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On the early identification and characterization of pear blister canker viroid, apple dimple fruit viroid, peach latent mosaic viroid and chrysanthemum chlorotic mottle viroid

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ABSTRACT

In the 90′s, pear blister canker viroid (PBCVd), apple dimple fruit viroid (ADFVd), peach latent mosaic viroid (PLMVd) and chrysanthemum chlorotic mottle viroid (CChMVd) were identified and characterized in the Ricardo Flores' laboratory. In these studies, the autonomous replication of these infectious RNAs and their involvement in the elicitation of diseases in their natural hosts were also shown. Their discovery was achieved by classical approaches based on the physical purification of the viroid RNAs from polyacrylamide gels followed by the sequencing of their genomic RNAs and by bioassays to assess their autonomous replication and the fulfillment of Koch's postulates. The molecular characterization of these four viroids, including the study of their sequence variability, contributed to the establishment of the concept of quasispecies for viroids and to the development of reliable molecular diagnostic methods that have facilitated the control of the diseases they caused. Most importantly, some of these viroids became valuable experimental model systems that are still used nowadays to study structural-functional relationships in RNAs and to dissect evolutionary and pathogenic pathways underlying plant-viroid interaction. The differences between early viroid discovery strategies, relying on biological and pathogenic issues, and the current high-throughput sequencing-based approaches, that frequently allow the discovery of new viroids and viroid-like RNAs in symptomless hosts, is also discussed, clarifying why the traditional molecular and biological studies mentioned above are still required to conclusively define the nature of any novel viroid-like RNA.

1. Introduction

Viroids are known as plant pathogens frequently inducing diseases in their host plants (Flores et al., 2005; Navarro et al., 2021). First viroids were discovered more than fifty years ago working on diseases of potato and citrus, which allowed identification of potato spindle tuber viroid (PSTVd, Diener, 1971) and citrus exocortis viroid (CEVd, Semancik and Weathers, 1972), respectively. Since then, more than 40 distinct viroids infecting several hosts, in which they may elicit a variety of symptoms of distinct intensities, have been reported. Conclusive identification of a viroid as the causal agent of a disease requires the fulfillment of Koch's postulates, which means that the viroid has to be isolated from the infected and symptomatic plants (generally by preparative gel electrophoresis) and inoculated (usually by mechanical means) in a non-infected host in which it must replicate and spread systemically, then causing the same symptoms observed in the original source (Di Serio et al., 2018). In the case of viroids, Koch's postulates can be fulfilled also by the inoculation of viroid RNAs (generally dimeric forms) derived from infectious clones. Such viroid RNAs may be generated by *in vitro* transcription or by *in vivo* transient expression after infiltration of *Agrobacterium tumefaciens* strains (agroinoculation) transformed with the corresponding viroid constructs (Hammond, 2022). These

Abbreviations: PBCVd, Pear blister canker viroid; ADFVd, Apple dimple fruit viroid; PLMVd, Peach latent mosaic viroid; CChMVd, Chrysanthemum chlorotic mosaic viroid.

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alternative approaches are very efficient and extremely useful, particularly when working with viroids accumulating at low concentrations in infected plants and, thus, not easily amenable for high-yield purification. During his scientific career, Ricardo Flores and members of his research group identified several new viroids and provided solid evidence for their role as causal agents of plant diseases. Here, we recapitulate the early experiments performed in the Ricardo Flores' laboratory at the end of the last century aimed at identifying and characterizing four new viroids. These viroids were shown to be the etiological agents of important diseases in their respective hosts: pear blister canker viroid (PBCVd), apple dimple fruit viroid (ADFVd), peach latent mosaic viroid (PLMVd) and chrysanthemum chlorotic mottle viroid (CChMVd). We also highlight how these viroids have been later deeply studied, becoming experimental model systems to further assess general molecular and biological features of nuclear and chloroplastic viroids.

2. Pear blister canker viroid

Among different reported bark disorders affecting pear trees, the so-called pear blister canker disease (PBC) was first described in the 60′s in France (Desvignes, 1970) as a bark alteration specifically induced in the pear cultivar (cv.) A20. This disease is associated with pustules or superficial cracks that evolve into blister and scaly cankers on stems/-branches, with leaves and fruits usually symptomless (Fig. 1A). PBC also causes decline and death in this sensitive indicator along years, but most commercial pear cultivars remain symptomless (Desvignes et al., 1999) though, eventually, a reduction in vigor and productivity can be observed. Pear blister canker viroid (PBCVd) is the causal agent of this specific bark alteration, and the number, intensity and size of the pustule bark cankers induced depends on the severity of the different viroid strains on cv. A20 (Fig. 1A). More than two decades after the first PBC description, Flores et al. (1991) suspected and proposed a viroid etiology for the disease based on the inability to cure infected plants by termotheraphy and the further identification of a small circular RNA in naturally infected and symptomatic pear trees that, apparently, were not infected by other viroids and or viruses. The inoculation of cucumber and pear seedling plants with purified preparations of this PBC-associated circular RNA, allowed them to confirm its autonomous replication, movement and accumulation *in planta,* thus showing that it was a *bona fide* viroid named pear blister canker viroid since then (Flores et al., 1991). Just one year later, the complete genome of PBCVd was sequenced by Hernández et al. (1992) by conventional molecular techniques showing that it is an RNA molecule of 315 nt for which a cruciform conformation was predicted according the criteria of minimum free energy. This viroid was found to contain the characteristic central conserved region (CCR) reported in apple scar skin viroid (ASSVd), the type member of the genus *Apscaviroid,* and a terminal conserved region (TCR) within its left-terminal domain, which is present in several viroids of the family *Pospiviroidae* (Hernández et al., 1992) (Fig. 2A).

The conclusive evidence that PBCVd is the causal agent of PBC disease was achieved by the fulfillment of Koch's postulates. Since PBCVd does not induce the typical PBC symptoms in young pear seedlings, buds of the indicator A20 were grafted on pear 'Fieudiere' seedlings that were previously inoculated with purified PBCVd RNA preparations (Ambrós et al., 1995a). One-two years later, PBC characteristic symptoms were observed on the A20 branches developed from the grafted buds, and PBCVd RNA was recovered from symptomatic tissues (Ambrós et al., 1995a). The same authors also provided the first insights into the genetic variability of PBCVd through the identification of different sequence variants (using conventional Sanger sequencing) that revealed the

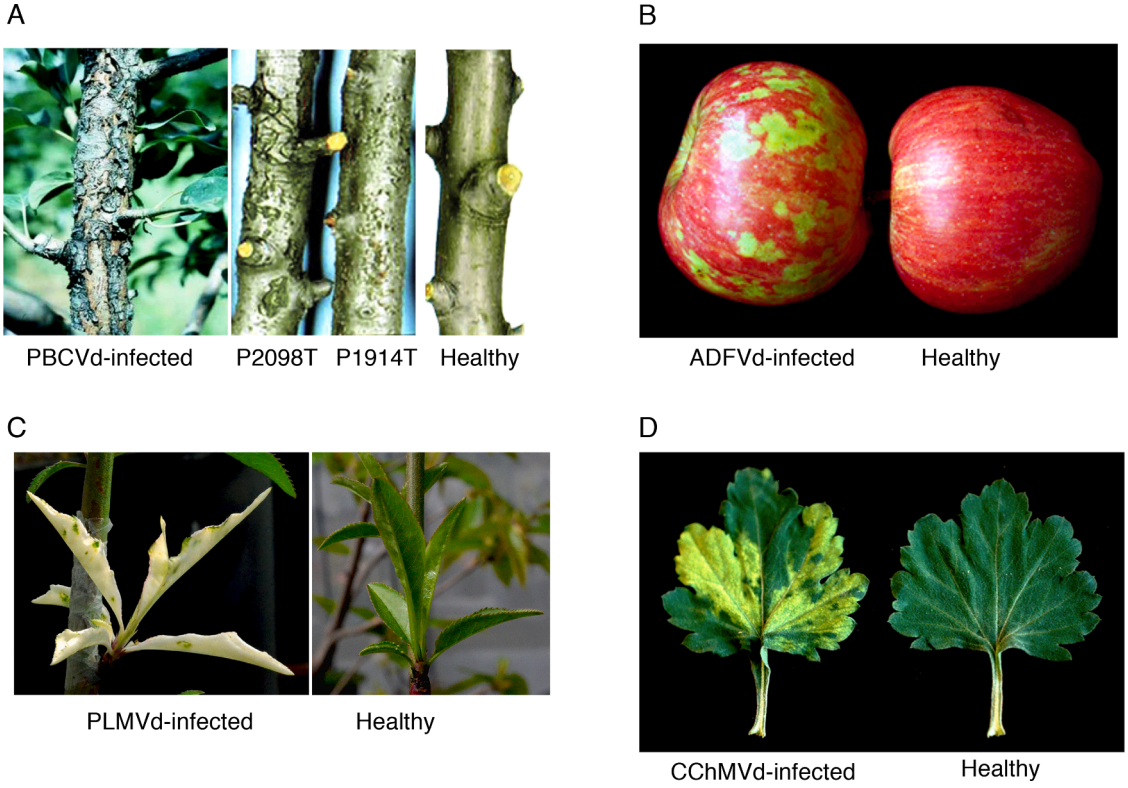

Fig. 1. (A) Symptoms induced by PBCVd on bark and stems of pear cv. A20 (on the left), symptoms induced by PBCVd isolates P2098T and P1914T, respectively (in the middle), healthy control (on the right) (Courtesy of J.C. Desvignes). (B) Symptoms induced by ADFVd on apple fruits cv. Starking Delicious (on the left) compared with healthy control (on the right). (C) Symptoms of severe albinism (peach calico) induced by PLMVd (isolate PC—C40) in peach cv. GF305 (on the left), healthy control (on the right). (D) Symptoms of leaf chlorosis induced by CChMVd (isolate S) in chrysanthemum cv. Bonnie Jean (on the left) compared with a healthy control (on the right).

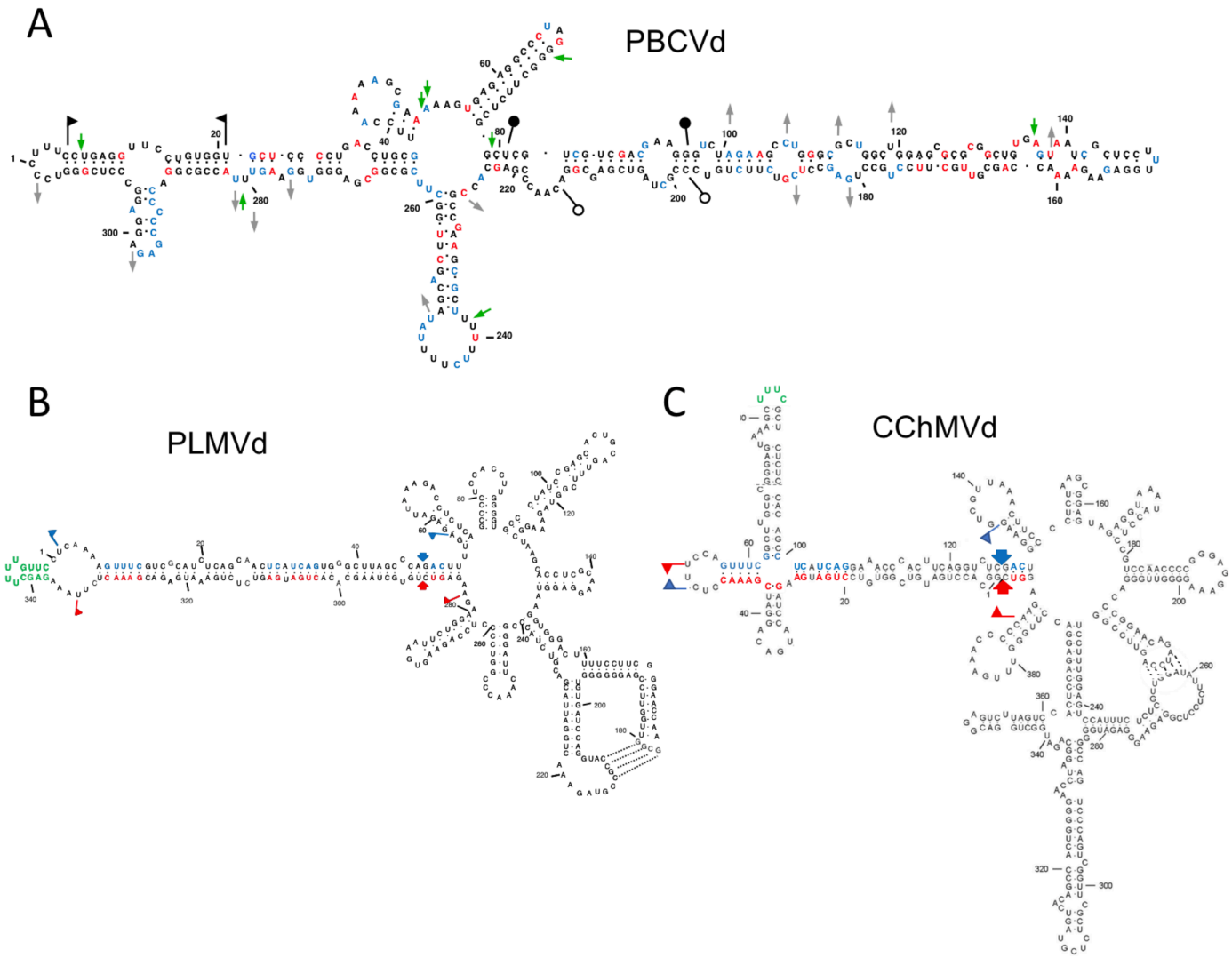

Fig. 2. Primary and secondary structure of lowest free energy for PBCVd reference variant NC_001830 (Hernández et al., 1992) (A), PLMVd variant PC-40 causing peach calico (B), and CChMVd symptomatic variant CM20 (C). In A, the terminal conserved region (TCR) is denoted by flags and the upper and lower strands of the central conserved region (CCR) by black and white circles, respectively. Positions of nucleotide insertions and deletions are marked by green arrows directed to or gray arrows starting from the sequence, respectively. Unique point mutations and other polymorphic positions, identified in the multiple sequence alignment performed with all the PBCVd variants deposited in databases, are denoted in red and blue colors, respectively. In B and C, sequences forming plus and minus hammerhead structures are delimited by flags, self-cleavage sites by arrows, and nucleotides conserved in most natural hammerhead structures are denoted in bold. Red and blue colors refer to plus and minus polarities, respectively. Sequences in green denote the pathogenicity determinant. The interaction between nucleotides in two loops forming a kissing-loop tertiary structure are indicated with broken lines.

quasiespecies nature of this viroid, as previously proposed for RNA viruses (Holland et al., 1982). A high number of point mutations affecting the PBCVd CCR have been reported (Ambros et al., 1995a; Yesilcollou et al., 2010), and many complete viroid variants from different isolates, hosts and geographic origin have been characterized (Hernández et al., 1992; Ambrós et al., 1995a; Joyce et al., 2006; Lolic et al., 2007; Kaponi et al., 2010; Yesilcollou et al., 2010; Nome et al., 2011; Elleuch et al., 2013), allowing the identification of 115 polymorphic positions distributed along the viroid genome and showing that the viroid size may range from 312 to 317 nt. Interestingly, most polymorphic positions map at loops and/or are compensatory mutations preserving the secondary structure foreseen for PBCVd (Fig. 2A) (Hernández et al., 1992 and data not shown). In this regard, a refined secondary structure, more closely related to the rod-like one proposed for other pospiviroids, was later on proposed for PBCVd based on data obtained by the SHAPE technology (Giguère et al., 2014).

Since PBCVd infections remain symptomless in many woody hosts and biological indexing takes so long, considerable efforts were done during the 90s in order to found affordable alternative sensible pear indicators for the viroid. They were identified in the pear varieties Fieud 37 and Fieud 110 that react faster, showing necrosis on petioles, and then on leaves and bark 3–4 months after inoculation (Desvignes et al., 1999). The availability of the first PBCVd sequence and cDNA clones by Hernández et al. (1992) enabled the development of rapid and sensitive viroid diagnostic methods based on dot-blot hybridization assays with specific riboprobes (Ambrós et al., 1995b). This diagnostic method allowed the detection of different PBCVd isolates varying in symptom intensity in cv. A20 and, moreover, helped to discard the involvement of PBCVd in other pear disorders such as bark split, bark necrosis and bud drop. In the following decades, new detection techniques were developed that included tissue-printing hybridization (Lolic et al., 2007), RT-PCR standard protocols (Faggioli and Ragozzino, 2002; Hassan et al., 2006), one-step multiplex RT-PCR (Ragozzino et al., 2004; Lin et al., 2012), standard and multiplex RT-PCR-probe capture hybridizations (Shamloul et al., 2002) and, more recently, quantitative RT-PCR (Malandraki et al., 2015; Beaver et al., 2022). The exploitation of

these techniques has allowed reliable and large-scaling of samples for fast diagnosis and/or PBCVd field survey studies.

PBCVd was first reported in nature in pear (*Pyrus communis* L.) and quince (*Cydonia oblonga*) and later in wild pear (*Pyrus amygdaliformis*) (Kyriakopoulou et al., 2001) and nashi (*Pyrus serotina*) (Joyce et al., 2006). Incidence vary strongly among countries, ranging from 10 % in France to more than 60 % in Greece (Di Serio et al., 2017). The viroid has been experimentally transmitted to other hosts and species of *Pyrus*, commercial pear cultivars, *Chaenomeles, Sorbus*, and *Malus* without symptom expression (Desvignes et al., 1999), and to cucumber (*Cucumis sativus*) which displays mild leaf symptoms or no symptoms (Flores et al., 1991). PBCVd was reported in wild and cultivated apples in Greece (Kaponi et al., 2010). PBCVd has been reported in several European countries (France, Spain, Italy, Greece, Bosnia-Herzegovina and Albania), Malta, North Africa (Tunisia), Turkey, Australia, China, Japan and the Americas (Argentina, Canada) (Desvignes, 1970; Hernández et al., 1992; Ambrós et al., 1995a; Loreti et al., 1997; Sano et al., 1997; Malfitano et al., 2004; Hassen et al., 2006; Joyce et al., 2006; Attard et al., 2007; Lolic et al., 2007; Di Serio et al., 2010; Kaponi et al., 2010; Yesilcollou et al., 2010; Navarro et al., 2011; Nome et al., 2011; Torchetti et al., 2012; Elleuch et al., 2013; Di Serio et al., 2017). Moreover, it is listed as a quarantine pest in some of these countries and is a regulated non-quarantine pest in the European Union. Due to the latency of the infection and the pear tolerance, it is likely that the real incidence of PBCVd has been underestimated.

Main transmission of PBCVd is through grafting or infected propagative material, while no animal vector or seed transmission has been reported. In fact, the spread of PBCVd along southern Europe could have been forced by using quince species, asymptomatically infected, as pear rootstocks of cultivated commercial varieties.

3. Apple dimple fruit viroid

Yellow-green spots, with a diameter of few mm and slightly depressed, were observed in 1995 on the red skin of apple fruits of cv. Starking Delicious trees grown in southern Italy. The spots were mainly located around the calyx and, if coalesced, generated deformed and extensively discolored fruits (Fig. 1B). The number of symptomatic plants was limited, but the symptoms were never observed before in the area. Since the observed fruit alterations resembled those induced by apple scar skin viroid (ASSVd) in certain apple cultivars (Hadidi et al., 1990), the involvement of a viroid as a potential causal agent was investigated (Di Serio et al., 1996). Early attempts to find a viroid-like RNA in symptomatic tissues were successful, identifying a circular RNA with a size, estimated by its electrophoretic mobility, smaller than that of ASSVd (329–300 nt). Northern-blot hybridization assays with ASSVd specific riboprobes showed that the nucleotide sequence of this circular RNA was only partially similar to ASSVd. Finally, cloning and sequencing of the reverse-transcribed and amplified cDNAs confirmed that the circular RNA identified in the Italian apple trees had a size (306–307 nt) and a nucleotide sequence different from ASSVd, with which it shared only 63.5 % identity (Di Serio et al., 1996). The name of apple dimple fruit viroid (ADFVd) was proposed for this new viroid because of its association with fruit symptoms in the original tree source. ADFVd showed the typical features of viroids classified in the genus *Apscaviroid,* including a quasi-rodlike conformation with the central and terminal conserved regions found in ASSVd and the other members of this genus. The autonomous replication (in the absence of any virus) of ADFVd was assessed through mechanical inoculation by stem slashing of purified ADFVd preparations into young apple seedlings of cv. Golden and by the subsequent viroid detection, based on Northern-blot hybridization assays, in the leaf tissue collected from the inoculated seedlings 10 months after inoculation (Di Serio et al., 2001). The viroid ability of inducing symptoms on apple fruits was tested by grafting bark tissues from these ADFVd-infected seedlings onto trees of the cv. Starkrimson. Development of fruit symptoms resembling those observed in the ADFVd-infected field trees, together with the identification of the viroid by Northern-blot hybridization in the inoculated trees, conclusively showed that ADFVd is the causal agent of apple dimple fruit disease. Bioassays were also extended to other cultivars, such as Starkrimson, Gala, Pink Lady and Braeburn, confirming that ADFVd generally induces symptoms milder than ASSVd in field conditions (Di Serio et al., 2001). In the same study, ADFVd did not cause any symptom in the apple cultivar Golden Delicious, which was identified as a potential symptomless host. Bioassays with ADFVd variants from Japan showed that other cultivars (such as Indo, Golden Mutsu) may remain symptomless after viroid infection. The same study extended the number of symptomatic cultivars to Fuji, Jonathan and Ohrin, pointing out that symptoms may vary depending on the cultivar. Symptoms sometimes resemble those induced by other viroids on apple trees, such as ASSVd and apple fruit crinkle viroid. Therefore, the possibility that this viroid is actually more widespread in Japan cannot be excluded because it could have been remained undetected in the symptomless cultivar or misidentified when diagnosis was based only on symptom expression. The pear indicator Fieud 37 was shown to be an experimental host of ADFVd, extending the potential host range of this viroid to tree species other than *Malus domestica* (Di Serio et al., 2001).

ADFVd has been reported in apple trees in Italy (Di Serio et al., 1996), Lebanon (Choueiri et al., 2007), Japan (He et al., 2010), and China (Ye et al., 2013). A study on the virome of fig trees performed by high-throughput sequencing (HTS) approach allowed the identification of ADFVd in a fig accession in Italy (Chiumenti et al., 2014). Amplification by RT-PCR, cloning and sequencing of the viroid full-length cDNA confirmed the presence of such a viroid in the fig accession. Due to the low accumulation of this viroid in this host, a nested RT-PCR protocol was developed that detected the viroid in several fig accessions in southern Italy. Recently, ADFVd has been reported in fig trees grown in Turkey, thus confirming that such a viroid has a natural host range than goes beyond apple tree species (Buzkan and Balsak, 2022). Interestingly ADFVd variants from fig trees identified in Italy and Turkey share 99% sequence identity with each other, and up to 100 % with other variants previously reported in Italy from apple trees (Buzkan and Balsak, 2022). Phylogenetic studies supported the clustering of ADFVd isolates from Japan in a clade different than that of variants from China and Italy (Chiumenti et al., 2014; Kasai et al., 2017), underlying a degree of sequence variability of this viroid higher than previously though.

There are still unanswered questions on the epidemiology of this ADFVd whose natural spread seems limited. On the one hand, the viroid did not spread from infected apple trees to neighboring or distant trees in the same field during a 3-year observation period (Malfitano et al., 2004). On the other hand, ADFVd transmission through seeds was excluded based on experimental trials (Malfitano et al., 2004). Therefore, the main spreading pathway of this viroid is human-assisted, likely through infected propagation material (Di Serio et al., 2017). The availability of efficient detection methods (Di Serio et al., 2017) facilitate the screening of propagation material to ascertain the absence of ADFVd.

4. Peach latent mosaic viroid

Peach latent mosaic (PLM) disease was first reported in France (Desvignes, 1976) and, on the basis of cross-protection assays (Desvignes, 1986), considered to be related to previously described diseases known as peach calico (PC) (Blodgett, 1944) and peach blotch (PB) (Willison, 1946) in the U.S., and peach yellow mosaic (PYM) (Kishi et al., 1973) in Japan. The term latent refers to the frequent lack of symptoms in leaves, with the first signs of disorder becoming apparent on the affected peach trees two years after planting. The pathological alterations may be diverse and include: delay in foliation, flowering and ripening, pink-broken lines on the rosaceous-white petals, deformation, discoloration and cracked sutures on fruits, bud necrosis, and rapid aging after the 5th year. When appearing, the phenotypical effects on

leaves may be variable and, moreover, fluctuating. In the greenhouse, isolates of PLM are graded as severe or latent depending on whether or not they incite leaf symptoms on seedlings of the peach indicator GF-305 (Desvignes, 1976, 1986)

Attempts to identify virions in diseased tissues were unsuccessful which, together with the high heat resistance of the agent, pointed to a viroid etiology. This became more plausible when a viroid-like RNA was detected in peach samples infected by isolates of the PLM agent but not in healthy controls (Flores and Llácer, 1988). Koch's postulates were fulfilled when the viroid-like RNA purified from a severe PLM isolate was inoculated on seedlings of the peach indicator GF-305 and induced the characteristic symptoms of the PLM disease, being possible to recover an RNA with identical physical properties from the inoculated plants. Such RNA was consequently called peach latent mosaic viroid (PLMVd) (Flores et al., 1990).

Once identified, efforts to determine the primary structure of PLMVd finally succeeded through a combination of direct RNA sequencing with base-specific RNases, design of specific oligodeoxynucleotides from RNA sequencing derived data, cDNA synthesis and cloning, and subsequent Maxam and Gilbert DNA sequencing. This first characterization of PLMVd, which corresponded to a typical severe isolate of the viroid, revealed a size of 338 nt and, remarkably, the presence of hammerhead ribozymes in the strands of both polarities (Hernández and Flores, 1992). This was not a trivial observation at that time since only another viroid, avocado sunblotch viroid (ASBVd), had been reported with such outstanding property (Hutchins et al., 1986). Thus, PLMVd discovery provided solid support to what was later named family *Avsunviroidae* (Flores et al., 2000). Initial experiments already showed the capacity of PLMVd hammerhead ribozymes to promote *in vitro* self-cleavage of PLMVd RNAs both during transcription and, also, in the complete absence of proteins, suggesting they play a role in the processing of oligomeric precursors *in vivo*. This conjecture was later supported by the finding of linear forms extracted from infected tissue with the termini expected from the hammerheads activity (Delgado et al., 2005). The initial predictions of PLMVd secondary structure of minimal free-energy revealed another distinguishing feature of the viroid: PLMVd was anticipated to adopt a branched conformation which was clearly different from the rod-like or quasi-rod-like structure of other known viroids (Fig. 2B). Data from *in vitro* experiments and natural sequence heterogeneity were consistent with this prediction and, moreover, the former pointed to the likely existence of a pseudoknot-like interaction between two hairpin loops of the branched conformation of the plus polarity strand (Bussière et al., 2000) (Fig. 2B), similarly to that found in related viroids (Gago et al., 2005; Serra et al., 2018). The same PLMVd strand contains additional elements of tertiary structure as revealed by UV irradiation (Hernández et al., 2006).

From its first characterization, many isolates and molecular variants of PLMVd have been described, ranging in size from 335 to 351 nt (*e.g.*, Ambrós et al., 1998; 1999; Malfitano et al., 2003; Rodio et al., 2006; Delgado et al., 2019; Glouzon et al., 2014; Wang et al., 2013). Indeed, this viroid shows an extraordinarily high sequence variability both within and between isolates. Each isolate is a complex mixture of molecular variants which may arise quickly from a single variant and which, altogether, may determine the phenotypical consequences on the host, as suggested by some early analyses at Ricardo's lab (Ambrós et al., 1998; 1999). Despite the viroid population complexity, studies aimed at identifying specific pathogenicity determinant(s) were conducted and they paid off in the case of PLMVd molecules causing peach calico (PC), an albino-variegated disease (Fig. 1C). An insertion of 12–14 nt in the loop closing the so-called hammerhead arm (Fig. 2B), was identified as being responsible of PC (Malfitano et al., 2003; Rodio et al., 2006; 2007). Afterwards, the mechanistic basis of the insertion effect was unveiled (Navarro et al., 2012) and related to the RNA silencing response that host triggers in an attempt to defeat viroids, including PLMVd (Martínez de Alba et al., 2002; Rodio et al., 2007; Navarro et al., 2012). More specifically, a small RNA (sRNA) derived from the pathogenic determinant targets for cleavage, via a RNA silencing mechanism, the mRNA of the chloroplastic heat shock protein 90, promoting its degradation (Navarro et al., 2012). Later on, PYM symptoms were associated to a single nucleotide forming part of a sRNA able to target another host mRNA, a thylakoid translocase subunit (Delgado et al., 2019).

The PLMVd infection was initially thought to be restricted to *Prunus* spp., where the incidence and economic impact may be remarkable in distinct parts of the world (Di Serio et al., 1999; Flores et al., 2003; 2006). In the last years the viroid has been detected in other fruit trees including apricot, plum, sweet cherry, pear and mango and, also, in grapevine (Kyriakopoulou et al., 2017). In addition, PLMVd infection in kaki and Johnsongrass has been recently reported (Oksal et al., 2021). However, information on the relative accumulation levels or the prevalence in these alternative hosts is lacking as the corresponding reports are usually limited to the detection of the viroid in one or few samples through RT-PCR.

To conclude, identification and characterization of PLMVd represented an important turning point in Ricardo's lab research lines and, more widely, in the field of viroids, opening up new investigation paths on structural, functional and evolutionary aspects of these minimal pathogenic RNAs.

5. Chrysanthemum chlorotic mottle viroid

More than 30 years passed from the first report of the chrysanthemum chlorotic mottle disease (CChM) (Dimock et al., 1971) to the identification and molecular characterization of its causal agent: the chrysanthemum chlorotic mottle viroid (CChMVd) (Navarro and Flores, 1997). CChM, first described in EEUU in greenhouse-cultivated chrysanthemum plants, is characterized by the presence of spotted chlorosis in the early-formed leaves that, at the end, lead to a more extended chlorosis on almost all the leaf surface (Fig. 1D). Additional symptoms included dwarfing and delay in flower development (Dimock et al., 1971). Based on the graft-transmissibility of the CChM and the absence of fungal or bacterial agents in the infected plants, a putative viral etiology was initially proposed by Dimock et al. (1971). However, the lack of virus-like particles in samples observed by electron microscopy as well as additional evidence (see below) supported that the causal agent of CChM could be a viroid, a new type of infectious agent discovered at that time (Diener, 1971; Semancik and Weathers, 1972). In fact, the infectivity of preparations from diseased plants was lost after RNase but not after DNase or proteinase treatments, the infectious agent was not sedimentable after high speed centrifugation, and it had a sedimentation coefficient in sucrose gradients of 6–14S (Romain and Horst, 1975), typical of small nucleic acids. However, all the attempts to identify a circular RNA in the preparations from diseased tissues using double PAGE electrophoresis, specifically developed for the detection of viroids, failed (Horst, 1975a). Therefore, in the absence of any additional information, the infectivity bioassay on sensitive chrysanthemum cultivars (such as Bonnie Jean, Yellow Delaware or Deep Ridge) was the unique tool for detecting the infectious agent of CChM. When Ricardo Flores decided to address this issue, the development of a reproducible bioassay on the natural host chrysanthemum cv. Bonnie Jean, in growth chambers under controlled conditions (28 °C with constant high light intensities), that allowed the expression of symptoms 8–10 days after inoculation, was determinant for the identification of the causal agent of CChM. Since circular and linear forms of viroids migrate separately in a denaturing 5 % PAGE, this approach was followed to associate nucleic acids migrating in a determined section of the gel with the infectivity. To this aim, RNA preparations from CChM-affected chrysanthemum plants were separated in a denaturing 5 % PAGE and the nucleic acids eluted from different gel sections were tested by bioassays. These preliminary experiments showed that infectivity was recovered only from a region where linear RNAs of about 400 nt migrated. To improve the resolution of the bands into the gel and in order to further separate the potential

infectious RNA from the co-migrating host RNAs, RNA preparations from healthy and CChM-affected chrysanthemum plants were examined in long denaturing PAGEs (sequencing gels). This new approach allowed the identification of a differential band corresponding to a linear RNA of about 400 nt, only present in CChM tissues which, when bioassayed after elution from the gel, was able to induce the typical symptoms of CChM in the chrysanthemum inoculated plants. Therefore, the agent of the disease was physically identified as a small RNA. Cloning and sequencing of the eluted differential RNA revealed that it had a sequence of 399 nt and that was predicted to fold in a branched conformation similar to that proposed for PLMVd (Fig. 2C). Interestingly, similarly to PLMVd, both polarity strands of this novel RNA contained hammerhead ribozyme structures that were active *in vitro* and *in vivo* (Navarro and Flores, 1997). *In vitro* dimeric head-to-tail transcripts of CChMVd were infectious and able to induce chlorosis in the inoculated chrysanthemum plants from which the viroid was recovered, thus confirming that this RNA replicated autonomously and fulfilling the Koch's postulates. Northern blot hybridization of RNA samples from infected plants separated on denaturing PAGE showed the existence of circular and linear forms of both polarity strands of the novel RNA, the hallmark of chloroplast-replicating viroids (Navarro and Flores, 1997). Altogether these experiments provided conclusive evidence that the agent of CChM is a viroid that was named chrysanthemum chlorotic mottle viroid (CChMVd) and classified as the third member of the family *Avsunviroidae*. CChMVd was the first member of this family infecting an herbaceous host.

The common properties shared with PLMVd, such as a branched secondary structure and insolubility in 2 M LiCl, led to the classification of these two viroids in the new genera *Pelamoviroid* (Navarro and Flores, 1997). Later on, a kissing-loop interaction between two loops in the secondary structure was identified in all members of this genera (Brussiere et al., 2000, Gago et al., 2005; Di Serio et al., 2018) that nowadays includes another viroid, apple hammerhead viroid (Serra et al., 2018).

The existence of an infectious non-symptomatic strain of the CChMVd was early predicted based on biological studies of cross-protection, which evidenced that a transmissible agent that protected against CChM was present in some asymptomatic plants (Horst, 1975b). Once the CChMVd sequence was available, CChMVd non-symptomatic variants (CChMVd-NS) were identified and characterized in plants preserved from infection of symptomatic (CChMVd-S) sequence variants. Comparison of CChMVd-NS and CChMVd-S sequence variants, together with bioassays with head-to tail dimeric transcripts of natural and mutated CChMVd molecules, lead to the identification of the determinant of pathogenicity (de la Peña et al., 1999; de la Peña and Flores, 2002). This motif mapped in the tetraloop at nucleotide positions 82–85 (UUUC) capping a hairpin in the proposed secondary structure of the CChMVd-S variants (Fig. 2C). Substitution of the tetraloop UUUC with GAAA (found in CChMV-NS) in CChMVd-S was enough to abolish the pathogenicity without altering viroid accumulation (de la Peña et al., 1999; de la Peña and Flores 2002). Further studies carried out during the last years showed that pathogenicity of CChMVd-S variants is likely induced through an RNA silencing-mediated mechanism activated by the infecting viroid (manuscript in preparation).

As with other viroids, the discovery of the CChMVd as the causal agent of the CChM allowed the development of efficient and rapid molecular detection methods necessary for disease control. Since CChMVd infects an herbaceous host and efficient and fast bioassays were developed, this viroid offered an advantageous system for the study of members of the family *Avsunviroidae* that, at that time, only included viroids infecting woody hosts (PLMVd and ASBVd). It is noteworthy that the system CChMVd-chrysanthemum has been successfully used to show the role of the tertiary interactions between the loops of the hammerhead ribozymes for both the *in vitro* self-cleavage activity and the *in vivo* viroid infectivity (de la Peña et al., 2003; Dufour et al., 2009). This viroid was also instrumental for the identification of the above-mentioned kissing-loop interaction between two loops in the branched secondary structure (also present in other members of the genus *Pelamoviroid*), which is critical for the *in vitro* folding and the *in vivo* viability of the viroid (Fig. 2c) (Gago et al., 2005). The CChMVd-chrysanthemum system was also used for the calculation of the mutation rate of a representative member of chloroplast-replicating viroids that resulted the highest known for a biological entity (Gago et al., 2009). Therefore, the discovery of the agent of a plant disease also provided a valuable model system to study structural,functional and evolutionary features of RNAs.

6. Concluding remarks

In the last century, most viroids were discovered in the frame of studies aimed at identifying the causal agents of virus-like diseases, the etiology of which was un proven notwithstanding the efforts to isolate the hypothetical disease-causing viruses. In several occasions, especially in the case of diseases affecting woody hosts, the hypothesis that a viroid could be involved derived from the unsuccessful attempt of sanitation of the propagation material through thermotherapy, because viroids were shown to be quite resistant to this treatment (Barba et al., 2017). Therefore, transmissibility by mechanical inoculation or grafting of the etiological agent(s) was generally established as a first step in the study of diseases possibly induced by viroids. By using symptomatic tissues and applying specific extraction protocols, viroids and viroid-like RNAs were first physically identified, generally through electrophoretic methods, and then characterized from a biological and molecular point of view with the determination of the full-length genomic sequence. This approach allowed mostly discovery and characterization of viroids causing plant diseases. Many of these efforts were made in Ricardo Flores' lab, as we have described in the previous sections in which we have focused the attention on the discovery and further analysis of four viroids affecting either fruit trees or ornamental plants: PBCVd, ADFVd, PLMVd and CChMVd.

In the current century, with the advent of HTS and its application for diagnostic purposes, the sequential steps leading to the identification of new viroids and viroid-like RNAs have been completely reversed, with determination of the genomic sequences of potential new viroids and viroid-like RNAs preceding their biological and molecular characterization. Sequenced reads from HTS of RNA libraries from any host, symptomatic or not symptomatic, can be *de novo* assembled in contigs and their homology with previously reported viroids and viroid-like RNAs can be searched in databases. Homology-independent bioinformatics tools to search for circular RNAs (Wu et al., 2012; Zhang et al., 2014) and/or for structural signatures of ribozymes in nucleic acids have been also developed (Ferbeyere et al., 1998; Jimenez et al., 2011; Weinberg et al., 2015; de la Peña et al., 2021), increasing the potentiality of finding new viroids and viroid-like RNAs that do not share any sequence homology with those previously reported. However, after the identification of potential new viroid-like RNAs *in silico*, the conclusive characterization of their biological nature needs to be ascertained by investigating whether they actually exist *in vivo* and are associated with the potential host, whether they are transmissible mechanically or by grafting, whether they replicate autonomously (in the absence of any helper virus), whether the existence of a host DNA counterparts can be excluded and, when they are associated with a disease, whether Koch's postulates can be fulfilled. Interestingly, most of the new viroids identified in the last ten years using these technologies were from non-symptomatic hosts, thus showing that previous approaches based on physical isolation of the viroid from diseased plants were certainly biased. Many new potential viroids and viroid-like RNAs are expected to be identified in the next future by the powerful HTS-based technology, but the traditional molecular and biological studies mentioned above are still required to conclusively define the nature of any novel viroid-like RNA.

CRediT authorship contribution statement

Beatriz Navarro: Conceptualization, Writing – review & editing. **Silvia Ambrós:** Conceptualization, Writing – review & editing. **Francesco Di Serio:** Conceptualization, Writing – review & editing. **Carmen Hernández:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

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.

Data availability

No data was used for the research described in the article.

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The work described in this paper reflects Ricardo Flores' willingness and commitment to substantially contribute to broadening the knowledge on exceptional biological entities, the viroids, unusual pathogens that, moreover, have been deemed as relics of precellular evolution. With this review, the authors, former Ph.D./postdoctoral students at Ricardo's lab, have intended to thank and recognize, once more, the invaluable guidance and lessons they got from him.

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