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In memoriam of Ricardo Flores: The career, achievements, and legacy of an inspirational plant virologist

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ABSTRACT

Ricardo Flores (1947–2020) focused his research on the identification, replication, pathogenesis, and evolution of viroids, the minimal non-protein-coding circular RNAs (250–400 nt) able to replicate and incite diseases in plants that are remarkable for being at the lowest step of the biological scale. He and his collaborators initially identified and characterized additional group members, adding six new ones to the family *Pospiviroidae*, and expanding the *Avsunviroidae* from one to four members. They showed that members of the second family "encode" ribozymes, a property that, together with others, makes them candidates for being the most primitive replicons that emerged on our planet 3500 million years ago. He also made important contributions regarding how viroids replicate, providing relevant data on the templates, enzymes, and ribozymes that mediate this process and on the mutation rate, which turned out to be the highest reported for any biological entity. More recently, he concentrated on the role that RNA silencing could play on viroid-host interactions, describing details of this process. Ricardo also worked on citrus tristeza virus, a widely different type of subcellular pathogen, and made important contributions on the structure, localization and functions of its unique p23 protein. His research has produced 170 original articles and reviews, according to Web of Science. He encouraged the scientific careers of a large number of researchers, and collaborated with many others, some of whom have recapitulated his scientific legacy in this review and contributed with other chapters in this special issue.

1. Introduction

Ricardo Flores, Research Professor at the Institute for Plant Molecular and Cell Biology (IBMCP), a research Center funded by both the Polytechnic University of Valencia (UPV) and the Spanish National Research Council (CSIC) passed away on December 20, 2020. This special issue of Virus Research, for which Flores was member of the Editorial Board from 2012 until 2020, is dedicated to his memory and his outstanding contributions in the field of plant virology. These were particularly relevant in the world of viroid RNAs, but also in the molecular biology of a very different microorganism, citrus tristeza virus (CTV), causing the most important viral disease of citrus. Ricardo was

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member of the Editorial Board of eight more journals (RNA Biology, 2004–2013; Frontiers in Microbiology, 2012–2020; Frontiers in Plant Science, 2015–2020; Archives of Virology, 2003–2008; Viruses, 2014–2020; Molecular Plant Pathology, 2003–2008; Journal of Plant Pathology, 2003–2020; Helyion-Elsevier, 2018–2020). He was Vice-president of the Spanish Society for Virology (2007–2013) and Honorary Member of the Hungarian Academy of Sciences (2007). Ricardo was not only an excellent researcher, but his curiosity also encompassed very diverse aspects of History and Art, which were his favorite subjects at coffee gatherings. This chapter, written by most of his PhD students, describes the main achievements of this inspirational scientist in the viroid RNA world.

2. The early years (1947–76)

Ricardo Flores was born in Almoradí, a small town of the Alicante province (Spain), and studied at the Jesuits' school of Alicante. He graduated in Agricultural Sciences in 1971 at UPV and two years later, he obtained his degree in Chemistry at the University of Valencia. With this background and dual vision, he joined the Institute of Agricultural Chemistry and Food Technology (IATA), created in 1966, a CSIC center, to develop his PhD research on CTV. Eduardo Primo Yúfera, the first Director of IATA favored the development of Biochemistry, a discipline that was still emerging in Valencia. Ricardo Flores combined enthusiasm and intellectual rigor to initiate this new biochemical approach in the Institute and studied the nucleoprotein particles associated with the tristeza disease of citrus (Flores et al., 1975), the main problem of the Valencian citrus industry at the time.

3. The postdoctoral years (1976–77, 1981)

After obtaining, under supervision of Prof. Vicente Conejero, his PhD degree in 1975, he got a post-doc position in the laboratory of Joseph S. Semancik at the University of California, Riverside, to study citrus exocortis viroid (CEVd), a viroid causing a serious disease to citrus grafted on sensitive rootstocks. In Riverside, he studied the properties of a cell-free system for the synthesis of CEVd (Flores and Semancik, 1982). Back in Valencia, he established his laboratory at the recently established Plant Molecular and Cellular Biology Unit of the IATA. His laboratory, together with those of Prof. Vicente Conejero at UPV and Dr. Nuria Durán-Vila at the Valencian Agricultural Research Institute (IVIA), would constitute a powerful nucleus of research on viroids in Spain, subsequently becoming world reference laboratories. In 1992 Ricardo was part of the founding team of the IBMCP, where he developed his research until his death. Many PhD students and post-docs had the opportunity of being involved in multifaceted and complex studies on viroids. He also established a permanent collaboration with the Virology, Molecular Biology and Plant Transformation laboratories of the IVIA to resume his former research on CTV. The main achievements on this topic are also discussed here.

4. Detecting and characterizing new viroids and viroid-like RNAs. The French and Italian connection

A relevant part of the work led by Ricardo Flores was focused on the detection and description of new viroid and viroid-like RNAs. This was a non-negligible challenge in the 80′s and 90′s when this kinds of projects started, since powerful and currently routine techniques, such as PCR (polymerase chain reaction) or NGS (next generation sequencing), were either not available or, at least, not yet affordable. With enthusiasm as the main tool, he encouraged his tiny team to become fishers in the small RNA world. Thanks to this initiative, a plethora of new viroid and viroid-like molecules came to light, starting with the early characterization, in the mid-80 s, of distinct isolates of CEVd, hop latent viroid (HLVd) or avocado sunblotch viroid (ASBVd) (Flores et al., 1985; García-Arenal et al., 1987; Pallas et al., 1987, 1988; Pallas and Flores, 1989), three entities already known at the time, and ending with *de novo* detection and sequencing of portulaca latent viroid in 2015 (Verhoeven et al., 2015). In this period, the work of his successive teams highly contributed to expand (and revisit) viroid phylogeny through reports on new members of the two viroid families, *Pospiviroidae* (nuclear viroids with a central conserved region and lacking ribozymes) and *Avsunviroidae* (chloroplastic viroids with hammerhead ribozymes and devoid of the central conserved region). This line of research resulted in the initial identification of peach latent mosaic viroid (PLMVd) (Hernández and Flores, 1992), pear blister canker viroid (PBCVd) (Hernández et al., 1992b), apple dimple fruit viroid (ADFVd) (Di Serio et al., 1996), chrysanthemum chlorotic mottle viroid (CChMVd) (Navarro and Flores, 1997), eggplant latent viroid (Fadda et al., 2003a), pepper chat fruit viroid (Verhoeven et al., 2009) and dahlia latent viroid (DLVd) (Verhoeven et al., 2013). Some viroid-like RNAs were also identified for the first time from carnation and cherry, though no proof of their autonomous replication, one of the defining characteristics of viroids, was obtained (Hernández et al., 1992a; Di Serio et al., 1997, 2006). Indeed, a DNA counterpart was found for the carnation viroid-like RNAs leading to the first report of a "retroviroid-like" element (Daròs and Flores, 1995b; Vera et al., 2000). The initial characterization of all mentioned viroids and viroid-like RNAs was usually limited to a single isolate of the corresponding agent but subsequent surveys allowed determination of the primary structure of a vast array of molecular variants which extended the quasispecies concept to viroid populations (Ambrós et al., 1995, 1998, 1999; Daròs and Flores, 1995a; de la Peña and Flores, 2002; Di Serio et al., 2002; Eiras et al., 2010; Fadda et al., 2003b; Malfitano et al., 2003; Messmer et al., 2017; Minoia et al., 2014b; Rodio et al., 2006) and, in many cases, paved the way for further studies aimed at unveiling biological and functional properties of the RNA.

Some of these investigations were conducted in close collaboration with other research groups. Special mention should be made in this context to the importance that the early collaboration of Ricardo with the French researcher Jean Claude Desvignes (Center Technique Interprofessionnel des Fruits et Légumes, CTIFL, Lanxade) (Fig. 1A) and later on with the Italian researcher Antonio Ragozzino (Fig. 1B) (University of Naples Federico II, Italy), had in the discovery of viroids and viroid-like RNAs. Both, Desvignes and Ragozzino had an agronomic background with a "clinical eye" for plant symptoms. Desvignes had categorized some fruit tree diseases of unknown etiology as likely caused by a pathogen "smaller than a virus" based on graft transmission experiments. This observation together with the difficulty of eradicating those diseases by thermotherapy, made him propose a viroid as etiological agent. This proposal reached the ears of Ricardo through Gerardo Llácer, a researcher of the IVIA (Fig. 2), and their joint efforts successfully culminated with the discovery of PLMVd and PBCVd at the beginning of the 90 s (Hernández and Flores, 1992; Hernández et al., 1992a). On his side, Ragozzino had excluded the association of three peach, apple and cherry diseases observed in Italy with known viruses. Instead, preliminary assays supported the involvement of viroids. Ragozzino informed Ricardo of these findings and this initial contact turned to be the first step of a long collaboration that allowed several Italian young students and fellows to enjoy Ricardo's mentoring and guidance during their PhD and post-doctoral studies. This intense research activity resulted in the discovery of ADFVd (Di Serio et al., 1996) and the identification of some PLMVd variants containing a specific pathogenic determinant as the causal agent of peach calico disease (Malfitano et al., 2003). In addition, the above-mentioned viroid-like RNAs from cherry were also characterized in the frame of such a fruitful collaboration (Di Serio et al., 2006; Minoia et al., 2014b). This extraordinary period in which a good number of new viroids were identified and characterized allowed, in collaboration with other colleagues such as Randles (Fig. 3A), Diener (Fig. 3B) and Bar-Joseph to propose a scheme for viroid classification and nomenclature (Flores et al., 1998) and later on a reassessment of the phylogenetic relationships of viroid and viroid-like satellite RNAs (Elena et al., 2001). More recently, Ricardo also

Fig. 1. (A) Ricardo Flores with Desvignes (left), Hernández (right) and two collaborators at the center Technique Interprofessionel des Fruits et Lègumes, Prigonrieux, La Force, France in 1991. (B) With Prof. Ragozzino (middle) and Dr. Di Serio (left) at Foundation of the European Society for Virology in 2008.

Fig. 2. Ricardo, wearing a cap, between Dr. Llácer and Dr. Pallas on the XXth international symposium on virus and virus-like diseases of temperature fruit crops celebrated in Antalya, Turkey, 2006. Photo courtesy of Roberto Michelluti.

contributed to the reevaluation of the species demarcation criteria in viroid taxonomy (Chiumenti et al., 2021).

5. Replicating the non-coding viroid RNA

Ricardo was always intrigued about the question of how viroids, being exclusively constituted by small non-coding RNAs, were able to complete complex infectious cycles when they managed to enter into the appropriate host plants. All along his career, he continuously sparked this debate within his laboratory in the search of host enzymes and structures involved in viroid replication. Indeed, in his postdoctoral stay in the Semancik's laboratory, Ricardo tried to understand how the CEVd RNAs were transcribed in the nucleus of the host plant *Gynura aurantiaca*. Sensitivity to low concentrations of the mycotoxin α-amanitin supported the role of a host DNA-dependent RNA polymerase, likely DNA-dependent RNA polymerase II, acting on a viroid RNA template (Flores and Semancik, 1982). This result, which was in agreement with a previous report obtained for potato spindle tuber viroid (PSTVd) in infected tomato protoplasts (Mühlbach and Sänger, 1979), highlighted that this central enzyme of host nucleic acid metabolism was involved in viroid replication. Ricardo followed the same strategy, using mycotoxins, to learn about the host enzyme involved in RNA transcription in the case of the viroids belonging to the family *Avsunviroidae* (Marcos and Flores, 1992), which later were definitively shown to accumulate (Bonfiglioli et al., 1994; Lima et al., 1994) and replicate through a symmetric rolling-circle mechanism (Daròs et al., 1994) in the chloroplasts of infected plants (Navarro et al., 1999). In contrast to a series of chloroplast genes, low concentrations of tagetitoxin did not affect the synthesis of ASBVd RNA strands in purified chloroplasts from infected avocados. This result suggested that the nuclear-encoded polymerase (NEP), which localizes in chloroplasts and resembles phage RNA polymerases, was required in ASBVd transcription (Navarro et al., 2000). This line of research coincided with the move to the new IBMCP institute in which Ricardo consolidated a large research group (Fig. 4).

Ricardo's interest in identifying host factors involved in viroid replication cycles led to an experimental strategy based on UV cross-linking to identify proteins that bind viroid RNAs during infection. This strategy allowed him to identify two closely related chloroplast RNA-binding proteins (PARBP33 and PARBP35) that bind ASBVd RNA in infected avocado tissues. In vitro processing analysis of ASBVd transcripts in the presence of PARBP33 showed that this protein behaves as an RNA chaperone that stimulates the hammerhead ribozyme-mediated

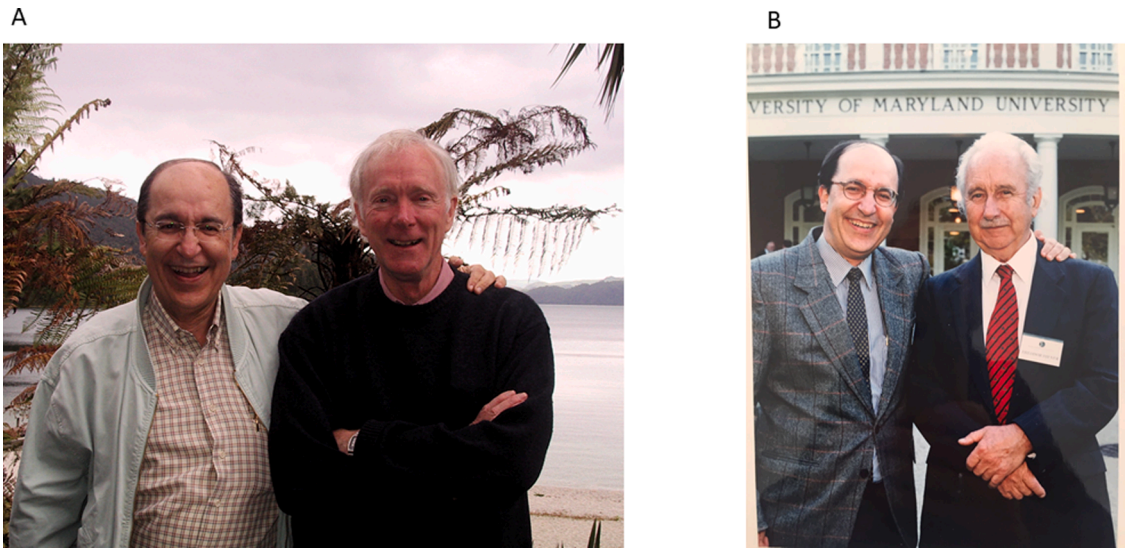

Fig. 3. (A) Ricardo and Randles at the Lake Okataina in Australasian Plant Virology Workshop in New Zealand. (B) With Diener at the University of Maryland.

Fig. 4. Ricardo and his research group at the IBMCP in 2005. Standing from left to right: Ahuir, Ricardo, de la Peña, Carbonell, Daròs, Gas and Martinez de Alba. Sitting, left to right are Delgado, Gago, Molina, and Marques.

self-cleavage. This result indicated that host proteins facilitated oligomeric ASBVd cleavage to unit-length (Daròs and Flores, 2002), despite being an RNA-based reaction. Another influential experimental system that was set up in his lab consisted of transgenic lines of the model plant *Arabidopsis thaliana* that expressed viroid dimeric transcripts (Daròs and Flores, 2004). Later, the use of this experimental system allowed characterizing the bonafide monomeric linear replication intermediate during CEVd replication, which contained 5′-phosphomonoester and 3′-hydroxyl termini. This intermediate with such terminal groups implied that a host enzyme, member of the RNase III family, was involved in the cleavage of multimeric RNAs during CEVd replication (Gas et al., 2007, 2008).

Regarding viroid replication, a mystery that really intrigued Ricardo was the identity of the enzyme involved in viroid circularization. This was a longstanding unanswered question until a combination of tomato protein chromatographic fractionation, mass spectrometry and silencing analyses allowed the identification of the tomato DNA ligase 1 as the host enzyme involved in PSTVd circularization (Nohales et al., 2012a). This result, in combination with that of DNA-dependent RNA polymerase II mentioned above, indicated that nuclear viroids managed to reprogram host DNA enzymes to act on viroid RNAs, using them as templates and substrates in a remarkable example of parasitic strategy. Coincidentally, Ricardo also participated this same year in the work that, using a combination of in vitro circularization assays and gene silencing analyses, showed the involvement of the chloroplastic isoform of tRNA ligase in the circularization of the RNAs of viroids belonging to the family *Avsunviroidae* (Nohales et al., 2012b).

6. Characterizing viroid ribozymes and other elements of tertiary structure of RNA

Catalytic RNAs or ribozymes were discovered in the 80 s, including the first small self-cleaving ribozyme, the hammerhead ribozyme (HHR). HHRs were reported in the circular (circ) RNA genomes of a plant virus satellite (Prody et al., 1986) and a viroid (Hutchins et al., 1986), indicating that self-cleaving motifs should play a key role in the replication of these minimal entities. At that time, Ricardo was mostly working with non-ribozyme containing viroids (family *Pospiviroidae*), but the existence of small ribozymes in other viroid-like entities quickly caught his attention and interest. In fact, the discovery of RNA catalysis had deep implications for the whole scientific community, offering strong support to a hypothesis for the origin of life: the RNA world. In this hypothetical world, first "living entities" would have been based on RNA acting as both the genetic material and the catalyst (Crick, 1968; Orgel, 1968; Woese, 1968), and those ancient ribozymes and RNA genomes would have remained in extant organisms. This way, the "weird" group of viroidal RNAs and their ribozymes were suddenly considered not only as a present agronomic threat but also as molecular fossils of the ancient RNA world (Flores et al., 2014, 2022).

Ricardo initially worked on the phytopathological features of the first viroid described with HHRs, the ASBVd (Marcos and Flores, 1992; Pallas et al., 1988), but later on, he became interested in the role of the ribozymes in the rolling-circle mechanism of replication (Daròs et al., 1994; Marcos and Flores, 1993). With the molecular characterization of a new HHR viroid associated with the peach latent mosaic disease (Hernández and Flores, 1992) and a circRNA with HHRs related to a carnation stunting syndrome (Hernández et al., 1992a), Ricardo's lab started a new era in the discovery of a new group of viroids and viroid-like RNAs with ribozymes. Newer examples of viroids and other circRNAs with HHRs were soon discovered and characterized in his group, such as CChMVd (Navarro and Flores, 1997), cherry small circular viroid-like RNA (csc RNA1) (Di Serio et al., 1997, 2006) or ELVd (Fadda et al., 2003a). As part of the molecular characterization of these novel agents, he always included an analysis of the in vitro self-cleavage capabilities of these RNAs, or even the role of host plant factors in ribozyme catalysis (Daròs and Flores, 2002).

Research in Ricardo's lab not only allowed a better understanding of the biology behind the ribozymes. The thorough analysis of naturally occurring HHRs vastly improved the basic knowledge of this model ribozyme. The plant-viroid system allowed both in vivo and in vitro approaches, revealing for example higher sequence flexibility in the HHR core (Ambrós and Flores, 1998). Especially fruitful was the study of CChMVd HHRs; the minus polarity ribozyme was found to harbor a pathogenicity determinant (de la Peña et al., 1999), whereas the ribozyme in the positive polarity taught us how to improve self-cleavage efficiency with a single nucleotide insertion in the HHR core (de la Peña and Flores, 2001). Studies with the ELVd ribozyme explained the evolutionary conservation of the trinucleotide sequence preceding the cleavage site (Carbonell et al., 2006). Moreover, in vitro studies in 2003 led to an unexpected discovery for a ribozyme thoroughly studied for almost 20 years. Since the HHR discovery, catalysis was analyzed using minimal variants lacking peripheral loops, but experiments using whole RNA motifs showed the key role of tertiary interactions between loops, unveiling the full catalytic power of the HHR (de la Peña et al., 2003). Structural characterization of the CChMVd loops done by nuclear magnetic resonance (NMR) analysis (Dufour et al., 2009) helped us to better understand these tertiary interactions in the HHR. All these data were crucial for the basic and applied research in the ribozyme field, and most importantly enabled the discovery of genomic HHRs across all kingdoms of life, including HHRs in human genome (Hammann et al., 2012).

The interest of Ricardo on RNA catalysis went even further, and he developed new biotechnological advances based on trans-acting ribozymes that included the tertiary stabilizing motifs (TSMs). In vitro and in vivo studies demonstrated their ability to cleave and interfere with PSTVd infection (Carbonell et al., 2011), supporting the idea that TSM-containing HHRs have potential to control pathogenic RNA replicons. In addition, the characterization performed by Ricardo's lab considering the HHRs in the context of viroid replication allowed them to be used as markers to demonstrate that these agents show the highest mutation rate reported for any biological entity (Gago et al., 2009).

Ricardo was also interested in finding interactions that could stabilize the viroid RNA and be relevant for its survival in the host. In silico predictions and natural variation identified a tertiary interaction in the CChMVd genome that is crucial for RNA folding and viroid viability (Gago et al., 2005). The conservation of similar interactions in other avsunviroids suggests that they are biologically relevant. UV cross-linking assays revealed another tertiary interaction within the PLMVd RNA, which connected the conserved residues U81 and the 3′-terminal C289. Since the initiation site of PLMVd minus-strand RNA maps at a double-stranded motif containing C289, the biological significance of this tertiary structure can be anticipated (Hernández et al., 2006).

More recently, Ricardo focussed on the whole structure of viroid genomes through SHAPE approaches (López-Carrasco and Flores, 2017a, 2017b). However, that was not enough, he really wanted to see viroids face to face, and atomic force microscopy (Moreno et al., 2019) allowed a last close sight to his long trip fellows, the viroids.

7. Viroid pathogenesis and RNA silencing

Ricardo Flores devoted major interest to the study of pathogenic processes induced by viroids. His research showed the fulfillment of Koch's postulates for several viroids, which were conclusively identified as the causal agents of plant diseases (reviewed by Di Serio et al., 2018). These kinds of studies were not limited to viroids infecting herbaceous hosts, like CChMVd (Navarro and Flores, 1997) and pepper chat fruit viroid (Verhoeven et al., 2009) that cause diseases in chrysanthemum and pepper, respectively, but were extended to several viroids infecting woody hosts, such as PLMVd (Hernández and Flores, 1992), PBCVd (Hernández et al., 1992b) and ADFVd (Di Serio et al., 2001), which were shown to be the agents of diseases in peach, pear and apple trees, respectively. The long time needed to complete these biological studies, especially in woody hosts, was never considered by Ricardo as an acceptable justification to elude this relevant step in the characterization of a viroid. In contrast, he believed that the efforts to fulfill Koch's postulates were beneficial also to develop appropriate experimental systems to further investigate the molecular mechanisms underlying viroid pathogenesis.

After early studies focusing on the possible association between the secondary structure of nuclear-replicating viroids and some pathogenic traits (Flores, 1984), Ricardo and his collaborators directed their attention to the pathogenesis induced by the chloroplast-replicating viroids CChMVd and PLMVd. The molecular determinants of severe chlorosis symptoms induced by some variants of these viroids in their respective natural hosts were mapped to a specific tetraloop in CChMVd (de la Peña et al., 1999, 2002) and to an insertion forming a short stem-loop in PLMVd (Malfitano et al., 2003). The association of variants bearing these determinants with the symptoms was studied in depth, highlighting the differential fitness and uneven distribution of symptomatic and non-symptomatic variants in the infected hosts (de la Peña et al., 2002; Rodio et al., 2006, 2007).

At the beginning of 2000, Ricardo started to investigate the role of RNA silencing in viroid-host interaction and showed that, similarly to nuclear-replicating viroids (Gómez et al., 2009; Itaya et al., 2001; Papaefthimiou et al., 2001), chloroplastic viroids are associated with viroid-derived small (s)RNAs resembling host-derived microRNAs (miRNAs) (Martinez de Alba et al., 2002), the key molecules of post-transcriptional RNA silencing. Later on, his group and others showed that viroids are both triggers and targets of RNA silencing (Carbonell et al., 2008; Di Serio et al., 2009, 2010; Gómez and Pallás,

2007; Itaya et al., 2007; Minoia et al., 2014a), and this conclusion motivated the subsequent development of different RNAi-based strategies for viroid control by several independent groups (Adkar-Purushothama et al., 2015; Carbonell and Daròs, 2017; Schwind et al., 2009).

This information was relevant showing the involvement of RNA silencing mediated by viroid-derived sRNAs as the primary cause of the pathogenic process triggered by severe PLMVd variants inducing peach calico disease (Navarro et al., 2012). The same mechanism has been more recently extended to a different chlorosis induced by other severe PLMVd variants bearing a different pathogenic determinant (Delgado et al., 2019) and to CChMVd (Serra et al., manuscript in preparation). Altogether, these data strongly support the involvement of a similar RNA silencing-based mechanism as the primary event eliciting chlorotic symptoms by several chloroplast-replicating viroids. The same initial event seems less likely in the case of symptoms induced by nuclear replicating viroids, such as the stunting and leaf curling induced by PSTVd in tomato and *Nicotiana benthamiana* plants (Flores et al., 2020; Navarro et al., 2021).

8. Not only viroids. The p23 protein of citrus tristeza virus

In 1996, Ricardo resumed his initial PhD research on CTV in close collaboration with colleagues in the IVIA and in the University of Florida, Lake Alfred. A first result of this collaboration was obtaining the full genomic RNA (gRNA) sequence of two mild CTV isolates from Spain and Florida (Albiach-Martí et al., 2000; Vives et al., 1999) that were essentially identical in spite of being separate for more than 30 years, suggesting that some virus genotypes are remarkably stable. Sequence comparisons suggested recombination events between genotypes, an important issue for CTV evolution (Martín et al., 2009). Analysis of genetic variation of the 3′ and 5′ gRNA ends revealed conservation of the first and wide variation in the second, with some isolates showing only 44% identity in their 5′UTR. All sequences studied belonged to one of three groups, with intra-group identity higher than 88% and between-group identities between 44 and 64%. However, the predicted secondary structure of the three types was very similar (López et al., 1998). This secondary structure was found critical for efficient virus replication (Gowda et al., 2003).

Because different studies suggested that the CTV-specific p23 protein likely evolved to regulate specific interactions between CTV and citrus (Flores et al., 2013), it became the main subject of Ricardo's CTV research. His laboratory showed that p23 has RNA-binding activity in a non-sequence specific mode, with the RNA-binding domain being located between amino acid (aa) positions 50 and 86, containing a zinc-finger motif and several basic aa's (López et al., 2000).

NGS and bioinformatic analyses of the sRNAs showed that CTV infection induces in citrus a strong RNA silencing reaction, with sRNAs covering the full gRNA. However, sRNA distribution was asymmetrical and presented a hotspot in the 2500 3′-terminal nucleotides, comprising the three CTV genes encoding viral suppressors of RNA silencing (VSR) (p25, p20 and p23). This sRNA distribution suggested that the DICER-LIKE (DCL) endoribonucleases 2 and 4 act on the double-stranded forms of both the gRNA and subgenomic RNAs (Ruiz-Ruiz et al., 2011).

Transgenic expression of the p23 VSR in sweet orange (CTV-susceptible) and sour orange (SO) (partially resistant) allowed CTV to escape from the phloem of both hosts, but facilitated systemic infection and increased virus titer only in SO, suggesting a differential interaction between p23 and host factors in both species (Fagoaga et al., 2011). Silencing of SO genes RNA-dependent RNA polymerase (RDR) 1, non-expressor of pathogenesis-related (NPR) genes 1, 3 and 4 and DCL2/DCL4 revealed that reduced expression of RDR1, NPR1 and DCL2/DCL4 increases CTV spread and accumulation, suggesting that both the salicylic acid-signaling and the RNA-silencing pathways are involved in SO resistance. Contrarily, silencing NPR3/NPR4 decreases CTV titer in SO, likely as a result of higher NPR1 accumulation enhancing the basal resistance (Gómez-Muñoz et al., 2017).

To investigate its subcellular localization, p23 or different mutants thereof fused with the green fluorescent protein (GFP) were agro-inoculated on *Nicotiana benthamiana*. P23 preferentially accumulated in the nucleolus and in plasmodesmata, with the nucleolar localization signal including the zinc-finger motif and some basic aa's within the 157 N-terminal residues, whereas plasmodesmatal localization requires the full 157-aa segment. Analysis of these mutants for VSR activity revealed that most of the protein regions are involved in RNA silencing. Expression of the same constructs from a potato virus X vector revealed that p23 is a pathogenicity determinant in *N. benthamiana*, with the pathogenicity motif being located in the 157 aa's at the N-terminus. Moreover, transgenic expression of p23 mutants in Mexican lime confirmed that the same p23 segment is the pathogenicity determinant in citrus (Ruiz-Ruiz et al., 2013). Constitutive expression of p23 from a mild or a moderate CTV isolate in transgenic citrus plants produced CTV-like symptoms and some non-specific aberrations, regardless of the isolate's pathogenicity characteristics. However, p23 expression under the control of a phloem-specific promoter incited only CTV-specific symptoms similar to those of the cognate CTV isolate (Soler et al., 2015), confirming that p23 is a pathogenicity protein when expressed in the phloem as in natural CTV infections.

Interaction of p23 with host factors was investigated by screening a *N. benthamiana* expression library using yeast-two-hybrid. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was identified as a potential interactor of p23, with interaction being confirmed by bimolecular fluorescence complementation (BiFC) assays. Moreover, CTV agro-inoculation on plants with reduced GAPDH expression achieved by virus-induced gene silencing showed reduced CTV accumulation, indicating that the p23-GAPDH interaction facilitates the CTV infection cycle (Ruiz-Ruiz et al., 2018). Following a similar approach, a new p23-interacting host factor has been discovered that also facilitates the infection process (Yang et al., 2021).

Searching for p23-induced CTV resistance was a last objective of Ricardo's cooperation. Transgenic lime plants expressing p23 in different constructs (sense, antisense or intron-hairpin) could not achieve full protection against CTV infection (Fagoaga et al., 2006; López et al., 2010). Contrastingly, transformation with an intron-hairpin construct carrying untranslatable versions of the three CTV VSR, yielded transgenic lines displaying full CTV resistance against the homologous isolate, but partial resistance when plants were inoculated with a heterologous CTV isolate, indicating that the resistance mechanism is sequence-dependent (Soler et al., 2012).

9. Final comments and introduction to the other chapters

Last year it was 50 years since Diener, of the U.S. Department of Agriculture (Beltsville, Maryland, U.S), discovered that the pathogenic agent of the potato spindle tuber disease was "a free RNA. . . much smaller than any viral genome", which he named viroid (Diener, 1971). Shortly afterwards, Joseph S. Semancik of the University of California (Riverside, U.S.) showed that the causal agent of the citrus exocortis disease was also a viroid (Semancik and Weathers, 1972). Only six years later Ricardo began to publish his early studies on viroids (Flores et al., 1978). Since then, with the exception of his valuable contribution to the CTV, Ricardo dedicated his whole scientific career elucidating the structure, pathogenesis and biology of these exciting small infectious RNAs. He passed on his enthusiasm to a large number of PhD students. Many of them have continued to work on viroids or in Plant Virology. This volume of Virus Research underlines the work of several of Ricardo Flores' former students as well as postdocs, and visiting scientists. This special issue updates the current knowledge on the different stages of the viroid infection cycle such as replication, movement, pathogenesis, epigenetics and interactions with host factors and highlights the value of viroid models in Virology, as a tribute to such an inspirational scientist.

CRediT authorship contribution statement

Vicente Pallas: Conceptualization, Writing – original draft. **Carmen Hernández:** Conceptualization, Writing – original draft. **Jose F. Marcos:** Writing – original draft. **Jose A. Daròs:** Conceptualization, Writing – original draft. **Silvia Ambrós:** Writing – review & editing. **Beatriz Navarro:** Writing – review & editing. **Jose A. Navarro:** Writing – review & editing. **Marcos de la Peña:** Conceptualization, Writing – original draft. **Selma Gago-Zachert:** Writing – review & editing. **Maria E. Gas:** Writing – review & editing. **Alberto Carbonell:** Writing – review & editing. **Carmelo López:** Writing – review & editing. **Angel E. Martínez de Alba:** Writing – review & editing. **Francesco Di Serio:** Conceptualization, Writing – original draft. **Pedro Moreno:** Conceptualization, Writing – original draft.

Declaration of Competing Interests

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

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