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Additional Information

In Memoriam of Ricardo Flores: The Career, Achievements, and Legacy of an inspirational plant virologist

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2

ABSTRACT

Ricardo Flores (1947-2020) focused his research on the identification, replication, pathogenesis and evolution of viroids -minimal non-protein-coding circular RNAs (250-400 nt) able to replicate and incite diseases in plants- which are 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 components. 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 in the particular case studied, 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. His research has produced 160 (WOS) original articles and reviews. He encouraged the scientific careers of a large number of researchers, some of whom have reviewed his scientific legacy in this review and contribute with other chapters in this special issue.

23 # 1. Introduction

24 Ricardo Flores, Research Professor at the Institute for Plant Molecular and Cell
25 Biology, a research center funded by both the Polytechnic University of Valencia
26 (UPV) and the Spanish Research Council (CSIC) passed away on December 23,
27 2020. This special volume of Virus Research, for which R. Flores was member of
28 the editorial board from 2012 until 2020, is dedicated to his memory and his
29 outstanding contributions in the field of plant virology, more specifically in the
30 world of viroid RNAs. Ricardo was member of the editorial board of eight more
31 journals (RNA Biology, 2004-2013; Frontiers in Microbiology, 2012-2020,
32 Frontiers in Plant Science (2015-202, Archives of Virology, 2003-08, Viruses,
33 2014-2020, Molecular Plant Pathology, 2003-08, Journal of Plant Pathology,
34 2003-2020 and Helyion-Elsevier, 2018-2020). He was Vice-president of the
35 Spanish Society for Virology (2007-2013) and Honorary Member of the
36 Hungarian Academy of Sciences (2007). Ricardo was not only an excellent
37 researcher, but also his curiosity encompassed very diverse aspects of history and
38 art, which were his favorite subjects of coffee gatherings. This chapter, written by
39 most of his PhDs, describes the main achievements of this inspirational scientist
40 in the viroid RNA world.

41 # 2. The Early Years (1947–76)

42 Ricardo Flores was born in Almoradí, a small town of the Alicante province
43 (Spain), and studied at the Jesuits of Orihuela. He graduated in Agricultural
44 Sciences in 1971 at the Polytechnic University of Valencia and two years later he
45 obtained his degree in Chemistry at the University of Valencia. This unique and
46 rich background was the key of Ricardo's many accomplishments. With this
47 background and dual vision, he joined the Institute of Agrochemistry and Food
48 Technology (IATA), created in 1966, a center of the Spanish National Research
49 Council (CSIC), to carry out his doctoral thesis. Eduardo Primo Yúfera, the first
50 Director of IATA favored the development of Biochemistry, a discipline that was
51 still incipient in Valencia. Ricardo Flores combined enthusiasm, intellectual rigor
52 and training in Agronomy to initiate this new biochemical approach in the
53 Institute and studied the nucleoprotein particles associated with the citrus
54 tristeza virus (CTV) to obtain his doctorate in 1975 (Flores et al., 1975).

55 # 3. The postdoctoral years (1976-77, 81)

56 After obtaining a PhD degree in 1975, with a dissertation on nucleoproteins
57 associated with citrus tristeza virus (CTV), he became a post-doc in the laboratory
58 of Joseph S. Semancik in Riverside, California, to study citrus exocortis viroid
59 (CEVd), a viroid causing a serious disease to citrus, the most relevant crop in his
60 native Valencia province in Spain. In Riverside, he studied the properties of a cell-
61 free system for synthesis of citrus exocortis viroid (Flores and Semancik, 1982).
62 Back in Valencia, he established his laboratory at the Plant Molecular and Cellular
63 Biology Unit of the IATA. His laboratory, together with that of Prof. Vicente

64 Conejero at the Polytechnic University of Valencia and the Dr. Nuria Durán-Vila
65 at the Valencian Agricultural Research Institute (IVIA), would constitute a
66 powerful nucleus of research in viroids in Spain, subsequently becoming world
67 reference laboratories. In 1992 Ricardo was part of the founding team of the Plant
68 Molecular and Cellular Biology Institute (IBMCP), where he developed his
69 research until the end of his days. Many PhD students and post docs had the
70 opportunity of being involved in multifaceted and complex studies on viroids.

71 4. Detecting and characterizing new viroids and viroid-like 72 RNAs. The French and Italian Connection.

73 A relevant part of the work led by Ricardo Flores was focused on the detection
74 and description of new viroid and viroid-like RNAs. This was a non-negligible
75 challenge at the eighties and nineties when this kind of projects started, since
76 powerful and currently routine techniques, such as PCR or NGS, were either not
77 available or, at best, not yet affordable. With enthusiasm as main tool, he
78 encouraged his, by that time, reduced team to pursue becoming fishers in the
79 small RNA world. Thanks to this initiative, a plethora of new viroid and viroid-
80 like molecules came to light, starting with the early characterization, in the mid-
81 80s, of distinct isolates of Citrus exocortis viroid, hop latent viroid or avocado
82 sunblotch viroid (Flores et al., 1985; García-Arenal et al., 1987; Pallás et al., 1987;
83 Pallás et al., 1988), two entities already known at the time, and ending with *de*
84 *novo* detection and sequencing of portulaca latent viroid in 2015 (Verhoeven et
85 al., 2015). In between these two periods, the work of his successive teams highly
86 contributed to expand (and revisit) viroid phylogeny through reports on new
87 members of the two viroid families, *Pospiviridae* (nuclear viroids with a central
88 conserved region and lacking ribozymes) and *Avsunviridae* (chloroplastic viroids
89 with hammerhead ribozymes and devoid of central conserved region). This line
90 of research resulted in the initial identification of peach latent mosaic viroid
91 (PLMVd) (Hernández and Flores, 1992), pear blister canker viroid (PBCVd)
92 (Hernández et al., 1992), apple dimple fruit viroid (Di Serio et al., 1996),
93 chrysanthemum chlorotic mottle viroid (Navarro and Flores, 1997), eggplant
94 latent viroid (Fadda et al., 2003a), pepper chat fruit viroid (Verhoeven et al.,
95 2009) and dahlia latent viroid (Verhoeven et al., 2013). Some viroid-like RNAs
96 were also identified for the first time from carnation and cherry, though no proof
97 of their autonomous replication, one of the defining characteristics of viroids,
98 were obtained (Hernández et al., 1992; Di Serio et al., 1997; 2006). Indeed, a DNA
99 counterpart was found for the carnation viroid-like RNAs leading to the first
100 report of a "retroviroid-like" element (Daròs and Flores, 1995; Vera et al., 2000).
101 The initial characterization of all mentioned viroids and viroid-like RNAs was
102 usually limited to a single isolate of the corresponding agent but subsequent
103 surveys allowed determination of the primary structure of a vast array of
104 molecular variants (Ambrós et al., 1995; 1998; 1999; Daròs and Flores, 1995; De
105 la Peña and Flores, 2002; Di Serio et al., 2002: Eiras et al., 2010; Fadda et al.,
106 2003b; Malfitano et al., 2003; Messmer et al., 2017; Minoia et al., 2014; Rodio et

107 al., 2006) that, in many cases, paved the way for further studies aimed at
108 unveiling biological and functional properties of the corresponding RNA.

109 Some of these investigations were conducted in close collaboration with other
110 research groups. Special mention should be made in this context to the
111 importance that the early collaboration of Ricardo with the French researcher
112 Jean Claude Desvignes (Centre Technique Interprofessionnel des Fruits et
113 Légumes, CTIFL, Lanxade) (Fig. 1A) and later on with the Italian researcher
114 Antonio Ragozzino (Fig. 1B)(University of Naples Federico II, Italy), had in the
115 discovery of viroids and viroid-like RNAs. Both, J. C. Desvignes and A. Ragozzino
116 had an agronomical background with a "clinical eye" for plant symptoms.
117 Desvignes, had categorized some fruit tree diseases of unknown etiology as likely
118 caused by a pathogen "smaller than a virus" on the basis of graft transmission
119 experiments. This observation together with the difficulty of eradicating those
120 diseases by thermotherapy, made him to propose a viroid as etiological agent.
121 This proposal reached the ears of Ricardo through Gerardo LLácer (a researcher
122 of Valencian Institute of Agrarian Research, IVIA, Fig. 2), and their joint efforts
123 successfully culminated with the discovery of PLMVd and PBCVd at the
124 beginning of the 90s (Hernández and Flores, 1992; Hernández et al., 1992). On
125 his side, Ragozzino had excluded the association of three peach, apple and cherry
126 diseases observed in Italy with known viruses. Instead, preliminary assays
127 supported the involvement of viroids. Ragozzino informed Ricardo of these
128 findings and this initial contact turned to be the first step of a long collaboration
129 that allowed several Italian young students and fellows to enjoy the Ricardo'
130 mentoring and guidance during their PhD and post-doctoral studies. This intense
131 research activity resulted in the discovery of ADFVd (Di Serio et al., 1996) and the
132 identification of some PLMVd variants containing a specific pathogenic
133 determinant as the causal agent of peach calico disease (Malfitano et al., 1993).
134 In addition, the above-mentioned viroid-like RNAs from cherry were also
135 characterized in the frame of such fruitful collaboration (Di Serio et al., 2006;
136 Minoia et al., 2014). This extraordinary period in which a good number of new
137 viroids were identified and characterized allowed, in collaboration with other
138 colleagues such as J. Randles (Fig. 3A), T.O. Diener (Fig. 3B) and M. Bar-Joseph
139 to propose a scheme for viroid classification and nomenclature (Flores et al.,
140 1998) and later on a reassessment of the phylogenetic relationships of viroid and
141 virod-like satellite RNAs (Elena et al., 2001). .

142 5. Replicating the non-coding viroid RNA

143 Ricardo was always intrigued about the question of how viroids, being exclusively
144 constituted by small non-coding RNAs, were able to complete complex infectious
145 cycles when they managed to enter into the appropriate host plants. All along his
146 career, he continuously sparked this debate within his laboratory in the search of
147 host enzymes and structures involved in viroid replication. Indeed, in his
148 postdoctoral stay in the laboratory of J.S. Semancik at the University of

149 California, Riverside, Ricardo tried to understand how citrus exocortis viroid
150 (CEVd) RNAs were transcribed in the nuclei of the host plant Gynura aurantica.
151 Sensitivity to low concentrations of the mycotoxin α-amanitin supported the role
152 of a host DNA-dependent RNA polymerase, likely DNA-dependent RNA
153 polymerase II, acting on a viroid RNA template (Flores and Semancik, 1982). This
154 result, which was in agreement with a previous report obtained for potato spindle
155 tuber viroid (PSTVd) in infected tomato protoplasts (Mühlbach and Sänger,
156 1979), highlighted that central enzymes of host nucleic acid metabolism were in
157 charge of viroid replication. Ricardo followed the same strategy, using
158 mycotoxins, to learn about the host enzyme involved in RNA transcription in the
159 case of the viroids belonging to the family Avsunviroidae (Marcos and Flores,
160 1992), which later were definitively shown to accumulate (Bonfiglioli et al., 1994;
161 Lima et al., 1994) and replicate through a symmetric rolling-circle mechanism
162 (Daròs et al., 1994) in the chloroplasts of infected plants (Navarro et al., 1999). In
163 contrast to a series of chloroplast genes, low concentrations of tagetitoxin did not
164 affect synthesis of avocado sunblotch viroid (ASBVd) RNA strands in purified
165 chloroplasts from infected avocados. This result suggested that the nuclear-
166 encoded polymerase (NEP), which localizes in chloroplasts and resembles phage
167 RNA polymerases, was required in ASBVd transcription (Navarro et al., 2000).
168 This line of research coincided with the move to a new institute in which Ricardo
169 consolidated a numerically important research group (Fig. 4).

170 Ricardo's interest in identifying host factors involved in viroid infectious cycles
171 led to an experimental strategy based on UV cross-linking to identify proteins that
172 bind viroid RNAs during infection. This strategy allowed to identify two closely
173 related chloroplast RNA-binding protein (PARBP33 and PARBP35) that bind
174 ASBVd RNA in infected avocado tissues. In vitro processing analysis of ASBVd
175 transcripts in the presence of PARBP33 showed that this protein behaves as an
176 RNA chaperone that stimulates the hammerhead ribozyme-mediated self-
177 cleavage. This result indicated that oligomeric ASBVd cleavage to unit-length,
178 despite being an RNA-based reaction, was facilitated by host proteins (Daròs and
179 Flores, 2002). Another influential experimental system that was set up in his lab
180 consisted of transgenic lines of the model plant Arabidopsis thaliana that
181 expressed viroid dimeric transcripts (Daròs and Flores, 2004). Later, the use of
182 this experimental system allowed characterizing the bonafide monomeric linear
183 replication intermediate in CEVd replication, which in contrast to expected
184 contained 5'-phosphomonoester and 3'-hydroxyl termini. This intermediate with
185 such terminal groups implied that a host enzyme, member of the RNase III
186 family, was involved in cleavage of multimeric RNAs during CEVd replication
187 (Gas et al., 2007, 2008).

188 Regarding viroid replication, a mystery that really intrigued Ricardo was the
189 identity of the enzyme involved in viroid circularization. This was a long-standing
190 unanswered question until a combination of tomato protein chromatographic
191 fractionation, mass spectrometry and silencing analyses allowed to identify

192 tomato DNA ligase 1 as the host enzyme involved in PSTVd circularization
193 (Nohales et al., 2012b). This result, in combination with that of DNA-dependent
194 RNA polymerase II mentioned above, indicated that nuclear viroids managed to
195 reprogram host DNA enzymes to act on viroid RNA templates and substrates in
196 a remarkable example of parasitic strategy. Coincidentally, Ricardo also
197 participated this same year in the work that, using a combination of in vitro
198 circularization assays and gene silencing analyses, showed the involvement of the
199 chloroplastic isoform of tRNA ligase in the circularization of the RNAs of viroids
200 belonging to the family Avsunviroidae (Nohales et al., 2012a).

201 6. Characterizing viroid ribozymes and other elements of 202 tertiary structure of RNA.

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

219 Ricardo initially worked on the phytopathological features of the first viroid
220 described with HHRs, the ASBVd, (Marcos and Flores, 1990; Pallas et al., 1988),
221 but later on, he got interested into the role of the ribozymes in the rolling-circle
222 mechanism of replication (Daròs et al., 1994; Marcos and Flores, 1993, 1992).
223 With the molecular characterization of a new HHR viroid associated to the peach
224 latent mosaic disease (Hernández and Flores, 1992) and a circRNA with HHRs
225 related to a carnation stunting syndrome (Hernández et al., 1992), Ricardo's lab
226 started a new era in the discovery of a new family of viroids with ribozymes: the
227 Avsunviroidae. Newer examples of viroids and other circRNAs with HHRs were
228 soon discovered and characterized in his group, such as CChMVd (Navarro and
229 Flores, 1997), csc viroid-like RNA (Di Serio et al., 1997) or ELVd (Fadda et al.,
230 2003b). As part of the molecular characterization of these novel agents, he always
231 included an analysis of the in vitro self-cleavage capabilities of these RNAs, or
232 even the role of host plant factors in ribozyme catalysis (Daròs and Flores, 2002).

233 But research in Ricardo's lab not only allowed a better understanding of the
234 biology behind the ribozymes. The thorough analysis of naturally occurring
235 HHRs vastly improved the basic knowledge of this model ribozyme. The plant-
236 viroid system nicely allowed both in vivo and in vitro approaches, revealing for
237 example a higher sequence flexibility in the canonical HHR core (Ambrós and
238 Flores, 1998). Especially fruitful was the study of CChMVd HHRs; the minus
239 polarity ribozyme was found to harbour a pathogenicity determinant (De la Peña
240 et al., 1999), whereas the ribozyme in the positive polarity taught us how to
241 improve self-cleavage efficiency with a single nucleotide insertion in the HHR
242 core (De la Peña and Flores, 2001). Studies with the ELVd ribozyme allowed to
243 explain the evolutionary conservation of the trinucleotide sequence preceding the
244 cleavage site (Carbonell et al., 2006). Moreover, in vitro studies in 2003 showed
245 an unexpected discovery for a ribozyme thoroughly studied for almost 20 years.
246 Since their discovery, HHR catalysis was analysed using minimal variants lacking
247 peripheral loops, but experiments using whole RNA motifs showed the key role
248 of tertiary interactions between loops, unveiling the full catalytic power of the
249 HHR (De la Peña et al., 2003). Structural characterization of the CChMVd loops
250 done by NMR analysis (Dufour et al., 2009) helped us to better understand these
251 tertiary interactions in the HHR. All these data were crucial for the basic and
252 applied research in the ribozyme field, and also derived in the discovery of
253 genomic HHRs across all kingdoms of life, including HHRs in our own genome
254 (Hammann et al., 2012).

255 But the interest of Ricardo on RNA catalysis went even further, and he developed
256 new biotechnological advances based on trans-acting ribozymes that included the
257 tertiary stabilizing motifs (TSMs). In vitro and in vivo studies demonstrated their
258 ability to cleave and interfere with PSTVd infection (Carbonell et al., 2011),
259 supporting the idea that TSM-containing HHRs have potential to control
260 pathogenic RNA replicons.

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

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

7. Viroid pathogenesis and RNA silencing.

Ricardo Flores devoted major interest to the study of pathogenic processes induced by viroids. Being the fulfilment of Koch's postulates one of the first steps in the study of pathogenesis (Di Serio et al., 2018), his research provided conclusive evidence of the viroid nature of several plant diseases. This kind of studies were not limited to viroids infecting herbaceous hosts, like CChMVd (Navarro and Flores, 1997) and PCFVd (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, 1992) 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 fulfil the 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 secondary structure of nuclear-replicating viroids and some pathogenic traits (Flores, 1984), Ricardo and his collaborators focused on 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; De la Peña et al., 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 deeply studied, 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; Rodio et al., 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 (Itaya et al., 2001; Papaefthimiou et al., 2001; Gómez et al., 2009), chloroplastic viroids are associated with viroid-derived small RNAs resembling host-derived microRNAs (miRNAs) (Martinez de Alba et al., 2002), the key molecules of post-transcriptional RNA silencing. Later on, his group showed that viroids are both triggers and targets of RNA silencing (Carbonell et al., 2008; Di Serio et al., 2009; Di Serio et al., 2010; Minoia et al., 2014), what motivated the subsequent development by several independent groups of different RNAi-based strategies for viroid control (Adkar-Purushothama et al., 2015; Carbonell and Daròs, 2017; Schwind et al. 2009).

This information was relevant to show the involvement of RNA silencing mediated by viroid-derived small RNAs as the primary cause of the pathogenic

317 process triggered by severe PLMVd variants inducing peach calico disease
318 (Navarro et al., 2012). The same mechanism has been more recently extended to
319 a different chlorosis induced by other severe PLMVd variants bearing a different
320 pathogenic determinant (Delgado et al., 2019) and to CChMVd (Serra et al.,
321 manuscript in preparation). Altogether these data strongly support the
322 involvement of a similar RNA silencing-based mechanism as the primary event
323 eliciting chlorotic symptoms by several chloroplast-replicating viroids. The same
324 initial event seems less likely in the case of symptoms induced by nuclear
325 replicating viroids, such as the stunting and leaf curling induced by PSTVd in
326 tomato and Nicotiana benthamiana plants (Flores et al., 2020; Navarro et al.,
327 2021).

328 8. Not only viroids. The p23 of Citrus Tristeza virus

329 In 1996 Ricardo Flores resumed his initial PhD research on Citrus tristeza virus
330 (CTV) in close collaboration with colleagues in Valencia (Spain) and Lake Alfred
331 (Florida). A first result of this collaboration was obtaining the full genomic RNA
332 (gRNA) sequence of two mild CTV isolates from Spain and Florida (Albiach-Martí
333 et al. 2000; Vives et al. 1999) that were essentially identical in spite of being
334 separate for more than 30 years, suggesting that some virus genotypes are
335 remarkably stable. Sequence comparisons suggested recombination events
336 between genotypes, an important issue for CTV evolution (Martín et al., 2009).
337 Analysis of genetic variation of the 3' and 5' gRNA ends revealed conservation of
338 the first and wide variation in the second, with some isolates showing only 44%
339 identity in their 5'UTR. All sequences studied belonged to one of three groups,
340 with intra-group identity higher than 88% and between-group identities between
341 44 and 64%. However, the predicted secondary structure of the three types was
342 very similar (López et al. 1998). This secondary structure was found critical for
343 efficient replication (Gowda et al., 2003).

344 Because different studies suggested that the CTV-specific p23 protein likely
345 evolved to regulate specific interactions between CTV and citrus (Flores et al.
346 2013), it became the main subject of Ricardo's CTV research. His laboratory
347 demonstrated that p23 has RNA-binding activity in a non-sequence specific
348 mode, with the RNA-binding domain being located between amino acid (aa)
349 positions 50 and 86, containing a zinc-finger motif and several basic aa (López et
350 al. 2000).

351 Deep sequencing and bioinformatic analyses of the small RNAs (sRNAs) showed
352 that CTV infection induces in citrus a strong RNA silencing reaction, with sRNAs
353 covering the full gRNA. However, sRNA distribution was asymmetrical and
354 presented a hotspot in the 2500 3'-terminal nucleotides, comprising the three
355 CTV genes encoding RNA silencing suppressors (RSS) (p25, p20 and p23). This
356 sRNA distribution suggested that the Dicer-like (DCL) ribonucleases 2 and 4 act
357 on the double stranded forms of both the gRNA and subgenomic RNAs (Ruiz-
358 Ruiz et al. 2013).

Transgenic expression of the p23 RSS in sweet orange (CTV-susceptible) and in sour orange (SO) (partially resistant) allowed CTV escaping 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 SO genes RDR1 (RNA-dependent RNA polymerase 1), NPR1, NPR3/NPR4 (non-expressor of pathogenesis-related genes 1, 3 and 4) and DCL2/DCL4, using an adequate virus vector, 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 agroexpressed in 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 within the 157 N-terminal residues, whereas plasmodesmatal localization requires the full 157-aa segment. Analysis of these mutants for RSS activity revealed that most protein regions are involved in RSS. Expression of the same constructs from a PVX vector revealed that p23 is a pathogenicity determinant in N. benthamiana, with the pathogenicity motif being located in the N-terminal 157 aa. 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 the isolate 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 detected as potential interactor with p23, with interaction being confirmed by bimolecular fluorescence complementation (BiFC) tests. Moreover, CTV agroinoculation of plants with GAPDH expression reduced 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

401 (sense, antisense or intron-hairpin) could not afford full protection against CTV
402 infection (Fagoaga et al. 2006; López et al. 2010). Contrastingly, transformation
403 with an intron-hairpin construct carrying untranslatable versions of the three
404 CTV RSS, yielded transgenic lines displaying full CTV resistance against the
405 homologous isolate, but partial resistance when plants were inoculated with a
406 heterologous CTV isolate, indicating that the resistance mechanism is sequence-
407 dependent (Soler et al. 2012).

408 ## 9. Final comments and introduction to the other chapters

409 Last year it was 50 years since Diener, of the U.S. Department of Agriculture
410 (Beltsville, Maryland, U.S), discovered that the pathogenic agent of the potato
411 spindle tuber disease was “a free RNA. . . much smaller than any viral genome”,
412 which he named viroid (Diener, 1971). Shortly afterwards, Joseph S. Semancik of
413 the University of California (Riverside, U.S.) showed that the causal agent of the
414 citrus exocortis disease was also a viroid (Semancik and Weathers, 1972). Only
415 six years later Ricardo began to publish his early studies on viroids (Flores et al.,
416 1978). Since then, with the exception of his valuable contribution to the CTV,
417 Ricardo dedicated his whole scientific career elucidating the structure,
418 pathogenesis and biology of these exciting small infectious RNAs. He passed on
419 his enthusiasm to a large number of PhD students. Many of them have continued
420 to work on viroids or in Plant Virology. This volume of Virus Research underlines
421 the work of several of Ricardo Flores’ former students as well as postdocs, and
422 visiting scientists. This special issue updates the current knowledge on the
423 different stages of the viroid infection cycle such as replication, movement,
424 pathogenesis, epigenetics and interactions with host factors and highlights the
425 value of viroid models in Virology, as a tribute to such an inspirational scientist.

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FIGURE LEGENDS

431 Figure 1. (A). Ricardo Flores with J.C. Desvignes (left), C. Hernández (right) and
432 two collaborators at the Centre Technique Interprofessionel des Fruits et
433 Lègumes, Prigonrieux, La Force, France in 1991. (B). With the Prof. A. Ragozzino
434 (middle) and Dr. F. Di Serio (left) at Foundation of the European Society for
435 Virology in 2008.

436 Figure 2. Ricardo, wearing a cap, between Dr. G. LLácer and Dr. V. Pallas on the
437 XXth international symposium on virus and virus-like diseases of temperature
438 fruit crops celebrated in Antalya, Turkey, 2006. Photo courtesy of Roberto
439 Michelluti.

440 Figure 3. (A). Ricardo and J. Randles at the lake Okataina in New Zeeland. (B)
441 With T.O. Diener at the University of Maryland.

442 Figure 4. Ricardo and his research group at the IBMCP in 2004. Standing from
443 left to right: A. Ahuir, D. Molina, E. Martinez de Alba, Ricardo, J.A. Daròs, A.
444 Carbonell. Sitting, left to right are S. Minoia, L. Covell, M.E. Gas and S. Gago.

445
446 Fig. 1.

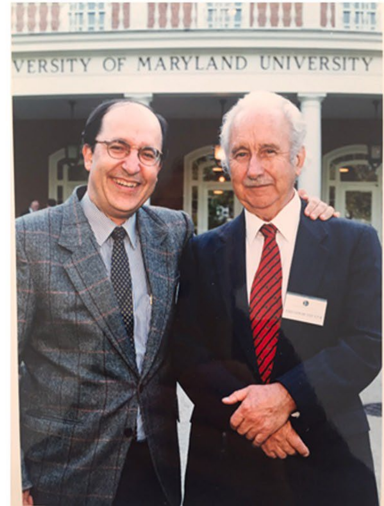
447

448 **Fig. 2.**

449

A

B



450

451 **Fig. 3.**

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454 **Fig. 4.**

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