



# Current view and perspectives in viroid replication

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Viroids are single-stranded circular noncoding RNAs that infect plants. The noncoding nature indicates that viroids must harness their RNA genomes to redirect host machinery for infection. Therefore, the viroid model provides invaluable opportunities for delineating fundamental principles of RNA structure-function relationships and for dissecting the composition and mechanism of RNA-related cellular machinery. There are two viroid families, *Pospiviroidae* and *Avsunviroidae*. Members of both families replicate via the RNA-based rolling-circle mechanism with some variations. Viroid replication is generally divided into three steps: transcription, cleavage, and ligation. Decades of studies have uncovered numerous viroid RNA structures with a regulatory role in replication and multiple enzymes critical for the three replication steps. This review discusses these findings and highlights the latest discoveries. Future studies will continue to elucidate regulatory factors and mechanism of host machinery exploited by viroids and provide new insights into host-viroid interactions in the context of pathogenesis.

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## Introduction

Viroids are single-stranded circular noncoding RNAs that replicate and systemically traffic in plants [1,2]. To date, there are more than 30 viroids grouped into two families, *Pospiviroidae* and *Avsunviroidae* [3]. Members of the two families are categorized by the distinct features in overall genome structures, replication sites and processes, and whether they possess ribozymes [1,2,4]. By and large, members of *Pospiviroidae* have a rod-shaped RNA genome, replicate via the asymmetric rolling-circle model in the nucleus, and do not have intrinsic ribozyme activity. PSTVd is the type species of *Pospiviroidae*. In contrast, members of *Avsunviroidae* adopt a highly branched

structure at one end of their RNA genome, replicate via the symmetric rolling-circle model in chloroplasts, and possess hammerhead ribozymes. Avocado sunblotch viroid (ASBVd) is the type species of *Avsunviroidae*.

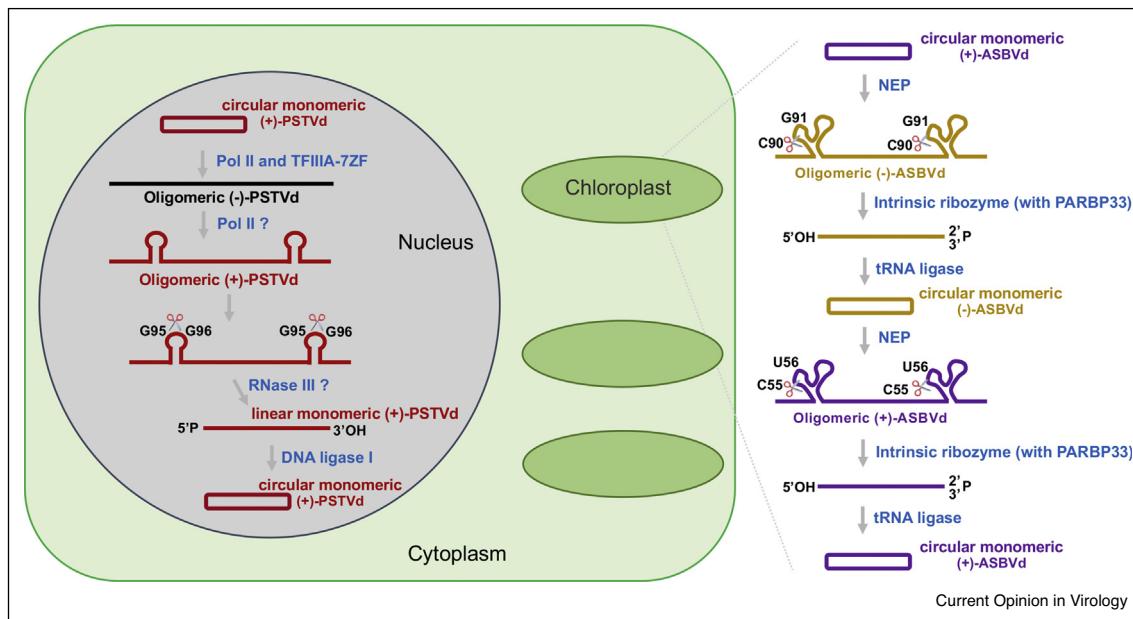
Viroids, as noncoding RNAs, must co-opt host factors to complete infection. Therefore, the viroid replication system offers excellent opportunities to unravel the function and mechanism of RNA-related host machinery. For instance, viroids of *Pospiviroidae* are substrates of DNA ligase I, uncovering an unexpected function of this deeply conserved enzyme [5••]. In addition, all viroids redirect host DNA-dependent RNA polymerases (DdRPs) for RNA-templated replication [1,2,4]. It was later found that the human Hepatitis delta virus exploits this RNA-dependent RNA polymerase activity of DdRPs for replication [6]. Moreover, this RNA-templated activity regulates gene expression in bacteria [7] and mammalian cells [8] as a widely used mechanism in gene regulation.

## Rolling-circle replication of viroids

After entering a cell through opportunistic mechanical wounds or with the aid of insect vectors, viroids traffic to specific organelles for replication. The replication process can largely be divided into three steps: transcription, cleavage, and ligation (Figure 1) [4]. Members of *Pospiviroidae* replicate in the nucleus starting from the circular (+)-RNA genome, via oligomeric (−)-RNA intermediates, to the generation of oligomeric (+)-RNAs. The oligomeric (+)-RNAs are cleaved into unit-length (+)-strands and ligated to yield progeny. Members of *Avsunviroidae* replicate in chloroplasts using circular (+)-RNA templates to generate oligomeric (−)-RNAs, which are cleaved and ligated to unit-length circular (−)-RNAs. Circular (−)-RNAs then serve as templates for producing oligomeric (+)-RNAs that are cleaved and subsequently ligated to generate unit-length progeny circular molecules.

The presence of circular viroids [9–11], longer-than-unit-length (−)-RNAs [12–19] and (+)-RNAs [12,15,17,18,20,21], as well as duplexes composed of one unit-length (+)-RNA and one longer-than-unit-length (−)-RNA [12,14,22,23] led to the rolling-circle replication model [12,14,17,23]. The finding that circular (−)-PSTVd does not exist established the asymmetric rolling-circle mechanism for members of *Pospiviroidae* [24]. The aforementioned double-stranded duplexes may serve as an efficient trigger of plant RNA silencing activity for small RNA (vd-sRNA) generation [25]. Interestingly, a novel homology-independent bioinformatics approach exploits

Figure 1



Rolling-circle replication mechanism.

PSTVd and ASBVd are selected to represent members of *Pospiviroidae* and *Avsuniviroidea*, respectively. Red and black lines refer to (+)-strands and (−)-strands of PSTVd, respectively. Purple and yellow lines refer to (+)-strands and (−)-strands of ASBVd, respectively. Pol II, DNA-dependent RNA polymerase II. NEP, nuclear-encoded plastid RNA polymerase. P, phosphate terminus. OH, hydroxyl terminus.

vd-sRNAs to assemble viroid genomes [26•]. Notably, some vd-sRNAs, derived from viroids of both families, are mapped to junction regions of oligomers, implying the existence of oligomeric duplexes during replication [26•].

### DNA-dependent RNA polymerases for RNA-templated transcription

Early studies reported that multiple polymerases could transcribe the PSTVd RNA genome *in vitro* [27–29]. However, mounting evidence supports DNA-dependent RNA Polymerase II (Pol II) as the authentic transcription enzyme for viroids of *Pospiviroidae*. First, purified tomato Pol II complex could transcribe the (+)-PSTVd RNA templates *in vitro* [27,30••,31•]. Second, a low concentration of α-amanitin, known to specifically impair Pol II activity, inhibits PSTVd transcription in nuclear extracts [32] and the transcription from (+)-PSTVd to (−)-PSTVd by partially purified Pol II [27]. In addition, the low concentration of α-amanitin inhibits the replication of PSTVd [33,34], cucumber pale fruit viroid [33], hop stunt viroid (HSVd) [35], and citrus exocortis viroid (CEVd) [36–39] in cells. Third, RNA-immunoprecipitation demonstrated that the largest subunit of Pol II interacts with both (−)-strands and (+)-strands of CEVd [40] and PSTVd [30••] *in vivo*. Furthermore, Pol II preferentially interacts with circular (+)-PSTVd in plants [30••]. Whether Pol II recognizes oligomeric (−)-strands as

templates remains unclear due to the discrepancy in the literature [34,41]. Notably, transcription is a continuous process *in vivo* as the products from circular (+)-strands readily serve as templates for producing oligomeric (+)-RNAs. Therefore, uncoupling the two steps is required to confirm the authentic enzyme using oligomeric (−)-RNAs as templates. For all members of *Avsuniviroidea*, their transcription is probably catalyzed by the single-subunit nuclear-encoded plastid RNA polymerase (NEP) [4], based on the observation that ASBVd *in vivo* replication is sensitive to NEP inhibitor targetoxin [42].

Transcription on viroid RNA templates starts at defined positions. Pol II initiates transcription at C1 or U359 position using circular (+)-PSTVd templates [32], whereas the initiation site from oligomeric (−)-PSTVd to oligomeric (+)-PSTVd awaits to be mapped. Moreover, the initiation sites of other viroids of *Pospiviroidae* remain to be elucidated. As the type species of *Avsuniviroidea*, the transcription initiation sites of ASBVd were mapped to U121 and U119 in (+)-strands and (−)-strands, respectively, both residing in the right terminal loop [43]. However, the *in vivo* initiation sites in another chloroplastic viroid, peach latent mosaic viroid (PLMVd), were mapped to C51 and A286 for the (+)-strand and (−)-strand templates, respectively [44,45]. The PLMVd initiation sites in both strands locate at similar double-stranded

motifs containing the conserved GUC triplet proximal to the cleavage sites [44,45]. Taken together, chloroplastic viroids adopt their specific strategies for transcription initiation during infection. Viroid transcription initiation at defined positions can be explained by the *de novo* transcription mode, akin to DNA-dependent transcription. In line with this assumption, a recent report provides empirical evidence supporting the *de novo* transcription of Pol II on PSTVd circular (+)-RNA templates [31\*].

### Regulatory mechanism underlying RNA-templated transcription

Recent studies unraveled the first host transcription factor dedicated to viroid RNA-templated transcription. Transcription factor IIIA (TFIIIA) was shown to directly bind PSTVd in a gel shift assay [46]. Following this work, a recent study showed that a splicing variant of TFIIIA, TFIIIA-7ZF, is the critical transcription factor for RNA-templated transcription catalyzed by Pol II [30\*\*]. TFIIIA-7ZF interacts with Pol II and both (+)-PSTVd and (-)-PSTVd RNAs, modulates PSTVd replication in plants and directly enhances Pol II processivity when transcribing PSTVd RNA templates [30\*\*]. It is noteworthy that TFIIIA-7ZF also binds with HSVd [47] and has been suggested to play a role in the replication of apple fruit crinkle viroid [48\*]. Thus, TFIIIA-7ZF-based replication catalyzed by Pol II is possibly conserved for members of *Pospiviroidae*.

Purified Pol II alone, without general transcription factors, cannot initiate DNA promoter-dependent transcription [31\*,49–51]. When mixing with TFIIIA-7ZF and circular PSTVd RNA templates, however, purified Pol II can initiate *de novo* transcription generating longer-than-unit-length products. This observation suggests that TFIIIA-7ZF acts as a general transcription factor in RNA-templated transcription [31\*]. Moreover, a conserved general transcription factor TFIIS, which is critical for DNA-dependent transcription, is dispensable for PSTVd RNA-templated transcription in cells. This observation implies that distinct Pol II machinery is formed for RNA-templated transcription [31\*]. It will be critical to dissect the functional components of Pol II and NEP machinery on viroid templates to achieve a comprehensive understanding of RNA-templated transcription.

Viroid RNA-templated transcription is error-prone. Chloroplastic viroids display the highest mutation rate among all biological entities [52\*\*,53\*], whereas nuclear-replicating viroids possess a mutation rate similar to some RNA viruses [53\*]. Mutations in accumulated (-)-PSTVd intermediates are nearly absent from the regions corresponding to the central conserved region (CCR) in (+)-PSTVd [54\*], reflecting the biased Pol II fidelity in transcribing distinct regions [54\*] or the selection pressure from RNA 3D structure-based constraint [55\*].

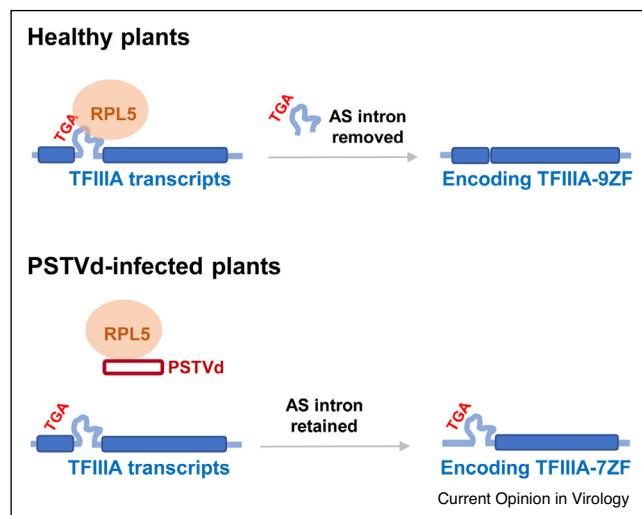
### Cleavage and ligation steps in viroid biogenesis

Members of *Pospiviroidae* do not possess intrinsic ribozymes, so they must rely on a host ribonuclease for cleavage. Using CEVd as a model, dimeric (+)-CEVd is cleaved between G96 and G97 in the CCR *in vivo*, specifically the upper strand of CCR [56]. The equivalent cleavage site on oligomeric (+)-PSTVd is mapped between G95 and G96 *in vitro* [57]. Thus, the cleavage site is probably conserved in members of *Pospiviroidae*. The cleavage products of CEVd possess a 5'-phosphomonoester and 3'-hydroxyl termini, hinting at the involvement of a host RNase III-type enzyme [4,58\*\*] yet to be identified. Interestingly, the ligation of PSTVd and several relatives are catalyzed by DNA ligase I [5\*\*]. To date, viroids of *Pospiviroidae* are the only known RNA substrates of DNA ligase I, raising the question of whether this enzyme accepts any endogenous RNA substrates and, if so, the biological significance.

ASBVd and all members of *Avsunviroidae* possess the intrinsic hammerhead ribozyme to cleave their oligomeric intermediates to unit-length linear products [2,4]. The cleavages occur between C55-U56 in (+)-ASBVd and between C90-G91 in (-)-ASBVd [59]. Despite possessing ribozyme activities, ASBVd interacts with a host factor, PARBP33, to enhance cleavage efficiency [60]. The cleavage products possess the 5'-hydroxyl and 2',3'-cyclic phosphodiester termini structures [59]. It is generally accepted that the chloroplastic tRNA ligase is responsible for the ligation of chloroplastic viroids [61\*\*]. Whether viroid replication impairs or reduces the endogenous function of this tRNA ligase as one of the causes of symptoms deserves further investigation.

### Critical RNA motifs that interact with host factors in replication

In the past 40 years, many RNA structures in various viroids have been found to be critical for one or multiple steps in infection. Some of those RNA structures are known to interact with host factors. Recently, the TFIIIA-7ZF binding site has been established as an RNA promoter [31\*]. This promoter resides at the lower strand of the left terminal region spanning loops 3–5 [30\*\*], which is proximal to the transcription initiation site [32], overlapping with the Pol II binding region [62], and critical for replication [63\*]. This promoter also overlaps with a GC box that was regarded as a potential promoter based on the mapping of the transcription initiation site and mutational analysis [32]. However, the left terminal region of PSTVd may fold into two distinct conformations [64–66]. Since it is unclear which confirmation is involved in the transcription initiation, the structural basis of this RNA promoter remains to be determined [47].

**Figure 2**

PSTVd modulating TFIIIA-7ZF expression through interaction with RPL5.

PSTVd directly binds RPL5 and reduces the intron removal of TFIIIA transcripts, resulting in optimized expression of TFIIIA-7ZF. AS, alternative splicing.

Genome-wide functional analysis has identified two regions in PSTVd genome critical for replication [63•]. One region, spanning loops 1–4, has been identified as the binding sites for TFIIIA-7ZF [30••] and Pol II [62]. The other region in the CCR, spanning loops 13–15, has been shown to be critical for RPL5 (ribosomal protein L5) binding [67••]. Notably, RPL5 is a splicing regulator suppressing the generation of TFIIIA-7ZF [68,69]. PSTVd interaction with RPL5 impairs the splicing regulation activity of RPL5 and is critical to modulate the expression of TFIIIA-7ZF, thereby influencing PSTVd transcription (Figure 2) [67••]. It awaits to be clarified whether related viroids of *Pospiviroidae* employ the same RPL5/TFIIIA-7ZF regulatory cascade in regulating replication.

The CCR is also critical for processing (i.e. cleavage and ligation) [4]. Particularly, hairpin I, which is conserved in all five genera in *Pospiviroidae*, is critical for cleavage [56]. Hairpin I is formed by the rearrangement of the upper strand of the CCR and consists of a tetraloop, an internal symmetric loop of 1–3 nt in each strand, and occasionally a 1 nt symmetric or asymmetric loop flanked by short stems [4]. Two adjacent hairpin I structures in oligomeric intermediates form a kissing loop via their palindromic tetraloops, providing a double-stranded structure for cleavage [56]. The cleavage results in two protruding nucleotides in each strand, which is the characteristic feature of RNase III activity [56]. The cleaved products then form the loop E structure for DNA ligase I-mediated ligation [56]. Since loop E is not conserved in

*Pospiviroidae*, it remains to be clarified whether DNA ligase I relies on loop E or a more general structure in the CCR.

## Summary and perspectives

Several seminal discoveries regarding viroid replication were reported in the last decade. Discoveries of ligases for members of both families and the terminal structures of cleavage products provide critical insights into the ligation steps. The dedicated transcription factor, TFIIIA-7ZF, has been uncovered to be critical for Pol II-catalyzed transcription using PSTVd RNA templates. PSTVd can modulate its replication through interaction with RPL5, leading to an optimized expression of TFIIIA-7ZF.

Because of technical constraints, genetic approaches were rarely applied in viroid research, which may be essential to corroborate the function of host factors harnessed by viroids. The progress in understanding the host machinery for viroids replication also presents new opportunities to elucidate the significance of such interactions in the context of viroid pathogenesis. It is well demonstrated that the viroid model can help dissect various RNA-related host machinery. From this prospect, future studies on RNA promoters and auxiliary factors dedicated to RNA templates may shed a novel light on RNA structure-function relationships.

## Conflict of interest statement

Nothing declared.

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