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Role of RNA silencing in plant-viroid interactions and in viroid pathogenesis

Francesco Di Serio [a,*], Robert A. Owens [b,1], Beatriz Navarro [a], Pedro Serra [c], Ángel Emilio Martínez de Alba [d], Sonia Delgado [e], Alberto Carbonell [c], Selma Gago-Zachert [f]

[a] Institute for Sustainable Plant Protection, National Research Council, Bari 70122, Italy
[b] Molecular Plant Pathology Laboratory, US Department of Agriculture, Agricultural Research Service, Beltsville, MD 20705, USA
[c] Instituto de Biología Molecular y Celular de Plantas (Consejo Superior de Investigaciones Científicas–Universitat Politècnica de València), Valencia 46022, Spain
[d] Institute for Agribiotechnology Research (CIALE), Department of Microbiology and Genetics, University of Salamanca, Villamayor 37185, Salamanca, Spain
[e] Instituto Agroforestal Mediterráneo (IAM-UPV), Camino de Vera, s/n 46022, Valencia, Spain
[f] Institute of Biochemistry and Biotechnology, Section Microbial Biotechnology, Martin Luther University Halle-Wittenberg, Halle/Saale 06120, Germany

A B S T R A C T

Viroids are small, single-stranded, non-protein coding and circular RNAs able to infect host plants in the absence of any helper virus. They may elicit symptoms in their hosts, but the underlying molecular pathways are only partially known. Here we address the role of post-transcriptional RNA silencing in plant-viroid-interplay, with major emphasis on the involvement of this sequence-specific RNA degradation mechanism in both plant antiviroid defence and viroid pathogenesis. This review is a tribute to the memory of Dr. Ricardo Flores, who largely contributed to elucidate this and other molecular mechanisms involved in plant-viroid interactions.

1. Introduction

RNA silencing is a sequence-specific RNA-mediated mechanism that occurs in most eukaryotes and regulates gene expression and transposon activity (Fire, 1999). RNA silencing occurs at the transcriptional level (transcriptional gene silencing, TGS), either preventing or dampening transcription through DNA methylation and chromatin modifications, or at the post-transcriptional level (post-transcriptional gene silencing, PTGS) through RNA cleavage (slicing) or translational repression (Baulcombe, 2004; Vaucheret, 2006). RNA silencing pathways are triggered by double-stranded RNAs (dsRNAs) or highly structured single stranded RNAs of cellular or exogenous origin, which are processed by RNase-III type enzymes of the DICER family into small RNA (sRNA) molecules (Bernstein et al., 2001; Fire et al., 1998; Hamilton and Baulcombe 1999). One of the strands of the sRNA duplex, the guide strand, is loaded onto an ARGONAUTE (AGO) protein to form the RNA-induced silencing complex (RISC) (Hammond et al., 2000, 2001); the other strand, called the star strand, is usually degraded. RISC specifically targets cognate nucleic acids based on the complementary to the guide strand.

In plants, RNA silencing pathways control such key biological processes as development, response to biotic and abiotic stresses and maintenance of genome integrity (Baulcombe, 2004). Plant sRNAs are typically divided in two classes, microRNAs (miRNAs) and small interfering RNAs (siRNAs), which differ in their biogenesis pathways and in the spectrum of their target transcripts (Axtell, 2013). miRNAs originate from endogenous transcripts with imperfect self-complementary fold-back structures that are processed by DICER-LIKE 1 (DCL1) and target other cellular transcripts (e.g. transcription factors). siRNAs may arise from transposons, centromeres, inverted repeats and invading nucleic acids (e.g. viral RNAs) and are generated by DCL2, DCL3, and DCL4 to silence their cognate targets (Bologna et al., 2014; Ding and Voinnet, 2007; Lopez-Gomollon and Baulcombe, 2022). RNA silencing can be amplified by the action of RNA-dependent RNA-polymerases (RDRs) that typically synthesize dsRNAs from target transcripts cleaved by sRNA/AGO complexes. The newly produced dsRNAs re-enter the RNA silencing cycle as they are processed by DCLs to generate a second pool of siRNAs named secondary siRNAs (Voinnet, 2008).

Here we assess the role of RNA silencing mechanism in viroid-host interactions with major emphasis on the involvement of the PTGS pathway in viroid pathogenesis.

* Corresponding author.
E-mail address: francesco.diserio@ipsp.cnr.it (F. Di Serio).
[1] Retired

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2. Viroids are targeted by RNA silencing

Viroids are plant infectious agents with small, circular, single-stranded, non-protein-coding RNA genomes (Flores et al., 2004; Navarro et al., 2021). Despite the lack of protein-coding capacity, viroids are able to infect higher plants, replicate autonomously and move throughout their hosts. Several of them are capable of inducing symptoms and causing diseases in multiple hosts (Kovalskaya and Hammond, 2014). Regarding replication, viroids are parasites of the cell's transcriptional machinery and are almost entirely dependent on the activity of host enzymes. According to the site of replication and accumulation of their genomes, structural features and replication mode, viroids are grouped into two families, *Pospiviroidae* and *Avsunviroidae*, including members that are located in nucleus and chloroplast, respectively (Flores et al., 2005).

Nuclear viroids share a central conserved region and adopt compact rod-like structures, while chloroplastic viroids contain hammerhead ribozymes motifs required for replication and their genomes fold into a less compact structure (Flores et al., 2009). Members of both families replicate by a rolling circle mechanism with RNA intermediates (asymmetric or symmetric in the case of *Pospiviroidae* and *Avsunviroidae,* respectively) that include dsRNA molecules, the main substrate of DCLs. Furthermore, viroid genomes possess strong secondary structures that could also be recognized by DCLs (Itaya et al., 2007). Consistent with these properties, the presence of viroid-derived sRNAs (vd-sRNAs) was first detected in plants infected with nuclear viroids (Itaya et al., 2001; Papaefthimiou et al., 2001) and later also in plants infected with peach latent mosaic viroid (PLMVd) and chrysanthemum chlorotic mottle viroid (CChMVd), two chloroplastic viroids (Martínez de Alba et al., 2002), indicating that members of both families are targets of the host RNA silencing machinery.

Although plant DCLs have all been assigned a nuclear localization based primarily on heterologous and/or overexpression studies (Fang and Spector, 2007; Xie et al., 2004), experiments based on immunolocalization assays revealed DCL4 antibody staining in both the nucleus and cytoplasm (Hoffer et al., 2011). In contrast, most PTGS components, including RDR6 and AGO1, are entirely or partly cytoplasmic (Derrien et al., 2012; Kumakura et al., 2009; Martínez de Alba et al., 2015). Thus, the detection of vd-sRNAs generated from viroids that replicate and accumulate in the chloroplast (Martínez de Alba et al., 2002) raises the question of whether vd-sRNA biogenesis may occur in this organelle. Because no DCL protein has been localized in the chloroplasts so far, the most likely scenario is that vd-sRNAs of chloroplastic viroids are originated in the cytoplasm during viroid movement from cell to cell, with the possible involvement of host RDRs generating viroid-derived dsRNAs that are likely targeted by cytoplasmic DCLs (Navarro et al., 2012). Overall, the mechanism of vd-sRNA biogenesis, especially in the case of chloroplast replicating viroids, remains still elusive.

The role of DCL enzymes in viroid infections was investigated by inoculating potato spindle tuber viroid (PSTVd) into *Nicotiana benthamiana* plants with reduced DCL activity. These experiments revealed that the combined activity of DCL2 and DCL3 is necessary for the plant's defence against PSTVd (Katsarou et al., 2016), while DCL4 plays a positive role in the infection, as viroid accumulation was reduced in plants with decreased levels of this protein (Dadami et al., 2013). Since levels of other nuclear viroids, including tomato apical stunt viroid (TASVd) and hop stunt viroid (HSVd), were also decreased in plants with reduced DCL4 expression, Katsarau et al. (2016) suggested that members of the *Pospiviroidae* family may have evolved to be primary targeted by the less detrimental DCL4. The involvement of DCLs in antiviroidal activity was further supported by the observation that a transgenic tomato line in which DCL2 and DCL4 were downregulated showed increased accumulation of PSTVd at early infection times, and developed lethal systemic necrosis accompanied by up-regulation of stress-responsive miRNAs and production of reactive oxygen species at later times (Suzuki et al., 2019).

Analysis in *N. benthamiana* plants infected with PSTVd provided the first direct evidence for the recruitment of vd-sRNAs by AGO proteins (Minoia et al., 2014). Immunoprecipitation of *Arabidopsis thaliana* (Arabidopsis) AGO proteins agroexpresssed in PSTVd-infected plants followed by deep-sequencing analysis revealed that vd-sRNAs are bound, with different affinities, by AGO1, AGO2, AGO3, AGO4, AGO5 and AGO9 (Minoia et al., 2014). As in the case of viral- and host-derived sRNAs, the sorting of the vd-siRNAs into the different AGO proteins depended on their 5′-terminal nucleotides, and, furthermore, AGO1, AGO2 and AGO3 preferentially associated with 21 and 22 nt-long vd-sRNAs while AGO4, AGO5 and AGO9 also bound those of 24 nt. The observation that vd-sRNAs are unevenly distributed along the viroid genome, accumulating in particular hotspots, suggested that the genomic RNA or its precursors are targeted by RISC. Consistent with this hypothesis, transient overexpression of AGO1, AGO2, AGO4 and AGO5 proteins in PSTVd-infected plants resulted in a reduction of viroid genomic RNA, supporting their role in antiviroid defence.

The role of plant RDRs in viroid infections has been analysed in several viroid/host pathosystems. RDR1 expression was reported to increase in tomato and cucumber plants infected with PSTVd and HSVd, respectively (Schiebel et al., 1993; Schiebel et al., 1998; Xia et al., 2017), suggesting its involvement in antiviroid defence (Sano, 2021). However, in other studies RDR1 expression in tomato remained unchanged upon infection by a different PSTVd strain (Zheng et al., 2017) or citrus exocortis viroid (Thibaut and Bragard, 2018), thus raising the possibility of differential responses depending on viroid-host combinations. In *N. benthamiana,* RDR1 expression is disrupted due to a natural sequence insertion that contains in-frame stop codons in the 5' portion of the respective ORF (Yang et al., 2004). Notably, accumulation of PSTVd and its vd-sRNAs was delayed in transgenic *N.benthamiana* plants overexpressing a functional *N. tabacum* RDR1 (Li et al., 2021). The same authors also showed that downregulation of *RDR1* in tomato correlated with an increased susceptibility to PSTVd infection, thus supporting the involvement of RDR1 in restricting the early viroid systemic invasion of the host. Silencing of RDR6 in transgenic *N. benthamiana* (NbRDR6i) plants resulted in increased accumulation of PSTVd genomic RNA early after infection and in invasion of the vegetative meristem by the viroid (Di Serio et al., 2010), showing that RDR6 activity restricts the systemic spread of PSTVd and its entry into the floral and vegetative meristems. Further evidence for a role of RDR6 in reducing PSTVd accumulation has been obtained by knocking-down its expression in wild-type *N. benthamiana* plants prior to viroid infection (Adkar-Purushothama and Perreault, 2019). In this later study, PSTVd genomic RNA was shown to accumulate to higher levels in RDR6-silenced plants than in control plants, reinforcing the idea that RDR6 is involved in the defensive pathway against viroid infections.

Finally, the discovery of vd-sRNAs in infected plants led to the hypothesis that, as reported for viruses (Tenllado and Diaz-Ruiz, 2001), co-inoculation with viroid-derived dsRNAs and vd-sRNAs might also protect plants against viroid infections. In agreement with the results obtained with viruses, co-inoculation of nuclear (citrus exocortis viroid, CEVd) or chloroplastic (CChMVd) viroid RNAs with their respective dsRNAs protected hosts from viroid infection, resulting in either non-infected plants or delayed onset of symptoms (Carbonell et al., 2008). Similar results were obtained when either the infectious viroid genomic RNAs were co-inoculated with a population of sRNAs derived from the corresponding *in vitro* processed sequences or when a construct allowing the expression of a hairpin RNA containing a partial sequence of PSTVd was co-infiltrated with an infectious PSTVd clone (Carbonell et al., 2008).

An independent study by Schwind et al. (2009) provided additional evidence that viroids can undergo sRNA-mediated degradation. These investigators showed that transgenic tomato plants expressing inverted repeats of the PSTVd sequence accumulated high levels of hairpin-derived sRNAs, that these plants were resistant to PSTVd infection, and that the degree of resistance was directly correlated with

the levels of sRNAs. In line with these results, citrus tristeza virus-encoded RNA silencing suppressor proteins, both during natural infections and when transgenically expressed, enhanced the accumulation levels of citrus dwarfing viroid (CDVd) in mexican lime, further confirming viroid targeting by the host post transcriptional RNA silencing (Serra et al., 2013).

More recent studies have demonstrated the feasibility of RNAi-based strategies for the control of viroid infections, both through the use of artificial microRNAs and synthetic trans-acting siRNAs (Carbonell and Daròs, 2017) and through the development of transgenic lines expressing non-infectious truncated viroid sequences (Adkar-Purushothama et al., 2015), thus providing evidence that engineering viroid resistance is feasible (Dalakouras et al., 2015; Flores et al., 2017).

3. RNA silencing plays a role in viroid pathogenesis

The discovery of vd-RNAs in tomato plants infected by PSTVd (Itaya et al., 2001; Papaefthimiou et al., 2001), a representative nuclear-replicating viroid, immediately raised the possibility that vd-sRNAs derived from nuclear-replicating viroids could guide the RNA silencing-mediated degradation of complementary and physiologically relevant mRNAs. Evidence supporting this hypothesis include (i) observations of typical PSTVd-induced symptoms in transgenic plants expressing non-replicating PSTVd hairpin RNAs (Wang et al. 2004) and (ii) the identification, in plants infected by PSTVd or related viroids, of specific vd-sRNAs that target for degradation several specific host mRNAs possibly involved in the elicitation of systemic symptoms (reviewed by Adkar-Purushothama and Perreault, 2020). Although the targeting of various host mRNAs by specific vd-sRNAs has been documented for several nuclear-replicating viroids, whether RNA silencing is actually responsible for the initial event in the elicitation of complex developmental disorders like the epinasty (leaf curling) or stunting induced by these viroids in some hosts seems difficult to ascertain. Indeed, different viroids or viroid variants frequently induce very similar symptoms in the same host, making it difficult to identify a single targeted gene initiating the pathogenic process and to discriminate between early and late effects (reviewed by Flores et al., 2020). In potato, PTGS of gene StTCP23, which encodes a transcription factor, is triggered by vd-sRNAs derived from the virulence modulating region of PSTVd, and downregulation of its expression results in tuber and plant developmental disorders very similar to those observed in PSTVd-infected potato plants, likely through the impairment of gibberellic acid (GA) biosynthesis and signaling pathways (Bao et al., 2019). Interpretation of these results may not be easily extended to other viroid-host combinations, however, because a recent attempt to identify vd-sRNA-targeted genes common to both tomato and *N. benthamiana* plants infected by the same PSTVd severe variant and showing similar developmental defects (stunting and epinasty) was unsuccessful (Navarro et al., 2021).

The situation is less complex for the chlorosis symptoms induced by specific variants of PLMVd, a chloroplast-replicating viroid (Hernández et al., 1992). In this case, viroid variants inducing a disease denoted as peach calico (PC), consisting of a severe chlorotic mosaic (albinism) in leaves, stems and fruits of peach trees, were shown to contain a specific pathogenicity determinant; i.e. a short 12–13 nt insertion (Malfitano et al., 2003). Furthermore, in a symptomatic host the severe PLMVd variants were strictly associated with the albino tissues which contain immature chloroplasts, while variants lacking the pathogenic determinant accumulated in green and infected tissues containing completely developed chloroplasts (Rodio et al., 2006; 2007). Importantly, vd-sRNAs with the pathogenic determinant were present only in albino tissues, where they specifically target chloroplastic heat shock protein 90 (cHSP90) mRNA for RNA silencing-mediated degradation (Fig. 1A, Navarro et al., 2012). cHSP90 is a nuclear-encoded protein that is translocated to plastids where it is involved in chloroplast development. Interestingly, Arabidopsis plants in which this gene is mutated exhibit a phenotype resembling that observed in PC albino tissues (Cao et al.,

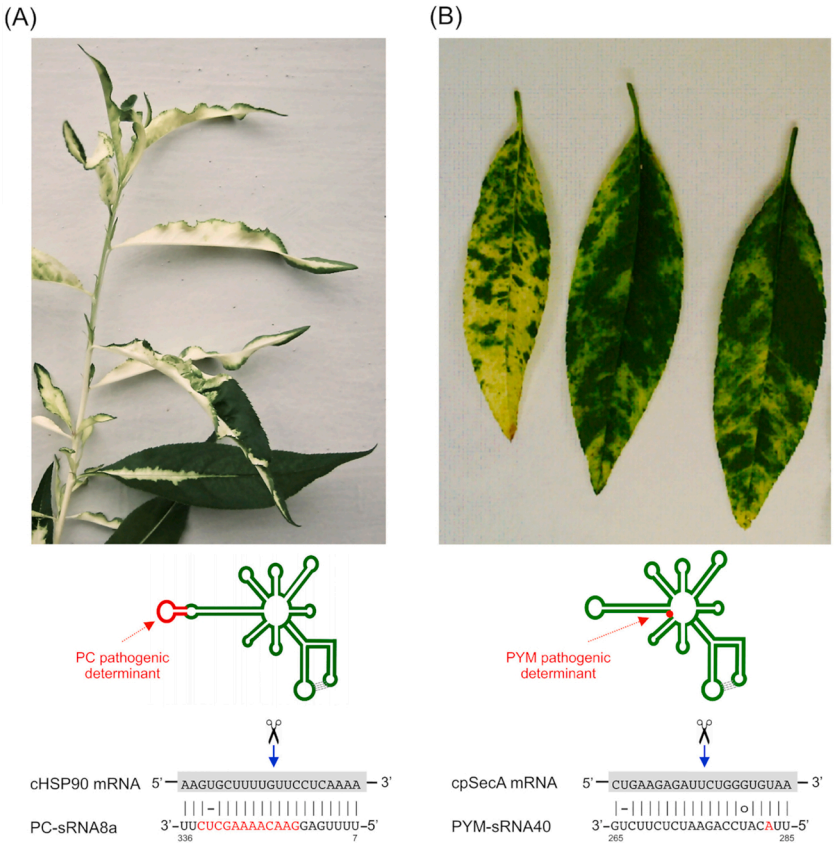

Fig. 1. (A) Upper panel: symptoms of peach calico (albinism) induced by PLMVd variants containing a pathogenic determinant consisting of a 12–13 nt insertion. Middle panel: pathogenic determinant of peach calico reported in red in the branched secondary structure of minimal free energy of PLMVd. Lower panel: hybrid formed between the vd-sRNA containing the PC determinant (in red) and the complementary host mRNA coding for cHSP90. **(B)** Symptoms of peach yellow mosaic (PYM) induced by PLMVd variants containing a pathogenic determinant mapped at a single nucleotide. Middle panel: pathogenic determinant of PYM indicated in red in the branched secondary structure of minimal free energy of PLMVd. Lower panel: hybrid formed between the vd-sRNA containing the PC determinant (in red) and the complementary host mRNA coding for cpSecA. (Figure reproduced from Navarro et al., 2020, FEMS Microbiology Reviews 44, 386–398 with permission).

2003). In the PC pathosystem, the close association of specific viroid pathogenic variants with the macroscopic symptoms facilitated the identification of the link between the primary molecular event (silencing of cHSP90 by vd-sRNAs containing the pathogenic determinant), a very specific cytopathic alteration (impairment of chloroplast development) and the early macroscopic symptom (albinism).

A similar mechanism has also been proposed for PLMVd variants causing peach yellow mosaic disease (PYM), which differs from PC because of the progression and the intensity of the mosaic that is a yellowing never evolving in albinism (Delgado et al., 2019). The pathogenic determinant of PYM has been mapped to a single nucleotide in the PYM-inducing PLMVd variants that, similarly to PC, were strictly associated with the symptomatic tissues. Moreover, it was shown that vd-sRNAs containing the pathogenic determinant directed the RNA silencing-mediated degradation of an mRNA that encodes a thylakoid translocase subunit (cpSecA) required for chloroplast development (Fig. 1B, Delgado et al., 2019). These findings show that PC and PYM, two different chlorosis syndromes caused by PLMVd variants with a specific pathogenic determinant, have different primary causes. Interestingly, an RNA silencing mechanism similar to that operating in PLMVd-induced chlorosis has been shown to operate in the elicitation of the leaf chlorosis caused by severe variants of CChMVd, another chloroplast-replicating viroid (Serra et al., manuscript in preparation), thus highlighting a common molecular pathway for the induction of chlorotic symptoms by members of the family *Avsunviroidae*. However, chlorotic symptoms are only one of the many alterations induced by these chloroplastic viroids in their hosts. For example, it is known that PLMVd may induce additional defects in peach fruits and petals, delay in flowering and fruit maturation and developmental alteration (open habit) of the infected trees (Desvignes, 1976). The molecular pathways underlying these symptoms are currently unknown and may not involve RNA silencing.

4. Other molecular pathways implicated in viroid pathogenesis

Viroid infections have long been recognized to cause far reaching changes in the regulation of host gene expression, an effect documented for several viroid-host combinations. Transcriptomic and proteomic studies have identified differential regulation of genes involved in many different molecular pathways upon viroid infection (reviewed by Owens et al., 2017). Genes implicated in plant hormone signaling, defence, protein metabolism, RNA binding, processing and modification were reported to be differentially expressed in viroid infected plants as compared with non-infected controls (Zheng et al., 2017; Štajner et al., 2019; Takino et al., 2019; Thibaut and Bragard, 2018; Wang et al., 2019). Notably, genes involved in plant innate immune responses are among those induced by viroid infection. Whether and how viroids might directly or indirectly trigger such immunity is not clear, however. Pathogen-associated molecular pattern (PAMP) or effectors, which are known to activate these plant defences against other pathogens, are recognized by host receptors (Bent et al.,2007), but if viroid RNAs (either genomic or replicative intermediates) may actually be perceived as a PAMP or as an effector triggering the immune response of the plant is not known.

The direct degradation of host-mRNAs by vd-sRNAs discussed in the previous section is likely only one of several molecular pathways involved in viroid pathogenesis. Other post-transcriptional events, such as changes in alternative splicing and the generation of phased secondary siRNAs with potential trans-acting functions, must also be considered as potential source of regulatory disruption (Zheng et al., 2017). In addition, viroids could reprogram host transcription through epigenetic mechanisms such as DNA methylation and histone modification (reviewed by Gómez et al., 2022).

An important early observation leading to the discovery of RNA-directed DNA-methylation (RdDM) was the fact that a replicating viroid (PSTVd) is able to induce the methylation of its corresponding transgene (Wassenegger et al., 1994; reviewed by Wassenegger and Dalakouras, 2021). Several studies highlighted a possible correlation between viroid infection and epigenetic alterations in the genome of either host plant (Castellano et al., 2015; 2016a; 2016b; Martínez et al., 2014; Lv et al., 2016) or a co-infecting DNA virus (Torchetti et al., 2016). Epigenetic changes in infected cucumber plants were correlated with a direct *in vivo* interaction between mature forms of hop stunt viroid (HSVd) with the histone deacetylase 6 (HDA6), an enzyme recognized as a component of the RdDM pathway (Castellano et al., 2016a).

A correlation between viroid infection status and hypermethylation of a trangene promoter has been reported in transformed *N. benthamiana* plants expressing GFP, with a potential role in this phenomenon proposed for the bromodomain containing viroid-binding protein VirP1 (Lv et al., 2016). Previous studies have identified other major roles in viroid infectivity for this protein (Kalantidis et al., 2007; Martínez de Alba et al., 2003). A generalized over-expression of genes involved in RdDM has also been reported in tomato plants infected by PSTVd (Torchetti et al., 2016). Taken altogether these studies support the ability of viroids to interfere with the epigenetic status of their hosts and consequently to modify the expression of many important genes. Although the underlying mechanism(s) is not completely known, several scenarios involving either direct or indirect interactions with viroid RNAs have been envisaged (Gómez et al., 2022).

And finally, changes in gene expression associated with viroid pathogenesis may be at least partially modulated through impairment of the host translational machinery. Evidence supporting this view includes i) viroid-induced alteration of pre-rRNA processing (Jakab et al., 1986), ii) changes in the expression of ribosomal genes and translation factors (Adkar-Purushothama et al., 2017; Lisón et al., 2013), and (iii) changes in ribosome biogenesis and functionality (Cottili et al., 2019) associated with certain viroid infections.

Taken together the effects described above depict a complex scenario for viroid pathogenesis in which a large variety of molecular mechanisms might simultaneously contribute to the development of the cytopathic effect and macroscopic alterations we observe in the infected and diseased hosts.

5. Concluding remarks

Viroids are both triggers and targets of post-transcriptional RNA silencing. The resulting vd-sRNAs may drive the sequence-specific degradation of the invasive viroid RNAs, thus having an antiviroid defence role. At the same time, certain vd-sRNAs may target host mRNAs for degradation and, when the encoded protein has a major physiological role, trigger or contribute to the development of specific symptoms. Therefore, the same molecular process (RNA silencing) may simultaneously act as both plant defence mechanism and pathogenic pathway in the plant-viroid interplay. Although RNA silencing has been shown to be the primary molecular lesion leading to the development of symptoms for certain viroids, it represents only one of several possible molecular mechanisms implicated in viroid pathogenesis. A broader view suggests that viroid disease is the resultant of a more complex network of molecular lesions and signalling elicited, either directly or indirectly, by these infectious non-coding RNAs.

CRediT authorship contribution statement

Francesco Di Serio: Conceptualization, Writing – review & editing. **Robert A. Owens:** Conceptualization, Writing – review & editing. **Beatriz Navarro:** Conceptualization, Writing – review & editing. **Pedro Serra:** Conceptualization, Writing – review & editing. **Ángel Emilio Martínez de Alba:** Conceptualization, Writing – review & editing. **Sonia Delgado:** Conceptualization, Writing – review & editing. **Alberto Carbonell:** Conceptualization, Writing – review & editing. **Selma Gago-Zachert:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

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