Viroid-host interactions: A molecular dialogue between two uneven partners

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Introduction

In terms of genome size, some plants are in the upper limit of the biological scale. In the other limit and in a certain way at the frontier of life are viroids, the minimal autonomous RNA replicons with a genome ten-fold smaller than that of the smallest known viral RNA. Despite being only composed by a tiny, circular, single-stranded RNA of 246-401 nt without mRNA activity, viroids store sufficient information to infect certain plants, to manipulate their genetic expression for producing a viroid progeny, and as a side effect to induce in most cases specific diseases (see for a review Diener, 2003). Due to their lack of protein coding ability, viroids are essentially parasites of the transcriptional machinery of their hosts, while viruses mostly parasitize the translational machinery. Therefore, viroid-host interactions display unique properties. Here we will focus on those underlying viroid invasion and pathogenesis, trying to frame them into the general scheme of RNA silencing, but for a better understanding we will first summarize how viroids replicate.

Subcellular localization and replication

The approximately 30 known viroids are classified into two families: *Pospiviroidae,* type species *Potato spindel tuber viroid* (PSTVd) (Diener, 1972, Gross et al. 1978), and *Avsunviroidae,* type species *Avocado sunblotch viroid* (ASBVd) (Hutchins et al. 1986). PSTVd and ASBVd replicate (and accumulate) in the nucleus and the chloroplast, respectively, with the available data indicating that the other members of both families behave as their corresponding type species. Additional criteria supporting this classification include the presence of a central conserved region in members of the family *Pospiviroidae*, and of hammerhead ribozymes in those of the family *Avsunviroidae* (which, therefore, are catalytic RNAs) (see for a review Flores et al. 2005).

In striking contrast to plant viruses that encode proteins mediating their replication and movement, viroids depend essentially on host factors for these purposes. Viroids replicate through an RNA-based rolling circle mechanism with three steps that, with some variations, are repeated for both polarity strands: i) synthesis of oligomeric strands by a host RNA polymerase that reiteratively transcribes the incoming circular template, to which the plus polarity is arbitrarily assigned, ii) processing to unit-length, which in the family *Avsunviroidae* is mediated by hammerhead ribozymes, and iii) circularization by an RNA ligase or by self-ligation (Branch and Robertson, 1984; Daròs et al. 1994). Remarkably, viroids modulate the template specificity of certain nuclear or chloroplastic DNA-dependent RNA polymerases to accept RNA templates (Muhlbach and Sänger, 1979; Navarro et al. 2000).

Movement

Following entry into the first cells that eventually become infected, viroids must recruit host proteins for intracellular, cell-to-cell and long-distance trafficking. Most studies in this area have been performed with PSTVd or *Hop stunt viroid* (HSVd), another member of the family *Pospiviroidae* infecting cucumber that is a convenient experimental system for this purpose. Whereas a specific and saturable receptor mediates the cytoskeleton-independent import of PSTVd plus strands into the nucleus, where processing to the final circular forms presumably occurs, there are no data on how members of the family *Avsunviroidae* travel across the chloroplast membrane. PSTVd seems to have a specific motif for transport through plasmodesmata because, when injected into mesophyll cells, moves cell-to-cell rapidly; members of the family *Avsunviroidae* most likely also follow

this route. Regarding long-distance movement, results with PSTVd and HSVd indicate that they are translocated through the phloem forming complexes with phloem proteins, and that this trafficking is sustained by viroid replication and governed by viroid RNA motifs and plant development (see for a review Ding et al. 2005). Mutational analysis of PSTVd has identified a motif that mediates its efficient trafficking from the bundle sheath into the mesophyll of *Nicotiana tabacum.* This motif, which is not required for trafficking in the reverse direction or between other cell types, is dependent on leaf developmental stages (Qi et al. 2004).

In situ hybridization of sections of PSTVd-infected tomato has revealed viroid signals in the vascular tissues as well as in other cells in the subapical region, but not in the shoot apical meristem (SAM) (Qi and Ding, 2003). This suggests that the surveillance system that restricts the entry of most RNA viruses into the SAM (Foster et al. 2002), also limits the PSTVd access to this plant compartment. However, the picture emerging from parallel studies with *Peach latent mosaic viroid* (PLMVd) (Hernández and Flores, 1992), a member of the family *Avsunviroidae,* appears very different. Certain PLMVd variants, exemplified by PC-C40, contain an insertion of 12-13 nt folding into a hairpin capped by a U-rich loop that is responsible for a variegated-albino phenotype known as peach calico (PC) (Malfitano et al. 2003; Rodio et al. 2006). Albino sectors of leaves infected with variant PC-C40 present malformed palisade cells and plastids resembling proplastids, in which processing of plastid rRNAs is impaired, thus blocking translation of plastid-encoded proteins. Northern-blot and *in situ* hybridizations have also revealed that PLMVd replicates in the albino leaf sectors and, most importantly, that it can bypass the RNA surveillance system, invade the SAM and induce alterations in proplastids (M.E. Rodio, S. Delgado, A. De Stradis, M.D. Gómez, R. Flores and F. Di Serio, unpublished data).

The involvement of the host RNA-dependent RNA polymerase 6 (RDR6) in restricting systemic spread of certain RNA viruses and precluding their invasion into the SAM has been recently documented in *N. benthamiana* plants in which RDR6 has been silenced (RDR6i plants) (Schwach et al. 2005; Qu et al. 2005). RDR6 is a key component of the RNA silencing pathway (see below), which has been implicated in an amplification circuit leading to the generation of the double-stranded RNA precursors of the secondary small interfering RNAs (siRNAs). Northern-blot and *in situ* hybridizations of wild-type and RDR6i plants inoculated with PSTVd indicate that an RDR6-based mechanism could also restrict systemic spread and accumulation of PSTVd (F. Di Serio, A.E. Martínez de Alba and R.

Flores, unpublished data). How members of the family *Avsunviroidae* evade this surveillance system remains an intriguing issue.

Pathogenesis

Lacking protein-coding capacity, the primary pathogenic effect of viroids must be exerted through the direct interaction of their genomic, or some viroid-derived RNAs, with a host protein or nucleic acid. Until recently, the genomic viroid RNA was regarded as primary pathogenic effector. Supporting this view, dissection of viroids genomes led to the identification of molecular determinants modulating their virulence. Such determinants have been mapped for PSTVd and other members of the family *Pospiviroidae* at small motifs within specific domains of their rod-like secondary structure (Gross et al. 1981; Visvader and Symons, 1986; see for a review Flores et al. 2005), or at loops and specific insertions in the branched conformation of representative members of the family *Avsunviroidae* like *Chrysanthemum chlorotic mottle viroid* (CChMVd) (Navarro and Flores, 1997; De la Peña et al. 1999) and PLMVd (see above). These determinants, by themselves or by inducing changes in the three-dimensional structure of the viroid RNA, could mediate specific RNA-protein interactions that may be the primary cause of viroid pathogenicity.

In the last few years, however, a new paradigm has emerged proposing that viroid symptoms could result from RNA silencing effects down-regulating the expression of certain host genes (Papaefthimiou et al. 2001; Wang et al. 2004). This hypothesis is based on the identification in plants infected by members of both families of viroid-specific small (21-24 nt) RNAs with the characteristic structural properties of the siRNAs (Papaefthimiou et al. 2001; Itaya et al. 2001; Martìnez de Alba et al. 2002) and, even if lacking conclusive evidence, it is intriguing because the same mechanism could mediate pathogenesis in both viroid families as well as in certain satellite RNAs (Wang et al. 2004). The hypothesis links the pathogenic effects elicited by viroids and some satellite RNAs with RNA-mediated post-transcriptional gene silencing (PTGS) (see for reviews Baulcombe, 2004; Brodersen and Voinnet, 2006). In the proposed mechanism, the viroid-specific siRNAs, generated by an enzyme of the RNase III class (Dicer, Dicer-like in plants) acting on the dsRNA replicative intermediates, the dsRNAs generated by RDR6 or the highly structured genomic RNA, mimic the effect of microRNAs (miRNAs) (Papaefthimiou et al. 2001; Wang et al. 2004). This special class of endogenous small regulatory RNAs guide a second RNA silencing key component, the RNA-induced silencing complex (RISC), for

degradation or translation arrest of host mRNAs (see for a review Kidner and Martienssen, 2005). The targeted host mRNAs, if they actually exist, remain to be identified (Flores et al. 2005). Furthermore, implicit in this hypothesis is the existence of siRNAs derived from the specific determinants of pathogenicity; these siRNAs, however, are not particularly abundant in the population of siRNAs recently cloned and sequenced from two representative members of the family *Pospiviroidae* (Itaya et al. 2007; Martín et al. 2007). Therefore, whether the primary effector of pathogenesis is the genomic RNA or the siRNAs, is still an open question.

Cross-protection

Observations indicating that pre-inoculation with a mild strain of a viroid confers resistance against subsequent challenge inoculation with a severe strain of the same or a closely-related viroid —the typical symptoms of the second strain are suppressed or attenuated for a certain time— were reported even before the fundamental differences between viroids and viruses were uncovered. These observations were then regarded as additional examples of cross-protection between viruses, in which phenomena of this class had been described previously. Protection against a virus can also be afforded by the transgenic expression of intact or truncated viral proteins, and of non-protein coding RNA sequences from the viral genome (protein- and RNA-mediated pathogen-derived resistance, respectively) (see for a review Hull, 2002). Because virus RNA-mediated cross-protection and PTGS are mechanistically related (Ratcliff et al. 1999), it is possible that cross-protection between viroids, which must be RNA-mediated because they lack protein-coding capacity, may also involve a PTGS mechanism. In this scenario, viroid cross-protection may be explained if it is assumed that the DCL-generated siRNAs from the pre-inoculated mild strain load and guide RISC for degrading the RNA of the challenging severe strain. This scheme is particularly attractive because provides a common interpretation for the cross-protection effects reported in members of both viroid families (Nibblett et al. 1978; De la Peña et al. 1999). However, additional studies are needed to proof that viroid cross-protection and PTGS are indeed mechanistically related.

Synergism

In contrast with the interfering effects observed between closely-related viroids infecting the same host, co-inoculation with two distantly-related viroids may result in a synergistic interaction leading to symptoms more

severe than those expected for purely additive effects of the two viroids (P. Serra, C.J. Barbosa, J.A. Daròs, R. Flores and N. Duran-Vila, unpublished data). Similar synergistic interactions between viruses have been known for a long time, and recently interpreted as the outcome of the combined action of the protein suppressors they encode to counteract the RNA silencing defensive response of their hosts. Because RNA silencing also controls plant development through endogenous miRNAs and siRNA, and because the defensive and the developmental pathways share common components, co-infection with two distinct viruses may lead to exacerbate symptoms as a result of their silencing suppressors impairing different steps of the RNA silencing pathways (see for a review MacDiarmid, 2005). A parallel explanation cannot be extrapolated to synergism between viroids because, lacking any mRNA activity, they do not encode silencing suppressors. However, certain plant RNA viruses not encoding protein suppressors do suppress RNA silencing as a consequence of sequestering for its replication host enzymes involved in the biogenesis of the siRNAs and miRNAs, the final effectors of RNA silencing (Takeda et al. 2005). Viroids may similarly interfere with the RNA silencing machinery of their hosts, with the synergistic effects of two unrelated co-infecting viroids arising from usurping for their replication more than one component of this machinery, thus compromising its normal physiological role. It is therefore possible that viroids, like viruses, may be suppressors of the RNA silencing machinery of their hosts, and that this machinery may mediate not only cross-protection but also synergism.

Evolution of structure

The finding of viroid-specific small RNAs structurally similar to typical siRNAs in plants infected by members of the two families, supports the view that viroids are inducers and targets (and perhaps even suppressors, see above) of the RNA silencing defensive response of their hosts. The strong secondary structure of the genomic viroid RNAs, resembling that of miRNA precursors, is the feature that most likely renders them susceptible to DCL as inferred from the observation that most of the siRNAs from two members of the family *Pospiviroidae* are of plus polarity (Itaya et al. 2007; Martín et al. 2007). The dsRNA replicative intermediates and the dsRNAs generated by RDR6 are potential sources of additional primary and secondary siRNAs, respectively. Are these RNAs *bona fide* siRNAs? PSTVd- and HSVd-specific siRNAs guide RISC to cleave viroid RNAs fused to mRNA reporters, although the mature viroid RNA transfected to *Nicotiana benthamiana*

protoplasts or expressed transgenically in this species is resistant to RISC-mediated degradation (Vogt et al. 2004; Gómez and Pallás, 2007; Itaya et al. 2007). However, other *in planta* experiments show that co-delivery of representative members of both families with their homologous dsRNAs (and in some cases with their homologous siRNAs generated *in vitro),* or co-agroinfiltration with hairpin constructs have negative effects on infectivity, suggesting that at least in some instances viroids are targeted by RISC (A. Carbonell, A.E. Martínez de Alba, R. Flores and S. Gago, unpublished data). It is thus conceivable that the need to evade their host RNA silencing machinery may have shaped viroid structure and evolution (Wang et al. 2004). More specifically, viroids could have evolved their secondary structure as a compromise between resistance to DCL and RISC, which act preferentially against RNAs with compact and relaxed secondary structures, respectively.

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