd elicits alterations in the DNA methylation patterns of the rRNA genes in host plants and results in increased accumulation of some of the rRNA-derived sRNAs [118]. HSVd caused demethylation of some of the rRNA genes leading to transcriptional reactivation of these genes, suggesting a novel molecular mechanism putatively involved in viroid pathogenicity. Moreover, it has been shown that the PSTVd induces degradation of ribosomal protein S3a-like mRNAs in infected tomato plants [119].

Further, an augmented number of differentially regulated genes was observed in peach plants doubly infected with both PLMVd and Prunus necrotic ringspot virus (PNRSV) when compared to those of single infections with either of the two viroids. The double infection also caused a synergistic impact on the peach fruit transcriptome [120]. The PLMVd (that replicates in chloroplasts) upon infection of *Prunus persica* (peach) induced the expression of six potential RNA-binding polypeptides, one of which is the elongation factor 1-alpha [eEF1A].

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