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Derailing the host machinery to achieve replication: how viroid and viroid-like RNAs successfully copy their genomes in hostile territory

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ABSTRACT

Circular infectious RNAs have been known for several decades. Their biology has been intriguing from the beginning, partly due to the antithesis between their efficiency and tiny size. Amongst infectious circular RNAs viroids hold a special place not only because they were the first to be characterized as such but also because they have been extensively studied as a group. Viroids do not encode proteins and therefore have to rely for their biological cycle on the host factors. As a result, the identification and functional characterization of host factors enabling their biological cycle has been of prime importance to the community. With the advent of high throughput sequencing technologies, viroid-like infectious RNAs have been found in plants, fungi, and animals, including mammals, making understanding their biology even more interesting and important. In this review, we summarize what is known about the replication of these tiny yet very efficient infectious RNAs.

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Introduction

Circular DNA and RNA infectious elements have been studied for many decades. With the advent of high-throughput sequencing methods, the number of these elements identified and the variety of biological groupings in which they are found have significantly increased [1–3]. Circular infectious RNAs have been categorized into four groups, as per Navarro and Turina's proposed classification [4]. Group I contains Ambiviruses, which infect fungi. These viruses have a 4–5 kb ambisense RNA with at least two open reading frames (ORFs), one of which produces an RNA-directed RNA polymerase (RdRp), and additionally have ribozymes in their RNA genome [5]. Group II, includes a small number of *Mitoviridae* viruses that have a circular genome of around 3 kb, containing ribozymes in their structure, and are capable of producing an RdRp [2,3]. While they primarily infect fungi, viruses of this family have also been identified in plants and invertebrates, suggesting that more circular Mitoviruses may be identified in the future [6,7]. However, we still know little about their biological functions as well as their replication mechanisms. Group III, comprises viruses with circular negative-sense RNA genomes of 1.2 to 1.7 kb, grouped in the *Kolmioviridae* family. The best-known member of this group, Hepatitis delta virus (HDV – species *Deltavirus italiense*), was identified in the 1970s. In 2024, between 15 and 20 million people were estimated to be infected with HDV, leading to liver cirrhosis and hepatocellular carcinoma [8]. HDV has a circular positive RNA of 1.7 kb (genomic HDV – gHDV); however, during infection, two additional RNAs are found in infected cells, the antigenomic circular RNA (agHDV) and the linear RNA or delta antigen mRNA of about 0.8 kb. This linear RNA is responsible for the production of a small protein named S-HDAg (195aa). In addition, editing of the antigenomic RNA by adenosine deaminase acting on RNA 1 (ADAR1) alters the linear RNA, driving the production of a second larger protein named L-HDAg (214aa) [9]. It is worth mentioning that a third small peptide named 'peptide-K' has been proposed, but no further information

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exists regarding its presence [10]. Even though HDV can replicate autonomously, infection requires a capsid/envelope from a helper virus (HV), usually (but not only) hepatitis B virus (HBV – species *Orthohepadnavirus hominoidei*). Nevertheless, in the last few years, many Delta-like (or HDV-like) viruses have been identified which can infect their host without the necessity of an HV [11]. Finally, Group IV contains elements of 200 to 800 nucleotides (nt), with or without coding proteins, often considered as 'subviral agents'. Some viroids and satellites are the major representatives of this group, but other agents like Zetaviruses and Obelisks are emerging as important players [2,12,13], yet with questionable infectious potential.

RNA satellites are relatively small RNAs related to a HV for their replication, encapsidation, and spread among hosts. The majority are linear but a small subset is circular, often termed sc-satRNAs [14,15]. In plants, they are associated with three viral genera (Sobemovirus, Nepovirus and Polerovirus), sharing no or minimal similarity with their HV or hosts. However, sc-satRNAs linked to sobemoviruses are encapsidated in their circular form within the virion, often termed virusoids, whereas those of the other two genera are usually linear and are circularized during infection [16]. Most satRNAs are non-coding with the exception of the satellite of rice yellow mottle virus (RYMV-species *Sobemovirus RYMV*), which produces a 16 KDa protein [17]. Indications about sc-satRNAs in species other than plants exists [18]. Usually, a sc-satRNA shares limited similarity with its HV and contains regions highly structured, with double-stranded regions interposing between bulges and loops, which resembles ribozymes [19].

Viroids are sub-cellular parasitic RNAs that reside in plant cells [14,20,21]. They are composed of a single RNA 'genome' of 246 to 401 nt that does not encode any peptides or proteins. Viroids were named based on their initial identification, which revealed symptoms and epidemiology similar to those of viruses. However, despite these similarities, viroids are a distinct group of parasitic RNAs, since the absence of a protective coat and their non-protein coding nature have resulted in a unique evolutionary path. Lacking innate protection and machinery, they almost entirely rely on host factors to fulfil their biological cycle in contrast to other more complex parasites. Specifically, they engage host factors to protect their RNA 'genome' from nucleases, replicate, move in short and long range, spread, and, in many cases, generate symptoms. There are two families of viroids: the *Pospiviroidae* family, which includes viroids that replicate in the nucleus through an asymmetric rolling circle replication, and the *Avsunviroidae* family, including viroids that replicate in the host chloroplasts through a symmetric rolling circle mechanism. The two viroid families differ in the presence or absence of a central conserved region (CCR) and/or hammerhead ribozyme, both involved in the replication process, as well as their replication pathways and replication sites within a cell [22,23].

The non-coding nature of viroids suggests that their structure plays a critical role in their biological cycle. In 1985, Keese and Symons proposed a model of five viroid domains for the rod-like structure of *Pospiviroidae*, based on sequence homology [24]. The Terminal Left domain (TL) contains an imperfect repeat that can form either a Y-shaped or rod-like structure [25]. The Pathogenicity domain (P) also known as virulence-modulating region affects the pathogenicity of the viroid [26]. The most conserved region is the Central domain (C) that forms a stable Hairpin I (HPI) [27] and contains a loop E motif. This loop E motif is a result of non-Watson–Crick pairings [28] and variations influence the infectivity in different plant species [29]. The Variable domain (V) contains a stable hairpin structure, known as Hairpin II (HPII) [30]. The Terminal Right domain (TR) also includes an internal loop with two Y–Y cis Watson–Crick pairs, an asymmetric internal loop with a cis Watson-Crick and a trans Watson-Hoogsteen pair and a stable hairpin loop with several bases stacked on top of each other for stability [31] In contrast, the *Avsunviroidae* family lacks the conserved motifs of the former family, but contains functional hammerhead ribozymes in both RNA polarities. Three members, Peach latent mosaic viroid (PLMVd), Chrysanthemum chlorotic mottle viroid (CChMVd), and Apple hammerhead viroid (AHVd), have a branched conformation stabilized by kissing-loops and pseudoknots, while Avocado sunblotch Viroid (ASBVd) and Eggplant latent viroid (ELVd) adopt quasi-rod-like conformations [32].

Viroids were long considered to be found exclusively in plants. However, it has been demonstrated that viroids of both *Pospiviroidae* and *Avsunviroidae* families are capable of replicating in three species of phytopathogenic fungi - *Cryphonectria parasitica*, *Valsa mali*, and *Fusarium graminearum* - even though the latter are not their natural hosts [18,33]. Interestingly, infection of *V. mali* with the Hop Stunt Viroid (HSVd) resulted in symptom induction [18]. It was also shown that ASBVd can replicate in the

cyanobacterium *Nostoc* sp. PCC 7120. Both positive and negative polarity RNA strands were detected by northern blot, confirming the presence of replication intermediates [34]. Replication of ASBVd does not impact the growth of Nostoc, but it rather coexists with the host's cellular functions without a phenotype [34]. Furthermore, metagenomic studies have shown that numerous viroid-like structures are present in various organisms, although limited information exists about their infectious capacities [2–4,35]. Viroid-like entities infecting fungi, have been identified with and without ribozymes present in their structure [35,36]. Circular RNAS (circRNAs) were isolated from *Botryosphaeria dothidea* (named BdcircRNAs), with a range of 157–450 nt, replicating in the nucleus, producing a phenotype in the fungus [36]. Nevertheless, their classification as viroids, or 'mycoviroids' as has been proposed by some researchers in the field, remains a subject of debate, as they lack a Central Conserved Region (CCR) and a typical hammerhead ribozyme (HHRz) domain. In any case, their significantly small size (157 nt) makes them the smallest known pathogens on earth. Finally, another interesting subgroup of viroid-like RNAs, named as retroviroids, are similar to viroids, but in addition to the genomic RNA, a DNA form also exists, integrated within the hosts' genome. Till today, only one member of this potential group is known, named carnation small viroid-like RNA (CarSV) [37–39], CarSV consists of a 275 nt circular RNA that harbours HHRz ribozyme structures in both polarities. In some cases, this viroid is associated with the carnation-etched ring virus (CERV – species *Caulimovirus incidianthi*) or the Alternanthera mosaic virus (AltMV – species *Potexvirus alternantherae*) [39]. CarSV involves a reverse transcription step during its biological cycle which still requires investigation. Given all the other peculiarities and the fact that CarSV does not undergo horizontal transmission, its classification as a viroid remains uncertain. The discovery of more retroviroids may help to resolve this issue.

Many aspects of viroid and viroid-like biology remain poorly understood. Currently, there is limited knowledge about their entry into cells, systemic movement, and intracellular mobility. Our understanding is primarily based on a few observations of their localization, cell-to-cell movement, and the small number of proteins associated with their RNA. Circular infectious RNAs share many similarities, with one of the most striking being the employment of a version of rolling circle replication. Although for some aspects of their replication cycle there is significant evidence, there are still other aspects that remain obscure. Since amongst these minimalist parasites there are some that cause significant losses in agriculture or health concerns in humans, there are not only scientific but also practical reasons that make their study important. In this review, we summarize the current understanding of viroid and viroid-like RNAs replication and highlight open questions.

The rolling circle replication mechanism

Circular pathogens typically use a form of rolling circle mechanism for their replication. Even though most of the information available today comes from viroids, it is plausible that this mechanism is used primarily because it allows the pathogen to quickly and efficiently produce multiple copies of their RNA using in most cases the host's cellular machinery. It is a mechanism that generates long multimeric RNA strands that can be processed into unit-length RNAs, ensuring the production of numerous copies coming from a single initiation event. The rolling circle replication is classified into three types: asymmetric, symmetric and double (Figure 1).

Asymmetric rolling circle replication

The asymmetric rolling circle mechanism has been mostly studied in *Pospiviroidae*. It was first described by Branch and Robertson in 1984, during their study on the family-type species Potato Spindle Tuber Viroid (PSTVd) [40]. The researchers used the term 'plus strand' RNA ((+) RNA) as a convention to describe the most abundant viroid RNA found in infected plants, while the term 'minus strand' ((-) RNA) was used for any nucleic acids complementary to a part or the full length of the (+) RNA. They had previously observed the presence of linear longer-than-unit viroid (-) RNA strands in RNA extracted from PSTVd infected plants, which varied in size and had extended double-stranded regions [41]. The detection and analysis of multimeric minus strands in viroid-infected cells led to the hypothesis that they play a significant role in viroid replication, serving as templates for the synthesis of plus strand RNAs. The fact that circular (–) RNA of PSTVd was not detected established the

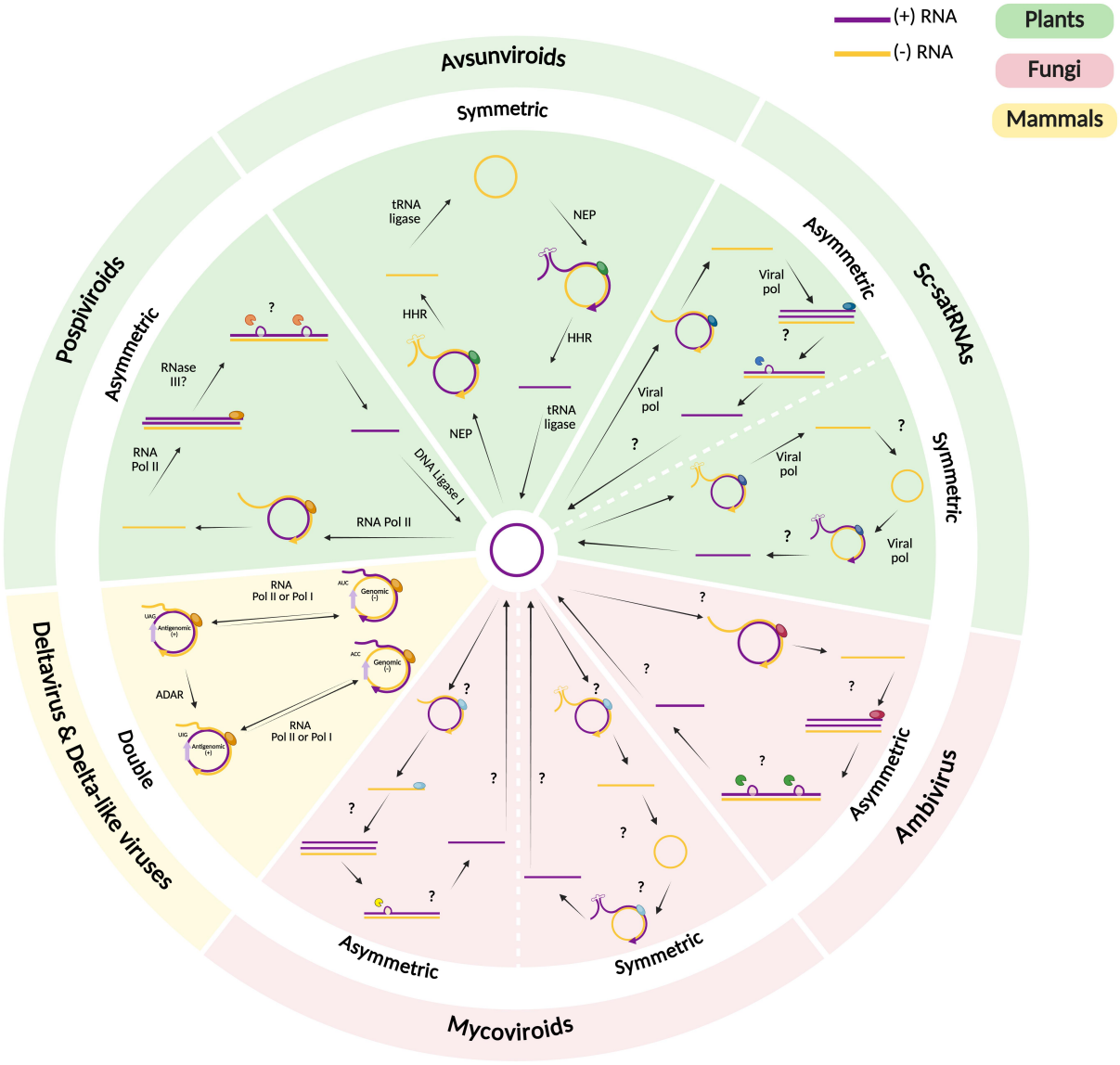

Figure 1. Schematic representation of various rolling circle mechanisms in different pathogens. Purple lines indicate the (+) RNA, while yellow lines represent the (-) RNA. Question marks ('?') denote potential factor(s) involved in each specific step. The background colour indicates the organism infected by the corresponding pathogen. Figure was created using BioRender (BioRender licence https://BioRender.com/s58m895).

asymmetric type of the single rolling circle replication for the members of *Pospiviroidae* family [40]. These findings led Branch and Robertson to propose a replication model based on viroid replication intermediate strands. Their model proposed a replication process that starts when the circular (+) RNA is transcribed into a longer-than-unit linear (-) RNA. Then, the linear (-) RNA acts as a template for synthesizing linear concatemeric (+) RNA. Following this, the concatemeric (+) RNA is cleaved into unit-length monomers by a host endonuclease. The unit-length monomers are subsequently ligated into circular RNA, completing the replication cycle [40].

Furthermore, it was shown that PSTVd can be successfully processed in yeast, although RNA cleavage happens at different sites compared to plants. Consequently, the resulting circular RNAs in yeast have some extra nucleotides that are not native to the viroid's typical structure in its natural host [42]. These non-native nucleotides are essentially additional RNA sequences resulting from the yeast's distinct processing methods. Thus, while the viroid can be processed and form circular RNAs in yeast, the resulting molecules are not identical to those formed in plant hosts. Nonetheless, the difference in PSTVd RNA cleavage sites in

yeast compared to plant hosts highlights the adaptability of viroids to exploit several host RNA processing pathways.

In the case of sc-satRNA the replication model is possibly more complex: Their highly structured circular RNA, the ribozymes usually found in their structure, and the presence of multimers during infection, suggest a rolling-circle mechanism for their replication [40]. The identification of both (+) and (-) circular RNAs implies that both symmetric and asymmetric rolling circle mechanisms can be at work in sc-satRNAs [43]. However, there are not many studies focused on elucidating the replication mechanisms of these pathogens. One of the challenges is that sc-satRNA replication is linked to the HV replication, and, therefore, it likely occurs in the cytoplasm, unlike most infectious circRNAs which typically replicate in the nucleus [44].

Symmetric rolling circle replication

The symmetric rolling circle has been mostly studied for members of the *Avsunviroidae* family and especially the type species ASBVd which was officially characterized and identified as the causal agent of avocado sunblotch disease in 1979 [45]. ASBVd has been detected in chloroplasts of infected avocado leaves by *in situ* hybridization techniques [46]. The replication occurs in chloroplasts of infected cells, as discovered by analysing RNA from infected avocado tissues and identifying circular RNAs of both positive and negative polarity [47]. The process starts when the monomeric circular (+) RNA serves as a template for synthesizing longer linear (-) RNA strands. The viroid's hammerhead ribozyme activity then self-cleaves these long (-) RNAs into monomeric linear forms. This self-cleavage relies on the presence of Mg^{2+} [48] and occurs both *in vitro* and *in vivo*, a fact that supports the role of hammerhead ribozymes in the replication cycle [49]. The monomeric linear (-) RNAs with 5′-hydroxyl and 2′,3′-phosphodiester termini are subsequently circularized to produce new circular (-) RNAs, which then act as templates for the synthesis of linear (+) RNA strands [50].

In 2011, it was reported that ASBVd can replicate in a nonconventional host, the yeast *Saccharomyces cerevisiae*, although not all steps of the replication process were described in detail [51]. ASBVd RNA strands of both polarities could perform the critical step of self-cleavage utilizing their own hammerhead ribozyme activity. However, the viroid monomeric RNA was found to be destabilized by both nuclear 3'and cytoplasmic 5' RNA degradation pathways in yeast, which shows that the viroid RNA in yeast faces a two-front attack: it is degraded from one end by enzymes in the cell nucleus and from the other end by enzymes in the cytoplasm [51]. This dual degradation makes it more challenging for the viroid RNA to remain stable and survive within yeast cells. These findings implied that viroids can also exploit the RNA processing pathways of other organisms. However, given that yeast cells do not contain chloroplasts, connecting these findings to the natural context of ASBVd processing in their plant hosts is not straightforward.

The rolling circle mechanism has been also identified in the replication of mycoviroids. Mycoviroids were shown to be resistant to DNAseI, RNAseIII and RNAseR enzymes, supporting their circular nature. Even though not much information can be found on this specific group of viroids, it has been proposed that they use a symmetric rolling circle mechanism for their replication. This was shown using a treatment with RNAseR combined with northern blot analysis, however to this date, no other information about the proteins involved in this mechanism are known [36].

Finally, some viruses also use the symmetric rolling circle for their replication, particularly Ambiviruses. Ambiviruses, now grouped in their own phylum named Ambiviricota, is a group of viruses containing a circular RNA with ribozymes in their structure and the ability to code for at least two ORFs in ambisense, one of which produces an RdRp [2–5]. The known members of the group are rapidly increasing since new viruses are being discovered as a result of metatranscriptomic analyses. These viruses are thought to replicate through a rolling circle mechanism, but this has been proven only for three members of the group: Cryphonectria parasitica ambivirus 1 (CpAV1- species *Orthounambivirus cryphonectriae*), Tullasnella ambivirus 1 (TuAV1 - species *Orthodumbivirus unatulasnellae*) and Tulasnella ambivirus 4 (TuAV4 - species *Orthoquambivirus unatulasnellae*) [2]. The circularity of the genomes of all three aforementioned viruses was initially suggested using specific bioinformatic tools and was then supported by northern blot analysis combined with RNAseR exonuclease experiments [2]. Even though it is suggested

that replication occurs through a symmetric rolling mechanism, since no (-) circular strand has been identified yet, direct proof is still missing.

Double rolling circle replication

HDV replicates using both genomic HDV (gHDV) and antigenomic HDV (gHDV) RNAs as templates, thus the name 'double rolling-circle mechanism' [9]. The discovery of this process came after many years of research and some parts of the mechanism are still unclear. Three HDV forms can be found during infection: the gHDV, the agHDV, and the linear RNA, with the expression level following this order [52,53]. However, only the gHDV was identified within a virion [52], suggesting that the other two forms were present only during the replication process. Furthermore, all forms were observed within the nucleus, further supporting a possible rolling mechanism like the one described for viroids. Nevertheless, the production of linear RNA as leading to the production of two proteins make this rolling circle mechanism different to that of viroids. The mechanism, as currently understood, works as follows: Upon entering cells, the gHDV, bound to the viral proteins, is transferred into the nucleus, probably due to the Nuclear Localization Signal (NLS) present in HDV proteins [54,55]. There, the gHDV is transcribed into a linear fragment by host Pol II, which continuously 'rolls' around the circular genome. This fragment is quickly self-cleaved, through ribozyme activity, and ligated creating the agHDV. Then, the produced agHDV is used as a template to produce new gHDV circular RNAs via the same rolling circle mechanism. The agHDV can be modified through a specific RNA editing mechanism, using ADAR proteins, which transform the UAG stop codon at position 1012 to a UIG codon, where 'I' stands for inosine, a nucleotide commonly found in tRNAs. This 'modified' agHDV is then used as a template to create a modified gHDV. The linear RNAs produced during this process mainly from gHDV, are capped and polyadenylated. The unmodified version is responsible for the production of the small S-HDAg and the modified for the production on the longer version named L-HDAg [9]. It is worth mentioning that the same type of replication mechanism has also been identified for Delta-like viruses like the rodent deltavirus (RDeV) [56].

Rolling circle replication in viroids: open questions

Host DdRps redirected as RdRps

Pospiviroidae replication takes place in the nucleoplasm [57]. Initial studies suggested that multiple polymerases derived from healthy plant tissue could transcribe PSTVd RNA genome *in vitro* [58–60] (Table 1). However, strong evidence indicates Pol II as the primary enzyme responsible for transcription. Several scientific findings support this hypothesis. First, it was demonstrated in early studies by assessing *in vitro* transcription of viroid RNA, that the fungal toxin α(alpha)-amanitin, a selective inhibitor of Pol II, has a negative effect on the replication of cucumber pale fruit viroid [61], PSTVd [61,62], HSVd [63], and citrus exocortis viroid (CEVd) [64,65]. In context with the above observations, low concentrations of α-amanitin inhibited *in vitro* transcription of (+) and (–) PSTVd strands by partially purified Pol II [59] and attenuated PSTVd transcription in tomato nuclear extracts [66]. Second, Pol II complex purified from healthy tomato tissue or wheat germ was able to *in vitro* synthesize full-length viroid copies, using (+) PSTVd RNA templates [59,67]. Third, RNA immunoprecipitation confirmed that the largest Pol II subunit interacts with strands of both polarities of CEVd [68] and PSTVd [67]*in vivo*. Notably, Pol II preferentially

Table 1. Proteins involved in rolling circle replication mechanisms in various subcellular parasitic RNAs.

Pathogen	Polymerase	cleavage	ligation
Pospiviroids	Pol II	RNAseIII	DNA ligase1
Avsunviroids	NEP polymerase	Ribozyme activity	tRNA ligase or self-ligation due to the ribozyme activity
Mycoviroids	?	?	?
Sc-satRNAs	HV polymerase	– Ribozyme activity with 2′-3′ cyclic monophosphate and a 5′ hydroxyl group ends -?	Host ligase (tRNA ligase?) or self-ligation due to the ribozyme activity
HDV	Pol II, Pol I, Pol III	Ribozyme activity with 2′-3′ cyclic monophosphate and a 5′ hydroxyl group ends	Host ligase or self-ligation due to the ribozyme activity
Ambiviruses	Viral RdRp (?)	Depending on the ribozyme of each virus	?

recognizes circular (+) PSTVd in plants [67]. Until recently, it was still unclear whether Pol II recognizes oligomeric (−) strands as templates, with conflicting reports in the literature [62,69]. However, it WAS demonstrated in a recent study that Pol II can also accept (−) strand viroid RNA as template for the production of (+) strand molecules [70].

Regarding transcription of the *Avsunviroidae* family, studies have demonstrated that purified chloroplasts from infected leaves are able to transcribe ASBVd RNAs of both polarities. Notably, tagetitoxin, which inhibits the function of the plastid-encoded chloroplastic RNA polymerase (PEP) but not the NEP, did not suppress ASBVd transcription [71]. This finding suggests that NEP is the primary RNA polymerase required for ASBVd replication [71].

Initial experiments about the sc-satRNA replication using both α-amanatin and actinomycin D with various sc-satRNA, clearly demonstrated that neither Pol II nor a cellular polymerase is involved in their replication, supporting the involvement of the HV viral protein [72,73]. This result was straightened later on with transfection experiments of protoplasts in the case of Cereal Yellow Dwarf Virus sc-satRNA replication.

Regarding viroid-like viruses using rolling circle mechanism, Pol II involvement in HDV replication processes remains a highly debated issue. There is multiple evidence suggesting Pol II implication in HDV replication. First, Pol II was shown to be necessary for *in vitro* partial viral RNA transcription [74]. Second, Pol II directly binds to the external loops of gHDV [74,75]. Third, S-HDAg has been shown to bind Pol II, enhancing HDV infection [76]. Fourth, the fraction of capped and polyadenylated linear RNAs produced are typical of Pol II activity. Finally, it was clearly shown in multiple scientific works (not all presented here) that the use of α-amanatin or anti-Pol II antibodies decreased HDV levels [74–78]. However, an involvement of the other human polymerases (Pol I and Pol II) has also been proposed, with a theory suggesting that agHDV is produced in the nucleoli [77–80]. The difficulty in identifying the principal components of the HDV double rolling-circle mechanism resides in the absence of a reliable infectious system, since until recently the presence of HBV was considered necessary for natural infection.

Finally, Ambiviruses encode their own RdRp (ORFA). Although it has not been conclusively proven, this protein may be involved in the proposed rolling-circle replication, similarly to viral polymerases used by circular RNA satellites. However, the participation of a second protein remains a possibility. Furthermore, due to the high variability of various ORFA domains between the known viruses of this family, various modes of action for this protein cannot be ruled out [2].

It is worth mentioning that redirecting host DNA-dependent RNA polymerases to transcribe RNA is a unique ability of viroids and viroid-like RNAs. In contrast, most viruses encode their own RdRp and entirely skip host-dependent transcription of their genome. The different strategy of viroids and viroid-like RNAs could probably be based on their lack of coding capacity, as well as their ability to mimick DNA structures that can be recognized as transcription templates. This dependency of viroids on host RNA polymerases has probably restricted their replication to specific organelles – nuclei and plastids – where their transcription machinery resides, and also highlights that redirecting cellular RdRp is something that probably need specific conditions to be achieved.

Transcription initiation sites

The transcription initiation process involves the recognition of the viroid RNA template and the binding of the host RNA polymerase to start RNA synthesis. Primer extension experiments with PSTVd in *Solanum tuberosum* cell extracts identified two specific transcription start sites (TSSs) at positions A111 and A325. These sites exhibit features such as the similarity of the six 5' – located nucleotides transcribed next, a GC-box placed in the same distance from the start site, and a GC-rich double-helical segment that suggest they may function as transcription start sites [66]. However, in 2006, these proposed start sites were disproven, as they were attributed to false-positive binding to endogenous nucleic acids rather than newly synthesized RNA by Pol II. To address this issue, a new approach was developed using nuclear extract from non-infected potato cell culture, allowing for efficient purification and discrimination of de novo-synthesized (-) strands from endogenous nucleic acids. Furthermore, primer extension analysis of the *de novo* synthesized (−) strands revealed that Pol II initiates transcription at C1 or U359, located in the hairpin loop of the left terminal domain of PSTVd (+) RNA [66]. *In planta* analyses of site-directed mutants [66], as well as

genome-wide mutational analysis [81], support the involvement of this left terminal loop in viroid infectivity [81,82]. This supports other studies showing that the GC-rich Hairpin II (HP II) metastable structural motif is valuable during the (-) strand replication and serves as transcription element for (+) strand synthesis. Moreover, in *N. benthamiana* protoplasts HP II mutations resulted in the elimination of circular PSTVd, providing additional support [30,81,83]. Nevertheless, further evidence is required to confirm this site, as well as to detect the transcription initiation site for (–) PSTVd oligomers, which remains unmapped. Additionally, the transcription start sites of other *Pospiviroidae* members are yet to be determined.

Regarding *Avsunviroidae* members, it was shown that the 5'-termini of primary transcripts have a triphosphate group that can be specifically labelled *in vitro* with [a-32P] GTP and guanylyltransferase. This property was used to investigate the initiation sites of ASBVd by *in vitro* capping and RNA ligase-mediated rapid amplification of cDNA ends assay (RLM-RACE) [84]. The ASBVd transcription initiation sites for both polarities were mapped to the right terminal loop, with U121 marking the start site for (+) strands and U119 for (–) strands, respectively [84]. A study addressing transcription initiation of another *Ansunviroidae* member, PLMVd, led originally to some controversy. Initially, primer extension experiments using *Escherichia coli* RNA polymerase to initiate transcription identified TSSs at positions A5, A6, A7, and G8 for the strands of plus polarity, and at positions A2, A337, A335, and G333 for the strands of minus polarity [85,86]. Later studies, however, employing various approaches including primer extension analyses, revealed C51 as the TSS for the plus strand and U286 as the TSS for the minus strand [87]. Further research using either circular or monomeric linear forms or subgenomic RNAs led to similar results with the identification of the same universal initiation site for each of the PLMVd strands shared by a broad range of viroid variants [88]. Notably, in this case the TSS of one strand is positioned near the self-cleavage site of the complementary strand. A combined approach of *in silico*, *in vitro* and *in vivo* assays mapping the secondary structure of ELVd revealed that the initiation start site of the (+) strand in the position U138 located in an asymmetric loop forming part of a hairpin. Thus, unlike other *Ansunviroidae* TSSs, the initiation site for transcription of (+) ELVd does not coincide with the hairpin equivalent of (-) ELVd initiation site. Furthermore, they are not located within or adjacent to conserved sequence or structural motifs, thus showing the adaptability of eggplant NEP (or some associated transcription factor) in template binding and transcription initiation [89]. The above observations suggest that different chloroplast-replicating viroids employ distinct strategies for transcription initiation.

Until today, there is limited information regarding the transcription initiation site of sc-satRNAs, which is, among other factors, also driven by their diversity. In the example of CYDV-PRV, the binding sites of the viral replicase in both polarities have been identified in specific loops, however the exact nucleotide of the initiation is still under investigation [90].

In the case of HDV, recent studies have elucidated the initiation points for the synthesis of the three RNA forms present during infection. Interestingly, the initiation sites of gHDV/agHDV and linear RNA are distinct. Pol II was shown to bind to both terminal loops of both polarities [75], with the initiation starting at position +1646nt for the most common strains of HDV [91]. However, the initiation of the linear mRNA was identified at position +1630 nt, further supporting the implication of other host polymerases [77] in HDV replication. The different initiation sites may also explain the varying abundance of these three RNA types, with gHDV being the most abundant, followed by agHDV, and finally linear RNA.

Regulation of transcription

Although Pol II is hijacked by viroids for the transcription of their RNA, it forms a complex with modified architecture, which has FEWER components compared to the respective complex transcribing DNA templates [70]. Purified Pol II alone, in the absence of transcription factors, cannot initiate DNA promoter-dependent transcription [92–94]. Analysis through nano-liquid chromatography-tandem mass spectrometry using a purified Pol II complex on RNA templates showed a modified Pol II architecture, which lacked Rpb4, Rpb5, Rpb6, Rpb7 and Rpb9 [95]. This version discriminates from the canonical 10- or 12-subunit Pol II cores observed on DNA templates. Importantly, Rpb9 is crucial for Pol II fidelity, thus its absence during viroid transcription by Pol II can be associated with the HIGHER mutation rate of viroids compared to endogenous transcripts [96]. Recent findings highlighted a host transcription factor dedicated

to viroid RNA-templated transcription. Transcription factor IIIA (TFIIIA) was demonstrated to directly bind PSTVd in a gel shift assay [97]. Furthermore, it was shown that a splicing variant of TFIIIA, named TFIIIA-7ZF, is the transcription factor necessary for Pol II-mediated RNA-templated viroid transcription [67]. TFIIIA-7ZF interacts with Pol II and PSTVd RNAs of both polarities. Additionally, TFIIIA-7ZF can directly enhance Pol II processing efficiency during *in vitro* PSTVd transcription, as well as modulate *in planta* PSTVd replication [67]. In context with the above findings, this transcription factor can also bind HSVd [98] and has been proposed to be involved in the replication of apple fruit crinkle viroid [99], implying that the mechanism dedicated to viroid RNA-templated transcription is possibly conserved among *Pospiviroidae* [100]. It is to note that other canonical transcription factors do not participate in viroid RNA synthesis by Pol II, suggesting a unique mechanism dedicated to viroid transcription [70]. An earlier study had shown that the plant ribosomal protein L5 (RPL5) regulates TFIIIA alternative splicing, favouring the production of the full-length protein TFIIIA-9ZF and reducing the accumulation of TFIIA-7ZF [101]. Interestingly, RPL5 interacts *in vitro* [78,82] and *in vivo* with PSTVd RNA through its CCR [96,102], leading to the modulation of TFIIIA-7ZF accumulation. Conversely, RPL5 overexpression has a negative effect on TFIIIA-7ZF expression and diminishes PSTVd accumulation. This finding suggests that PSTVd establishes an interaction with RPL5 and manipulates the alternative splicing of TFIIIA, thus autonomously modulating its replication [96,102]. Even though these two factors have been identified as important for the initiation step, it cannot be excluded that additional factors are implicated in this process. Furthermore, it is yet unclear whether this unique regulatory mechanism is conserved in other *Pospiviroidae* members.

For *Avsunviroidae* members, the involvement of transcription factors or other proteins in the regulation of transcription remains unknown. Further studies are necessary to identify the additional components of Pol II and NEP machinery involved in viroid transcription. For Mycoviruses or Ambiviruses, there is currently no information on the regulatory mechanisms of their transcription.

In the case of HDV, both its encoded proteins can regulate its replication, although in a completely different manner. S-HDAg is secreted in the initial steps of infection and forms nuclear speckles with HDV RNA [103]. This protein enhances Pol II binding and therefore promotes gHDV replication. As the infection progresses, a second, longer protein is produced (L-HDAg) by an RNA editing mechanism. L-HDAg competes with the replication complex, inhibiting it and enhancing virion encapsidation [55,104]. The competition between these two proteins produced by the same pathogen during different stages of infection further highlights the complex regulation dynamics during the rolling circle mechanism.

Cleavage dynamics during replication

While there is significant progress in understanding the replication process of *Pospiviroidae*, there are still open questions at various steps of viroid replication, including the concatemer cleavage step. *Pospiviroidae* viroids are not known of catalysing self-cleavage [105], therefore they rely on host enzymes for the digestion of oligomers to monomers. Given the non-coding nature of viroids, their structure is of great importance for their biological cycle. It is now well-understood that the loop motifs present in viroids play critical roles in their replication and stability [106]. Since 1997, Baumstark and colleagues have proposed a mechanism for the cleavage and processing of *Pospiviroidae* RNA, emphasizing on the structural dynamics. They suggested that the RNA is initially cleaved within the stem of a GNRA tetraloop structure. This cleavage induces a local conformational change, switching the tetraloop to a loop E motif. The loop E motif stabilizes the 5' end through base-pairing, enabling a second cleavage. This second cleavage produces unit-length linear intermediates with aligned 3' and 5' ends. Viroid-expressing *Arabidopsis thaliana* (*A. thaliana*) plants were used to map the cleavage sites, showing that cleavage occurs within conserved double-stranded RNA structures, like hairpin I [107]. Specifically, in *A. thaliana* expressing dimeric CEVd (+) RNA, it was shown that the (+) CEVd is cleaved between positions G96 and G97 in the central conserved region *in vivo*, resulting in products with 5′-phosphomonoester and free 3′-hydroxyl termini. Based on this finding, it was proposed that an RNAse III-type enzyme must be involved in this mechanism, though direct evidence is lacking.

One significant limitation of the existing studies is the use of *A. thaliana* plants, which are not naturally infected by viroids. Although there is evidence that *A. thaliana* has the necessary enzymes for*Pospiviroidae*

replication, the universality of these findings is not clear, as all experiments used genetically engineered Arabidopsis plants that express dimeric transcripts of nuclear viroids, such as CEVd and HSVd. Furthermore, the RNase III-like enzyme responsible for the cleavage remains unidentified, and additional research in natural hosts is needed to validate these assumptions. Recent studies have demonstrated that Dicer-like (DCL) enzymes, which are a group of RNase III-like enzymes, are primarily involved in the host defence mechanism against viroids rather than the direct cleavage of viroid intermediate RNAs during replication [108–110]. *N. benthamiana* DCL enzymes generate viroid-derived small interfering RNAs (vd-sRNAs), however they do not appear to play a role in processing the viroid replication-intermediate RNAs. DCL4 has been identified as the ribonuclease primarily processing viroid RNA, resulting in minimal damage. In contrast, the combination of DCL2 and DCL3 leads to more effective viroid targeting [108].

Unlike nuclear-replicating viroids, the cleavage of replication-intermediates of *Avsunviroidae*, HDV and Ambiviruses of both polarities is carried out by ribozymes. Ribozymes are currently classified into 21 distinct groups depending on their structure and mode of action [111], with five groups reported for circular RNAs (hammerhead-HHRz, hairpin-HRPz, HDV ribozyme -DVRz, twister ribozyme TWRz and Varkud Satellite ribozyme-VSRz) [112]. Avsunviroids contain cis-acting HHRz [48,113]. (+) RNA and (-) RNA of ASBVd exhibit differences in electrophoretic mobility under native conditions, indicating distinct structural properties and are not cleaved with the same efficiency [114]. The cleavage reaction requires divalent metal ions, particularly magnesium ions (Mg^{2+}), which play a crucial role in the catalysis [48]. Although the hammerhead is self-cleaving, it has been shown that chloroplast proteins can bind viroid RNA and facilitate its HHRz-mediated self-cleavage [115]. UV-irradiation of avocado leaves infected with ASBVd led to the identification of two closely related chloroplast RNA-binding proteins, PARBP33 and PARBP35, which interact with the viroid. PARBP33 specifically acts as an RNA chaperone, stimulating the hammerhead-mediated self-cleavage of multimeric ASBVd transcripts *in vitro* [115]. The self-cleavage of *Avocado Sunblotch Viroid* (ASBVd) by the hammerhead ribozyme (HHRz) is also influenced by how the viroid RNA self-assembles. Using Small Angle Neutron Scattering (SANS) and structural modelling, it has been demonstrated that the transition between dimer and monomer forms of the RNA is a key step for efficient self-cleavage [116]. Detailed RNA structure modelling has indicated that interactions between RNA molecules, inter- or intramolecular, can either stabilize or destabilize the active form required for self-cleavage [116].

Similar to the hammerhead ribozyme activity observed in avsunviroids, and in spite of limited information on sc-satRNAs, it appears that most sc-satRNAs contain at least one ribozyme. This ribozyme is responsible for the cleavage of the concatemers during replication, leaving 5' hydroxyl and 2'_3' cyclic phosphodiester ends, as expected. Evidence for this cleavage has been shown since 1986 with studies on both natural or *in vitro*-generated satellite oligomers [117,118]. However, not all sc-satRNAs contain ribozymes in both polarities, further supporting the idea that some sc-satRNAs use a symmetric and others asymmetric rolling circle for their replication.

HDV and HDV-like agents also contain a DVRz. DVRzs are sequences of approximately 85 nt folding in a unique secondary structure, consisting of four double-stranded regions, three single-stranded domains, and two loops. These sequences are present in both gHDV and agHDV with small differences in their structure. The self-cleavage occurs immediately upstream of the sequence, releasing a 2'-3' cyclic monophosphate and a 5' hydroxyl group [119]. Recent studies showed that the DVRz is rather conserved among various available strains and that even small modifications influence its autocatalytic activity [120]. Particularly, C75 (C76 for the agHDV) was shown to be of critical importance, since mutations inhibited HDV infection [121]. However, HDV mutants that do not contain any DVRz were identified in patients receiving antiviral therapy, suggesting that HDV replication is DVRz-independent under certain circumstances [122]. Curiously, DVRz has also been identified as potential Internal ribosomal entry sites (IRES), thus promoting the translation of open reading frames (ORF), however further experiments are needed to elucidate the exact mechanism [123].

Finally, Ambiviruses also use the self-cleavage process during their replication. However, different types of ribozymes have been identified, sometimes more than one in the same genome. CpAV1 has two HHRzs, TuAV1 contains one HHRz, and TuAV4 comprises one HHRz and one HRRz. Using *in vitro*-transcribed RNAs, the self-cleavage was tested and found identical to the prediction cleavage site. In addition, *in vivo* experiments in infected Tulasnella and Cryphonectria parasitic extracts combined with 5' RACE further

supported these functional ribozymes [2], as well as their putative role in a possible symmetric rolling-circle mechanism. Nevertheless, the exact mechanism of ribozyme-mediated self-cleavage is still poorly understood and more studies are necessary to clarify the exact mechanism behind this process.

Ligation of linear monomers during rolling circle replication

In vitro tests using PSTVd RNA oligomers showed no evidence of self-cleavage and circularization, even under conditions where self-splicing of group I introns, and self-cleavage of other viroid-like tobacco ringspot virus and ASBVd was observed [105]. Diener originally proposed a model for viroid cleavage-ligation, where complementarity facilitated an enzyme to cleave the RNA into monomeric segments and ligate them into covalently circular viroid molecules [107].

Experiments in 1981, using wheat germ extracts showed that PSTVd employs host ligases for circularization. It was speculated that this would likely be an RNA ligase, as natural linear PSTVd molecules from plants were circularized by wheat germ extract. Cleaved circular viroid molecules generated by ribonuclease T1, producing 5'–hydroxyl and 3'-phosphate or cyclic 2“,3”-phosphate termini, could be re-circularized with wheat germ extract, whereas those from nuclease S1, with 5“–phosphate and 3”-hydroxyl termini, could not, suggesting that unit-length viroids contain either 3“–phosphate or cyclic 2”,3'-phosphate termini [124]. Monomeric PSTVd RNAs with a 2‘,3'-cyclic phosphate were generated *in vitro* by RNase T1 from *Aspergillus oryzae*, which cleaved precursor RNA and ligated 5'-OH and 2‘,3'-cyclic phosphate ends to form circular RNA in the presence of spermine or magnesium ions. Circularization required the 2‘,3'-cyclic phosphate, as phosphatase had no effect, while mild HCl hydrolysis inhibited circularization. These findings suggest that degradative endoribonucleases can also act as RNA ligases, promoting intramolecular ligation and potentially playing a role in viroid processing *in vivo* [125]. Biophysical, biochemical, and infectivity studies of longer-than-unit-length transcript mutants revealed a specific secondary structure crucial for RNase T1 cleavage and ligation, exposing both cleavage sites for efficient processing [126]. Advancing from previous studies that used a non-host enzyme (RNase T1), nuclear extracts from non-infected potato cells were tested. Full-length PSTVd transcripts with 17 extra nucleotides of the CCR showed only one of four possible structures, the 'extended middle structure' where the 5'– and 3'-ends branch out from the rod-like structure at nucleotide 87 could be circularized properly [127].

Baumstark et al. proposed that the conformational transition from the tetraloop structure to the loop E structure should be irreversible due to the dissociation of the 5' cleavage fragment, acting as the driving force from cleavage to ligation [128]. Moreover, mutational analysis using PSTVd mini RNA (domains and characteristics described in section 2.4) showed that an unpaired nt in loop E, distinct from the conserved loop E motif, is essential for ligation [129]. This hypothesis was additionally supported by *in vivo* experiments analysing 16 mutants of transgenic *A. thaliana* expressing dimeric (+) transcripts of CEVd. Mutations outside loop E that affected or abolished the reaction suggest that ligation depends on nucleotides from both CCR strands, including those within loop E and adjacent regions. Since loop E is present only in the genera *Pospiviroid* and *Cocadviroid* within the family *Pospiviroidae*, other genera in this family may rely on alternative motifs [130]. The existence of 5′-phosphomonoester and 3′-hydroxyl termini was verified by 5' and 3' RACE experiments (rapid amplification of 5′ and 3′ cDNA ends) and *in vitro* ligation assays using linear CEVd (+) RNA resulting from the cleavage of dimeric transcript from the same *A. thaliana* transgenic plants [131]. Gas et al. suggested that if an RNase III mediates cleavage, an RNA ligase capable of joining 5'-phosphomonoester and free 3'-hydroxyl termini would be required. In this scenario, this ligase would belong to a different class to the one represented by the wheat germ RNA ligase [130,131]. Later studies employing both *in vitro* and *in vivo* approaches showed that the long-sought enzyme was DNA ligase1 [132]. Testing tomato protein fractions with high viroid-circularizing activity and T4 RNA ligase as a control confirmed the presence of an enzyme responsible for the final circularization step, as activity was lost after proteinase K digestion and thermal inactivation. Protein extracts could only ligate monomeric linear PSTVd RNA opened between positions G95 and G96 with 5-phosphomonoester and 3-hydroxyl termini, but not other terminal groups or opened positions. Testing different nucleoside triphosphates or combinations showed an ATP requirement. Hypothesizing that the enzyme may follow the conventional nucleotidyltransferase mechanism, [α- [32] P]ATP was added to the reaction and the radiolabeled proteins were analysed by mass spectrometry revealing a protein similar to *A. thaliana* DNA ligase

1. Purifying the tomato DNA ligase 1 ortholog confirmed its efficiency in circulizing the linear PSTVd RNA precursor and had the same exquisite substrate specificity as the tomato extract. In *N. benthamiana* plants, *in vivo* experiments where the endogenous protein was silenced using either a hairpin construct or VIGS co-expressed with infectious dimeric PSTVd RNA, resulted in significantly lower circular-to-total RNA ratios. Since the *in vivo* technique did not completely abolish PSTVd circularization, this could be due to incomplete silencing or the possibility that another host DNA ligase compensates for the silenced DNA ligase 1 [132]. Recently, it was shown that DNA ligase 1 and PSTVd may colocalize with the nucleolus *in N. benthamiana* protoplasts, where (+) PSTVd RNA is highly concentrated, suggesting that they form a biomolecular condensate for RNA processing [133]. Open questions remain: Does DNA ligase 1 rely on loop E or a different CCR structure, as loop E is present only in the genera *Pospiviroid* and *Cocadviroid*? Since DNA ligase 1 activity was not completely abolished, allowing some circularization, an additional technique may be necessary to unequivocally confirm it as the sole ligase.

In *Avsunviroidae*, unit-length PLMVd transcripts with 2‘,3’-cyclic phosphate and 5’-hydroxyl termini self-ligate with 5% efficiency *in vitro* compared to 50% when wheat germ RNA ligase was used. Self-ligation experiments showed a linear product increase over time independent of substrate concentration. The addition of ATP had no effect, suggesting the 2’,3’-cyclic phosphate contributes to phosphodiester bond formation. Previous experiments showed that 5’−phosphorylated or 3’-dephosphorylated linear transcripts could self-ligate with or without ATP, supporting the assumption that both 5’-hydroxyl and 2‘,3’-cyclic phosphate groups are essential for self-ligation. The phosphodiester bond formed during self-ligation was analysed based on the ratio of 2‘,5’ to 3‘,5’ isomers, with approximately 30% 3‘,5’ and 70% 2‘,5’ bonds produced [118]. Similar experiments by Côté and Perreault, using complete enzymatic RNA hydrolysis coupled with thin layer chromatography (TLC) fractionation, showed that 2‘,5’-phosphodiester bonds were present in at least 96% of the transcripts [134]. Self-cleavage assays of transcripts with a 2‘,5’ or 3‘,5’ phosphodiester bond at the self-ligation site showed that this bond inhibits further self-cleavage. Additionally, incubation with a nucleus extract revealed that host enzymes, including the intron-debranching enzyme, cannot alter or cleave this bond [135]. These results suggest the presence of mostly 2‘,5’ isomers, which are not self-cleaved in hammerhead circular RNA [135]. Autoradiography and TLC confirmed 2‘,5’-phosphodiester bonds at the PLMVd ligation site. One proposed mechanism involves a host 2‘,5’ RNA ligase, similar to an *E. coli* enzyme that ligates tRNA halves *in vitro*, while an alternative hypothesis suggests self-ligation. Self-ligation is supported by: i) the minimal requirements, which only involve the correct alignment of strand ends produced by hammerhead self-cleavage; ii) the ligation site being located in a stable stem; and iii) the fact that this mechanism is still used today, indicating that selective pressure favours its preservation [134]. The low efficiency of self-ligation *in vitro* could indicate that the reaction needs a specific chloroplastic biochemical environment, which is challenging to reproduce in a laboratory setting. Mutational analysis in transplastomic *Chlamydomonas reinhardtii* showed that ELVd cleavage and ligation have different sequence requirements as two deletion mutants that cleaved efficiently exhibited ligation defects. Specifically, a quasi-double-stranded structure in the central region of the ELVd molecule, containing the ligation site within an internal loop motif, appears to play a role in ELVd circularization within the chloroplast [136]. The enzymatic circularization during the replication of *Avsunviroidae* family members was reported by Nohales *et al*., involving a chloroplastic isoform of the plant tRNA ligase from eggplant [137]. A recombinant version of this enzyme was purified and validated for its *in vitro* efficiency in catalysing the circularization of monomeric linear ELVd RNA of both polarities resulting from self-cleavage. Additionally, the same enzyme successfully circularized monomeric linear (+) and (-) RNAs of ASBVd, PLMVd, and CChMVd viroids. However, it was unable to circularize similarly produced linear ELVd (+) RNA opened at different positions within the circular molecule, suggesting specificity for physiological monomeric linear (+) ELVd (A333-G1) substrate [137] and supporting the demand of a quasi-double-stranded structure [121]. *In vivo* experiments of dimeric (+) ELVd transiently expressed in *N. benthamiana* pre-inoculated with a VIGS vector to induce silencing of endogenous tRNA ligase, resulted in a decreased ratio of circular/linear RNA. RNA ligation mediated by plant tRNA ligases produces 3’,5’ phosphodiester or 2’-phosphomonoester junctions. However, 2’-phosphomonoester is absent in ASBVd and ELVd circular RNAs accumulating in infected tissues [137]. To investigate the sequence and structural requirements for tRNA ligase-mediated ELVd circularization, an *E. coli* co-expression system was used to analyse mutations affecting the ligation site of the ELVd quasi-double-stranded structure. Eggplant tRNA ligase efficiently circularized all mutations except those in the ribozyme domain, highlighting its dual role in

RNA cleavage and circularization [138]. This hypothesis was supported by results using another *E. coli* co-expression system of tRNA ligase and longer than unit (+) ELVd containing two copies of the hammerhead ribozyme and appropriate termini. Ligation relied on ribozyme sequences at 5'-hydroxyl and 2',3'-cyclic termini, with increased circularization in variants with rearranged ligation positions when these sequences were present. *In silico* analysis revealed structural similarities between the hammerhead ribozyme catalytic pocket and the anticodon loop of tRNAs, both featuring a U-turn of the phosphodiester backbone. Mutagenesis in the ribozyme domain supports the role of the conserved U-turn loop residues in ligation, suggesting that viroid ribozymes mimic tRNA structures to recruit host tRNA ligase for circularization [139]. Although it demonstrated the importance of tRNA ligase in viroid replication, it remains uncertain whether a host 2',5' RNA ligase exists or how 2',5'-phosphodiester bonds are formed at the PLMVd ligation site in infected peach leaves.

There are two potential mechanisms for the ligation step of sc-satRNAs, both independent of the virus. The first involves self-ligation facilitated by ribozymes within the sequence. This has been demonstrated in the case of the tobacco ringspot virus satellite (sTRV) [140]. However, differences in the ligation efficiency of ribozymes have been observed. Studies indicate that sTRV contains a hammerhead ribozyme (HHRz) in its plus strand and a hairpin ribozyme (HRPz) in its minus strand. *In vitro* circularization experiments have shown that HRPz exhibits higher ligation efficiency than HHRz [141,142]. The presence of these ribozymes in different strand polarities suggests that variations in rolling circle replication efficiency may occur not only between different pathogens but also within the same pathogen[146]. Additionally, it has been proposed that RNA folding could influence ligation efficiency, implying that environmental factors may significantly impact the replication efficiency of a pathogen [143]. The second possible ligation mechanism involves a host RNA ligase. In the case of sc-satRNAs associated with Solanum nodiflorum mottle virus (SNMV, species Sobemovirus SNMOV) and Velvet tobacco mottle virus (VTMoV, species Sobemovirus VTMOV), tRNA ligase has been suggested as a potential candidate [144]. However, this hypothesis is based solely on the observed RNA termini and lacks direct experimental evidence. For HDV, two main hypotheses exist regarding the ligation of HDV monomers. The first suggests self-ligation. Sharmeen et al. (1989) conducted an experiment using self-cleaved HDV RNA produced *in vitro*, incubating it for 2 days at 4°C in the presence of ethylenediamine to assess ligation efficiency [145]. Their findings indicated that HDV RNA could undergo ligation through its ribozyme activity, a capability observed in various ribozymes [146]. However, the self-ligation hypothesis has been contested by more recent studies, particularly the work of Reid and Lazinski [147]. In their research, earlier experiments were replicated, but the observed ligation efficiency was insufficient. Notably, when they conducted *in vivo* experiments, HDV mutants lacking delta virus ribozymes (DVRz) were still efficiently ligated in Huh7 cells, suggesting the involvement of a host factor. This hypothesis is further supported by the presence of HDV mutants lacking DVRz in infected patients[105]. Despite this evidence, the specific host factor responsible for ligation remains unidentified, and conclusive proof for this theory is still lacking.

Conclusions

The rolling circle replication mechanism is a highly efficient and versatile strategy employed by various circular pathogens, including viroids, satellite RNAs, and certain viruses, to replicate their genomes. This mechanism, classified into asymmetric, symmetric, and double types, allows these pathogens to exploit host cellular machinery to produce multiple copies of their RNA from a single initiation event.

Nevertheless, open questions remain regarding the specific host factors involved in transcription initiation, cleavage, and ligation during rolling-circle replication. This is particularly obvious in the cases of Ambiviruses and sc-satRNAs but it is also true for the better studied viroids and HDV. For instance, while studies suggest initiation sites at specific positions for (+) PSTVd RNA, the exact transcription start site (TSS) for (–) PSTVd oligomers remains unmapped, and the TSSs for other *Pospiviroidae* members have yet to be determined. Finally, the enzyme performing the cleavage step of the concatemeric intermediates has still to be identified. For *Avsunviroidae* members, although initiation sites have been mapped for ASBVd, other members (e.g. PLMVd and ELVd) show variable or controversial TSS locations. Finally, there are also multiple open questions regarding HDV replication such as the exact roles of Pol II (and possibly Pol I) in HDV replication and HDV transcription initiation, as well as the packaging of only the (-) strand.

Future research should focus on identifying the specific host enzymes and regulatory factors involved in each step of rolling circle replication, as well as exploring the evolutionary implications of these mechanisms. By elucidating these processes, we can gain deeper insights into the biology of circular pathogens and potentially develop targeted strategies to control their spread and impact on agriculture and human health.

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Data availability statement

No data were generated for this work.

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