Letters

Viroids: a light in the darkness of the *lncRNA*-directed regulatory networks in plants

The recent explosion in the number of detected long (> 100–200 nts) RNAs with no apparent protein-coding potential (*lncRNAs*) has raised substantial doubts about the role played by these intriguing transcripts in complex organisms (Clark *et al.*, 2012; Rinn & Chang, 2012). Although a vast majority of *lncRNAs* may possibly be '*junk*' transcripts generated as sub-products of cell activity, a significant number of plant *lncRNAs* has been shown to exhibit biological-like properties, such as tissue-specific expression (Sugiyama *et al.*, 2003), sub-cellular compartmentalization (Campalans *et al.*, 2004) and/or association with stress response (Franco-Zorrilla *et al.*, 2007; Heo & Sung, 2010) which argues against the possibility of representing transcriptional '*noise*'. Recognition that *lncRNAs* play a role as potent and specific regulators of gene expression in almost all the species studied to date has fuelled speculation that *lncRNAs* might be a hidden layer of regulation closely related to the fine control of diverse aspects of plant physiology, such as development, morphogenesis and stress response (Ben Amor *et al.*, 2009; Chinnusamy & Zhu, 2009; Au *et al.*, 2011; Röther & Meister, 2011).

In plants, and unlike mammalians, *lncRNAs* have been less extensively studied, and their identification and functional elucidation occur rather by chance than by systematic and/or directed screenings. Consequently to date, their mechanisms of action and potential targets are largely unknown (Au *et al.*, 2011; Bardou *et al.*, 2011). The lack of studies on *lncRNA*-directed processes in plants can be attributed to the fact that most of these regulatory pathways represent unconventional phenomena which are generally associated with both subtle phenotypic changes, which complicate the identification of related mutants (Mattick, 2009) and lack of molecular probes that help shed light on these functional networks.

Viroids are a class of sub-viral plant-pathogenic long noncoding RNAs (240–400 nt) composed of a circular single-stranded molecule. Viroid infection comprises a series of coordinated steps involving: (1) intracellular compartmentalization for replication; (2) export to neighboring cells; and (3) entry to vascular tissue for long-distance trafficking to distant plant organs (Ding, 2009). Since they lack protein-coding activity, viroids are compelled to subvert endogenous *lncRNA*-directed regulatory routes to complete their life cycle in the infected cell. In the last years, several research groups have employed host-viroid interactions to study diverse plant cellular biology aspects and to provide evidence that this experimental system constitutes a valuable tool that has contributed to impel our understanding of *lncRNA*-directed mechanisms in plants. Here, we briefly summarize the more relevant findings obtained with this pathogenic model in terms of *lncRNA* trafficking, processing and *lncRNA*-induced development alterations (Fig. 1), thus supporting the view that viroid research could emerge as a promissory source of alternative concepts related with *lncRNA* metabolism in plants.

Intracellular *lncRNA* trafficking

Viroids possess two well-determined replication sites in the infected cell: the nucleus for *Pospiviroidae* and chloroplasts for *Avsunviroidae*. Consequently, subverting the pathways regulating the *lncRNA* compartmentalization into both cellular organelles is the first obstacle that viroids face after host entry. Pioneer works using labeled and/or chimerical transcripts have determined that the subcellular localization of viroids from the *Pospiviroidae* family is mediated in *cis* by RNA sequences or structural motifs, which are required for the nuclear import by a specific receptor via a cytoskeleton-independent route (Woo *et al.*, 1999; Zhao *et al.*, 2001). Alternatively, a viroid-binding protein with a nuclear localization signal has been identified that could be involved in the nuclear compartmentalization of the *Potato spindle tuber viroid* (PSTVd) in infected cells (Martínez de Alba *et al.*, 2003), and has revealed the existence of host factors capable of regulating the nuclear targeting of *lncRNAs* in the plant cell.

More recently, by using a combined cytoplasmic and nuclear expression approach it was shown that *Eggplant latent viroid* (ELVd) transcripts traffic from the cytoplasm into the nucleus, and subsequently from there into chloroplasts, which suggests a novel route to explain *Avsunviroidae* selective sub-cellular compartmentalization (Gómez & Pallás, 2012a). On this hypothetical pathway, once the ELVd invades the cell cytoplasm, it is imported into the nucleus. Next, the viroid uses this organelle as a sub-cellular port to be launched into the chloroplast, where replication occurs (Gómez & Pallás, 2012b). These results support the existence of a yet uncharacterized plant signaling mechanism mediated by noncoding RNAs that is able to regulate '*in cis*' the selective import of nuclear transcripts into chloroplasts. We proposed that this pathway may constitute a novel mechanism capable of regulating the accumulation of the nuclear-encoded proteins in chloroplasts alternatively to that based on signal-peptides (Gómez & Pallás, 2010a,b). Interestingly, it has been recently suggested that this *lncRNA*-directed mechanism could also mediate the protein import into the cyanobacterial endosymbiont/plastids of the rhizarian amoeba *Paulinella chromatophora* (Mackiewicz *et al.*, 2012). Furthermore, in parasitic plants showing major reductions in chloroplast genome including 13 out of 30 tRNAs genes, the chloroplastic translation processes are maintained, implying that these structural *lncRNAs* must be imported from outside the plastid (Bungard, 2004). Finally, the recent observation that noncoding

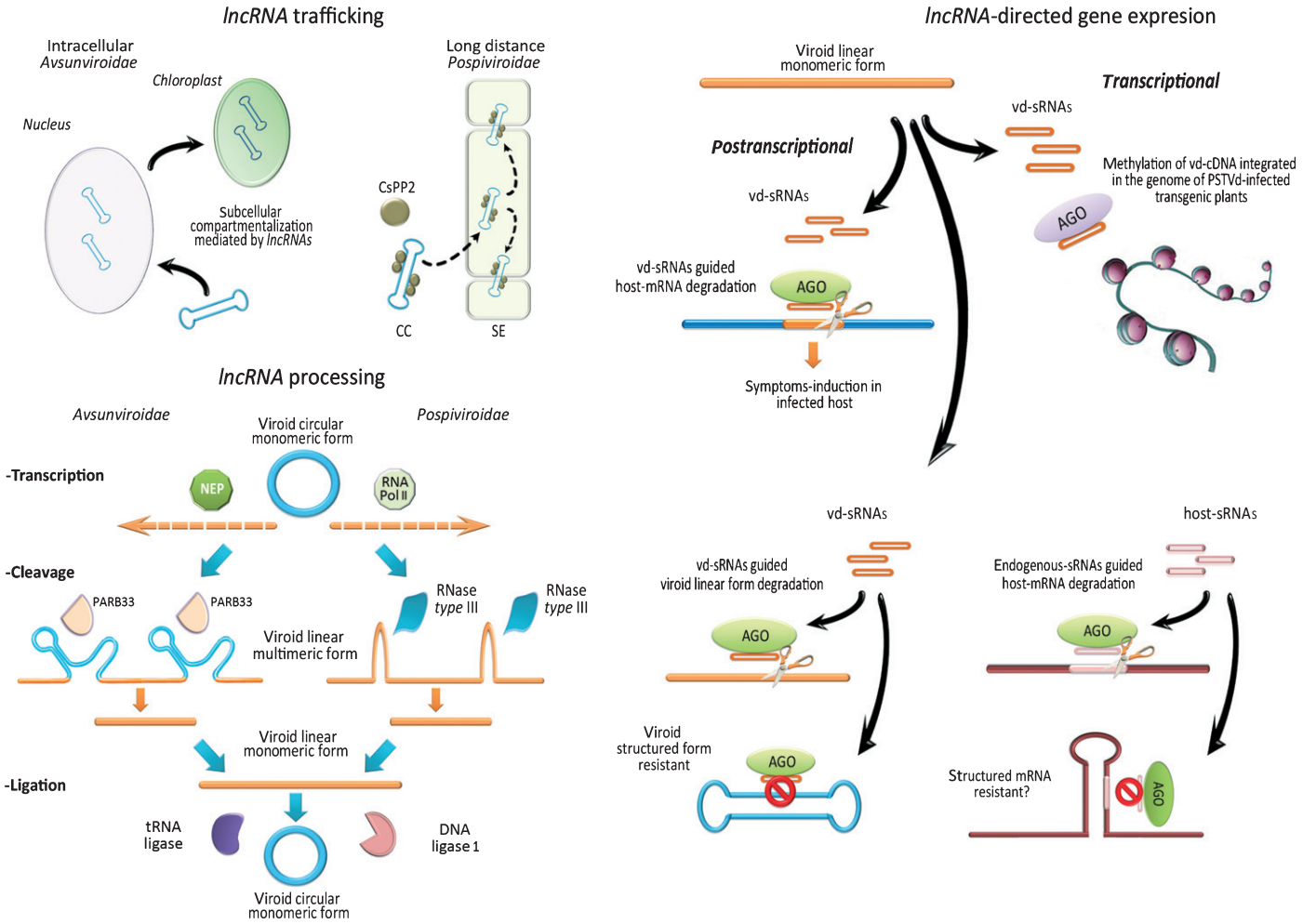

Fig. 1 Simplified representation of different viroid RNA-directed processes that can shed light on the potential parallel *lncRNA*-mediated mechanisms in plants. *lncRNA* trafficking (upper left panel): to explain its compartmentalization into chloroplasts it was proposed that after cytoplasm-invasion the ELVd is imported into the nucleus of the infected cell. Then, the viroid RNA uses this organelle as a port for delivery into chloroplasts for replication. Conversely, it has been shown that CsPP2 (a translocatable RNA-binding phloem protein from *Cucumis sativus*) is able to form an *in vivo* ribonucleoprotein complex in the phloem and mediate the vascular movement of HSVd in cucumber plants. *lncRNA* processing (lower left panel): the plant-endogenous factors known to be involved in the different viroid-replication steps are described: the RNA polymerase II and the nuclear-encoded polymerase (NEP) for transcription of *Pospiviroidae* and *Avsunvirodae*, respectively; the chloroplast protein PARBP33 that enhances the self-cleavage of ABSVd; the type III-RNase that mediates the processing of multimeric forms and the plant tRNA ligase and DNA ligase that mediate the circularization of linear intermediates during *Avsunviroidae* and *Pospiviroidade* replication, respectively. *lncRNA*-directed gene expression (right panel): infected plants accumulate vd-siRNAs that guide in *trans* the RISC-mediated cleavage of partially homologous host-transcripts inducing plant symptoms. The structured viroid mature-forms are resistant to RNA silencing-mediated degradation. The possibility that endogenous mRNAs could generate compacted secondary structures to modulate their susceptibility to sRNA-mediated degradation is also represented. At the transcriptional level, it was shown that vd-sRNAs induce the DNA methylation in transgenic *Nicotiana benthamiana* plants expressing PSTVd-cDNA. The different aspects regarding these mechanisms are broadly explained in the text. vd-sRNAs, viroid-derived sRNAs; CC, companion cells; SE, sieve elements; AGO, Argonaute.

transcripts (acting as RNA-intermediates) could mediate the transference of DNA between mitochondrial and plastid genomes in carrot (*Daucus carota*) (Iorizzo *et al.*, 2012), suppose that the subcellular-traffick of endogenous *ncRNAs* could be a non-rare event in plants.

Although the cell components and the functional mechanism directing the specific nuclear and/or chloroplastic localization remain to be elucidated, we considered that such approaches, supported in the host–viroid interaction emerge as a starting point to provide insights into the poorly understood mechanism of *lncRNA* compartmentalization in plant cells.

lncRNA processing

After completing the compartmentalization step, viroids should subvert the plant cell machineries involved in RNA processing to guarantee their replication during a process comprising three sequential host-mediated events: (1) transcription; (2) cleavage; (3) ligation.

Earlier works have shown that representative *Pospiviroidae* can redirect RNA Polymerase II activity to transcribe an *lncRNA* template instead of DNA (Mühlbach & Sänger, 1979; Flores & Semancik, 1982), thus providing preliminary evidence that viroid

replication would emerge as a nonconventional cellular process. Next, by using PSTVd as a model, it has been suggested that *Pospiviroidae* cleavage is mediated by a host type-III RNase, which recognizes and cuts a quasi-double-stranded RNA structure produced during nuclear viroid transcription (Gas *et al.*, 2007). Finally in more recent work, the same group has shown that, in the final replication process step, PSTVd transcripts are able to reprogram host DNA ligase 1 to act as a true *lncRNA* ligase that catalyzes their circularization (Nohales *et al.*, 2012a). These results compose a scenario in which *Pospiviroidae* emerge as RNA parasites that are able to recruit, subvert and redirect DNA-dependent factors to promote their accumulation in infected cells (Nohales *et al.*, 2012a). Moreover, it is possible to speculate that the unexpected activities for both RNA polymerase II and DNA ligase 1 represent only a few pieces of a much more complex puzzle of the endogenous-*lncRNA* metabolism in plants that were brought to light by studying viroid replication.

Avsunviroidae are the unique pathogenic RNAs known to replicate in chloroplasts. Plastids of flowering plants contain at least two types of RNA polymerases (RNAPs): one is the plastid-encoded eubacteria-type RNAP (PEP) and the other is the nuclear-encoded phage-type RNAP (NEP). Strong evidence from differential ABSVd transcription assays supports the involvement of the NEP in the transcription of chloroplastic viroids (Navarro *et al.*, 2000). Other data, however, obtained by *in vitro* transcription using *Escherichia coli* RNAP holoenzyme, suggest that the involvement of PEP in PLMVd replication should not be excluded (Pelchat *et al.*, 2001, 2002). Cleavage of the RNA intermediates is autocatalytic and mediated by the hammerhead ribozymes embedded in the viroid genome. However, the identification of a chloroplast protein (PARBP33) that is able to bind ABSVd *in vivo* and to enhance their cleavage *in vitro* suggests that specific host factors can modulate this step in viroid replication (Daròs & Flores, 2002). The finding that the *Avsunviroidae* form ribozymes to catalyze the self-cleavage has attracted much attention to these *lncRNAs* and has bolstered their search in other organisms. The discovery in *Arabidopsis* of two transcripts (*Ara1* and *Ara2*) with predicted ribozyme-like structures, cleavage activity *in vitro* and showing tissue-specific accumulation suggests that this class of catalytic *lncRNAs* can exist in plants (Przybilski *et al.*, 2005). Finally, recent findings obtained using combined *in vitro* and *in vivo* assays have allowed to propose that a chloroplastic isoform of the plant tRNA ligase is the host component mediating the circularization of linear intermediates during *Avsunviroidae* replication (Nohales *et al.*, 2012b). It is significant to note that *tRNAs* are a class of structural housekeeping *lncRNAs*, hence the demonstration that *Avsunviroidae* recruits a cell factor involved in the processing of endogenous *lncRNAs* offers perspectives on using a viroid–host model to help to understand some regulatory mechanisms directed by *lncRNAs* in plants.

Long-distance *lncRNA* trafficking

Currently, it is accepted, that besides assimilates and other nutrients the phloem transports systemic 'signals' thought to be involved in regulating numerous aspects of plant development (Atkins *et al.*, 2011), supporting a new paradigm whereby proteins and RNAs may operate as signaling complexes within the vascular system to form a long-distance regulatory network (Lough & Lucas, 2006; Banerjee *et al.*, 2009; Ham *et al.*, 2009; Li *et al.*, 2011). Among the different RNA types that have been shown to move through the phloem both coding (mRNAs; Hannapel, 2010) and noncoding (sRNAs; Buhtz *et al.*, 2010) RNAs have been described. To date, there are not experimental data supporting the systemic signaling mediated by *lncRNAs*. However, the fact that this evidence has not been obtained yet, does not mean that *lncRNAs* could not be systemically transported to exert their functions in different tissues where they are synthesized. In this scenario, the significant progress made in the knowledge of the intercellular and vascular movement of the viroid–RNA can shed light on the structural requirements of plant-endogenous *lncRNAs* for vascular entry and systemic trafficking (Wang & Ding, 2010). Studies on viroid–host interactions have revealed that diverse RNA motifs mediate the traffic of viroid–RNA from bundle sheath to mesophyll cells or from bundle sheath to phloem (Zhong & Ding, 2008). The same authors expose that structurally conserved similar motifs in rRNAs have been demonstrated to serve as protein-binding sites. Thus, it is reasonable to suppose that identifying the cell components responsible for the viroid trafficking may help us to understand the potential systemic regulation processes directed by *lncRNAs* that are expected to exist in plants (Poltronieri & Santino, 2012). Phloem protein 2 from cucumber (CsPP2) was the first host factor reported to bind *Hop stunt viroid* (HSVd) (Gómez & Pallás, 2001) and PSTVd (Owens *et al.*, 2001) RNAs *in vitro* and was proposed as a potential candidate to mediate long-distance viroid-trafficking in plants. In subsequent studies, it was demonstrated that viroid–CsPP2 complexes exist in the phloem exudates of the infected plant and are translocated from the stock to scions in grafts assays, which reinforce the involvement of CsPP2 in the systemic movement of HSVd (Gómez & Pallás, 2004). Following this experimental approach, two translocatable proteins (CmelLec17 and Cmel14) capable of binding both HSVd and *Avocado sun blotch viroid* (ABSVd) RNA were identified in melon phloem exudates (Gómez *et al.*, 2005). In ongoing studies using HSVd as a probe, we have observed the existence of RNA-phloem protein complexes in representative members of the family *Cucurbitaceae* (M. Totosa, V. Pallás, & G. Gómez, unpublished data), thus suggesting that the *lncRNA*-binding proteins are, at least in cucurbits, common phloem components.

In order to maintain their global homeostasis, plants have evolved a phloem-mediated long-distance RNA-signaling pathway implicated in the noncell autonomous regulation of diverse developmental processes. Recently, it has been shown that the tissue-specific trafficking and accumulation of transcript BEL5 (involved in tuber induction in potato) are controlled by its 3′ untranslated region, which reveals that essential aspects of this signaling network may be controlled in *cis* by RNA sequences lacking protein-coding capacity (Hannapel, 2010). Unraveling the mechanisms controlling viroid trafficking may provide

insights into a better understanding of the key aspects of the *lncRNAs*-directed regulatory mechanisms in this vascular information way.

lncRNA-directed gene expression

Accumulating evidence supports the idea that *lncRNAs* can exploit a wide range of mechanisms to modulate the gene expression (Wang & Chang, 2011), playing for example, a relevant role as precursor of sRNAs that once loaded into Argonaute (AGO) proteins function as key effectors in both transcriptional (Wierzbicki, 2012) and post-transcriptional (Röther & Meister, 2011) gene regulation. One of the mechanisms controlling genome activity at transcriptional level is known in plants as RNA-directed DNA methylation (RdDM). In this process *lncRNAs* give rise to siRNAs that target complementary genomic regions mediating the establishment of DNA methylation and histone modifications. These chromatin modifications repress subsequent transcription thus preventing gene expression within regulated regions (Wierzbicki, 2012). However, if the targeted molecule is RNA, there can be post-transcriptional gene silencing via RNA cleavage, translational repression or mRNA destabilization in a process known as post-transcriptional RNA silencing (Melnyk *et al.*, 2011).

Although the basis of viroid pathogenesis remains unclear, this process could be envisioned as a result of alterations in plant gene expression induced by viroid interference in *lncRNAs*-directed regulatory networks at both the transcriptional and post-transcriptional levels. Perhaps the most representative example of this issue can be found in the discovery of the RNA-directed DNA-methylation (RdDM) phenomenon. This *lncRNA*-directed mechanism, which controls plant genome activity by means of transcriptional silencing (Wierzbicki, 2012), was first observed when studying viroid infection in PSTVd-expressing transgenic tobacco plants (Wassenegger *et al.*, 1994). In this work full and partial-length PSTVd cDNAs were introduced into the tobacco genome via the Agrobacterium-mediated transformation. Southern blot analysis revealed that after viroid infection plant-integrated PSTVd-specific sequences become fully methylated, whereas the flanking genomic plant DNA remain unaltered. These findings demonstrated that in the infected plants exists a sequence-specific mechanism of '*de novo*' methylation of genes induced by these pathogenic *lncRNAs*. Subsequent studies have revealed that diverse genes exhibit transcriptional alteration during infection (Itaya *et al.*, 2002; Tessitori *et al.*, 2007), which is further evidence to support the existence of a link between viroid pathogenesis and transcriptional regulation in plants.

The possibility that viroid-derived small RNAs (vd-sRNAs) can guide the post-transcriptional silencing of host mRNAs, inducing metabolic alterations phenotypically expressed as symptoms, was initially exposed in 2001 (Papaefthimiou *et al.*, 2001) and later extended (Wang *et al.*, 2004; Gómez *et al.*, 2009). Two lines of evidences obtained in both the *Pospiviroidae* and *Avsunviroidae* families support this hypothesis. First, by using graft inoculation assays it was shown that in HSVd-infected *Nicotiana benthamiana* plants, the symptoms expression is dependent on *RDR6* (Gómez *et al.*, 2008), this being a key component of many aspects of RNA silencing mainly involved in the biogenesis of small RNAs. More recently, Navarro *et al.* (2012) determined that two PLMVd-derived sRNAs direct the cleavage of the mRNA encoding the chloroplastic heat-shock protein 90 (cHSP90) in infected peach plants, thus implicating RNA silencing in the modulation of the host gene expression by a viroid. In addition, it has been recently shown that siRNAs derived from the Y-sat (a *ncRNA* satellite of the *Cucumber mosaic virus* – CMV) regulate the accumulation of a host-transcript in infected *N. tabacum* plants (Shimura *et al.*, 2011).

A yet unexplored way to explain the modulation of the host gene expression exerted by these pathogenic *lncRNAs* could be the possibility that the linear viroid-transcripts generated at some stage in replication can act as *molecular sponges* that are able to capture and inactivate partially homologous plant-endogenous sRNAs in a process similar to the described for the fine regulation of *PHO2* mRNA by hybrid miR399/*lncRNA-IPS1* in response to phosphate starvation in *Arabidopsis* (Franco-Zorrilla *et al.*, 2007). Another potential point connecting viroid biology and gene expression control arises from the demonstration that their highly structured mature forms are resistant to RNA silencing-mediated degradation (Gómez & Pallás, 2007; Itaya *et al.*, 2007). These observations permit us to hypothesize about the possibility that, by means of changes in their structural complexity, plant-endogenous RNAs can regulate their susceptibility to RNA silencing, thus revealing a potential strategy to the RNA-directed post-transcriptional modulation of gene expression in plants.

Overview

By assuming that *lncRNAs* play an important role as cell biology ribo-regulators, the next frontier is the functional characterization of the complex interplay between *lncRNAs* and their regulatory targets. This conceptual upheaval requires heterodox viewing and the implementation of novel research models. At least, three possible functional characteristics should be fulfilled by these intriguing transcripts, these being: synthesis and processing; specific sub-cellular localization and long-distance trafficking; and the ability to modulate gene expression. Keeping in mind that it is necessary to be cautious when assuming functional parallelisms, we expected that the study of the viroid–host interaction focusing on plant cell biology could provide useful tools to satisfy these requirements. Altogether, the earlier results support the notion that viroids can be used as molecular lanterns that could contribute to shed light on, at least in part, the dark *lncRNA*-directed mechanism involved in the fine regulation of plant cell biology. In our opinion, the plant–viroid interaction emerge as a highly tractable model that can contribute to tackle the challenge of unraveling how *lncRNAs* control fundamental aspects of plant physiology, such as development and stress response.

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