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Charith Raj Adkar-Purushothama, Jean-Pierre Perreault

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Charith Raj Adkar-Purushothama[1,2] | **Jean-Pierre Perreault**[2]

[1]MYM Nutraceuticals Inc, Vancouver, British Columbia, Canada

[2]RNA Group/Groupe ARN, Département de Biochimie, Faculté de médecine des sciences de la santé, Pavillon de Recherche Appliquée au Cancer, Université de Sherbrooke, Québec, Canada

Correspondence
Charith Raj Adkar-Purushothama, MYM Nutraceuticals Inc, 250–1095 West Pender Street, Vancouver, BC V6C 1T2, Canada.
Email: charith.adkar@usherbrooke.ca; charith.adkar@mym.ca
Jean-Pierre Perreault, RNA Group/Groupe ARN, Département de Biochimie, Faculté de médecine des sciences de la santé, Pavillon de Recherche Appliquée au Cancer, Université de Sherbrooke, 3201 rue Jean-Mignault, Sherbrooke, Québec, J1E 4K8, Canada.
Email: jean-pierre.perreault@usherbrooke.ca

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Abstract

Viroids are one of the most enigmatic highly structured, circular, single-stranded RNA phytopathogens. Although they are not known to code for any peptide, viroids induce visible symptoms in susceptible host plants that resemble those associated with many plant viruses. It is known that viroids induce disease symptoms by direct interaction with host factors; however, the precise mechanism by which this occurs remains poorly understood. Studies on the host's responses to viroid infection, host susceptibility and nonhost resistance have been underway for several years, but much remains to be done in order to fully understand the complex nature of viroid–host interactions. Recent progress using molecular biology techniques combined with computational algorithms, in particular evidence of the role of viroid-derived small RNAs in the RNA silencing pathways of a disease network, has widened the knowledge of viroid pathogenicity. The complexity of viroid–host interactions has been revealed in the past decades to include, but not be limited to, the involvement of host factors, viroid structural complexity, and viroid-induced ribosomal stress, which is further boosted by the discovery of long noncoding RNAs (lncRNAs). In this review, the current understanding of the viroid–host interaction has been summarized with the goal of simplifying the complexity of viroid biology for future research.

This article is categorized under:
    RNA in Disease and Development > RNA in Disease

KEYWORDS

viroids, viroid-host interactions, viroid structure, viroid induced RNA silencing, vd-sRNA, gene silencing

1 | INTRODUCTION

To date, viroids are the smallest known infectious agents. The first viroid to be identified and characterized was the potato spindle tuber viroid (PSTVd) (Diener, 2003). The degenerative disease of Irish potato in North America was called as potato spindle tuber disease as infected plants were typically associated with the symptoms of elongated tubers (Martin, 1922). For over 40 years, a virus was suspected as being the causative agent. In 1967, Theodor Diener together with William Raymer obtained convincing evidence that PSTVd is a protein-free RNA 50–80 times smaller than the smallest viral genomes. In 1971, Diener proposed the term "viroid" to denote a small, low molecular weight, plant pathogenic RNA molecule (Diener, 1971, 2018; Diener & Raymer, 1967). The viroid concept was further supported by the discoveries of the citrus exocortis viroid (CEVd), which causes the citrus exocortis disease of citrus (Sänger, 1972; Semancik & Weathers, 1972), and the chrysanthemum stunt viroid (CSVd) (Diener & Lawson, 1973; Hollings & Stone, 1973). Both PSTVd and CEVd exhibited the characteristics of being both double-stranded (ds) and single-stranded (ss) RNA molecules. For instance, CEVd was eluted

from methylated albumin and CF-11 cellulose columns as a double-stranded molecule. The eluted molecule was susceptible to both ribonuclease (RNase) and formaldehyde treatment, but was resistant to diethyl pyrocarbonate (DEPC) inactivation (Semancik, 1970; Semancik, Morris, & Weathers, 1973; Semancik & Weathers, 1972).

Further characterization revealed that viroids are circular, single-stranded, noncoding plant pathogenic RNA molecules of 246 to 401 nucleotides (nt) in length (Ding, 2009; Flores, Hernández, Martínez de Alba, Daròs, & Di Serio, 2005). Since the discovery of PSTVd, several thousand viroid sequence variants have been made available in public databanks such as the National Centre for Biotechnology Information (NCBI). The majority of known viroid cause diseases in economically important crops such as potato, tomato, hop, coconut, grapevine, apple, avocado, and peach, to name but a few examples (Kovalskaya & Hammond, 2014). Viroid disease symptoms greatly depend on the viroid strain, the type and severity of the diseases that they cause, and on the host they infect. With the advancements in plant pathogen diagnosis technology, new viroids, and their distribution in new geographic areas, are constantly being reported. For example, the application of RNA-Seq and computational algorithms revealed the presence of both grapevine hammerhead viroid-like RNA (GHVd-like RNA) and grapevine latent viroid (GLVd) in grapevine samples, while *apple* hammerhead viroid-like RNA (AHVd-like RNA) was detected in deep sequence data obtained from apple samples (Wu et al., 2012; Zhang et al., 2014). More recently, *grapevine yellow speckle viroid* 1 (GYSVd-1), hop stunt viroid (HSVd), and Australian grapevine viroid (AGVd) were reported to have infected grapevines in India, indicating the distribution of viroids in new geographical regions (Adkar-Purushothama, Kanchepalli, Yanjarappa, Zhang, & Sano, 2014; Sahana et al., 2013).

With the discovery of viroids, one of the most interesting topics in the study of pathogen–host interactions is to understand how such a small RNA (sRNA) molecule could induce disease. Plant viruses have the ability to use proteins of viral origin to help in their invasion of the host. As a noncoding RNA molecule, viroids are instead solely dependent on their structure and genome information for this (Ding & Itaya, 2007; Heinlein, 2015; Qi et al., 2004; Tabler & Tsagris, 2004; Taliansky, Torrance, & Kalinina, 2008). Even before the concept of noncoding RNAs being key components of the regulation mechanisms of development and defense processes, comparison of the properties of PSTVd with those of conventional plant viruses suggested that the genome of PSTVd was an abnormal regulatory RNA and not a functional mRNA (Diener, 1971). With the greater understanding of the functions of the noncoding RNAs, viroids emerged as the most productive tool with which to understand the fundamental principles of noncoding RNAs in life processes (Ding & Itaya, 2007). As the field of plant virology developed, our understanding of the molecular properties of viroid RNA, its pathogenicity, and its interaction with the host plant progressed significantly. Studies on understanding viroid pathogenicity took a sudden turn in 2004 when transgenic tomato plants possessing an inverted repeat hairpin construct of PSTVd were found to exhibit viroid-associated symptoms (Wang et al., 2004). Around the same time, several researchers noticed the presence of sRNAs similar to the invading viroid genome in plants (Itaya, Folimonov, Matsuda, Nelson, & Ding, 2001; Machida, Yamahata, Watanuki, Owens, & Sano, 2007; Markarian, Li, Ding, & Semancik, 2004; Martínez de Alba, Flores, & Hernández, 2002; Matoušek et al., 2007; Papaefthimiou et al., 2001; Teruo Sano & Matsuura, 2004).

In the recent years, several articles have reviewed in detail certain aspects of viroids: their mode of replication, their trafficking, their structure, and their disease controlling strategies in host plants (Flores et al., 2005; Gago-Zachert, 2016; Kovalskaya & Hammond, 2014; Steger & Riesner, 2018; Tsagris, Martínez de Alba, Gozmanova, & Kalantidis, 2008). Although the last decade has emphasized understanding viroid biology and viroid–host interactions using molecular biology, computational biology, and both biochemical and biotechnological tools to elucidate viroid pathogenicity, a summarized description of these different works is lacking. Hence, the main focus of this review is on the mechanism by which these noncoding RNA molecules induce disease symptoms in host plants.

2 | VIROID STRUCTURE AND PATHOGENICITY

As a naked RNA pathogen, its secondary structure plays a crucial role in the viroid's life cycle, specifically in successful invasion of the host plant, replication, pathogenesis, and transport. Hence, the elucidation of the secondary structures of viroids is of paramount importance in understanding the host-viroid interaction. Although computer-assisted, thermodynamic-based predictions of the secondary structures of viroids are widely used, structural elucidation in vitro and/or *in cellulo* is more biologically relevant to the viroid life cycle.

Several biochemical, microscopic, molecular biology, and plant assays on PSTVd, CEVd, and CSVd have helped elucidate that viroids are single-stranded, covalently closed naked RNA molecules (Ding, 2009; Flores et al., 2005). Using this information, five structural and functional domains were proposed for PSTVd and related viroids (Keese & Symons, 1985;
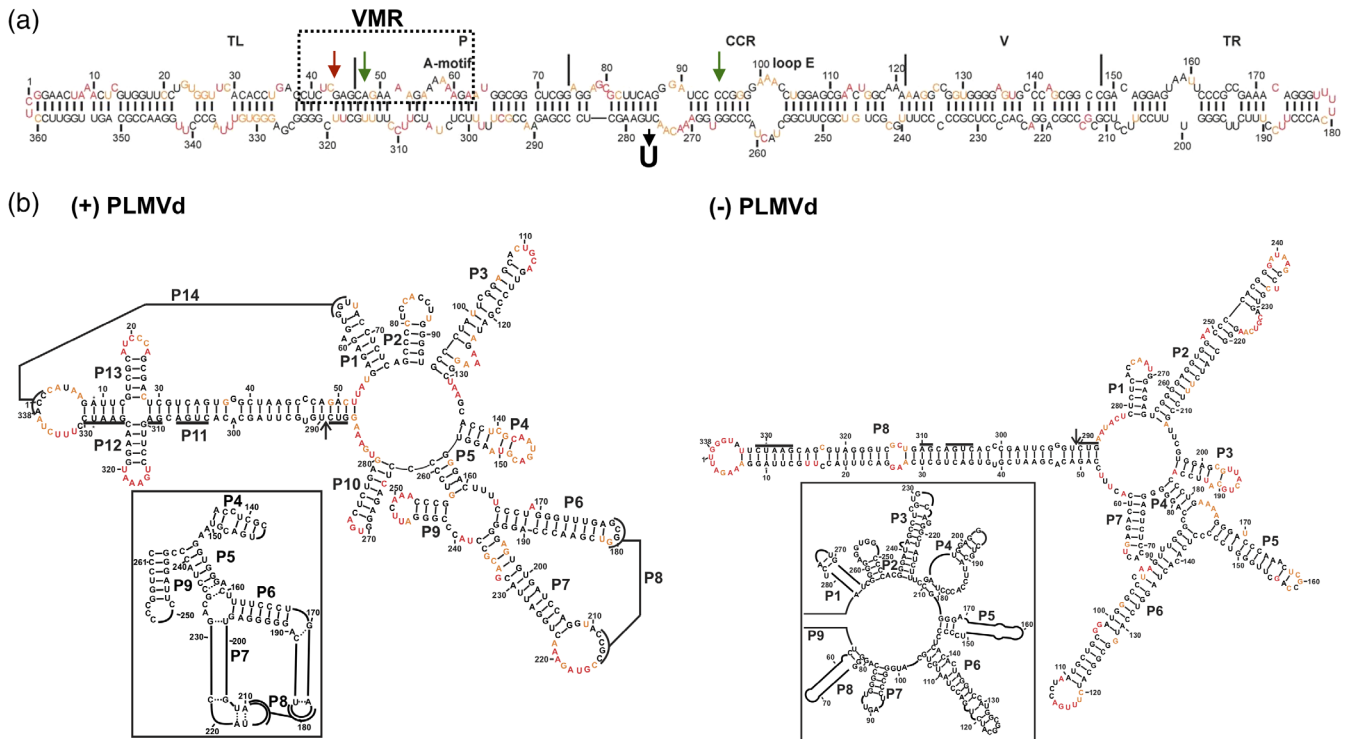
FIGURE 1 The most stable secondary structures of potato spindle tuber viroid (PSTVd) and peach latent mosaic viroid (PLMVd). The final structural models of PSTVd (a) and both the (+) and (−) polarities of PLMVd (b) as obtained by inputting the selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) data into the RNAstructure software. The nucleotides in black denote those of low SHAPE reactivities (0–0.40), those in orange are of intermediate reactivities (0.40–0.85) and those in red are highly reactive (.0.85). The different structural/functional motifs are delimited by full lines, and the presence of both the A-motif and the loop E are noted on the PSTVd structure. The nucleotides in the box denote the virulence-modulating region (VMR). The insertions (red arrows), deletions (green arrows) and substitutions (black arrows) of the nucleotides that are known to determine disease severity are shown on the secondary structure of PSTVd. The nucleotides involved in the formation of the hammerhead are underlined in the PLMVd structures, and the cleavage sites are indicated by an arrow. The asterisks in the hammerhead region of (b) denote the nucleotides mutated in order to avoid self-cleavage during the in vitro SHAPE analysis

TABLE 1 Characteristic features used for the classification of viroids

	Family	
Features	*Pospiviroidae*	*Avsunviroidae*
Structure of mature viroid	Rod-like	Y-shaped or branched
Structural/Functional domains	Terminal left (TL), Pathogenicity (P), Central (C), Variable (V) and Terminal right (TR)	N.A.
Ribozyme activity	No	Yes
Replication site in host	Nucleolus	Chloroplast
Replication mode	Asymmetric rolling circle	Symmetric rolling circle
Enzymes involved	RNA-templated RNA transcription	RNA-templated RNA transcription
	DNA-dependent	Nuclear-encoded chloroplastic
	RNA polymerase II	RNA polymerase
Host range	Depending on viroid species either broad or narrow	Narrow

Note: Modified from Tsagris et al. (2008).
N.A., not applicable.

Sano, Candresse, Hammond, Diener, & Owens, 1992). These domains are: the Terminal Left (TL), the Pathogenicity (P), the Central (C), the Variable (V), and the Terminal Right (TR) domains (see Figure 1). Two stretches of conserved nucleotides within the C domain forms the central conserved region (CCR). The C domain is capable of forming two or more

alternative structures that may regulate replication (Owens & Hammond, 2009). The presence or the absence of the C domain classifies viroids into two families, the *Pospiviroidae* and the *Avsunviroidae*. The details of the structural and functional features used for viroid classification are summarized in Table 1.

The families are further divided into genera based on the RNA structural features and phylogenetic relationship among the species. Most of the known viroids are grouped under the family *Pospiviroidae*, whose type species is PSTVd (Di Serio et al., 2014). Specifically, members of the family *Pospiviroidae* divided into genera based on sequence homologies in the CCR, on the terminal conserved hairpin and on the terminal conserved region (Flores et al., 2005). Hence, the family *Pospiviroidae* includes five genera, the *Pospiviroid*, the *Hostuviroid*, the *Cocadviroid*, the *Apscaviroid*, and the *Coleviroid* (Di Serio et al., 2014; Giguère, Adkar-Purushothama, & Perreault, 2014). The viroid species that lack a CCR are grouped under the family *Avsunviroidae*, whose type species is the avocado sunblotch viroid (ASBVd) (Hutchins, Rathjen, Forster, & Symons, 1986). Other members of the family include the peach latent mosaic viroid (PLMVd), the chrysanthemum chlorotic mottle viroid (CChMVd), and the eggplant latent viroid (ELVd) (Fagoaga, 1994; Hernández & Flores, 1992; Navarro & Flores, 1997). Members of the family *Avsunviroidae* are further divided into three genera namely, *Avsunviroid*, *Pelamoviroid*, and *Elaviroid*

TABLE 2 Current classification of viroids and viroid-like RNAs

Family	Genus	Species	Abbreviation
Pospiviroidae	*Pospiviroid*	*Potato spindle tuber viroid*	**PSTVd**
		Tomato apical stunt viroid	TASVd
		Tomato chlorotic dwarf viroid	TCDVd
		Tomato planta macho viroid	TPMVd
		Columnea latent viroid	CLVd
		Citrus exocortis viroid	CEVd
		Chrysanthemum stunt viroid	CSVd
		Pepper chat fruit viroid	PCFVd
		Iresine viroid I	IrVd-1
		*Portulaca latent viroid**	PoLVd
	Hostuviroid	*Hop stunt viroid*	**HSVd**
		Dahlia latent viroid	DLVd
	Cocadviroid	*Coconut cadang-cadang viroid*	**CCCVd**
		Coconut tinangaja viroid	CTiVd
		Citrus bark cracking viroid	CBCVd
		Hop latent viroid	HLVd
	Apscaviroid	*Apple scar skin viroid*	ASSVd
		Apple dimple fruit viroid	ADFVd
		Pear blister canker viroid	PBCVd
		Citrus bent leaf viroid	CBLVd
		Citrus dwarfing viroid	CDVd
		Citrus viroid V	CVd-V
Pospiviroidae	*Apscaviroid*	*Citrus viroid VI*	CVd-VI
		Citrus viroid OS	CVd-OS
		Australian grapevine viroid	AGVd
		Grapevine yellow speckle viroid 1	GYSVd-1
		Grapevine yellow speckle viroid 2	GYSVd-2
		*Apple fruit crinkle viroid**	AFCVd
		*Grapevine yellow speckle viroid 3**	GYSVd-3

(Continues)

TABLE 2 (Continued)

Family	Genus	Species	Abbreviation
		*Grapevine latent viroid**	GLVd
		*Persimmon latent viroid**	PLVd
		*Persimmon viroid 2**	PVd-2
	Coleviroid	**Coleus blumei viroid 1**	**CbVd-1**
		Coleus blumei viroid 2	CbVd-2
		Coleus blumei viroid 3	CbVd-3
		*Coleus blumei viroid 4**	CbVd-4
		*Coleus blumei viroid 5**	CbVd-5
		*Coleus blumei viroid 6**	CbVd-6
Avsunviroidae	*Avsunviroid*	**Avocado sun blotch viroid**	**ASBVd**
	Pelamoviroid	**Peach latent mosaic viroid**	**PLMVd**
		Chrysanthemum chlorotic mottle viroid	CChMVd
	Elaviroid	**Eggplant latent viroid**	**ELVd**
		Grapevine hammerhead viroid-like RNA*	GHVd-like RNA
		Apple hammerhead viroid-like RNA*	AHVd-like RNA

*Unassigned viroids and viroid-like RNAs.

[Correction added on 18 November 2019 after first online publication: a footnote has been added in Table 2.]

based on the morphological and structural features of hammerhead as well as viroid RNA (Di Serio et al., 2014). Strands of both polarities of all the members of the family *Avsunviroidae* exhibit self-cleavage activity through the use of hammerhead ribozymes (Flores, Daròs, & Hernández, 2000). A detailed classification of viroids is presented in Table 2.

The development of biochemical techniques such as the selective 2'-hydroxyl (2'-OH) acylation analyzed by primer extension (SHAPE) (Wilkinson, Merino, & Weeks, 2006) has allowed researchers to elucidate the structures of viroid in in vitro and/or *in cellulo*. Briefly, in the SHAPE reaction, the 2'-OH group of the unbound (i.e., single-stranded) ribose residues of the RNA molecule reacts with an electrophilic reagent, forming a chemical adduct on the nucleotide. This is followed by a primer extension step using reverse transcriptase, which is unable to pass through the chemical adduct. The positions and signal strengths of these nucleotides are then determined by electrophoresis. The resulting data is normalized, and is then used for secondary structure predictions using structure prediction software (Giguère & Perreault, 2017). Comparison of the in vitro SHAPE-based structure with that obtained from the classical enzymatic probing for (+) PLMVd showed almost similar structures (Figure 1b) (Bussiere, Ouellet, Cote, Levesque, & Perreault, 2000; Dubé, Baumstark, Bisaillon, & Perreault, 2010; Giguère, Adkar-Purushothama, Bolduc, & Perreault, 2014). The subsequent application of SHAPE-based structure predictions on several PLMVd variants revealed new structural motifs such as pseudoknots and a cruciform (Dubé, Bolduc, Bisaillon, & Perreault, 2011). The computer-assisted structure prediction for ASBVd revealed a quasi-rod-like secondary structure. Interestingly, the SHAPE-based secondary structure elucidation of both (+) and (−) transcripts of ASBVd revealed that the two polarities fold into different structures, which explains their observed differences in both electrophoretic mobility under native conditions and in their thermal denaturation profiles (Delan-Forino et al., 2014). More recently, the application of the RNA ligase-mediated rapid amplification of complementary DNA (cDNA) ends (RLM-RACE) combined with SHAPE on RNA preparations obtained from ELVd-infected tissues revealed that the transcription initiation sites of ELVd strands map to different sequence/structural motifs in in vivo derived RNA conformations (López-Carrasco et al., 2016). The application of high-throughput selective 2'-hydroxyl acylation analyzed by primer extension (hSHAPE) data in computer-assisted structure prediction software has revealed the presence of two different structures for the (+) and (−) strands of the members of the *Avsunviroidae* family (Giguère, Adkar-Purushothama, Bolduc, & Perreault, 2014). Furthermore, some members of the of *Pospiviroidae* family showed deviation from the classical rod-like structure. For instance, both the columnea latent viroid (CLVd) and the citrus *viroid* OS (CVd-OS) showed branching as opposed to the thermodynamically predicted rod-like structures (Giguère, Adkar-Purushothama, & Perreault, 2014; López-Carrasco & Flores, 2017). Following the elucidation of the structure of a sequence variant for all viroids from the *Pospiviroidae*, the structural hallmarks permitting the identification of each genus were proposed (Giguère & Perreault, 2017).

Recently, the direct visualization of the native structures of three viroid RNAs (PSTVd, *Pospiviroidae* family; PLMVd and ELVd, *Avsunviroidae* family) at a single-molecule resolution of viroid RNA three-dimensional (3D) structure was studied using atomic force microscopy (AFM) at 0 and 4 mM Mg^{2+} (Moreno et al., 2019). Results confirmed the stabilizing role of tertiary structures such as kissing-loop interactions of PLMVd, and their functional; conformations. These studies illuminate the structural complexity of these quasi-species RNA *in planta*.

Along with the determination of the structural motifs of PSTVd, research was also focused on understanding the role of the different regions on the viroid's pathogenicity using variants of PSTVd on sensitive tomato cultivars such as Rutgers. The results showed that the nucleotides located within the P and C domains play crucial roles in the viroid-induced symptoms in plants (Dickson, Robertson, Niblett, Horst, & Zaitlin, 1979; Visvader & Symons, 1985). A change in one to three nucleotides in the virulence-modulating region (VMR) of the P domain of PSTVd has been shown to affect the symptom severity, presumably by affecting the ability of the VMR to interact with unidentified host factors (Figure 1a) (Schnölzer, Haas, Ramm, Hofmann, & Sänger, 1985; Wassenegger et al., 1996). However, other data obtained with CEVd does not support this correlation (Visvader & Symons, 1985). Experiments with series of interspecific chimeras formed between CEVd and the tomato apical stunt viroid (TASVd) revealed the involvement of the TL rather than the P domain in symptom severity, although the symptom induction was not completely independent of viroid titer. This led to the identification of three discrete sequences regions that may correspond to these pathogenicity determinants (Sano et al., 1992). In another study, a single nucleotide change in the C domain of PSTVd (U257A) converted an intermediate strain to a lethal strain that induced severe growth stunting and a flat top of the tomato shoot (Qi & Ding, 2003). The presence of such pathogenicity determinants outside of the P domain in the other members of the *Pospiviroidae* family has also been reported elsewhere. For example, sequence changes at three sites of the P and C domains (specifically the nucleotides located at positions 87, 197, and 216) in the coconut cadang-cadang viroid (CCCVd) induced a severe lamina-depleting symptom (Rodriguez & Randles, 1993), while a cluster of six specific changes in the V
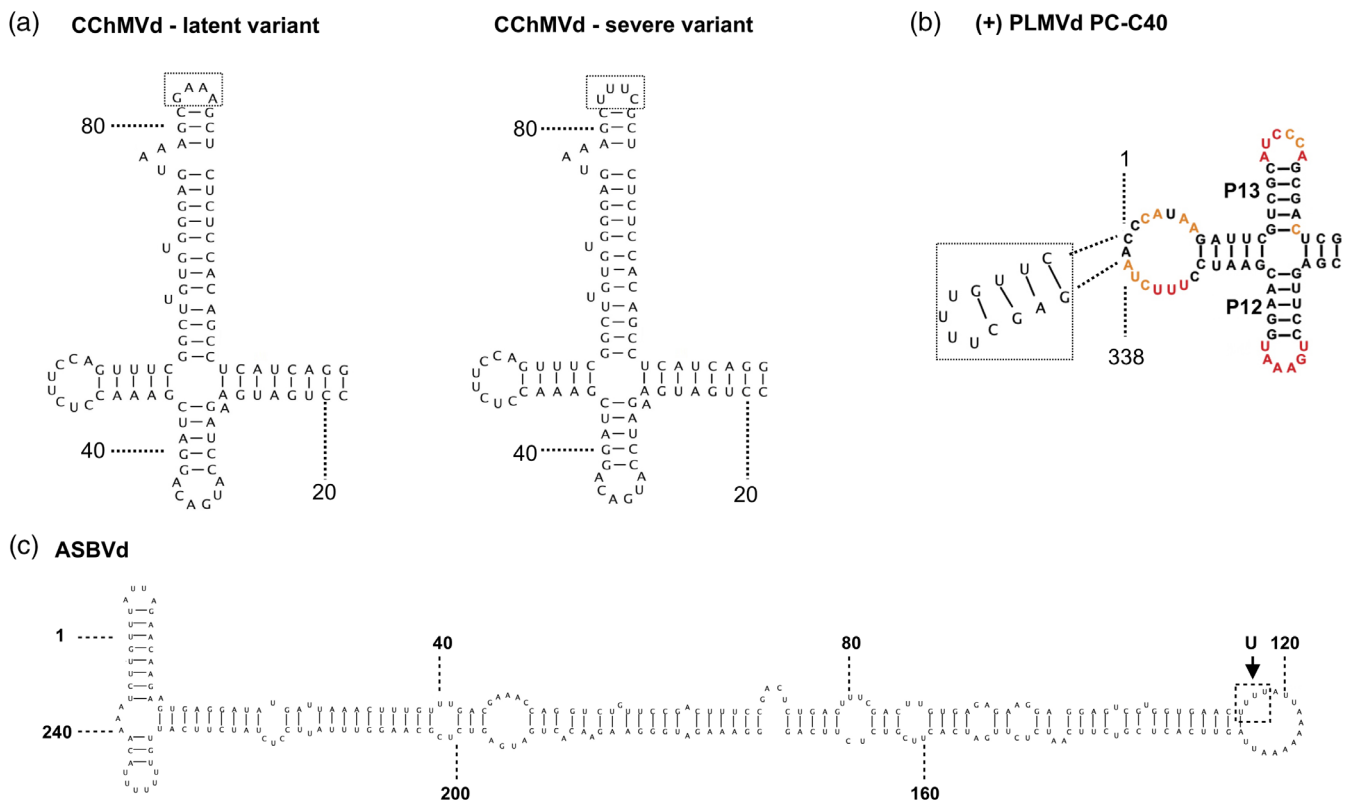

FIGURE 2 Motifs of the members of the *Avsunviroidae* family involved in pathogenesis. The final structural motifs of the (+) polarities of chrysanthemum chlorotic mottle viroid (CChMVd) (a), the (+) polarity of peach latent mosaic viroid (PLMVd) PC-C40 (b) and the (+) polarity of avocado sunblotch viroid (ASBVd) (c) were predicted using the mfold web tool (Zuker, 2003). The nucleotides that are involved in the pathogenesis are shown in the boxes. In (a) the change in the tetraloop nucleotide sequence from GAAA to UUUC converts a latent variant of CChMVd to a severe symptomatic CChMVd one on susceptible chrysanthemum cultivars. In (b) the presence of a 12-nt hairpin in PLMVd induces the calico effect (bleaching of leaves) in susceptible peach cultivars. In (c) the addition of a U between the bases located at positions 115 and 118 is associated with leaf variegation and bleaching in ASBVd infected avocado plants

domain of the HSVd-related group II citrus viroids caused symptoms of cachexia in citrus trees (Reanwarakorn & Semancik, 1998). Alternatively, the primary cause of viroid pathogenicity was correlated to the specific conformation of the VMR by comparing the most stable structures of PSTVd variants of different pathogenicities, suggesting an interaction between the VMR region and a host protein (Hammond, 1992; Owens et al., 1996; Schmitz & Riesner, 1998; Steger et al., 1984). This observation was supported by the drawing of a correlation between PSTVd virulence and the activation of the p68 protein kinase (Diener, Hammond, Black, & Katze, 1993). However, recent in vivo structure data of PSTVd in *Nicotiana benthamiana* leaves showed that the RNA accumulates as a "naked" form rather than being tightly associated with protecting host proteins (López-Carrasco & Flores, 2017). Mutagenic studies on two isolates of PSTVd which replicate and induce disease symptoms differentially in tomato plants (Tsushima, Tsushima, & Sano, 2016) showed that changing position 201 from U, G, or A to C can affect the replication of the viroid as this mutation changes the loop structure at the right boundary of the RY-motif's consensus sequence. The loop structure may also influence recognition by Virp1, the viroid-binding protein (Martínez de Alba, Sägesser, Tabler, & Tsagris, 2003). Furthermore, mutations in both the TL and the P domains influenced symptom induction, while mutations in the V and TR domains had minimal influence on both the systemic accumulation and symptom expression (Tsushima et al., 2016).

As with the members of the *Pospiviroidae* family, the motifs of the members of the *Avsunviroidae* family involved in pathogenesis are shown. For example, mutating the GAAA in the tetraloop of the CChMVd (Figure 2a) to UUUC switches the symptoms from latent to severe on susceptible chrysanthemum cultivars without affecting the viroid's titer (De la Peña & Flores, 2002; De la Peña, Navarro, & Flores, 1999). The presence of a 12-13-nt hairpin in PLMVd induces peach calico (PC) (Figure 2b) (Malfitano et al., 2003). A single sequence change (especially the insertion of U between bases 115 and 118 nt) in the right terminal loop of ASBVd leads to a more open structure and is associated with leaf variegation and bleaching (Figure 2c) (Schnell, Kuhn, Olano, & Quintanilla, 2001; Semancik & Szychowski, 1994).

Over the years, the structure of viroids has always attracted much of attention from the research community (for a recent review see Steger & Perreault, 2016). Today the secondary structure of at least one sequence variant of each viroid species has been elucidated in solution. Moreover, the effect or contribution of some mutants to the secondary structure has been revealed. However, the knowledge at the nucleotide level and kinetically preferred folding in terms of the structure/function relationship remains weak, even more so when considering that a viroid species may fold into different secondary structures which have been poorly studied. Furthermore, not only the sequence differences on viroid RNA molecule lead to difference in their thermodynamically stable secondary structures, but also quite different, kinetically preferred structures with biologicals functions have been noted elsewhere (Loss, Schmitz, Steger, & Riesner, 1991; Repsilber et al., 1999). For instance, formation of a thermodynamically metastable structure containing a hairpin II (HPII) in the (−) strand of PSTVd (replication intermediate) is critical for the replication and infectivity of the viroid (Loss et al., 1991). Detailed analysis of formation of metastable structures by sequential folding during transcription revealed that, viroids are able to use both metastable and stable structures for their biological functions (Repsilber et al., 1999).

3 | VIROID PATHOGENICITY AND HOST COMPONENTS

Since viroids are noncoding RNA molecules, disease induction must be the result of the direct interaction of the genomic RNA with certain host factors. Hence, for many years, research was focused on identifying the host proteins that interact with the viroid RNA. One early study demonstrated the ability of circular PSTVd to interact with several histone and nuclear proteins of tomato (Wolff, Gilz, Schumacher, & Riesner, 1985). Although a 43-kDA protein was isolated from cellular complexes by combining ultra violet crosslinking and RNase digestion in PSTVd infected plants, the cellular function of this protein was not determined (Klaff et al., 1989). Another experiment showed the interaction of the wheat germ RNA polymerase II with the terminal loops of PSTVd (Goodman, Nagel, Rappold, Klotz, & Riesner, 1984). An immunoprecipitation assay demonstrated the binding of a tomato bromodomain-containing protein, Virp1, to the 71-nt bulged TR hairpin of PSTVd (Martínez de Alba et al., 2003). This interaction was found to play an important role in the systemic spread of the viroid RNA (Maniataki, Martínez de Alba, Sägesser, Tabler, & Tsagris, 2003). Additionally, the phloem RNA binding protein PP2 (Phloem Protein 2) isolated from *Cucumis sativus* (CsPP2) appears to be involved in the long distance trafficking of HSVd as it forms a ribonucleoprotein complex with the viroid RNA (Gómez & Pallás, 2001, 2004). Recently, the enhanced efficiency of the apple scar skin viroid (ASSVd) transfers through the Whitefly *Trialeurodes vaporariorum* (*Tv*) in the presence of CsPP2 has been observed. The binding of CsPP2 with ASSVd was determined by electrophoretic mobility shift assay (EMSA) and North-Western hybridization assays (Walia, Dhir, Zaidi, & Hallan, 2015). Up to now, Virp1 and CsPP2 are the two best characterized proteins that are implicated in *Pospiviroidae* translocation. For *Avsunviroidae*, experiments involving UV-irradiated avocado leaves infected with ASBVd resulted in the detection of ASBVd–host protein interactions (Daros, 2002). Tandem mass spectrometry analysis has revealed the involvement of two closely related chloroplast RNA-binding

proteins (PARBP33 and PARBP35) in the hammerhead ribozyme-mediated self-cleavage of multimeric ASBVd transcripts (Daros, 2002).

The first evidence that viroids are able to regulate protein phosphorylation came from experiments with both PSTVd and the CEVd. It was demonstrated that PSTVd could stimulate the phosphorylation of a tomato protein associated with a double-stranded RNA-stimulated protein kinase activity (Hiddinga, Crum, Hu, & Roth, 1988). Specifically, PSTVd infected tomato plants have exhibited a differentially phosphorylated host-encoded protein p68. Protein p68 is similar to the dsRNA-dependent protein kinases of mammalian systems which regulate both protein synthesis and virus replication. In another experimental setup, mild and severe strains of PSTVd reconfirmed the differential activation of p68 and suggested a role for p68 in viroid pathogenesis. The incubation of the mammalian homolog of this protein with PSTVd strains of various pathogenicities lead to differential activation levels, thus supporting an implication for its involvement in viroid pathogenicity (Diener et al., 1993; Langland, Jin, Jacobs, & Roth, 1995). Surprisingly this differential activation of p68 was observed with PSTVd strains that differed by only one two-nucleotide inversion (UUC→CUU) in the lower pathogenicity region of the viroid. That said, these two variants showed minor changes in their secondary structures (Diener et al., 1993). Subsequently, the upregulation of the transcription of a newly described protein kinase (the PKV protein) was observed during PSTVd infection in tomato (Hammond & Zhao, 2000). The authors found that the level of transcription was higher in the plants infected by either a severe or an intermediate PSTVd strain, as compared to what is seen with either infection by a mild strain or in a healthy plant. PKV is similar to the mammalian cyclic nucleotide-dependent kinase, implying involvement in the transduction of extracellular signals. The modification of the transcription of this gene could have a great influence on viroid-induced symptom development. Even CEVd is able to both induce and reduce the in vitro phosphorylation of diverse proteins during the infection at the onset of symptom appearance (Vera & Conejero, 1989). Here, the authors noted that the modifications were higher in the presence of Mn^{2+}, illustrating the importance of the Mn^{2+}-dependent protein kinase activity in the phosphorylation modifications of the host's proteins. It is unclear how viroid infection modifies protein phosphorylation, but it is clear that these modifications might have a critical effect on several biological pathways. Other approaches examining the involvement of host proteins during viroid infection using comparative protein and transcript analyses have revealed that, irrespective of the viroid species, viroids induce "pathogenesis-related" (PR) proteins during infection (Owens & Hammond, 2009). Moreover, PSTVd infection in tomato induces an array of altered expression levels of genes related to stress, growth, development, and defense (Owens, Tech, Shao, Sano, & Baker, 2012).

Meanwhile, mass spectrometric analysis of the proteins extracted from peach (*Prunus persica*) trees infected with the chloroplast-replicating PLMVd revealed the presence of six putative RNA-binding proteins (Dubé, Bisaillon, & Perreault, 2009). One of the RNA-binding proteins detected in this study was the elongation factor 1-alpha (eEF1A) that had previously been, shown to be involved in both the replication and the translation of RNA viruses by interacting directly with the either RNA molecule or viral RNA-dependent RNA polymerase (Bastin & Hall, 1976; Joshi, Ravel, & Haenni, 1986; Yamaji et al., 2006). In case of PSTVd, a nuclear replicating viroid, replication start site is located in the hairpin loop of the TL region of the secondary structure and utilizes DNA-dependent RNA polymerase II for transcription (Kolonko et al., 2006). in vitro studies revealed that, the ribosomal protein L5 (RPL5) and transcription factor IIIA (TFIIIA) from *Arabidopsis thaliana* bind to the (+) strand of the PSTVd indicating the role of these two proteins in viroid replication (Eiras, Nohales, Kitajima, Flores, & Daròs, 2011). This is further supported by the findings where *N. benthamiana* canonical 9-zinc finger (ZF) Transcription Factor IIIA (TFIIIA-9ZF) and its variant TFIIIA-7ZF interacted with (+) strand of PSTVd whereas only TFIIIA-7ZF found to interact with (−) strand of PSTVd. *In planta* experiments revealed that the expression of *TFIIIA-7ZF* directly correlates with PSTVd replication (Wang et al., 2016). More recently, in vitro and in vivo studies revealed that, PSTVd favor the expression of TFIIIA-7ZF, thereby promoting its replication by directly interacting with a splicing regulator, RPL5 with CCR, which is critical for PSTVd replication (Dissanayaka Mudiyanselage, Qu, Tian, Jiang, & Wang, 2018; Jiang et al., 2018).

4 | EFFECT OF VIROID INFECTION ON HOST PROTEIN EXPRESSION

The early descriptions of viroid RNA led to the proposal of it functioning as an abnormal regulatory RNA (Diener, 1971). Even though viroids are very simple in terms of both structure and genome, they induce complex host responses, including varying disease symptoms by simple changes in their genomes. A strain of viroid with a few differences in its nucleotide sequence can cause totally different symptoms in infected host plants depending on the cultivar (Itaya, Matsuda, Gonzales, Nelson, & Ding, 2002). Early studies on the leaf proteins in PSTVd infected tomato plants revealed a prominent increase in the PR protein called P14 (Henriquez & Sänger, 1984). Comprehensive analysis of the differential gene expression of tomato plants infected with mild and severe strains of PSTVd revealed that they alter the expression of both common and unique

TABLE 3 Host proteins affected during viroid infection

Function	Protein	Viroid	Host	Reference
Defense	Pathogenesis-related protein P69 family, P23, PR1	CEVd	*Solanum lycopersicum*	Lisón et al. (2013)
	Superoxide dismutase [Fe]; [Cu-Zn], chloroplastic	CEVd	*Solanum lycopersicum*	Lisón et al. (2013)
	Glucan endo-1,3-beta-glucosidase B family P14.	PSTVd	*Solanum lycopersicum*	Henriquez and Sänger (1984)
	Peroxidase	CEVd	*Solanum lycopersicum*	Gadea et al. (1996)
	Proteinase inhibitor	CEVd	*Solanum lycopersicum*	Gadea et al. (1996)
Replication, transcription and translation	Elongation factor 1-alpha	CEVd	*Solanum lycopersicum*	Lisón et al. (2013)
	40S ribosomal (S3)-like protein	CEVd	*Solanum tuberosum*	Lisón et al. (2013)
	Ribosomal protein S6	CEVd	*Nicotiana tabacum*	Lisón et al. (2013)
	Protein p68	PSTVd	*Solanum lycopersicum*	Hiddinga et al. (1988)
	Elongation factor 1-alpha (eEF1A)	PLMVd	*Prunus persica*	Dubé et al. (2009)
Metabolism	Oxygen-evolving enhancer protein 2 (OEE2), chloroplastic	CEVd	*Solanum lycopersicum*	Lisón et al. (2013)
	Photosystem II oxygen-evolving complex protein 3 (OEE3)	CEVd	*Solanum lycopersicum*	Lisón et al. (2013)
	Oxygen-evolving enhancer protein 2 (OEE2), chloroplastic	CEVd	*Solanum tuberosum*	Lisón et al. (2013)
Other/unknown function	Xyloglucan endotransglycosylase LeXET2	CEVd	*Solanum lycopersicum*	Lisón et al. (2013)
	SKP1 component, SCF ubiquitin ligase	CEVd	*Nicotiana tabacum*	Lisón et al. (2013)
	Protein kinase gene, *pkv*	PSTVd	*Solanum lycopersicum*	Hammond and Zhao (2000)
	Lipoxygenase	CEVd	*Solanum lycopersicum*	Gadea et al. (1996)
	Desaturase-like enzyme	CEVd	*Solanum lycopersicum*	Gadea et al. (1996)

Abbreviations: SKP1, S-phase kinase-associated protein 1; SCF, SKP, Cullin, F-box containing complex.

genes that are involved in defense response, stress response, cell wall structure and chloroplast function, among others (Itaya et al., 2002). The alteration and differential expression of host genes upon viroid infection was further supported by a combination of microarray and large-scale RNA-sequence analyses conducted on two different tomato plant cultivars (cv. Rutgers and cv. Moneymaker) infected with PSTVd (Owens et al., 2012). The PSTVd sensitive cv. Rutgers showed changes in the mRNA levels of more than half of the approximately 10,000 genes present on the array. However, both the cv. Rutgers and cv. Moneymaker (asymptomatic host) exhibited a down-regulation of the genes involved in the biogenesis of chloroplasts and effects on the mRNAs involved in the biosynthesis of gibberellin and other hormones involved in the signaling pathways.

A differential hybridization experiment of defense-related genes in viroid infected tomato plants revealed the characteristic expression pattern for peroxidase, a desaturase-like enzyme, a lipoxygenase, and a proteinase inhibitor in aerial tissues upon CEVd infection in tomato plants (Gadea, Mayda, Conejero, & Vera, 1996). Proteomic analysis by both two-dimensional gel electrophoresis and mass spectrometry of tomato plants infected with CEVd revealed the differential expression of certain proteins, 45 of which were identified and classified based on their functions (Lisón et al., 2013). Interestingly, some proteins that are involved in translation showed alteration in their expression upon CEVd infection. Additionally, the double infection of peach plants with *Prunus necrotic ringspot virus* (PNRSV) and PLMVd led to a higher number of differentially regulated genes as compared to either of the single infections. The characterization of the differentially expressed genes in double infected plants revealed a synergistic effect on the host's transcriptome in peach fruit (Herranz et al., 2013). Table 3 summarizes the host protein components that are altered during viroid infection.

5 | VIROID INFECTION AND GENE SILENCING

The early demonstration of the post-transcriptional gene silencing (PTGS) of a viroid molecule was reported in a PSTVd over-expression system (Wassenegger, Heimes, Riedel, & Sänger, 1994). In this study, the methylation of PSTVd DNA incorporated into the tobacco genome was observed after autonomous viroid RNA replication had taken place in the plants. The authors hypothesized that the mechanism of the de novo methylation of the genes was induced and targeted in a sequence-specific manner by the viroid RNAs. However, this experimental system did not confirm whether or not the methylation was associated with PTGS (Pélissier & Wassenegger, 2000). In 2001, two separate groups reported the detection of viroid-specific sRNAs of approximately 25-nt in PSTVd infected plants (Itaya et al., 2001; Papaefthimiou et al., 2001). Although the authors used different variants of PSTVd in the infection assay, no apparent correlation was observed between the degree of virulence of the viroid isolate and quantity of viroid-specific sRNAs recovered. Subsequently, the viroids that replicate and accumulate in the chloroplast were shown to be the targets of PTGS as Northern blot hybridization analysis revealed the presence of viroid-derived sRNAs (vd-sRNAs) in both PLMVd and CChMVd infected peach and chrysanthemum plants, respectively (Martínez de Alba et al., 2002). In order to examine the role of viroids in gene silencing, fragments of PSTVd cDNA were tagged at the 3' end with the green fluorescent protein (GFP) and then were expressed in tobacco plants. The difference in GFP expression was monitored in PSTVd-infected and PSTVd noninfected plants. The transgenic plants infected with PSTVd showed suppression in GFP expression as well as the accumulation of PSTVd-specific small interfering RNA (siRNA) but not in PSTVd noninfected plants indicating the de novo methylation of PSTVd-specific part of the transgene (Vogt et al., 2004).

RNA silencing (RNA interference, RNAi) is a potent antiviral defense mechanism in plants and animals against either double-stranded or highly structured RNA pathogens. The signature of this defense response is the production and accumulation of 21- to 24-nt long sRNAs that are specific to the invading RNA pathogen. The detection of vd-sRNAs suggested that viroids can trigger the RNA silencing of host genes irrespective of their site of replication (Itaya et al., 2001; Martínez de Alba et al., 2002; Papaefthimiou et al., 2001). These studies further confirmed the production of vd-sRNA from both the (+) and (−) strands of the viroid RNA. Upon infection, these viroid molecules are processed by RNase III-type ribonucleases (i.e., DICER or DICER-LIKE), resulting in the production of siRNAs of 21–24 nt in length (Dadami et al., 2013). Several research groups, using various viroid–host combinations, observed the accumulation of vd-sRNAs in viroid infected plants (Bolduc, Hoareau, St-Pierre, & Perreault, 2010; Ivanova et al., 2014; Navarro et al., 2009; Sano & Matsuura, 2004; Tsushima, Adkar-Purushothama, Taneda, & Sano, 2015; Tsushima et al., 2011). The profiling of such vd-sRNAs by next-generation sequencing revealed that the genomic (+) strands of the viroid produce more sRNA than do the antigenomic (−) strands. Such a differential accumulation can be attributed to the lower accumulation levels of the (−) strands in the viroid infected plants as these (−) strands are only produced as a replication intermediate (Hutchins et al., 1986; Wang et al., 2011). Although these vd-sRNAs are found to be biologically active in guiding the RNA-induced silencing complex (RISC)-mediated cleavage, the replication of PSTVd was resistant to the RNA silencing mechanism. By molecular biology and biochemical analyses it was found that the secondary structure of the mature PSTVd is critical to its resistance to the RISC-mediated cleavage as viroids are devoid of silencing suppressor activities like those viruses possess (Itaya et al., 2007). These observations support the hypotheses that viroid infection triggers RNA silencing, and that vd-sRNA plays a role in disease symptom induction in host plants. However, it has also been shown that plants infected with HSVd exhibited the transcriptional reactivation of ribosomal RNA (rRNA) genes during viroid infection by the demethylation of some rRNA genes (Martinez, Castellano, Tortosa, Pallas, & Gomez, 2014). This resulted in the increased transcription of rRNA precursors during infection. Although this study reports a new mechanism associated with viroid pathogenicity, further work is required in order to confirm the involvement of viroids in host gene modification.

6 | POTENTIAL TARGETS OF Vd-sRNAs

Although RNA silencing provides multilayer protection to hosts against invading RNA pathogens, some pathogens overcome this protection by counteracting the RNA silencing machinery either by possessing or triggering RNA silencing suppressor activities. That said, to date viroids are not known to either possess or induce any of the known RNA silencing suppressors of host plant. Hence, one of the viroid pathogenicity hypotheses is that the vd-sRNA resulting from host's RNA silencing machinery might target host mRNA and trigger a host signal cascade, eventually leading to the induction or the exhibition of disease symptoms (Navarro et al., 2012). This hypothesis is further supported by the fact that viroids are noncoding RNA pathogens which with minor sequence variations can produce different symptoms in infected plants. Additionally, several studies suggested that most of these vd-sRNAs originate from specific regions of the viroid molecules that demonstrated

TABLE 4 List of proven vd-sRNA targets and their experimental host plants

vd-sRNA sequence (3'-5')	Target gene	Viroid	Host	Reference
UCCUUGUUUUCAAGAACUUCU	EF-1-alpha	PLMVd	Peach	Navarro et al. (2012)
UUCUUGAAAACAAGGAGUUUU	Heat shock protein 90			
CGGAAGAAAAAGAAAAAGAAC	Nicotiana soluble inorganic pyropho- sphatase (siPPase)	PSTVd	Nicotiana benthamiana, Nicotiana tabacum	Eamens et al. (2014)
UGUUUCUAUCUCUUUUUCUC	WD40-repeat protein (SolWD40-repeat)	TPMVd	Tomato	Aviña-Padilla et al. (2015)
AAAAAGAAAAGACGAGUCCUC	Callose synthase 11-like; Callose synthase 12-like	PSTVd	Tomato	Adkar-Purushothama et al. (2015a)
GCUCCUUCCGUGGGCUUCUU	Chloride channel protein CLC-b-like mRNA			Adkar-Purushothama, Iyer, and Perreault (2017)
CUUUUCUUUUUCUUCCGCCG	40S ribosomal protein S3a-like			
GGAAGAAAAAGAAAAGACGA	LRR-RSTPK; P14KA1			Adkar-Purushothama and Perreault, (2018)
CCGUGAGGGUGGCAGGAAAA	STPK			
AAAUCGGGAACCUUGGCGUCA	RSTPK, PPR, VMP1			
UCAAGGCUCCUUGGUUGACGC	FRIGIDA-like protein 3 mRNA (FRL3)			Adkar-Purushothama, Sano, and Perreault (2018)
GACGUCCGUCAUUUCUUUUUU	Translational activator gene 1	CEVd	Tomato	Thibaut and Claude (2018)
UCCCCGCUCCGCGAAGUCGCU	Argonaute2a			
GUGGACUGGGACGUCCGUCAU	Putative RNA-binding protein			
CGUGGUGUCCUUGGAGUUCUU	Epoxide hydrolase			
CCCUUUGGACCUCCUUCAGCU	Transmembrane 9 superfamily member			

potential binding sites in the host genome (Machida et al., 2007; Wang et al., 2011). The profiling of vd-sRNA data obtained from PSTVd-infected tomato plants revealed the presence of vd-sRNA hotspots on both the (+) and (−) strands of PSTVd. Although the vd-sRNA accumulation patterns from different research group showed little variation, similar hotspot regions were found across the viroid strains and host cultivars (Adkar-Purushothama et al., 2015b; Diermann, Matoušek, Junge, Riesner, & Steger, 2010). The presence of such hotspots indicates the RNA silencing susceptible regions on the viroid's secondary structure (Tsushima et al., 2015; Wang et al., 2011). Analysis of the deep-sequencing data of vd-sRNA obtained from PSTVd infected tomato plants (cv. Rutgers) revealed that the majority of the vd-sRNAs detected were derived from the pathogenicity-modulating domain of PSTVd. Similarly, PLMVd, a member of the *Avsunviroidae* family, also generated vd-sRNA hotspot regions during infection (Navarro et al., 2012).

Interestingly, a PLMVd variant induces an albinism (i.e., PC) in susceptible host plants only if it contains a specific 12–13-nt hairpin insertion (Malfitano et al., 2003). Taking advantage of this sequence dependent symptom induction, and using deep sequencing, semiquantitative RT-PCR and RNA ligase-mediated rapid amplification of cDNA ends (RACE), the authors determined that two PLMVd-sRNAs containing the PC-associated insertion (PC-sRNA8a and PC-sRNA8b) target the mRNA encoding the chloroplastic heat-shock protein 90 (cHSP90) for cleavage, thus emphasizing the role of RNA silencing in viroid-induced symptom expression (Navarro et al., 2012). On the other hand, by expressing the vd-sRNA derived from PSTVd that was predicted to target the callose synthase gene in an artificial microRNA experiment in a transient expression system, the direct interaction between vd-sRNA and the predicted host target sequence was demonstrated (Adkar-Purushothama et al., 2015a). As the callose synthase gene is involved in the host defense mechanism, PSTVd mutants incapable of targeting callose synthase mRNAs failed both to accumulate and to induce disease symptoms as compared to what is seen with the wildtype. Additionally, the expression of a sequence corresponding to the PSTVd VMR that was predicted to target a soluble inorganic pyrophosphatase (siPPase) on an artificial miRNA in both *Nicotiana tabacum* and *N. benthamiana* induced abnormal phenotypes that are closely similar to what is seen in PSTVd infected plants (Eamens, Smith, Dennis, Wassenegger, & Wang, 2014). Using computer-based target prediction algorithms coupled with molecular biology experiments, it was shown that a single vd-sRNA is capable of inducing the RNA silencing of more than one host defense gene in a PSTVd-tomato combination (Adkar-Purushothama & Perreault, 2018). Several research groups have demonstrated the involvement of vd-sRNAs in the down regulation of host genes using different approaches (summarized in Table 4) (Adkar-Purushothama et al., 2017, 2018; Aviña-Padilla et al., 2015; Aviña-Padilla, Rivera-Bustamante, Kovalskaya, & Hammond, 2018; Thibaut & Claude, 2018; Wang et al., 2011).

7 | INDIRECT EFFECT OF VIROIDS ON HOST GENES AND MIRNAS

In plants, *RNA-dependent RNA* (*RdR*) polymerases have been shown to be essential for virus-induced PTGS (Dalmay, Hamilton, Rudd, Angell, & Baulcombe, 2000; Mourrain et al., 2000; Vance & Vaucheret, 2001). So far, six *RdRs* have been isolated from plants: *RdR1* to *RdR6* (Wassenegger & Krczal, 2006). Of these, the *RdR6* gene has been found to respond the most to stress in *Arabidopsis* (Willmann, Endres, Cook, & Gregory, 2011). In order to demonstrate the role of RdR6 in viroid-induced symptoms, a symptomatic transgenic line of *N. benthamiana* that expresses and processes HSVd under both different growth conditions and in grafting assays with the RdR6 silenced line (*rdr6i-Nb*) was analyzed (Gómez, Martinez, & Pallas, 2008). Interestingly, while the stocks were symptomatic, the scion of the *rdr6i-Nb* plants were devoid of symptoms even though they exhibited the accumulation of HSVd. Analysis of PSTVd-infected RdR6 compromised plants revealed increased amounts of both PSTVd and vd-sRNAs (Adkar-Purushothama & Perreault, 2019; Di Serio, Martinez de Alba, Navarro, Gisel, & Flores, 2010). It should be noted that RdR6 is also known to be involved in the production of secondary siRNAs and in the phenomenon called transitivity (Brodersen & Voinnet, 2006; Schwach, Vaistij, Jones, & Baulcombe, 2005; Vaistij, Jones, & Baulcombe, 2002). Gómez et al. hypothesized the production of *trans*-acting small interference RNAs (tasiRNA), or phased secondary interference RNAs (phasiRNA), in viroid replication and pathogenesis (Gómez, Martínez, & Pallás, 2009). More recently, comprehensive transcriptome analyses of data obtained from PSTVd infected tomato plants not only revealed the genome-wide changes in the alternative splicing of host protein-coding genes, but also changes in the activities of a host miRNA and the induction of both phasiRNAs and the immune response of the host plants (Zheng, Wang, Ding, & Fei, 2017). This nonspecific alteration of the host genome is further supported by a separate study where the examination of the parallel analysis of RNA ends (PARE) library obtained for PSTVd infected tomato plants revealed the widespread cleavage of the host mRNAs in locations other than the vd-sRNA binding site, demonstrating that viroid-infection induced the vd-sRNA independent degradation of endogenous mRNAs (Adkar-Purushothama et al., 2017). A similar widespread silencing of host genes was observed in viroid infected *Arabidopsis* (Cao et al., 2014). In this study, it was observed that the induction of antiviral
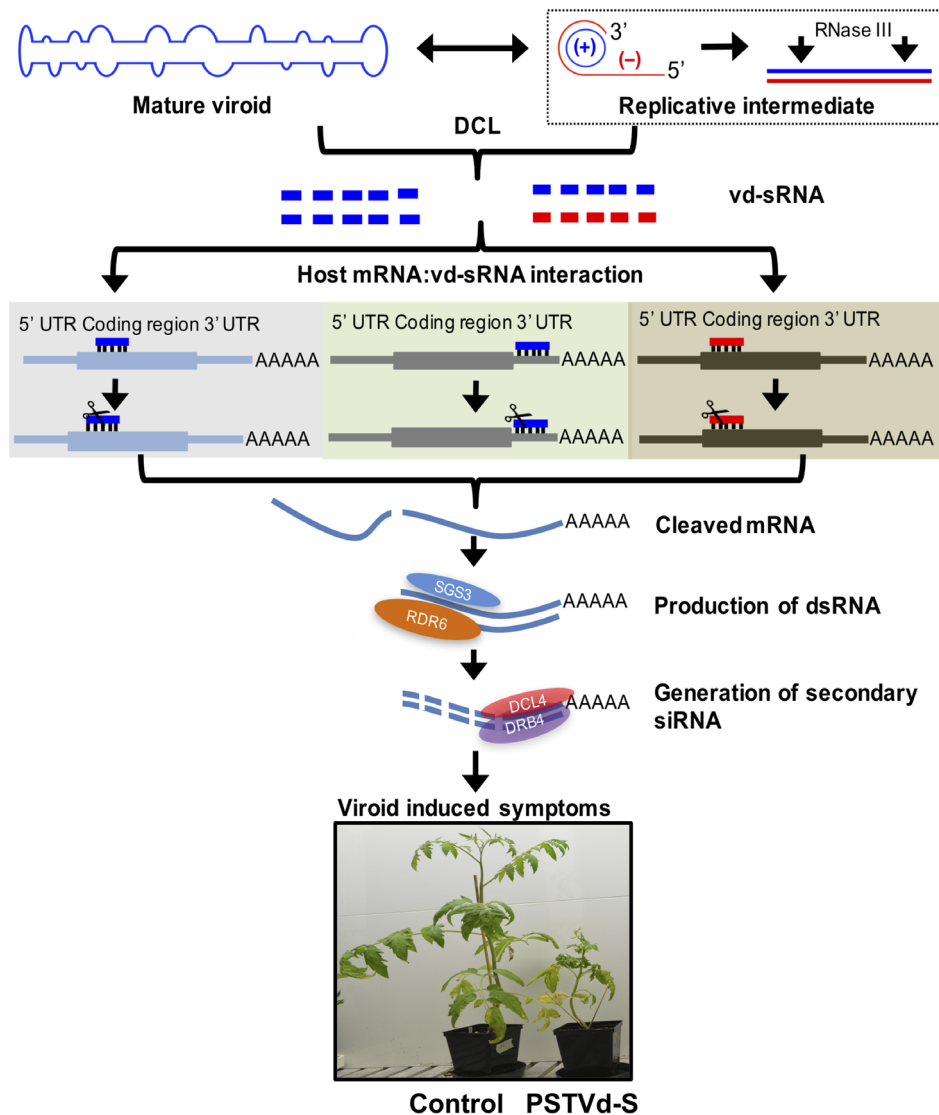
FIGURE 3 Putative working model for the production of secondary small interfering RNA (siRNA) and viroid-induced symptoms. The circular viroid molecule, upon infection, replicates in the host plant via a rolling circular mechanism. Due to both the high degree of internal base-pairing and the nuclear dsRNA intermediate formed during the viroid's replication (Flores et al., 2005), it induces the host's RNA silencing machinery through the activity of the DICER-*like* (DCL) *RNase* III-type ribonucleases, and results in the production of siRNAs called viroid-derived sRNA (vd-sRNA). The RNA-induced silencing complex recruits these vd-sRNAs in order to direct the degradation of complementary RNAs. This results in the accumulation of aberrant host mRNAs which lack either the 5'-cap or the 3'-poly(A)-tail. These aberrant mRNAs might act as the templates for RDR6 in the presence of SGS3, resulting in the production of dsRNA. This phased production of host dsRNA serves as a substrate for RNA silencing through the DCL4 and DRB4 complexes. This results in the production of several secondary siRNAs that are complementary to the host's RNA, and the cycle thus continues. In a nutshell, the RNA silencing initiated by the viroid is multiplexed by the host's RDR6 to act on the host's own mRNAs, resulting in symptom expression

RNA silencing in *Arabidopsis* was associated with the *RdR1*-dependent production of virus-activated siRNAs (vasiRNAs) which target hundreds of host genes through the RNA silencing machinery. Previous studies have shown that the induction of *RdR1* by PSTVd in tomato plants is an antiviroid defense mechanism (Schiebel et al., 1998). Although further research is needed in this area, it appears obvious that viroid infection triggers the production of secondary siRNAs that act on the host mRNAs that are not direct targets of the vd-sRNAs. The proposed model for the production of viroid-induced secondary siRNA, and its role in symptom induction, is shown in Figure 3.

Several reviews have discussed the role of alteration in miRNA expression during virus infection, which in turn modulates symptom production and other pathogen–host interactions (Huang, Yang, Lu, & Zhang, 2016; Ruiz-Ferrer & Voinnet, 2009; Skalsky & Cullen, 2010). As described elsewhere, PSTVd infected tomato plants demonstrated the accumulation of some host

miRNAs that are involved in leaf development (Diermann et al., 2010). Additionally, analysis of the deep-sequencing data obtained from PSTVd infected tomato plants demonstrated the modulation of the host miRNA during viroid infection (Wang et al., 2011). These viroid-induced alterations of miRNAs were further confirmed elsewhere by focusing on the 36 recognized tomato miRNAs included in miRbase (Owens et al., 2012). Northern blot hybridization analysis was performed in order to demonstrate the down-regulation of the two most abundantly expressed miRNAs, miR159, and miR166. More recently, the analysis of the deep-sequencing data obtained from a PSTVd variant infected tomato plant's leaves and stems revealed alteration in the miRNAs involved in diverse functions such as disease resistance (e.g., miR482a, miR482b, miR6023, miR6026), leaf development (e.g., miR319) and leaf curling (e.g., miR172a, miR172b) (Tsushima et al., 2015).

8 | CONCLUSION AND FUTURE PERSPECTIVE

Since the discovery of viroids in 1967, several groups of researchers around the globe have used various viroid-host combinations in order to understand both a viroid's interaction with the host's components and its consequences in inducing disease symptoms. The early days of viroid research were focused on the role of the structural/functional motifs in the viroid-induced symptoms. Later, the focus shifted toward studying the viroid's interactions with the host's proteins. With advancements in molecular biology, and with the development of bioinformatic tools, viroid research has been reshaped in recent years focusing on the transcriptomic analysis of viroid infected plants. The previous demonstration of the accumulation of vd-sRNA in viroid infected plants, and the possible role of the host's RNA silencing machinery, led to speculation of the direct and/or indirect involvement vd-sRNA in viroid-induced disease symptom expression. The recent *in planta* experiments aimed at defining the vd-sRNA-induced RNA silencing of host mRNAs helped define the vd-sRNA-induced regulation of the host's normal gene expression in a sequence-specific manner, and could explain why a minor sequence variation between viroid strains could induce an array of symptoms in susceptible host cultivars. However, further analyses are required in order to clearly demonstrate the mechanism behind the widespread genome degradation observed in viroid infected plants.

Although circular RNA (circRNA) transcripts were identified long ago, they had previously been considered as being either artifacts derived from aberrant RNA splicing, or as being specific to viral pathogens such as the viroids and the Hepatitis delta virus. Consequently, the knowledge on circRNA has remained limited for almost 20 years. The recent discoveries of both circRNA in mammalian brain cells, and of their regulator functions, provided new momentum for circRNA research (Jeck et al., 2013). Although the translation of circRNAs remained a controversial theory, later studies showed both the in vitro and in vivo translation of endogenous circRNAs, thus providing mechanistic evidence of their translation (Legnini et al., 2017; Vogelstein et al., 2013). Despite viroids lack of any protein coding capacity (Ding & Itaya, 2007), studies have shown that viroid infection could either affect the host's translational machinery, or could regulate translation (Lisón et al., 2013). More recently, analyzing the polysome fractions extracted from CEVd infected tomato plants and PSTVd infected tomato plants and *N. benthamiana* plants revealed the presence of viroid molecule in the ribosomal fraction of viroid infected plants. This study clearly demonstrated that viroids induce ribosomal stress by direct interference with the host translation machinery (Cottilli et al., 2019). However, in-depth studies using advanced technologies such as polysome extraction coupled with deep sequencing is worthwhile in order to characterize the regions of viroid that interacts with host translation machinery. This could open-up new avenues in the study of the viroid–host interactions. On the other hand, examination of the direct interaction of vd-sRNAs, or of aberrant viroid RNA molecules, with host proteins could yield a new picture of viroid's pathogenicity.

Thermodynamic folding of sequenced viroid RNA has helped in understanding the two-dimensional structure of the mature viroid molecule for many years. However, structure prediction of the most stable viroid sequence in vitro using hSHAPE has revealed the actual folding of viroid molecule in solution. This is further enhanced by the direct visualization of the viroid RNA by AFM. The application of these techniques *in planta* will bring further insights into viroid biology, and should help explain the structures that govern the stability of viroid RNAs inside the cell. Furthermore, this could elucidate what permits the mature viroid to escape the host's RNA silencing machinery even though viroid infection triggers the host's RNA silencing. Clearly, the 50 years of research on viroid biology has contributed a lot to the understanding of the viroid–host interactions. The remaining challenge is to bring together all of the previous research data in order to understand the mode of action of these lncRNA molecules. Even though they look simple both genetically and structurally, they are much more complex than what was originally imagined.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Charith Raj Adkar-Purushothama: Conceptualization; visualization; writing-original draft, review, and editing. **Jean-Pierre Perreault**: Conceptualization; funding acquisition; visualization, writing-original draft, review, & editing.

ORCID

Charith Raj Adkar-Purushothama https://orcid.org/0000-0001-8868-6008
Jean-Pierre Perreault https://orcid.org/0000-0002-4559-5541

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