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Viroids,infectious long non-coding RNAs with autonomous replication

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Highlights

- Plant endogenous long non-coding RNAs are regulators of gene expression
- Endogenous lncRNAs are involved in crucial plant processes like flowering and stress responses
- Viroids are plant pathogens with non-protein coding genomes
- Viroids can induce symptoms by down-regulation of host transcripts

Abstract

Transcriptome deep-sequencing studies performed during the last years confirmed that the vast majority of the RNAs transcribed in higher organisms correspond to several types of non-coding RNAs including long non-coding RNAs (lncRNAs). The study of lncRNAs and the identification of their functions, is still an emerging field in plants but the characterization of some of them indicate that they play an important role in crucial regulatory processes like flowering regulation, and responses to abiotic stress and plant hormones. A second group of lncRNAs present in plants is formed by viroids, exogenous infectious subviral plant pathogens well known since many years. Viroids are composed of circular RNA genomes without protein-coding capacity and subvert enzymatic activities of their hosts to complete its own biological cycle. Different aspects of viroid biology and viroid-host interactions have been elucidated in the last years and some of them are the main topic of this review together with the analysis of the state-of-the-art about the growing field of endogenous lncRNAs in plants.

Keywords: viroid; long non-coding RNAs

1. Introduction: long non-coding RNAs (lncRNAs) in plants

Opposite to RNAs with protein-coding capabilities (messenger RNAs, mRNAs) non-coding RNAs (ncRNAs) are defined based on their inabilityto code for proteins. In recent years our knowledge about endogenous ncRNAs increased exponentially due to the development and application of next-generation sequencing tools. Transcriptome deep-sequencing analysisconfirmed that only a small fraction of transcribed RNAs correspond to mRNAs, whereas the vast majority consists of several types of non-coding RNAs including long non-coding RNAs (lncRNAs). lncRNAs are arbitrarily defined as RNA transcripts longer than 200 nucleotides (nt) without protein coding capacity (Mercer et al., 2009) and are important riboregulators of gene expression (Wilusz et al., 2009). They have been well characterized in the last years in animals, where they exert crucial functions in the regulation of important cellular processes (Rinn and Chang, 2012). In plants the study of lncRNAs is still an emerging field, but the characterization of a few of them indicates the important role that they play in regulatory processes like flowering regulation and responses to abiotic stress and to plant hormones (Ariel et al., 2015).

Different form the endogenous lncRNAs there are lncRNAs of exogenous origin (Flores et al., 2014) that have been well known in plants since many years. This group is comprised by viroids, infectious plant pathogens with RNA genomes and lacking protein-coding capacity (Katsarou et al., 2015). Consistent with the absence of protein-coding capacity, viroidssubvert enzymatic activities of their hosts for its own benefice. Viroids are restricted to higher plants in which they replicate and accumulate in the nucleus (nuclear viroids) or in the chloroplasts (chloroplastic viroids) (Flores et al., 2004). Different aspects of viroid biology have been elucidated and some of them are the main topic of this review together with the analysis of the state-of-the-art of the growing field of endogenous lncRNAs in plants.

2. Endogenous plant lncRNAs

In the recent years, with the advance in massive sequencing techniques, it was discovered that a huge fraction of the human genome is transcribed (Consortium et al., 2007). These transcribed regions include a high number of non-protein-coding RNAs that comprisea group of diverse transcripts that can be classified according to their location, length and biological functions (Liu et al., 2015). ncRNAs encompass those with housekeeping and regulatory functions. The first group includes ncRNAs involved in protein synthesis and mRNA maturation, like ribosomal RNAs, transfer RNAs, small nuclear RNAs and small nucleolar RNAs, whereas the second group comprises small and long ncRNAs. small ncRNAs are 20-30 nt long and consist of microRNAs (miRNAs) and small

interfering RNAs (siRNAs), which play a role in transcriptional and posttranscriptional regulation of gene expression (Gomes et al., 2013). Long non-coding RNAs (lncRNAs) are defined as RNA molecules longer than 200 nt without protein coding capacity (Rinn and Chang, 2012). The size limit is arbitrarily fixed to differentiate them from small ncRNAs and some housekeeping ncRNAs. Many of these lncRNAs accumulate to significant levels and, like mRNAs, are capped, polyadenylated and spliced (Hu et al., 2012). The function of several lncRNAs has been established during the last years in mammals. They are involved in regulation of important cellular processes including X-chromosome inactivation and imprinting, and in modulation of cellular responses to developmental conditions or environmental signals (Hu et al., 2012). Besides their role in regulating development, alterations in their correct expression are related with several diseases like cancer, Alzheimer and diabetes (for more detailed information see Rinn and Chang, 2012).

In plants, the role of lncRNAs is less known but there are several essential processes in which they take part.Work performed mainly in the model plant *Arabidopsis thaliana* allowed to establish that, in addition to their role as precursors of miRNAs, lncRNAs are also involved in the regulation of crucial processes such as flowering and responses to abiotic stresses like phosphate starvation (for a recent review see Ariel et al., 2015). In order to exert their functions, lncRNAs form ribonucleoprotein complexes with several proteins modulating chromatin modifications in response to cold and to hormones, and mediate alternative splicing hijacking RNA-binding proteins (Figure 1). lncRNAs are also involved in modulation of gene expression by sequestering miRNAs and in processes related with RNA degradation and protein translation stability. In plants,similar to mammals, several lncRNAs are capped and polyadenylated suggesting that they are transcribed by theDNA-dependent RNA polymerase II. Additionally, other plant lncRNAs are transcribed by the RNA polymerase III (Wu et al., 2012a) orby RNA polymerases II and V (Ariel et al., 2014). Considering their location relative to neighbouring protein-coding genes they are classified in 1) intergenic lncRNAs (lincRNAs), 2) intronic lncRNAs, 3) natural antisense lncRNAs(lncNATs) and 4) promoter lncRNAs (Ariel et al., 2015).

Genome wide analysis of lncRNAs mediated responses to several growth and biotic and abiotic stresses were recently performed in *A. thaliana* (Liu et al., 2012; Wang et al., 2014a).Liu and collaborators (2012)carried out an integrative analysis of tilling array data sets that allowed the identification of more than 6000lincRNAs. In order to validate the newly identified lincRNAs the authors designed and produced a custom long-oligonucleotide array including more 3700 lincRNA sequences. Results from experiments performedwith the array indicated that, 1) the expression level of the lincRNAs is 30- to 60-fold lower than mRNAs expression, 2) the lincRNAs show an organ preferential expression pattern, 3)the expression of several of them is induced in response to abiotic stresses (cold and high-salt) and to hormone treatment (abscisic acid, ABA) (Liu et al., 2012).In a

recent publication, a similar approach was used to identify lncNATs. The authors designed anarray to detect the expression of sense and antisense transcripts and to compare their responses to dark and light conditions (Wang et al., 2014a). Results from ChIP-seq (chromatin immunoprecipitation combined with massive DNA sequencing) data, led them to establish that NATs are associated with positive histone marks, suggesting that antisense transcripts could be involved in histone modification of sense transcripts in response to light (Wang et al., 2014a).

Large-scale analyses of NATs have also been performed in other species including important crops like wheat (*Triticum aestivum*) (Coram et al., 2009), sugarcane (*Saccharum officinarum*) (Lembke et al., 2012), rice (Lu et al., 2012), soybean (*Glycine max*) (Zheng et al., 2013) and turnip rape (*Brassica rapa* L.) (Yu et al., 2013). Additionally, *nat*-siRNAs (small interfering RNAs derived from NATs) have been identified in species like barley (*Hordeum vulgare* L.) (Held, et al., 2008), rice (Zhou et al., 2009), soybean (Zheng et al., 2013), cassava (*Manihot esculenta*) (Quintero et al., 2013) and turnip rape (Yu et al., 2013), suggesting that gene expression regulation mediated by NATs and *nat*-siRNAs is widely widespread in plants.

Additionally to these genome-wide analysis, several functional studies demonstrated the role of lncRNAs in the regulation of processes that are crucial for plant development and life cycle. Flowering is one of the most important processes during plant life, especially in annual species like *A. thaliana*. Plants sense the seasonal changes in temperature and day length to control the transition from the vegetative stage to the flowering period (Levy et al., 2002). In several species exposition to long periods of cold (vernalization) promotes flowering, thus ensuring that seed production occurs in spring and summer. In *A. thaliana*, the target of the vernalization process is *Flowering locus C* (*FLC*), a gene encoding a MADS-box protein that is the main flowering repressor (Sheldon et al., 2000). Vernalization decreases *FLC* transcripts and protein levels in a stable manner, and gene activity is restored again in the next generation (Sheldon et al., 2000). Two lncRNAs of *A. thaliana*, *COOLAIR* and *COLDAIR*, are involved in flowering control by regulating *FLC* expression. Both lncRNAs are expressed in response to cold and are essential for initiation and maintenance of *FLC* repression *via* histone 3 methylation by the Polycomb Repressive Complex 2 (PRC2). *COOLAIR* is expressed as a population of lncRNA RNAs antisense to *FLC* (Swiezewski et al., 2009), whereas *COLDAIR* is an intronic lncRNA transcribed from the first intron of *FLC* (Heo and Sung, 2011). *COLDAIR* interacts with the protein CURLY LEAF (CLF), which is part of the PCR2 complex, and guides the complex to the *FLC* promoter for initiating repression by trimethylation of histone 3 at lysine 27 (H3K27me3) (Heo and Sung, 2011) (Figure 1a). The consequent repression of *FLC* expression induces flowering after winter. Silencing of *COLDAIR* results in unstable repression of *FLC* and delay in flowering after vernalization (Heo and Sung, 2011), indicating the relevance of the lncRNA in this process.

Another important process in which lncRNAs play a role is the regulation of responses to phosphate starvation. Phosphorus is a key component of nucleic acids and phospholipids, is essential for plant growth and development and is present in the soil in the form of inorganic phosphate (Pi) or organophosphate. In order to find adequate sources of phosphate plants need to explore the soil by modifying and adapting their root architecture to improve phosphate acquisition (Peret et al., 2014). *A. thaliana* responds to phosphate starvation reducing the primary root growth and increasing the growth and number of lateral roots and roothairs, ,allowing the plant to explore a more extensive volume of soil. In addition to these local responses, the plantimplements systemic responses, including phosphate transport, recovery and recycling, to maintain phosphate homeostasis(Peret et al., 2011). The role of ncRNAs in the systemic responses became more evident during the last few years. In *A. thaliana* early responses to phosphate starvation in roots include the increased expression of the microRNA399 (miR399) (Fujii et al., 2005) that guides the cleavage of *PHO2* mRNA. *PHO2* encodes an E2 ubiquitin conjugase-related protein (Aung et al., 2006), that affects shoot Pi content and Pi remobilization*via* polyubiquitination of the PHOSPHATE TRANSPORTER 1.4 (PHT1.4) (Huang et al., 2013) by the E3 ligase NITROGEN LIMITATION ADAPTATION (NLA) and its subsequent degradation by the 26 S proteasome (Park et al., 2014).During phosphate deprivation, the *NLA* and *PHO2* transcripts are targeted for cleavage by miR827 and miR399, respectively, allowing PHT1.4 accumulation and a consistent increase in phosphate uptake (Park et al., 2014). In a late response, PHO2 expression is regulated by a mechanism known as target mimicry involving the lncRNA *INDUCED BY PHOSPHATE STARVATION1* (*IPS1*) (Franco-Zorrilla et al., 2007). *IPS1* is complementary to miR399 but contains a mismatch in the miRNA cleavage site that abolishes cleavage and avoids miR399 recycling (Figure 1 b). In this way miR399 is “sequestered” by *IPS1*, the *PHO2* mRNA is therefore protected from cleavage and is translated restoring the initial conditions.Another lncRNA involved in phosphate starvation response was recently identified in rice (*Oryza sativa*) (Jabnoune et al., 2013). In this specie the *OsPHO1;2* gene, which encodes for the phosphate transporter OsPHO1;2 responsible for phosphate loading into the xylem vessels, is overlapped by the lncNAT*cis-nat OsPHO1;2* (Secco et al., 2010).The expression of *PHO1;2* is not affected in response to phosphate starvation, whereas the expression of *cis-nat OsPHO1;2* is strongly and specifically increased (Secco et al., 2010).The steady state level of OsPHO1;2 follows the expression profile of *cis-nat OsPHO1;2*, indicating the existence of a positive relation between the lncNATexpression and the transporteraccumulation (Jabnoune et al., 2013).The exact mechanism of OsPHO1;2 regulation by *cis-nat OsPHO1;2* is still unknown, but unexpectedly the *cis-nat OsPHO1;2*RNA was found associated to the polysomal fraction in phosphate deprivation conditions suggesting that the increase of *cis-nat OsPHO1;2* expression enhances *OsPHO1;2* mRNA translation (Jabnoune et al., 2013) (Figure 1 c, right).

lncNATs can also act following the canonical mechanism in which the expression of antisense transcripts induces the down-regulation of the complementary target (Borsani et al., 2005; Zubko and Meyer, 2007). In a recently published work it has been demonstrated that the expression of the Heat shock factor (HSFB2α) of *A. thaliana* is controlled by a lncNAT. This lncNAT, called *asHSFB2α,* is expressed in leaves in response to heat stress, whereas both genes (*HSFB2α* and *asHSFB2α*) are expressed in normal conditions in the gametophyte (Wunderlich et al., 2014). Overexpression of any of both genes leads to down-regulation of the other one (Figure 1 c, left). The consequences of this imbalance are reduction in biomass production at early developmental stages and failures in female gametophyte development (Wunderlich et al., 2014), indicating the importance of a balanced expression of both members of the gene pair.

In recent publications, the role of lincRNAs from *A. thaliana* in regulation of photomorphogenesis (Wang et al., 2014 b), in alternative splicing modulation (Bardou et al., 2014) and in structural changes at the chromatin level in response to plant hormones (Ariel et al., 2014) was established.*HIDDEN TREASURE 1* (*HID1*), an intergenic lncRNA member of a polycistronic cluster of lncRNAs, promotes photomorphogenesis (growth in response to light) under continuous red light (Wang et al., 2014b). *HID1* forms part of large nuclear ribonucleoprotein complexes and associates with chromatin at the first intron of the *PHYTOCHROME INTERACTING FACTOR 3* (PIF3) gene repressing its transcription (Figure 1 a). PIF3 is a transcription factor essential for photomorphogenesis repression (Kim et al., 2002) whose expression is increased in *hid1* mutants producing hyposensitive phenotypes in the presence of continuous red light (Wang et al., 2014b). Interestingly,*HID1* is evolutionary conserved in land plants and the phenotype of the *hid1* mutant of *Arabidopsis* can be restored by expressing *OsHID1*, the rice ortholog of *HID1*, indicating that the function of this lncRNA may be conserved in monocots and dicots (Wang et al., 2014 b). Two lncRNAs, *EARLY NODULIN 40* (*ENOD40*) from *Medicago truncatula* and *ALTERNATIVE SPLICING COMPETITOR* (*ASCO*) from Arabidopsis are involved in modulation of alternative splicing. Expression of *ASCO*affects alternative splicing by interaction with nuclear speckle RNA-binding proteins, interfering with specific changes required for organogenesis and altering lateral roots formation in response to auxin (Bardou et al., 2014) (Figure 1 d). *APOLO*, a long intergenic non-coding RNA from *A. thaliana* is also involved in regulation of responses to auxin (Ariel et al., 2014). This lncRNA regulates the expression of *PINOD* (*PID*), a gene that encodes a regulatory kinase that determines the localization of the auxin transporter *PIN-FORMED 2* (*PIN2*) (Friml et al., 2004). *APOLO* is transcribed by the RNA polymerases II and V in response to auxin, inducing the formation of a chromatin loop that includes the promoter of *PID*. Changes in chromatin topology, modulated by *APOLO* expression and including RNA-dependent DNA methylation, active DNA demethylation and Polycomb complexes

activity, control the loop dynamics and *PID* expression in response to auxin (Ariel et al., 2014) (Figure 1 a).

3. Exogenous plant lncRNAs: Viroids

Viroids are plant subviral pathogens with small, circular, single-stranded and non-protein coding RNA genomes (Flores et al., 2004). The size of viroid genomes is comprised between 246 (*Avocado sunblotch viroid*, ASBVd) and 401 nucleotides (*Chrysanthemum chlorotic mottle viroid*, CChMVd). Although viroids can be considered lncRNAs based on their inability to code for proteins and on their genome size (Gomez and Pallas, 2013), they differ from the endogenous "canonical" lncRNAs in several aspects. In contraposition to the endogenous lncRNAs, viroids are capable of autonomous replication, have an exogenous origin and several of them are able to induce diseases in their host plants, and these differences must be considered when comparisons among both groups of lncRNAs are performed.

Viroids are restricted to higher plants including dicots and monocots, in which several of them induce symptoms and cause diseases that range from moderate to extremely severe (Kovalskaya and Hammond, 2014). Despite the lack of a protein-coding capability viroids replicate and move systemically in their host plants (Ding, 2009). For this aim viroids subvert several host proteins for their own replication and movement inside the host (Ding, 2010; Flores et al., 2009; Flores et al., 2015). Early work demonstrated that viroid genomes accumulate in infected plant tissues together with RNA replication intermediates (Grill and Semancik, 1978). The more abundant (genomic) strand was arbitrarily defined as plus (+) strand, whereas the less abundant one, present only as multimeric form in one group of viroids (see below), was defined as the (-) polarity strand (Branch and Robertson, 1984).

Viroids were discovered more than 50 years ago, when *Potato spindle tuber viroid* (PSTVd) was identified as the causal agent of a disease inducing tuber deformation in potato plants (Diener, 1972). Since then more than 30 additional viroids have been identified (Di Serio et al., 2014) and this number continues increasing as illustrated by the recent detection of new viroids in several species like persimmon (*Diospyros kaki*) (Nakaune and Nakano, 2008), sweet pepper (*Capsicum annuum* L.) (Verhoeven et al., 2009), coleus (*Coleus blumei*) (Hou et al., 2009), grapevine (*Vitis vinifera*) (Jiang et al., 2012), dahlia (*Dahlia* spp.) (Verhoeven et al., 2013) and portulaca (*Portulaca* spp.) (Verhoeven et al., 2015). Additionally, the use of next generation sequencing methods and the development of computational algorithms enabled the discovery of new viroids by assembly of circular genomes

from small RNAs sequencing data in grapevine (Wu et al., 2012b), fig (*Ficus carica* L) (Chiumenti et al., 2014), and grapevine and apple (*Malus domestica*) (Zhang et al., 2014).

3.1 Classification and characteristics of both viroid families

Viroids are classified into two families based on three major features: 1) subcellular localization and replication site, 2) presence or absence of hammerhead ribozymes and 3) structural properties (Kovalskaya and Hammond, 2014) (Figure 2). Members of the family *Pospiviroidae* (named based on the family type viroid PSTVd) replicate and accumulate in the nucleus, exhibit a conserved central region (CCR) and lack hammerheads motifs. Additionally, and due to the presence of self-complementary regions in their sequences, they adopt compact rod-shape structures. Members of the family *Avsunviroidae*, the type species of which is ASBVd, replicate and accumulate in chloroplasts, do not have a CCR but contain hammerhead ribozyme motifs in both the genomic and the antigenomic strands produced during replication, and their genomes adopt less compact folding (Flores et al., 2009). According to current taxonomy, the family *Pospiviroidae* includes 28 members that are distributed into five genera (*Pospiviroid*, *Hostuviroid*, *Cocadviroid*, *Apscaviroid* and *Coleviroid*) whereas the family *Avsunviroidae* comprises four members that are grouped into three genera (*Avsunviroid*, *Pelamoviroid* and *Elaviroid*)(Di Serio et al., 2014).

3.2 Structure

Members of thefamily *Pospiviroidae* fold into compact rod-shaped structures due to the high degree of sequence complementarity of their genomes (Flores et al., 2004).Nuclear viroids have a central conserved region (CCR) and a terminal conserved region (TCR) or a terminal conserved hairpin (TCH). Sequence differences in the CCR and the presence or absence of TCR and TCH are the features considered for the assignment of nuclear viroids to different genera. The rod-like structures adopted by members of the family *Pospiviroidae* contain five structural and functional domains, the central (C), pathogenic (P), variable (V) and terminal left (T_L) and right (T_R), respectively (Keese and Symons, 1985).The CCR is located in the C domain while the TCR and TCH are contained within the T_L domain (Flores et al., 2004).

Rod-like structures of members of the family *Pospiviroidae* were first deduced from the high degree of complementarity of their sequences and later with the help of software programs,like Mfold (Zuker, 2003), that allowed the prediction of secondary structures based on thermodynamic approaches. The application of other techniques, including electronic microscopy, for the analysis of highly purified viroids confirmed the rod-like nature of native viroids (Sanger et al., 1976). Resolution of the structure of several nuclear viroids including three variants of *Citrus exocortis viroid* (CEVd) was recently performed by “selective 2′-hydroxyl acylation analyzed by primer extension” (SHAPE)

(Xu et al., 2012). This technique allows the characterization of the RNA structure at single-nucleotide resolution; it is based on the specific reaction of an hydroxyl-selective electrophile (N-methylisatoic anhydride, NMIA) with the 2´-hydroxil groups of conformationally flexible or disordered nucleotides that are subsequently identified by primer extension (Merino et al., 2005). Advantages of this technique are that NMIA shows good reactivity towards all four RNA nucleotides (Merino et al., 2005) and that it can be adapted to a high-throughput format in which the process can be accelerated by the use of fluorescent nucleotides and capillary electrophoreses (Wilkinson et al., 2008) and by software systems that allow a rapid analysis of the raw data (Vasa et al., 2008).Thus, in concordance with previous data, results of structure analysis of transcripts corresponding to several nuclear viroids by high throughput SHAPE (hSHAPE) confirmed that viroids from the family *Pospiviroidae* fold into rod-like structures (Xu et al., 2012). Moreover, these approaches enabled the identification of structure motifs that are characteristic and specific for each genus (Giguere et al., 2014b).

The only chloroplastic viroid with a structure resembling the rod-like folding of the nuclear viroids is ASBVd (Symons, 1981). Nevertheless, the ASBVd structure additionally contains a bifurcated left-terminal domain (Gast et al., 1996; Navarro and Flores, 2000; Symons, 1981). The predicted *in silico* structure of *Eggplant latent viroid* (ELVd), which was deduced from minimum free energy calculations, showed a quasi-rod like-like conformation with the presence of two bifurcations at the terminal domains (Fadda et al., 2003). This structure is consistent with the majority of the nucleotide exchanges that are detected in natural sequence variants, which, mainly consist of compensatory mutations or co-variations (Fadda et al., 2003). Using the same RNA folding methods, multibranched structures were predicted for the remaining chloroplastic viroids, *Peach latent mosaic viroid* (PLMVd) and CChMVd (Hernandez and Flores, 1992; Navarro and Flores, 1997). The structure predictions of these two viroids were additionally supported by sequence data of several natural variants that were isolated from infected plants (Flores et al., 2012). The sequence analysis of these two viroids revealed that variability mainly accumulated in RNA loops. If present in the stems, variability is found as co-variations or compensatory mutations that preserve the structure of the molecules (Flores et al., 2004; Flores et al., 2012). Nuclease analysis and oligonucleotide binding shift assays confirmed the predicted structure of PLMVd *in vitro* and additionally indicated the presence of an interaction between two hairpin loops (Bussiere et al., 2000). This tertiary interaction allows the formation of a pseudoknot that may stabilize the viroid branched structure *in vivo*. The presence of a similar loop-loop interaction in CChMVd, which together with PLMVd is a member of the genus *Pelamoviroid*, was identified by *in silico* approaches in concert with sequence analysis of natural CChMVd variants. In the latter case, the mutations that were detected support the idea that the proposed interaction indeed exists in *in vivo* (Gago et al., 2005). The putative kissing-loop interaction was further assessed

in vivo by combining site-directed mutagenesis, bioassays and viroid progeny analysis. These data revealed that disruption of this interaction abolishes, or drastically reduces, the viroid viability (Gago et al., 2005). Additional *in vitro* analysis demonstrated that the interaction is critical for the *in vitro* folding and, like in the case of PLMVd (Dube et al., 2010), occurs only in the viroid (+) strand (Gago et al., 2005). The determination of the structures of both polarity strands for all members of the family *Avsunviroidae*was recently performed using high-throughput SHAPE (Giguere et al., 2014a). The results of these analyses indicated that, for each viroid, the (+) and (-) strands adopt a different conformation and allowed the prediction of additional tertiary interactions not identified before (Giguere et al., 2014a).

3.3 Replication

Viroids of both families replicate via a rolling-circle mechanism with only RNA intermediates (Figure 3). The more abundant genomic strand (+) strand serves as template for the generation of the complementary (-) strand, which in turn, acts as a template for the synthesis of additional (+) strands (Flores et al., 2004). The rolling circle mechanism of replication involves three steps: 1) the generation of longer-than-units molecules with the genomic circular single-stranded (+) RNA used as template, 2) the processing of the multimeric sequences to unit length molecules and 3) the circularization of the linear monomers to generate the mature viroid molecules (Flores et al., 2004; Grill and Semancik, 1978). In the case of the nuclear viroids all steps are performed by host enzymes whereas in the case of the chloroplastic viroids the processing of the oligomers generated during the first stage of replication is a self-catalysed process, which is carried out by hammerhead ribozyme motifs contained in the (+) and (-) strands.Nuclear viroids follow an asymmetric variant of the rolling-circle mechanism in which the (-) oligomers are not processed but instead are used as templates for the synthesis of (+) oligomeric molecules that are processed to unit-length strands and circularized. The chloroplastic viroids follow a symmetric variant of the mechanism in which both (+) and (-) oligomers are processed to generate (+) and (-) unit-length monomeric forms that are circularized and used as templates for the next round of replication. As a consequence of these differences in plants infected with nuclear viroids, the detected circular form corresponds only to (+) polarity strands (Rohde and Sanger, 1981), while, in contrast, for the chloroplastic viroids both, (+) and (-) circular molecules are observable *in vivo*(Fadda et al., 2003; Navarro and Flores, 1997).

3.3.1 Synthesis of oligomeric viroidal molecules

As already mentioned, the templates of the initial step of replication are the most abundant monomeric circular forms that are assigned by convention the plus (+) polarity. These circular molecules are transcribed by host DNA-dependent RNA polymerases generating oligomers of complementary (-) polarity. In the case of the members from the family *Pospiviroidae*this step is

catalysed by the DNA-dependent RNA polymerase II, which normally catalyses the synthesis of messenger RNAs (Kovalskaya and Hammond, 2014). Experimental evidence that RNA pol II is involved in the replication of nuclear viroids was obtained by the observation that low concentration (i.e., in the nanomolar range) of α-amanitin, a fungal toxin that inhibits the enzyme, block the transcription of (+) and also (-) viroid strands *in vitro* as well as *in vivo* (Muhlbach and Sanger, 1979). Moreover, viroid strands of both polarities have been recovered by immunoprecipitation with an antibody against the largest subunit of the RNA polymerase II (Warrilow and Symons, 1999).

Chloroplastic viroids are replicated in a similar form, but in this case by the nuclear encoded polymerase (NEP) that is present in chloroplasts (Flores et al., 2009). Two DNA-dependent RNA polymerases exists in chloroplasts, NEP that resembles phage RNA polymerases and consist in only one unit, and the multisubunit plastid-encoded RNA polymerase (PEP), which derives from the cyanobacterial enzyme (Williams-Carrier et al., 2014). If the activity of PEP is blocked with tagetitoxin, the *in vitro* transcription of chloroplastic genes is affected. However, the synthesis of ASBVd strands in chloroplastic preparations of avocado (*Persea americana*) leaves infected with the viroid is not affected (Navarro et al., 2000),indicating that this enzyme is not involved in replication of chloroplastic viroids. Additionally, the accumulation of typical NEP transcripts and viroid strands is particularly high in leaf areas showing albinism caused by viroid infection with specific PLMVd variants in peach (*Prunus persica*) (Rodio et al., 2006) suggesting that NEP is involved in viroid replication. This hypothesis is supported by the observation that in the same areas processing and accumulation of chloroplastic rRNA precursors is abolished and the translation of plastid-encoded proteins (e.g. PEP) is impaired (Rodio et al., 2007).

3.3.2 Cleavage of the oligomeric molecules to unit-length monomers

In the second step of replication the oligomers generated by the respective RNA polymerases are cleaved to unit length molecules. As outlined, members of the family *Pospiviroidae* follow an asymmetric variant of the rolling-circle mechanism in which the (-) oligomeric molecules generated by the RNA pol II are not cleaved but instead serve as templates for the synthesis of (+) oligomers. The enigma of the cleavage site on the (+) oligomers as well as of the enzyme involved in this process was solved recently (Gas et al., 2007; Gas et al., 2008) by the use of transgenic *A. thaliana* (a non-host plant) lines expressing dimeric constructs of (+) polarity of viroids of three different genera of the family *Pospiviroidae* (Daros and Flores, 2004). Thus, mapping of the processing site of the dimeric constructs expressed in *A. thaliana* combined with data obtained from infectivity analysis of CEVd mutants indicated that the substrate for *in vivo* cleavage is a conserved double-stranded structure formed in the oligomeric sequence (for more details see Gas et al., 2008). The termini of the monomeric lineal (+) strands generated after cleavage turned out to be 5´ phosphomonoester (5´-P)

and 3´ hydroxyl (3´-OH) (Gas et al., 2008), which strongly indicate that the enzyme involved in the processing of (+) oligomeric sequences is a member of the type III RNase family (Gas et al., 2008). These enzymes act on double-stranded or highly structured RNAs and generate 5´-P and 3´-OH termini (MacRae et al., 2008).

In the case of chloroplastic viroids both, the (+) and the (-) strands contain hammerhead domains (Daros et al., 1994; Fadda et al., 2003; Hernandez et al., 1992; Hutchins et al., 1986; Navarro and Flores, 1997). Hammerheads are small RNAs motifs that are able to catalyse the cleavage of the RNAs in which they are included and were first described in viroids and viral satellite RNAs (Hutchins et al., 1986; Prody et al., 1986). The self-cleavage activity of the hammerheads depends on the correct folding of the RNA molecules in which they are contained, which is achieved during transcription (Carbonell et al., 2006). Additionally, tertiary interactions between structural elements that are present in the full-length hammerhead sequence are known to stabilize the active conformation and to support high cleavage rates at physiological concentration of Mg^{2+} (De la Peña et al., 2003; Khvorova et al., 2003). In all the chloroplastic viroids, the oligomeric (-) RNAs synthetized during the first part of the rolling-circle are cleaved by the hammerhead domain present in the (-) strand to produce linear full-length (-) monomers. Subsequently, these are ligated to generate circular (-) full-length molecules, which in turn, serve as templates for the synthesis of oligomeric (+) strands. During this second replication step, the oligomeric (+) strands are also cleaved by the (+) strand hammerhead domain. The unit-length (+) monomers are ligated to generate (+) genomic forms, which may start another replication cycle (Flores et al., 2009). The presence of circular monomeric molecules of both polarities [(+) and (-)] is a hallmark of the members of the family *Avsunviroidae* and indicates that a symmetric variant of the rolling-circle mechanism,which requires both types of templates, is followed. Overall, it is important to note that cleavage of the oligomeric forms into monomeric unit-length molecules, which are later processed to generate the circular forms of both polarities, is autocatalytic and mediated by the hammerheads present in the viroid strands of (+) and (-) polarity, respectively. It is also important to highlight that this is the only replication step that is not catalysed by host enzymes but by the viroid RNA itself (Flores et al., 2001).

3.3.3 Circularization of the unit-length monomers to generate mature viroids

The third step of replication consists in the circularization of the monomeric unit-length molecules *via* ligation of the ends generated by oligomeric cleavage. Identification of the enzymes involved in this process was recently achieved (Nohales et al., 2012a; Nohales et al., 2012b). Earlier *in vitro* assays using as substrate monomeric (+) linear forms of PSTVd purified from infected plants and a wheat germ extract containing tRNA ligase showed the ability of the latter to circularize the monomeric molecules (Branch et al., 1982). This result suggested a tRNA ligase being responsible for

the circularization of viroid monomers *in vivo*. RNA ligases of this type require 5´-hydroxyl and 2´,3´-cyclic phosphodiester ends (Konarska et al., 1981). However, based on recent results, these ends are not present in the monomeric (+) linear forms of several members of the family *Pospiviroidae* [CEVd (Semancik and Weathers, 1972), *Hop stunt viroid*, HSVd (Ohno et al., 1983), and *Apple scar skin viroid*, ASSVd (Hashimoto and Koganezawa, 1987)] derived from the expression of dimeric constructs of these viroids in *A. thaliana* (Gas et al., 2008). The processed monomeric forms, which were produced by site-specific cleavage of a dsRNA structure formed during the transcription of the dimeric constructs *in vivo*, showed 5'-P and 3'-OH termini, which are characteristic of cleavage mediated by an RNase III-like enzyme (Gas et al., 2008). These results strongly indicate the existence of a second plant RNA ligase involved in the ligation of these termini (Flores et al., 2009). A recent study that applied a combination of *in vitro* and *in vivo* approaches demonstrated that the RNA ligase activity responsible for circularization of nuclear viroids resides in the plant DNA ligase I, normally catalyzing formation of a phosphodiester bond between 5´-P and 3´-OH termini in DNA molecules (Wu et al., 2001), but redirected to ligate RNA molecules (Nohales et al., 2012a). This enzyme requires ATP for adenylation, is essential for *A. thaliana* (Taylor et al., 1998) and is involved in repair of single- and double-strand breaks (Waterworth et al., 2009). Nohales and collaborators demonstrated that the DNA ligase I of tomato (*Solanum lycopersicum* L.) catalyzes the circularization of monomeric linear forms of PSTVd with 5´-P and 3´-OH groups *in vitro*, and that silencing of the homologue enzyme in *Nicotiana benthamiana* decreases the accumulation of PSTVd and reduces the ratio of monomeric circular to total monomeric forms of the viroid RNA (Nohales et al., 2012a). Considering that the cleavage mechanism seems to be conserved among members of the family *Pospiviroidae* (Gas et al., 2007), it is reasonable to assume that the ligation step for all family members follows a common mechanism.

As explained above, during the replication of members of the family *Avsunviroidae*, the monomeric forms of both polarities generated by the catalytic self-cleavage activity of hammerhead ribozymes contains 5´-OH and 2´,3´-cyclic phosphodiester termini (Hutchins et al., 1986). The self-ligation capacity of hairpin ribozymes in certain viroid-like satellite RNAs (Buzayan et al., 1986) fueled the idea that ligation in chloroplastic viroids was mediated by the their own hammerhead domains. The main concern burdening this hypothesis is the low efficiency of self-ligation activity of hammerheads compared with their self-cleavage. Interestingly, the RNA termini generated by hammerhead mediated cleavage are compatible with the ligase specificity of the wheat germ tRNA ligase (Makino et al., 2005) but this enzyme is mainly localized in the nucleus where it is involved in tRNA maturation (Englert and Beier, 2005). However, in *A. thaliana* and rice the tRNA ligase, as well as the 2´-phosphotransferase (that removes the 2´-phosphomonoester group generated by the tRNA ligase), display signal peptides at their N-termini directing their transit to chloroplasts and

proplastids, respectively (Englert et al., 2007).The chloroplastic localization of both enzymes, together with the specificity of the tRNA ligase for termini similar to the ones generated by the hammerhead ribozymes, thus gave a first hint about the potential role of these enzymes in the ligation of chloroplastic viroids (Flores et al., 2009). Along the same line, studies of cleavage and ligation of ELVd mutants in the green alga *Chlamydomonas reinhardtii* pointed to a tRNA ligase present in the chloroplasts as responsible for ligation of the members of the family *Avsunviroidae* (Martinez et al., 2009). In further support of these data, recent work demonstrated that a recombinant version of the chloroplastic isoform of the tRNA ligase of eggplant (*Solanum melongena* L.), the host plant of ELVd, catalyzes the circularization of (+) and (-) monomeric forms of all the members of the family *Avsunviroidae* (Nohales et al., 2012b). The observed activity was highly specific, as indicated by the absence of ligation of monomeric forms of ELVd opened at positions differing from the hammerhead cleavage site on the viroid molecule (Nohales et al., 2012b). Additionally, silencing of the endogenous tRNA ligase of *N. benthamiana* produces a significant decrease of circular forms of ELVd derived from transient expression of a dimeric construct in the same plant (Nohales et al., 2012b). Altogether, these results indicate that the chloroplastic isoform of the host tRNA ligase is responsible for the circularization of the monomeric (+) and (-) molecules of members of the family *Avsunviroidae*.

In summary, viroids are parasites that recruit crucial components of the replication and transcription machinery of the host cell to achieve their own replication. Nuclear viroids redirect the activities of the DNA-dependent RNA polymerase II and DNA ligase I to function on viroid RNA templates and substrates, respectively. Chloroplastic viroids, in turn, utilize enzymes present in the chloroplast: the NEP polymerase for rolling-circle replication of the circular (+) and (-) RNA strands, and the tRNA ligase to circularize the resulting unit-length monomers of both polarities.

3.4 Viroid-host interactions

3.4.1 Viroid interactions with plant proteins

Viroids of both families are known to interact with host proteins and, through this way, they may induce symptoms. Early studies demonstrated that circular or linear PSTVd molecules interact with histones, unknown nuclear proteins and wheat germ RNA polymerase II (Goodman et al., 1984; Wolff et al., 1985). Another protein that was found to interact with PSTVd is the tomato viroid RNA-binding protein 1 (VirP1), a member of a family of transcriptional regulators associated with chromatin remodelling, which was identified in a RNA-ligand screen (Martinez de Alba et al., 2003). VirP1 contains a bromodomain and binds specifically to a conserved motif present in the right terminal domain of PSTVd (Maniataki et al., 2003; Martinez de Alba et al., 2003). The interaction between the viroid RNA and VirP1 mediates viroid systemic spreading in the host plant and is abolished in the

absence of the conserved motif (Maniataki et al., 2003). Transgenic *N. tabacum* and *N. benthamiana* plants in which the expression of the orthologous Virp1 gene was supressed *via* RNA silencing were infected neither by PSTVd nor by HSVd, indicating a major role of Virp1 in viroid infection (Kalantidis et al., 2007). Experiments with protoplasts isolated from the suppressed plants revealed that viroid replication is abolished (Kalantidis et al., 2007) and hence suggested that the failure of viroid infection in the transgenic plants may be a consequence of the absence of replication.

Viroids can also form ribonucleoprotein complexes with host proteins as it was demonstrated for HSVd and the cucumber phloem protein 2 (CsPP2), a phloem located lectin (Gomez and Pallas, 2001). Grafting experiments demonstrated that the complex is translocated from the rootstock into the scion and that viroid molecules contained in the complex induce symptoms in the scion. These results indicate that CsPP2 is involved in the systemic movement of the viroid molecules in the infected plants (Gomez and Pallas, 2004). CsPP2 is one of the more abundant proteins in phloem exudates from cucumber (*Cucumis sativus*), contains an RNA binding motif and interacts *in vitro* (Gomez and Pallas, 2001; Owens et al., 2001) and *in vivo*(Gomez and Pallas, 2004) with HSVd, but also with other RNA molecules. Due to this last characteristic, the role of CsPP2 in pathogenesis is yet uncertain (Owens and Hammond, 2009).

Interactions with host proteins were also identified for chloroplastic viroids. ASBVd interacts with two nuclear-encoded chloroplastic proteins, PARBP33 and PARBP35 (Daros and Flores, 2002). Both are RNA-binding proteins of a family whose members are involved in stabilization, maturation and editing of chloroplastic transcripts. Both proteins were identified in aducts stabilized by UV-irradiation of leaves from infected avocado plants. PARBP33 act as RNA chaperone and stimulates the catalytic activity of ASBVd hammerheads *in vitro*, increasing the self-cleavage of multimeric ASBVd transcripts (Daros and Flores, 2002). Considering the previously described catalytic activity of the hammerhead structures, this finding supports the view that PARBP33 and PARBP35 may be involved in viroid replication rather than in disease induction (Owens and Hammond, 2009). Mass-spectrometry analysis of viroid RNA-cellular protein complexes that were originally identified by Northwestern from partially purified peach leaf extracts allowed the identification of other six putative RNA-binding proteins including the elongation factor 1-alpha (eEF1A) (Dube et al., 2009). The interaction between PLMVd and purified eEF1A was confirmed *in vitro* by several techniques, including electrophoresis mobility shift assays, and immunoprecipitation of eEF1A from crude extracts of infected peach leaves co-precipitated the PLMVd RNA. While these data indicate the existence of an interaction between PLMVd and eEF1A *in vivo*, the role of eEF1A in viroid replication and/or viroid-mediated disease is not clear (Dube et al., 2009).

3.4.2 Epigenetic changes induced by viroid infections

When tobacco plants were transformed with the cDNA of PSTVd, the whole cDNA sequences were fully methylated, but only, if transformation was followed by an infection with the replicative viroid (Wassenegger et al., 1994). In contrast, in plants in which PSTVd replication was undetectable the genome-integrated PSTVd cDNAs remained unmethylated (Wassenegger et al., 1994). These results indicated that viroid replication is involved in RNA-directed methylation of the viroid sequences integrated in the host genome (Wassenegger et al., 1994). Along the same line, infection of tobacco plants with PSTVd was shown to suppress the simultaneous expression of a transgene, which consisted of the green fluorescent protein (GFP) reporter fused to a non-infectious sequence of PSTVd (Vogt et al., 2004). Interestingly, this suppression correlated with a *de novo* methylation that affected only the PSTVd-specific part of the transgene (Vogt et al., 2004). Although,these data indicate that viroid replication is involved in RNA-directed induction of methylation of viroid cDNA sequences integrated in the host genome the biological relevance of these interesting observations is still unclear (Dalakouras et al., 2013; Vogt et al., 2004).

More recently it was reported that viroid infection may also induce changes in the DNA methylation pattern of host rRNA encoding-genes (Martinez et al., 2014). Thus, cucumber plants infected with HSVd showed increased accumulation of rRNA precursors and high levels of small RNAs derived from rRNAs. Methylation analysis of a region within the genomic 45S rDNA indicated that during HSVd infection the number of methylated cytosine residues decreased and that this correlated with the transcriptional activation of normally silenced rDNAs (Martinez et al., 2014). In sum, these findings provide important insights on altered host gene expression in response to viroid infection. However, it is still uncertain if these effects are directly or indirectly associated with viroid replication (Minoia et al., 2014).

3.4.3 Plant responses to viroid infection

During infections of plant cells with subcellular pathogens that contain or consist of an RNA genome the plant cell is exposed to double-stranded (ds) RNA molecules. dsRNA is either present in highly structured regions of the pathogen genomes (Molnar et al., 2005)or is generated in the course of the genome replication (Mlotshwa et al., 2008).dsRNA acts as a pathogen-associated molecular pattern (PAMP) that is detected by the cellular RNA silencing machinery, which is an inducible defence mechanism activated in response to the presence of foreign nucleic acids. As part of this machinery, Dicer-like proteins (DCL) initially process the dsRNA into small duplexes of 21, 22 or 24 base pairs containing 2 nt long overhangs at their 3´ ends called small interfering RNAs (siRNAs) (Axtell, 2013). During infections with RNA viruses, virus-derived siRNAs (vsiRNAs) accumulate in the infected tissues and get incorporated in Argonaute (AGO) endonucleases, which are part of endogenous RNA-induced silencing complexes (RISC). Following removal of one of the vsiRNAs

strands, the remaining strand directs RISC to the complementary cognate viral RNAs, which then are cleaved or inactivated by AGO (Mlotshwa et al., 2008). Viruses have evolved different strategies in order to escape from this mechanism of plant defence. Proteins with silencing suppressor function (viral suppressors of RNA silencing, VSRs) are encoded in almost all plant viral genomes. These VSRs, without obvious sequence similarities and with different origins, block the RNA interference pathway at different steps by interacting with key components of the silencing machinery (Burgyan and Havelda, 2011). In close analogy with infections caused by viruses,viroid-derived small interfering RNAs (vd-siRNAs) are detectable in several plants infected with nuclear or chloroplastic viroids (Bolduc et al., 2010; Di Serio et al., 2009; Di Serio et al., 2010; Itaya et al., 2001; Landry and Perreault, 2005; Machida et al., 2007; Martin et al., 2007; Martinez de Alba et al., 2002; Martinez et al., 2010; Navarro et al., 2012; Navarro et al., 2009; Papaefthimiou et al., 2001) indicating that viroid molecules are also targets of the plant-silencing machinery. The absence of protein coding capacity in viroids excludes the possibility of a protein-mediated silencing suppressor activity. However, it was proposed that viroids may escape RISC due to the compact structures of their genomes (single-stranded, closed and with a high degree of secondary structure) (Gomez and Pallas, 2007; Itaya et al., 2007; Wang et al., 2004). This hypothesis is supported by the observation of vd-derived siRNAs in the cytoplasm but not in the nucleus (where the viroid replicates and accumulates) of PSTVd-infected plants(Denti et al., 2004). On the other hand, it was found that the co-inoculation of viroid infectious sequences together with dsRNAs molecules of the same sequence induces resistance and/or a delay in symptoms development (Carbonell et al., 2008) indicating that a “pre-activation” of the RNA interference by dsRNA molecules is enough to control the infection. Similar results were obtained when the viroid RNA was co-inoculated together with a population of small RNAs derived from viroid sequences processed *in vitro* (Carbonell et al., 2008). In the same line the transient co-expression in *N. benthamiana* of a hairpin RNA (hpRNA) containing part of the PSTVd sequence reduces the accumulation of PSTVd in the agroinfiltrated and also in the upper non-infiltrated leaves (Carbonell et al., 2008). The inverted sequences that form the hairpin construct are complementary and, accordingly, produce dsRNAs*in vivo* that are efficiently processed to generate siRNAs that are loaded into RISC, which in turn, targets the complementary RNAs for cleavage and degradation. Stable expression of virus specific hairpin constructs hence is a frequently used tool for the generation of plants that are resistant against infections with the corresponding virus (Mitter and Dietzgen, 2012). Transgenic tomato plants that express inverted repeats of PSTVd were found to accumulate high levels of hairpin-derived siRNAs and were resistant to PSTVd infections (Schwind et al., 2009). Importantly, the resistance of these transgenic lines correlated with the levels of siRNAs derived from the hairpin construct (Schwind et al., 2009). This result is in close agreement with the previous ones (Carbonell et al., 2008) showing that the effect of dsRNAs and small RNAs is sequence-specific and

dose dependent. Taken together these studies indicate that the mature viroid molecules is targeted by the plant silencing machinery and that RNA interference can be used to produce viroid-resistant plants.

This last hypothesis is further strengthened by recent results from Minoia and collaborators (Minoia et al., 2014), who transiently expressed tagged AGO proteins from *A. thaliana* in *N. benthamiana* plants that were infected with PSTVd. Subsequent immunoprecipitation of the AtAGO proteins and deep sequencing analysis of the bound siRNAs showed that, as with viral vsiRNAs, binding essentially depends on the size and the identity of the 5´ terminal nucleotide of the vd-siRNAs (Minoia et al., 2014). Thus, AGO1, AGO2 and AGO3 preferentially bind vd-siRNAs of 21 and 22 nt whereas AGO4, AGO5 and AGO9 are loaded with vd-siRNAs of 24 nt. vd-siRNAs with uracil (U) or cytosine (C) at their 5´ end were mainly bound by AGO1 and AGO5, while vd-siRNAs with 5´ adenine (A) were bound by AGO2 and AGO4 (Minoia et al., 2014). Interestingly, transient expression of AGO1, AGO2, AGO4 and AGO5 also causes a reduction in the levels of genomic PSTVd RNAs in infected tissue indicating that vd-siRNAs are not only loaded into RISC but also capable of guiding RISC to the cognate targets (Minoia et al., 2014). The current status accordingly is that in close analogy to the silencing mechanisms of viral RNAs, DCLs presumably process the viroid RNAs and produce different types of vd-siRNAs. The latter are loaded into the different AGO proteins and some of them may mediate the slicing of the mature viroid genomes in the cytosol during their transit to plasmodesmata to move to neighbouring cells or into the vascular system to be transported to distant organs.

3.4.4 Molecular mechanisms of symptoms induction

During infection of host plants several viroids produce different types of symptoms. Their severity is a consequence of the interactions between viroid and host genomes and they are similar to those originated from viral infections, including stunting, dwarfing, leaf epinasty, chlorotic or necrotic spots, vein clearing, cankers, malformations of flowers, fruits and tubers and, in the case of extremely virulent strains, plant death (Kovalskaya and Hammond, 2014). Additionally, some viroids like ELVd (Fadda et al., 2003)or a nuclear viroid recently discovered in portulaca plants (Verhoeven et al., 2015), are asymptomatic. On the other hand, infections of different strains of the same viroid may be symptomless or induce mild to severe symptoms in the same host plant. This is, for example, the case with natural sequence variants of two of the chloroplastic viroids, CChMVd (De la Peña et al., 1999) and PLMVd (Flores et al., 2006).

Several determinants of pathogenicity have been identified in nuclear and chloroplastic viroids. Detailed analysis of diverse PSTVd strains showed that minor sequence changes cause dramatic

effects on symptom expression (Dickson et al., 1978) leading to the identification of "pathogenicity domains" in this and other related viroids (Keese and Symons, 1985). Thus, besides its functional relevance, the central conserved region (CCR) of PSTVd plays also an important role in pathogenesis. Qi and Ding demonstrated that the transversion of the U at position 257 to A converted the intermediate PSTVd strain into a lethal strain that induces severe growth reduction and death of the infected plants (Qi and Ding, 2003). Surprisingly, changes of the same nucleotide (U257) to C or G did not induce these severe symptoms (Qi and Ding, 2003). Along the same line, cells from tomato plants infected with the U257A mutant showed a strong reduction in size and significantly decreased transcripts levels of *LeExp2*(Qi and Ding, 2003),an expansin involved in cell expansion that is expressed in fast growing sectors of hypocotyls, stems and leaves (Reinhardt et al., 1998). Considering that viroid titer is not affected in the U257A variant, the alterations in plant growth and development does not appear to be linked to an increase in replication (Qi and Ding, 2003). Similar analyses of sequence variants present in plants infected with other nuclear viroids, like HSVd and *Coconut cadang-cadang viroid* (CCCVd), or with chloroplastic viroids, like ASBVd, CChMVd and PLMVd identified additional pathogenicity determinants. HSVd variants found in trees showing cachexia symptoms contain six characteristic changes in the sequence of the variable (V) domain (Reanwarakorn and Semancik, 1999). In subsequent works, the individual role of these nucleotides in pathogenesis were analyzed by site-directed mutagenesis studies supporting that the expression of cachexia symptoms is modulated by a single nucleotide change (Serra et al., 2008).CCCVd infects coconut palm plants and is the agent of an extremely severe disease that caused the death of about 30 million of palms plants in the Philippines (Haseloff and Symons, 1982; Randles et al., 1988). Severe symptoms are associated with one or two changes at three sites in the P and C domains of this viroid (Rodriguez and Randles, 1993). In ASBVd, the accumulation of mutations in the right terminal loop of the rood-shaped conformation leads to a more relaxed structure and to a reduction in symptoms severity, namely from severe bleaching to symptomless sectors in the infected leaves (Semancik and Szychowski, 1994). Comparisons of viroid sequences of CChMVd that were isolated from asymptomatic and symptomatic chrysanthemum plants showed that changes in a tetraloop are responsible for these differences in symptom expression. The substitution by site-directed mutagenesis of the UUUC loop-sequence by a GAAA sequence present in the non-symptomatic variants was enough to change the symptomatic phenotype to a non-symptomatic one without affecting the accumulation of the viroid in the infected plants (De la Peña et al., 1999). Vice versa, replacement of the non-symptomatic tretraloop sequence GAAA by the UUUC sequence in the non-symptomatic variant CMNS25-1 was sufficient to induce chlorosis in chrysanthemum plants, confirming the role of the tetraloop sequence in symptoms development (Misceo, personal communication). In PLMVd, some infected peach trees showed an extreme chlorosis known as

"peach calico" that is absent in trees infected with asymptomatic isolates. Sequencing of PLMVd variants from symptomatic leaves indicated the existence of two groups, one of which contains an insertion of 12 to 13 nucleotides that generates a hairpin structure capped by a U-rich loop. By mechanical inoculation of peach trees, it was shown that only plants infected with the sequence containing the insertion developed symptoms and that the insertion was conserved in the viroid progeny (Malfitano et al., 2003). In turn, deletion of the insertion from the calico sequence by site-directed mutagenesis abolished the induction of the albino-variegated symptoms in infected trees without affecting viroid replication, indicating that the insertion is responsible for the symptomatology (Malfitano et al., 2003).

In many cases the way in which the pathogenicity determinants of viroids may induce symptoms development is still unknown. Interestingly, some of the likely players are supposed to be vd-siRNAs and the plant RNA silencing response. Since the identification of vd-siRNAs in infected plants, it was postulated that the pathogen-derived siRNAs could act like the endogenous miRNAs and siRNAs by targeting host mRNAs for cleavage or translation inhibition. In support of this hypothesis, a correlation between symptom severity and vd-siRNA accumulation was reported with different viroids (Gomez et al., 2008; Markarian et al., 2004; Matousek et al., 2007; Wang et al., 2004), and several pre-miRNAs and mRNAs potentially targeted by vd-siRNAs were identified (Diermann et al., 2010; Wang et al., 2011). For example, the analysis of vd-siRNAs from the virulent PSTVd strain AS1, which causes severe symptoms including dwarfing and necrosis in infected tomato plants, revealed that these map, at high frequency, to the domain that modulates pathogenicity in PSTVd (Diermann et al., 2010). Furthermore, in plants infected with PSTVd, the accumulation of certain endogenous miRNAs involved in leaf development was suppressed, suggesting that viroid-derived symptoms may be caused by this suppression (Diermann et al., 2010). In another work, changes in the expression levels of more than one hundred tomato genes containing potential binding sites for vd-siRNAs from PSTVd were assessed by microarray analysis. The mRNAs of 18 of these genes, including two genes involved in gibberellin or jasmonic acid biosynthesis, contain potential binding sites for PSTVd-derived siRNAs in their open reading frames, and were identified to be down-regulated early during infection (Wang et al., 2011). Hop (*Humulus lupulus*) plants infected with HSVd show changes in petiole coloration associated with changes in phenylpropanoid metabolites. The expression of a gene encoding the naringenin-chalcone synthase H1, a key enzyme for the phenylpropanoid synthesis, was highly reduced in infected plants leading to propose that this decrease in expression is due to the action of vd-siRNAs (Fussy et al., 2013).

Significant evidence on the role of vd-siRNAs that can target plant transcripts was recently provided (Navarro et al., 2012). These authors identified an endogenous target of two vd-siRNAs,

derived from the insertion present in the symptomatic PLMVd variants causing peach calico. Both vd-siRNAs target the mRNA of the chloroplastic heat-shock protein 90 (cHSP90) for cleavage indicating that the viroid can modulate the expression of this host gene by RNA silencing. A vd-siRNA mediated down-regulation of cHSP90, a protein involved in chloroplast biogenesis and signal transduction between the nucleus and chloroplasts, is consistent with previously identified malformations of the chloroplasts in calico tissues (Navarro et al., 2012; Rodio et al., 2007). There are also indications that vd-siRNAs derived from various PLMVd regions may target host mRNAs, suggesting that they could induce symptoms by affecting several peach genes (Di Serio et al., 2012; Navarro et al., 2012). In concordance with this, PSTVd-derived small RNAs, when expressed as artificial microRNAs (amiRNAs) in *N. tabacum* and *N. benthamiana*, induce abnormal phenotypes (Eamens et al., 2014). In particular, the expression of one amiRNA corresponding to sequences within the PSTVd virulence modulating region induces phenotypes resembling the symptoms observed in PSTVd infected plants (Eamens et al., 2014). In the same experiments the phenotype severity correlated with accumulation of the amiRNA and with down-regulation of the expression of the *in silico* predicted target, a soluble inorganic pyrophosphatase (siPPase) from *Nicotiana*. These data strongly suggest that symptoms development in viroid infected plants results from the silencing of the host gene by vd-siRNAs (Eamens et al., 2014). *In silico* predictions of complementarity between putative vd-siRNAs and coding regions in the plant genome has enabled the identification of potential host genes involved in symptom development in viroid infected plants (Avina-Padilla et al., 2015).Such an analysis resulted, for example, in the identification of a family of vd-siRNA that are conserved in seven viroid species infecting tomato. The predicted target for this vd-siRNA is a gene encoding for a conserved tomato WD40-repeat protein (SolWD40-repeat). WD40-repeat proteins are conserved in several species and are involved in different processes including cell cycle control, histone modifications, tolerance to abiotic stress, plant growth and pollen viability. Validation of the putative target in tomato plants infected with *Tomato planta macho viroid* (TPMVd) revealed that the expression of the *SolWD40-repeat* encoding gene was down-regulated and that the corresponding mRNA was cleaved at the predicted target site, confirming that the production of the vd-sRNA in the infected plants leads to the down-regulation of the endogenous mRNA by RNA silencing (Avina-Padilla et al., 2015).

4. Future perspectives and concluding remarks.

During several years one of the main limitations for the study of plant responses to viroids was the inability of *A. thaliana*, a widely used model plant for which a high number of molecular tools are available, to be infected by these pathogens. Although all the attempts to transmit viroids to Arabidopsis were unsuccessful, analysis of transgenic *A. thaliana* lines expressing dimeric constructs of several nuclear viroids confirmed that Arabidopsis has the enzymatic machinery for viroid

replication (but not for movement), indicating the potential of this species to address questions related with viroids biology (Daros and Flores, 2004). Despite viroid mobility being impaired, the transgenic plants were used to identify the processing site of the oligomeric intermediates generated during replication of nuclear viroids and to characterize the ends of the processed molecules (Flores et al., 2008; Gas et al., 2007; Gas et al., 2008). The increase of available sequenced genomes, including those from natural host plants, will facilitate the understanding about viroid-host interactions. In this direction, the release of the complete sequence of the peach genome combined with previous deep-sequencing results allowed the identification of potential vd-siRNAs targets in peach and its ulterior validation (Navarro et al., 2012). Bioinformatics approaches using viroid sequences and the complete genomes from *A. thaliana* and tomato allowed the identification of potential vd-siRNAs and their putative targets (Diermann et al., 2010). Using a similar strategy and including the sequences of 30 members of the family *Pospiviroidae*, potential targets of vd-siRNAs were identified in *A. thaliana* and validated in tomato plants infected with TPMVd (Avina-Padilla et al., 2015).

The study and characterization of endogenous lncRNAs in plants is an emerging field of high relevance for our understanding about regulatory mechanisms of gene expression (Ariel et al., 2015). Considering that viroids are able to modulate expression of endogenous protein-coding genes (Avina-Padilla et al., 2015; Eamens et al., 2014; Navarro et al., 2012) it is of great interest to elucidate the relationship between exogenous and endogenous lncRNAs in plants and to decipher their interactions. These studies are of especial interest if it is considered that members of the family *Pospiviroidae* replicate in the nucleus in which transcription happens and where several endogenous lncRNAs are localized. Additionally, endogenous lncRNAs have been also identified in the chloroplasts (Georg et al., 2010; Hotto et al., 2010), suggesting that interactions between both types of lncRNAs, endogenous and infectious exogenous, may also occur in these organelles.

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References

Ariel, F., Jegu, T., Latrasse, D., Romero-Barrios, N., Christ, A., Benhamed, M., Crespi, M.,;1; 2014. Noncoding transcription by alternative RNA polymerases dynamically regulates an auxin-driven chromatin loop. Mol. Cell 55, 383-396.

Ariel, F., Romero-Barrios, N., Jegu, T., Benhamed, M., Crespi, M.,;1; 2015. Battles and hijacks: noncoding transcription in plants. Trends Plant Sci. 20, 362-371.

Aung, K., Lin, S.I., Wu, C.C., Huang, Y.T., Su, C.L., Chiou, T.J.,;1; 2006. pho2, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. Plant Physiol. 141, 1000-1011.

Avina-Padilla, K., Martinez de la Vega, O., Rivera-Bustamante, R., Martinez-Soriano, J.P., Owens, R.A., Hammond, R.W., Vielle-Calzada, J.P.,;1; 2015. In silico prediction and validation of potential gene targets for pospiviroid-derived small RNAs during tomato infection. Gene 564, 197-205.

Axtell, M.J.,;1; 2013. Classification and comparison of small RNAs from plants. Annu. Rev. Plant Biol. 64, 137-159.

Bardou, F., Ariel, F., Simpson, C.G., Romero-Barrios, N., Laporte, P., Balzergue, S., Brown, J.W., Crespi, M.,;1; 2014. Long noncoding RNA modulates alternative splicing regulators in Arabidopsis. Dev. Cell 30, 166-176.

Bolduc, F., Hoareau, C., St-Pierre, P., Perreault, J.P.,;1; 2010. In-depth sequencing of the siRNAs associated with peach latent mosaic viroid infection. BMC Mol. Biol. 11, 16.

Borsani, O., Zhu, J., Verslues, P.E., Sunkar, R., Zhu, J.K.,;1; 2005. Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis. Cell 123, 1279-1291.

Branch, A.D., Robertson, H.D.,;1; 1984. A replication cycle for viroids and other small infectious RNA's. Science 223, 450-455.

Branch, A.D., Robertson, H.D., Greer, C., Gegenheimer, P., Peebles, C., Abelson, J.,;1; 1982. Cell-free circularization of viroid progeny RNA by an RNA ligase from wheat germ. Science 217, 1147-1149.

Burgyan, J., Havelda, Z.,;1; 2011. Viral suppressors of RNA silencing. Trends Plant Sci. 16, 265-272.

Bussiere, F., Ouellet, J., Cote, F., Levesque, D., Perreault, J.P.,;1; 2000. Mapping in solution shows the peach latent mosaic viroid to possess a new pseudoknot in a complex, branched secondary structure. J. Virol. 74, 2647-2654.

Buzayan, J.M., Gerlach, W.L., Bruening, G.,;1; 1986. Satellite tobacco ringspot virus RNA: A subset of the RNA sequence is sufficient for autolytic processing. Proc. Natl. Acad. Sci. U.S.A. 83, 8859-8862.

Carbonell, A., De la Peña, M., Flores, R., Gago, S.,;1; 2006. Effects of the trinucleotide preceding the self-cleavage site on eggplant latent viroid hammerheads: differences in co- and post-transcriptional self-cleavage may explain the lack of trinucleotide AUC in most natural hammerheads. Nucleic Acids Res. 34, 5613-5622.

Carbonell, A., Martinez de Alba, A.E., Flores, R., Gago, S.,;1; 2008. Double-stranded RNA interferes in a sequence-specific manner with the infection of representative members of the two viroid families. Virology 371, 44-53.

Chiumenti, M., Torchetti, E.M., Di Serio, F., Minafra, A.,;1; 2014. Identification and characterization of a viroid resembling apple dimple fruit viroid in fig (Ficus carica L.) by next generation sequencing of small RNAs. Virus Res. 188, 54-59.

Consortium, E.P., Birney, E., Stamatoyannopoulos, J.A., Dutta, A., Guigo, R., Gingeras, T.R., Margulies, E.H., Weng, Z., Snyder, M., Dermitzakis, E.T., Thurman, R.E., Kuehn, M.S., Taylor, C.M., Neph, S., Koch, C.M., Asthana, S., Malhotra, A., Adzhubei, I., Greenbaum, J.A., Andrews, R.M., Flicek, P., Boyle, P.J., Cao, H., Carter, N.P., Clelland, G.K., Davis, S., Day, N., Dhami, P., Dillon, S.C., Dorschner, M.O., Fiegler, H., Giresi, P.G., Goldy, J., Hawrylycz, M., Haydock, A., Humbert, R., James, K.D., Johnson, B.E., Johnson, E.M., Frum, T.T., Rosenzweig, E.R., Karnani, N., Lee, K., Lefebvre, G.C., Navas, P.A., Neri, F., Parker, S.C., Sabo, P.J., Sandstrom, R., Shafer, A., Vetrie, D., Weaver, M., Wilcox, S., Yu, M., Collins, F.S., Dekker, J., Lieb, J.D., Tullius, T.D., Crawford, G.E., Sunyaev, S., Noble, W.S., Dunham, I., Denoeud, F., Reymond, A., Kapranov, P., Rozowsky, J., Zheng, D., Castelo, R., Frankish, A., Harrow, J., Ghosh, S., Sandelin, A., Hofacker, I.L., Baertsch, R., Keefe, D., Dike, S., Cheng, J., Hirsch, H.A., Sekinger, E.A., Lagarde, J., Abril, J.F., Shahab, A., Flamm, C., Fried, C., Hackermuller, J., Hertel, J., Lindemeyer, M., Missal, K., Tanzer, A., Washietl, S., Korbel, J., Emanuelsson, O., Pedersen, J.S., Holroyd, N., Taylor, R., Swarbreck, D., Matthews, N., Dickson, M.C., Thomas, D.J., Weirauch, M.T., et al.,;1; 2007. Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. Nature 447, 799-816.

Coram, T.E., Settles, M.L., Chen, X.,;1; 2009. Large-scale analysis of antisense transcription in wheat using the Affymetrix GeneChip Wheat Genome Array. BMC Genomics 10, 253.

Dalakouras, A., Dadami, E., Wassenegger, M.,;1; 2013. Viroid-induced DNA methylation in plants. Biomol. Concepts 4, 557-565.

Daros, J.A., Flores, R.,;1; 2002. A chloroplast protein binds a viroid RNA in vivo and facilitates its hammerhead-mediated self-cleavage. EMBO J. 21, 749-759.

Daros, J.A., Flores, R.,;1; 2004. Arabidopsis thaliana has the enzymatic machinery for replicating representative viroid species of the family Pospiviroidae. Proc. Natl. Acad. Sci. U.S.A. 101, 6792-6797.

Daros, J.A., Marcos, J.F., Hernandez, C., Flores, R.,;1; 1994. Replication of avocado sunblotch viroid: evidence for a symmetric pathway with two rolling circles and hammerhead ribozyme processing. Proc. Natl. Acad. Sci. U.S.A. 91, 12813-12817.

De la Peña, M., Gago, S., Flores, R.,;1; 2003. Peripheral regions of natural hammerhead ribozymes greatly increase their self-cleavage activity. EMBO J. 22, 5561-5570.

De la Peña, M., Navarro, B., Flores, R.,;1; 1999. Mapping the molecular determinant of pathogenicity in a hammerhead viroid: a tetraloop within the in vivo branched RNA conformation. Proc. Natl. Acad. Sci. U.S.A. 96, 9960-9965.

Denti, M.A., Boutla, A., Tsagris, M., Tabler, M.,;1; 2004. Short interfering RNAs specific for potato spindle tuber viroid are found in the cytoplasm but not in the nucleus. Plant J. 37, 762-769.

Di Serio, F., De Stradis, A., Delgado, S., Flores, R., Navarro, B.,;1; 2012. Cytopathic Effects Incited by Viroid RNAs and Putative Underlying Mechanisms. Front. Plant Sci. 3, 288.

Di Serio, F., Flores, R., Verhoeven, J.T., Li, S.F., Pallas, V., Randles, J.W., Sano, T., Vidalakis, G., Owens, R.A.,;1; 2014. Current status of viroid taxonomy. Arch. Virol. 159, 3467-3478.

Di Serio, F., Gisel, A., Navarro, B., Delgado, S., Martinez de Alba, A.E., Donvito, G., Flores, R.,;1; 2009. Deep sequencing of the small RNAs derived from two symptomatic variants of a chloroplastic viroid: implications for their genesis and for pathogenesis. PLoS ONE 4, e7539.

Di Serio, F., Martinez de Alba, A.E., Navarro, B., Gisel, A., Flores, R.,;1; 2010. RNA-dependent RNA polymerase 6 delays accumulation and precludes meristem invasion of a viroid that replicates in the nucleus. J. Virol. 84, 2477-2489.

Dickson, E., Diener, T.O., Robertson, H.D.,;1; 1978. Potato spindle tuber and citrus exocortis viroids undergo no major sequence changes during replication in two different hosts. Proc. Natl. Acad. Sci. U.S.A. 75, 951-954.

Diener, T.O.,;1; 1972. Potato spindle tuber viroid. 8. Correlation of infectivity with a UV-absorbing component and thermal denaturation properties of the RNA. Virology 50, 606-609.

Diermann, N., Matousek, J., Junge, M., Riesner, D., Steger, G.,;1; 2010. Characterization of plant miRNAs and small RNAs derived from potato spindle tuber viroid (PSTVd) in infected tomato. Biol. Chem. 391, 1379-1390.

Ding, B.,;1; 2009. The biology of viroid-host interactions. Annu. Rev. Phytopathol. 47, 105-131.

Ding, B.,;1; 2010. Viroids: self-replicating, mobile, and fast-evolving noncoding regulatory RNAs. Wiley interdiscip. Rev. RNA 1, 362-375.

Dube, A., Baumstark, T., Bisaillon, M., Perreault, J.P.,;1; 2010. The RNA strands of the plus and minus polarities of peach latent mosaic viroid fold into different structures. RNA 16, 463-473.

Dube, A., Bisaillon, M., Perreault, J.P.,;1; 2009. Identification of proteins from prunus persica that interact with peach latent mosaic viroid. J. Virol. 83, 12057-12067.

Eamens, A.L., Smith, N.A., Dennis, E.S., Wassenegger, M., Wang, M.B.,;1; 2014. In Nicotiana species, an artificial microRNA corresponding to the virulence modulating region of Potato spindle tuber viroid directs RNA silencing of a soluble inorganic pyrophosphatase gene and the development of abnormal phenotypes. Virology 450-451, 266-277.

Englert, M., Beier, H.,;1; 2005. Plant tRNA ligases are multifunctional enzymes that have diverged in sequence and substrate specificity from RNA ligases of other phylogenetic origins. Nucleic Acids Res. 33, 388-399.

Englert, M., Latz, A., Becker, D., Gimple, O., Beier, H., Akama, K.,;1; 2007. Plant pre-tRNA splicing enzymes are targeted to multiple cellular compartments. Biochimie 89, 1351-1365.

Fadda, Z., Daros, J.A., Fagoaga, C., Flores, R., Duran-Vila, N.,;1; 2003. Eggplant latent viroid, the candidate type species for a new genus within the family Avsunviroidae (hammerhead viroids). J. Virol. 77, 6528-6532.

Flores, R., Delgado, S., Gas, M.E., Carbonell, A., Molina, D., Gago, S., De la Peña, M.,;1; 2004. Viroids: the minimal non-coding RNAs with autonomous replication. FEBS Lett. 567, 42-48.

Flores, R., Delgado, S., Rodio, M.E., Ambros, S., Hernandez, C., Serio, F.D.,;1; 2006. Peach latent mosaic viroid: not so latent. Mol. Plant Pathol. 7, 209-221.

Flores, R., Gago-Zachert, S., Serra, P., Sanjuán, R., Elena, S.F.,;1; 2014. Viroids: survivors from the RNA world? Annu. Rev. Microbiol. 68, 395-414.

Flores, R., Gas, M.E., Molina-Serrano, D., Nohales, M.A., Carbonell, A., Gago, S., De la Peña, M., Daros, J.A.,;1; 2009. Viroid replication: rolling-circles, enzymes and ribozymes. Viruses 1, 317-334.

Flores, R., Hernandez, C., De la Peña, M., Vera, A., Daros, J.A.,;1; 2001. Hammerhead ribozyme structure and function in plant RNA replication. Methods Enzymol. 341, 540-552.

Flores, R., Minoia, S., Carbonell, A., Gisel, A., Delgado, S., Lopez-Carrasco, A., Navarro, B., Di Serio, F.,;1; 2015. Viroids, the simplest RNA replicons: How they manipulate their hosts for being propagated and how their hosts react for containing the infection. Virus Res., in press; doi: 10.1016/j.virusres.2015.02.027.

Flores, R., Serra, P., Minoia, S., Di Serio, F., Navarro, B.,;1; 2012. Viroids: from genotype to phenotype just relying on RNA sequence and structural motifs. Front. Microbiol. 3, 217.

Franco-Zorrilla, J.M., Valli, A., Todesco, M., Mateos, I., Puga, M.I., Rubio-Somoza, I., Leyva, A., Weigel, D., Garcia, J.A., Paz-Ares, J.,;1; 2007. Target mimicry provides a new mechanism for regulation of microRNA activity. Nat. Genet. 39, 1033-1037.

Friml, J., Yang, X., Michniewicz, M., Weijers, D., Quint, A., Tietz, O., Benjamins, R., Ouwerkerk, P.B., Ljung, K., Sandberg, G., Hooykaas, P.J., Palme, K., Offringa, R.,;1; 2004. A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. Science 306, 862-865.

Fujii, H., Chiou, T.J., Lin, S.I., Aung, K., Zhu, J.K.,;1; 2005. A miRNA involved in phosphate-starvation response in Arabidopsis. Curr. Biol. 15, 2038-2043.

Fussy, Z., Patzak, J., Stehlik, J., Matousek, J.,;1; 2013. Imbalance in expression of hop (Humulus lupulus) chalcone synthase H1 and its regulators during hop stunt viroid pathogenesis. Journal of Plant Physiol. 170, 688-695.

Gago, S., De la Peña, M., Flores, R.,;1; 2005. A kissing-loop interaction in a hammerhead viroid RNA critical for its in vitro folding and in vivo viability. RNA 11, 1073-1083.

Gas, M.E., Hernandez, C., Flores, R., Daros, J.A.,;1; 2007. Processing of nuclear viroids in vivo: an interplay between RNA conformations. PLoS Pathog. 3, 1813-1826.

Gas, M.E., Molina-Serrano, D., Hernandez, C., Flores, R., Daros, J.A.,;1; 2008. Monomeric linear RNA of citrus exocortis viroid resulting from processing in vivo has 5'-phosphomonoester and 3'-hydroxyl termini: implications for the RNase and RNA ligase involved in replication. J. Virol. 82, 10321-10325.

Gast, F.U., Kempe, D., Spieker, R.L., Sanger, H.L.,;1; 1996. Secondary structure probing of potato spindle tuber viroid (PSTVd) and sequence comparison with other small pathogenic RNA replicons provides evidence for central non-canonical base-pairs, large A-rich loops, and a terminal branch. J. Mol. Biol. 262, 652-670.

Georg, J., Honsel, A., Voss, B., Rennenberg, H., Hess, W.R.,;1; 2010. A long antisense RNA in plant chloroplasts. New Phytol. 186, 615-622.

Giguere, T., Adkar-Purushothama, C.R., Bolduc, F., Perreault, J.P.,;1; 2014a. Elucidation of the structures of all members of the Avsunviroidae family. Mol. Plant Pathol. 15, 767-779.

Giguere, T., Adkar-Purushothama, C.R., Perreault, J.P.,;1; 2014b. Comprehensive secondary structure elucidation of four genera of the family Pospiviroidae. PLoS ONE 9, e98655.

Gomes, A.Q., Nolasco, S., Soares, H.,;1; 2013. Non-coding RNAs: multi-tasking molecules in the cell. Int. J. Mol. Sci. 14, 16010-16039.

Gomez, G., Martinez, G., Pallas, V.,;1; 2008. Viroid-induced symptoms in Nicotiana benthamiana plants are dependent on RDR6 activity. Plant Physiol. 148, 414-423.

Gomez, G., Pallas, V.,;1; 2001. Identification of an in vitro ribonucleoprotein complex between a viroid RNA and a phloem protein from cucumber plants. Mol. Plant-Microbe Interact. 14, 910-913.

Gomez, G., Pallas, V.,;1; 2004. A long-distance translocatable phloem protein from cucumber forms a ribonucleoprotein complex in vivo with Hop stunt viroid RNA. J. Virol. 78, 10104-10110.

Gomez, G., Pallas, V.,;1; 2007. Mature monomeric forms of Hop stunt viroid resist RNA silencing in transgenic plants. Plant J. 51, 1041-1049.

Gomez, G., Pallas, V.,;1; 2013. Viroids: a light in the darkness of the lncRNA-directed regulatory networks in plants. New Phytol. 198, 10–15.

Goodman, T.C., Nagel, L., Rappold, W., Klotz, G., Riesner, D.,;1; 1984. Viroid replication: equilibrium association constant and comparative activity measurements for the viroid-polymerase interaction. Nucleic Acids Res. 12, 6231-6246.

Grill, L.K., Semancik, J.S.,;1; 1978. RNA sequences complementary to citrus exocortis viroid in nucleic acid preparations from infected Gynura aurantiaca. Proc. Natl. Acad. Sci. U.S.A. 75, 896-900.

Haseloff, J., Symons, R.H.,;1; 1982. Comparative sequence and structure of viroid-like RNAs of two plant viruses. Nucleic Acids Res. 10, 3681-3691.

Hashimoto, J., Koganezawa, H.,;1; 1987. Nucleotide sequence and secondary structure of apple scar skin viroid. Nucleic Acids Res. 15, 7045-7052.

Held, M.A., Penning, B., Brandt, A.S., Kessans, S.A., Yong, W., Scofield, S.R., Carpita, N.C.,;1; 2008. Small-interfering RNAs from natural antisense transcripts derived from a cellulose synthase gene modulate cell wall biosynthesis in barley. Proc. Natl. Acad. Sci. U.S.A. 105, 20534-20539.

Heo, J.B., Sung, S.,;1; 2011. Vernalization-mediated epigenetic silencing by a long intronic noncoding RNA. Science 331, 76-79.

Hernandez, C., Daros, J.A., Elena, S.F., Moya, A., Flores, R.,;1; 1992. The strands of both polarities of a small circular RNA from carnation self-cleave in vitro through alternative double- and single-hammerhead structures. Nucleic Acids Res. 20, 6323-6329.

Hernandez, C., Flores, R.,;1; 1992. Plus and minus RNAs of peach latent mosaic viroid self-cleave in vitro via hammerhead structures. Proc. Natl. Acad. Sci. U.S.A. 89, 3711-3715.

Hotto, A.M., Huston, Z.E., Stern, D.B.,;1; 2010. Overexpression of a natural chloroplast-encoded antisense RNA in tobacco destabilizes 5S rRNA and retards plant growth. BMC Plant Biol. 10, 213.

Hou, W.Y., Sano, T., Li, F., Wu, Z.J., Li, L., Li, S.F.,;1; 2009. Identification and characterization of a new coleviroid (CbVd-5). Arch. Virol. 154, 315-320.

Hu, W., Alvarez-Dominguez, J.R., Lodish, H.F.,;1; 2012. Regulation of mammalian cell differentiation by long non-coding RNAs. EMBO Rep. 13, 971-983.

Huang, T.K., Han, C.L., Lin, S.I., Chen, Y.J., Tsai, Y.C., Chen, Y.R., Chen, J.W., Lin, W.Y., Chen, P.M., Liu, T.Y., Chen, Y.S., Sun, C.M., Chiou, T.J.,;1; 2013. Identification of downstream components of ubiquitin-conjugating enzyme PHOSPHATE2 by quantitative membrane proteomics in Arabidopsis roots. Plant Cell 25, 4044-4060.

Hutchins, C.J., Rathjen, P.D., Forster, A.C., Symons, R.H.,;1; 1986. Self-cleavage of plus and minus RNA transcripts of avocado sunblotch viroid. Nucleic Acids Res. 14, 3627-3640.

Itaya, A., Folimonov, A., Matsuda, Y., Nelson, R.S., Ding, B.,;1; 2001. Potato spindle tuber viroid as inducer of RNA silencing in infected tomato. Mol. Plant-Microbe Interact. 14, 1332-1334.

Itaya, A., Zhong, X., Bundschuh, R., Qi, Y., Wang, Y., Takeda, R., Harris, A.R., Molina, C., Nelson, R.S., Ding, B.,;1; 2007. A structured viroid RNA serves as a substrate for dicer-like cleavage to produce biologically active small RNAs but is resistant to RNA-induced silencing complex-mediated degradation. J. Virol. 81, 2980-2994.

Jabnoune, M., Secco, D., Lecampion, C., Robaglia, C., Shu, Q., Poirier, Y.,;1; 2013. A rice cis-natural antisense RNA acts as a translational enhancer for its cognate mRNA and contributes to phosphate homeostasis and plant fitness. Plant Cell 25, 4166-4182.

Jiang, D., Sano, T., Tsuji, M., Araki, H., Sagawa, K., Purushothama, C.R., Zhang, Z., Guo, R., Xie, L., Wu, Z., Wang, H., Li, S.,;1; 2012. Comprehensive diversity analysis of viroids infecting grapevine in China and Japan. Virus Res. 169, 237-245.

Kalantidis, K., Denti, M.A., Tzortzakaki, S., Marinou, E., Tabler, M., Tsagris, M.,;1; 2007. Virp1 is a host protein with a major role in Potato spindle tuber viroid infection in Nicotiana plants. J. Virol. 81, 12872-12880.

Katsarou, K., Rao, A.L., Tsagris, M., Kalantidis, K.,;1; 2015. Infectious long non-coding RNAs. Biochemie, in press; doi: 10.1016/j.biochi.2015.05.005.

Keese, P., Symons, R.H.,;1; 1985. Domains in viroids: evidence of intermolecular RNA rearrangements and their contribution to viroid evolution. Proc. Natl. Acad. Sci. U.S.A. 82, 4582-4586.

Khvorova, A., Lescoute, A., Westhof, E., Jayasena, S.D.,;1; 2003. Sequence elements outside the hammerhead ribozyme catalytic core enable intracellular activity. Nat. Struct. Biol. 10, 708-712.

Kim, T.H., Kim, B.H., von Arnim, A.G.,;1; 2002. Repressors of photomorphogenesis. Int. Rev. Cytol. 220, 185-223.

Konarska, M., Filipowicz, W., Domdey, H., Gross, H.J.,;1; 1981. Formation of a 2'-phosphomonoester, 3',5'-phosphodiester linkage by a novel RNA ligase in wheat germ. Nature 293, 112-116.

Kovalskaya, N., Hammond, R.W.,;1; 2014. Molecular biology of viroid-host interactions and disease control strategies. Plant Sci. 228, 48-60.

Landry, P., Perreault, J.P.,;1; 2005. Identification of a peach latent mosaic viroid hairpin able to act as a Dicer-like substrate. J. Virol. 79, 6540-6543.

Lembke, C.G., Nishiyama, M.Y., Jr., Sato, P.M., de Andrade, R.F., Souza, G.M.,;1; 2012. Identification of sense and antisense transcripts regulated by drought in sugarcane. Plant Mol. Biol. 79, 461-477.

Levy, Y.Y., Mesnage, S., Mylne, J.S., Gendall, A.R., Dean, C.,;1; 2002. Multiple roles of Arabidopsis VRN1 in vernalization and flowering time control. Science 297, 243-246.

Liu, J., Jung, C., Xu, J., Wang, H., Deng, S., Bernad, L., Arenas-Huertero, C., Chua, N.H.,;1; 2012. Genome-wide analysis uncovers regulation of long intergenic noncoding RNAs in Arabidopsis. Plant Cell 24, 4333-4345.

Liu, X., Hao, L., Li, D., Zhu, L., Hu, S.,;1; 2015. Long Non-coding RNAs and Their Biological Roles in Plants. Genomics Proteomics Bioinformatics, in press; doi: 10.1016/j.gpb.2015.02.003.

Lu, T., Zhu, C., Lu, G., Guo, Y., Zhou, Y., Zhang, Z., Zhao, Y., Li, W., Lu, Y., Tang, W., Feng, Q., Han, B.,;1; 2012. Strand-specific RNA-seq reveals widespread occurrence of novel cis-natural antisense transcripts in rice. BMC Genomics 13, 721.

Machida, S., Yamahata, N., Watanuki, H., Owens, R.A., Sano, T.,;1; 2007. Successive accumulation of two size classes of viroid-specific small RNA in potato spindle tuber viroid-infected tomato plants. J. Gen. Virol. 88, 3452-3457.

MacRae, I.J., Ma, E., Zhou, M., Robinson, C.V., Doudna, J.A.,;1; 2008. In vitro reconstitution of the human RISC-loading complex. Proc. Natl. Acad. Sci. U.S.A. 105, 512-517.

Makino, S., Sawasaki, T., Endo, Y., Takai, K.,;1; 2005. Purification and sequence determination of an RNA ligase from wheat embryos. Nucleic Acids Symp. Ser. 49, 319-320.

Malfitano, M., Di Serio, F., Covelli, L., Ragozzino, A., Hernandez, C., Flores, R.,;1; 2003. Peach latent mosaic viroid variants inducing peach calico (extreme chlorosis) contain a characteristic insertion that is responsible for this symptomatology. Virology 313, 492-501.

Maniataki, E., Martinez de Alba, A.E., Sagesser, R., Tabler, M., Tsagris, M.,;1; 2003. Viroid RNA systemic spread may depend on the interaction of a 71-nucleotide bulged hairpin with the host protein VirP1. RNA 9, 346-354.

Markarian, N., Li, H.W., Ding, S.W., Semancik, J.S.,;1; 2004. RNA silencing as related to viroid induced symptom expression. Arch. Virol. 149, 397-406.

Martin, R., Arenas, C., Daros, J.A., Covarrubias, A., Reyes, J.L., Chua, N.H.,;1; 2007. Characterization of small RNAs derived from Citrus exocortis viroid (CEVd) in infected tomato plants. Virology 367, 135-146.

Martinez de Alba, A.E., Flores, R., Hernandez, C.,;1; 2002. Two chloroplastic viroids induce the accumulation of small RNAs associated with posttranscriptional gene silencing. J. Virol. 76, 13094-13096.

Martinez de Alba, A.E., Sagesser, R., Tabler, M., Tsagris, M.,;1; 2003. A bromodomain-containing protein from tomato specifically binds potato spindle tuber viroid RNA in vitro and in vivo. J. Virol. 77, 9685-9694.

Martinez, F., Marques, J., Salvador, M.L., Daros, J.A.,;1; 2009. Mutational analysis of eggplant latent viroid RNA processing in Chlamydomonas reinhardtii chloroplast. J. Gen. Virol. 90, 3057-3065.

Martinez, G., Castellano, M., Tortosa, M., Pallas, V., Gomez, G.,;1; 2014. A pathogenic non-coding RNA induces changes in dynamic DNA methylation of ribosomal RNA genes in host plants. Nucleic Acids Res. 42, 1553-1562.

Martinez, G., Donaire, L., Llave, C., Pallas, V., Gomez, G.,;1; 2010. High-throughput sequencing of Hop stunt viroid-derived small RNAs from cucumber leaves and phloem. Mol. Plant Pathol. 11, 347-359.

Matousek, J., Kozlova, P., Orctova, L., Schmitz, A., Pesina, K., Bannach, O., Diermann, N., Steger, G., Riesner, D.,;1; 2007. Accumulation of viroid-specific small RNAs and increase in nucleolytic activities linked to viroid-caused pathogenesis. Biol. Chem. 388, 1-13.

Mercer, T.R., Dinger, M.E., Mattick, J.S.,;1; 2009. Long non-coding RNAs: insights into functions. Nat. Rev. Genet. 10, 155-159.

Merino, E.J., Wilkinson, K.A., Coughlan, J.L., Weeks, K.M.,;1; 2005. RNA structure analysis at single nucleotide resolution by selective 2'-hydroxyl acylation and primer extension (SHAPE). J. Am. Chem. Soc. 127, 4223-4231.

Minoia, S., Carbonell, A., Di Serio, F., Gisel, A., Carrington, J.C., Navarro, B., Flores, R.,;1; 2014. Specific argonautes selectively bind small RNAs derived from potato spindle tuber viroid and attenuate viroid accumulation in vivo. J. Virol. 88, 11933-11945.

Mitter, N., Dietzgen, R.G.,;1; 2012. Use of hairpin RNA constructs for engineering plant virus resistance. Methods Mol. Biol. 894, 191-208.

Mlotshwa, S., Pruss, G.J., Vance, V.,;1; 2008. Small RNAs in viral infection and host defense. Trends Plant Sci. 13, 375-382.

Molnar, A., Csorba, T., Lakatos, L., Varallyay, E., Lacomme, C., Burgyan, J.,;1; 2005. Plant virus-derived small interfering RNAs originate predominantly from highly structured single-stranded viral RNAs. J. Virol. 79, 7812-7818.

Muhlbach, H.P., Sanger, H.L.,;1; 1979. Viroid replication is inhibited by alpha-amanitin. Nature 278, 185-188.

Nakaune, R., Nakano, M.,;1; 2008. Identification of a new Apscaviroid from Japanese persimmon. Arch. Virol. 153, 969-972.

Navarro, B., Flores, R.,;1; 1997. Chrysanthemum chlorotic mottle viroid: unusual structural properties of a subgroup of self-cleaving viroids with hammerhead ribozymes. Proc. Natl. Acad. Sci. U.S.A. 94, 11262-11267.

Navarro, B., Gisel, A., Rodio, M.E., Delgado, S., Flores, R., Di Serio, F.,;1; 2012. Small RNAs containing the pathogenic determinant of a chloroplast-replicating viroid guide the degradation of a host mRNA as predicted by RNA silencing. Plant J. 70, 991-1003.

Navarro, B., Pantaleo, V., Gisel, A., Moxon, S., Dalmay, T., Bisztray, G., Di Serio, F., Burgyan, J.,;1; 2009. Deep sequencing of viroid-derived small RNAs from grapevine provides new insights on the role of RNA silencing in plant-viroid interaction. PLoS ONE 4, e7686.

Navarro, J.A., Flores, R.,;1; 2000. Characterization of the initiation sites of both polarity strands of a viroid RNA reveals a motif conserved in sequence and structure. EMBO J. 19, 2662-2670.

Navarro, J.A., Vera, A., Flores, R.,;1; 2000. A chloroplastic RNA polymerase resistant to tagetitoxin is involved in replication of avocado sunblotch viroid. Virology 268, 218-225.

Nohales, M.A., Flores, R., Daros, J.A.,;1; 2012a. Viroid RNA redirects host DNA ligase 1 to act as an RNA ligase. Proc. Natl. Acad. Sci. U.S.A. 109, 13805-13810.

Nohales, M.A., Molina-Serrano, D., Flores, R., Daros, J.A.,;1; 2012b. Involvement of the chloroplastic isoform of tRNA ligase in the replication of viroids belonging to the family Avsunviroidae. J. Virol. 86, 8269-8276.

Ohno, T., Takamatsu, N., Meshi, T., Okada, Y.,;1; 1983. Hop stunt viroid: molecular cloning and nucleotide sequence of the complete cDNA copy. Nucleic Acids Res. 11, 6185-6197.

Owens, R.A., Blackburn, M., Ding, B.,;1; 2001. Possible involvement of the phloem lectin in long-distance viroid movement. Mol. Plant-Microbe Interact. 14, 905-909.

Owens, R.A., Hammond, R.W.,;1; 2009. Viroid pathogenicity: one process, many faces. Viruses 1, 298-316.

Papaefthimiou, I., Hamilton, A., Denti, M., Baulcombe, D., Tsagris, M., Tabler, M.,;1; 2001. Replicating potato spindle tuber viroid RNA is accompanied by short RNA fragments that are characteristic of post-transcriptional gene silencing. Nucleic Acids Res. 29, 2395-2400.

Park, B.S., Seo, J.S., Chua, N.H.,;1; 2014. NITROGEN LIMITATION ADAPTATION recruits PHOSPHATE2 to target the phosphate transporter PT2 for degradation during the regulation of Arabidopsis phosphate homeostasis. Plant Cell 26, 454-464.

Peret, B., Clement, M., Nussaume, L., Desnos, T.,;1; 2011. Root developmental adaptation to phosphate starvation: better safe than sorry. Trends Plant Sci. 16, 442-450.

Peret, B., Desnos, T., Jost, R., Kanno, S., Berkowitz, O., Nussaume, L.,;1; 2014. Root architecture responses: in search of phosphate. Plant Physiol. 166, 1713-1723.

Prody, G.A., Bakos, J.T., Buzayan, J.M., Schneider, I.R., Bruening, G.,;1; 1986. Autolytic processing of dimeric plant virus satellite RNA. Science 231, 1577-1580.

Qi, Y., Ding, B.,;1; 2003. Inhibition of cell growth and shoot development by a specific nucleotide sequence in a noncoding viroid RNA. Plant Cell 15, 1360-1374.

Quintero, A., Perez-Quintero, A.L., Lopez, C.,;1; 2013. Identification of ta-siRNAs and cis-nat-siRNAs in cassava and their roles in response to cassava bacterial blight. Genomics Proteomics Bioinformatics 11, 172-181.

Randles, J.W., Rodriguez, M.J., Imperial, J.S.,;1; 1988. Cadang-cadang disease of coconut palm. Microbiol. Sci. 5, 18-22.

Reanwarakorn, K., Semancik, J.S.,;1; 1999. Correlation of hop stunt viroid variants to cachexia and xyloporosis diseases of citrus. Phytopathology 89, 568-574.

Reinhardt, D., Wittwer, F., Mandel, T., Kuhlemeier, C.,;1; 1998. Localized upregulation of a new expansin gene predicts the site of leaf formation in the tomato meristem. Plant Cell 10, 1427-1437.

Rinn, J.L., Chang, H.Y.,;1; 2012. Genome regulation by long noncoding RNAs. Annu. Rev. Biochem. 81, 145-166.

Rodio, M.E., Delgado, S., De Stradis, A., Gomez, M.D., Flores, R., Di Serio, F.,;1; 2007. A viroid RNA with a specific structural motif inhibits chloroplast development. Plant Cell 19, 3610-3626.

Rodio, M.E., Delgado, S., Flores, R., Di Serio, F.,;1; 2006. Variants of Peach latent mosaic viroid inducing peach calico: uneven distribution in infected plants and requirements of the insertion containing the pathogenicity determinant. J. Gen. Virol. 87, 231-240.

Rodriguez, M.J., Randles, J.W.,;1; 1993. Coconut cadang-cadang viroid (CCCVd) mutants associated with severe disease vary in both the pathogenicity domain and the central conserved region. Nucleic Acids Res. 21, 2771.

Rohde, W., Sanger, H.L.,;1; 1981. Detection of complementary RNA intermediates of viroid replication by Northern blot hybridization. Biosci. Rep. 1, 327-336.

Sanger, H.L., Klotz, G., Riesner, D., Gross, H.J., Kleinschmidt, A.K.,;1; 1976. Viroids are single-stranded covalently closed circular RNA molecules existing as highly base-paired rod-like structures. Proc. Natl. Acad. Sci. U.S.A. 73, 3852-3856.

Schwind, N., Zwiebel, M., Itaya, A., Ding, B., Wang, M.B., Krczal, G., Wassenegger, M.,;1; 2009. RNAi-mediated resistance to Potato spindle tuber viroid in transgenic tomato expressing a viroid hairpin RNA construct. Mol. Plant Pathol. 10, 459-469.

Secco, D., Baumann, A., Poirier, Y.,;1; 2010. Characterization of the rice PHO1 gene family reveals a key role for OsPHO1;2 in phosphate homeostasis and the evolution of a distinct clade in dicotyledons. Plant Physiol. 152, 1693-1704.

Semancik, J.S., Szychowski, J.A.,;1; 1994. Avocado sunblotch disease: a persistent viroid infection in which variants are associated with differential symptoms. J. Gen. Virol. 75, 1543-1549.

Semancik, J.S., Weathers, L.G.,;1; 1972. Exocortis virus: an infectious free-nucleic acid plant virus with unusual properties. Virology 47, 456-466.

Serra, P., Gago, S., Duran-Vila, N.,;1; 2008. A single nucleotide change in Hop stunt viroid modulates citrus cachexia symptoms. Virus Res. 138, 130-134.

Sheldon, C.C., Rouse, D.T., Finnegan, E.J., Peacock, W.J., Dennis, E.S.,;1; 2000. The molecular basis of vernalization: the central role of FLOWERING LOCUS C (FLC). Proc. Natl. Acad. Sci. U.S.A. 97, 3753-3758.

Stefanovic, A., Ribot, C., Rouached, H., Wang, Y., Chong, J., Belbahri, L., Delessert, S., Poirier, Y.,;1; 2007. Members of the PHO1 gene family show limited functional redundancy in phosphate transfer to the shoot, and are regulated by phosphate deficiency via distinct pathways. Plant J. 50, 982-994.

Swiezewski, S., Liu, F., Magusin, A., Dean, C.,;1; 2009. Cold-induced silencing by long antisense transcripts of an Arabidopsis Polycomb target. Nature 462, 799-802.

Symons, R.H.,;1; 1981. Avocado sunblotch viroid: primary sequence and proposed secondary structure. Nucleic Acids Res. 9, 6527-6537.

Taylor, R.M., Hamer, M.J., Rosamond, J., Bray, C.M.,;1; 1998. Molecular cloning and functional analysis of the Arabidopsis thaliana DNA ligase I homologue. Plant J. 14, 75-81.

Vasa, S.M., Guex, N., Wilkinson, K.A., Weeks, K.M., Giddings, M.C.,;1; 2008. ShapeFinder: a software system for high-throughput quantitative analysis of nucleic acid reactivity information resolved by capillary electrophoresis. RNA 14, 1979-1990.

Verhoeven, J.T., Jansen, C.C., Roenhorst, J.W., Flores, R., De la Peña, M.,;1; 2009. Pepper chat fruit viroid: biological and molecular properties of a proposed new species of the genus Pospiviroid. Virus Res. 144, 209-214.

Verhoeven, J.T., Meekes, E.T., Roenhorst, J.W., Flores, R., Serra, P.,;1; 2013. Dahlia latent viroid: a recombinant new species of the family Pospiviroidae posing intriguing questions about its origin and classification. J. Gen. Virol. 94, 711-719.

Verhoeven, J.T., Roenhorst, J.W., Hooftman, M., Meekes, E.T., Flores, R., Serra, P.,;1; 2015. A pospiviroid from symptomless portulaca plants closely related to iresine viroid 1. Virus Res. 205, 22-26.

Vogt, U., Pelissier, T., Putz, A., Razvi, F., Fischer, R., Wassenegger, M.,;1; 2004. Viroid-induced RNA silencing of GFP-viroid fusion transgenes does not induce extensive spreading of methylation or transitive silencing. Plant J. 38, 107-118.

Wang, H., Chung, P.J., Liu, J., Jang, I.C., Kean, M.J., Xu, J., Chua, N.H.,;1; 2014a. Genome-wide identification of long noncoding natural antisense transcripts and their responses to light in Arabidopsis. Genome Res. 24, 444-453.

Wang, M.B., Bian, X.Y., Wu, L.M., Liu, L.X., Smith, N.A., Isenegger, D., Wu, R.M., Masuta, C., Vance, V.B., Watson, J.M., Rezaian, A., Dennis, E.S., Waterhouse, P.M.,;1; 2004. On the role of RNA silencing in the pathogenicity and evolution of viroids and viral satellites. Proc. Natl. Acad. Sci. U.S.A. 101, 3275-3280.

Wang, Y., Fan, X., Lin, F., He, G., Terzaghi, W., Zhu, D., Deng, X.W.,;1; 2014b. Arabidopsis noncoding RNA mediates control of photomorphogenesis by red light. Proc. Natl. Acad. Sci. U.S.A. 111, 10359-10364.

Wang, Y., Shibuya, M., Taneda, A., Kurauchi, T., Senda, M., Owens, R.A., Sano, T.,;1; 2011. Accumulation of Potato spindle tuber viroid-specific small RNAs is accompanied by specific changes in gene expression in two tomato cultivars. Virology 413, 72-83.

Warrilow, D., Symons, R.H.,;1; 1999. Citrus exocortis viroid RNA is associated with the largest subunit of RNA polymerase II in tomato in vivo. Arch. Virol. 144, 2367-2375.

Wassenegger, M., Heimes, S., Riedel, L., Sanger, H.L.,;1; 1994. RNA-directed de novo methylation of genomic sequences in plants. Cell 76, 567-576.

Waterworth, W.M., Kozak, J., Provost, C.M., Bray, C.M., Angelis, K.J., West, C.E.,;1; 2009. DNA ligase 1 deficient plants display severe growth defects and delayed repair of both DNA single and double strand breaks. BMC Plant Biol. 9, 79.

Wilkinson, K.A., Gorelick, R.J., Vasa, S.M., Guex, N., Rein, A., Mathews, D.H., Giddings, M.C., Weeks, K.M.,;1; 2008. High-throughput SHAPE analysis reveals structures in HIV-1 genomic RNA strongly conserved across distinct biological states. PLoS Biol. 6, e96.

Williams-Carrier, R., Zoschke, R., Belcher, S., Pfalz, J., Barkan, A.,;1; 2014. A major role for the plastid-encoded RNA polymerase complex in the expression of plastid transfer RNAs. Plant Physiol. 164, 239-248.

Wilusz, J.E., Sunwoo, H., Spector, D.L.,;1; 2009. Long noncoding RNAs: functional surprises from the RNA world. Genes Dev. 23, 1494-1504.

Wolff, P., Gilz, R., Schumacher, J., Riesner, D.,;1; 1985. Complexes of viroids with histones and other proteins. Nucleic Acids Res. 13, 355-367.

Wu, J., Okada, T., Fukushima, T., Tsudzuki, T., Sugiura, M., Yukawa, Y.,;1; 2012a. A novel hypoxic stress-responsive long non-coding RNA transcribed by RNA polymerase III in Arabidopsis. RNA Biol. 9, 302-313.

Wu, Q., Wang, Y., Cao, M., Pantaleo, V., Burgyan, J., Li, W.X., Ding, S.W.,;1; 2012b. Homology-independent discovery of replicating pathogenic circular RNAs by deep sequencing and a new computational algorithm. Proc. Natl. Acad. Sci. U.S.A. 109, 3938-3943.

Wu, Y.Q., Hohn, B., Ziemienowic, A.,;1; 2001. Characterization of an ATP-dependent type I DNA ligase from Arabidopsis thaliana. Plant Mol. Biol. 46, 161-170.

Wunderlich, M., Gross-Hardt, R., Schoffl, F.,;1; 2014. Heat shock factor HSFB2a involved in gametophyte development of Arabidopsis thaliana and its expression is controlled by a heat-inducible long non-coding antisense RNA. Plant Mol. Biol. 85, 541-550.

Xu, W., Bolduc, F., Hong, N., Perreault, J.P.,;1; 2012. The use of a combination of computer-assisted structure prediction and SHAPE probing to elucidate the secondary structures of five viroids. Mol. Plant Pathol. 13, 666-676.

Yu, X., Yang, J., Li, X., Liu, X., Sun, C., Wu, F., He, Y.,;1; 2013. Global analysis of cis-natural antisense transcripts and their heat-responsive nat-siRNAs in Brassica rapa. BMC Plant Biol. 13, 208.

Zhang, Z., Qi, S., Tang, N., Zhang, X., Chen, S., Zhu, P., Ma, L., Cheng, J., Xu, Y., Lu, M., Wang, H., Ding, S.W., Li, S., Wu, Q.,;1; 2014. Discovery of replicating circular RNAs by RNA-seq and computational algorithms. PLoS Pathog. 10, e1004553.

Zheng, H., Qiyan, J., Zhiyong, N., Hui, Z.,;1; 2013. Prediction and identification of natural antisense transcripts and their small RNAs in soybean (Glycine max). BMC Genomics 14, 280.

Zhou, X., Sunkar, R., Jin, H., Zhu, J.K., Zhang, W.,;1; 2009. Genome-wide identification and analysis of small RNAs originated from natural antisense transcripts in Oryza sativa. Genome Res. 19, 70-78.

Zubko, E., Meyer, P.,;1; 2007. A natural antisense transcript of the Petunia hybrida Sho gene suggests a role for an antisense mechanism in cytokinin regulation. Plant J. 52, 1131-1139.

Zuker, M.,;1; 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31, 3406-3415.

Figure Captions

Figure 1 Mechanisms of lncRNA function. (a) Several lncRNAs interact with proteins forming ribonucleoprotein complexes that affect gene expression by chromatin modifications in response to cold (Heo and Sung, 2011; Swiezewski et al., 2009) and light (Wang et al., 2014b). (b) lncRNAs regulate gene expression at the post-transcriptional level by sequestering miRNAs, producing an increase in the stability of the mRNA targeted by the miRNA (Franco-Zorrilla et al., 2007). (c) The co-expression of natural antisense long non-coding RNAs and the complementary transcripts leads to RNA degradation (left) (Wunderlich et al., 2014), in a similar way as proposed for natural antisense transcripts involving two protein-coding genes (Borsani et al., 2005) or to an increase of the complementary mRNA translation by an unknown mechanism (right) (Jabnoune et al., 2013). (d) lncRNAs modulate alternative splicing in response to auxin (a plant hormone) by hijacking nuclear splicing factors (Bardou et al., 2014). lncRNAs and mRNAs are indicated in blue and red, respectively. Thin lines represent untranslated regions (UTRs) and introns. Proteins or protein complexes are represented by incomplete ovals.

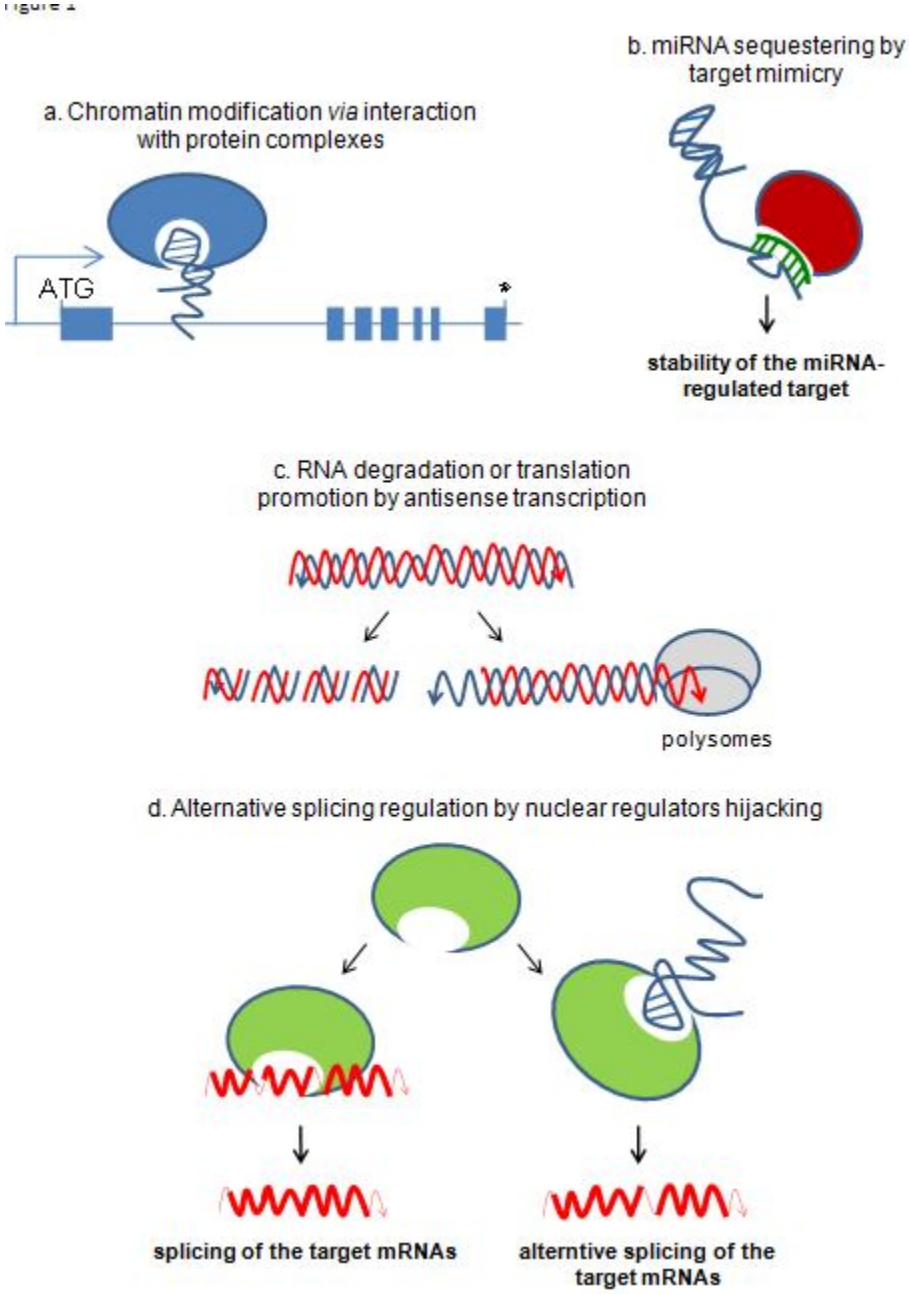

Figure 2 Structural and functional features of the two viroid families. The name of each family derives from the type species, *Pospiviroidae* and *Avsunviroidae* from *Potato spindle tuber viroid* and *Avocado sunblotch viroid*, respectively. (Figure based in Flores et al., 2014).

	Family *Pospiviroidae*	Family *Avsunviroidae*
Conserved regions and domains	central conserved region (CCR)	hammerhead ribozymes
Structure	rod-like	quasi rod-like or branched
Subcellular localization and replication	nucleus	chloroplasts
Type species	*Potato spindle tuber viroid* (PSTVd)	*Avocado sunblotch viroid* (ASBVd)

Figure 3 Replication of viroids from the families*Pospiviroidae* and *Avsunviroidaevia* the rolling circle-mechanism. Nuclear (left) and chloroplastic (right) viroids follow the asymmetric or the symmetric variants of the rolling circle mechanism, respectively. Strand polarities are included and cleavage reactions are indicated with arrowheads. Abbreviations: Rz, hammerhead ribozyme; NEP, nuclear encoded polymerase. It is important to notice that the representation of the cleavage step was simplified: cleavage by RNase III enzymes occurs in double-stranded structures of the (+) oligomer (for more details see Flores et al., 2012) and self-cleavage happens co-transcriptionally during replication of members of the families *Pospiviroidae* and *Avsunviroidae*, respectively. (Figure based in Ding and Itaya, 2007 and Flores et al., 2009).